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Relationship between antioxidant and anxiolytic activity of standardized extracts of *Melissa officinalis* and *Rosmarinus officinalis.*

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Abstract

All diseases, especially those neurological pathologies (Depression, anxiety, Alzheimer and Parkinson's diseases, etc) are described to be associated to oxidative stress. An increased concentration of metal ions in neurons of patients could be, at least in part, the cause of this oxidative stress. Reduced metal ions generate ROS through Haber-Weiss and/or Fenton reactions. By other hand, psychotropic effects of diverse plants have been described, between them *Melissa officinalis* and *Rosmarinus officinalis*. Pharmacological studies about the beneficial effects of these plants are scarce, especially those about *Rosmarinus officinalis*. In this work, a correlation between biological antioxidant activity of these plants and their anxiolytic activity was studied. Results show that this correlation seems to occur, and the pharmacokinetic and pharmacodynamic importance of this phenomenon is discussed.

Keywords: *Melissa; Rosmarinus; officinalis;* antioxidant; anxiolytic; activity.

Introduction

Psychiatric disorders such as depression and anxiety affect a large population with a wide range of ages. In these last years, an increase in consumption of anti-psychotic drugs by young and old has occurred [1]. It is well known that the consumption of psychotropic drugs is associated with a variety of side effects, such as anti-cholinergic effects (dry mouth, blurring, constipation, agitation, and confusion), cardiovascular effects (orthostatic hypotension, tachycardia, and arrhythmia) and weight gain, with the added risk of suffering obesity and diabetes [2].

There is evidence associating oxidative stress to the phenomena of depression and anxiety [3]. Patients with depressive disorders exhibit a high correlation between plasmatic levels of malondialdehyde (MDA) and superoxide dismutase (SOD) activity in erythrocytes, phenomena correlated with the severity of the depressive disorder [4]. Otherwise, it has been demonstrated that anxiety disorders, including panic attacks and obsessive-compulsive disorder (OCD), are associated to an increase in the products of lipid peroxidation, specifically MDA [5]. In addition, patients suffering severe depressive disorders show modified plasmatic levels of glutathione reductase (GR) and MDA, as well as erythrocytes levels of glutathione peroxidase (GSH-Px), SOD and MDA, compared to healthy subjects [6].

These evidence have elicited the development of novel antioxidant therapeutic strategies to treat neurodegenerative and psychiatric diseases [3, 7].

Medicinal plants, specially their leaves, have a high content of antioxidants, particularly, polyphenols. Therefore, during the last years the search for new therapies for psychiatric diseases based on medicinal plants has increased [8]. To respect, leaves preparations of *Melissa officinalis* and *Rosmarinus officinalis* have shown to have antioxidant activity, as well as psychotropic activity [9, 10].

Melissa officinalis is an aromatic plant belonging to Lamiaceae family, widely distributed in Mediterranean zones. The leaves of this plant have been used in popular medicine for their sedative, spasmolytic and antibacterial properties. Moreover, aqueous extracts of Melissa officinalis showed anti-inflammatory properties [9]. Also, hydroalcoholic extracts of the leaves of this plant have shown antidepressive-like and anxiolytic effects, comparable to Diazepam in rats [11]. The authors suggest that some of the components of the Melissa officinalis extracts would exert an effect in the γ-aminobutyric acid transaminase (GABA-T) activity, increasing the inhibitory effect of GABA on the neuronal excitability.

Rosmarinus officinalis is a plant belonging to Lamiaceae family, original from the Mediterranean rocky coast zone of Europe and Africa. This plant is well known for its culinary properties. Traditionally, its leaves have been used as spasmolytic, liver protector and anti-inflammatory. An antinociceptive effect was demonstrated in rodents [10]. Previous studies in our laboratory indicated that rats treated with an standardized hydroalcoholic extract of

Rosmarinus officinalis presented antidepressive and anxiolytic effects comparable to Fluoxetine and Diazepam [12]. These comparative effects suggest an increase in the inhibitory effect of GABA-T, in addition to a possible inhibitory effect on the presynaptic serotonin reuptake [13].
There is scarce evidence of the responsible compounds of the *Melissa officinalis* and *Rosmarinus officinalis* extracts exerting the psychotropic activity. It should be noted that neurologic mediators of psychotropic activities are principally amines. Previous studies in our laboratory showed that a hydroalcoholic extract of *Rosmarinus officinalis* was able to inhibit the N-demethylation of aminopyrine, a reaction catalysed by oxidative system of cytochrome P450 [14]. This enzymatic system is mainly responsible of the biotransformation of lipophilic xenobiotics, an essential physicochemical property for every active principles acting in neurons exerting psychotropic and antioxidant activity; they must cross the brain-blood barrier, a phenomenon that depend of lipophilicity of such compounds.
Summarizing, pharmacological studies related to psychotropic effects of preparations of *Melissa officinalis* and *Rosmarinus officinalis* are scarce. Moreover, a diversity of commercial leaf preparations of this plant exist but without any pharmacological nor posology studies related to them. In addition, patients suffering affective disorders show altered levels of lipid peroxidation and on the activity of some antioxidant enzymes [5]. Therefore, it was decided to study the biological antioxidant capacity of standardized dried extracts of *Melissa officinalis* and *Rosmarinus officinalis* and correlate this antioxidant capacity to the behavioural effects on Sprague-Dawley rats under anxiety test.

