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Antioxidant activities of *Ptychopetalum olacoides* ("muirapuama") in mice brain

I.R. Siqueira[b,c], C. Fochesatto[a], I.L.S. Torres[b], A.L. da Silva[b], D.S. Nunes[d], E. Elisabetsky[b,c], C.A. Netto[a,c,*]

[a]*Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600, Prédio Anexo, 90035-003 Porto Alegre RS, Brazil*
[b]*Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, sala 202, 90050-170 Porto Alegre RS, Brazil*
[c]*PPG-Fisiologia, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite, 500, 90050-170 Porto Alegre RS, Brazil*
[d]*Departamento de Química, Universidade Estadual de Ponta Grossa, Ponta Grossa, PR, Brazil*

Abstract

Ptychopetalum olacoides (PO) roots are used by Amazonian peoples to prepare traditional remedies for treating various central nervous system conditions in which free radicals are likely to be implicated. Following the identification of PO ethanol extract (POEE) free-radical scavenging properties in vitro, the aim of this study was to verify the in vivo antioxidant effect of POEE. Aging mice (14 months) were treated (i.p.) with saline, DMSO (20%) or POEE (100 mg/kg body wt.), and the hippocampi, cerebral cortex, striata, hypothalamus and cerebellum dissected out 60 min later to measure antioxidant enzyme activities, free-radical production and damage to macromolecules. POEE administration reduced free-radical production in the hypothalamus, lead to significant decrease in lipid peroxidation in the cerebral cortex, striatum and hypothalamus, as well as in the carbonyl content in cerebellum and striatum. In terms of antioxidant enzymes, catalase activity was increased in the cortex, striatum, cerebellum and hippocampus, while glutathione peroxidase activity was increased in the hippocampus. This study suggests that POEE contains compounds able to improve the cellular antioxidant network efficacy in the brain, ultimately reducing the damage caused by oxidative stress.

Keywords: *Ptychopetalum olacoides*; Marapuama; Muirapuama; Antioxidant activity; Brain; Aging

Introduction

Among the numerous hypotheses developed to understand the aging process, the free-radical theory of aging has become especially prominent (Harman, 1956). Free radicals, such as hydrogen peroxide, superoxide anion, hydroxyl, alkoxyl and peroxyl radicals, are continuously produced during oxidative metabolism; in excess they may lead to direct oxidation of critical biological molecules (e.g., membranous lipid peroxidation, specific protein destruction and DNA strand breaks). Accordingly, cell defense mechanisms to reduce oxidative damage include enzymatic systems: superoxide dismutase (SOD) converts superoxide radicals into H_2O_2; catalase

*Corresponding author. Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Rua Eurípedes M. Duarte 10, Nonoai, 305, 90830-250, Porto Alegre RS, Brazil.
Tel.: +55 51 316 5577; fax: +55 51 316 5540.
E-mail address: alex@prograd.ufrgs.br (C.A. Netto).

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(CAT) is responsible for detoxification of hydrogen peroxide (H_2O_2); and glutathione peroxidase (GPx) breaks down peroxides, notably those derived from the oxidation of membrane phospholipids. Non-enzymatic antioxidants, such as vitamins E and C, glutathione and carotenoids, also play important roles in antioxidant defense mechanisms (Halliwell, 1992). Oxidative stress, the result of an imbalance between free-radical-generating and free-radical-scavenging systems, has been associated with various pathologies, including those affecting the central nervous system (Halliwell, 1992).

Medicinal plants are commonly used for treating CNS disorders in traditional medicinal systems in Brazil. Alcoholic infusions of *Ptychopetalum olacoides* Bentham (PO, Olacaceae), known as ''muirapuama'' and ''marapuama'', are consumed in the Amazon for the treatment of CNS-related conditions or during highly stressful periods (Elisabetsky, 1987; Grenand et al., 1987; Siqueira et al., 1998); the frequency of elders and patients recovering from pathologies associated with damage to the central nervous system (such as stroke) among its users are of particular interest (Elisabetsky and Siqueira, 1998). This plant is also widely employed due to its alleged aphrodisiac effects. This species is currently included in dozens of herbal drugs or multivitamin dietary supplements available all around the world that are claimed to enhance sexual, physical and cognitive performance.

