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Protective effect of supercritical fluid rosemary extract, *Rosmarinus officinalis*, on antioxidants of major organs of aged rats

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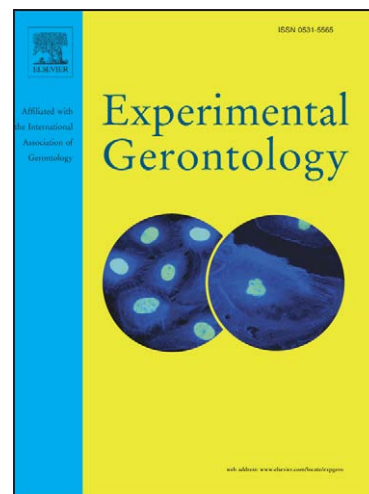
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7 antioxidants of major organs of aged rats.  
8

9 **Running title:** Effects of rosemary extract in aged rats

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**Abstract**

Rosemary leaves, "*Rosmarinus officinalis*", possess a variety of antioxidant, anti-tumoral and anti-inflammatory bioactivities. We hypothesized that rosemary extract could enhance antioxidant defenses and improve antioxidant status in aged rats.

This work evaluates whether supplementing their diet with super critical fluid (SFE) rosemary extract containing 20% antioxidant carnosic acid (CA) reduces oxidative stress in aged rats.

Aged Wistar rats (20 months old) were included in the study. Rats were fed for twelve weeks with a standard kibble (80%) supplemented with turkey breast (20%) containing none or one of two different SFE rosemary concentrations (0.2% and 0.02%). After sacrifice, tissue samples were collected from heart and brain (cortex and hippocampus). Enzyme activities of catalase (CAT), glutathione peroxidase (GPX), superoxide dismutase (SOD) and nitric oxide synthase (NOS) were quantitatively analyzed. Lipid peroxidation and levels of reactive oxygen species (ROS) were also determined.

Rosemary decreased lipid peroxidation in both brain tissues. The levels of catalase activities in heart and cortex were decreased in the rosemary-treated groups. The SFE rosemary treated rats presented lower NOS levels in heart and lower ROS levels in hippocampus than the control rats.

Supplementing the diet of aged rats with SFE rosemary extract produced a decrease in antioxidant enzyme activity, lipid peroxidation and ROS levels that was significant for catalase activity in heart and brain, NOS in heart, and LPO and ROS levels in different brain tissues. These observations suggest that the rosemary supplement improved the oxidative stress status in old rats.

**Key words:** Oxidative stress, ageing, SFE rosemary, carnosic acid, *Rosmarinus officinalis*.

## Introduction

Oxidative stress results from an imbalance between the cellular production of reactive oxygen species (ROS) and the antioxidant mechanisms that remove them (Halliwell, 1992). Oxidative stress can damage the cell through the oxidization of cellular elements like membrane lipids, proteins and DNA. Numerous studies have reported correlations between age and the accumulation of oxidative damage in cellular macromolecules (Floyd and Hensley, 2002; Stadtman, 2001). For example, aged rats exhibit increased lipid peroxidation (Calabrese et al., 2004, Devi and Kiran, 2004; Gupta et al., 1991; Murray and Lynch, 1998; O'Donnell and Lynch, 1998) and protein oxidation (Cini and Moretti, 1995; Forster et al., 1996; Sohal et al., 1994) in their brains. Additionally, it has been demonstrated that aged rats have increased oxidative damage in both nuclear and mitochondrial DNA (Hamilton et al., 2001). The strong correlation between increased age and oxidative stress and the accumulation of oxidative damage has suggested the oxidative stress hypothesis of ageing (Beckman and Ames, 1998). The increase in age-related oxidative damage could result from a multitude of factors, including a decrease in antioxidant defenses.

Several antioxidant mechanisms counterbalance the potential deleterious effects of ROS. Among the enzymatic ROS scavengers are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) (Brigelius-Flohe, 1999; Halliwell, 1991). There are also hydrophilic radical scavengers such as ascorbate (Vitamin C), urate, and glutathione, as well as lipophilic radical scavengers such as tocopherol (Vitamin E), carotenoids and flavonoids. Therefore, dietary supplementation with antioxidants could conceivably protect against the molecular effects of lipid peroxidation, free radicals and ROS and also delay the progress of many chronic diseases (Lai et al., 2001; Gulcin et al., 2003). Research into the characteristics of traditional herbs has revealed several interesting bioactive compounds. Consequently, compounds (particularly from natural sources) with potentially antioxidative properties are being sought.

