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Research Paper

Central nervous system activity of the proanthocyanidin-rich fraction obtained from *Croton celtidifolius* in rats

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Abstract

Objectives The aim of the present study was to evaluate the possible neurobehavioural effects in rats of the proanthocyanidin-rich fraction (PRF) isolated from the bark of *Croton celtidifolius* (Euphorbiaceae).

Methods Adult Wistar rats were treated with the PRF (0.3–30 mg/kg) and evaluated in different behavioural paradigms classically used for the screening of drugs with psychoactive effects.

Key findings Acute intraperitoneal (i.p.) administration of PRF decreased spontaneous locomotor activity (open field arena and activity cage), enhanced the duration of ethyl ether-induced hypnosis, increased the latency to the first convulsion induced by pentylenetetrazole (60 mg/kg, i.p.) and attenuated apomorphine-induced (0.5 mg/kg, i.p.) stereotyped behaviour. In lower doses, PRF (0.3 or 3 mg/kg, i.p.) increased the frequency of open arm entries in the elevated plus-maze test.

Conclusions The present findings suggest that the systemic administration of PRF induces a wide spectrum of behavioural alterations in rats, consistent with the putative existence of hypnosedative, anticonvulsant and anxiolytic compounds.

Keywords behaviour; catechin; central actions; *Croton celtidifolius*; proanthocyanidin; rats

Introduction

Several medicinal plants are traditionally used for their psychoactive effects. Despite this, the scientific community – as opposed to folk medicine practitioners – has given little attention to the therapeutic usefulness of such plants.[1] On the other hand, some clinical studies have endorsed the therapeutic benefits of medicinal plants in the treatment of psychiatric conditions, including affective, cognitive and sleep disorders.[2]

Croton celtidifolius Baill (Euphorbiaceae) is a native plant of the Brazilian Atlantic Forest, frequently found in the Southern region.[3] This plant is popularly known as 'Sangue-de-Adáve' in folk medicine, and the infusion of its bark is indicated for the treatment of inflammatory and ulcerative diseases.[4] During the last 10 years, we have extensively studied the pharmacological properties of the hydroalcoholic extract, fractions, sub-fractions and compounds isolated from the bark of this plant. Our previous findings indicated a series of biological activities of *C. celtidifolius* that includes antiedematogenic, antioxidant, anti-inflammatory, superoxide dismutase enzyme activity modulatory[5] and antinociceptive effects.[6]

Phytochemical studies have already characterised some of the compounds present in *C. celtidifolius*, including cyclitols (1L-1-O-methyl-myo-inositol and neo-inositol) and sitosterol,[7] catechins, gallocatechins and proanthocyanidins,[4] alkaloids and saponins.[8] More recently, we have focused our studies on the proanthocyanidin-rich fraction (PRF) isolated from the ethyl acetate soluble fraction (EAF) of *C. celtidifolius* bark. We have demonstrated that the PRF presents antinociceptive effects against chemical and thermal noxious stimuli, with involvement of capsaicin-sensitive C-fibres and dopamine D_2 receptors.[9,10] Moreover, PRF induces endothelium-dependent vasorelaxant effects via the NO–cGMP pathway and

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hyperpolarisation due to the activation of Ca^{2+}-dependent K^+ channels in the small-resistance arteries of rats.[11,12]

Catechins, the main constituents of the PRF of *C. celtidifolius*, are a group of naturally occurring flavonoids that share the flavan (3,4-dihydro-2-phenyl-2H-1-benzopyran) basic backbone.[13] (+)-Catechin and (–)-epicatechin are also the building blocks of most proanthocyanins (oligomers or polymers of flavan-3-ol units).[14] Besides the classical biological and pharmacological activities described for several flavonoids, such as anti-inflammatory and antioxidant properties,[15,16] many studies have reported their effects on the central nervous system (CNS) as analgesics, anxiolytics and sedatives.[16,17]

Considering the aforementioned pharmacological profile of the PRF and that some catechins are psychoactive,[18] in the present study we investigated the possible neurobehavioural effects in rats of the acute intraperitoneal (i.p.) administration of the PRF isolated from *C. celtidifolius* bark. We used a battery of behavioural paradigms that are well validated for the screening of compounds with psychoactive properties.