Materials and Methods

Reagents

Catechin ((2R, 3S)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol), sodium ascorbate, DTNB (5,5'-ditiobis-(2-nitrobenzoico) acid), bovine serum albumin (Fraction IV) were purchased from Sigma-Aldrich. Trichloroacetic acid (TCA), thiobarbituric acid (TBA), cooper sulphate ($CuSO_4$) and Folin-Ciocalteu's Reagent were purchased from Merck (Chile). Diazepam, reference drug (positive control) for anxiolytic activity, was obtained from F. Hoffmann-La Roche, Basel, Switzerland. All other reagents were of analytical degree.

Extracts of Melissa officinalis and Rosmarinus officinalis

Standardized dried extracts of leaves of *Melissa officinalis* and *Rosmarinus officinalis* were graciously provided by Ximena Polanco Laboratory (Santiago, Chile). These extracts were obtained by hydroalcoholic extraction of leaves of these plants, and then dried. The detailed procedures of extraction are private property.
Dried extracts of *Melissa officinalis* and *Rosmarinus officinalis* were freshly dissolved in 0.9% saline and administered intraperitoneal (6.25, 12.5, 25 and 50 mg/Kg i.p.), in a volume of 1mL/Kg bodyweight [15]. Control animals were injected with an equal volume of physiological saline solution as negative control or Diazepam (1mg/Kg) as positive control. Administered doses of the extracts were determined in preliminary tests.

Animals

Adult male Sprague Dawley rats (220g – 250g) were maintainedin groups of 7-8 per cage, at the vivarium of the Faculty of Medicine, Universidad de Chile. Animals were fed with a normal pellet diet and water *ad libitum* and maintained in 12h light:dark cycles at 22±1 C. All procedures were performed according to the protocols approved by the Ethical Committee of Faculty of Medicine of the Universidad de Chile. Likewise, all experiments on animal behaviour were conducted according to the international standards of animal wellness, recommended by the Society of Neuroscience (USA) [16]. Both number of animals and time of treatment, corresponded to the minimum required to obtain significant data. Each animal was treated and analysed only once. Rats were randomly assigned to each pharmacological treatment.

Isolation of rat liver microsomes

Microsomal fraction was prepared by differential centrifugation as previously described inLetelier et al., 2010[17]. Protein determination was performed according to Lowry et. al, 1981[18] using BSA as standard.

Polyphenol Determination

Determination of total polyphenols in herbal extracts was performed according to described method inLetelier et al, 2008[19]. Linearity of this method was analysed using a calibration curve with catechin as standard (Data not shown).

Oxidative conditions

Through Haber Weis and/or Fenton reactions, transition metal, in their reduced form generates ROS [20, 21]. Therefore, 25ηM $CuSO_4$ and 1mM ascorbate were used as generating system of superoxide anion ($O_2^{\cdot -}$).

Microsomal lipid peroxidation

Cu^{+2}/ascorbate-induced microsomal lipid peroxidation was estimated by determining thiobarbituric acid reactive substances (TBARS) according toLetelier at., 2005[22].

Microsomal thiols determination

Determination of microsomal thiols was performed according toLetelier et al., 2005[22]. This spectrophotometric technique quantifies released TNB after reaction of DNTB with thiolic groups of the sample.

Chelating capacity of Melissa officinalis and Rosmarinus officinalis extracts

Chelation activity was determined by the capacity of extracts to modify the spectra of Cu^{+2}. For this, the spectrum of 0.5mM $CuSO_4$ was determined between 200 to 600nm in the presence and absence of the herbal extracts. Blanks contained all reagents with the exception of 0.5mM $CuSO_4$.

N-demethylation of aminopyrine

The N-demethylation activity of cytochrome P450 system was determined according toLetelier et al., 2009[23]. Enzymatic activity was expressed as nmol of generated formaldehyde/min/mg of microsomal protein.

Spontaneous motor activity

Thirtyminutes after thetreatment with the extracts (6.25, 12.5, 25 and 50 mg/kg i.p.), ratswere individually placed in a Plexiglas cage (30x30x30cm) and located inside a soundproof chamber. Diazepam or saline solutions were used as reference drugs(positive and negative controls, respectively). The floor of the cage was an activity platform connected to an amplifier and an electromechanical counter. Total motor activity was monitored every 5min during a period of 30min. Each animal was observed continuously and directly via a video camera connected to a computer where every measurement was recorded. Videos were used for posterior reanalysis when needed.

Elevated plus-maze

This test has been validated to measure anxiety in rodents [24]. The apparatus consisted in two open arms (50x10cm), two enclosed arms (50x10x20cm) and a central platform (10x10cm), disposed in such way that the two arms of each type were opposite to each other. The maze is elevated 100cm above the floor. Immediatelyafter the motor activity register, each rat was placed in the central platform of the maze facing one of the enclosed arms. During the 5min test period, the number of entries and the time spent in each arm (open or enclosed) were registered [25]. The entry into an arm was definedas the point when the animal places the four paws onto the arm.