*Rosemarinus officinalis* leaves possess a variety of bioactive agents, including antioxidants (Auroma et al., 1996; Ramirez et al., 2006), anti-tumorals (Singletary et al., 1996) and anti-inflammatories (Altinier et al., 2007). The main relevant constituents are composed of vast numbers of polyphenolics, including carnosic acid, carnosol, rosemarinic acid, ursolic acid, etc (Ramirez et al., 2006; Richeimer et al., 1996; Seronans et al.,

2000). Among these, carnosic acid (a phenolic diterpene compound), and carnosol are the most potent antioxidant constituents (about 90% of antioxidant activity). The disadvantages of CA, however, are its thermal-, photo-, and oxidation-labile physico-chemical properties. The problems derived from its physico-chemical properties can be avoided using super critical fluid extraction to produce rosemary extract, which, because of the low temperatures at which it operates, is very suitable for the extraction of thermolabile compounds like antioxidants (Ramirez et al., 2006). Furthermore, CA has antimicrobial activity (Moreno et al., 2006; Oluwatuyi et al., 2004), can inhibit lipid absorption in humans (Ninomiya et al., 2004) and is a free radical scavenger, due to its phenolic skeleton (del Bano et al., 2003; Masuda et al., 2001, 2002). Nevertheless, little information is available about the dietary value of rosemary extract in regard to its antioxidant activity and possible application in inhibiting oxidative stress in aged individuals.

The present study was designed to determine whether two concentrations (0.2 and 0.02%) of a rosemary extract obtained via the supercritical fluid extraction method (Ramirez et al., 2006) and containing 20% CA (with high free radical scavenging capacity) could enhance antioxidant status in aged rats. We focused our attention on its effects on the activities of catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) as well as on the levels of nitric oxide synthase activity (NOS), ROS and lipid peroxidation.

## Material and Methods

### Animals

Male Wistar rats between 20 and 21 months old (550–700 g body wt.), were obtained from Harlam (Barcelona, Spain) and acclimated for at least 1 week prior to experimental use. Animals were individually housed with free access to food and water, and maintained under a normal light–dark cycle in the Experimental Surgery Service of La Paz Hospital. The present study was approved by the Institutional Animal Ethics Committee (IAEC), La Paz Hospital, Madrid, Spain, in accordance with the Spanish law for the protection of experimental animals and other research purposes: RD 1201/2005.

Rats were distributed randomly into three groups of ten animals each. Table I shows the different groups and diets.

### Diets

Animals were fed standard kibble (80%) plus turkey breast in pellet form (20%, donated by

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3 FRIAL, S A (Madrid, Spain) for one week to accustom the animals to the diet. Afterwards,  
4 animals were fed the following twelve weeks with kibble plus the meat alone or  
5 supplemented with either 0.2% or 0.02% SFE rosemary extract (see Table I). Frial, SA  
6 (Madrid Spain), a Spanish food manufacturer, provided turkey breast pellets that were  
7 plain or supplemented with 0.2% or 0.02% SFE rosemary extract. Body weight did not  
8 show significant differences between groups at any time during the study.  
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### 11 12 **Sample collection and tissue preparation**

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16 At twelve weeks on the diet, rats were killed by decapitation under light anesthesia with  
17 isoflurane (Forane<sup>®</sup>, at 2%). Heart and brain were quickly removed. Cerebral cortex and  
18 hippocampus were isolated (Cini and Moretti, 1995), weighed and rinsed several times in  
19 ice-cold ACSF medium. Afterwards all samples were washed in an ice-cold phosphate  
20 buffer saline (PBS). The washed samples were immediately immersed in liquid nitrogen  
21 and stored at  $-70^{\circ}$  C until biochemical analysis.  
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27 On the day of use, the frozen tissue samples were quickly weighed and homogenized 1:10  
28 in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and  
29 centrifuged at  $12000 \times g$  for 15 min at  $4^{\circ}$  C. The supernatants were separated and used  
30 for protein determination, enzyme activity assay, lipid peroxidation and ROS  
31 measurement.  
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### 36 **Protein assay**

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38 The total protein concentrations in the tissue homogenates were measured by Bradford's  
39 method (Pierce, BCA Protein Assay kit, USA) using bovine serum albumin as standard.  
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### 43 **Catalase activity assay**

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45 Catalase activity was spectrophotometrically measured in tissue homogenates as in  
46 (Cohen, G. 1970), following the manufacturer's protocol (Catalase Assay kit" Cayman<sup>®</sup>  
47 Chemical, USA). This method uses a formaldehyde solution as standard. The absorbance  
48 of standard and samples was read at 540 nm with a plate reader (Biotek Instruments, Inc.  
49 Vermont, USA). Catalase activity was expressed in nmol/min/mg of protein.  
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### 54 **SOD activity assay**

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3 Total SOD activity (cytosolic and mitochondrial) was determined as in (Paoletti, F., 1990)  
4 following the manufacturer's instructions (Superoxide Dismutase assay kit" Cayman®  
5 Chemical, USA). Supernatants were desalted by passage through a Sephadex G-25  
6 column. The SOD samples were also treated with a mixture of ethanol-chloroform (2:1,  
7 v/v) and distilled water to eliminate hemoglobin and red blood cells and then read on plate  
8 reader (Biotek Instruments, Inc. Vermont, USA) at 450 nm. Results are expressed as units  
9 per mg of protein (U/mg of protein).  
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### 14 **GPX activity assay**

