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Full Length Research Paper

Anxiolytic and sedative effects of essential oil from *Casimiroa pringlei* on Wistar rats

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The genus *Casimiroa* (Rutaceae) is native from Mexico and Central America, and *Casimiroa pringlei* (S. Watson) Engl. has been employed since prehispanic times in infusions and macerations to induce sleep and as an anxiolytic. Considering the folk use of *C. pringlei*, the purpose of the study was to evaluate the behavioral effects of essential oil from *C. pringlei* (EO*Cp*) on an animal model. After EO*Cp* extraction, two doses were orally administered to groups of Wistar rats, and their effects were compared with those produced by a conventional anxiolytic (bromazepam, 0.023 mg/kg) and by an anxiogenic compound (caffeine, 20 mg/kg). In the Irwin test, EO*Cp* (795 mg/kg) induced similar behavioral, sensory and motor effects to those induced by bromazepam, and significantly different from the effects of caffeine administration. While in the elevated plus-maze EO*Cp* increased exploration of the open arms without decreasing spontaneous activity in the open field, a higher dose (1000 mg/kg) significantly reduced open field activity. The hole-board test confirmed a significant anxiolytic effect of EO*Cp*. These findings demonstrate the sedative and anxiolytic properties of *C. pringlei* in rats, and they represent the first evaluation of its traditional use as a medication.

Key words: Anxiolytics, *Casimiroa pringlei*, sedatives.

INTRODUCTION

Among the great diversity of Mexican flora, more than 4000 species are considered as medicinal plants, which are routinely or sporadically employed by more than 90 % of the Mexican population to alleviate different diseases (Aguilar et al., 1994; Argueta et al., 1994). *Casimiroa edulis* Llave and Lex. (Rutaceae), known as "zapote blanco", is an example of a medicinal plant that has been employed since prehispanic times throughout the colonial period to induce sleep and as an anxiolytic (Lozoya and Enriquez, 1981). The genus *Casimiroa* is native to Mexico and Central America, where it grows in subtropical and temperate climates (Pennington and Sarukhan, 1998). Ten species constitute this genus, and most of them both wild and cultivated, are found in Mexico (Vera-Caletti and Vibrans 2002). Recently, it has been reported that the ethanol and water-extracts from bark and leaves of *C. edulis* contain active compounds able to produce hypotensive and anticonvulsant effects in rats and guinea pigs (Magos et al., 1999; Garzon et al., 1999). Although approximately 25 compounds have been already isolated from *C. edulis* extracts, there are no reports about the biological effect of any of them (Lozoya and Enriquez, 1981; Navarro et al., 1995).

Concerning the folk use of the genus *Casimiroa* to treat anxiety, its evaluation would provide valuable information to suggest alternatives to conventional treatments. Benzodiazepines, the drugs most frequently prescribed to treat anxiety, can be associated with undesirable side effects such as tolerance and dependency. According to Akhondzadh et al. (2001), 25 - 30% of the patients fail to respond to the treatments. In this respect, it has been demonstrated that the sedative and anticonvulsant properties of *Matricaria recutita* L-(camomille), *Passiflora*

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incarnata L. (passion flower), *Piper methysticum* Forst *(*kava) *and Valeriana officinalis* L. (valerian) arise from their interaction with the GABAergic system (Wasowski et al., 1995; Zanoli, 2000; Parra et al., 2002), the main source of inhibition in the central nervous system (CNS). Since *Casimiroa* displays similar anxiolytic and sedative properties*,* there is a great interest to investigate the behavioral effects, and the CNS targets of the compounds contained in this medicinal plant.

Besides *C. edulis* other species of *Casimiroa* are also employed in traditional medicine displaying similar sedative properties. They have been employed in Mexican folk medicine, as infusions and macerations, in medicinal baths against anxiety and insomnia (Martínez, 1967; Standley, 1924). One of these species is *C. pringlei,* a small tree that can reach up to 4 m height and it is widely distributed along the states of central Mexico (Standley, 1924; Pennington and Sarukhan, 1998). Therefore, the purpose of this study was to evaluate the anxiolytic and sedative properties of *Casimiroa pringlei* on an animal model. For that purpose, essential oil obtained from *C. pringlei* was administered to adult male rats per oral route. Bromazepam was employed as positive control for anxiolytic effects, while caffeine was used as a reference point for anxiogenic effects.

