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Effects of chronic administration of *Melissa officinalis* L. extract on anxiety-like reactivity and on circadian and exploratory activities in mice

Alvin Ibarra [a], Nicolas Feuillere [b], Marc Roller [b], Edith Lesburgere [c], Daniel Beracochea [c,*]

[a] Naturex Inc, 375 Huyler St, South Hackensack, NJ 07606, USA
[b] Naturex SA, Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France
[c] CNIC-Centre de Neurosciences Intégratives et Cognitives, UMR CNRS 5228-Université de Bordeaux1 et 2, Avenue des Facultés 33405 Talence-cédex, France

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ABSTRACT

This study aimed to determine the effects of chronic (15 consecutive days of treatment) per os administration of *Melissa officinalis* L. extract (Cyracos®, Naturex) on anxiety-like reactivity in mice. As measured by HPLC, Cyracos® contains significant amounts of rosmarinic acid and the triterpenoids oleanolic acid and ursolic acid, which inhibit gamma-aminobutyric acid transaminase (GABA-T) activity and increase GABA levels in the brain (Awad et al., 2007; Awad et al., 2009).

Thus, we evaluated Cyracos® use in independent groups of C57BL/6 mice with regard to anxiety-like reactivity in an elevated plus maze and an open field task. We found that Cyracos® significantly reduced anxiety-like reactivity in the elevated plus maze dose-dependently, but no significant effect was observed in the open field task. Parallel experiments in independent groups of mice showed that the Cyracos® dose at which it exerted anxiolytic-like effects in the elevated plus maze did not alter exploratory or circadian activities. Therefore, our results demonstrate that Cyracos® has anxiolytic-like effects under moderate stress conditions and does not alter activity levels.

Introduction

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the human central nervous system. The GABAergic system represents a promising future target of new pharmacological strategies that treat anxiety (Domschke and Zwanzger, 2008).

Recent evidence suggests that *Melissa officinalis* L. extract, which contains rosmarinic acid and the triterpenoids oleanolic acid and ursolic acid, inhibits gamma-aminobutyric acid transaminase (GABA-T) activity (Awad et al., 2007; Awad et al., 2009). GABA-T inhibition increases the availability of GABA in the brain.

The GABAergic system has been widely demonstrated to regulate cognitive function (Menzies et al., 2007; Lewis et al., 2008) and emotional behavior (Radley et al., 2009; Thoeringer et al., 2009). Consequently, most of the anti-anxiety effects of benzodiazepines are due to increases in GABAergic neurotransmission (Beracochea, 2006 for review), and direct modulation of the GABAergic system reduces anxiety-like reactivity in rodents (Zhang and Cranney, 2008; Lalonde and Strazielle, 2009).

Due to the ability of *Melissa officinalis* L. to inhibit GABA-T activity (Awad et al., 2007; Awad et al., 2009) and increase the availability of GABA in the brain, we hypothetized that *Melissa officinalis* L. extract reduces anxiety-like reactivity under stressful situations. In this study, we tested this hypothesis by measuring anxiety-like reactivity in mice that were treated chronically with *Melissa officinalis* L. extract during exercises, such as the elevated plus maze and the open field task. In addition, we also determined the effects of the *Melissa officinalis* L. extract on circadian and exploratory activities.

Materials and methods

Extract preparation

Aerial parts of *Melissa officinalis* L. were collected from the Maine-et-Loire region, France. A voucher specimen was deposited at Naturex Italy (Reference: 1MLS11, Lot number: M080077; Via Galileo Ferraris, 44, 21042 Carono pertusella, VA, Italy). The plant material was authenticated using macroscopic, microscopic, and high-performance thin-layer chromatography techniques (Reich and Schibli, 2007).

The *Melissa officinalis* L. extract was generated through an industrial process at Naturex SA (Cyracos®, Reference: 503078,

* Corresponding author.
E-mail address: d.beracochea@cnic.u-bordeaux1.fr (D. Beracochea).

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Lot number: 064/76/A8; Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France), in which the aerial parts were mixed in a dynamic extractor with an ethanol:water (30:70) solution at a ratio of 1:8 for 2 h at 70 °C. This step was repeated twice.

After filtration, the 2 pools were combined, and the clarified solution was concentrated under a vacuum at 45 °C and spray-dried to obtain a fine powder. The moisture content in the powder extract was less than 8%. After extraction, the sample was analyzed for pesticide (USP-31-NF26 S1, 2008) and heavy metal (Method 993.14 AOAC, 2005) content at Covance Laboratories (Madison, WI, USA) for compliance.