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17 GPX activity was assayed as in (Paglia, D. E., et al. 1967) following the manufacturer's  
18 instructions (Glutathione Peroxidase assay kit" Cayman® Chemical, USA). This method is  
19 based on the oxidation of NADPH to NAD<sup>+</sup>, which is accompanied by a decrease in  
20 absorbance at 340nm. GPX activity was measured by initiating the reaction with 2.4 mM  
21 cumene hydroperoxide. One unit is defined as the amount of enzyme that oxidizes 1 μmol  
22 of NADPH per min at 25°C. The absorbance was read every minute at 340 nm using a  
23 plate reader (Biotek Instruments, Inc. Vermont, USA) to obtain at least 5 time points. The  
24 GPX activity was calculated in nmol/min/mg of protein.  
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### 32 **Oxide Nitric Synthase activity**

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34 NOS activity was measured in all tissues according to the method described in (Nims  
35 R.W., 1995) and following the manufacturer's instructions in "Nitrate/Nitrite Colorimetric  
36 Assay Kit (LDH method)" (Cayman® Chemical, USA). Prior to assay homogenated  
37 samples were filtered using a 30 kDa molecular weight cut-off filter. The sample volume for  
38 assay was a maximum of 40μl of the filtrate. A nitrate standard curve was included in order  
39 to quantitate sample nitrate + nitrite concentrations. The adsorbancies were read at 540  
40 nm using a plate reader (Biotek Instruments, Inc. Vermont, USA). Results are expressed in  
41 μM units/mg of protein.  
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### 49 **Lipid Peroxidation: Determination of malondialdehyde (MDA)**

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51 Heart and brain lipid peroxidation was determined by measuring the level of MDA using  
52 the Ohkawa technique (Ohkawa, H., 1979) following the manufacturer's instructions  
53 (TBARS assay kit" Cayman® Chemical, USA). This method is based on the reaction of  
54 thiobarbituric acid with malonyl dialdehyde MDA. MDA dilutions ranged between 0 and 50  
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3  $\mu\text{M}$  were used as standards. Results were measured on a 532 nm plate reader (Biotek  
4 Instruments, Inc. Vermont, USA). MDA concentration was expressed as  $\mu\text{M}$  of MDA/mg of  
5 protein.  
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### 8 9 **Assay for Reactive Oxygen Species (ROS assay)**

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11 A fluorometric assay (Socci, D. J., 1999) was used to determine the relative levels of  
12 different reactive oxygen species. This assay measures the oxidative conversion of stable  
13 2', 7'-dichlorodihydrofluorecein diacetate ( $\text{H}_2\text{DCFDA}$ , Invitrogen, Molecular Probes) to  
14 the highly fluorescent 2', 7'-dichlorofluorescein in the presence of peroxides, especially  
15 hydrogen peroxide. Fractions from tissue homogenates were loaded with 40  $\mu\text{l}$  of 1.25 mM  
16 2', 7'- dichlorodihydrofluorecein diacetate in methanol. A remaining fraction with  
17 methanol was used as a blank to control for tissue autofluorescence. All samples were  
18 incubated for 15 min in a 32° C water bath. Fluorescence was determined at four time  
19 points postincubation (0, 30, 60, 90 min) using a plate reader (Biotek Instruments, Inc.  
20 Vermont, USA), at 510 nm excitation, with 528 nm emission. Blank readings were  
21 subtracted from loaded sample readings, with values indexed per milligram of total protein.  
22 Readings at 60 minutes post incubation were considered optimal.  
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### 32 **Statistical analyses**

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34 All calculations were performed using SPSS 11.0 statistical software. Data from different  
35 groups were statistically analysed and compared using analysis of variance (ANOVA)  
36 followed by post hoc analysis using the Dunnett and Tukey tests. *P*-values less than 0.05  
37 were considered statistically significant. Results were expressed as means  $\pm$  S.D.  
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## 42 **Results**

### 43 **Lipid Peroxidation**

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45 The results in heart did not show a significant effect from SFE rosemary extract on lipid  
46 peroxidation in both groups with a  $F(2, 29) = 2.63$ ;  $p < 0.131$ .  
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50 However, the rosemary extract supplement did significantly decrease lipid peroxidation in  
51 the cortex and hippocampus with  $F(2, 29) = 14.31$ ;  $p < 0.001$  for the former and  $F(2, 29) =$   
52  $9.8$ ;  $p < 0.001$  for the latter. The effect was stronger in the animals treated with 0.2%  
53 ( $p < 0.001$ ) than in the animals treated with 0.02% rosemary extract ( $p < 0.029$ ), in  
54 comparison to the controls. The hippocampus only showed significantly decreased  
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3 peroxidation in the 0.2% rosemary treated rats ( $p<0.001$ ). Rats receiving the 0.02%  
4 rosemary supplement did not show a significant decrease ( $p<0.08$ ). Lipid peroxidation  
5 levels are represented in a bar diagram for cortex (figure 1A) and hippocampus (figure 1B)  
6 and measured as MDA concentration in ( $\mu\text{M}$ )/ mg of protein.  
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### 10 **CAT, GPX and SOD Activity**