MATERIALS AND METHODS

Animals

Male Wistar rats (300 - 350 g) were installed in an appropriate room with inverted light-dark cycle (lights on at 1900 h, lights off at 0700 h), food and water *ad libitum* and mean temperature 28°C. All experiments were performed in the dark phase (between 0900 and 1600 h) and according to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (2003). In order to assess the non-toxic doses for the rats, we carried on the LD_{50} studies in male Balb C mice (30 - 40 g) (Guidelines for testing of chemicals, 2000), which were treated under the same approved guidelines and housed separately in the same animal facilities. The reason to employ mice in this protocol was the lower amount of essential oil required for this species, along the full range of doses, due to its lower size and weight.

Plant collection

Approximately 80 kg of *C. pringlei* young branches were collected from Guadalcázar, San Luis Potosí, Mexico, from July to December 2005. One specimen was registered and stored as reference at the herbarium SLPM (San Luis Potosí, México)"Isidro Palacios" from Instituto de Investigación de Zonas Desérticas de la UASLP (Universidad Autónoma de San Luis Potosí) (specimen number 37573). Leaves were detached from the branches and stored at 4°C in dark polyethylene bags until the extraction procedures.

Extraction of essential oil

Leaves of *C. pringlei* were triturated in a common blender and placed in boiling water (ratio 4 L water/500 g leaves); the essential oil was obtained by means of steam distillation, followed by extraction with a mixture of ethyl ether: water (ratio 6.7:1). The extract was vacuum evaporated, dehydrated with sodium sulfate and stored in the dark in a tightly closed bottle until used in the assays with animals (Ponce-Monter et al., 2008). The obtained amount of essential oil (EO*Cp*) was 0.4 ml /kg of leaves (0.04%).

Drugs

Bromazepam (LEXOTAN®, Roche, México) was administered as a standard anxiolytic compound, while caffeine (MERCK®, Darmstadt) was used as an anxiogenic reference point. In both cases, the vehicle was distilled water and administration was orally by means of an intragastric intubation needle. In order to avoid the use of a non polar vehicle for *C. pringlei* essential oil, this was administered alone. The maximum dosage for a treatment was 0.5 ml.

Acute toxicity study

A total of 24 mice were randomly assigned to eight groups or treatments, with three animals each. They were dosed with either vehicle (corn oil) or EO*Cp* at logarithmic doses 316, 500, 795, 1000, 2000, 3000 and 5000 mg/kg. The animals were returned to their home cages and observed 30 min, 1, 2, 3, 4 and 24 h after administration.

Irwin test

This test was employed to obtain a semi-quantitative assessment of the behavioral, neurological, autonomic and toxic effects of EO*Cp*. The animals were evaluated 30 min, 1, 2, 6 and 24 h after the treatment administration. The test was evaluated according to Irwin (1962) in blind fashion, employing three groups of seven rats each, the group that received the essential oil (795 mg/kg) plus the two reference groups, namely bromazepam and caffeine.

Elevated plus-maze test

In order to test the anxiolytic and sedative properties of EO*Cp*, the animals were tested in an elevated maze (Lister 1987; Yilmazer-Hande et al., 2003). The maze consist of two open arms (60 x 10 cm) facing each other, and two closed arms (60 x 10 x 24 cm walls), placed perpendicularly to the open arms. This apparatus was fabricated in acrylic; the arms originated in a central platform and it was placed at 65 cm height over a dark surface (Kalueff, 2004). Alter placing the animal in the center of the maze the following parameters were recorded by an observer, unaware of the treatment received by each animal, during a 5 min test period: number of entries into any of the arms and total time spent in either open or closed arms. Entry into an arm was considered only when the animal placed the four paws inside the arm. After each test, the maze was thoroughly cleaned with diluted ethanol (Carvalho-Netto and Souza, 2004).

Open field test

Following the elevated plus-maze test, the animals were transferred to the open field apparatus and examined during 5 min, in order to contrast the spontaneous activity of the animals with their behavior in the maze. This apparatus consisted on one squared meter area with 35 cm height walls, divided in 25 identical squares (Pellow et al., 1985; Carvalo-Freitas and Costa, 2002; Molina-Hernández et al., 2004). The same observer recorded the number of squares crossed, number of rearings and number of times that the animals

displayed grooming behavior. A square crossed was counted when the animal placed the four paws into the delimited area.