Analysis of hydroxycinnamic acids

An HPLC method was developed to analyze rosmarinic acid and total hydroxycinnamic acid content in the *Melissa officinalis* L. extract. Total hydroxycinnamic acid derivatives were expressed as total rosmarinic acid.

In this procedure, the standard (rosmarinic acid 4957S, Extrasynthese, France) and the *Melissa officinalis* L. extract were dissolved in HPLC-grade water. The HPLC system that we used was an Agilent 1100, equipped with a UV detector at 330 nm. The stationary phase was a Zorbax® SB C_{18} column (5 µm, 4.6 mm ID x 250 mm) that was thermostated at 35 °C. The mobile phases were (A) acetonitrile (Sigma-Aldrich, USA) and (B) water with 0.01% H_3PO_4 with a rate flow of 0.7 ml/min. A solution of 5% A and 95% B was maintained for 10 min and switched to 30% A and 70% B 20 minutes later, followed by a step gradient to 100% A 5 minutes later. Then, the system was reequilibrated to the initial composition.

The peak of rosmarinic acid appeared at approximately 15.8 min in the chromatogram. The value for total hydroxycinnamic acids was determined by integrating all of the peaks from 8 min to 22 min (Carnat et al., 1998). Fig. 1A shows the chromatogram of hydroxycinnamic acid derivatives in the *Melissa officinalis* L. extract, which contained 9.32% rosmarinic acid and 16.62% total hydroxycinnamic acids.

Analysis of triterpenes

Oleanolic and ursolic acids were analyzed by HPLC. The standards, oleanolic acid (0041S, Extrasynthese, France) and ursolic acid (0037S, Extrasynthese, France), and the *Melissa officinalis* L. extract were dissolved in HPLC-grade acetone. The HPLC system was an Agilent 1100, equipped with a UV detector at 210 nm. The stationary phase was a Luna® C_{18} column (3 µm, 4.0 mm ID x 150 mm), thermostated at 30 °C. The mobile phases were (A) acetonitrile and (B) water with 0.1% H3PO4. Ninety percent A and 10% B were eluted under isocratic conditions.

Fig. 1B shows the chromatogram of triterpenes in the *Melissa officinalis* L. extract, which contained 0.17% oleanolic acid and 0.47% ursolic acid.

Behavioral studies

Animals

We used 6-week-old male C57 Bl/6 Jico mice from Iffa-Credo, Lyon (France). On arrival, mice were housed together in colony cages (40 cm long x 25 cm high x 20 cm wide), matched for weight, and placed in an animal room (ambient temperature, 22°C; automatic light cycle, on: 7 AM, off: 7 PM) with *ad libitum* access to food and water. They remained in collective cages for at least 16 weeks.

At least 2 weeks before treatment and behavioral testing began, mice were housed individually, with *ad libitum* access to food and water. In all experiments, the mice were at least 24 weeks old at the time of the behavioral tests.

Emotional reactivity

The elevated plus maze. The plus maze, which was composed of grey Plexiglas, consisted of 4 arms that were arranged in the shape of a cross. Each arm was 67 cm long, 7 cm wide, and 80 cm above the ground. The 4 arms were joined at the center by a 7-cm square platform. Two opposite arms of the plus maze were "closed" in by 24-cm-high sidewalls but open on the top, and the other arms did not have sidewalls; these walls did not extend the center of the maze.

At the beginning of each test, mice were placed in the center of the maze in a cylinder (8 cm in diameter, 17 cm high) for 30 sec. Then, the cylinder was removed, and mice were allowed to explore all arms of the maze freely for 6 min. Activity and latencies were measured using semiautomatic counters and timers. An entry was counted only when a mouse entered an arm with all 4 feet.

Two measures of "anxiety" were assessed: the ratio of time in the open arms divided by the total time in all arms of the maze (time ratio), and the ratio of entries into open arms divided by the total number of entries into all arms (ratio entries). Smaller ratios reflect greater anxiety in a mouse (Pellow and File, 1986; Beracochea and Krazem, 1991; Krazem et al., 2001).

The open field task. A circular open field chamber was constructed from Plexiglas, measuring 100 cm in diameter and surrounded by a 15-cm-high wall. The floor was composed of white Plexiglas. A camera that was above the apparatus was connected to a computer screen, which divided the apparatus floor into 64 zones. Two lamps, 2 m above the apparatus, provided 600 lux of illumination, distributed equally over the entire surface of the apparatus.

