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Preliminary Studies on the Protective Effect of *Rosmarinus Officinalis* on Astrocytes

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Abstract

Rosmarinus officinalis L (Rosemary) is an aromatic species spontaneously growing in the Mediterranean area endorsed with antioxidant activity mainly related to its polyphenolic composition. The aim of this study was to evaluate the protective activity of *R. officinalis* on astrocytes culture (U-373 MG) submitted to the oxidative damage induced by H_2O_2 when compared with the positive control, Trolox. *R. officinalis* aerial parts were collected in central Spain and extracted with methanol and concentrated until dryness. First, antioxidant activity was assayed by the ORAC method. Then, the lack of cell toxicity was observed for concentrations ranging from 12.5 to 200 µg/mL of rosemary extract for 24h (MTT -3(4,5-dimethyltiazol-2-il)-2,5-diphenyltetrazolium- reduction assay with and without oxidative injury). Antiradical and antioxidant activities were evaluated by the 2′,7′-dichlorofluorescein (DCFH)- DA assay and reduced glutathione (GSH) levels. Oxidative damage induced by 1mM H_2O_2 was shown as a significant increase in ROS production and a decrease in reduced glutathione (GSH) levels. Rosemary extract did not alter ROS production when administered alone or previous to the oxidative insult. Cells treated with 50 µg/mL of rosemary extract were able to recover GSH levels and the oxidized/reduced glutathione ratio close to control cells. Results demonstrated that the protective effect shown by *R. officinalis* extract through its antioxidant ability may involve other mechanisms different from a direct effect on ROS production or modulation of the glutathione activity.

Keywords: *Rosmarinus officinalis*; Oxidative Stress; ROS; MTT

Abbreviations: ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; ORAC: Oxygen Radical Absorbance Capacity; GSH: Glutathione; INIA: Institute of Agricultural and Food Technology; DMEM: Dulbecco´s Modified Eagle´s Medium; FBS: Foetal Bovine Serum; DMSO: Dimethyl sulphoxide; DCF: Dichlorofluorescein

Introduction

Different pro-oxidant compounds in the form of reactive oxygen species (ROS) such as hydrogen peroxide, nitric oxide, superoxide and the highly reactive hydroxyl radicals and reactive nitrogen species (RNS) are naturally generated in biological systems. Its production is counteracted by the intrinsic antioxidant defense, both enzymatic and non-enzymatic, which protects against free radicals and the subsequent cell damage [1]. Oxidative damage occurs as an imbalance between the production of ROS and the ability of intrinsic antioxidant systems, to scavenge these radicals. Oxidation of macromolecules such as proteins, lipids and DNA may lead to cell degeneration and death due to an increase in the release of apoptotic inducing factors [2,3]. Brain is especially sensitive to oxidative stress because of the high proportion of unsaturated fatty acids, the high metabolic rate, the low antioxidants proportion and the slow cellular regeneration. Neurodegenerative diseases such as Alzheimer's, Parkinson or amyotrophic lateral sclerosis have been found to be directly related to oxidative stress increase, elderly being the main risk factor for the development of these kind of diseases, together with toxic metabolic or infectious processes [4-7]. *Rosmarinus officinalis L.* (Lamiaceae) is an ever green plant spontaneously growing in the Mediterranean area. Aerial parts of rosemary are rich in polyphenolic compounds endorsed with antioxidant activity [8-12]. In continuation with our research line, *R. officinalis* methanolic extract was assayed on the human astrocyte glioblastoma, which is known as a useful model for the study of astrocyte functions under both physiological and pathological conditions, with the aim of assessing the mechanism of action of the antioxidant ability. In this study, the antioxidant capacity was first evaluated in the *R. officinalis* methanolic extracts by the oxygen radical absorbance

capacity (ORAC) method [13]. Briefly, sample of Trolox was mixed with fluorescein in a 96-multiwell plate and the AAPH added. AAPH was used to generate peroxyl radicals that oxidize fluorescein, causing a decrease in fluorescence (excitation wavelength 485nm and emission wavelength 528nm) which is measured every 4 seconds for 90 minutes at 37 CºC. Then, the effect of Rosemary methanol extract on cell viability was tested in the MTT assay at different concentrations on the human astrocyte glioblastoma U373. Finally, GSH and GSSG/GSH ratio levels were tested to determine whether rosemary extract may influence on this antioxidant defence activity. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of vitamin E, was chosen as a positive control in all the assays conducted in this work. Trolox is able to decrease ROS production, to prevent cytotoxicity in human cancer cell lines and to rescue cells from apoptotic death [14,15].

