Antidepressant- and anxiolytic-like activities of an oil extract of propolis in rats

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A R T I C L E   I N F O

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A B S T R A C T

Purpose: Propolis biological effects are mainly attributed to its polyphenolic constituents such as flavonoids and phenolic acids that were recently described in the chemical composition of an extract of propolis obtained with edible vegetal oil (OEP) by our group. The aim of this study was to evaluate the effect of OEP on the behavior of rats.

Materials and methods: An in vivo open field (OF), elevated Plus-maze (EPM), and forced swimming (FS) tests were performed to evaluate locomotor activity, anxiolytic- and antidepressant effects of the extract. Besides, oxidative stress levels were measured in rat blood samples after the behavioral assays by evaluation of the Trolox equivalent antioxidant capacity (TEAC) and nitric oxide levels.

Results: OEP increased locomotion in the OF test (50 mg/kg) and central locomotion and open arm entries in the OF and EPM tests (10–50 mg/kg) and decreased the immobility time in the FS test (10–50 mg/kg). Moreover, OEP reduced nitric oxide levels in response to swim stress induced in rats.

Conclusion: OEP exerted stimulant, anxiolytic and antidepressant effects on the Central Nervous System and antioxidant activity in rats, highlighting propolis as a potential therapeutic compound for behavior impairment of anxiety and depression.

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Introduction

Aqueous or ethanolic extracts of propolis (EEP) are widely used as alternative medicinal products due to their antimicrobial and anti-inflammatory properties as well as their ability to enhance bodily resistance to illness. In bee hives, propolis is primarily used to maintain an aseptic environment, which is essential to colony survival. Sforcin and Bankova (2011) discussed the potential of propolis for the development of new drugs, but there is a substantial lack of clinical research in pharmacologic studies. Many studies have reported the antimicrobial, antioxidant, anti-tumor, and anti-inflammatory effects of propolis (Menezes, 2005; Alencar et al., 2007; Carvalho et al., 2011). However, the effect(s) of propolis on the Central Nervous System (CNS), such as depressant and anxiolytic effects, have been poorly reported.

Several studies have reported pharmacological activities associated with the EEP. Indeed, recent studies reported that the oil extract of Brazilian propolis (OEP) had antifungal and antibacterial activities, mainly against Gram-positive bacteria in vitro (Buriol et al., 2009; Finger et al., 2013) and anti-tumoral activity in mice in vivo (Carvalho et al., 2011). Concerning its effect on the CNS, several studies have suggested that the EEP and its phenolic compounds have neuroprotective effects in both in vitro and in vivo models (Amodio et al., 2003; Kwon et al., 2004; Shimazawa et al., 2005; Liu et al., 2008; Gao et al., 2008; Nakajima et al., 2009). Mahmoud et al. (2000), showed that caffeic acid phenethyl ester (CAPE), a component of propolis, exhibited neuroprotective effects against oxidative damage in an induced model of ischemia and in neurodegenerative disorders including Alzheimer’s disease (Menezes, 2005; Mahmoud et al., 2000; Chen et al., 2008).
In addition, *in vitro* and *in vivo* studies have shown that propolis flavonoids produce neuronal protection against brain damage induced by several neurotoxins (for review see Dovichi and Lajolo, 2011). The effect can be explained by a dual mechanism, such as the antioxidant and anti-inflammatory effects of propolis, in models of Alzheimer’s disease (Youdim and Joseph, 2001). Studies suggest that flavonoids could be an alternative therapy for neurodegenerative disorders due to their ability to modulate neuronal function (Gualaldo et al., 2000; Dhawan et al., 2001; Watanabe et al., 2001; Dhawan et al., 2004; Johnston and Beart, 2004). Regular intake of these compounds has been shown to improve cognitive functions; this effect is most likely mediated through protection of neurons and/or by improvement of neuronal function (Commenges et al., 2000; Vauzour et al., 2008). Thus, an important point in propolis research refers to its antioxidant and CNS properties, including modification of behavior and mood (Gualaldo et al., 2000; Dhawan et al., 2001, 2004).

Because oil extracts have advantages over the more commonly used EEP, especially in terms of flavor, it is important to assess their pharmacological potential. Moreover, recently our group described the chemical composition of OEP that presents substances with biological activity (Finger et al., 2013). In the present study, we aimed to investigate, for the first time, the effect of the OEP on the CNS of rats by behavioral tests. Additionally, the antioxidant activity of OEP was evaluated *in vivo.