We have previously reported that a specific ethanol extract (POEE) of PO roots possesses various central nervous system (CNS) activities (Siqueira et al., 1998), including mild anxiogenic effect in the hole-board test, improvement of long-term memory retrieval in the adult and aged mice step down paradigm (da Silva et al., 2002, 2004), and that it inhibits AChE (in vitro and ex vivo assays, Siqueira et al., 2003), suggesting that improvement in cholinergic function might be a neurochemical correlate of the extract's behavioral effects.

A substantial antioxidant property could be related to some of the therapeutic properties claimed to be associated with marapuama, as radical scavengers reverse the loss of spatial memory and decrease damage to brain proteins in aged gerbils and rats (Carney et al., 1991; Socci et al., 1995). Indeed, we recently reported a marked free-radical scavenging property of POEE in several in vitro assays (Siqueira et al., 2002). The aim of the present study was to evaluate the effects of POEE treatment on the oxidative status in different brain areas of aging mice.

Materials and methods

Plant material. Roots of *Ptychopetalum olacoides* Bentham (PO, Olacaceae) were collected in the State of Pará (Brazil) and identified by Mr. N. Rosa (voucher MG 108036, Goeldi Museum herbarium).

Chemicals. Thiobarbituric acid and Trolox were obtained from Merck, and ABAP from Wako Chemicals Inc. (USA). 2′-7′-dichlorofluorescein diacetate (DCFH-DA), 2′-7′-dichlorofluorescein (DCF), trichloroacetic acid (TCA), 2,4-dinitrophenylhydrazine (DNPH), guanidine hydrochloride, phenylmethylsulfonyl fluoride (PMSF), 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol) and hydrogen peroxide stock solution were purchased from Sigma Chemical Co.

Preparation of ethanol extract. The extractive procedure has been detailed elsewhere (Siqueira et al., 1998); briefly, milled roots were extracted (Sohxlet) with ethanol and dried in vacuo, yielding a brown syrup (6%). For assays, POEE was diluted in 20% DMSO.

HPLC analysis. Analytical HPLC was carried out on a HP 1100 system equipped with a photodiode array detector (Agilent Technologies). The extract was analyzed on a Zorbax extended C18 column (250 × 4.6 mm i.d.) with the gradient: MeOH-H_2O gradient (10:90–100:0). The flow rate was 1 ml/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 200 and 500 nm. Injection 20 ml (5 mg/ml). Results are presented in Fig. 1.

Animals and Treatment. Swiss albino male adult mice (CF1 strain), 14 months of age, housed with access to food and water ad libitum, and light-dark cycles of 12 h, were used. DMSO 20% or POEE 100 mg/kg body wt. were administered intraperitoneally (0.1 ml/10 g body wt.); this dose was chosen because it proved to lessen the cognitive deficit of aging animals in an inhibitory avoidance task (da Silva et al., 2004).

Preparation of brain samples. Mice were decapitated 60 min after drug administration; the brain was quickly excised. Hippocampi, cerebral cortex, striata, hypothalamus and cerebellum were dissected out, and instantaneously placed in liquid nitrogen. Brain tissues were homogenized in 10 vol. of ice-cold phosphate buffer (0.1 M, pH 7.4) containing KCl 140 mM, EDTA (1 mM) and phenylmethylsulfonyl fluoride (1 mM). The homogenate was centrifuged at 960 × *g* for 10 min; the supernatant was used for the assays.

Free radical content. To assess the free-radical levels we used 2′-7′-dichlorofluorescein diacetate (DCFH-DA) as a probe (Sriram et al., 1997).