Statistical analysis

Antioxidant activity data was analysed using t-student test and regression analysis. Behavioural data were analysed using one-way analysis of variance (ANOVA) followed by Newman-Keuls's Multiple Comparison test. In behavioural evaluation, results represent the mean of at least eight independent experiment ± SEM.Prism Graph Pad Software was used for all statistical analysis. A probability level of 0.05 or less was accepted as significant.

Results

In order to correlate the antioxidant capacity of herbal extracts with the presence of antioxidants, the concentration of polyphenols of *Melissa officinalis and Rosmarinus officinalis* was determined. Dried extract of *Melissa officinalis* contained 4 times less polyphenols than that of *Rosmarinus officinalis*:25.5±0.69µg *vs* 110.1±24.79µg of catechin equivalents/mg of extract, respectively.

Antilipoperoxidative effect of herbal extracts

Both dried extracts inhibited the microsomal lipid peroxidation in a dose- response manner. The EC_{50} values obtained from semilogarithmic curves were 24.9µg for *Melissa officinalis* and 3.9µg for *Rosmarinus officinalis* (Figure 1). This data indicates that *Rosmarinus officinalis* extract is approximately 6-fold better antilipoperoxidant agent than *Melissa officinalis* extract.

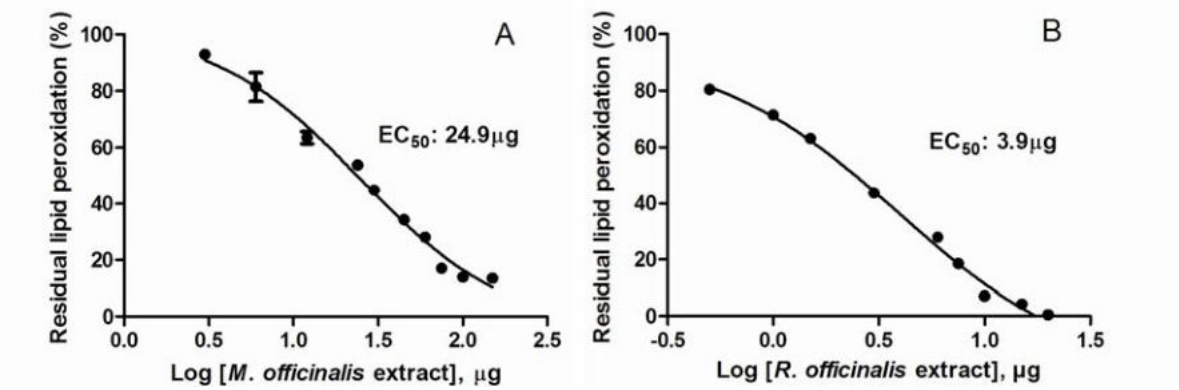

Figure 1. Lipid peroxidation of microsomes in presence of herbal extracts.

Microsomes (0.5mg/mL) were incubated for 5min with different concentrations of *Melissa officinalis* (3.0 -150µg) (Figure 1A) and *Rosmarinus officinalis* (0.5 - 20µg) (Figure 1 B) extracts. Percentages of inhibition were calculated considering as 100% lipid peroxidation measured in the absence of extracts. Assay conditions as described in Methods. All values represent the mean of at least four independent experiment ± S.E.

Prevention of microsomal thiols oxidation in presence of herbal extracts

Preincubation of microsomes with herbal extracts 5min before adding Cu^{+2}/ascorbate system, inhibited the microsomal thiols oxidation in a dose-response manner (Figure 2). EC_{50} values to prevent the oxidation of microsomal thiols were approximately 26.9µg and 9.56µg for Melissa officinalis and Rosmarinus officinalis, respectively, (Figure 2).

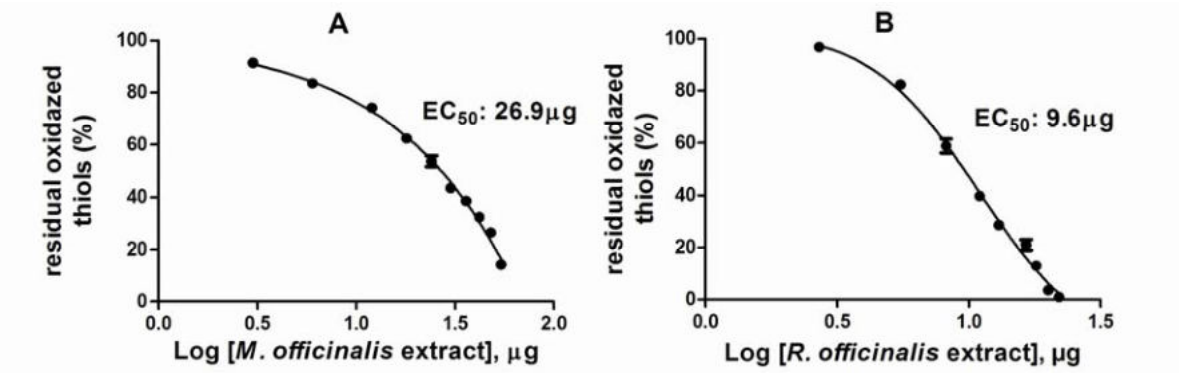

Figure 2. Thiols oxidation of microsomes in presence of herbal extracts.