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12 In heart tissue, ANOVA analysis of data showed significantly decreased CAT activity in  
13 rosemary treated animals  $F(2, 29)=6.815$ ;  $p<0.003$ . Results for catalase activity are shown  
14 in a bar diagram (figure 2A) with catalase activity measured as  $\text{nmol}/\text{min}/\mu\text{g}$  of protein.  
15 Differences between groups were at the  $p<0.05$  level with the 0.2% rosemary extract and  
16 the  $p<0.003$  level with the 0.02% rosemary extract. The levels of catalase activity were  
17 higher in controls than in either study group. Data also showed a significant effect from  
18 SFE rosemary extract on CAT activity in the cortex  $F(2, 29)=5.56$ ;  $p<0.007$ . Figure 2B  
19 shows the results in the different study groups. The cortical effect was significant with both  
20 0.2% rosemary extract ( $p<0.05$ ) and 0.02% rosemary extract ( $p<0.017$ ). However, ANOVA  
21 data did not show a significant effect from rosemary extract on CAT activity in the  
22 hippocampus when the three groups were compared  $F(2, 29)=0.421$ ;  $p<0.663$ .

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GPX activity in heart, cortex or hippocampus was not affected by rosemary extract at  
either concentration ( $F(2, 29)=0.477$ ,  $p<0.63$ ;  $F(2, 29)=0.42$ ;  $p<0.66$  and  $F(2, 29)=1.00$ ;  
 $p<0.38$  respectively).

The ANOVA for SOD activity did not show an effect from rosemary extract on enzyme  
activity in any of the three tissues: heart, cortex or hippocampus ( $F(2, 29)=0.940$ ;  $p<0.40$ ,  
 $F(2, 29)=0.158$ ;  $p<0.855$  and  $F(2, 29)=3.56$ ;  $p<0.54$ , respectively). Table II gives the mean  
and SD for SOD and GPX, which showed no significant differences.

### 45 **NOS activity**

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Heart NOS activity levels were significantly decreased by SFE rosemary extract  $F(2,$   
 $29)=4.4$ ;  $p<0.029$ . Interestingly, when we compared each group individually against the  
others we observed a statistical difference between control and rosemary extract at 0.02%.  
( $p<0.05$ ). Figure 3 shows a bar diagram for NOS activity in the different study groups. NOS  
activity is expressed as the concentration of nitrate+nitrite in  $\mu\text{M}$  units/  $\mu\text{g}$  of protein.  
However the ANOVA did not show a significant effect from rosemary extract on enzyme

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3 activity in hippocampus ( $F(2, 29)=0.111$ ;  $p<0.89$ ). Table II shows the non-significant  
4 values for NOS.  
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### 6 7 **ROS levels**

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9 ANOVA data did not show a significant effect from SFE rosemary extract on ROS levels in  
10 heart or cortex, ( $F(2, 29)=2.86$ ;  $p<0.08$  for the former and  $F(2, 29)=0.39$ ;  $p<0.68$  for the  
11 second tissue). In hippocampus, however ANOVA did show that the rosemary extract  
12 significantly decreased ROS levels ( $F(2, 29)=26.21$ ;  $p<0.001$ ). The effect of 0.2%  
13 rosemary extract was ( $p<0.001$ ) stronger than that of 0.02% rosemary extract ( $p<0.002$ ).  
14 The figure 4 bar diagram shows the results for ROS levels in this tissue in the different  
15 study groups, expressed as fluorescence units per mg of total protein.  
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### 22 **Discussion**

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24 The aging process itself exemplifies the cumulative effect of deterioration caused by free  
25 radicals in cells and tissues. Although most tissues, including brain and heart, have built-in  
26 mechanisms to counteract these radicals, the antioxidant defense is overwhelmed by the  
27 aging process, disease aside. The role of enzymatic antioxidants in ageing is  
28 controversial. Normal ageing is accompanied by a decline in antioxidant defenses  
29 reflected in reduced glutathione in blood and different tissues of animals and humans  
30 (Muthuswamy, et al., 2006). In fact, supplementing the diet with natural compounds with  
31 antioxidant properties has been shown to be beneficial (De la Fuente and Victor, 2000).  
32 Nevertheless, it has recently been hypothesized that antioxidants do not seem to control  
33 the kinetics of ageing (Barja, 2004). Many of these antioxidant enzymes are induced by  
34 stress, so higher levels would indicate either a better protection or, alternatively, a greater  
35 need for antioxidant defenses. In fact, the antioxidant mechanisms are a harmonic system  
36 and the interactions among them are complex.  
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48 In this work we have analyzed whether two different areas of the brain, namely the  
49 cerebral cortex and the hippocampus respond to the effects of rosemary extract in an  
50 aged animal model using an extract containing 20% CA and obtained with the  
51 supercritical fluid extraction method (Ramirez et al., 2006) at two concentrations (0.2% and  
52 0.02%). Additionally, we also included heart tissue to evaluate the possible effects on  
53 cardiovascular oxidative stress status in aged rats.  
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3 Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of  
4 polyunsaturated lipids. Only low levels of lipid peroxides occur in body tissues under  
5 physiological conditions. An excessive generation of free radicals leads to peroxidative  
6 changes that ultimately result in enhanced lipid peroxidation (Rikans and Hornbrook,  
7 1997). We inferred lipid peroxidation from the level of MDA, which is a secondary product  
8 of lipid peroxidation, and is used as an indicator of tissue damage (Ohkawa et al., 1979).  
9 Ageing has been reported to be associated with increased disruption of lipid membranes  
10 leading to a subsequent formation of peroxide radicals (Niki et al., 1993). The available  
11 literature indicates that the brain has higher levels of lipids and consumes a greater  
12 quantity of oxygen than other organs, and in fact some studies have reported an increase  
13 in lipid peroxidation with ageing (Farooqui et al., 1987; Jayakumar et al., 2007; Serrano  
14 and Klann, 2004). In our results, the level of membrane lipid products in brain was  
15 significantly lower in the groups receiving the rosemary extract. The two concentrations of  
16 rosemary extract (0.2% and 0.02%) had a significant antioxidant lipid effect in cortex, but,  
17 and surprisingly, only the lowest concentration (0.02%) of rosemary extract had a  
18 significant effect in hippocampus. These data corroborate findings in earlier investigations  
19 where antioxidants and administration of a natural grape seed extract decreased lipid  
20 peroxidation in the brain of aged rats (Arivazhagan and Panneerselvam, 2000; Balu et al.,  
21 2005). The observed reduction in MDA levels in aged rats following the administration of  
22 rosemary extract suggest *Rosmarinus officinalis*, may have an antioxidant effect in the  
23 brain.