Hole- board test

The animals were subjected to the hole-board test during 5 min. The evaluations were performed in two sets of independent groups. In one set of four groups (caffeine, bromazepam, and 795 or 1000mg/k of EO*Cp*) the test was performed before the administration of the treatments and 60 min later. In the second set, the testing was done before and 120 min after administration of the drugs. This apparatus consisted of a 72 x 72 cm plastic square with 28 cm height walls. The floor had 16 evenly spaced holes (4 and 3 cm of diameter and depth, respectively). The animal was placed in the center of the square and allowed to explore the area by dipping its head into the holes. By introducing its head in the hole, the two eyes of the rat disappeared from the view of the observer for several seconds. Each time the animal performed such an exploration, the observer registered the event and its duration (Takeda et al., 1998).

Experimental design and statistical analyses

Most of the data were categorical, and few of the continuous variables showed normal distribution and variance homogeneity, according to Shapiro Wilk and Levene tests, respectively (Bland, 2000). The comparisons among experimental groups were then performed by nonparametric analysis through Kruskal-Wallis test followed by a two-tailed Mann Whitney test (Bland, 2000) for multiple comparisons. The significance level for the Kruskal-Wallis test was $P < 0.05$, while for the *pos hoc* Mann Whitney test the Bonferroni correction was applied, namely, this level was considered as a fraction of 0.05, depending on the number of comparisons made against the anxiogenic control (Sankoh et al., 1997; Bland, 2000). For the Irwin test, the significance level was $P < 0.025$ (two comparisons), for the elevated plus-maze, open field and hole-board tests, $p < 0.017$ (three comparisons).

Tests for the reference point drugs

In order to obtain positive controls for both anxiolytic and anxiogenic reactions to the administration of EO*Cp*, dose-response curves for oral caffeine and bromazepam were tested in the elevated plus-maze and in the open field apparatus. The data for the elevated plus-maze are reported as percentage of time spent in the open arms. Thus, a control animal would prefer the closed arms, which results in a minor time spent in the open arms, while the administration of an anxiolytic would decrease that preference and even reverse the natural avoidance of open spaces (Lister 1987). By contrast, the anxiogenic effect is reflected in more time spent in the closed arms. Experiments were performed in groups of seven animals that were evaluated at 30, 60, 120, and 180 min after administration of the drugs. Control animals were treated with distilled water.

Caffeine

Based on the literature about caffeine effects on rats, the doses tested were 10, 20, 40 and 80 mg/kg (Bhattacharya et al., 1997; Silva and Frussa-Filho, 2000; Montenegro et al., 2005).

Bromazepam

According to literaturee reports (Bourin et al., 1 992; Silva and Frussa-Filho, 2000; Montenegro et al., 2005; Puga et al., 2005; Schuck et al., 1998), 0.023, 0.049 and 0.092 mg/kg were administered to groups of seven rats.

RESULTS

Acute toxicity study

At doses of EO*Cp* over 2000 mg/kg the animals showed a general depression of CNS functions, ranging from important decrease of locomotive activity to death. At lower doses, the animals displayed only passivity and somnolence as compared to controls. The LD_{50} for oral administration of EO*Cp* was 2433 mg/kg, calculated with the method of Litchfield and Wilcoxon (1949). This value indicates that the EO*Cp* is moderately toxic (Franck, 1992). Based on this study, the dose of 795 mg/kg was selected for the Irwin test, and a further dose of 1000 mg/kg was added for the evaluation of anxiolytic and sedative effects of EO*Cp* on behavioral tasks.

General effects of EOCp

Behavioral, sensory and motor effects of EO*Cp* were similar to those induced by bromazepan, and significantly different from the effects observed under caffeine administration. Table 1 summarizes the significant changes observed in this experiment at 60 and 120 min after administration of the drugs. Sensory responses were attenuated in presence of EO*Cp*, as well as spontaneous activity and alertness. No signs of CNS excitation such as tremors, convulsions, stereotypical movements or exophtalmos were observed under any treatment, neither autonomic responses such as piloerection or sialorrhea. Besides the decrease reported in grip strength and body tone, motor parameters such as righting reflex, posture and limb orientation, among others explored in the Irwin test, were unaltered. For both CNS depressants, bromazepam and EO*Cp*, behavioral, sensory and motor responses remained attenuated 6 hours after administration of the drugs. By contrast, all animals displayed normal scores in all parameters evaluated 24 h after administration of the drugs.

Tests for the reference point drugs

Caffeine: The administration of caffeine significantly reduced the preference for the open arms at 60 min ($H (3 N = 27) = 10.3$, $P < 0.05$), 120 min ($H (3 N = 27) = 15.2$, $P < 0.05$) and 180 min ($H (3 N = 28) 9.4$, $P < 0.05$; Figure 1A). This effect was significant for the 20 mg/kg dose at 120 and 180 min ($P = 0.006$) while in the open field this dose was associated with a significant increment of spontaneous activity only during 30 min after administration, while higher doses (40 and 80 mg/kg) induced a decrease of locomotor activity, during 2 and 3 h after administration of the drug (Figure 1B).

