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In vitro evaluation of antioxidant, anxiolytic and antidepressant-like effects of the *Bellis perennis* extract

Thiago Henrique Costa Marques,[1] Cássio Herbert Santos de Melo,[2] Rivelilson Mendes de Freitas*,[2]

[1]Departamento de Bioquimica e Farmacologia, Programa de Pós-graduação em Farmacologia, Universidade Federal de Piauí, Brazil,
[2]Departamento de Química, Universidade Federal de Piauí, Brazil.

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Abstract: Acute toxicity, antioxidant activity *in vitro* and general pharmacological effects of the flower crude ethanolic extract of *Bellis perennis* L., Asteraceae, a popular medicine used in South America, were investigated in mice. The oral route LD50 value was found 2.31 g/kg. Oral administration at doses 50, 100 and 150 mg/kg of the extract neither caused significant changes in general behavior nor led to toxic symptoms. Anxiolytic-like properties were studied in the open field test and the possible antidepressant-like actions were evaluated in the forced swimming test (FST). There is a significant decrease in the number of crossings at all dosages mentioned above, but no sedative effects at any dosages when compared to controls. In the FST, the extract dosage of 150 mg/kg was effective in reducing immobility, along with a significant increase in swimming time. The ethanolic extract showed strong antioxidant potential in vitro, through the removal capacity against hydroxyl radicals and nitric oxide as well as prevented the formation of reactive substances to thiobarbituric acid (TBARS). Together, these results indicate that the ethanolic extract has effect on central nervous system, which might due to its antioxidant property, as demonstrated *in vitro* methods used. These results suggest that some of the components in ethanolic extract of *B. perennis*, such as alkaloids, phenolic compounds and flavonoids may have antioxidant, anxiolytic and antidepressant-like properties. Additional investigations are in progress.

Introduction

Bellis perennis L., Asteraceae, is a perennial native herbaceous plant in Brazil, Europe, Turkey, Cyprus, Syria, and Azerbaijan (Avato & Tava, 1995). It has been used in folk medicine in the treatment of rheumatism and as an expectorant (Schopke et al., 1991; Hansel et al., 1992), antiecchymotic (Avato & Tava, 1995), to alleviate stomachache and has antiulcerogenic (Yazıcıoğlu & Tuzlacı, 1995), antifungal (Desevedavy et al., 1989) and anticonvulsant effects (Marques et al, 2011).

Numerous psychoactive plants with effects on central nervous system have been studied. Extensive preclinical *in vitro* and *in vivo* studies have been conducted to validate phytotherapies of plants extracts (Kumar, 2006). Although it is possible to isolate single active compound, it is more common for plant extracts to contain many potential psychoactive components (Sarris et al., 2011). At clinical application, co-administration of herbal medicine and Complementary and Alternative Medicine is prevalent amongst sufferers of anxiety and depression.

Mood, anxiety, and sleep disorders are prevalent and highly comorbid psychiatric conditions (Kessler et al., 2005) that have been treated with botanical medicines since antiquity. Data from a nationally representative sample of 2055 people interviewed during 1997-1998 revealed that 57% of those suffering anxiety attack, and 54% of those with severe depression reported using herbal medicine and Complementary and Alternative Medicine therapies during the previous 12 months to treat these disorders (Kessler et al., 2001). In addition, depression, which is thought to result from brain biochemical changes, is a common disease of adulthood (Richelson, 1991; Greenberg et al., 1993; Judd, 1995; Baker et al., 2000). This affective disorder afflicts about 5.1 to 15.7% of the adult population in the Brazil at any specific time (Mello et al, 2007). Plant extracts are some of the most attractive sources of new drugs, and have been shown to produce promising results for the treatment

of depression (Ernst, 1995; De Smet & Nolen, 1996).

Depression is often referred to as the common cold of psychopathology (Charney & Weissman, 1988; Gilbert, 1992). In the United States, more than 14% of adults are 18 years-old and older and 13.7% of youth's ages 12-17 had at least one major depressive episode in their lives (Substance Abuse and Mental Health Services Administration, 2006). Besides those clinical depressions, everybody experiences depressive symptoms, such as feeling of sadness, worthlessness, helplessness and loneliness in some degree (Munoz, 1987).

