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Anti-stress effects of the "tonic" *Ptychopetalum olacoides* (Marapuama) in mice

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ABSTRACT

With the recognition that high levels of sustained stress are associated with the natural course of countless illnesses, effective anti-stress agents have gained importance. Improved endurance to particularly stressful periods is one of the medicinal claims for Marapuama (*Ptychopetalum olacoides* Bentham, PO), a popular Amazonian herbal. The purpose of this study was to evaluate if PO possesses anti-stress properties. To this end, an extract from PO (POEE) was evaluated on anxiety and glucose levels in mice submitted to the unpredictable chronic mild stress (UCMS) paradigm. POEE did not present anxiolytic effects, but was able to prevent ($p<0.01$) the UCMS-induced anxiety as assessed by the light/dark test (time spent in the lit area, POEE 100 and 300 mg/kg 235.9 ± 20.6 s and 250.4 ± 17.4 s, respectively, compared to DMSO 104.7 ± 24.4 s). Likewise, although POEE did not induce noticeable effects on glycemia, it effectively ($p<0.01$) prevented the UCMS-induced hyperglycemia (POEE 100 and 300 mg/kg 106.4 ± 6.7 mg/dl and 107.3 ± 3.3 mg/dl, respectively, compared to DMSO 134.6 ± 5.9 mg/dl). Additionally, POEE (50-200 mg/kg i.p. and 800 mg/kg p.o.) significantly ($p<0.01$ and $p<0.05$, respectively) increased the time to hypoxia-induced convulsion (by 38%, 51%, 59% and 27%, respectively for i.p. and p.o. treatments). The data indicate that POEE counteracts some of the effects brought about by chronic stress. This study combined with the identified antioxidant and neuroprotective properties, as well as the claimed benefits associated with stressful periods suggest that *Ptychopetalum olacoides* (Marapuama) might possess adaptogen-like properties.

Introduction

In Brazil "tonics" are used in order to increase physical endurance, ameliorate performance in mental tasks, improve memory, or are even indicated for periods of convalescence from debilitating diseases or sudden weight loss. While tonics may be similar to fortifiers, energizers, or restoratives (Mendes and Carlini 2007), some are regarded as having specific qualities, such as for instance "sexual tonics". Although tonics may be used in a vitamin-like fashion simply to ensure "good health", some are rather used as stimulants (e.g, "rebite", "arrebite", popular amongst truck drivers), some are clearly meant to improve physical and mental performance (being popular in the fitness center scenario and amongst students, respectively), while others are used in association with periods of illness, either to help convalescence or to minimize the harsh effects of remedies (popular amongst older people) (Elisabetsky and Siqueira 1998). Examples of plants traditionally used as "tonics" in Brazil include *Heteropterys aphrodisiaca* O Mach. ("nó-de-cachorro") (Malpighiaceae), *Paullinia cupana* Kunth ("guaraná") (Sapindaceae), and *Mauritia flexuosa* L. ("buriti") (Aracaceae) (for review see Mendes and Carlini 2007).

Based on the original ideas of Lazarev (1947), Wagner and colleagues defined adaptogens as "substances which elicit in an organism a state of non-specifically raised resistance allowing them to counteract stressor signals and to adapt to exceptional strain" (Wagner et al. 1994). More recently, adaptogens have been defined as "natural metabolic regulators that increase the ability of the organism to adapt to environmental factors and to avoid damage from such factors" (Panossian et al. 1999a). A revised and expanded interpretation of the concept and associated therapeutic outcomes has been thoroughly discussed (Olalde Rangel 2005a, b, c). The general purposes of a treatment with adaptogens include to decrease reactivity to stress, to diminish stress triggered reactions (alarm phase of the stress response), and to prevent or at least delay the state of exhaustion (conferring protection against long-term stress) (Panossian et al. 1999a). The mode of action of adaptogens is not completely understood, but the modulation of catecholamines and other stress mediators

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(e.g., cortisol and nitric oxide) has been proposed (Panossian et al. 1999b). In addition, antioxidant, immunomodulator, hypoglycemic, hypocholesterolemic, and other non-specific properties may be involved in the overall adaptogenic effect (Panossian et al. 1999c; Rege et al. 1999; Davydov and Krikorian 2000).