Table 1. Irwin test parameters where EOCp elicited different responses to those associated with an ansiogenic drug.

Profile	Parameters	Treatments and time after administration					
		795 mg/kg	Bromazepam 0.023 mg/kg	Caffeine 20 mg/kg	795 mg/kg	Bromazepam 0.023 mg/kg	Caffeine 20 mg/kg
		60 min			120 min		
		(median and interquartile range)					
Behavioral	Spontaneus activity	3.7 2.6-4.7	1.0-3.3	6.5 5.2-7.8*	3.2 2.1-4.4	2.0 0.5-0.8	6.5 5.2-7.9*
	Arousal	2.5 - 4.6	2.6 1.5-3.7	6.4 5.2-7.5*	3.6 2.6-4.5	2.0 1.0-2.9	6.6 5.6-7.5*
Sensory	Touch	2.9 1.4-4.2	3.0 1.4-4.5	6.4 5.1-7.7*	2.9 1.7-4.0	2.9 1.7-4.0	5.7 4.6-6.7*
	Toe-pinch	4.0 3.2-4.8	3.8 2.9-4.7	5.6 4.5-6.7	3.6 2.6-4.5	3.1 2.2-4.0	5.6 4.6-6.5*
	Pinna	3.1 2.3-4.0	2.6 1.7-3.6	5.2 4.2-6.3*	2.0 0.8-3.2	2.2 1.0-3.5	5.0 3.4-6.5*
	Noise	2.7 2.1-3.3	2.3 1.7-2.9	5.6 4.2-6.9	2.9 2.0-3.7	3.1 2.3-4.0	5.4 4.2-6.7*
Motor	Grip strength	4.7 3.8-5.6	3.5 2.5-4.4	5.7 4.4-6.9*	4.0 2.8-5.2	4.0 2.8-5.2	5.2 3.8-6.7
	Muscle tone.	3.9 3.3-4.3	3.5 2.0-9-4	5.3 4.7-5.9*	3.7 3.1-4.3	3.1 2.5-3.7	4.8 4.8-6.0*

* $p < 0.025$ compared with EOCp. Kruskal Wallis followed by Mann-Whitney test. No significant differences were found between EOCp and Bromazepam.