At the start of each trial, animals were placed in the center of the apparatus in a small cylinder to orient them randomly. Following a 30-sec delay, the cylinder was removed, and the subjects were allowed to freely explore the apparatus for 10 min. Two measures were recorded: the initial time to reach to the periphery from the center of the apparatus, and the total number of zones that were crossed by the subjects. These measures are influenced benzodiazepine administration (Krazem et al., 2001).

Activity

The light/dark activity meter system. (Imetronic, France). A metal rack was built (1.80 m high, 1 m wide, 0.6 m deep) with 8 boxes. Each box was composed of a sliding floor, a detachable cage, and infrared captors and equipped with a water bottle and a food trail at the front of the box.

The rack was connected to an electronic interface to communicate with a computer. The software managed the 8 boxes and stored data on time (1 sec < time unit < x hours), duration (from 1 minute to many days), and locomotor activity. Further, the time that the box was lit could be scheduled. In our experiment, animals were placed in the box for 24 hours, which was lit from 7 AM to 7 PM.

The four-hole board. The 4-hole board apparatus was used to measure the exploratory activity of mice during a 6-minute period. The 4-hole board apparatus was enclosed by grey Plexiglas walls (45 x 45 x 30 cm high). The floor of the board was also composed of grey Plexiglas. On the floor, 4 holes (3 cm in diameter, 2.5 cm deep) were located in each corner 6 cm away from the sidewalls. The apparatus was placed in a room that was exposed to 10-dB background noise, and a light was centered over the apparatus, providing 40 lux of intensity. The apparatus was cleaned with 95% ethanol and water before each behavioral test.

Photocells, placed in each hole, were used to measure the number of head-dips in each hole and the total number of head-dips in the 4 holes. At the beginning of the test, mice were placed