Ptychopetalum olacoides Bentham (PO) (Olacaceae), known as Marapuama in Brazil and Muirapuama in other South American countries, is found in medicinal plant markets or as herbals in diverse formulations, usually sold to increase physical, mental and/or sexual performance. In the Amazon, where its use originates, Marapuama is especially used by the elderly to treat a condition recognized as "nerve weakness" (Elisabetsky and Siqueira 1998). The syndrome includes lassitude, a general lack of desire or motivation, tremors and sexual impotence as prominent symptoms (Siqueira et al. 1998). The species is also used by anyone (regardless of age) for enduring periods of high physical (such as gold miners) or mental (such as students in test periods) demand. The roots are usually prepared in "*cachaça*" (the national distilled spirit obtained from sugar cane) or wine and sold as a "*garrafadas*" (a local jargon, where *garrafa* is the substantive for bottle to which the adjective suffix *ada* is added; plant material usually cut down in pieces are left in the bottle filled with wither wine or cachaça, circa 750 ml). The formula is usually drunk daily before meals, an usual dose roughly equivalent to 60 ml of a "*garrafada*". We have shown that a standardized ethanol extract of *P. olacoides* (POEE) is promnesic (da Silva et al. 2004, 2007) counteracts several types of amnesia (da Silva et al. 2008), and has neuroprotective (Siqueira et al. 2004), antioxidant (Siqueira et al. 2007), and antidepressant properties (Piato et al. 2008, 2009).

Given the traditional uses of PO, the antidepressant effects identified for POEE, and the relationship between stress and depression (Sapolsky et al. 2000), the present study aimed to evaluate whether POEE counteracts stress-induced effects. To this end, we evaluated the effects of POEE on anxiety and glucose levels in mice submitted to chronic mild stress. Additionally, POEE was evaluated in relation to mice endurance against hypoxia.

Materials and Methods

Animals

Two months old male CF1 mice (40-50 g) were used for hypoxia, glycemia and light/dark experiments with acute treatments. BALB/c male mice with two months of age (25-35 g) were used for the unpredictable chronic mild stress (UCMS). Mice were obtained from Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), and housed in our own animal facility (22 ± 1 °C, 12 hr-light-dark cycle, free access to food [Nuvilab CR1] and water) for at least two weeks before experiments (non stress period). The project was approved by the University ethics committee (approval # 2006543).

Extract

Roots of *P. olacoides* were collected near the Capim River (Pará, Brazil), and identified by Nelson Rosa (voucher MPEG n° 108.036, Goeldi Museum). Ground *P. olacoides* roots (2.5 kg) were extracted with ethanol (12 l) in Soxhlet (40 h), and evaporated under reduced pressure to yield POEE (6 g of extract per 100 g of drug). The HPLC of the extract used in this study was previously published at Siqueira et al. (2007); the HPLC analysis was conducted using HP 1100 system equipped with photodiode array detector [Agilent Technologies]; the extract was analyzed on a Zorbax extended C18 column [250 × 4.6 mm] with the MeOH-H2O gradient [10:90–100:0]; solvents were used in HPLC grade with high purity. The flow rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra were recorded between 200 and 500 nm. Injection volume was 20 ml [5 mg/ml]). The presence of lipids, flavonoids, methylxanthines (Montrucchio et al. 2002), diterpenes (Tang et al. 2009a, b) have been reported for *P. olacoides*, although in the latter case the plant identification is doubtful. Despite older and confusing references to "muyrapuamin" (Peckolt 1901; Duke 1992), the species and this POEE extract is devoid of alkaloids.

