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Anxiolytic-like Effects of *Tilia petiolaris* DC. Inflorescences Infusion

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SUMMARY. Infusions of the inflorescences of *Tilia* are widely used for their tranquilizing properties around the world and have been regarded as non-toxic. Despite the widespread use of the tea of silver Linden in folk medicine, the number of scientific studies for the evaluation of its therapeutic utilization is limited. In an attempt to add experimental confirmation to its popular medicinal use, the central nervous system related effects of the chronic ingestion of the infusion of *Tilia petiolaris* DC. inflorescences were evaluated in the holeboard, locomotor activity and light-dark tests in mice. The infusion induced significant increases in the exploration of holes and in the time spent head-dipping in the holeboard assay. Moreover, it significantly increased the time spent in the light area in the light-dark test. These results suggest that the infusion of *Tilia petiolaris* DC. inflorescences exerts an anxiolytic-like activity in mice.

INTRODUCTION

Anxiety is a subjective emotional state of uneasiness, not pleasant and even fearful. When anxiety reaches pathological levels the subject experiences behavioral changes, apprehension, motor troubles, sweating, and hypertension. Traditional medicine offers many treatments for this ailment, most of them based on herbal preparations.

Infusion of the inflorescences of *Tilia* is widely used for its tranquilizing properties around the world, but the number of scientific studies that evaluates their therapeutic use is scarce.

We have already proved that a partially purified ethyl ether fraction of *Tilia tomentosa* inflorescences, administered intraperitoneally (i.p.) in mice, had anxiolytic effects [1]. Another studies documented that aqueous extracts of Linden produced sedative effects in mice [2]. Recently, the anxiolytic and sedative effects of *Tilia americana* var. *mexicana* extracts were demonstrated [3-7].

Tilia petiolaris DC. is a deciduous tree native to South–East Europe and Western Asia. This tree is commonly known as Pendant Silver Linden or Weeping White Linden. In a previous work we isolated and identified, from an ethanolic extract of *Tilia petiolaris* DC. inflorescences, three flavonoid glycosides; quercetin 3-O-glucoside-7-O-rhamnoside, kaempferol 3-O-glucoside-7-O-rhamnoside and isoquercitrin, with clear central nervous system (CNS) depressant actions [8].

In an attempt to add experimental confirmation to the popular medicinal use of this plant, the infusion of the aerial parts of *Tilia petiolaris* DC. was evaluated for its pharmacological activity on the CNS in mice, using various behavioral models.

MATERIALS AND METHODS

Plant Material

Dry *Tilia petiolaris* DC. (Tiliaceae) inflorescences were obtained from a local commercial

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source. Its identification was carried out by Ing. G. Giberti from the Botany Museum of the School of Pharmacy and Biochemistry of Buenos Aires, where a sample of plant material was deposited (number 2697) for future reference.

Preparation of the plant infusion

Ten g of powdered *Tilia petiolaris* DC. inflorescences were infused into 500 ml of boiling water for 5 min, and filtered though Whatman N° 4 paper. The infusion (INF) thus obtained yielded an amount of dry residue of 4 mg/ml.

An evaluation of the flavonoids profile in INF by thin layer chromatography on silica gel polyester sheets, with 254 nm fluorescent indicator (SIGMA), developed with chloroform/methanol/water (8:2:0.2 v/v) as the solvent and stained using a general indicator for flavonoids (2-aminoethyl diphenylborinate, 0.3 % in methanol) and analytical HPLC analyses (LKB Pharmacia apparatus and C18 reversed phase Vydac columns) revealed the presence of quercetin 3-O-glucoside-7-O-rhamnoside, kaempferol 3-O-glucoside-7-O-rhamnoside and isoquercitrin, among others. These results are in accordance with those of Atoui *et al.* [9].