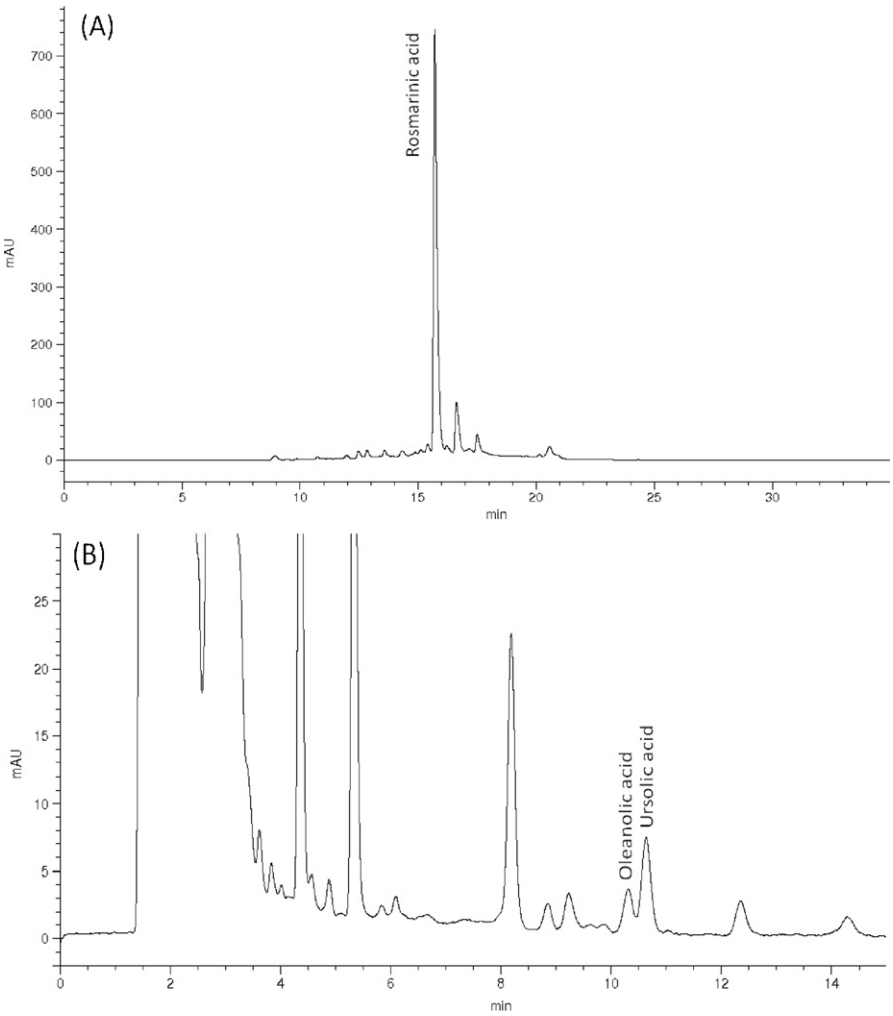

Fig. 1. (A) Chromatogram of hydroxycinnamic acids and (B) triterpenoids in *Melissa officinalis* L. extract (Cyracos®, Naturex).

in a small cylinder (16 cm in diameter, 13 cm high) for 10 sec, which was removed to orient the mouse randomly in the apparatus.

Experimental schedule

All experiments were performed using independent groups of mice. Except for the light/dark activity meter recordings, which lasted 24 hours, all behavioral testing took place from 8 AM to 1 PM. In all experiments, animals were not deprived of food.

Behavioral studies were performed in 2 phases. In the first phase, 3 doses of Cyracos® were administered (120 mg/kg, 240 mg/kg, and 360 mg/kg) for the elevated plus maze and the light/dark activity meter apparatus task. In a second phase, according to the results of the first phase, 2 doses of Cyracos® were studied (240 mg/kg and 360 mg/kg) in the elevated plus maze and open field task, as well as for exploratory behavior in the 4-hole board.

Cyracos® administration

Cyracos® was dissolved in water and administered per os on 15 consecutive days. Each dose of Cyracos® was prepared fresh daily. The doses were 120 mg/kg, 240 mg/kg, and 360 mg/kg. Four days before the start of the treatment, mice received water per os daily to become accustomed to the experimenter and the per os gavage procedure. Control mice received water on an identical schedule. The last per os Cyracos® dose (15th day) was delivered 1 hour before behavioral testing began.

Statistical analysis

Statistical analyses were performed using Statview® software. Behavioral data were analyzed primarily with one-way factorial analysis of variance (ANOVA) using "Doses" as the factor, followed by post hoc Dunnett test. Results were considered significant when the "p" value was less than 0.05.

Results

Behavior phase 1

Elevated plus maze

The activity ratio (Fig. 2A) differed significantly between the groups ($F(3,28) = 9.4$; $p = 0.0002$), wherein compared with vehicle (0.37 ± 0.02), Cyracos® increased the activity ratio at the 240-mg/kg (0.43 ± 0.012; $p = 0.03$) and 360-mg/kg doses (0.47 ± 0.018; $p = 0.004$); in contrast, the lower dose (120 mg/kg) had no significant effect (0.35 ± 0.017; ns). Latency ratios were statistically similar between the groups (Fig. 2B) ($F(3,28) = 2.7$; $p = 0.06$), although the 240-mg/kg (0.36 ± 0.027) and 360-mg/kg groups (0.36 ± 0.019) induced a small increase in the ratio

compared with vehicle (0.28 ± 0.028). The lower Cyracos® dose (120 mg/kg) had no significant effect (0.29 ± 0.027).

Total activity differed significantly between the groups ($F(3,28)=3.69$; $p=0.02$), due primarily to the higher (360 mg/kg) Cyracos® dose (30.0 ± 1.54; $p < 0.05$ compared with vehicle: 24.0 ± 1.03); the other doses of Cyracos® did not elicit any significant effects (120 mg/kg, 25.3 ± 1.34; 240 mg/kg, 27.0 ± 1.38).

The total times that were spent visiting the arms of the maze were similar between groups ($F(3,28) < 1.0$) (120 mg/kg: 189.7 ± 10.21 sec; 240 mg/kg: 203.6 ± 12.8 sec; 360 mg/kg: 191.5 ± 9.2 sec; vehicle: 195.6 ± 11.0 sec).

Light/Dark activity

The total amount of activity over 24 hours did not differ significantly between the groups (Fig. 3A) ($F(3,28) < 1.0$). Specifically, the Cyracos® groups, at 120 mg/kg (n=8; 434.8 ± 54.9), 240 mg/kg (437.0 ± 64.2), and 360 mg/kg (434.5 ± 119.5), experienced comparable total activities relative to vehicle (598.1 ± 93.1; ns for all comparisons). Moreover, no significant difference was observed during the 12-hour light phase (Fig. 3B) ($F(3,28) < 1.0$) or the 12-hour dark phase of the cycle (Fig. 3C) ($F(3,28) < 1.0$).