Lipid peroxidation assay. The formation of thiobarbituric acid reactive substances (TBARS) was based on Bromont et al. (1989).

Determination of protein carbonyl levels. Protein carbonyl content was determined as described previously (Levine et al., 1990).

Total antioxidant reactivity (TAR) assay. The TAR assay is based on luminol-enhanced chemiluminescence (CL) induced by an azo initiator (Lissi et al., 1995; Desmarchelier et al., 1997).

Superoxide dismutase (SOD) activity. SOD acti vity was determined using a RANSOD kit (Randox

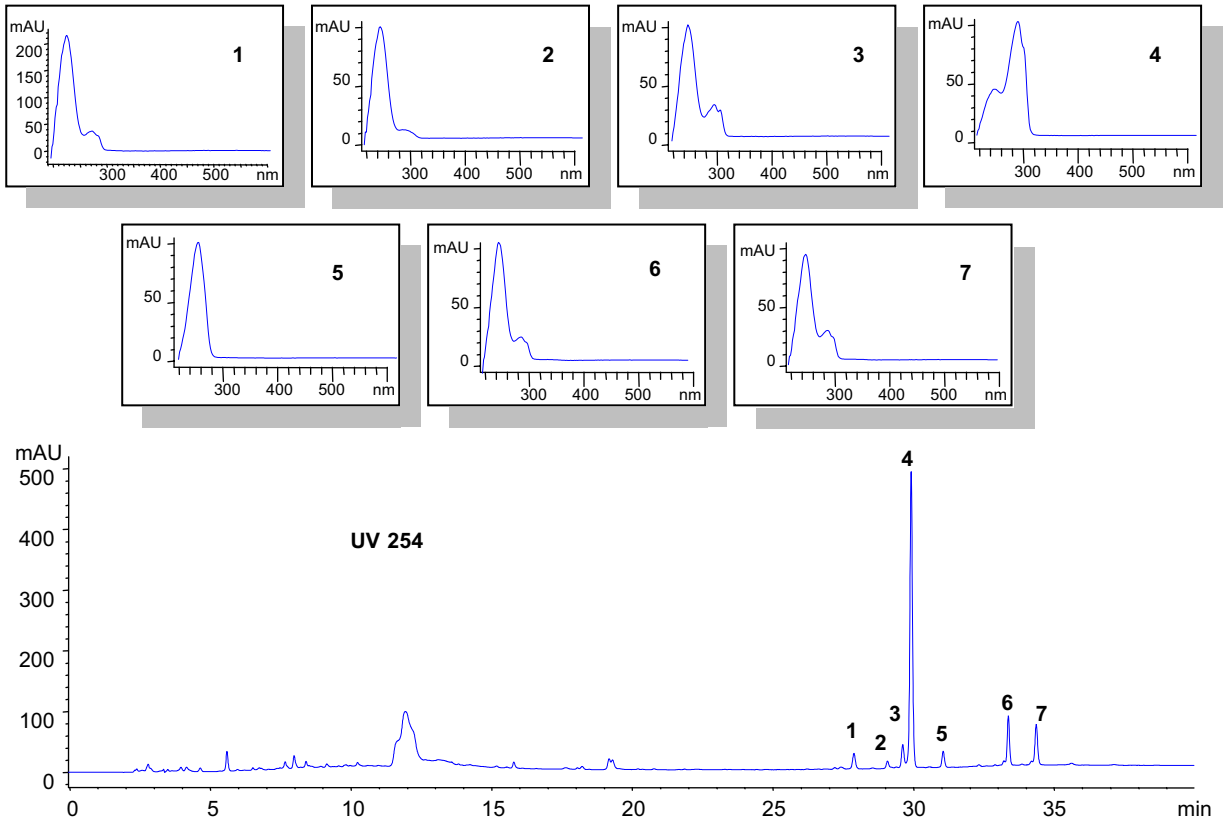

Fig. 1. HPLC/UV analysis of ethanol extract from *Ptychopetalum olacoides* (POEE).