Animals

Adult male Swiss mice weighing 25-30 g were used in the assays. Animals were housed in groups of five in a controlled environment (20-23 °C), with free access to food and liquid (water, a solution of diazepam (DZ) or INF); and maintained on a 12h/12h day/night cycle, light on at 06:00 AM except for the light/dark assay where the light was on at 6:00 PM. Housing, handling, and experimental procedures complied with the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication N° 85-23, revised 1985) and CICUAL (Institutional Committee for the Care and Use of Laboratory Animals, University of Buenos Aires, Argentina). All efforts were taken for minimize animal suffering and the number of animals used was the minimum number consistent with obtaining significant data. The animals were randomly assigned to any treatment group and were used only once. The behavioral experiments were performed between 10:00 AM to 2:00 PM.

Experimental procedure

Three different groups of mice were treated in parallel with water (vehicle, VEH), INF or a solution of DZ (Roche Diagnostics, Argentina), a reference compound, as their sole source of drinking fluid for 10 days before performing the tests.

The volume of liquid consumed daily by mice was 150 ml/kg, and no significant differences were found among all the experimental groups. Thus, the dose of INF ingested per day was 600 mg of dry residue/kg, which corresponds to a tea prepared with 3 g of dry inflorescences/kg. This value was calculated taking into account that the concentration of INF was 4 mg of dry residue/ml. The concentration of the solution of DZ was 0.1 mg/ml, so the dose of DZ was 15 mg/kg per day.

Pharmacological studies

The holeboard assay

This assay was conducted in a walled black Plexiglass arena with a floor of 60 cm x 60 cm and 30 cm high walls, with four centered and equally spaced holes in the floor, 2 cm in diameter each as previously described by us [10]. The number of holes explored, the time spent head-dipping and the number of rearings were measured during 5 min. An increase in head-dipping behavior indicates an anxiolytic effect in animals [11]. The number of rearings, also, reflects an exploration component and is influenced by the level of anxiety; therefore, it can be recorded as an anxiety index [12]. Meanwhile, a decrease in all the parameters measured reveals a sedative behavior [13].

Light/dark transition test

The light/dark box consisted of a fully automated Plexiglass box monitored by computer with two compartments, distinguished by wall color, illumination and size; one light area (30 cm long x 21 cm wide x 21 cm height) illuminated by a 60-W light (400 lx) in the ceiling of the compartment and with white walls, and a smaller dark compartment (14 cm long x 21 cm wide x 21 cm height) with black walls and not illuminated. An opening door (6 cm x 3 cm) located in the center of the partition at floor level connected the two compartments.

On the walls and along the longest axis of the box, 16 infrared emitting diodes and 16 infrared detectors were arranged in perfectly aligned pairs (five in the dark area and eleven in the light compartment). The model is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior in response to novel environment and light. The experiments were per-

formed between 10:00 AM to 2:00 PM, in the middle of the dark phase. Animals were placed in the center of the dark area facing the wall opposite to the door. To reduce any neophobic response to the test situation, the light–dark compartments are previously dirty with mice other than those used during the test. Mice are always tested in a soiled apparatus, and there is no cleaning between trials. The following parameters were recorded during 5 min: 1) latency time of the first crossing to the light compartment, 2) the number of crossings between both compartments, 3) the total time spent in the illuminated zone of the cage, 4) the overall movements in both areas expressed as a function of the time spent in the compartment under consideration [14].

Assessment of locomotor activity

All mice were individually exposed to the locomotor activity assay after completing the holeboard test. The spontaneous locomotion activity was measured in a box made of Plexiglass, with a floor of 30 cm by 15 cm and 15 cm high walls as previously described by us 10. The locomotor activity was expressed as total light beam counts per 5 min.

Statistical analyses

The mean number of responses for each group and for each test was calculated, and the final results were expressed as mean ± SEM (standard error of the mean). All data were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparisons between treatments and vehicle were made using Dunnett's multiple comparison test (Prism 5.00, GraphPad Software). For the analysis of movements in both compartments in the light-dark assay, data collected were expressed as movement by unity of time (movements/time spent in the area) to avoid false interpretation of results [14].