Ethnobotanical researches are often significant in revealing locally important plant species, especially for the discovery of crude drugs (Ferreira et al., 2009; Costa Júnior et al., 2010). The documentation of traditional knowledge, especially on the medicinal uses of plants has provided many important modern drugs (Cox, 2000; Flaster, 1996). The modern pharmacopoeia still contains 25% of the drugs derived from plants and many others are synthetic analogues base on plant prototype compounds. So, the traditional medicine still remains the main resource for a large majority of the people in developing countries for their primary health care (Danøe & Bøgh, 1999; World Health Organization, 2002; Ferreira et al., 2008). More than 50,000 flowering plants are used for medicinal purposes across the world (Govaerts, 2001; Schippmann et al., 2002).

Because medicinal plants continue to be culturally suitable as treatments for several illnesses, it is important to record their uses and perform studies about their toxicity and pharmacological activities to ensure their efficacy and safety (Andrade-Cetto, 2009). Pharmacological studies involving medicinal plants have been the topic of several analyses in scientific meetings in Brazil. However, this issue is still polemic, especially when the question analyzed deals with strategies to be employed in this kind of study, the extracts to be prepared, the animal models to be used, the reproducibility of this studies and, finally, the posology employed by folk medicine (Sousa-Brito, 1996).

The objective of the present work was to determine the antioxidant activity *in vitro*, the acute toxicity (LD50) as well as evaluate the effects produced by the acute administration of ethanolic extract of flowers from *B. perennis* using the open field and forced swimming tests in order to assess the anxiolytic and antidepressant activities of this medicinal plant, in attempting to clarify their mechanism of action.

Materials and Methods

Drugs

The drugs used were reserpine, paroxetine, imipramine, and polyoxyethylene-sorbitan monolated (Tween 80) purchased from Sigma (USA), flumazenil and diazepam from Cristália (Brazil). These agents were administrated by intraperitoneally (*i.p.*) route at a dose volume of 0.1 mL/10 g.

Plant material and extract preparation

The plant was collected in October 2010, in Pacoti, state of Ceará, Brazil. The voucher specimen has been identified and deposited at the Graziela Barroso Herbarium at the Federal University of Piauí (voucher number 27276).

The flowers collected from *Bellis perennis* L., Asteraceae, were dried at 55 ºC under shade and powdered mechanically. Crush yielded of flowers (310 g) was extracted with hexane (57%, w/w), followed by ethanol (EtOH) (3.2%, w/w) in a Soxhlet apparatus (8 h for each solvent). The extract was concentrated in a vacuum evaporator. The dried extract was kept at 4 ºC in refrigerator in the air tight bottles until use.

Phytochemical screening of ethanolic extract of flowers from B. perennis

The ethanolic extract was screened for the presence of unsaturated sterols, triterpenes, alkaloids, flavonoids, lactones/esters, and protein/amino acids and carbohydrates and/or glycosides (Stahl, 1969).

Determination of antioxidant activity of ethanolic extract of flowers from B. perennis *against hydroxyl radical formation*

The formation of hydroxyl radical ($OH^{\bullet}$) was quantified using the oxidative degradation of 2-deoxyribose (Lopes et al., 1999). The principle of the antioxidant activity of ethanolic extract of flowers from *B. perennis* was measured using 2-deoxyribose degradation. It was determined the level of malondialdehyde (MDA) produced by its condensation with 2-thiobarbituric acid (TBA). Briefly, reactions were initiated by adding Fe^{2+} 6 mM final solutions containing 2-deoxyribose 5 mM, H_2O_2 100 mM and phosphate buffer (pH 7.2) 20 mM. To determine the antioxidant activity of ethanolic extract at concentrations of 10, 25, 50, 100 and 150 mg/mL against the formation of the hydroxyl radical. The absorbance was measured at 532 nm and results were expressed as percentages relative to the reaction standard.