Material and Methods

Plant material

The bark of *C. celtidifolius* Baill was collected in March, 2008, from the forest surrounding the city of Orleans (Santa Catarina, Brazil). A voucher specimen (document number 31272) was identified and deposited at the Department of Botany of the Universidade Federal de Santa Catarina (UFSC, Florianópolis, Brazil).

Air-dried powdered bark of *C. celtidifolius* was extracted by consecutive percolation with an aqueous solution containing 80% ethanol at room temperature. The solvent was removed by vacuum and the crude extract was dissolved in acetone to obtain a saturated solution. A large volume of water was then added, causing precipitate formation. This precipitate was subsequently removed by filtration and the solution was transferred to a rotavapor for acetone evaporation. The resultant aqueous solution was extracted with ethyl acetate, which generated the PRF after organic solvent removal. This method provided a yield of 0.56% of PRF from the original air-dried plant material.

Chemical characterisation of the proanthocyanidin-rich fraction

Phytochemical investigation using column chromatography fractionation followed by nuclear magnetic resonance (NMR) spectral analysis of the isolated compounds, together with micellar electrokinetic chromatography (MEKC) analyses, showed that the PRF contains 27.4% catechin, 1.3% epicatechin, 6.4% gallocatechin, 16.7% catechin-(4α→8)-catechin, 4.9% gallocatechin-(4α→8)-catechin, and 43.3% proanthocyanidin oligomers. Further thiolysis of the PRF following MECK demonstrated that proanthocyanidin oligomers were mainly composed of catechin and epicatechin units in the ratio of 1.5 to 1.[12]

Animals

A total of 260 male Wistar rats (3 months old, 250–350 g) were used in the experiments. Animals were maintained on a 12-h light–dark cycle (lights on at 6:00h) at constant room temperature (22 ± 2°C) and they were housed in groups of five animals per cage with free access to food and water. All animals were allowed to adapt to the laboratory conditions for at least 1 week before the behavioural assessment. All procedures used in the present study complied with the guidelines on animal care of the local Ethics Committee on the Use of Animals (CEUA/UFSC), which follows the NIH publication *Principles of Laboratory Animal Care*. Each animal was used just once and all efforts were made to use the minimum number of animals required to obtain consistent experimental data.

Drugs

Ethyl ether was purchased from Vetec Química Fina Ltda., Brazil. Pentylenetetrazole (Sigma-Aldrich Co., USA) and diazepam (Dienpax, Sanofi-Winthrop Lab., Brazil) were dissolved in 0.9% saline. Apomorphine (Sigma-Aldrich Co., USA) was dissolved in a solution containing 0.9% saline with 0.1 mg/ml ascorbic acid. Haloperidol (Sigma-Aldrich Co., USA) was dissolved in a single drop of 1N HCl and diluted with saline. PRF from *C. celtidifolius* was dissolved in saline (0.9% NaCl).

Treatment schedules

PRF (0.3, 1, 3, 10 or 30 mg/kg) was injected by the intraperitoneal (i.p.) route in a volume of 0.1 ml/100 g of body weight, 30 min before testing. Diazepam (0.25, 1.5 or 5 mg/kg, i.p.) or haloperidol (5 mg/kg, i.p.) were used as reference drugs (positive controls). Control solutions consisted of the PRF vehicle (saline) and were injected via the same administration route, volume and treatment schedule. All drug doses used were selected according to previous literature.[9,10,19,20]

Behavioural tests

The animals were submitted to different tests classically used for the screening of psychoactive drugs, such as the open field, activity cage, ethyl-ether-induced hypnosis, apomorphine-induced stereotyped behaviour, elevated plus-maze, pentylenetetrazole-induced convulsions and rectal temperature. All tests were carried out during the light period of the cycle (between 10:00 and 17:00h), and were performed on different days, with independent groups of animals. The experiments were conducted in a sound-attenuated room under low-intensity light (12 lux). Behaviour was monitored through a video camera positioned above the testing apparatus and the images were analysed online, in an adjacent room, by an experienced experimenter who was unaware of the experimental groups. Each experimental group consisted of six to ten animals.