**Materials and methods**

**Propolis origin and extract preparation**

*Samples*: propolis samples were collected in 2006 and supplied by Campolin & Schmidt Company from Prudentópolis city (Paraná State, Brazil). Propolis was stored at −18 °C until extraction.

*Extracts*: Fifty grams of propolis was extracted in a shaker with 500 ml of canola oil for 24 h at room temperature. After that period, the extract was filtered and partitioned in an 80:20 (v/v) methanol:water solution. The aqueous methanolic phase was dried in a rotatory evaporator to yield the OEP as described by Finger et al. (2013).

**Animals**

Two-month-old female Wistar rats weighing 150–300 g were obtained from the Federal University of Pará (UFPA) and kept in collective cages (5 animals per cage). Animals were maintained in a climate-controlled room with a 12-h reverse light/dark cycle (lights on 7:00 AM) and food and water *ad libitum*. All procedures were approved by the Ethics Committee on Experimental Animals of the Federal University of Pará under license number BIO-046-12 and followed the guidelines suggested by the NIH Guide for the Care and Use of Laboratory Animals.

Oxidative stress evaluation experiments were designed in the *In Vitro* Activities Laboratory, and behavioral assays were performed in the Laboratory of Pharmacology of Inflammation and Behavior at the Federal University of Pará. After the behavioral assays, the animals were euthanized by cervical displacement. OEP was dissolved in hydroalcoholic solution plus Tween 80 (5%) and intraperitoneally (i.p.) administered at doses of 10 mg/kg, 30 mg/kg, and 50 mg/kg. All OEP doses were administered 1 h before the behavioral tests. For the antioxidant analyses, the animals’ blood samples were obtained by puncturing the retro-orbital plexus.

The positive control treatments included diazepam (DZP: 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one, Diazepam® from Hipolabor Laboratory, Brazil) and fluoxetine (FXT: N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxyl]propan-1-amine hydrochloride, Fluxene® from Eurofarma Laboratory, Brazil).

Troxol equivalent antioxidant capacity (TEAC) and nitric oxide levels were measured in the blood samples of rats treated with saline (behavioral training stress [BTS] group) or OEP (BTS + OEP) and subjected to a forced swimming test for 5 min. The control group animals were not subjected to behavioral activities (basal group).

**Behavioral assays**

Animals were divided into five groups (n = 8–10 animals per group), defined as follows: A – negative control group (vehicle), received hydroalcoholic solution plus Tween 80 (5%) i.p.; B – positive control group, received 10 mg/kg DZP i.p. (anxiety-like tests) or 10 mg/kg FXT i.p. (depressant-like test); C – received 10 mg/kg OEP i.p.; D – received 30 mg/kg OEP i.p.; and E – received 50 mg/kg OEP i.p.

**Open field (OF) test**

OEP, the hydroalcoholic solution plus Tween 80 (5%) or the DZP were administered 60 min before the behavioral tests. Rats were placed individually in the center of a wooden arena (100 cm × 100 cm × 40 cm) divided into 25 quadrants to evaluate the number of sections visited by the animal over a period of 5 min. The test was videotaped and analyzed by Any Maze Stoelting software (USA). The parameters total distance traveled, time spent in the central area, and number of grooming events were measured (Zeef et al., 2012). The placement of one, two or three paws in a square followed by a return to the previous square was not considered a crossing.

**Elevated plus maze (EPM) test**

Following the open field (OF) test, the animals were subjected to the elevated plus maze (EPM) test, which consists of a plus-shaped wooden maze with two opposite open arms (50 cm × 10 cm) and two enclosed arms (50 cm × 10 cm × 40 cm) spreading out from a central platform (10 cm × 10 cm) elevated at a height of 50 cm from the floor. The animals were individually placed in the center of the EPM, facing one of the enclosed arms, and were allowed to explore the apparatus for 5 min following the procedure described by Pellow et al. (1985). The following parameters were measured: (a) frequency of open arm entries (OAE), (b) frequency of enclosed arm entries (EAE), (c) open arm time (OAT), and (d) enclosed arm time (EAT). The %OAE and %OAT were calculated according to the formula open/total × 100. An entry was counted whenever the animal placed four paws in an arm of the maze. An anxiogenic effect is defined as a decrease in the % OAE and/or % OAT.

**Forced swimming (FS) test**

Following the EPM test, the animals were subjected to the forced swimming (FS) test. Rodents were individually dropped into cylindrical tank (50 cm in diameter; 70 cm high) containing water at 23 ± 1 °C and were monitored for 5 min in inescapable conditions. Immobility time was recorded during the last 3 min. The first 2 min were considered habituation. The rats were judged as immobile whenever they stopped swimming and floated in an upright position for 2 s, making only small movements to keep their head above the water level. An antidepressant-like effect was considered present when lower immobility was observed compared to the control group. The number of climbing events was measured to evaluate motor conditions (Porsolt et al., 1977).
After the behavioral tests, blood samples were collected for determination of oxidative stress.