Determination of antioxidant activity of ethanolic extract of flowers from B. perennis *scavenger against thiobarbituric acid reactive species (TBARS)*

The thiobarbituric acid reactive species assay was used to quantify lipid peroxidation and

adapted according to the method previously described (Esterbauer & Cheeseman, 1990). Lipid peroxidation was induced by adding 0.1 mL of the dihydrochloride of 2,2'-azobis-2-aminopropane (AAPH) 0.12 M. The absorbance of the concentrations of 10, 25, 50, 100 and 150 mg/mL of the ethanolic extract from *B. perennis* was measured using a spectrophotometer Biospectro SP 220 to 532 nm. The results were expressed as percentage of thiobarbituric acid reactive species (TBARS) formed compared to that produced by AAPH alone (reaction medium).

Determination of antioxidant activity of ethanolic extract of flowers from B. perennis *scavenger against nitric oxide (NO) content*

Nitric oxide was generated by decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4) by Griess reaction (Basu & Hazra, 2006). The absorbance of the concentrations of 10, 25, 50, 100 and 150 mg/mL of the ethanolic extract from *B. perennis* was measured using a spectrophotometer Biospectro SP 220 to 540 nm. The results were expressed as percentage of nitrite formed compared to the sodium nitroprusside (NPS) alone (reaction medium).

Animals

Male Swiss mice (25-30 g, two months-old), were used. The animals were randomly housed in appropriate cages at 26±1 °C under 12 h light/dark cycle (lights on 06:00 am-18:00 pm) with access to food (Purina®) and water *ad libitum*. All experiments were carried out 8 am in a quiet room. Experimental protocols and procedures were approved by the Ethics Committee on Animal Experiments at the Federal University of Piauí (CEEA/UFPI # 077/2010). All procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Behavioral screening

Behavioral screening was performed following parameters described by Almeida et al. (1999) and animals were observed at fourteen days consecutive after oral route (*p.o.*) administration of ethanolic extract from *B. perennis* (50, 100 and 150 mg/kg, *p.o.*). During fourteen consecutive days, the animals were observed to detect general signs of toxicity: piloerection, prostration, writhing, increased evacuation, grooming, discrete groups, dyspnea, sedation, analgesia and palpebral ptosis. The spontaneous locomotor activity of the animals was assessed in a cage activity (50 × 50 × 50 cm) after 30 min of the last dose of treatment (Asakura et al., 1993).

Acute toxicity (LD50) and effect on general behaviour

The acute oral LD50 and the general behaviour activity of the crude ethanolic extract were evaluated in Swiss adult albino mice of both sex and weighing between 25 and 30 g. A 12 h dark-light cycle, 26±1 °C temperature and 50-60% humidity were provided. The animals received standard food and before the experiments they were fasted overnight with water *ad libitum.* The ethanolic extract was suspended in 0.05% Tween 80, dissolved in 0.9% saline and administered orally in growing doses (500, 1000, 2000 and 3000 mg/kg). Mice were kept under observation for fourteen days (Hamilton et al., 1977).

Open field test

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), diazepam (1.0 mg/kg, *i.p.*), flumazenil (2.5 mg/kg, *i.p.*), and ethanolic extract from *B. perennis* (50, 100 and 150 mg/kg, *i.p.*), that received treatment acute with single dose before of open field test. The open-field arena was made of acrylic (transparent walls and black floor, 30×30×15 cm), divided into nine squares of equal areas. The lighting in the center of the open field used was 7 lux. The open-field was used to evaluate the exploratory activity of the animal (Archer, 1973). After 30 min the mouse was placed individually into the center of the arena, and allowed to explore it freely. The observed parameters were: ambulations (the number of squares crossed with all four paws), numbers for grooming and rearing, recorded for 5 min testing period.

Experimental protocols

Animals were treated with ethanolic extract and submitted to the open-field test 30 (*i.p.*) min later. Another group of animals was submitted to the forced swimming test. Controls were administered with vehicle (0.05% Tween 80 dissolved in 0.9% saline; vehicle group). In order to elucidate the possible mechanism involved in the anxiolytic effects of the ethanolic extract from *B. perennis*, we used diazepam and flumazenil administered alone or associated with ethanolic extract in the open-field test.