Behavior Phase 2.

Open field. No significant difference was observed between groups with regard to the time that was spent crossing the maze from the center to the peripheral wall (Fig. 4A) ($F(2,27)=2.16$; $p=0.13$). The 240-mg/kg (N=10; 58.1 ± 3.7 sec) and 360-mg/kg (N=10; 53.2 ± 1.8 sec) Cyracos® groups did not differ significantly from vehicle (N=10; 63.7 ± 4.56 sec; ns for both comparisons). Moreover, the number of crossed zones (Fig. 4B) was similar between the Cyracos® groups ($F(2,27)=1.8$; $p=0.18$) at 240 mg/kg

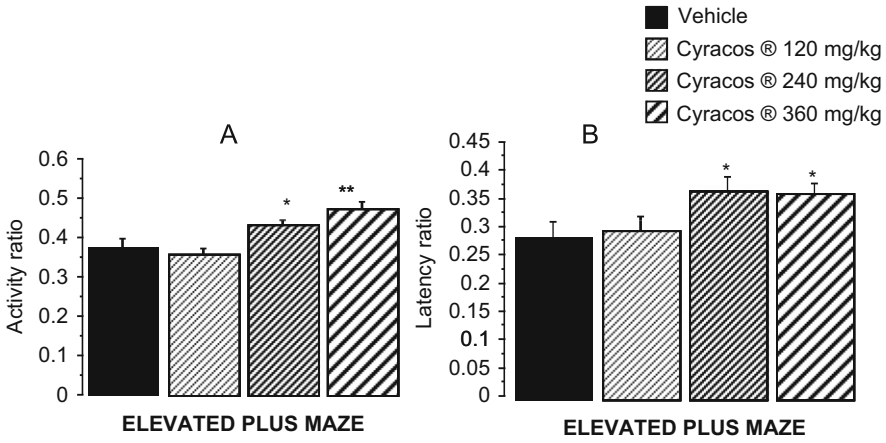

Fig. 2. Effects of Cyracos® on activity ratio (A) and latency ratio (B) in the elevated plus maze of phase 1. Cyracos® dose-dependently increased both ratio. * and ** denote $p < 0.05$ and 0.01, respectively, compared with vehicle.

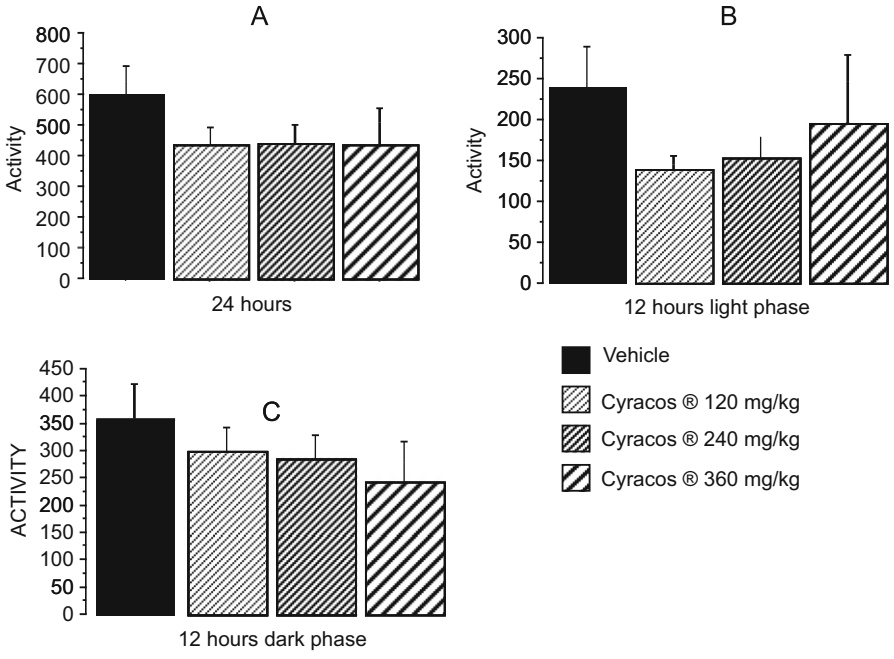

Fig. 3. Effects of Cyracos® on circadian activity over 24 hrs (A) or during the 12-hr light phase (B) or the 12-h dark phase (C) in the light/dark activity meter system. Cyracos® produced no significant changes in activity levels compared with vehicle.