Spontaneous motor activity

Two tests were used to assess the effects of PRF (1–30 mg/kg, i.p.) on locomotor and exploratory activities of rats: the open field and the activity cage. The open field was used to investigate the effects of PRF on locomotor and exploratory activities induced by a novel environment.[21] The apparatus, made of wood and covered with impermeable Formica, had a 100 × 100 cm white floor (divided by black lines into 25 20 × 20 cm squares) and 40-cm high white walls. Each rat

was placed in the centre of the open field and allowed to freely explore the apparatus for 5 min. The total number of squares crossed and total number of rearings (vertical activity) were recorded during the open field test.

In order to complement the investigation of the effects of PRF on general locomotor activity of rats, an independent group of animals was tested on the activity cage as described previously.[22] The activity cage (70 × 30 × 22 cm) had a steel grid floor and was equipped with three parallel horizontal infrared beams positioned 3 cm above the floor and spaced evenly along the longitudinal axis. There was a digital counter that recorded photocell beam interruptions. Total locomotor activity was monitored every 5 min during a 30-min period. Haloperidol (5 mg/kg, i.p.) was used as the positive control in both experiments.

Ethyl ether-induced hypnosis

The animals were treated with PRF (3, 10 or 30 mg/kg, i.p.), and 30 min later they were placed in a glass container (30 × 20 cm) saturated with 10 ml of ethyl ether (10 min of saturation) as described previously.[23,24] The latency to the loss of righting reflex and the total duration of sleep were recorded using stopwatches. Sleeping time was measured by the loss of righting reflex, with the recovery of this reflex considered the hypnosis endpoint as previously described.[25] Diazepam (0.75 mg/kg, i.p.) was used as the positive control.

Apomorphine-induced stereotyped behaviour

The animals were isolated in Plexiglas cages for 45 min before injection of PRF (3, 10 or 30 mg/kg, i.p.), followed 30 min later by an injection of apomorphine (0.5 mg/kg, s.c.). Behaviour was observed for 10 s every 10 min for 90 min post apomorphine injection in wire mesh cages (15 × 31 × 26 cm). Stereotyped behaviour was scored according to the scale proposed by Setler *et al.*:[26] 0 = asleep or stationary, 1 = active, 2 = predominantly active but with bursts of stereotyped sniffing, rearing, or head-bobbing but with locomotor activity still present, 3 = constant stereotyped activity such as sniffing, rearing, or head-bobbing but with locomotor activity still present, 4 = constant stereotyped activity limited to one location, 5 = constant stereotyped activity but with bursts of licking and/or gnawing and biting, 6 = continual licking and/or gnawing of cage grids. The total sum of stereotypy scores obtained for each animal during the 90-min observation period was used to obtain the median value of stereotypy scores within each group. Haloperidol (5 mg/kg, i.p.) was used as the positive control.

Elevated plus-maze test

The effects of PRF (0.3, 1 or 3 mg/kg, i.p.) on anxiety-like behaviour were assessed using the elevated plus-maze test.[27] The apparatus was made of wood and consisted of two open arms (50 × 10 cm each) surrounded by a 1-cm high Plexiglas ledge, and two enclosed arms (50 × 10 × 40 cm each), elevated 50 cm above the floor. The intersection of the four arms (central platform) measured 10 × 10 cm. Each animal was placed in the centre of the maze facing an enclosed arm, and was observed for 5 min. The parameters of anxiety-like behaviour were defined as the percentage of open-arm entries relative to the total number of arm entries, and the percentage of time spent on open arms in relation to the total time spent on both arms. An arm entry was recorded when a rat placed all four paws onto an arm. An increase in these parameters indicated an anxiolytic-like effect.[27,28] The total number of closed-arm entries was used as an index of locomotor activity.[29] Diazepam (0.25 mg/kg, i.p.) was used as the positive control drug.

Pentylenetetrazole-induced convulsions

The animals were treated with PRF (3, 10 or 30 mg/kg, i.p.) and 30 min later their received a single injection of pentylenetetrazole (60 mg/kg, i.p.) for induction of seizures.[30] The following parameters were observed: total of deaths per group, total of animals which developed seizures per group, latency to and duration of the first clonic or tonic–clonic convulsion as well its duration (cut-off time: 1800 s). Diazepam (5 mg/kg, i.p.) was used as the positive control.