Forced swimming test (FST)

Mice were randomly allocated to the following groups: control (0.05% Tween 80, dissolved in 0.9% saline, *p.o.*), imipramine (50 mg/kg, *i.p.*), paroxetine (20 mg/kg, *i.p.*), reserpine (0.25 mg/kg, *i.p.*), and ethanolic extract from *B. perennis* (50, 100 and 150 mg/kg, *i.p.*), that received treatment acute with dose

single before of behavioural test. This test is the most widely used and recognized pharmacological model (Porsolt et al., 1977a) for assessing antidepressant activity (Porsolt et al., 1977b; Porsolt et al. 1978). The development of immobility when mice were placed inside an inescapable cylinder filled with water reflects the cessation of persistent escape-directed behavior. Briefly, mice were individually placed in a circular tank (46 cm tall×20 cm in diameter) filled with tap water (26±1 °C) to a depth of 20 cm and left there for 5 min. During this period the behavior of the animals was recorded by an observer. Mice were considered immobile when remained floating without struggling and making only slight movements necessary to maintain the head above the water.

Experimental protocols

Animals were treated with ethanolic extract and submitted to the forced swimming test 30 (*i.p.*) min later. Controls were administered with vehicle (0.05% Tween 80 dissolved in 0.9% saline; vehicle group). In order to elucidate the possible mechanism involved in the antidepressant effect of the ethanolic extract, imipramine and paroxetine alone or associated with ethanolic extract were used in the forced swimming test.

Imipramine was chosen because this drug is a classical antidepressant that acts through noradrenergic and serotonergic pathways. Paroxetine, another antidepressant drug, is more selective, and acts predominantly through serotonergic pathways. Both antidepressants were then used, alone as positive controls or in combination with higher dose of ethanolic extract to investigate alteration/interference of imipramine or paroxetine on the antidepressant effect of ethanolic extract. Reserpine, a drug known to cause depletion of biogenic amines (noradrenaline, dopamine and serotonin) from storage granules, was also used to evaluate the participation of those amines in the ethanolic extract antidepressant effect. In a combination protocol, imipramine, paroxetine or reserpine was administered 10 min before ethanolic extract, and the test was performed 30 min later. The dose of imipramine, paroxetine and reserpine was determined from previous studies (Campos et al., 2004; Wesolowska et al., 2006) and pilot experiments.

Statistical analyses

The data obtained were evaluated by one-way analysis of variance (ANOVA) followed by *t*-Student-Neuman-Keuls as *post hoc* test. Differences were considered to be statistically significant when $p<0.05$.

Results

The phytochemical screening of ethanolic extract from *B. perennis*, Asteraceae, revealed the presence of alkaloids, phenolic compounds, flavonoids, carbohydrates, steroids and proteins.

The ethanolic extract (10, 25, 50, 100, 150 mg/mL) produced a significant decrease in TBARS production at all concentrations tested, while Trolox produced a decrease of 42.64% in the production of TBARS (Table 1).

Table 1. Antioxidant activities *in vitro* of the ethanolic extract from *Bellis perennis*.

Groups	Dose (mg/mL)	Percentage *in vitro* scavenging activities		
		Nitric oxide	Hydroxyl radical	TBARS assay*
System	-	99.90±1.35	100.20±1.34	99.90±1.32
Ethanolic extract	10	65.30±2.87[a]	87.23±1.29[a]	76.16±2.70[a]
	25	59.01±3.68[a]	83.30±2.64[a]	76.08±5.98[a]
	50	53.66±3.93[a]	81.49±4.03[a]	72.73±7.09[a]
	100	52.59±3.84[a]	69.64±2.27[a]	67.50±1.74[a]
	150	51.91±2.38[a]	58.85±2.54[a]	32.44±4.24[a]
Trolox	0.45	42.54±2.82[a]	28.94±0.96[a]	57.36±6.48[a]

