Preliminary Studies on the Protective Effect of Rosmarinus Officinalis on Astrocytes

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Submission: March 03, 2016; Published: March 22, 2016

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$_2$O$_2$ 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$_2$O$_2$ 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 °C. 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 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 10⁴ 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).
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 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 200 µg/mL; the lowest (3.13 and 6.25 µg/mL) and the highest (400-800 µg/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 200 µg/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 H₂O₂ 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). H₂O₂ 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. The protective effect of rosemary on GSH and GSSG concentration was determined in cells treated with 1mM H₂O₂ or 1 mM H₂O₂ 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 H₂O₂ 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 GSG/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 anti-radical and antioxidant activities by partially recovering GSH levels. These results may contribute to the knowledge of the mechanism effect, although further 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 compounds or extract for 24h.
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 H2O2 or 1mM H2O2 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 groups.

| Compound                      | GSH (n mol/mg Protein) | GSSG (n mol/mg Protein) | RI= GSSG/(GSSG+GSH) |
|-------------------------------|------------------------|-------------------------|---------------------|
| Control                       | 29.28±                  | 20.35±                  | 0.41±               |
| 1mM H2O2                     | 22.52±                  | 26.55±                  | 0.54±               |
| 12.5 µg/ml + 1mM H2O2        | 25.12±                  | 22.50±                  | 0.47±               |
| 25 µg/ml + 1mM H2O2          | 24.60±                  | 22.50±                  | 0.48±               |
| 50 µg/ml + 1mM H2O2          | 25.19±                  | 21.35±                  | 0.46±               |
| 100 µg/ml + 1mM H2O2         | 26.05±                  | 24.07±                  | 0.48±               |
| 0.5mM Trolox                  | 38.10±                  | 32.70±                  | 0.45±               |

References

1. Singh RP, Sharad S, Kapur S (2004) Free radicals and oxidative stress in neurodegenerative diseases: Relevance of dietary antioxidants. J Ind Acad Clin Med 5(3): 218-225.
2. Barnham KJ, Masters CL, Bush AI (2004) Neurodegenerative diseases and oxidant stress. Nat Rev Drug Discov 3(3): 205-214.
3. Emerit J, Ideas M, Bricaire F (2004) Neurodegenerative diseases and oxidative stress. Biomed Pharmacother 58(1): 39-46.
4. Gandhi S, Abramov AY (2012) Mechanism of oxidative stress in neurodegeneration. Oxid Med Cell Longev.
5. Gutierrez-Merino C, Lopez-Sanchez C, Lagoa R, Samhan-Arias AK, Bueno C, et al. (2011) Neuroprotective actions of flavonoids. Curr Med Chem 18(9): 1195-1212.
6. Halliwell B (2012) Free radicals and antioxidants: updating a personal view. Nutr Rev 70(5): 257-265.
7. Taupin P (2010) A dual activity of ROS and oxidative stress on adult neurogenesis and Alzheimer’s disease. Cent Nerv Syst Agents Med Chem 10(1): 16-21.
8. Abuashwashi M, Palomino O, Gómez-Serranillos MP (2014) Variability in the polyphenolic composition and antioxidant ability of wild Rosmarinus officinalis L collected in Spain. Planta Med 80.
9. Almela L, Blas Sánchez-Muñoz, Fernández-López JA, Roca MJ, Rabe V (2006) Liquid chromatographic-mass spectrometric analysis of phenolics and free radical scavenging activity of Rosemary extract from different raw material. J Chromatogr A 1120(1-2): 221-229.
10. Collins MA, Charles HP (1987) Antimicrobial activity of Carnosol and urosalic acid: two anti-oxidant constituents of Rosmarinus officinalis. J Food Microbiol 43: 311-315.
11. Moreno S, Scheyer T, Romano CS, Nojnov AA (2006) Antioxidant and antimicrobial activities of rosemary extracts linked to their polyphenol composition. Free Radic Res 40(2): 223-231.
12. Papageorgiou V, Mallouchos A, Komiitis M (2008) Investigation of the Antioxidant behavior of Air- and Freeze- Dried Aromatic plant materials in relation to their phenolic content and vegetative cycle. J Agric Food Chem 56(14): 5743-5752.
13. Dávalos A, Gómez-Cordovés C, Bartolomé B (2004) Extended applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. J Agric Food Chem 52(1): 48-54.
14. Kello M, Drutovic D, Chripkova M, Pilatova M, Budovska M, et al. (2014) ROS-Dependent Antiproliferative Effect of Brassinin Derivative Homobrassinin in Human Colorectal Cancer Caco2 Cells. Molecules 19(8): 10877-10897.
15. Schoeneberger H, Belz K, Schenk B, Fulda S (2014) Impairment of antioxidant defense via glutathione depletion sensitizes acute lymphoblastic leukemia cells for Smac mimetic-induced cell death. Oncogene 34(31): 4032-4043.
16. Mosmann T (1983) Rapid colorimetric assay for cellular growth
and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65(1-2): 55-63.

17. Le Bel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′-7′-Dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 5(2): 227-231.

18. Hissin PJ, Hilf R. (1976) A fluorometric method for the determination of oxidizes and reduced glutathione in tissues. Anal Biochem 74(1): 214-226.

19. Martín MA, Ramos S, Mateos R, Marais J, Bravo L, et al. (2015) Chemical characterization and chemo-protective activity of cranberry phenolic extracts in a model cell culture. Response of the antioxidant defenses and regulation of signaling pathways. Food Res Int 71: 68-82.

20. Matés JM, Pérez-Gómez C, Núñez de Castro I (1999) Antioxidant enzymes and human disease. Clin Biochem 32(8): 595-603.

21. Sayre LM, Smith MA, Perry G (2001) Chemistry and biochemistry of oxidative stress in neurodegenerative disease. Curr Med Chem 8(7): 721-738.