Drugs

Imipramine HCl and diazepam were acquired from Sigma (USA) and dissolved in saline (0.9% NaCl) and propylene glycol (20% PPG), respectively. POEE was dissolved in 20% DMSO. All drugs were given as 0.1 ml/10 g body weight. The doses used for positive controls were selected from the studies on which the methodologies were based.

Unpredictable chronic mild stress model

The UCMS protocol was based on Yalcin et al. (2005), except that in this study anxiety rather than depression related parameters were assessed (Mineur et al. 2006). At the end of a two-week non stress, drug-free period, mice were assigned to different experimental groups in a semi-randomized fashion, so that mean body weights were comparable in all groups. From this point on, mice assigned to experimental groups were maintained in individual cages and on an inverted light-dark cycle; a non stress control group remained in the same room, housed 4-5 mice/cage, and was not submitted to stressors. Following the two-week non stress period, for 6 weeks mice were subjected several times a day to one of the following stressors: damp sawdust (90-180 min), 3 sawdust changes (30-60 min), sawdust-free cage (90-180 min), sawdust-free cage with 200 ml water (90-180 min), social stress, transfer to a new, clean cage, 45° cage tilting (90-180 min), 15 min of cat meowing, inversion of the light-dark cycle (for 48 h in a different room), and several 30 min periods of light during the dark phase. To prevent habituation and maintain the aspect of unpredictability, the timing of all stressors and stressor sequences was changed weekly. After two weeks of stress, drugs were given daily for the remaining 4 weeks. Imipramine was used as control in order to differentiate antidepressant from anti-stress effects. Imipramine (20 mg/kg) and saline were injected intraperiotoneally (i.p.), whereas POEE (50, 100 and 300 mg/kg) and DMSO were given orally (p.o.). All drugs were given as 0.1 ml/10 g body weight.

Light/dark

48 h after the end of the UCMS and 24 h after the last drug administrations, mice were submitted to the light/dark procedure (Li and Quock 2001). The apparatus consisted of a rectangular wood box (46 × 27 × 30 cm), divided into one small (18 × 27 cm) and one large (27 × 27 cm) areas, with a door-like opening (7.5 × 7.5 cm) in the center of the separation. The small compartment was painted in black and light-free, whereas the large one was white and brightly lit with two 60 W cold light sources. Each animal was individually placed in the center of the bright compartment (facing away from the door) and the following parameters were noted for 5 min: latency to the first crossing from one compartment to the other, time spent in the light compartment and the number of crossings between the light and dark

compartments. The test was performed in a quiet and darkened room (red bulb), and mice were kept in this room for at least 1 h before the session. In order to check if POEE shows anxiolytic properties independent of previous stress, additional experiments were done after acute treatments. To allow comparison with literature and previous data of our group, CF1 (instead of BALB/c) mice were used. Groups of mice (n = 8) were treated once intraperiotoneally with saline, 20% PPG, diazepam 1 mg/kg, imipramine 20 mg/kg , or orally with 20% DMSO, POEE 50, 100 and 300 mg/kg; after 30 min (i.p.) or 90 min (p.o.) animals were placed in the light/dark box.

Glycemia

Glucose measurements were taken 72 h after the end of the UCMS, using a commercial glucometer tape (Accu-Chek Active® Roche), with a blood drop drawn from the tail. Measurements were taken between 13:00 h and 14:00 h, after 5 hours of fasting (food and water). To verify if POEE had an effect *per se* on glycemia, groups of mice (n = 8) were treated once with saline, imipramine 20 mg/kg (i.p.), 20% DMSO or POEE 50, 100 and 300 mg/kg (p.o.); blood samples were collected after 30 min (i.p.) or 90 min (p.o.).

Endurance to Hypoxia

The method was based on Caillard et al. (1975). Animals received saline, 20% DMSO, POEE 50, 100 and 200 mg/kg (i.p.) or POEE 400 and 800 mg/kg (p.o.). After 30 min (i.p.) or 90 min (p.o.), the endurance to hypoxia was determined by placing mice individually in air-tight jars (300 ml). Endurance to hypoxia was defined as the latency to onset of convulsions, recorded with stop watches.