Microsomes (0.5mg/mL) were preincubated for 5min with different concentrations of *Melissa officinalis* (3-54µg) (Figure 2A) and *Rosmarinus officinalis* (2.7-22µg) (Figure 2B). Percentage of residual oxidized thiols was calculated considering as 100% of microsomal oxidized thiols those measured in the presence of Cu^{2+}/ascorbate and absence of herbal extracts. Assay conditions as described in Methods. Values represent the mean of at least four independent experiments ± S.E.

Melissa officinalis and Rosmarinus officinalis extracts prevent the decreasing of microsomal thiols in the presence of Cu^{+2}

Microsomal membrane contains thiolic proteins such as GHS-transferase, Cytochrome P450 monooxygenase and UDP-glucuronyltransferase. Presence of cooper ions in micromolar concentrations could affect these proteins because thiolic groups are able to chelate these ions [17], [23]. Cooper ions diminished approximately in 80% the total content of microsomal thiols and both herbal extracts partially prevented this phenomenon; *Rosmarinus officinalis* extract provoked the greatest effect. Thereby, maximum concentration assayed of *Melissa officinalis* and *Rosmarinus officinalis* extracts (100µg of extract/ 250µg of

microsomal protein) increased 15% and 30% the residual microsomal thiols concentration, respectively (Figure 3).

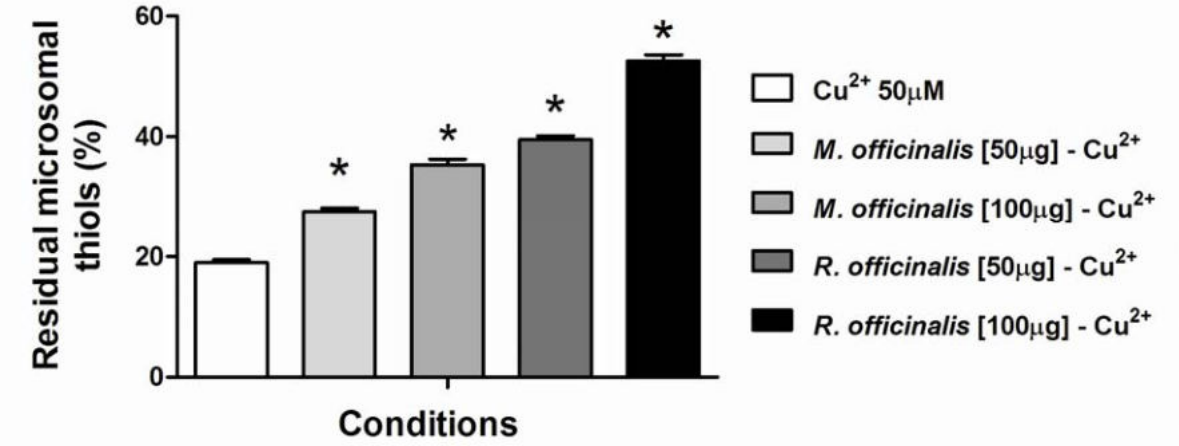

Figure 3. Microsomal thiols in the presence of Cu^{2+}.

[Microsomal protein]: 0.25mg/mL. [$CuSO_4$]: 50µM. Assay conditions as described in Methods. All values represent the mean of at least four independent experiments ± S.E. (*) Values significantly different to control value ($p<0.05$).

Cu^{+2} - Chelating capacity of herbal extracts

The addition of *Melissa officinalis* extract (300µg and 500µg) to $CuSO_4$ solution did not modify significantly the characteristic absorbance at 241nm of Cu^{+2}, but a new peak at 370nm appeared. The absorbance at this wavelength seems to depend on the *Melissa officinalis* concentration (Figure 4). Ratio of *Melissa officinalis* concentration tested (500µg/300µg) was 1.67, value similar to that obtained from the maximum absorbances measured at 370nm in the presence of both *Melissa officinalis* concentrations tested: 0.215/0.138 = 1.56. In the same conditions, *Rosmarinus officinalis* extract (300µg and 500µg) revealed new absorbance peaks at 294nm, 384nm and 434nm (Figure 4). Absorbance at 241nm, characteristic of Cu^{+2}, seemed to depend of *Rosmarinus officinalis* extract concentrations tested. In this case, ratio calculated between extract concentrations tested (500µg/300µg) was 1.67, similar to ratio obtained from the experimental absorbances at 241nm: 0.849/0.496=1.71 and 384nm: 0.351/0.20= 1.74.