*TBARS - Thiobarbituric acid reactive species; Antioxidant activities of ethanolic extract on TBARS level, NO- and OH-scavenging activities *in vitro*. Control means basal NO production of vehicle (Tween 80 0.05% dissolved in 0.9% saline) in the absence of a NO generator source (without SNP); SNP group reflects nitrite production by sodium nitroprusside alone, considered 100% of NO production. The effect of different concentrations of ethanolic extract against SNP was determined by the Griess method. Hydroxyl radical scavenging activity was quantified using 2-deoxyribose oxidative degradation *in vitro*, which produces malondialdehyde by condensation with 2-thiobarbituric acid (TBA). System is MDA production from 2-deoxyribose degradation with $FeSO_4$ and H_2O_2 alone. Other groups denote MDA production by $FeSO_4$ and H_2O_2 in the presence of different concentrations of ethanolic extract (10, 25, 50, 100 and 150 mg/mL). Lipid extracted from egg yolk was subjected to oxidative damage by incubation with AAPH and the ability of different concentrations of ethanolic extract to prevent TBARS formation was analysed. Control means basal lipid peroxidation with vehicle alone (Tween 80 0.05% dissolved in 0.9% saline); AAPH alone group is considered 100% of oxidative damage. Values represent mean±S.E.M., n=10, experiments in duplicate. [a]$p<0.001$ versus System; (ANOVA and t-Student-Neuman-Keuls as *post hoc* test).

The results presented in Table 1 show that the ethanolic extract (10, 25, 50, 100, 150 mg/mL) produced hydroxyl radical removal at all concentrations tested when compared to the system group. The concentration of 150 mg/ml of the ethanolic extract produced the largest increasing in removal of hydroxyl radical when compared to other concentrations. Trolox already produced hydroxyl radical removal (71.06%).

The ethanolic extract (10, 25, 50, 100, 150 mg/mL) was able to reduce nitrite production at all concentrations tested, as shown in Table 1. The Trolox, a synthetic analogue of α-tocopherol used as standard antioxidant, decreased by 57.46% the production of nitrite.

Ethanolic extract from *B. perenni*s at doses of 50, 100 and 150 mg/kg (*p.o.*) showed no behavioral changes in animals during 14 days of treatment. The oral route LD50 value was 2.31 g/kg of body weight (1.76-3.03 g/kg - 95% confidence limit). Oral administration of doses up to 2.0 g/kg did not show any toxic symptom in mice.

Ethanolic extract from *B. perennis* at doses of 50, 100 and 150 mg/kg showed no sedative effects as assessed by the open-field test (Figure 1). Significant effects were detected with at doses tested that produced similar percentages of decrease (57, 58 and 55%, respectively) in the number of crossings ($p<0.05$). The number of rearing in ethanolic extract groups was reduced of 59, 55 and 56% with at doses 50, 100 and 150 mg/kg ($p<0.05$), respectively, whose results were similar to those observed with diazepam (positive control). The numbers of crossings, grooming and rearings in diazepam group were decreased ($p<0.05$). In addition, the number of grooming behavior in ethanolic extract groups was increased of 166, 181 and 216% with at doses 50, 100 and 150 mg/kg respectively, when compared to the diazepam group ($p<0.05$; Figure 1).

Flumazenil was used for evaluating the mechanism of action about the ethanolic extract anxiolytic effect. So, 15 min after the flumazenil injection, ethanolic extract (150 mg/kg) was administered, and under these conditions, animals' behavior was similar to the alone ethanolic extract 150 group, indicating that their action mechanism cannot have involvement of GABAergic system (Figure 1).

The ethanolic extract possible antidepressant effect was studied in the forced swimming test (Table 2). Under this condition, ethanolic extract was used at doses (50, 100 and 150 mg/kg, *p.o.*), since at these doses the sedative and anxiolytic effects of ethanolic extract do not mask the antidepressant activity. Significant decreases of 24 and 28% in the immobility time were also observed after ethanolic extract administration (150 mg/kg) when compared to the ethanolic extract 50 mg/kg and 100 mg/kg groups, respectively (Table 2, $p<0.05$).