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37 When we examined heart tissue we did not observe significant differences between the  
38 rosemary extract treated and the control groups, although levels of lipid peroxidation were  
39 lower in the former. Recent studies report increased MDA levels in the heart of aged rats  
40 (Jayakumar et al., 2007; Sivonova et al., 2007). Therefore, rosemary has some antioxidant  
41 effect in heart tissue, which somehow reverses the lipid peroxidation observed in aged  
42 individuals, but it is not enough to be deemed significant in our study.

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47 Living tissues are endowed with innate antioxidant defence mechanisms, including the  
48 CAT, SOD and GPX. CAT has been shown to be responsible for the detoxification of  
49 significant amounts of  $H_2O_2$  (Cheng et al., 1981). SOD catalyzes the removal of  
50 superoxide radical  $O_2^-$ , which would otherwise damage the cell membrane and biological  
51 structures. GPX catalyses the reduction of  $H_2O_2$  to  $H_2O$  and  $O_2$ . In general, a reduction in  
52 the activity of these enzymes is associated with an accumulation of highly reactive free  
53 radicals, and can lead to deleterious effects such as loss of cell membrane integrity and  
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3 function (Reedy and Lokesh, 1992 ; Sheela and Angusti, 1995). We found diminished  
4 levels of CAT activity in brain and heart tissues in both rosemary extract treated groups,  
5 and this decrease was statistically significant in heart and cortex but not in the  
6 hippocampus. This decrease in the enzymatic activity was significant in heart with both  
7 rosemary extract concentrations (0.02%, 0.2%), but only the higher concentration (0.2%)  
8 had an effect in cortex. The available information in literature about age-related changes in  
9 rodent antioxidant enzymes indicates that catalase activity is increased with ageing and  
10 catalase activity changes are more selective in tissues such as heart and brain (Calabrese  
11 et al., 2004). The lower CAT activity levels observed here in the rosemary-treated animals  
12 are related to the effect of the supplement. In agreement with the literature our results  
13 could suggest that rosemary extract somehow reverses the effects of aging on catalase  
14 activity in heart and cortex, indicating the antioxidant potential of the rosemary extract.  
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18 The findings on SOD and GPX activities were not statistically significant, although they did  
19 show some reduction in these enzymatic activities in the rosemary treated groups in brain  
20 and heart. The available information about the activity and expression of GPX and SOD is  
21 contradictory, with decrease, no changes and increases with ageing in different tissues,  
22 among them, heart and brain, all being reported (Benzi and Moretti, 1995; Carrillo et al.,  
23 1992; Dođru-Abbasođlu et al., 1997; Danh et al., 1986; Gupta et al., 1991; Scarpa et al.,  
24 1987; Warner, 1994). In fact, changes in antioxidant enzyme activity need to be carefully  
25 interpreted, taking into consideration the facts that these changes are markedly variable  
26 depending on sex as well as the organs and brain regions examined and even on the  
27 animal model or analytic techniques employed in different studies.  
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31 There are two main forms of NOS in the heart, inducible NOS (iNOS) located in the  
32 myocytes, and endothelial NOS (eNOS), located in endothelial cells (Bolli, 2001). NOS has  
33 a dual cardioprotective role: initiator and mediator. The mechanism by which NOS protects  
34 heart tissue has not yet been totally clarified, although a number of different mechanisms  
35 have been suggested, the most important being antioxidant action (Bolli, 2001). We could  
36 not find significant differences in the levels of the enzyme activity in the brain regions  
37 analyzed. Our results found NOS decreases in heart tissue in the groups treated with  
38 rosemary extract (0.02%) and these differences with the control group were statistically  
39 significant. The available information indicates that aged Wistar rats show increased  
40 levels of NOS (Cernadas et al., 1998; Zieman et al., 2001). Notably, recent studies on the  
41 functional role of NOS in cardioprotection report that augmented NO levels are related to  
42 heart failure, whereas NOS decreases levels of NO activity and protects this tissue.  
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3 Furthermore, dietary deficiencies of oligoelements like copper can raise NOS levels and  
4 there is a clear relationship with the appearance of heart tissue abnormalities and elevated  
5 NOS levels (Saari et al., 2007). Therefore, our results could indicate a protective action by  
6 rosemary extract in heart tissue through decreases in NOS activity levels, and  
7 subsequently, NO production.  
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11 In general, the ageing process is related with an increase in the production of the different  
12 reactive oxygen species (Hagen et al., 2002). Our results showed no significant  
13 differences in ROS levels between rosemary extract treated groups and the control group  
14 in either heart or cortex. However, the group comparison in the hippocampus showed  
15 significantly lower ROS levels in this structure with both concentrations of rosemary  
16 extract. We therefore suggest that rosemary extract has an antioxidant effect as a free  
17 radical scavenger in this organ. This agrees with a growing number of studies showing that  
18 natural extracts and phytochemicals have a positive impact on brain ageing through their  
19 action on ROS, particulally in the hippocampus (Bastianetto and Quirion, 2002).  
20