Rectal temperature

Rectal temperature was measured with a siliconised thermostat probe inserted 2 cm into the rectum of the animals as described previously.[31] Temperature recording was performed with a Dixtal digital thermometer. The probe was held in the rectum until a stable rectal temperature was measured for 20 s, with an accuracy of 0.1°C. The rectal temperature was recorded twice: before (T1) and 1 h after (T2) treatment. The temperature variation (ΔT) was estimated as ΔT = T2 – T1 for each animal.

Statistical analysis

Parametric data are presented as mean ± SEM, and the statistical analysis was carried out using one-way analysis of variance (ANOVA). Following significant ANOVAs, multiple post-hoc comparisons were performed using Dunnett's test. The effects of positive control drugs were compared separately against the vehicle control group using unpaired Student's *t*-test. Non-parametric data are presented as median values (25th and 75th percentile) and the statistical analysis was carried out using the Kruskal–Wallis test followed by Dunn's test. The effects of positive control drugs were compared separately against the vehicle control group using a Mann–Whitney test. The minimum accepted level of significance was $P \leq 0.05$. All analyses were performed using the Statistica software package (Stat Soft Inc., Tulsa, Oklahoma, USA).

Results

Effects of PRF on spontaneous motor activity

One-way ANOVA revealed a significant effect for the PRF treatment on the total number of squares crossed ($F(4,32) = 16.54$; $P \leq 0.001$) and total number of rearings ($F(4, 32) = 17.12$; $P \leq 0.001$)) in the open field (Table 1). Dunnett's test indicated that high doses of PRF (10 and 30 mg/kg, i.p.) decreased the locomotor activity of the rats in the open field, while an intermediate PRF dose (3 mg/kg, i.p.) increased locomotion. Moreover, Student's *t*-test indicated significant differences between vehicle- and haloperidol-

Table 1 Effects of the proanthocyanidin-rich fraction on the locomotor activity of rats evaluated in open field and activity chamber

	Open field arena	Activity chamber	
Treatment	Crossings	Rearing	Crossings
Saline	64.90 ± 8.45	13.80 ± 1.53	211.47 ± 17.44
PRF 1 mg/kg i.p.	80.00 ± 4.21	20.43 ± 2.48	178.17 ± 30.25
PRF 3 mg/kg i.p.	94.00 ± 5.03*	23.33 ± 3.05*	137.75 ± 21.36
PRF 10 mg/kg i.p.	37.83 ± 5.34*	6.50 ± 1.61*	111.75 ± 31.47*
PRF 30 mg/kg i.p.	20.12 ± 7.98*	3.50 ± 1.43*	31.12 ± 7.18*
Haloperidol 5 mg/kg i.p.	10.17 ± 1.99**	5.53 ± 1.23**	14.83 ± 2.89**

Proanthocyanidin-rich fraction (PRF) was administered at 1–30 mg/kg i.p. Evaluation for 5 min in open field and 30 min in activity chamber. Haloperidol (5 mg/kg, i.p.) was used as standard drug. Each value represents the mean ± SEM. of 6–10 rats. *$P \leq 0.05$ as compared to saline (one-way ANOVA followed by Dunnett's test). **$P \leq 0.05$ as compared to saline (Student's *t*-test).