Statistical analysis

Results are expressed as mean ± S.E.M. Data were analyzed by one-way ANOVA followed by Duncan post hoc. SPSS 11.0 for Windows was used for the statistical analysis. Significance was set at $p<0.05$.

Results

As expected, the UCMS protocol was anxiogenic, with mice spending nearly 50% less time in the lit compartment in comparison to the non stress group ($F_{6,57} = 49.6$, $p<0.01$,

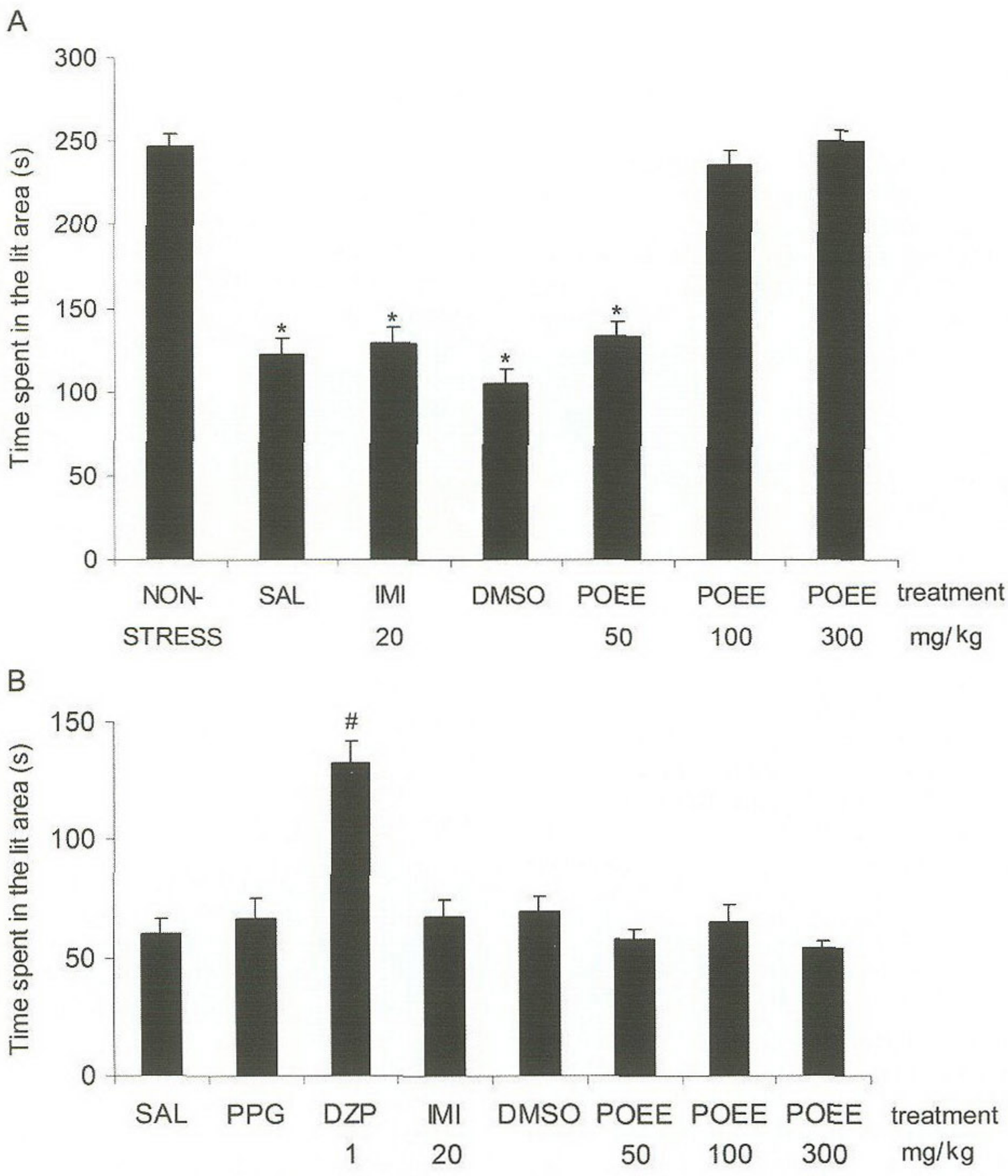

Fig. 1. Effects of POEE, imipramine and diazepam on the light/dark test. Fig. 1A: BALB/c mice submitted to UCMS. Fig. 1B: CF1 mice not submitted to UCMS, but to single drug treatment. Saline = SAL, imipramine = IMI, and diazepam = DZP. Mean ± S.E.M. (n = 8-10). (A) *$p<0.01$ x non-stress group and (B) #$p<0.01$ x controls, ANOVA/Duncan.