21 As far as we know this is the first work that shows antioxidant effects of rosemary extract  
22 in aged rats (in vivo). In conclusion, in aged rats rosemary extract consumption decreases  
23 cerebral catalase activity, lipid peroxidation and ROS levels, thus protecting the brain, and  
24 decreases the activity of catalase and NOS in the heart, protecting this organ. The effects  
25 of the two rosemary concentrations were different in the three tissue types since lipid  
26 peroxidation, ROS levels and enzymatic activities were different in both treated groups.  
27 Further studies are required to determine the causes behind the distinct effects of the two  
28 different rosemary extract concentrations and it remains to be seen whether a similar  
29 protective effect can be demonstrated in aged humans. Thus, rosemary extract could be  
30 incorporated to the diet as a nutritional supplement, to augment the body's defenses  
31 against oxidative stress.  
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### Legends to the figures.

**Figure 1.** Bar diagram showing the lipid peroxidation in the different groups in cortex (A) and hippocampus (B). # indicates significant at  $p < 0.001$  level (control vs 0.2% rosemary extract) and \* at  $p < 0.03$  level (control vs 0.02% rosemary extract). Bar diagram represents the mean and SD values for each group.

**Figure 2.** Bar diagram showing the catalase activity in the different groups in heart (A) and cortex (B). # indicates significant at  $p < 0.05$  level (control vs 0.2% rosemary extract) and \* at  $p < 0.017$  level (control vs 0.02% rosemary extract). Bar diagram represents the mean and SD values for each group.

**Figure 3.** Bar diagram showing the NOS activity the different groups in heart. \* indicates significant at  $p < 0.05$  level (control vs 0.02% rosemary extract). Bar diagram represents the mean and SD values for each group.

**Figure 4.** Bar diagram showing the ROS levels in the different groups in hippocampus. # indicates significant at  $p < 0.001$  level (control vs 0.2% rosemary extract) and \* at  $p < 0.002$  level (control vs 0.2% rosemary extract). Bar diagram represents the mean and SD values for each group.

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10 **Tables**

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13 **Table I.** Different groups, the characteristics of the diet and the meat food composition.  
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Groups		Extract concentration (%)
1	Control (n=10)	0
2	C. A. Ext. 20% (n=10)	0.2
3	C. A. Ext. 20% (n=10)	0.02
Functional meat food composition		Values per 100gr
1	Energy (Kcal)	107
2	Proteins (g)	19.0
3	Carbohydrates (g)	1.0
4	Fat (g)	2.0
5	Saturated (g)	0.7
6	Monosaturated (g)	0.8
7	Polyunsaturated (g) ( $\omega$ -3+ $\omega$ -6)	0.5
8	Na <sup>+</sup> (g)	0.5
9	Ratio ( $\omega$ -3/ $\omega$ -6)	0.5

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**Table II.** Statistical descriptive data of SOD, GPX and NOS activity in heart, cortex and hippocampus.

Tissue	Groups	SOD	GPX	NOS
heart	Control	47.50±6.3	21.14±3.8	5.70±0.9 <sup>a</sup>
	Rosemary 0.02%	44.85±6.2	17.75±2.9	3.87±0.9 <sup>b</sup>
	Rosemary 0.2%	41.90±5.9	18.11±3.0	5.50±1.1 <sup>a</sup>
cortex	Control	35.73±4.9	74.60±9.9	11.40±1.7
	Rosemary 0.02%	32.87±4.6	67.75±8.9	11.10±1.7
	Rosemary 0.2%	31.61±4.2	68.76±8.8	10.78±1.5
hippocampus	Control	32.67±5.0	56.70±7.1	14.29±2.1
	Rosemary 0.02%	31.40±4.7	48.12±6.7	15.35±3.5
	Rosemary 0.2%	30.00±4.5	52.44±7.0	14.9±3.2

Values are mean ± S. D. of ten animals per group. SOD activity is expressed as units per mg of protein. GPX activity is expressed as nmol/min/mg of protein. NOS activity is expressed as  $\mu\text{M}$  units per mg of protein. Values with different superscripts between groups in each column are significantly different ( $p < 0.05$ ).