Materials and Methods

Plant material and extraction process

Aerial parts of *R. officinalis* spontaneously growing in Spain were harvested during flowering in May, 2004. Samples were identified by the Department of Aromatic and Medicinal Plants Research, National Institute of Agricultural and Food Technology (INIA). A voucher specimen was deposited for internal control at the INIA (Madrid, Spain). Samples were dried in an oven at 35°C, grind down and sieved through a 2 mm mesh, and kept protected from light and moisture until use. 60 mg of each sample was extracted with 20 ml Methanol for one hour, under shaking. The suspension was then filtered through one filter paper; 10ml Methanol were added to the sample and filter again over the first methanolic extract. The extract was left for overnight to dry and stored at 5ºC and protected from light until use.

Reagents

Dulbecco´s modified Eagle´s medium (DMEM), RPMI1640 medium, Foetal bovine serum (FBS), PBS, Gentamicin were purchased from Gibco (Invitrogen, Paisley, UK). Dimethyl sulphoxide (DMSO), Hydrogen peroxide solution (30% w/w), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,7-dichloro-dihydrofluorescein di acetate (DCFH-DA), AAPH were obtained from Sigma-Aldrich (St Louis, MO, US

Cell culture

Human astrocytoma U373 MG line was obtained from Cell Culture and Biological Resources Unit at Alcalade Henares University (Madrid, Spain). Cells were grown in a humidified incubator at 5% CO2 and 95% air at 37ºC in Dulbecco's Modified Eagle's medium (DMEM) piruvate free,from Invitrogen (Madrid, Spain),, supplemented with 10% fetal bovine serum (FBS) (Biowhitaker) and 50mg/l of each one of the following antibiotics: gentamicin, penicillin and streptomycin.

ORAC assay

Sample of Trolox was mixed with fluorescein in a 96-multiwell plate and the AAPH added. AAPH was used to generate peroxyl radicals that oxidize fluorescein causing a decrease in fluorescence (excitation wavelength 485nm and emission wavelength 528nm) which is measured every 4 seconds for 90 minutes at 37 °C in a multiwell plate reader (FLUO star OPTIMA fluorimeter, BMG LABTECH). Results calculate the relationship of the areas under the curve between blank and samples and are expressed as micromoles of Trolox equivalents per gram.

MTT assay

Cell viability (cell growth inhibition) was determined by MTT assay [16] with some modification. Cells were incubated in 96-well plates, at density of 5 x 10^4 cells/well for 24h, then the cells were treated with different concentrations of the Romero extracts (range from 3.13 to 800 µg/ml) for another 24h. Triton X-100 5% was used as a negative control, finally 2mg/ml MTT was added and the plate were incubated for 1 h at 37 °C, then the form azan crystal formed were dissolved by adding DMSO and the absorbance was measured at 550 nm using Digiscan 340 microplate reader (ASYA Hitech GmbH, Eugendorf, Austria). For all the experiments, every sample was analyzed in triplicate, with four plates for each condition.

Intracellular ROS production assay

ROS production was evaluated by the DCFH-DA assay [17] with some modification, This assay is based on the oxidation of the non fluorescent compound 2′,7′-dichlorofluorescein (DCFH) into the fluorescent compound dichlorofluorescein (DCF) in presence of ROS. Cells were incubated in 96-well plate for 24h and 50µl of 2'', 7'-dichlorofluorescin diacetate (DCFH-DA) at a concentration of 10 µM were added for 30 min at darkness. Then, cells were treated with different concentrations of rosemary extract and the generation of ROS was measured for 2h in a microplate fluorescence reader (FLx800, Bio-Tek Instrumentation) with excitation at 480 nm and emission at 510 nm.

Determination of the Glutathione levels

The GSH and GSSG levels were determined according to the method of Hissin and Hilf [18]. Determination of GSH was performed by adding 50 µL of the sample to a mixture of150 µL of 0.1 M sodium phosphate buffer (pH 8.0) and 20 µL of o-phthaldehyde (1mg/mL methanol). The determination of GSSG was conducted by mixing 50 µL of the sample and 3 µL of N-ethylmaleimide for 30 min in darkness before adding 150 µL of 0.1 N NaOH (pH 12) and 20 µL of o-phthaldehyde (1mg/mL, methanol). Finally, both preparations were incubated for 15 min at room temperature in darkness, and fluorescence was measured at an emission wavelength of 485 nm and an excitation wavelength of 528 nm with a microplate fluorescence reader (FLx800, Bio-Tek Instrumentation).

