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PHARMACOLOGICAL PROPERTIES OF TILIA EUROPEAE AQUEOUS EXTRACT: SCREENING ANXIOLYTIC/SEDATIVE ACTIVITY IN MICE

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Abstract

The extracts or infusions obtained from inflorescences of Tilia europeae have been used in popular medicine as a sedative/anxiolytic drug. The purpose of the present work was to evaluate these possible properties of this extract, by using different behavioural models.

The extract, orally administered to mice, prolonged sleeping time, changed the level of anxiety, affected curiosity and locomotor activity and showed miorrelaxant properties.

Key words: Tilia europeae , anxiolytic effect, sedation, locomotion, exploration

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INTRODUCTION

Tilia europeae, an hibrid of Tilia cordata and Tilia platyphyllos Scop., is one of the lime flower species that are used as traditional medicinal plants in Latin America as sedatives and tranquilizers [1, 2]. For this purpose, the infusion of their inflorescences is used to prepare a tea.

Little is knwon about the mechanism(s) of action of Tilia europeae extracts. Our group of work studied the effect of tilia aqueous extracton the GABA A receptorcomplex and found that this extracts displayed the [3H]muscimol bound to crude synaptic membranes, inhibited the [3H]-flunitrazepam binding and stimulated the uptake of 36Cl by synaptoneurosomes isolated from rat cortices [3]. The last effect was blocked by picrotoxin what suggest that this extract contains some compound with affinity for the competitive binding site of the GABA A receptor-complex [4].

The present study was designed to evaluate the action of this aqueous extract on the central nervous system and its possible anxiolytic/sedative and miorrelaxant properties using different models based on mice behaviour.

There are numerous ways of interpreting the anxiety models but according some authors they can be split effectively into main camps: "ethological" models depending on the response of naive animals to an unfamiliar situation; and "trained" models depending on the response to punishment, usually but not always signalled. The "ethological" models included the elevated plus maze and the black white box. The reflex condititioner may be a good way to test the response to punishment. The methods using aversive brain stimulation included the object-burying test.

To evaluate the coordenation of the motor activity we used the Boissier's chimney [5]. The anxiolytic activity was studied using the elevated plus maze [6], the object-burying [7] and hole-board tests [8]. The potentiation of pentobarbital sleeping time [9] gived us the sedative properties and the wire test [10] the miorrelaxant activity. We also used the reflex conditioner [11] as a model of conflict schedules.

MATERIALS AND METHODS

1. Tilia extract preparation: Aqueous extract (3%) was prepared from powdered dessicated inflorescences of Tilia europeae obtained at the Botanic Garden of Coimbra and identified by the Botanic Institute of the University of Coimbra. The inflorescences were dried at room temperature and then kept at -20°C.

To prepare the aqueous extract, the dried material was crushed in a mechanical mill and passed through a 20 mesh sieve. The resulting powder (3 g) were resuspended in 100 ml of boiled distilled water; after cooling, the infusion was filtered under vacuum. The contents of hydrocinnamic acids (caffeic acid as standard) [12] and flavonoids (rutine as standard) [13] were determined. The extracts used in the experiments contained 0.84 ± 0.05 mg of caffeic acid and 0.11 ± 0.03 mg of rutin per ml of extract.

2. Animals

Adult male albino Wistar rats weighing 180-250 g were used in the automatic reflex conditioner.Mice weighing 25-30 g were used in the other experiments.

The animals were housed in colony cages at an ambient temperature of $25 \pm 2^{\circ}$ C and relative humidity of 50 ± 10 with a light-dark cycle of 12h each. They were fed standard pellet chow and water ad libitum. In order to avoid the interference by the circadian rhytm in central nervous system amine content an d turnover, all the experiments were performed at same time of the day (8.00-12.00 am.).

3.1 Elevated plus maze

The elevated plus maze test is a rodent model that is used extensively in the discovery of novel anxiolytic drugs. The maze was made of wood and had two open arms (50x10 cm; illuminated by two red lights) and two enclosed arms of the same size with walls 40 cm high; it was elevated 50 cm above the ground . 45 min after oral tilia extract (10, 15 or 20 ml/kg) or 20 min after intraperitoneal diazepam (0.5 or 0.8 mg/kg) administration, each animal was placed in the central square (10x10 cm) and allowed 5 min to freely explore the maze; the number of entries and time spent on open and closed arms were scored [6].