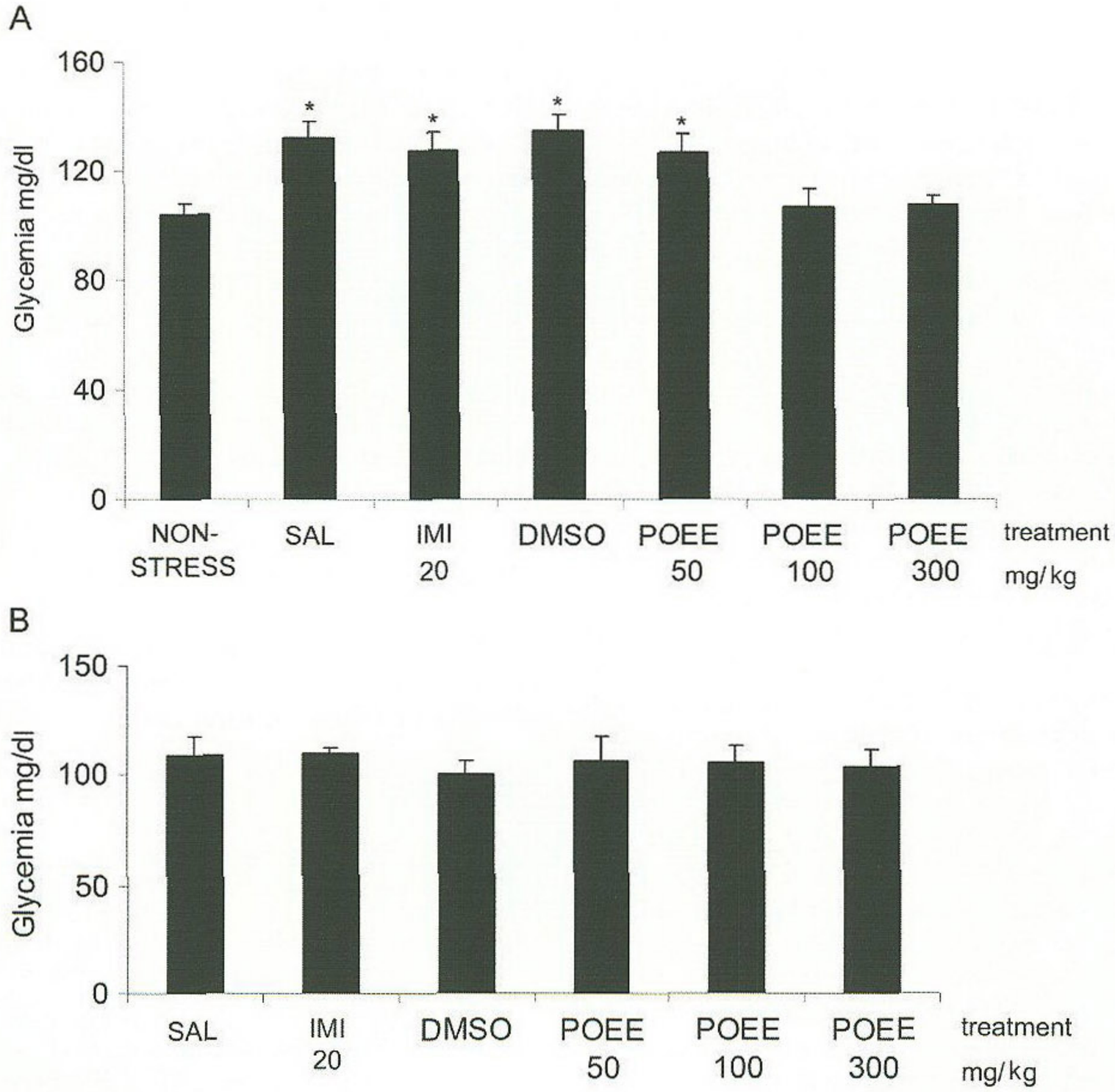

Fig. 2. Effects of POEE and imipramine on glycemia. Fig. 2A: BALB/c mice submitted to the UCMS. Fig. 1B: CF1 mice not submitted to UCMS, but to single drug treatment. Imipramine = IMI. Mean ± S.E.M. (n = 8-10). *p<0.01 x non-stress group, ANOVA/Duncan.