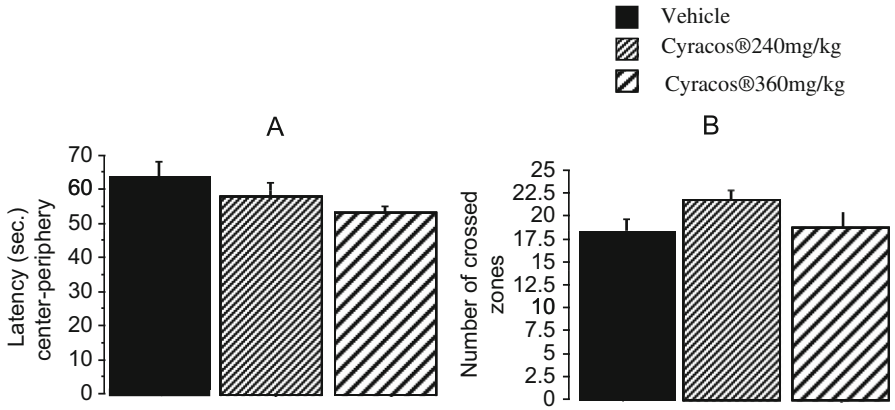

Fig. 4. Effects of Cyracos® on the latency during movement from the center to the periphery (A) and on the number of zone crossed (B) in the open field. Cyracos® produced no significant changes in performance compared with vehicle.

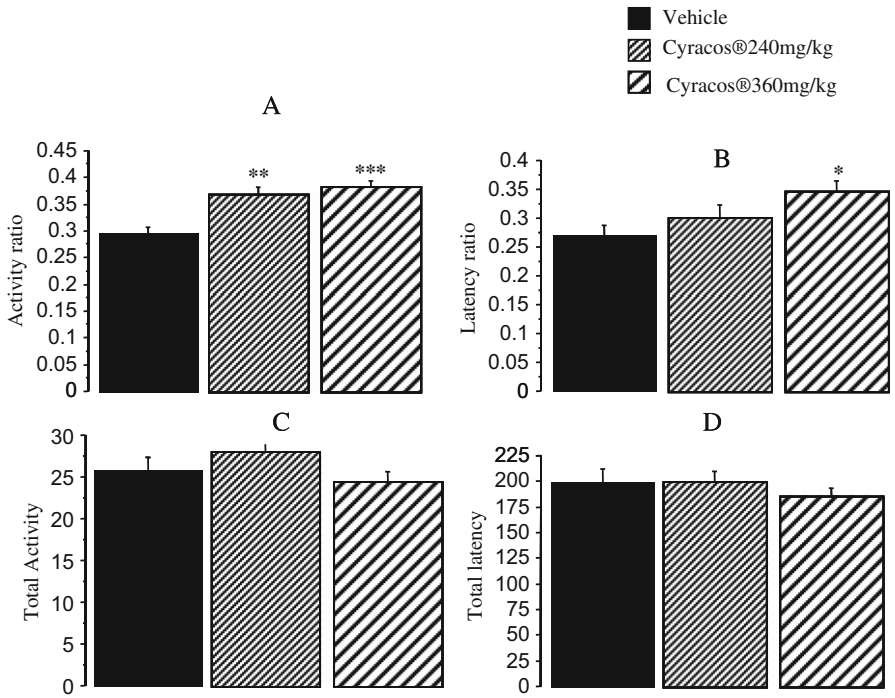

Fig. 5. Effects of Cyracos® on activity ratio (A) and latency ratio (B) in the elevated plus maze of phase 2. Cyracos® dose-dependently increased both ratios. *: $p < 0.05$: **: $p < 0.01$; ***: $p < 0.001$, respectively, compared with vehicle. No significant differences were observed compared with vehicle in the total number of entries into the 4 arms of the maze (C) or in the total time spent in these 4 arms (D).

(21.7 ± 3.7) and 360 mg/kg (18.8 ± 1.6) and vehicle (18.4 ± 1.2; ns in both comparisons).

Elevated plus maze. The activity ratio (Fig. 5A) differed significantly between groups ($F(2,27) = 9.4$; $p = 0.0002$). Specifically, compared with vehicle (n=10; 0.29 ± 0.12), Cyracos® increased the activity ratio at 240 mg/kg (0.36 ± 0.014; $p = 0.0004$) and 360 mg/kg (N=10; 0.38 ± 0.012; $p = 0.001$).