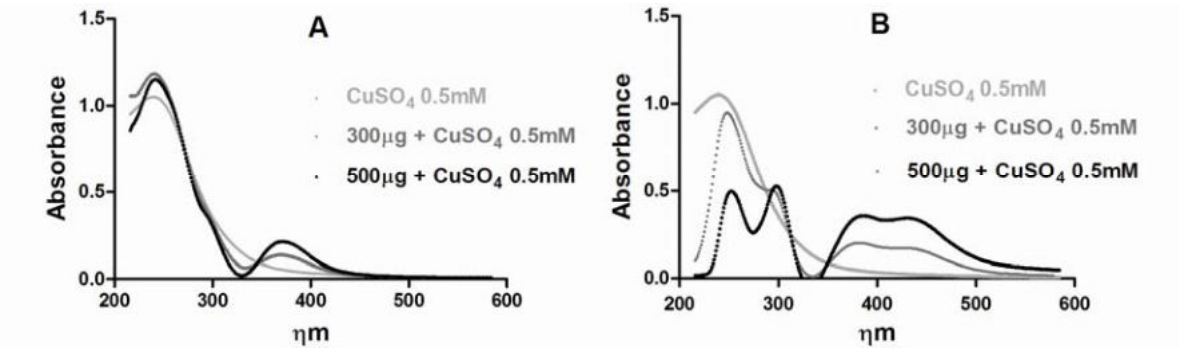

Figure 4. Experimental Absorbance spectrum of $CuSO_4$ solution in the presence of *Melissa officinalis* (A) and *Rosmarinus officinalis* (B) extracts. Assay conditions as described in Methods.

Inhibition of aminopyrine N-demethylation by herbal extracts

Previous data indicate that *Melissa officinalis* and *Rosmarinus officinalis* extracts tested present psychotropic activities, mainly for the presence of amines [14, 26]. For

that, the effect of this herbal extract on N-demethylation activity of Cytochrome P450 system was determined. *Melissa officinalis* and *Rosmarinus officinalis* extracts inhibited the in 23% and 43%, respectively (Figure 5).

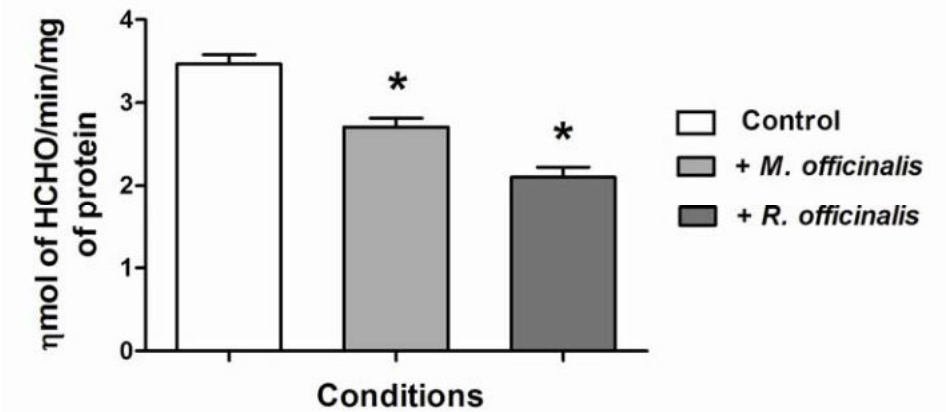

Figure 5. N-demethylation of aminopyrine.

[*Melissa officinalis*]:50µg/mg of microsomal protein; [*Rosmarinus officinalis*]: 50µg/mg of microsomal protein. Assay conditions as described in Methods. All values represent the mean of at least four independent experiments ± S.E. (*) Values significantly different to control value ($p<0.05$).

Spontaneous motor activity after administration of herbal extracts to rats

Administration of *Melissa officinalis* and *Rosmarinus officinalis* extracts to rats decreased the spontaneous motor activity as a dose-response manner (Figure 6). One-way ANOVA revealed significant effects of treatment on total motor activity after administration of *Melissa officinalis* $F(5,42)=19.24$ ($p<0.0001$)and *Rosmarinus officinalis* , $F(5,42)=17.85$ ($p<0.0001$). Subsequent Newman-Keuls test demonstrated that each extract induced a significant decrease in total motor activity at all doses tested, except for 6.25mg of *Melissa officinalis,* which was not different from saline solution (negative control). Nevertheless, the overall effects of the highest doses of *Melissa officinalis* (25 and 50mg/Kg) and *Rosmarinus officinalis* (12.5, 25 and 50mg/Kg) were similar to Diazepam 1mg/Kg ($p>0.05$) (Figure 6).

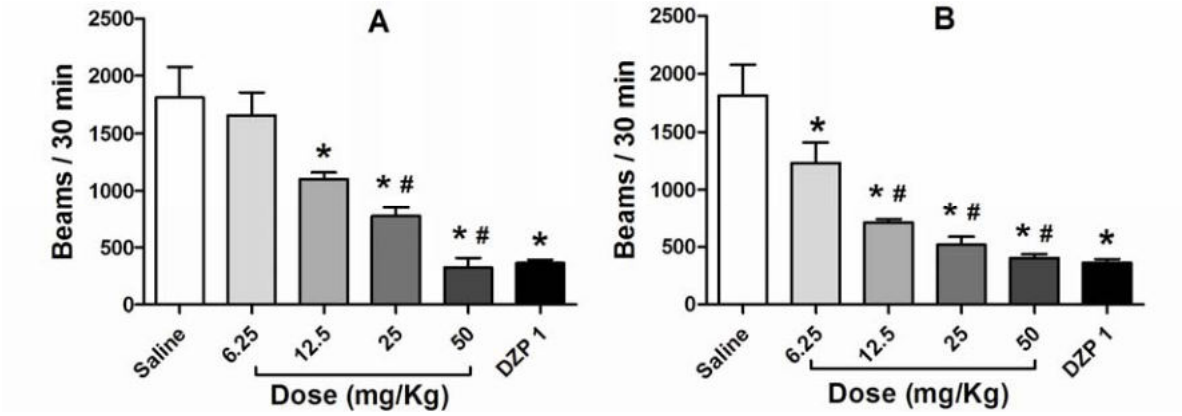

Figure 6. Spontaneous motor activity.