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Statistical analysis

Stat graphics Centurion 16.1.15 (XV) was used. One-way analysis of variance (ANOVA) followed to Fishers least significant difference (LSD) test was applied to obtain the differences between samples. $p<0.05$ was considered as statistically significant.

Results and Discussion

Results showed a showed a strong antioxidant activity by the ORAC method, with a value of 3.03±0.15 µmol TE/mg (value is mean ± SD, n=3). The direct effect of Rosemary extraction cell viability (MTT) showed no statistically significant differences on cell survival with respect to the control group (untreated cells) for concentrations between 12.5 and 200µg/mL; the lowest (3.13 and 6.25µg/mL) and the highest (400-800µg/ml) concentrations induced a decrease in cell survival although far away from the levels achieved with the toxic alone (Triton) (Figure 1A). Thus, concentrations ranging from 12.5 to 200µg/mL were chosen for the following assays. Pretreatment of cells with doses of 12.5, 25, 50 and 100 µg/mL of the extract for 24h before H_2O_2 exposure was able to significantly recover cell viability when compared to the negative control, Triton (Figure 1B). To test the effect of different concentrations on intracellular ROS levels, doses of 12.5, 25, 50 and 100 µg/mL of the extract were added and evaluated by the DCFH-DA assay (Figure 2A). H_2O_2 as the oxidant insult caused an increase in ROS levels by 117% when compared to control cells. Rosemary extract did not increase ROS concentration, this indicating no cellular stress or oxidative damage which could influence the functional conditions of cells. Pretreatment of the cells with the methanolic extract previous to oxidative insult, ROS levels were also inferior to those achieved by untreated cells, although no statistically significant differences were found (Figure 2B). Therefore, neuronal cells treated with the *R. Officinalis* extract seem to be in a favourable condition to face an oxidative challenge. Then the protective effect of rosemary on GSH and GSSG concentration was determined in cells treated with 1mMH2O2 or 1 mM H_2O_2 plus noted concentrations of extract or Trolox as a positive control (Table 1). A slight depletion of intracellular GSH levels was observed when 1 mM H_2O_2 was added for 24 h to astrocytes; co-treatment with 0.5mM Trolox completely prevented the depletion of GSH. Co treatment with different rosemary concentrations partially recovered GSH levels, the strongest effect found with 50 µg/mL rosemary extract. Although the GSH recover was no statistically significant, the ratio GSSG/GSH was closer to untreated cells (0.46 vs 0.41, respectively). The role of reduced Glutathione (GSH) as the main non-enzymatic antioxidant defence is due to the reaction with free radicals and the repair of free radical induced damage through electron-transfer reactions. Moreover, the loss of cellular GSH seems to have an important role in apoptotic signalling [19-21]. Therefore, maintaining GSH concentration above a critical threshold while facing a stressful situation represents a crucial advantage for cell survival. In conclusion, the results obtained in this work support previous data on the antioxidant effect of *R. officinalis* [10,11]. Rosemary methanolic extract was not toxic on the assayed cell line and exerted moderate antiradical and antioxidant activities by partially recovering GSH levels. These results may contribute to the knowledge of the mechanism effect, althoughfurther experiments are needed to assess and define the molecular mechanism of action involved in such antioxidant effect in order to confirm *R. officinalis* as a potential therapeutics within those diseases in which oxidative stress plays a crucial role.

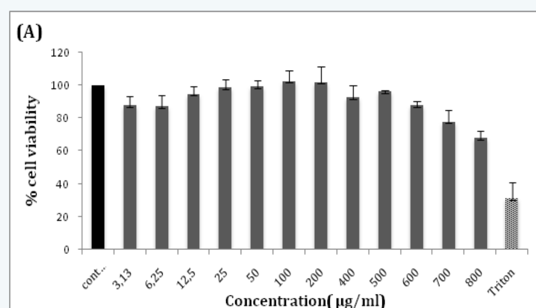

Figure 1A: Effect of R. officinalis methanolic extract on cell viability compared to positive control (0.5mM Trolox). Direct effect: U-373MG cells were treated with different concentrations of the compounds or extract for 24h.