3.2 Hole-board test

The hole-board test described by Boissier and Simon [8] evaluates the possible anxiolytic/sedative drug effect, by measuring the number of head dips of mice on a wood slab, before and after drug administration (45 min after oral 10ml/kg aqueous tilia extract administration; 20 min after intraperitoneal 0.25 mg/kg diazepam administration). The wood slabe, 50 cm above the ground, contained 16 inspection holes, 3 cm diameter, spaced 5 cm between them. The number of head dips of mice was counted during 5 min before and after drugs administration. The results were expressed as % of reduction of number of head dips after drug administration.

3.3 Pentobarbital-sleeping time

The potentiation of pentobarbital-sleeping time after drug administration was used to evaluate the hypnotic/sedative activity of the extract. Mice were treated with the tilia aqueous extract (10 ml/kg p.o.) or vehicle (water p.o.) and one hour later with pentobarbital (30 mg/kg i.p.). Diazepam (0.25 mg/kg, i.p) was administered 20 min before pentobarbital and the time interval between the loss and the recovery of the righting reflex (sleeping time) was measured [9].

3.4 Horizontal wire test

This test consisted of a horizontally strung wire (1mm diameter, 15 cm long) placed 20 cm from the table. Mice were lifted by the fore-feet and the time they spent to reach equilibrium with the hind-feet was scored. The increase of this time could indicate muscle relaxant or sedative action. After two trials performed at 5-min intervals, the test took place. A myorelaxant drug, like diazepam will impair mice to grasp the wire. Generally, this state of muscle relaxation is commonly associated with sedation.

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After vehicle, diazepam or tilia extract administration, the mice were individually placed on a gauge copper wire, the time they spent to reach equilibrium with the hind-feet was scored. The increase of this time could indicate muscle relaxant or sedative action [10].

3.5 Motor coordination

The Boissier's "chimney" test [5] was used. In this test the ability of the mice to climb backwards in a vertically hold tube is observed. Animals that employ more than 30 sec to reach the upper edge of a 25 cm long glass tube, are considered as incoordinated. The test was carried out one, 45 min after treatment. Diazepam (0.25 mg/kg, i.p) was used as a positive control.

3.6 Object-burying test

Rodents have a natural defence reaction to strange and dangerous objects in spaying bedding material over the object, leading to coverage of the object. One of the procedure is to use objects that provoke burying spontaneously [7]. For this experiment a plexiglas cage of 23x17x14 cm with a smooth lid punctured by small ventilation holes and 25 glass marbles of 1.5 cm diameter are required. The floor of the cage is covered with a fresh 5 cm layer of sawdust or other loose bedding material. The marbles are placed in contact with each other in the middle of the cage; then the mouse is placed into the cage for 30 min, after which time is removed and the burying response is quantified by counting the number of marbles that are more than two-thirds covered with sawdust.

Mice will bury the glass marbles and the behaviour is particularly vigorous in novel cages with clean bedding material.

The influence of the extract (0.18, 0.36 and 0.72 g/kg) was investigated by oral administration 30 min before placing them each in a cage with marbles and the results compared with them obtained in the negative (vehicle) and positive (diazepam) controls.

3.7 Automatic reflex conditioner

This fully automated apparatus is designed to study conditioned reflexes (avoidance reaction) in rats and mice. It consists of a programming/recording unit and a cage divided into two sections by partition with an intercommunicating door at floor level. The cage is made of PERSPEX sheets and provided with acoustic and visual stimulators, which supply conditioning stimuli. The operator can switch on either the visual or acoustic stimulator, or both, at will.

The "reinforcement" consists of an electricl stimulus applied to the floor bars of the cage by a special "static scrambler" circuit.

The programming/recording unit supplies the stimuli, the magnitude and duration of which can be varied, and records the animal's response via a writing mechanism which discriminates between responses caused by acoustic and visual stimuli (conditioned reflexes) and those requiring an electrical stimulus as well (reinforcements).

The acoustic stimulator comprise a loudspeaker mounted on the side of the cage, powered by a suitable low-frequency generator located in the programming/recording unit. A knob regulates the intensity of the sound, which is monitored on an arbitrary scale.

Voltage varies from 0 to 150 V and is applied to the cage floor bars.