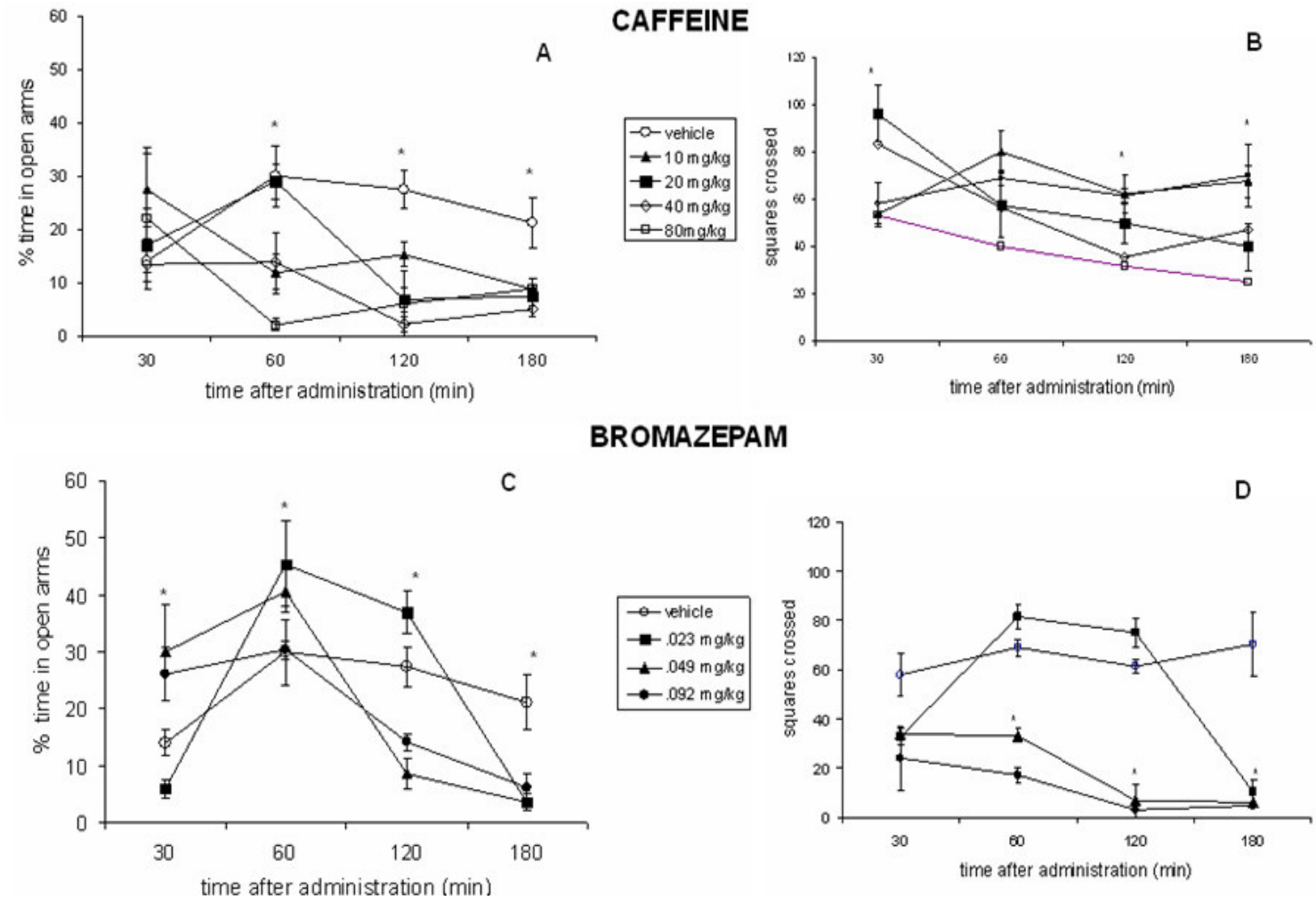

Figure 1. Dose-response curves for caffeine and bromazepam. Each point represents the mean ± SEM of six or seven rats. The tests were performed sequentially, first in the elevated plus-maze and then in the open field. Asterisks represent a significant difference among treatments calculated by means of the Kruskal- Wallis test. The group that received 0.023 mg/kg bromazepam showed an increase of permanence in open arms ($p < 0.05$) at 30, 60 and 180 min, while 20 mg/kg of caffeine were associated to decreased permanence at 120 min ($p < 0.05$, Mann – Whitney test). No changes in locomotive activity were observed at these doses at 60 and 120 min after drugs administration.

Bromazepam: The anxiolytic effect of the 0.023 mg/kg dose, reflected as preference for the open arms, was recorded at all observation times (H (3 N = 28) ranged from 8 to 12.4, P values between 0.046 and 0.006; Mann Whitney comparison versus control P ≤ 0.05 at all four observation points). Three hours after the drug administration, all groups displayed an apparent preference for the closed arms (Figure 1C). The open field test revealed that the 0.023 mg/kg dose induced a significant decrease of locomotive activity at 1 and 3 hours after administration (Figure 1 D).

Based on this validation, a caffeine dose of 20 mg/kg, and a bromazepam dose of 0.023 mg/kg were selected due to their anxiogenic and anxiolytic effects, respecttively, which were significantly accompanied by fewer changes of locomotive activity than at the higher doses. No significant treatment effects were observed on grooming behavior and rearing counts; only bromazepam reduced significantly the number of rearings at higher doses not further employed in the study (data not shown).

Anxiolytic and sedative effects of EOCp

Once determined the depressing effects of EO*Cp* on the CNS, anxiolytic and sedative properties were evaluated by means of two behavioral tasks. In addition to the dose employed in the Irwin test, a higher dose was assayed (1000 mg/kg) in order to observe whether it caused a greater inhibition of CNS functions. The comparative effects of the two EO*Cp* doses in terms of preference for open arms are displayed in Figure 2A. Bromazepam and the lower EO*Cp* dose significantly increased the time spent in the open arms at 60 min (H(4 N = 30) = 11.8, p = 0.019) and at 120 min after administration of the drugs (H (4 N = 35) = 17.8, P = 0.0014), while the response to highest EO*Cp* dose was not significantly different from the caffeine control. In order to disclose whether the animals entered less to the open arms due to lower motor activity, the open field test was performed immediately. Figure 2D shows a significant reduction of locomotive activity in the group of animals exposed to the highest EO*Cp* dose (H (4 N = 35) = 21.9, P = 0.0002, Mann Whitney test P < 0.017). At 60 min the decrease of locomotive activity was remarkable for this group (Figure 2B), although it was statistically similar to the caffeine control (P = 0.06). Confirming the sedative effect of EO*Cp*, the number of rearings displayed by the animals exposed to the higher EO*Cp* dose decreased significantly at 60 min after administration, and this diminution was even more remarkable at 120 min (Figure 2C and F), when the lower dose was also associated with a significant diminution of rearings, even regarding the bromazepam group (H (4 N = 35) = 21.8, P = 0.0002, Mann Whitney test P < 0.017 for all three comparisons). The number and duration of grooming events remained constant within treatments (data not shown).