RESULTS

Figures 1A and 1B show the effects of INF and DZ in the holeboard and the locomotor activity tests, respectively. ANOVA indicated a significant difference on the number of holes explored [$F(2,25) = 4.83$, $p < 0.05$], the time spent head-dipping [$F(2,25) = 8.94$, $p < 0.01$], and the locomotor activity counts [$F(2,25) = 4.09$, $p < 0.05$] but the number of mouse rearings was unaffected [$F(2,25) = 3.04$, $p = 0.0654$]. The anxiolytic-like effect of INF was evidenced in the holeboard test (Dunnett's procedure) by an increment in the exploration of holes ($p < 0.05$) and in the time spent head-dipping ($p < 0.01$) (Fig. 1A). Otherwise, INF caused no changes in the locomotor activity of mice (Fig. 1B). Comparatively, mice treated with a solution of DZ showed an augmented exploration on holes ($p < 0.05$, Fig. 1A) and on the locomotor activity counts ($p < 0.05$, Fig. 1B) suggesting an anxiolytic-like performance.

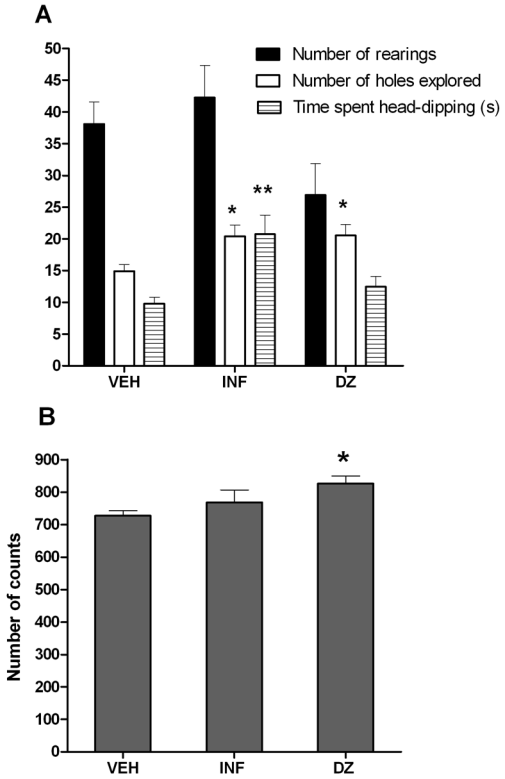

Figure 1. Effect of *Tilia petiolaris* DC. inflorescences (INF) on the holeboard and locomotor activity tests in mice. Results are expressed as mean ± S.E.M. of **A**) holeboard parameters; **B**) spontaneous locomotor activity counts; registered in 5 min sessions. * $p < 0.05$, ** $p < 0.01$, significantly different from vehicle; Dunnett's multiple comparison test after ANOVA (n = 8–11 mice/group).

Results of the light-dark test are shown in Figure 2. In this assay ANOVA indicated a significant difference on the time spent in the light area [$F(2,20) = 4.88$, $p < 0.05$], the number of inter-compartment transitions [$F(2,20) = 7.56$, $p < 0.01$], the number of movements/unity of time in the light [$F(2,20) = 3.62$, $p < 0.05$] and in the dark area [$F(2,20) = 5.77$, $p < 0.05$] and in the total ambulatory counts [$F(2,20) = 4.20$, $p < 0.05$]. The latency to enter the light area [$F(2,20) = 2.70$, $p = 0.092$] was not significantly affected. Comparisons between the vehicle control group and experimental groups (Dunnett's test) indi-