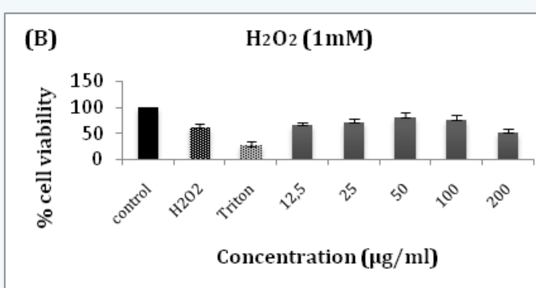

Figure 1B: Effect of R. officinalis methanolic extract on cell viability compared to positive control (0.5mM Trolox). Protective effect: U-373MG cells were treated with different concentrations of the methanolic extract for 24h. Then cells were washed and 1mM H2O2 was added to all the cultures except for controls for 3h. Cell viability is expressed as a percentage of MTT levels. Values are means ± SD (n=3, 3 replicates). *p< 0.05

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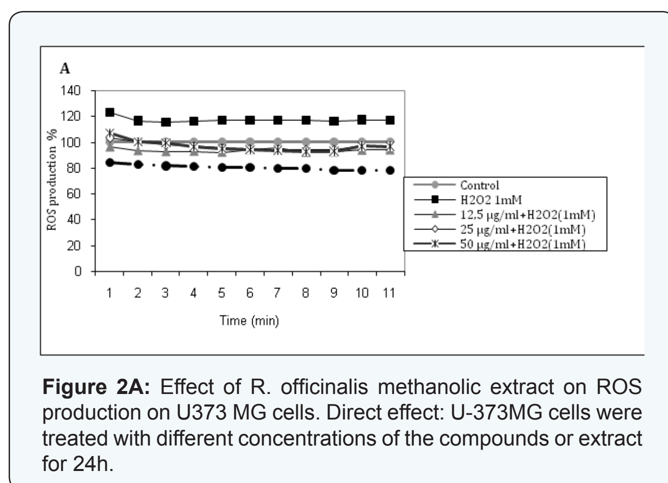

Figure 2A: Effect of R. officinalis methanolic extract on ROS production on U373 MG cells. Direct effect: U-373MG cells were treated with different concentrations of the compounds or extract for 24h.

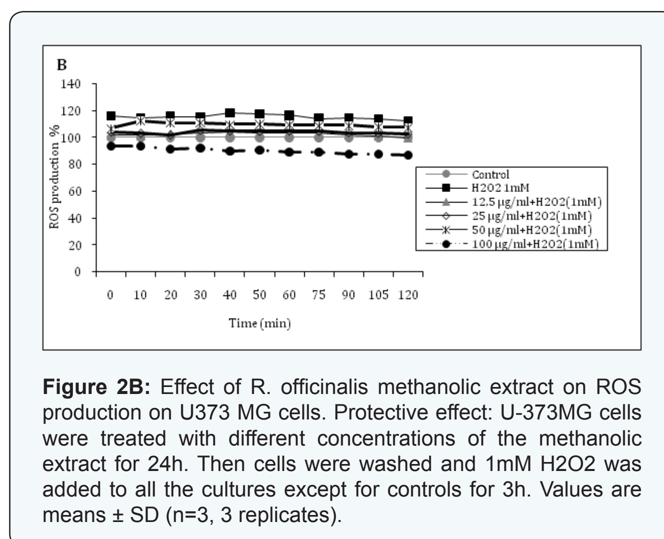

Figure 2B: Effect of R. officinalis methanolic extract on ROS production on U373 MG cells. Protective effect: U-373MG cells were treated with different concentrations of the methanolic extract for 24h. Then cells were washed and 1mM H2O2 was added to all the cultures except for controls for 3h. Values are means ± SD (n=3, 3 replicates).

Table 1: Protective effect of *R. officinalis* methanolic extract on the antioxidant defenses of U373 MG cells. GSH and GSSG were determined in cells treated with 1mM H_2O_2 or 1mM H_2O_2 plus noted concentrations of the positive control 0.5mM Trolox or *R. officinalis* extract. Values are means ± SD, n=3. Values are expressed as redox index (RI). Different letters indicate statistically significant differences (p<0.05) among grous groups.

Compound	GSH (n mol/mg Protein)	GSSG (n mol/mg Protein)	RI= GSSG/ (GSSG+)
Control	29.28[a]	20.35[a]	0.41[a]
1mM H_2O_2	22.52[a]	26.55[a]	0.54[a]
12.5µg/ml + 1mM H_2O_2	25.12[a]	22.50[a]	0.47[a]
25µg/ml + 1mM H_2O_2	24.60[a]	22.50[a]	0.48[a]
50 µg/ml + 1mM H_2O_2	25.19[a]	21.35[a]	0.46[a]
100 µg/ml + 1mM H_2O_2	26.05[a]	24.07[a]	0.48[a]
0.5mM Trolox	38.10[b]	32.27[b]	0.45[b]

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