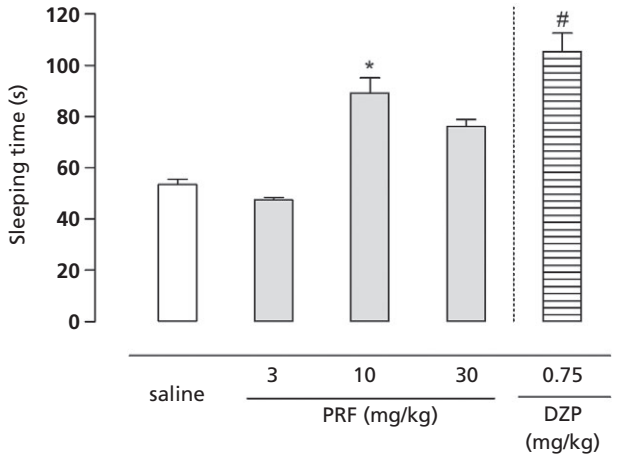

Figure 1 Effects of the proanthocyanidin-rich fraction from *Croton celtidifolius* on ethyl ether-induced hypnosis. Proanthocyanidin-rich fraction (PRF) was administered at 3–30 mg/kg i.p. Sleep time was used as the comparison parameter. Diazepam (DZP) at 0.75 mg/kg i.p., was used as the positive control. Each value represents the mean ± SEM. of 6–10 animals. *$P < 0.05$ as compared to saline (one-way ANOVA followed by Dunnett's test). #$P \leq 0.05$ as compared to saline (Student's *t*-test).

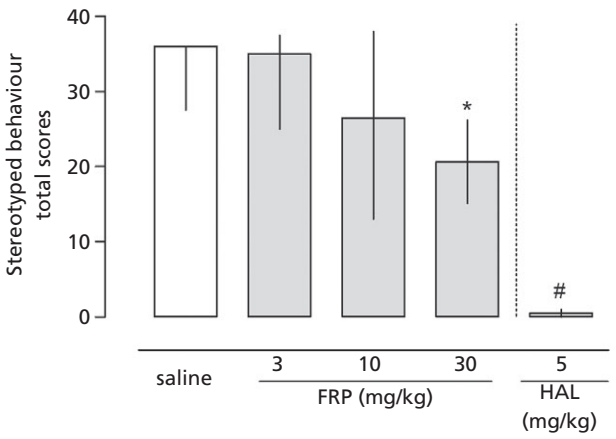

Figure 2 Effects of the proanthocyanidin-rich fraction from *Croton celtidifolius* on apomorphine-induced stereotyped behaviour. Proanthocyanidin-rich fraction (PRF) was administered at 3–30 mg/kg i.p. The sum of scores of stereotyped behaviours was used as the comparison parameter. Haloperidol (HAL) at 5 mg/kg i.p. was used as the positive control. Data are reported as the median (25th and 75th percentile) of 6–10 animals. *$P \leq 0.05$ as compared to saline (Kruskal–Wallis ANOVA followed by Dunn's test). #$P \leq 0.05$ as compared to saline (Mann–Whitney test).

treated groups in the total squares crossed ($t = 4.90$; $P \leq 0.001$) and rearings ($t = 3.85$; $P \leq 0.01$).

In the activity cage, one-way ANOVA revealed a significant effect for the PRF treatment ($F(4,40) = 10.60$; $P \leq 0.001$) in the total number of crossings (Table 1). Dunnett's test indicated that high doses of PRF (10 and 30 mg/kg, i.p.) decreased the locomotor activity of rats in this test. Haloperidol (5 mg/kg, i.p.) also reduced locomotion ($t = 7.01$; $P \leq 0.001$).

Effects of PRF on ethyl ether-induced hypnosis

One-way ANOVA revealed a significant effect for the PRF treatment on the duration of the hypnosis ($F(3,28) = 4.02$; $P \leq 0.05$) (Figure 1). However, PRF treatment did not alter the latency to the loss of the righting reflex ($P > 0.05$, data not shown). Dunnett's test indicated that PRF (10 mg/kg, i.p.) increased the duration of ethyl ether-induced hypnosis. Diazepam (0.75 mg/kg, i.p.) increased the total sleep duration compared to the control group ($t = -3.02$; $P \leq 0.01$).

Effects of PRF on apomorphine-induced stereotyped behaviour

As illustrated in Figure 2, the Kruskal–Wallis analysis revealed a significant effect for the PRF treatment on the total score of stereotyped behaviours ($H = 8.86$; $P \leq 0.05$). The subsequent Dunn's test indicated that PRF decreased the total score for apomorphine-induced stereotyped behaviours only at the highest dose tested (30 mg/kg, i.p.). Haloperidol (5 mg/kg, i.p.) significantly reduced the total score of stereotyped behaviours ($t = 0.00$; $P \leq 0.001$).