**Measurement of total antioxidant status (TAS)**

The total antioxidant status (TAS) is a sensitive and reliable marker for detecting in vivo oxidative stress markers that may not be detectable through the measurement of a single, specific antioxidant (Cohen et al., 2009). In this study, rats were pretreated with OEP (10 mg/kg, 30 mg/kg, and 50 mg/kg) or vehicle 60 min before the behavioral tests. After the behavioral tests (about 5 h), blood samples were collected to evaluate TAS by measuring the TEAC levels using the method developed by Rufino et al. (2007). In this assay, 2,2-azinobis(3-ethylbenzothiazoline, 6-sulfonate) (ABTS2) is incubated with persulfate to produce ABTS+. This species is blue-green. Antioxidants present in the sample cause a reduction in absorption proportional to their concentration. The antioxidant capacities of the samples are expressed as TEAC using a calibration curve plotted with different amounts of Trolox, and their absorbance measured at 740 nm (Re et al., 1999).

**Determination of serum nitric oxide concentration**

The nitrate ($\text{NO}_3^-$) present in the serum samples was converted to nitrite with nitrate reductase, and the nitrite concentration was determined using the Griess method (Granger et al., 1999). Briefly, 100 μl of the supernatant samples was incubated with an equal volume of Griess reagent for 10 min at room temperature. The absorbance was measured on a plate scanner (Spectra Max 250; Molecular Devices, Menlo Park, CA, USA) at 550 nm. The nitrite ($\text{NO}_2^-$) concentration was determined using a standard curve generated using sodium nitrite (NaNO₂). Nitrite production is expressed per μM.

**Statistical analysis**

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons of behavior or oxidative stress test results. $p$ values less than 0.05 ($p < 0.05$) were considered to be indicative of significance.

**Results**

**OEP increases ambulation in the OF test**

The locomotor activity of each group in the open field arena (for 5 min) is summarized in Fig. 1. Acute OEP administration at 50 mg/kg ($p < 0.001$) increased the distance traveled on the apparatus, which was similar to that of the positive control DZP ($p < 0.001$). Post hoc comparisons indicated that doses below 50 mg/kg of OEP (10 mg/kg and 30 mg/kg) did not alter locomotion.

**OEP showed an anxiolytic-like effect in experimental models of anxiety**

The effects of acute OEP administration evaluated in the OF test revealed that propolis increased the percentage of central quadrants crossed at all doses (10 mg/kg, 30 mg/kg, and 50 mg/kg) in a dose-dependent manner (Fig. 2, Panel A). One-way ANOVA revealed that the other anxiety parameters, such as the percent of time spent in the central area (Fig. 2, Panel B) and the number of grooming events (Fig. 2, Panel C), were also altered, demonstrating anxiolytic-like effects at a dose of 50 mg/kg.

Anxiolytic-like effects of OEP were also observed in the EPM test (Fig. 3). A subsequent Tukey’s test indicated that the open arm entries increased for all propolis extract doses tested (Fig. 3, Panel A; $p < 0.001$). However, the time spent in the open arms only increased at OEP doses higher than 10 mg/kg (Fig. 3, Panel B; $p < 0.001$ and $p < 0.05$, respectively).

**OEP presents antidepressant-like effect in rodents**

The effect of OEP administration on antidepressant-like activity evaluated by the FS test is illustrated in Fig. 4. One-way ANOVA revealed that OEP reduced the immobility time at all doses tested (Fig. 4, Panel A) without affecting motor parameters such as the number of climbing events (Fig. 4, Panel B).

**OEP showed antioxidant activity in rodents behavioral training stress**

The behavioral training stress group (BTS group) showed an increase of antioxidant (TAS) and pro-oxidant (NO) parameters compared to the basal group. The TEAC levels induced in the BTS group were not altered by pretreatment with any dose of OEP (Fig. 5, panel A). However, OEP was able to significantly decrease the NO levels in the serum of BTS-animals treated with doses of 10 mg/kg, 30 mg/kg and 50 mg/kg (Fig. 5, panel B).

**Discussion**

In the present study, we investigated whether OEP exerts behavioral and oxidative stress effects. We report that acute OEP administration causes behavioral effects as hyperlocomotion, anxiolytic- and antidepressant-like effects based on specific behavioral tests (OF, EPM, and FS). Oxidative biochemical analysis demonstrates that OEP reverses the negative effects of behavioral stress.