Laboratories, USA) which is based on the procedure described by Delmas-Beauvieux et al. (1995).

Catalase (CAT) activity. CAT activity was determined according to Aebi (1984).

Glutathione peroxidase (GPx) activity. GPx activity was determined according to Wendel (1981). The activity of selenium-dependent GPx was measured taking *tert*-butyl-hydroperoxide as the substrate.

Protein assay. The total protein concentration was determined (Bradford, 1976).

Statistical analysis. Data were analyzed using ANOVA with post hoc analysis by Duncan's multiple-range test. Results are expressed as mean ± standard error of the mean (SEM).

Results

A single i.p. administration of 100 mg/kg body wt. POEE induced changes in most of the studied indexes of oxidative stress; different patterns were found at different brain regions. A significant decrease ($F_{(2,11)} = 3.781$; $p < 0.05$) in free radical levels (DCF formation) was observed in the hypothalamus (Table 1). For oxidative-induced protein damage, CARB content was decreased in the cerebellum ($F_{(2,15)} = 3.431$; $p = 0.05$), and striatum ($F_{(2,17)} = 5.374$; $p = 0.018$) (Table 1). Lipoperoxidation (TBARS levels, Fig. 2) was decreased in the cortex ($F_{(2,17)} = 4.201$; $p = 0.033$), striatum ($F_{(2,15)} = 5.973$; $p = 0.013$) and hypothalamus ($F_{(2,10)} = 4.241$; $p < 0.05$). No changes in TAR levels were observed in the brain regions examined (data not shown). In terms of antioxidant enzyme activities, there were no changes in SOD activity (Fig. 3A), GPx activity was increased only in the hippocampus ($F_{(2,10)} = 7.384$; $p < 0.01$, 33%, Fig. 3B), and CAT activity was enhanced in the striatum ($F_{(2,11)} = 6.35$; $p = 0.015$, 33%), cerebellum ($F_{(2,13)} = 4.229$; $p < 0.04$, 30%), and hippocampus ($F_{(2,11)} = 3.781$; $p < 0.05$, 47%) of POEE treated mice (Fig. 3C).

Discussion and conclusions

The working hypothesis of this study was that the therapeutic properties traditionally claimed for PO could be associated with its ability to increase antioxidant capacity, therefore attenuating free-radical generation and the consequent damage to lipids and proteins. Because the endogenous antioxidant defenses are not always completely effective, as is the case in normal aging and neurodegenerative diseases, it has been proposed that exogenous antioxidants could effectively restrain the cumulative effects of oxidative damage.

Table 1. Effects of POEE (100 mg/kg body wt.) on free radical production (DCFH-DA as probe), and protein oxidative damage (carbonyl content) in brain regions from aging mice

	DCF (nmol DCF/mg protein)			CARB (nmol DNPH/mg protein)		
	Saline	DMSO	POEE	Saline	DMSO	POEE
Cerebellum	1.88 ± 0.11	1.84 ± 0.06	2.13 ± 0.11	1087 ± 115	1189 ± 253	622 ± 62[a]
Frontal cortex	1.87 ± 0.39	1.40 ± 0.36	1.63 ± 0.44	1520 ± 224	1842 ± 172	1774 ± 244
Striatum	2.21 ± 0.15	2.24 ± 0.24	2.55 ± 0.23	2411 ± 76	2010 ± 299	1164 ± 337[a]
Hypothalamus	2.83 ± 0.12	2.71 ± 0.18	2.37 ± 0.15[b]	1143 ± 183	1477 ± 285	1704 ± 620
Hippocampus	1.68 ± 0.24	1.36 ± 0.17	1.41 ± 0.17	2856 ± 584	1926 ± 428	2075 ± 317

Data are expressed as a mean ± SEM for six to eight experiments.
[a]As compared to saline and DMSO groups.
[b]As compared to saline group, ANOVA followed by Duncan's test ($p < 0.05$).