Finally, to confirm the anxiolytic effects of EO*Cp*, the hole-board test was applied to another set of experimental groups. In this test, the administration of 795 mg/kg EO*Cp* significantly increased the number of hole explorations by animals. In parallel, accumulated time of these head-dippings increased, compared to their own basal performance. Two hours after administrations of the drugs, there were no significant differences on head-dippings between groups (Figure 3).

DISCUSSION

The results of this study performed through four experimental approaches demonstrate that the administration of non-toxic EO*Cp* doses to Wistar rats reduces anxiety, sensory responses and spontaneous activity. These results were obtained by means of classical paradigms for the search of effects of different substances of CNS (Schmitt and Hiemke, 1998; Kalueand Tuohimaa, 2004). As mentioned above, one important shortcoming of phytochemical research in animals is the administration route of plant extracts, which makes difficult the extrapolation from bench findings to clinical application. Most of the studies employ intraperitoneal administration (Herrera-Ruiz et al., 2007; Emamghoreishi et al., 2005; Deciga-Campos et al., 2004; Jaffary et al., 2004; Vasconcelos, 2004) which by-passes a series of pharmacodynamic steps that may be essential for the therapeutic effects on humans, such as absorption, transport and even initial metabolic transformations (Takeda et al., 1998; Mora et al., 2005). For this reason, the evaluation of EO*Cp* effects was performed after the validation of anxiogenic and anxiolytic effects of reference drugs at different doses and times following oral administration. We consider that this strategy allows a closer approach to the traditional use of medicinal plants.

The anxiolytic and sedative effects dependence on dose and time is clearly shown in Figures 2 and 3. While both doses were associated with an anxiolytic effect by increasing the exploration of the open arms after 60 min, only the highest dose decreased activity in the open field. Two hours after administration of both doses the animals almost did not explore the maze and were practically asleep, as noted in the number of squares crossed and number of rearings recorded, which indicates a predominant sedative effect. According to Takeda et al. (1998), the hole-board allows a simultaneous assessment of anxiolytic and sedative properties of a drug. Thus, typical anxiolytics increase the number and duration of head-dippings at doses that do not produce sedation. The EO*Cp* dose of 795 mg/kg increased the number of head-dippings 60 min after administration, but not at the second hour, while the highest dose decreased this variable at both times recorded (Crawley, 1985; da Silva and Elisabetsky, 2001; Sonavane, et al., 2002; Suárez et al., 2003, 2004).

Testing other species of Rutaceae, it has been reported that the essential oil of *Citrus aurantium* L. increases the