The pharmacological effects of propolis have been attributed to the presence of flavonoids, phenolic acids and ester derivatives (Lu et al., 2004), and to the presence of terpenes (Silva et al., 2005; Albuquerque et al., 2007). Our research group recently described the chemical composition of the OEP employed in the behavioral tests in the present study (Carvalho et al., 2011; Finger et al., 2013). Carvalho et al. (2011) reported that this sample of propolis collected in 2006 in Prudentópolis city ( Paraná State, Brazil) presented several constituents that were identified by liquid chromatography/electrospray ionization mass spectrometry in negative-ion (ESI(-))-MS/MS that provides fingerprinting characterization through its characteristic profiles of...
3,4-dihydroxy-5-prenyl-cinnamic acid, 3-prenyl-4-hydroxycinnamic acid and (E)-3-[4-hydroxy-3-{(E)-4-(2,3-dihydrocinnamoyloxy)-3-methyl-2-butenyl]-5-prenylphenyl]-2-propenoic acid. In addition, we suggested that OEP has several advantages over the common EEP, such as the possibility of new pharmaceutical presentations for topical or internal applications. Because of the absence of ethanol in its formulation, edible OEP is expected to be better tolerated (Finger et al., 2013).

Although the biological activities of propolis have been described (Menezes, 2005; Alencar et al., 2007; Carvalho et al., 2011), its effects on the CNS as a neuroprotector and causative agent of alterations in behavior has been poorly described (Shimazawa et al., 2005; Nakajima et al., 2007; Izuta et al., 2008; Lee et al., 2013). In this study, we demonstrated that acute OEP administration in animals produces anxiolytic-like activity in both the OF and EPM tests and is associated with an increase in spontaneous locomotor behavior. Anxiolytic-like activity began at doses of 10 mg/kg, similar to the DZP group. However, at doses of 30 and 50 mg/kg,
this effect was more pronounced than that of the DZP group. These data indicate that OEP is more potent than a classic anxiolytic drug by the parameters measured in the OF test, such as the amount of time spent in the central area and reduced grooming. Despite the potent anxiolytic-like effect, the dose of 50 mg/kg resulted in hyperlocomotion, which was evident by the increased ambulation measured in the arena. These findings showed that the OEP had anxiolytic-like effects at all dosages tested and in both the OF and EPM models in a manner similar to the positive control, DZP.

Recently, Li et al. (2012), reported that propolis essential oil (PEO) at a dose of 100 mg/kg can readily mitigate anxiety-like behavior and inhibit hypothalamic-pituitary-adrenal (HPA) axis hyperactivity and lipid peroxidation in brain tissue. However, in the spontaneous locomotor activity test, PEO showed no significant effect on motor parameters, which indicated the ability of the essential oil to mitigate anxiety without causing sedation. These authors also showed that the PEO significantly decreased the plasma levels of cortisol (CORT), adrenocorticotropic hormone (ACTH) and malondialdehyde (MDA), whereas it increased the activity of superoxide dismutase (SOD) in restraint-stressed mice. Thus, these data strongly suggest that PEO has therapeutic effects on anxiety through antagonizing the hyperfunction of hypothalamic-pituitary-adrenal (HPA) axis and enhancing antioxidation mechanisms in brain tissue (Li et al., 2012).

Prut and Belzung (2003), reported that anxiolytic-like drugs induce an increase in central locomotion in animals. In addition, other authors showed that these drugs diminish grooming in the OF test (Moody et al., 1993; Prut and Belzung, 2003). Our results demonstrated that 50 mg/kg OEP promotes an increase in locomotion during the OF test that is similar to the DZP group. These results are different from those of others who used protocols with EEP and PEO (Li et al., 2012; Lee et al., 2013). The mechanism of action involved in this effect needs to be elucidated. Altogether, our results suggest that the effect of OEP is related to its anxiolytic-like property because it diminishes risk-avoidance behavior, promoting exploration of the environment.