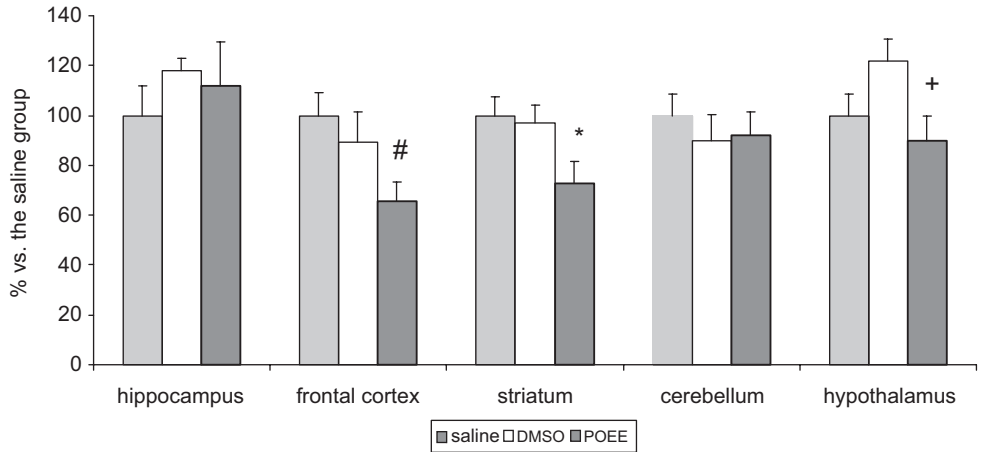

Fig. 2. Effects of POEE (100 mg/kg body wt.) on lipid damage (thiobarbituric acid reactive substance, TBARS) in brain regions from aging mice. Data expressed as percentage of saline (mean ± SEM, 6–8 experiments). The absolute mean TBARS values for saline group were 0.34 ± 0.03 (cerebellum), 0.76 ± 0.07 (frontal cortex), 0.81 ± 0.06 (striatum), 0.34 ± 0.03 (hypothalamus), 0.76 ± 0.09 (hippocampus) (pmol MDA /mg protein). ANOVA followed by Duncan's test ($p < 0.05$), * as compared to saline and DMSO groups, # as compared to saline group, + as compared to DMSO group.

Confirming previously reported results of in vivo and in vitro activities (da Silva et al., 2002; Siqueira et al., 1998, 2003), this study shows that an acute treatment with POEE can improve the protective defenses against oxidative stress in specific brain areas. This is an important step toward verifying a physiologically relevant contribution. The data presented here show a decrease in free-radical generation in the hypothalamus (but not cortex, striatum or hippocampus) obtained from POEE-treated aged mice. POEE also reduced the levels of two markers of oxidative stress: the formation of TBARS, an index of lipid peroxidation, was reduced in the frontal cortex, striatum and hypothalamus, and the carbonyl content, an index of oxidative damage to proteins, was diminished in the cerebellum and striatum.

It is conceivable that POEE may contribute to the maintenance of neural membrane stability by inhibiting lipid peroxidation. Lipid peroxidation affects membrane integrity and is accompanied by the generation of chemically reactive aldehyde products; these are thought to contribute significantly to the pathological effects of lipid peroxidation by covalently modifying cellular macromolecules, especially proteins. Carbonyl derivatives are formed by the attack of highly reactive free radicals to amino acid residues, or by reacting with sugars and/or products of sugar oxidation, or with lipid peroxidation products. The oxidative modification of proteins leads to structural changes and a consequent inactivation of many enzymes (Levine et al., 1990). The decline in oxidized proteins observed in brain areas of POEE-treated animals could be the direct result of reduced lipid peroxidation; in the case of the hypothalamus, such decline could also be related to an efficient scavenging of free radicals, as expressed by DCF levels.