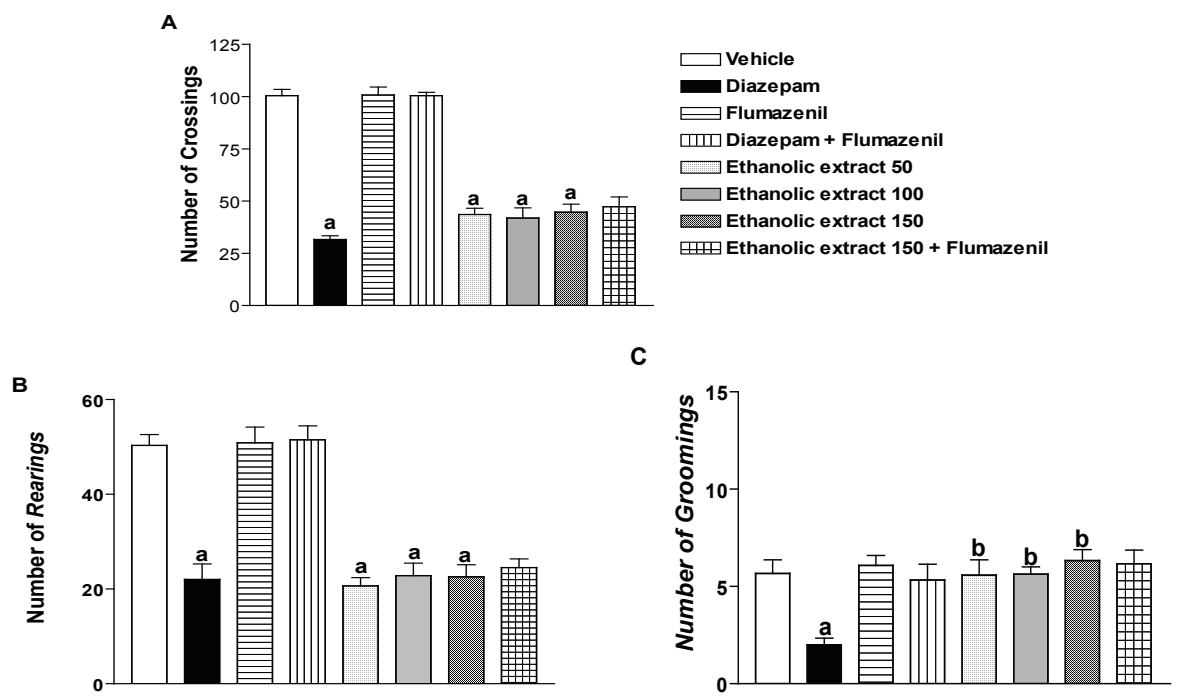

Figure 1. Effects of ethanolic extract from *Bellis perennis* in the open field test, in mice. A. Number of crossings; B. Number of rearing; C. Number of grooming. Control: tween 80 0.05% dissolved in saline 0.9%; ethanolic extract, 50, 100 and 150 mg/kg (*p.o.*); diazepam, 1 mg/kg (*i.p.*); flumazenil, 2.5 mg/kg (*i.p.*). Drugs were administered 10 min before ethanolic and the test performed 30 min later. [a]$p<0.05$ as compared to controls; [b]$p<0.05$ as compared to diazepam group (ANOVA followed by t-Student-Newman-Keuls as *post hoc* test).

Table 2. Antidepressant effects of ethanolic extract from *B. perennis* in the forced swimming test, in mice, and the possible involvement of noradrenergic and/or serotonergic systems.

Treatments	Time of Immobility (s)	Values of Immobility (%)
Vehicle (24)	195.00± 2.60	-
Ethanolic extract 50 (12)	81.90± 9.70[a]	↓58a
Ethanolic extract 100 (12)	85.70± 9.50[a]	↓56[a]
Ethanolic extract 150 (12)	62.40± 13.40[a]	↓68[a] ↓24[c] ↓28[d]
Imipramine 50 (12)	82.20± 1.80[a]	↓58[a]
Imipramine 50+ethanolic extract 150 (12)	20.67± 1.76[a,b]	↓67[a] ↓75[b]
Paroxetine 20 (12)	72.20±1.80[a]	↓58[a]
Paroxetine 20+ethanolic extract 150 (12)	66.15±17.10	NS
Reserpine 0.25 (12)	192.20±1.80	NS
Reserpine 0.25+ethanolic extract 150 (12)	189.60± 15.00	NS

Experiments performed as described in materials and methods. Ethanolic extract was administered oral route. Values are the mean±SEM. of the immobility time. In parenthesis is the number of animals per group. Drugs were administered 10 min before ethanolic extract and the test performed 30 min later. [a]$p<0.05$ and [b]$p<0.01$ as compared to controls (ANOVA followed by *t*-Student-Newman-Keuls as *post hoc* test). [c]$p<0.05$ and [d]$p<0.01$ as compared to ethanolic extract 50 mg/kg and 100 mg/kg groups (ANOVA followed by *t*-Student-Newman-Keuls as *post hoc* test).