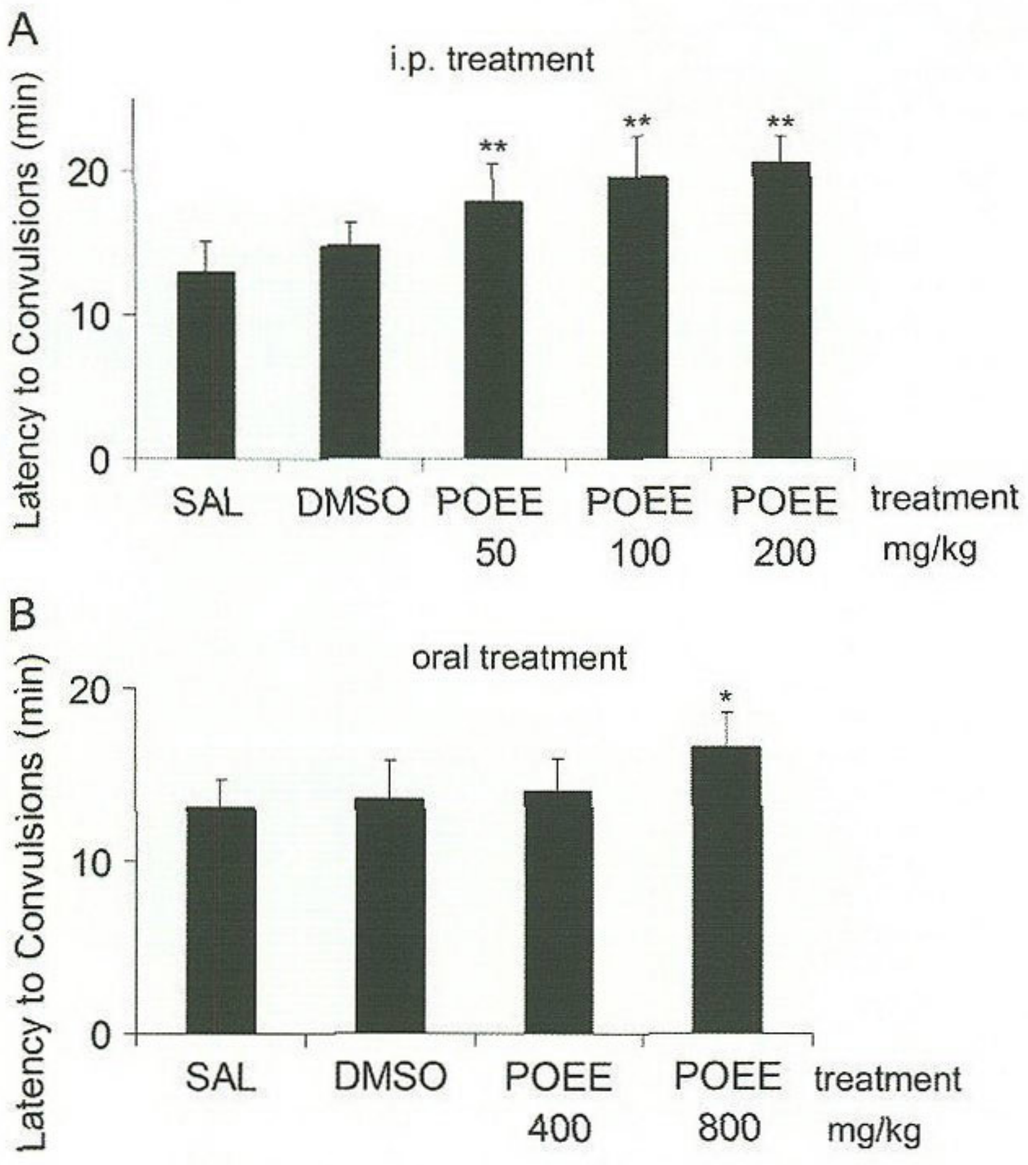

Fig. 3. Effects of POEE on mice' endurance to hypoxia. Mean ± S.E.M. (n = 6). *p<0.05 and **p<0.01 x controls, ANOVA/Duncan.

Fig. 1A). Treatment with POEE 100 and 300 mg/kg (but not 50 mg/kg) prevented the increase in anxiety. DMSO and imipramine were devoid of effects. No differences were observed regarding number of crossings ($F_{6,57} = 1.0$, $p>0.05$) or latency to first crossing ($F_{6,57} = 0.7$, $p>0.05$) (data not shown). Fig. 1B shows that only acute diazepam significantly altered the time spent in the lit compartment ($F_{7,63} = 13.8$, $p<0.01$). As with the UCMS, no differences were noted regarding the other parameters ($F_{7,63} = 1.1$ $p>0.05$ for number of crossings, $F_{7,63} = 0.7$, $p>0.05$ for latency to first crossing).

Glycemia levels were significantly higher in mice submitted to UCMS ($F_{6,59} = 6.4$, $p<0.01$, Fig. 2A). Glycemia levels of animals treated with POEE 100 and 300 mg/kg (but not POEE 50 mg/kg, or imipramine) were comparable to those of the non stress group. No differences were noted in the glycemia of acutely treated animals ($F_{5,47} = 0.2$, $p>0.05$, Fig. 2B).

Endurance to hypoxia was significantly increased (38-59%) by POEE at all three i.p. doses ($F_{4,28} = 10.9$, $p<0.01$, Fig. 3A,), and the highest oral dose of POEE (26.7% increase, $F_{3,23} = 3.7$, $p<0.05$, Fig. 3B).

Discussion

It is widely accepted that stress contributes to the pathogenesis of a variety of diseases, including psychiatric disorders. A clear relationship with stress has been established for endocrine disorders (including diabetes), immunosuppression, sexual and cognitive dysfunctions, peptic ulcer, hypertension and heart diseases, ulcerative colitis, anxiety, and depression (Sapolsky et al. 2000; Chandola et al. 2008). The response to acute stress is adaptive (allostasis), and appears to have restricted harmful effects since an array of physiological, biochemical and endocrine responses are set in motion to maintain homeostasis (Tsigos and

Chrousos 2002; McEwen 2008). Nevertheless, sustained chronic stress appears to induce the syndrome described by Selye (1936), a state of exhaustion leading to deregulation of stress mediators and pathologies (McEwen 2008). For instance, high levels of stress have been shown to be associated with hippocampus and prefrontal cortex neuronal atrophy (with consequences for memory and executive function) (McEwen 2008), as well as amygdala hypertrophy (associated with fear, anxiety, aggression, and first depressive episode) (Frodl et al. 2003).