(A) Treatmentwith *Melissa officinalis* extract (B) Treatmentwith *Rosmarinus officinalis* extract. Saline: Animals treated with saline solution (negative control). DZP 1: Diazepam (1mg/Kg) (positive control). All values represent the mean obtained from behaviour

evaluation of eight animals ± SEM. (*) Values significantly different to negative control (saline) (p<0.05), (#) Values statistically equal to Diazepam (p>0.05); p values were obtained by ANOVA followed by Newman-Keuls's Multiple Comparison test.

Number of entries and time spent on open arms in the elevated plus-maze

Administration of Melissa officinalis and Rosmarinus officinalis extracts increased the number of entries and time spent on open arms in the elevated plus-maze. One-way ANOVA analysis revealed significant effects of Melissa officinalis extract F(5,42)=19.03 (p<0.0001), and Rosmarinus officinalis extract F(5,42)=17.31 (p<0.0001) on the percentage of entries into the open arms. (Figure 7). Newman-Keuls analysis indicated that the effect of treatment on the percentage of entries into the open arms was only significant on the highest dose tested of *Melissa officinalis* (50mg/Kg) (p<0.0001). *Rosmarinus officinalis* treatment in both higher doses (25 and 50 mg/kg) was significant respect to control saline, and the highest dose was similar to Diazepam (1 mg/kg).(Figure 7).

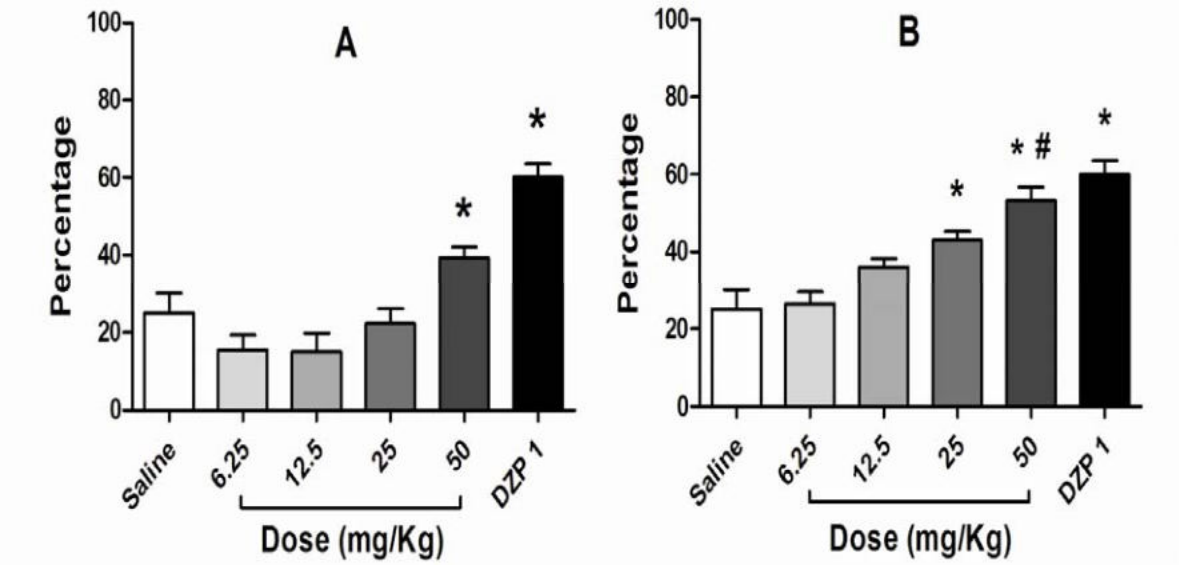

Figure 7. Percentage of entries into openarms.

(A) Treatmentwith *Melissa officinalis* extract; (B) Treatmentwith *Rosmarinus officinalis* extract. Saline: Animals treated with saline solution (negative control). DZP 1: Diazepam (1mg/Kg of animal weight) (positive control). All values represent the mean obtained from behaviour evaluation of eight animals ± SEM. (*) Values significantly different to negative control (saline) (p<0.05), (#) Values statistically equal to Diazepam (p>0.05); p values obtained by ANOVA followed by Newman-Keuls's Multiple Comparison test.