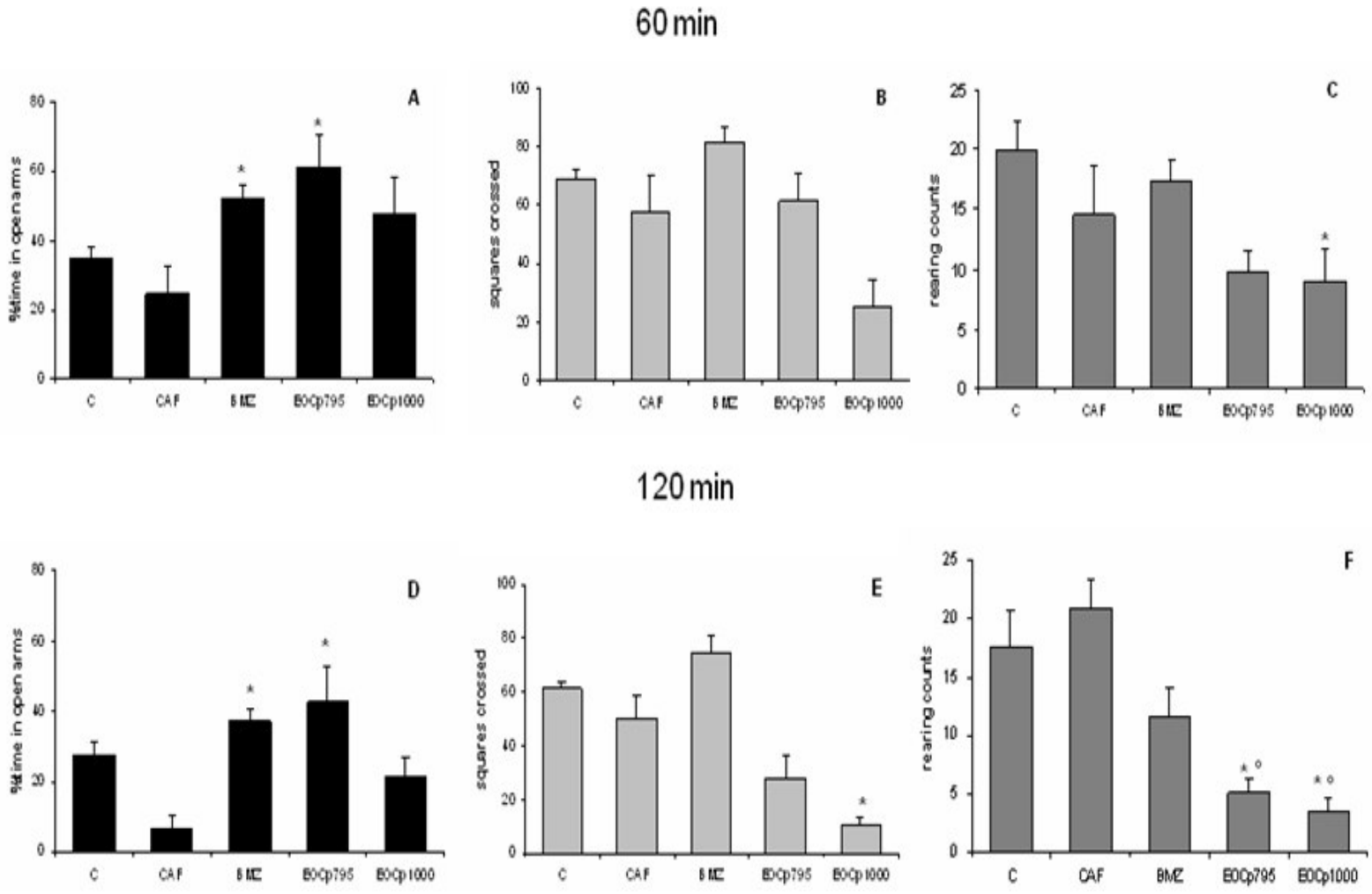

Figure 2. Effect of two doses of essential oil of *Casimiroa pringlei* (EOCp) on three behavioral parameters, 60 and 120 min after administration of the treatments. A and D. Percentage of time spent in open arms of the elevated plus-maze. B and E. Locomotive activity assessed during 5 min in the open field. C and F. Number of exploratory rearings displayed in the open field during 5 min. Each column or point represents the mean ± SEM of six or seven rats. Data were analyzed by means of Kruskal-Wallis test followed by Mann-Whitney U test. Asterisks on top of the bars indicate significant differences ($P < 0.017$) from caffeine. On F degree represents a significant difference from the bromazepam group.

sleeping time induced by barbiturics, when employed in animal models of anxiety at a dose of 1 g/kg of body weight (Carvalho-Freitas and Costa, 2002); while Mora et al. (2005) demonstrated the anxiolytic and sedative properties of *C. edulis* hydro-alcoholic extract. Also, a reduced locomotion and the reversal of desipramine antidepressive effect were observed in rats exposed to an extract of *C. edulis* (Molina-Hernández et al., 2004). Finally, through the joint administration to rats of dopaminergic and serotonergic drugs with lemon essential oil, Komiya et al. (2006) suggested that the anxiolytic and antidepressant properties of this essential oil derive from its ability to suppress dopaminergic activity and to enhance serotonergic functions. These results, however, do require further confirmation.

Our findings represent the first evaluation of the medicinal potential of *C. pringlei*, which is widely distributed in the central and northern states of Mexico and traditionally employed against insomnia and anxiety (Standley, 1924). Based on these results, it becomes relevant to isolate and characterize the main EO*Cp* components, not only because of its pharmaceutical importance *per se*, but also considering their possible use as precursors in the development of new drugs. It is worth to remind that although the important progress of organic synthesis and hemi-synthesis processes, most of the biologically active compounds used in the pharmaceutical industry are still obtained from natural sources (Newman and Cragg, 2007).