Despite the fact that TAR levels (indicators of antioxidant reactivity) of brain samples were not affected by acute treatment with POEE, DCF formation (measure of free radical formation) was found to be reduced only in the hypothalamus. Data indicate that the highly active antioxidant compound(s) responsible

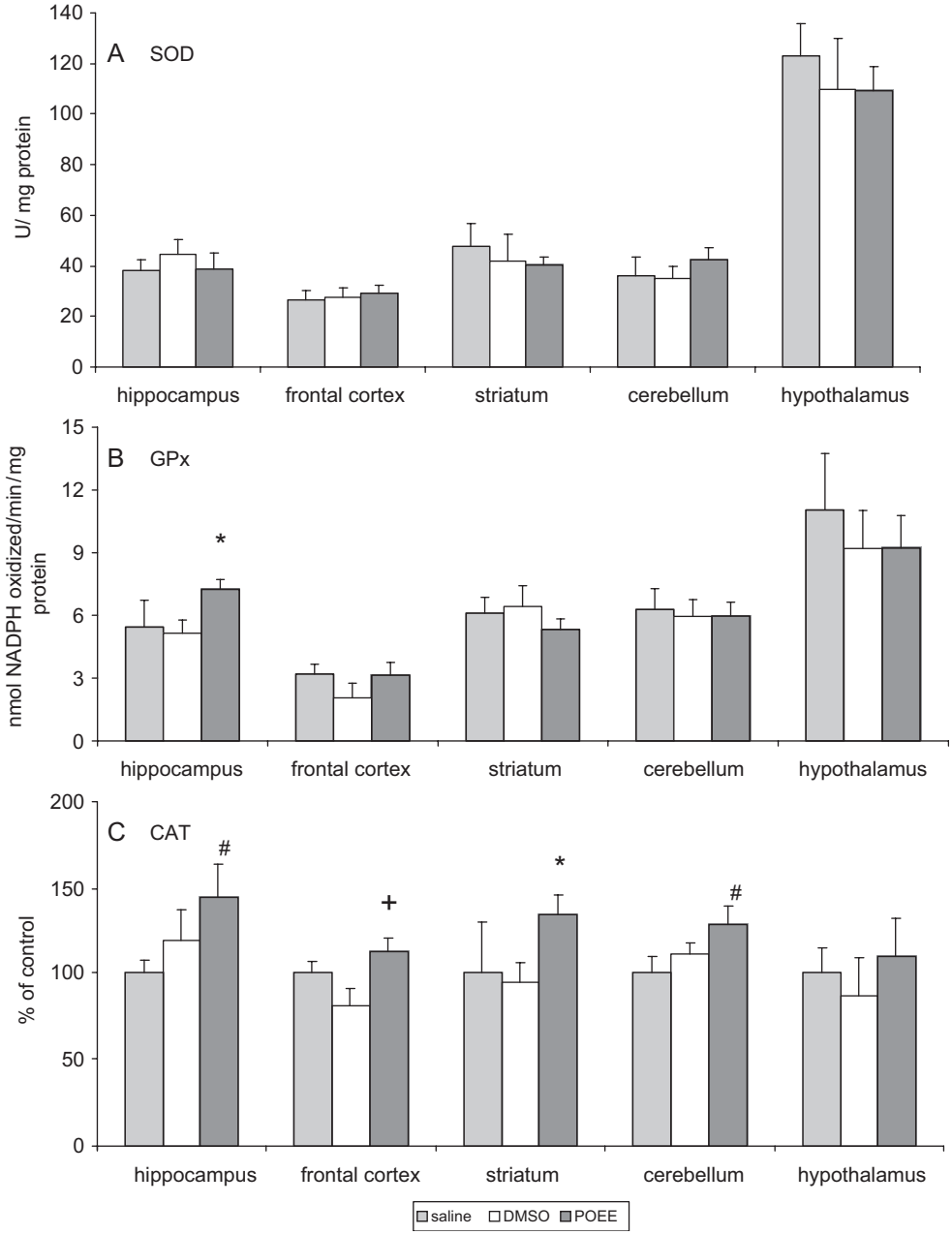

Fig. 3. Effects of POEE (100 mg/kg body wt.) on antioxidant enzymes (SOD 2A, CAT 2B, and GPx 2C) activities in brain regions from aging mice. The mean CAT values from saline groups were 677 ± 59 (cerebellum), 315 ± 19.15 (frontal cortex), 396 ± 113 (striatum), 525 ± 72 (hypothalamus), and 322 ± 21 mU/mg protein (hippocampus). Data expressed as mean $\pm$ SEM, 6–8 experiments. Anova followed by Duncan's test ($p < 0.05$), * as compared to saline and DMSO groups, # as compared to saline group, + as compared to DMSO group.

for the high TAR levels observed with POEE experiments in vitro (Siqueira et al., 2002) did not reach the brain areas studied, at least in significant amounts. The significant decrease in free-radical content found only in the hypothalamus suggests the presence of antioxidants unable to cross the BBB, since the structure lacks this barrier (Weindl and Joynt, 1973). Interestingly, several groups have suggested that the hypothalamus is a crucial area in mediating the age-related decline of physiological functions and alterations of biological rhythms (Meites et al., 1987; Carlson and Sawada, 1996; Hofman, 1997).

The diverse pattern of effects obtained in various brain areas can be interpreted as the result of a distinct distribution of active compound(s), as well as to specificities in regional antioxidant organization, since it has well recognized that the susceptibility to oxidative stress varies among brain regions (Candelario-Jalil et al., 2001a; Homi et al., 2002). Bickford (1993), Bickford et al. (1992) have suggested that the motor-dependent

learning deficits observed in aged rats is strongly related with losses in cerebellar function, which can be modified by nutritional intervention with antioxidants (Bickford et al., 2000). It is therefore noteworthy that the oxidative-protective effects of POEE seem to be particularly relevant in the cerebellum, striatum and hypothalamus.