The counter operates at a frequency of 12.5 pulses per second on 50-cycle mains or 15 pulses on 60-cycle mains, mains frequency being divided electronically by four. It comes into operation when the acoustic and /or visual stimulus starts, and stops when the animal goes through the door.

By the end of the session it will have computed the sum of the animal's waiting times (latencies) in 1/12.5 (or 1/15) of a second [11].

The response pen is given by o movie across the width of the paper from the moment the conditioning stimulus that is switched on the instant the animal goes through the door. The height of the peaks provides an accurate picture of the conditioning process. The sum of waiting times is therefore indicated by the counter and by the sum of the heights of the peaks. The counter gives a graphic picture of the entire session and shows the degree of learning.

Data handling and statistics. Data represet the mean \pm S.E.M. Differences between grups were evaluated by ANOVA analysis to determine statistical significance; unless otherwise indicated *P < 0.05 was considered statistically significant.

Results

In all experiments, diazepam (0.25-0.8 mg/Kg) was used as reference drug.

Performance of mice (n=20) following oral administration of vehicle and tilia extract and i. p.

administration of diazepam on the elevated plus maze is shown in Figs. 1 and 2.

On the open arms, tilia extract (15 and 20 ml/kg) and diazepam (0.5 and 0.8 mg/kg) increased the number of entries compared to control animals. Only 20 ml/kg tilia extract and 0.8 mg/kg diazepam increased the time spent on this area. Tilia 10 ml/kg did not change these parameters



Figure 1. Effects of tilia extract and diazepam on the behaviour of mice in the elevated plus maze. The open arms entries (A) and the time spent on the open arms (B) were scored during 5 min. Mice were pretreated with tilia extract or water (45 min before the test, p.o) or diazepam (20 min, i.p.). Data represent the mean \pm S.E.M. n = 5-11. *P< 0.05 (ANOVA) compared to control.

On the closed arms, tilia increased the number of entries but it did not change the time spent on this area. On the contrary, tilia (20 ml/kg) and diazepam did not change the number of entries, compared to the control group, and decreased the time spent on this area. Tilia 10 ml/kg did not significantly changed these parameters

On the hole board test low dose of tilia extract (10ml/kg) and diazepam (0.25 mg/kg) decreased the



Figure 2. Effects of tilia extract and diazepam on the behaviour of mice in the elevated plus maze. The closed arms entries (A) and the time spent on the closed arms (B) were scored during 5 min. Mice were pretreated with tilia extract or water (45 min before the test, p.o) or diazepam (20 min i.p.). Data represent the mean \pm S.E.M. n = 5-11. *P< 0.05 (ANOVA) compared to control.

number of head dips of mice compared with control (vehicle) (Fig. 3).



Figure 3. Reduction (%) of number of head dips of mice on the hole board test after 10ml/kg tilia extract (n=35) or 0.25 mg/kg diazepam (n=10) administration. The number of head dips were counted during 5 min before and after drug administration. Mice were treated with tilia extract or water (n=22), 45 min. before the test, p.o, or diazepam (20 min i.p.). *P< 0.05 (ANOVA) compared to control.

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Pentobarbital sleeping time of mice treated with tilia (10 ml/kg) and diazepam (0.25 mg/kg) was significantly higher compared to controls (Fig. 4). These results suggest that tilia extract potentiated the barbiturate induced sleep, displaying anxiolytic/sedative effect.



Figure 4. Effects of tilla extract or diazepam on pentobarbital sleeping time. Mice were treated with tilla aqueous extract (n=31) or vehicle (10 ml/kg p.o., n=24) and one hour later pentobarbital (30 mg/kg, i.p.); diazepam (0.25 mg/kg, i.p., n=24) was administered 20 min before pentobarbital. The time interval between the loss and the recovery of the righting reflex (sleeping time) was measured. Data represent the mean \pm S.E.M. *P< 0.05 (ANOVA) compared to control.

On the horizontal wire test, tilia extract (10 and 20 ml/kg) and diazepam (0.5 mg/kg) decreased the ability of mice to reach the wire with the hind-feet, increasing the suspended time compared to controls producing a clear-cut myorelaxant effect (Table I).