In addition, our data showed that the OEP has antidepressant-like activity in depressive-like rat models (Porsolt swim test). Similarly, Lee et al. (2013), reported that the EEP also showed antidepressant-like activity without locomotor alteration in the OF test starting at 100 mg/kg. This dose was higher than that used in our study, which started at 30 mg/kg. However, in the Lee protocol, propolis did not alter spontaneous locomotion in the OF models, while in our study an increase in locomotion was noted at a dose of 50 mg/kg. These authors also suggested that the mechanisms of the antidepressant effect might be related to modulation of the hypothalamic-pituitary-adrenal (HPA) axis mediated by an increase in glucocorticoid receptor (GR) function. This receptor plays an important role in the therapeutic action antidepressants (Lee et al., 2013). Our study demonstrated that OEP produces higher effects in the CNS than the EEP tested by Lee and colleagues. However, this difference may be because the chemical composition of propolis may be different, and the extraction solvent might also affect the potency of the activity in the CNS. Vegetable oils should be more effective than ethanol in extracting the lipophilic substances of propolis, which are capable of crossing the blood–brain barrier.
protection, and propolis exerts an antioxidant effect in the in vivo model of cerebral injury. The antioxidant properties of propolis extracts may be related to their chemical composition, such as the content of flavonoids (including flavones, flavonols, flavanones and dihydroflavonols) and other phenolic compounds (mainly substituted cinnamic acids and their esters) (Moreno et al., 2000; Banskota et al., 2001; Kumazawa et al., 2004). These properties have been assessed through different chemical assays, such as DPPH radical and superoxide anion scavenging assays (Banskota et al., 2000; Russo et al., 2004; Izuta et al., 2009) and determination of the inhibition of DNA cleavage induced by hydrogen peroxide (Russo et al., 2004).

In terms of CNS activity, several studies have demonstrated that the caffeic acid phenethyl ester (CAPE) found in propolis has neuroprotective potential against the oxidative damage induced by ischemia–reperfusion and cytotoxic, inflammatory and autoimmune processes (Wei et al., 2004; Ilhan et al., 2004; Tsai et al., 2006). Some studies showed that CAPE and alpha-tocopherol suppressed ischemia–reperfusion-induced cerebral lipid peroxidation and injury. CAPE protected the spinal cord from ischemia–reperfusion injury (Ilhan et al., 1999; Irmak et al., 2003). Other studies reported that CAPE was able to inhibit reactive oxygen species (ROS) production at the transcriptional level through the suppression of nuclear factor kappa B (NF-κb) activation and by directly inhibiting inducible nitric oxide synthase (iNOS) (Ilhan et al., 2004). In addition, Wei et al. (2004) showed that CAPE blocks hypoxia–ischemia–induced neuronal death through inhibiting inflammation and mitochondrial cytochrome C release. Furthermore, CAPE was also found to downregulate the expression of c-fos, that is a conventional marker for neuronal activation (Noh et al., 2012) involved in dysfunction of HPA axis and related to behavior disorders, which may explain the antidepressant-like activity observed (Ha et al., 2009). The suppression of c-fos induced by propolis may be indicative of the complex behavioral responses elicited by behavioral tests such as the FS test. This observation may provide insights into the neural circuits that may be involved in mediating the HPA axis response to the stress induced by the behavior test, mainly through the release of neurochemical mediators (Lee et al., 2013). In this regard, previous studies have shown that elevated corticosterone levels in response to stress are correlated with increased c-fos expression. Antidepressant drugs reduce c-fos protein overproduction and decrease neural activity, modulating HPA-axis activity (Yau et al., 1995; De Kloet et al., 1998). In addition, western blot analysis revealed that there was a molecular link between the antidepressant-like activity of propolis extract and GR function in the hippocampus (Lee et al., 2013).

Other studies found that flavonoids were able to promote the maintenance and functionality of the substantia nigra pars compacta and striatum in rodents administered 6-hydroxydopamine (6-OHDA) in a Parkinson’s disease model (Youdim et al., 2001; Youdim and Stephenson, 2004). Orsatti and Sforcin (2012), observed that propolis exerts immunomodulatory activity in chronically stressed mice, upregulating Toll-like receptor (TLR)-2 and TLR-4 mRNA expression. These receptors are known pattern recognition receptors (PRRs) of the innate immune system that initiate and propagate an inflammatory response, inducing the synthesis and release of cytokines, crossing blood–brain barrier and affecting structures related to behavior, such as the hippocampus and amygdala. Pro-inflammatory cytokine inhibition blocks disorders behaviors. Together, these data suggest that the immune system plays a role in the brain in terms of behavioral regulation, possibly connected to survival. Thus, drugs with anti-inflammatory activities may have behavioral effects through modulation of immune pathways (Orsatti and Sforcin, 2012).

**Conclusion**

In conclusion, our data showed that OEP exerted hyperlomocorticoid and anxiolytic- and antidepressant-like effects in the CNS in different animal models. Moreover, the extract also showed antioxidant activity after stress induced by the forced swim test.

**Conflict of interest**

None declared.