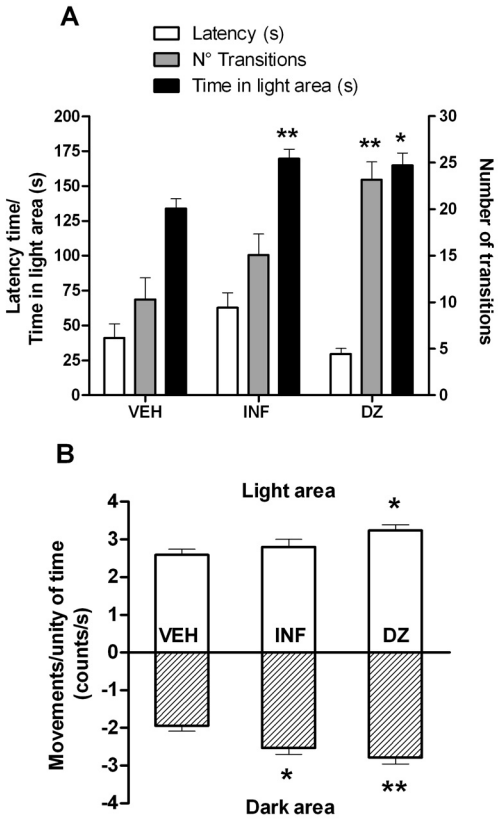

Figure 2. Effect of *Tilia petiolaris* DC. inflorescences (INF) on the light-dark test in mice. Results are expressed as **A**) mean ± S.E.M. of the latency to enter the light area, the number of inter-compartment transitions, the time spent in the light area; **B**) mean ± S.E.M. of the number of movements/unity of time in the light and in the dark area; registered in 5 min sessions. * $p < 0.05$, ** $p < 0.01$, significantly different from vehicle; Dunnett's multiple comparison test after ANOVA (n = 7–8 mice/group).

cated that DZ significantly increased the number of transitions and the time spent in the light area ($p < 0.01$ and $p < 0.05$, respectively, Fig. 2A), the number of movements/unity of time in the light and in the dark area ($p < 0.05$ and $p < 0.01$, respectively, Fig. 2B). Meanwhile, INF caused a noticeable increment in the number of transitions and significantly increased both, the time spent in the light area ($P < 0.01$, Fig. 2A) and the number of movements/unity of time in the dark area ($P < 0.05$, Fig. 2B), evidencing an anxiolytic-like activity.

DISCUSSION

To determine the effect of the regular intake of a tea of *Tilia,* an infusion of *Tilia petiolaris* DC. inflorescences was administered for 10 days prior to behavioral testing in mice, thus closely mimicking the oral route conditions in humans. These effects were evidenced in popular assays usually employed to measure behavior of mice.

It is well known that drugs that affect general motor function could affect holeboard and light-dark performance, so, a screening of locomotor activity appears to be necessary for eliminating false-positive results. Our evaluation showed that INF produced no changes in the spontaneous locomotor activity of mice.

In the holeboard assay INF produced a significant increment in the exploration of holes and in the time spent head-dipping, without any change in the number of rearings. The incremented exploration activity in a novel environment, like the holeboard box, is an indication of anxiolytic behavior of mice [15]. In the light-dark assay INF caused a significant increase in both, the time spent in the light area and the number of movements/unity of time in the dark area, but no changes were observed in the other parameters. The enhanced time spent in the light compartment is considered to be the more reliable indicator to assess anxiolytic-like activity [14]. The anxiolytic-like action evidenced by INF in these studies was comparable to that observed for DZ.

Although we have previously determined the presence of CNS depressant flavonoid glycosides in an ethanolic extract of the inflorescences of *Tilia petiolaris* DC. [8], and these compounds were also found here present in INF, the anxiolytic effects exerted by the tea seem to contradict the depressant actions shown by the isolated compounds. Plant extracts containing so many different constituents can exhibit a variety of effects, something contradictory, depending on the chemical composition, route of administration and duration of the treatment. Furthermore, *in vivo* effects of extracts do not always correlate directly with the effects of isolated compounds, due to issues of bioavailability, metabolism and dosage changes.

The pharmacological profile of the infusion of *Tilia petiolaris* DC. inflorescences reported here revealed that the chronic treatment of mice that ingested this tea produced an anxiolytic-like activity that validates its traditional use.

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