Augmenting body defenses against free radicals seems to be another interesting approach for treating neurodegenerative disorders (Nikolov and Richardson, 1998). The ability of POEE to enhance brain CAT activity is of importance because catalase has very low activity in the brain (Benzi and Moretti, 1995). Additionally, it is relevant that catalase (mitochondrial and cytosolic) has been found to enhance respiration maintaining adequate ATP levels (Rodriguez et al., 2000). It has been shown that a persistent and intense decrease in hippocampal GPx and glutathione reductase activities occurs in experimental models of induced neuronal damage (Candelario-Jalil et al., 2001b). Therefore, the POEE-induced enhancement of CAT and GPx activities in some brain structures might be an important mechanism to avoid hydrogen peroxide accumulation.

At this point, neither the active compound(s) nor the exact mechanism(s) by which POEE exerts its antioxidant activities are completely known. Nevertheless, β-sistosterol and lupeol, both present in *P. olacoides*, have been shown to possess antioxidant properties. Beta-sistosterol has been reported to inhibit lipid peroxidation in vitro, at low concentrations (van Rensburg et al., 2000), while lupeol is associated with lipid-peroxide reduction (Nagaraj et al., 2000).

Our results are consistent with previous studies reporting neuroprotection by antioxidants, as well as improvements in age-related neurological decline found with other herbal drugs (Arivazhagan et al., 2001; Ward et al., 2002). In situations of oxidative stress, especially in the brain, it is desirable that a combination of complementary antioxidant properties occur. Our previous studies indicate that *P. olacoides* possesses compound(s) with multiple and complementary modes of action; in this case, free-radical scavenging and increased activity of antioxidant enzymes might be important neuroprotective properties.

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