Effects of PRF on the elevated plus-maze test

Table 2 summarises the effects of PRF on anxiety-like responses in the elevated plus-maze test. One-way ANOVA revealed effect of PRF on the percentage of open-arms entries ($F(3,26) = 3.86$; $P \leq 0.05$), without modifying the percentage of open-arms time ($P > 0.05$), and on the total number of enclosed-arms entries ($P > 0.05$). Dunnett's test indicated that PRF (0.3 and 3 mg/kg, i.p.) increased the percentage of open-

Table 2 Effects of the proanthocyanidin-rich fraction on the behaviour of rats evaluated in elevated plus-maze

Treatment	% Open-arms time	% Open-arms entries	Total number of enclosed arms entries
Saline	24.65 ± 6.21	33.22 ± 5.33	8.37 ± 0.62
PRF 0.3 mg/kg i.p.	44.01 ± 5.96	50.13 ± 3.42*	6.33 ± 0.76
PRF 1 mg/kg i.p.	34.25 ± 5.12	43.74 ± 2.40	7.25 ± 0.56
PRF 3 mg/kg i.p.	41.81 ± 5.77	45.44 ± 3.25*	6.37 ± 0.68
Diazepam 0.25 mg/kg i.p.	61.03 ± 9.97**	59.36 ± 9.05**	6.57 ± 2.02

Proanthocyanidin-rich fraction (PRF) was administered at 0.3 – 3 mg/kg i.p. and rats were assessed in the elevated plus-maze for 5 min. Diazepam (0.25 mg/kg, i.p.) was used as the standard drug. Each value represents the mean ± SEM. of 6–10 rats. *$P \leq 0.05$ as compared to saline (one-way ANOVA followed by Dunnett's test). **$P \leq 0.05$ as compared to saline (Student's *t*-test).

Table 3 Effects of the proanthocyanidin-rich fraction on the behavioural parameters of rats evaluated by pentylenetetrazole-induced convulsions and rectal temperature

	Pentylenetetrazole-induced convulsions				Rectal temperature
Treatment	First seizure duration (s)†	Latency to first convulsion (s)†	Development of convulsion	Death	ΔT (°C)
Saline	10.50 (7–12.75)	68 (51–83.50)	6/6	3/6	0.25 ± 0.22
PRF 3 mg/kg i.p.	10.50 (7.50–12.75)	566.50 (58–1480)	5/6	1/6	0.00 ± 0.16
PRF 10 mg/kg i.p.	8.0 (4.5–13)	484 (98–1800)*	5/6	1/6	–0.45 ± 0.31
PRF 30 mg/kg i.p.	8.5 (5.25–11.25)	537 (237–1095)*	5/6	0/6	–1.28 ± 0.21**
Diazepam 5 mg/kg i.p.	0.0 (0.0-0–0)***	1800 (1800–1800)***	0/6	0/6	—
Diazepam 1.5 mg/kg i.p	—	—	—	—	–1.35 ± 0.55****

Proanthocyanidin-rich fraction (PRF) was administered at 3–30 mg/kg i.p. Each value represents the mean ± SEM of 6–10 rats. *$P \leq 0.05$ as compared to saline (Kruskal–Wallis ANOVA followed by Dunn's test). **$P \leq 0.05$ as compared to saline (one-way ANOVA followed by Dunnett's test). ***$P \leq 0.05$ as compared to saline (Mann–Whitney test). ****$P \leq 0.05$ as compared to saline (Student's *t*-test). †Data are reported as the median (25th and 75th percentiles).

arms entries, suggesting an anxiolytic-like effect. As expected, diazepam (0.25 mg/kg, i.p.) increased the percentage of open-arms time ($t = 3.39$; $P \leq 0.05$) and entries ($t = 3.39$; $P > 0.05$) without altering the total number of enclosed-arms entries ($t = 1.33$; $P = 0.23$).

Effects of PRF on pentylenetetrazole-induced convulsions

Kruskal–Wallis ANOVA revealed a significant effect for the PRF treatment ($H = 13.19$; $P \leq 0.005$) on the latency to the first convulsive episode induced by pentylenetetrazole (60 mg/kg, i.p.). However, PRF treatment did not alter the duration of the first convulsion ($P > 0.05$) (Table 3). Dunn's test confirmed that PRF (10 or 30 mg/kg, i.p.) increased the latency to the first convulsion and reduced the number of deaths. Moreover, diazepam (5 mg/kg, i.p.) completely prevented the convulsion episodes induced by pentylenetetrazole.