Notably, the between-group difference in latency ratio reached statistical significance (Fig. 5B) ($F(2,27) = 2.7$; $p = 0.04$). The chief difference was observed at the higher dose (360 mg/kg; 0.34 ± 0.20; $p < 0.05$ compared with vehicle: N=10: 0.27 ± 0.016), whereas the 240-mg/kg dose (0.29 ± 0.024) did not differ from vehicle (ns).

The total activity (Fig. 5C) and total time that was spent visiting the arms (Fig. 5D) were not significantly different between the groups ($F < 1.0$ in all analysis).

Exploratory activity in the four-hole board. The total number of head-dips (Fig. 6A) did not differ significantly between groups ($F(2,27) < 1.0$). Compared with vehicle (n=10; 17.7 ± 0.5), Cyracos® induced no significant change at the 240-mg/kg (17.6 ± 1.6; ns) or 360-mg/kg dose (N=10; 16.0 ± 1.1; ns).

The total time that was spent head-dipping (Fig. 6B) was similar between the groups ($F(2,27) = 2.59$; $p = 0.09$); compared with vehicle (n=10; 5.3 ± 0.4 sec), Cyracos® had no significant effect at the 240-mg/kg (4.7 ± 0.7 sec; ns) or 360-mg/kg dose (N=10; 3.7 ± 0.3 sec; ns).

Discussion

Two mechanisms by which *Melissa officinalis* L. extract acts have been postulated: through its 1. cholinergic properties, as measured by acetylcholinesterase (AChE) inhibition and choliner-

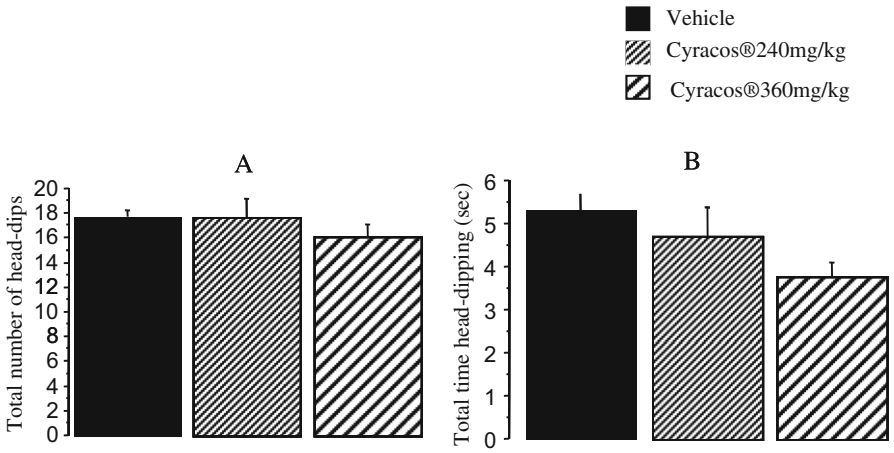

Fig. 6. Effects of Cyracos® on exploratory activity in the hole-board. No significant differences were observed in Cyracos®-treated mice compared with vehicle in the total number of head-dips (A) or total time spent head-dipping (B).

gic receptor-binding capacity (nicotinic and muscarinic receptors), and 2. GABAergic properties, through inhibition of gamma-aminobutyric acid transaminase (GABA-T).

Damage to the cholinergic system in the brain has been proposed to effect the memory deficits that are associated with Alzheimer disease (Parihar and Hemnani, 2004). Kennedy et al. (Kennedy et al., 2003) did not find any inhibition of AChE that was responsible for acetylcholine reductions in the human brain. In other tests that evaluated cholinergic receptor-binding activity, they found negligible nicotinic- and low muscarinic-binding. Moreover, in a separate human study, the same group found that 600 mg of Melissa extract increased calmness and reduced alertness (Kennedy et al., 2004). This pattern of mood modulation led them to propose that the GABAergic system is a mechanism by which *Melissa officinalis* L. exerts its anxiolytic effects.