The results showed that ethanolic extract presents a significant antidepressant effect at doses of 50, 100 and 150 mg/kg, suggested by the respective decrease in 58, 56 and 68% of the time of immobility. The association of ethanolic extract 150 mg/kg with imipramine showed a greater decrease of the immobility time in 67 ($p<0.01$) and 75% ($p<0.01$), respectively, in comparison with the groups treated with ethanolic extract alone (150 mg/kg) or imipramine (50 mg/kg) alone. However, the association of ethanolic extract with paroxetine did not alter the effect observed with ethanolic extract or paroxetine alone ($p>0.05$), suggesting that the serotonergic system is not involved in the ethanolic extract antidepressant effect. On the contrary, the ethanolic extract activity was totally blocked by the previous administration of reserpine. These data suggest that the noradrenergic system participates in the ethanolic extract antidepressant action.

Discussion

In this paper, we reported a preparation of the ethanolic extract from *Bellis perennis* L., Asteraceae. So, we utilized the method previously described by Costa Júnior and coworkers (Costa Júnior et al., 2011), and in our study the antioxidant activity *in vitro* and some of the neuropharmacological properties of this extract were analyzed. Since that the determination of preliminary acute toxicity indicated LD_{50} values of 2.31 g/kg, we evaluated the behavioral effects of ethanolic extract at lower doses.

Firstly, in our study we evaluated the antioxidant activity of ethanol extract on TBARS assay, which is a method used to quantify lipid peroxidation during oxidative stress. The ethanolic extract was able to prevent lipid peroxidation induced by this method. This result suggests that the ethanolic extract may exert protective against lipid peroxidation.

In our study also showed that the ethanolic extract produces the removal of the hydroxyl radical, resulting in a significant antioxidant activity against this radical. Finally, we evaluated the antioxidant effects of ethanolic extract against NO production. The ethanolic extract in different concentrations tested produced action scavenger of NO, leading to decreasing the nitrite production, suggesting antioxidant activity *in vitro*.

This antioxidant activity *in vitro* may be associated with the effects on central nervous system that has been demonstrated in our experiments performed *in vivo*. The central effects of the ethanolic extract from *B. perennis* were evaluated. Ethanolic extract was firstly evaluated on the open-field test, which provides a good indication of the animal's emotional state (Hall, 1936). The results showed that ethanolic extract was able significantly decrease not only the number of crossings, but also the number of rearing that is indicative of a possible sedative effect and muscle relaxant.

Our results in open-field test suggest a possible sedative effect for the ethanolic extract of *B. perennis*. However, the action mechanism of the sedative effect of this compound has not been elucidated yet. Probably, this effect could be produced through an effect upon the neuronal excitability by ionic channel modulation, since the voltage gated K^+ channels from the Kv family are involved in the neuronal signaling (Costa et al., 1996).

The grooming behaviour is related to arousal following exposure to stress. This grooming behaviour can to be elicited from paraventricular nucleus of the hypothalamus due to high activation of hypothalamic-pituitary-adrenocortical axis (HPA) (Do-Rego et al., 2005). The absence of effect of ethanolic extract from *B. perennis* in the doses tested on grooming behaviour suggests that this extract does not exert its pharmacological activity mediated by activation of the HPA system, since it does not was detected any change in the number of groomings of animals treated with the extract.

Increased number of lines crossed (horizontal

movement) is an indicative of central nervous system stimulant properties. Similarly, the rearing (vertical movements) is an indicative of tendency of locomotion (Vogel, 2002). The treatment with diazepam, an anxiolytic drug, decreased all these parameters significantly when compared with vehicle group. Treatment with ethanolic extract at doses of 50, 100 and 150 mg/kg significantly decreased rearing and ambulatory behavior ($p<0.05$) well. These findings suggest a possible anxiolytic effect of the extract. In our behavioral tests we observed that animals treated with ethanolic extract remained longer and made a greater number of crossings in the central area, suggesting a possible anxiolytic effect for the specie studied.