Figure 1

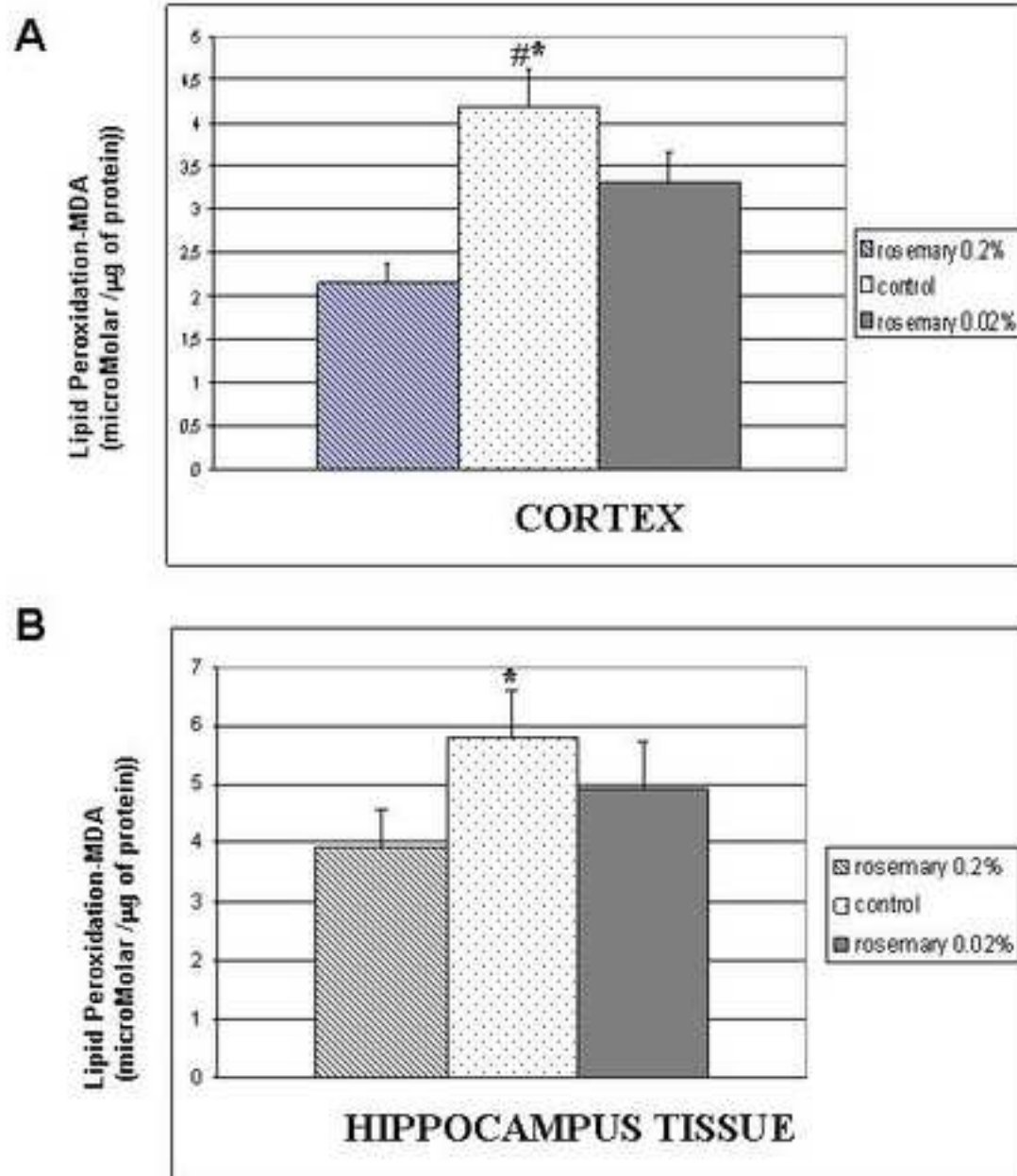
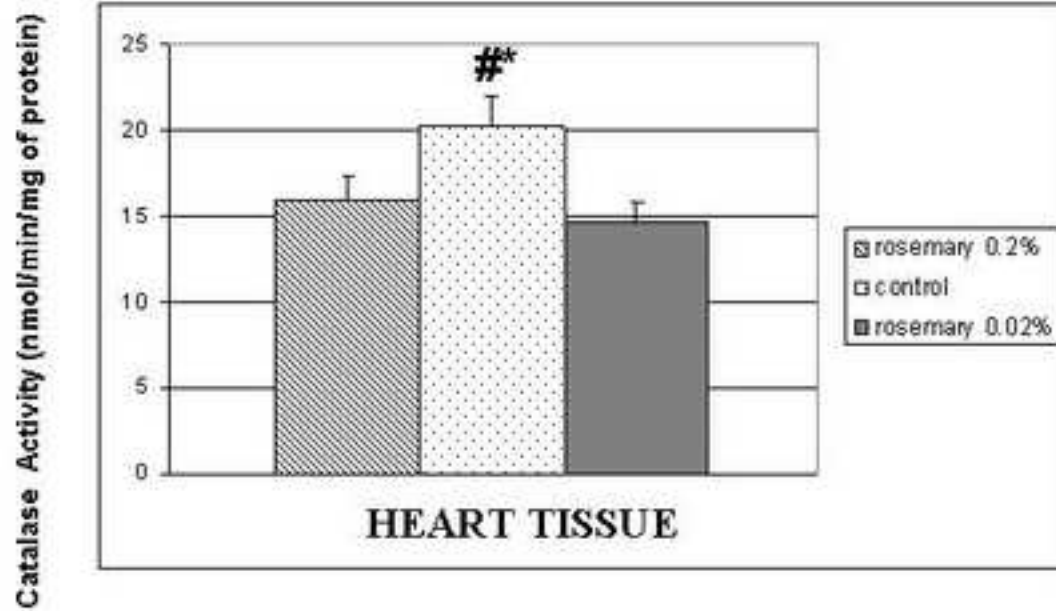