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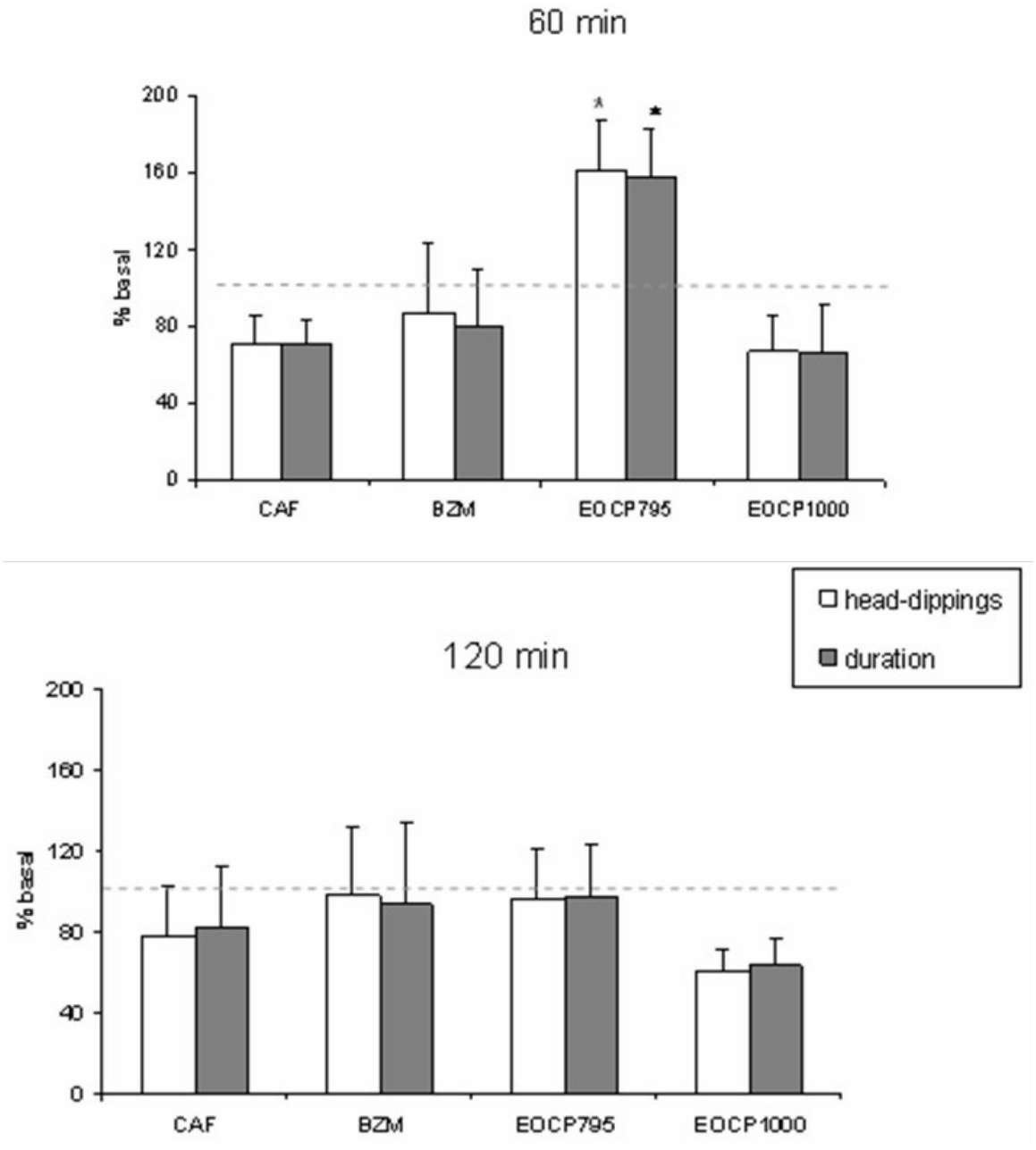

Figure 3. Head-dipping behaviors tested in the hole-board during 5 min. Basal measurements were performed before administration of treatments. The columns represent the observations 60 or 120 min later, expressed as percentage of basal values. * $P < 0.017$ vs caffeine, Kruskal-Wallis followed by Mann-Whitney U tests, N = 6 for each group.

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CONFLICT OF INTEREST

The authors have no conflict of interest. This research received funding only from the Universidad Autónoma de San Luis Potosí.

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