Flumazenil reversed only the diazepam effect but do not change ethanolic extract effects, indicating that both drugs do not possess a similar mechanism of action. In order to study the possible anxiolytic effect of ethanolic extract, the elevated-plus-maze test was used, and the results showed that ethanolic extract did not increase the time of permanence and it did not alter the number of entrances in the open arms, representing a negative response (data not shown). Our results point out that the antianxiety activity of extract in study no involves the GABAergic system.

Several neurotransmitters are believed to be involved in the pathophysiology of depression including serotonin, noradrenalin and dopamine (Dailly et al., 2004; Moltzen & Bang-Andersen, 2006). The monoamine hypothesis is based on the assumption that depression is due to deficiency of one or another of these neurotransmitters (Hindmarch, 2002; Rang et al., 2007).

Preparations from traditional medicinal plants are often used for neurodegenerative disorders covering a broad area of different diseases. Schmidt and coworkers (Schmidt et al., 2009) reported these aspects on screening studies focusing on 24 extracts prepared from twelve plants which are used in traditional South Brazilian medicine. The plant species belong to different families and are expected to contain different effective compounds. The use is often based on folklore without any scientific evidence of efficacy and the effective compounds are unknown.

Traditional anxiety models include exploration-based paradigms (*e.g.*, open field, holeboard, elevated plus maze, light-dark box, mirrored chamber, social interaction tests) and conditioned or unconditioned threat response. Popular experimental models of depression include "despair" paradigms (such as Porsolt´s forced swim, tail suspension tests and learned helplessness), as well as olfactory bulbectomy, maternal/social deprivation and "anhedonic" chronic mild stress (Kaluef et al., 2007).

Antidepressants compounds are known to act by several distinct mechanisms at the receptor level, probably also stimulating similar pathways at the sub-cellular level (Yildiz et al., 2002). Ethanolic extract was also able to decrease the immobility time of mice (50 100 and 150 mg/kg) in the forced swimming test. At higher doses (150 mg/kg), the antidepressant effect was not masked by sedative and hypnotic actions of the drug. The ethanolic extract effect was increased by treatment with imipramine, a tricyclic antidepressant that blocks the reuptake of both serotonin and norepinephrine. However, there were no alterations after association ethanolic extract with paroxetine, a known selective serotonin reuptake inhibitor. In our experiments were associated effective doses of ethanolic extract with paroxetine or imipramine in order to clarify the extract's mechanism of action. In addition to imipramine, a synergist effect could be observed, with increased reduction in immobility time. The synergistic profile, however, was not observed when paroxetine was administered in association with ethanolic extract, suggesting that both paroxetine and ethanolic extract no share a common mechanism of action, once the co-administration of effective doses of both resulted in no synergistic effect.

Additionally, ethanolic extract effects were totally blocked by the reserpine pretreatment, a known inhibitor of the vesicular catecholamine transporter (that facilitates vesicular storage). A similar process occurs at storage sites for serotonin, what can finally result in biogenic amine depletion (Henry et al., 1987). Furthermore, this finding suggests that the antidepressant effect of ethanolic extract is probably related, at least in part, to the increase in central nervous system noradrenergic activity.

Besides, preliminary studies of acute toxicity in mice revealed that high doses of this extract (2.0 g/kg *p.o.*) do not have lethal effects and are well tolerated. In conclusion, our results evidence that the ethanolic extract of the flowers from *B. perennis* possesses antioxidant *in vitro* activity and we suggest antidepressant properties that need further investigation in other animal models of depression. However, further studies are necessary to confirm and extend these results. Somehow, the findings presented here are relevant because they validate the folk use of *B. perennis*, a medicinal plant used in South America.

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***Correspondence**

Rivelilson Mendes de Freitas
Programa de Pós-graduação em Ciências Farmacêuticas, Universidade Federal do Piauí
Campus Universitário Ministro Petrônio Portella, Bairro Ininga, 64.049-550 Teresina-PI, Brazil
rivelilson@pq.cnpq.br
Tel.: +55 86 3215 5870