Effects of PRF on rectal temperature

One-way ANOVA revealed a significant effect for the PRF treatment on the rectal temperature ($F(3,20) = 8.59$; $P \leq 0.001$) (Table 3). Dunnett's post-hoc test confirmed that PRF decreased the rectal temperature of rats at the highest dose tested (30 mg/kg, i.p.). Diazepam (1.5 mg/kg, i.p.) also reduced the rectal temperature compared to the control group ($t = 5.14$; $P \leq 0.005$) (Table 3).

Discussion

In the present study, we investigated whether the PRF from *C. celtidifolius* displays psychoative actions in rats, since previous findings from our group have indicated the involvement of central pathways in its antinociceptive properties.[9] Taken together, the present findings demonstrate for the first time that the PRF displays a wide spectrum of neurobehavioural effects in rats. The high doses of PRF (10–30 mg/kg, i.p.) tested induced behavioural responses suggestive of CNS-depressant activity, while a moderate dose (3 mg/kg, i.p.) of the present fraction induced motor stimulation and anxiolytic-like effects when the animals were tested in the open field and the elevated plus-maze, respectively.

The evaluation of spontaneous motor activity in rodents has been used to analyse the putative central effects of drugs,[32] and agents that suppress motor activity may display sedative actions.[33] In the present study, the locomotion of independent group of animals was measured using two tests – activity cage and open field arena – aimed at evaluating the effects of PRF on general locomotion (long session, 30 min) and locomotion induced by novel environment (short session, 5 min), respectively. We demonstrated that a single i.p. administration of PRF to rats caused a dose-dependent decrease in the spontaneous motor activity observed in the activity cage. However, in the open field arena, the analysis revealed a hermetic-like biphasic dose response, in which lower doses of PRF significantly increased the locomotion and exploratory

behaviours while the higher doses decreased it. Such a biphasic curve, which is quite common among benzodiazepine agonists,[34,35] could be attributed to a reduction in anxiety in the new environment at lower doses and to a sedative effect at higher doses, which leads to a reduction in exploratory behaviour.

In order to further confirm this sedative effect of the PRF of *C. celtidifolius*, we performed an ethyl-ether-induced hypnosis test. In this test, it is known that a decrease in sleep latency and an increase in sleeping time are classically related to CNS-depressant drugs.[36] Ethyl-ether-induced hypnosis follows the same principle as hypnosis induced by barbiturates.[28] However, due to the possibility of false positives due to pharmacokinetic interactions occurring when barbiturates are used as hypnotic agents[37,38] and the fact that many phenolic compounds are known to inhibit enzymes and proteins,[39] we chose to use ethyl ether as the sleep inducer, since this agent is not metabolised in the liver. Our results showed that PRF prolonged ethyl-ether-induced sleep in rats, confirming its hypnosedative property.

To evaluate if the sedative profile of PRF could be due to neuroleptic activity, we analysed the effect of this fraction on apomorphine-induced stereotyped behaviour. The property of a drug to antagonise apomorphine-induced stereotyped behaviour in rodents has been correlated with potential antipsychotic actions.[40] Our findings showed that the highest dose of PRF (30 mg/kg, i.p.) tested was able to decrease the apomorphine-induced stereotyped behaviour, an effect completely prevented by a typical antipsychotic, haloperidol, which is a dopamine D_2 receptor antagonist.[41] The effects of PRF seem to be specific, since it promotes behavioural responses in a dose-related manner and no adverse events, such as writhing-induced hypoactivity and diarrhoea, were observed after the i.p. treatments.