Furthermore, the time spent into the open arms after the administration of herbal extracts also increased. One-way ANOVA revealed a significant effect into the time spent on open arms after the administration of Melissa officinalis F(5,42)=17.64 (p<0.0001), and Rosmarinus officinalis F(5,42)=5.109 (p<0.005) (Figure 8). Subsequent Newman-Keuls analysis indicated that the effect of both extracts on the percentage of time spent into the enclosed arms was significant on the highest dose of both extracts tested (50mg/Kg) (p<0.05). However, no significant difference between other doses of Rosmarinus officinalis (12.5, 25 and 50mg/Kg) and Diazepam 1mg/Kg were observed (Figure 8).

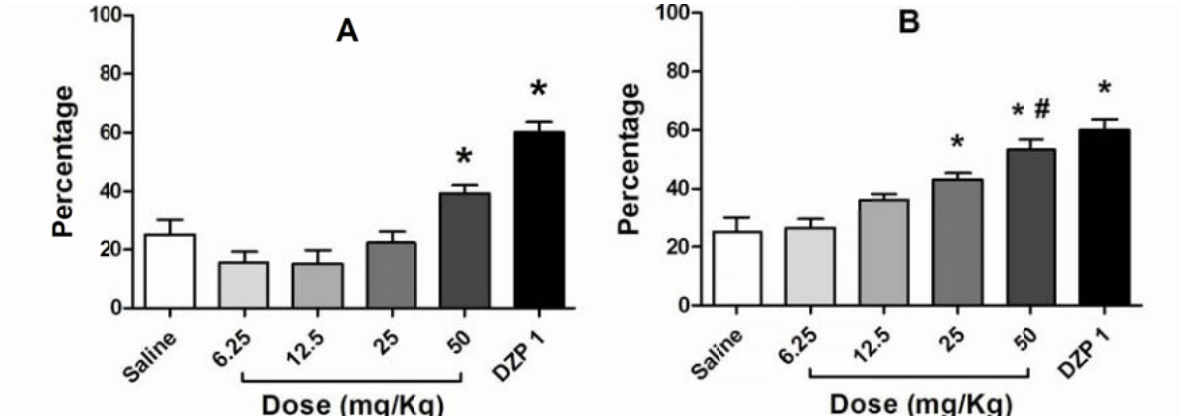

Figure 8. Percentage of time spent into open arms.

(A) Treatmentwith *Melissa officinalis* extract (B) Treatmentwith *Rosmarinus officinalis* extract. Saline: Animals treated with saline solution (negative control). DZP 1: Diazepam (1mg/Kg) (positive control). All values represent the mean obtained from behaviour evaluation of eight animals ± SEM. (*) Values significantly different to negative control (saline) ($p<0.05$), (#) Values statistically equal to Diazepam ($p>0.05$); p values obtained by ANOVA followed by Newman-Keuls's Multiple Comparison test.

Correlation between antioxidant capacity and anxiolytic effect of the herbal extracts

In order to correlate the antioxidant capacity of the herbal extracts and their anxiolytic effects, the ratio of the assayed parameters in the presence of both herbalextracts was calculated (Table 1).

Rosmarinus officinalis extract contained 4.3 times the concentration of polyphenols compared to *Melissa officinalis* extract. Likewise, the EC_{50} values obtained in the assays of prevention of oxidation of lipids and thiols of microsomal membranes were 6.4 and 2.8 times higher for *Melissa officinalis* compared to *Rosmarinus officinalis* extract.

Anxiolytic effect of the herbal extracts was measured through the elevated plus-maze. The increasing on the number of entries and time spent into the open arms of the maze are indicators of diminishing of anxiety in rat [25]. According to this, both parameters showed that *Rosmarinus officinalis* extract effect was 1.5 times higher than *Melissa officinalis*.

Table 1. Ratio of results obtained in presence of *Rosmarinus officinalis* and *Melissa officinalis.*

Assayed parameter	Rosmarinus officinalis Melissa officinalis
Concentration of polyphenols[1]	4.3
Antilipoperoxidative effect[2]	6.4
Prevention of oxidation of microsomal thiols[2]	2.8
Prevention of oxidation , in the presence of Cu^{2+}, of microsomal thiols[3]	2.0
Increasing in the number of entries into the open arms of the pluz-maze[4]	1.4
Increasing in the time spent into the open arms of the pluz-maze[4]	1.6

[1] Values were calculated from results.
[2] Values were calculated using EC_{50} obtained in the prevention of oxidation of lipids and thiols of microsomes, figure 1 and 2.
[3] Value obtained from Cu^{2+} chelation assays from figure 3.
[4] Values were calculated considering the maximum observed effect, obtained with the dose 50mg/Kg of each extract (Figure 7 and 8).