We here show that POEE, although devoid of acute anxiolytic effect, was able to prevent chronic stress-induced anxiety (light/dark). Likewise, although POEE did not have a noticeable effect on glycemia, it effectively prevented the chronic stress-induced hyperglycemia. Moreover, POEE significantly increased mice endurance to hypoxia across a range of doses, given i.p. or orally. Overall, the data indicate that POEE is useful to counteract some deleterious effects of chronic stress; in other words, it can be argued that POEE possesses an adaptogen-like profile.

A series of stress models has been used to investigate the physiological consequences of stress and/or evaluate anti-stress agents. Unfortunately, however, the literature lacks standardized and well characterized assessment paradigms. The most frequently used animal protocols are swimming endurance (Singh et al. 2001; Kannur et al. 2006), hypoxia (Singh et al. 2001), immobilization (Singh et al. 2001; Siripurapu et al. 2005), cold stress (Kannur et al. 2006) and chronic stress (Siripurapu et al. 2005; Bhattacharya and Muruganandam 2003; Rai et al. 2003a; Singh et al. 2005). Animals exposed to chronic stress present behavioral changes associated with depression and anxiety (Mineur et al. 2006), as well as increased serum corticosterone (Ibarguen-Vargas et al. 2008) and glucose (Rai et al. 2003a). The reduction of UCMS-induced hyperglycemia by POEE treatment is similar to that reported for other adaptogen plants such as *Panax ginseng* C.A.Mey. (Rai et al. 2003b) and *Withania somnifera* L. (Indian ginseng) (Bhattacharya and Muruganandam 2003). Although the mechanism by which adaptogens prevent hyperglycemia is not clear, cortisol is known to enhance liver gluconeogenesis and reduce cellular glucose uptake (Surwit and Schneider 1993). We have previously reported that POEE treatment prevents the UCMS-induced increase in corticosterone in mice (Piato et al. 2008).

When mice are exposed to a hypoxic environment, brain noradrenaline is significantly decreased (Georgiev et al. 1995), and a state of oxidative stress is established (Morin et al. 2001). POEE-induced resistance to hypoxia may therefore be at least partially explained by its capacity to potentiate noradrenergic activity by beta receptors (Siqueira et al. 1998; Piato et al. 2008), combined with its antioxidant properties (Siqueira et al. 2007). The data here reported are consistent with the neuroprotective effects of POEE found with hippocampal slices submitted to oxygen and glucose deprivation (Siqueira et al. 2004).

Correlating the doses used in animal experiments to those used traditionally by local communities is not a trivial issue. Nevertheless, we believe that the doses used in this study, likewise in our previously reported data with this extract, bear relevance to traditional use. An estimate of 150 g of crude material/750 ml garrafada is not unrealistic given that the specific weight of this crude drug is high (hence the *tupi* name of "hard wood"); this relation is similar to the drug/solvent relation used to prepare POEE. Since actual reliable data on the extraction profile of traditional garrafadas is not available, if the same 6% yield (drug/extract) is used for calculation, a 60 ml dose corresponds to an intake of 750 mg/dose in a 70 kg user. Considering that due to pharmacokinetics specificity doses used in mice are significantly higher than those used in humans (Terpstra 2001), we believe that the range of doses used in these studies are well relevant to traditional use.

Stress management is not an easily accomplished task (see http://www.isma.org.uk). Although benzodiazepines have significant anti-stress activity in acute animal models of stress (Calil and Marcondes 2006; Singh and Kumar 2008), long-term use of low to moderate therapeutic doses is more often than not associated with dependence (Allison and Pratt 2003). Benzodiazepine withdrawal syndrome and increased risk of accidents due to motor impairment are limitations to their continued use (Haro et al. 2003). Chronic treatment with adaptogens appears to have a stress-protective effect and to lead to a long-lasting state of non-specific resistance, resulting from adaptive changes of the organism (Panossian and Wagner 2005). Further experiments are necessary to typify POEE as an adaptogen, and evaluate its benefits in stress management strategies.

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