Based on the biphasic curve observed on the open field, we next evaluated the possible anxiety-like effects of PRF. The elevated plus-maze is the most popular test for identifying new benzodiazepine-like anxiolytic agents and is based on the fact that rodents are extremely averse to open spaces.[42] Increased exploration in the open areas of the elevated plus-maze suggests an anxiolytic-like effect.[27] We demonstrated that lower doses of PRF significantly increase the percentage of entries in the open arms and that they tend to increase the percentage of time spent on the open arms of the elevated plus-maze, indicating a putative anxiolytic-like profile. These results were not due to general locomotor activity stimulation, since PRF did not alter the number of entries into the enclosed arms, a well-accepted index of motor activity in this test.[28]

The pentylenetetrazole-induced convulsions test is one of the main acute experimental models used in the preliminary screening of potential anticonvulsant drugs.[43] Moreover, it is believed that pentylenetetrazole exerts its action by antagonising the $GABA_A$–receptor complex.[44] Here, we demonstrated that PRF pretreatment increased the latency to the first convulsive episode, suggesting a partial anticonvulsant effect. It has been suggested that hypothermia may be useful in reducing seizures and associated brain damage and that hyperthermia should be avoided in the status epilepticus.[45,46] The beneficial effect of hypothermia in attenuating pentylenetetrazole-induced kindling has been previously demonstrated.[47] Our findings showed that the highest tested dose of PRF (30 mg/kg, i.p.) was able to promote a significant hypothermic effect, a well-known common effect of benzodiazepines,[48] which we again demonstrated here when administering the standard drug diazepam.

Interestingly, one of the main compounds present in the PRF from *C. celtidifolius*, the catechins, have already been associated with the CNS-depressant effects of plant extracts. For example, Chang *et al*. demonstrated that the activity of the locus coeruleus is inhibited by various catechins,[49] which may be one of the underlying mechanisms accounting for the sedative and/or anxiolytic effects observed in the present behavioural tests. However, the molecular bases for these actions are not yet fully understood. Some possibilities include direct interaction with voltage-gated channels and neurotransmitter receptors,[49] such as $GABA_A$ receptors, the μ-opioid receptor and the α2-adrenoceptor.[50–52]

As mentioned above, several well-known flavonoids – like the catechins – exert CNS effects, acting as analgesics, anxiolytics and sedatives.[16,17] Some of the pharmacological properties exhibited by the flavonoids have been correlated with good affinities for the benzodiazepine binding site at the $GABA_A$ receptors,[53,54] suggesting a pharmacological mechanism for the present results that needs to be further investigated in future studies. The particular effects of flavonoids at the $GABA_A$ receptors have been widely reported.[17,55] Fernandez *et al*. demonstrated that flavan-3-ol derivatives are positive modulators of $GABA_A$ receptors, with higher efficacy for the α_2 subtype and anxiolytic action in mice.[13] Moreover, several studies demonstrated anxiolytic actions[18] and sedative/hypnotic actions of some catechins, partially through the $GABA_A$ receptors.[56]

Besides the interaction with the GABAergic system, some catechins are also described as interacting with the glutamatergic system. Bae *et al*. reported that (–)-epigallocatechin gallate may inhibit spontaneous excitatory synaptic transmission, due to a direct antagonism of glutamate AMPA receptors, and it also reduces the glutamate-induced calcium influx in PC12 cells.[57,58]

The CNS actions of some flavonoids are also supported by studies of bioavailability, which demonstrate that flavonoids, including catechins, are membrane-permeable and cross the blood–brain barrier, accumulating in the brain following systemic administration.[59,60] Furthermore, flavonoids, including epicatechin, can be transformed *in vivo* by glucuronidation.[61] Corroborating these findings, a previous study has demonstrated the presence of epicatechin glucuronide and 3′-O-methyl epicatechin glucuronide in the rat brain after oral ingestion of epicatechin.[62] Thus, it is possible that catechin glucuronides may exert extracellular effects, such as those involving receptor binding. For example, apigenin, naringenin, kaempferon and quercetin-3-O-β-D-glucoside bind to benzodiazepine-binding sites of different receptors, including the $GABA_A$ receptor.[18,19]

Conclusions

In summary, the present findings indicate that the proanthocyanidin-rich fraction isolated from *Croton celtidifolius* bark promotes hypnosedative, anticonvulsant, antipsy-

chotic and anxiolytic-like effects in rats. These effects seem to be linked to the presence of proanthocyanidins and catechins in this fraction, but further studies are necessary to confirm the effects of the isolated compounds. The search for a pharmacological mechanism will certainly start with candidate targets within the GABAergic system, in line with previous evidence.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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