Gamma-aminobutyric acid (GABA) is the chief inhibitory neurotransmitter in the human central nervous system. It has been suggested that the GABAergic system represents a promising target for new pharmacological strategies for the treatment of anxiety (Domschk and Zwanzge, 2008). In a preliminary screen of 10 anxiolytic commercial plant extracts, Awad et al. (Awad et al., 2007) found that aqueous extract of *Melissa officinalis* L. exhibited the greatest inhibition of GABA transaminase (GABA-T) activity (IC50 = 0.35 mg/ml). The inhibition of GABA-T increases the available amount of GABA in the brain. In a further study, the same group isolated and identified rosmarinic acid and the triterpenoids ursolic acid and oleanolic acid as potent *in vitro* inhibitors of GABA-T (Awad et al., 2009). In *in vitro* assays, rosmarinic acid inhibited GABA-T by 40% at 100 ug/ml, oleanolic acid inhibited GABA-T by 20% at 10 ug/ml, and ursolic acid inhibited GABA-T by 20% at 100 ug/ml. They used standards, not compounds isolated from the *Melissa officinalis* L. extract. The authors proposed conducting animal studies to confirm the GABA-T inhibitory capacity of *Melissa officinalis* L. extract and isolated compounds.

Because the increase in GABAergic neurotransmission was associated with reduced anxiety, the behavioral study that has been described in this report was aimed at determining the anti-anxiety effects of *Melissa officinalis* L. extract. We found that Cyracos® produced no changes in performance in light/dark activity compared with vehicle, as measured over 24 hours, exploratory activity in the hole-board, or the open field task. In contrast, 240 mg/kg and 360 mg/kg Cyracos®, but not 120 mg/kg, decreased activity and latency ratios during the elevated plus maze. Notably, the results for the elevated plus maze were observed in 2 independent experiments, demonstrating that this effect is robust, even though the decrease in activity ratio was more obvious in both experiments compared with the latency ratio.

The anti-anxiety effects of *Melissa officinalis* L. extract in the elevated plus maze were comparable with those following the administration of benzodiazepines in this task – ie, activity and latency ratios increased compared with vehicle (Beracochea et al.; Krazem et al.). A similar effect was observed after administration of tiagabine (a selective GABA transporter-1 inhibitor) (Thoeringer et al., 2009).

In contrast, no behavioral difference was observed in the open field test, another task that evaluates the anti-anxiety effects of compounds. The disparate effects of *Melissa officinalis* L. extract in the anxiety tasks might be linked to the difference in anxiety that is experienced in the 2 tests. The open field task is more stressful compared with the elevated plus maze task. To this end, we measured plasma corticosterone concentrations in independent groups of C57 mice in preliminary experiments, after which they were subjected to the open field or elevated plus maze task; we found that plasma corticosterone levels increased significantly after the open field task compared with the elevated plus maze (Pierard and Beracochea, 2009, unpublished results).

Based on these findings, the anti-anxiety effects of *Melissa officinalis* L. extract might depend on relative plasma and brain concentrations of corticosterone during the 2 tests. In support of this model, some studies have observed interactions between the GABAergic system and the hypothalamo-pituitary-adenocortical (HPA) axis (Calogero et al., 1988; Herman et al., 2004; Thoeringer et al., 2009). Thus, whereas a small increase in GABAergic activity that results from chronic *Melissa officinalis* L. extract intake might be sufficient to significantly reduce moderate anxiety levels, it could be inefficacious during more stressful situations, depending on relative stress-induced HPA responses.

Regardless of the biological mechanisms that sustain the anti-anxiety effects of the *Melissa officinalis* L. extract, which remain to be further determined, the sparing of exploratory activity in the hole-board task and of circadian activity in the light/dark system demonstrates that the anti-anxiety activity of chronic *Melissa officinalis* L. extract intake during the elevated plus maze does not depend on general exploratory or circadian functions.

In conclusion, our study shows that Cyracos® has anti-anxiety effects in the elevated plus maze, which might be linked to the inhibitory activity of Cyracos® and its components on GABA-T,

resulting in an increase in the availability of GABA in the brain. These effects were observed in the absence of any impairment in activity.

Authors' disclosure statement

Naturex is involved in the research/development and marketing/sales of Cyracos® as an ingredient for the food, nutraceutical, and cosmetic industries. Therefore, Naturex has a commercial interest in this publication. The Centre de Neurosciences Intégratives et Cognitives (CNIC), the conducting laboratory, was paid by Naturex to perform and report the scientific work, which formed the basis of this publication. CNIC and Naturex declare that the data in this publication represent a true and faithful representation of the work that was performed.

Acknowledgement

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