Discussion

Folk medicine has used different types of plants to treat various diseases [8]. Particularly, preparations of *Melissa officinalis* and *Rosmarinus officinalis* had shown to have psychotropic effects [11, 14], which implies the presence of lipophilic compounds capable of crossing the blood brain barrier. In regard, the standardized dried extracts of *Melissa officinalis* and *Rosmarinus officinalis* inhibited the N-demethylation of aminopyrine, reaction catalysed by Cytochrome P450 system. The feature of compounds metabolized by this oxidative system is its lipophilicity. Then, the inhibitory effect on N-demethylation of aminopyrine observed may be due to the presence of lipophilic compounds in the extracts (Figure 5)

The brain constitutes only 2% of total body weight; however, it utilizes 20% of total oxygen taken up by the body. In addition, brain tissues are rich in fatty acids and have deficient antioxidant mechanisms, therefore the brain is highly susceptible to damage generated by oxidative stress [27]. It has been reported in the literature that a large number of psychiatric and neurodegenerative diseases are associated with oxidative stress [1, 3, 7]. The dried extracts of *Melissa officinalis* and *Rosmarinus officinalis* inhibited the lipid peroxidation of rat liver microsomes, and prevented the oxidation of microsomal thiols indicating the presence of antioxidant compounds in the extracts (Figure 1). Regarding this, polyphenols, compounds recognized by their antioxidant activity are especially abundant in leaves. The mechanisms involved in the antioxidant activity developed by herbal extracts are diverse and not all of them can be tested *in vitro* assays, as for example induction of enzymes such as SOD, catalase and lipid peroxidase, Therefore, the true contribution of herbal preparations to cellular antioxidant capacity is only measurable *in vivo.*

In neurons of patients suffering of Alzheimer and Parkinson's diseases have been observed an accumulation of metallic ions [28]. These ions are able to induce the generation of reactive oxygen species and to bound to aminoacid residues containing oxygen, nitrogen and sulphur [17]. Both phenomena are involved in the toxicity of free metallic ions present in the cell [17, 28]. The standardized dried extracts of *Melissa officinalis* and *Rosmarinus officinalis* were able to chelate Cu^{+2} ions (Figure 4), which added to their antioxidant activity, reflected in the inhibition of oxidation of lipids and thiols compounds present in the microsomal membrane (Figure 1 and 2), ensures cell protective ability of these extracts.

Moreover, affective disorders, such as depression and anxiety, have been described to be associated with oxidative stress [29]. Melissa officinalis has been widely described as anxiolytic [11]; however, studies related to the psychotropic properties of Rosmarinus officinalis are scarce. Since these activities are associated to oxidative stress and both extracts showed different antioxidant activities, behavioural studies were conducted in order to measure their anxiolytic effect.

Anxiolytic effect was evaluated in Sprague Dawley rats through the elevated plus-maze test [24]. Increasing in the number of entries and time spent into the open arms of the maze was higher in rats administered with the Rosmarinus officinalis extract compared to those administered with Melissa officinalis (Figure 7 and 8). Moreover, this difference was also observed in the antioxidant activity. The anxiolytic effect exerted by both extract would be caused to the presence of active principles interacting directly with GABA Receptors. There is evidence indicating that certain compounds present in Melissa officin*alis* and *Rosmarinus officinalis* (e.g. rosmarinic acid) have an inhibitory effect on the activity of GABA-T [11], [13]. This effect should increase brain GABA availability, which decrease the anxiety of the patient.

Furthermore, both extracts diminished the spontaneous motor activity, measured as locomotor activity, rearing and grooming behaviours. Interestingly, this phenomenon was higher in those rats administered with *Melissa officinalis*. This result seems to indicate that *Melissa officinalis* has a more sedative effect than *Rosmarinus officinalis.* Benzodiazepines, used as classic anxiolytics, generate an anxiolytic effect coupled with a sedative effect because the inhibitory effect exerted in $GABA_A$ receptor. Recently, it was demonstrated that this sedative effect occurs specifically in the 1 subtype $GABA_A$ receptor [30]. According to this, anxiolytic and sedative effect exerted by both herbal extracts would be caused to the presence of compounds able to act on $GABA_A$ receptor.

On the other hand, classical treatments used in patients suffering of psychiatric and neurodegenerative diseases show moderate to severe adverse effects, which affect the quality of life of patients. The doses administrated in the behavioural studies did not show adverse effects, measured through changes in the clinical semiological parameters (data not shown).

These results allow proposing the development of new phytodrugs from standardized dried extracts of *Melissa officinalis* and *Rosmarinus officinalis*. Co-therapies using phytodrugs could help to decrease the doses of synthetic drugs currently in use, so reducing their adverse effects. New pharmacological studies are necessary however, to obtain an effective and safe phytodrug. These studies and the dose to provoke the desired effect are required before clinical trials begin.

Conclusions

We demonstrated that *Melissa officinalis* and *Rosmarinus officinalis* extracts presents an anxiolytic effect. This pharmacological effect seems to be caused by the presence of compounds able to act into GABAergic system, and to act through the antioxidant activity of these extracts.

List of abbreviations

ANOVA: Analysis of Variance
GR: glutathione reductase
GSH-Px: Glutathione peroxidase
GABA: γ-amino butyric acid
GABA-T: γ-amino butyric acid transaminase
$GABA_A$: γ-amino butyric acid receptor subtype A.
i.p.: intraperitoneal
MDA: malondialdehyde
OCD: obsessive-compulsive disorder
SOD: Superoxide dismutase

Authors' contributions

MELconceived of the study, and participated in its design and coordination and helped to draft the manuscript.
CGG carried out the behavioural studiesand helped to draft the manuscript
CLVB carried out the antioxidant assays.
GDV participated in the design of the behavioural study and performed the statistical analysisof it.

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