	V/	
Drugs	Time (sec)	
Control (water)		
(n= 35)	2.1 ± 0.5	
Tilia 10 ml/kg		
(n=10)	6.9 ± 2.5 *	
Tilia 20 ml/kg		
(n=10)	>30	
Diazepam 0.5 mg/kg		
(n=20)	5.2 ± 1.5 *	

The results of the tilia extract effects on the motor coordination shows a decrease after tilia and diazepam administration more evident for diazepam.Mice were treated with tilia aqueous extract, vehicle or diazepam (0.25 mg/Kg, i.p.).

Table II		
Drugs	Time (seg.)	
Controlo		
(n= 30)	5.52 ± 0.72	
Tilia 10 ml/kg		
(n=30)	10.6 ± 1,2 *	
Diazepam 0.5 mg/kg		
(n=30)	> 30 *	

The results of the object-burying test are present in the Fig. 5. A significant decrease in the number of marbles that are more than two-thirds covered with sawdust was observed in the animal groups traeted with 10 mg/Kg tilia extract (n=20) and diazepam (n=20) compared with the control group (n=25).



Figure 5. Effects of tilia extract or diazepam on the object-burying test. Mice were treated with tilia aqueous extract (n=9), vehicle (n=9) or diazepam (0.25 mg/kg, i.p., n=24) and 5 min later the number of hidden glass marbles were counted. Data represent the mean \pm S.E.M. *P< 0.05 (ANOVA) compared to control.

The avoidance reaction in the group tracted with tilia extract (n=7) did not changed related with the counter and the sum of the heights of the peaks obtained in the control group (n=7), (Fig. 6).



Discussion

Pharmacological screening was undertaken to evaluate the possible anxiolytic/sedative and relaxant effects that are described for Tilia europeae in folk medicine. The same preparations popularly used were tested by oral administration.

Modification of pentobarbital-sleeping time after the administration of Tilia europeae was used to verify the sedative activity of the plant. It was observed that tilia had some central nervous system depressant effects, since it potentiated pentobarbital-sleeping time of mice. The depressant effects could be partially dependent on the presence of benzodiazepine-like compound [2]. In fact, some authors have shown that the extracts of inflorescences of Tilia species that inhibited the binding of 3H-flunitrazepam to synaptic membranes of rat brain, contained these BZD-like compounds

The main finding of the present study is that Tilia europeae aqueous extract had anxiolytic effects in the elevated plus maze; the fear of open spaces is the predominant anxiogenic stimulus in the elevated plus maze. So, tilia extract, like diazepam, has anxiolytic activity once it increases the time spent on the open arms and the number of entries into this area.

The anxiolytic effect was observed yet with the hole board and object-burying tests in which we obtained a decrease of the curiosity and the number of marbles covered in the animals received the extract. These that tilia extract results suaaest has an anxiolytic/sedative effects. The results obtained with the wire test suggests muscle relaxation properties. Like diazepam (0.5 mg/Kg i.p.) tilia extract (10 and 20 ml/Kg o.r.) provoked decrease in muscle tone. This data suggests that at low doses both tilia extract (10 ml/Kg) and diazepam (0.5 mg/Kg) have anxiolytic effects with generating sedation and muscle relaxation, which are dose-dependents. This is according other studies that showed that a complex fraction containing unidentified constituints, administered intraperitoneally to mice, had anxiolytic effects measured by pharmacological tests [4].

The tilia-induced sedation is difficult to explain principally because we used a crude aqueous extract. We do not believe that tilia extract enhanced the barbiturate effect by slowing the metabolism of pentobarbital. It is well known that pentobarbital had a short-lasting anaesthetic effect to the immediate redestribution from brain into musculature rather than to rapid metabolic destrution. Instead, we think of a positive interaction of tilia at the barbiturate binding site of the GABAA-benzodiazepine receptor complex what is according the findings of our work group [3].

The reflexive behavior and conditioned reflexes has been utilized in research on the behavioral effects of drugs. For instance, considerable use has been made of a procedure for the study of anxiolytic drugs in which a stimulus paired with the delidery of electric schock enhances the response to a loud auditory stimulus that elicits a startle response [11]. No significant differences were observed in the group treated with the extract related with the control group what indicate any direct depressor effect.

In conclusion, using specific behavioural animal models, our results support the suggestion that tilia extract, like diazepam, can reduce anxiety with sedation and muscle relaxation.

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