Figure 2

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Catalase Activity (nmol/min/mg of protein)

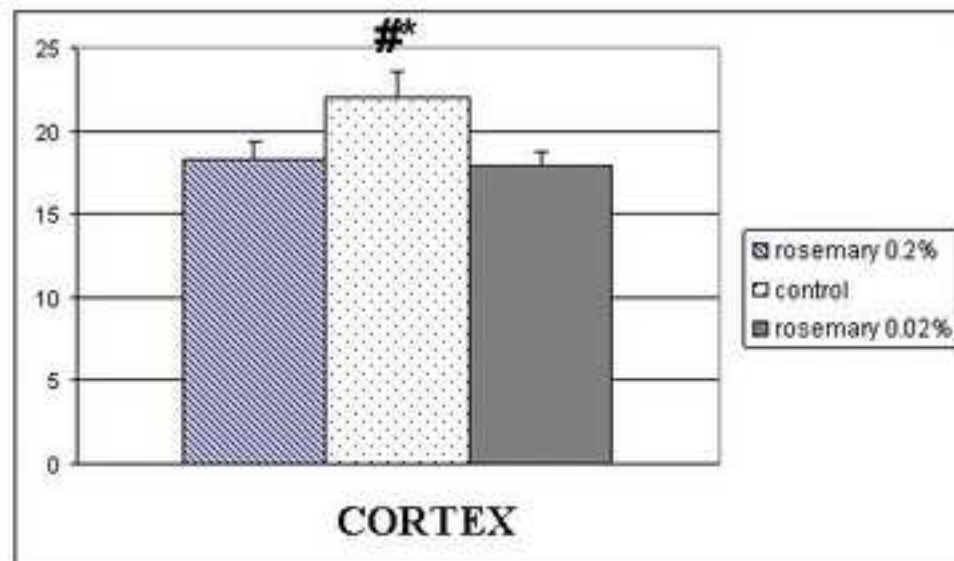


Figure 3

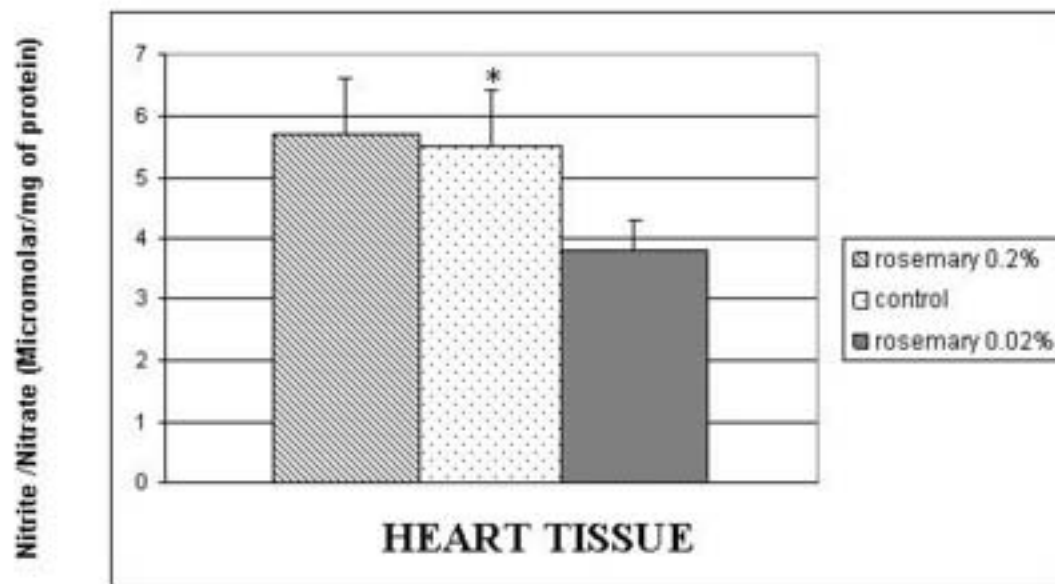


Figure 4

