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Rosmarinus officinalis L. Essential Oil Protects Astrocytes against Oxidative Damage

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Abstract

Rosmarinus officinalis L. (Rosemary) is an aromatic species spontaneously growing in the Mediterranean area. The antioxidant ability of essential oils has led to experimental approach in neurodegenerative or cancer diseases that imply oxidative processes. The aim of this study is to assess the *in vitro* antioxidant and neuroprotective potentials of the essential oil from *R. officinalis* collected in Spain, using a H_2O_2-induced oxidative stress model in the astrocytoma U373-MG human cell line. Aerial parts of *R. officinalis* were collected in central Spain and essential oil obtained by Clevenger distillation. Essential oil composition was assessed by Gas Chromatography (CG). The antioxidant ability was first evaluated by the Oxygen Radical Absorbance Capacity (ORAC) and the free radical scavenging activity (DPPH) methods. Cellular viability was assessed by MTT method assay after 24h pretreatment with oil in order to measure protection from H_2O_2 in astrocytes. Furthermore, the intracellular ROS formation (DCFH-DA method), Caspase-3 activity (fluorometric assay) and reduced-glutathione (GSH) levels were determined. *R. officinalis L.* essential oil showed *in vitro* antioxidant activity through the ORAC and DPPH assays. No cell toxicity was observed for concentrations ranging from 1.0-12.5µg/ml essential oil. These concentrations also exerted a protective effect on astrocytes simultaneously treated with 1mM H_2O_2. Oxidative damage by 1mM H_2O_2 was shown as a significant decrease in GSH concentration. *R. officinalis* essential oil at 12.5µg/ml recovered GSH levels close to control values. A slight decrease in the activation of caspase -3 previously induced by 1mM H_2O_2 was also shown at 5 µg/ml. *R. officinalis* essential oil protects astrocytes against oxidative damage mainly by modulating GSH levels and caspase-3 activity.

Keywords: Rosemary; Essential oil; GC, Astrocytes; Antioxidant; ORAC; DPPH; MTT; ROS; GSH; Caspase 3

Abbreviations: GC: Gas Chromatography; ORAC: Oxygen Radical Absorbance Capacity; ROS: Reactive Oxygen Species; FR: Free Radicals; FDA: Food and Drug Administration; FEMA: Fragrance and Essence Manufacturers Association; DPPH: *2, 2-Diphenyl-1-picrylhydrazyl*; FBS: Foetal Bovine Serum; DMSO: Dimethyl sulphoxide; LRI: Linear Retention Indices; DMEM: Dulbecco's Modified Eagle's medium; FBS: Fetal Bovine Serum; DCF: Dichlorofluorescein; DCFH-DA: *2'', 7'-Dichlorofluorescin Diacetate*

Introduction

Medicinal plants are a great source of new active compounds and mechanisms of action to treat human diseases. Among them, the antioxidant activity is one of the main studied effects due to the relationship between oxidative stress and reactive oxygen species (ROS) and several pathologies development. Nowadays, one of the priorities in pharmacological research is the obtention of new agents capable of prevent or at least decrease the impact of these oxidation mediators at physiological level by acting on different biochemical or pharmacological targets. Oxidative reactions are physiological processes aimed to release different substances which are needed in cellular metabolism. These reactions involve the transfer of electrons and may generate compounds known as ROS, among which there are the free radicals (FR) and oxygen derived molecules with high reactivity. Oxidative stress is defined as the imbalance between the production of free radicals and the ability of a biological system to quickly detoxify the reactive intermediates and repair the damage caused at protein, lipid and DNA levels [1]. Antioxidant compounds are defined as those which at low concentration are capable of preventing oxidative damage mediated by free radicals trough different pathways such as a direct uptake of ROS, modulation of enzyme activity or chelating metal ions (Fe^{+3}, Cu^{+}), among others [2].

Rosmarinus officinalis L. (Lamiaceae), commonly known as rosemary, is an aromatic shrub spontaneously growing in the Mediterranean regions. Aerial parts and essential oil of

Rosemary have been used in folk medicine since ancient times for the treatment of asthenia, hypotension, chronic weakness, as carminative and to improve peripheral circulation, among others. Essential oil was described for colic, nervous disorders and painful or delayed menses; also women used it for minor menstrual complaints [3]. Nowadays, traditional use in the European Union is recognized for *R. officinalis* essential oil for two different therapeutic indications: symptomatic relief of dyspepsia and mild spasmodic disorders of the gastrointestinal tract (oral use); and relief of minor muscular and articular pain and in minor peripheral circulation disorders (cutaneous use and bath additive). Rosemary leafis rich in polyphenolic compounds with antioxidant activity [4,5]. Phenolic compounds such as flavonoids and phenolic acids exert broad pharmacological effects such as antiproliferative, antitumour, antinflammatory, apoptosis-inducing and antioxidant activities which provide important health benefits related to metabolic syndrome, cancer, brain health and immune system [6]. *In vitro* studies previously published with rosemary show that its antioxidant properties result mainly from the phenolic diterpenes, such as carnosic acid, carnosol and rosmanol [7].

The essential oil obtained from Rosemary aerial parts shows a complex composition which varies according the plant geographic origin or phenological stage [8-12]. According to the structure of the carbon skeleton of the main constituents of the essential oil, three biogenetic types have been described: *eucalyptol* type (Italy, Morocco and Tunisia), *camphor-borneol* type (Spain) and *α-pinene-verbenone* type (France, Corsica). Rosemary oil is listed as Safe by the Fragrance and Essence Manufacturers Association of the USA (FEMA) and listed for food use by the US Food and Drug Administration (FDA). In this sense, rosemary essential oil is used as a spice, flavouring and preservative agent, as oxidation is one of the main causes of chemical deterioration which could lead to rancidity, deterioration of nutritional value and even safety of foods [13].

The leaves contain 0.5 to 2.5 % of a volatile oil, consisting of 0.8-6 % esters and 8-20 % free alcohols [14]. This essential oil is a colourless or pale yellow liquid with a camphoraceous taste and contains monoterpenes, phenols, sesquiterpenes, monoterpenoid ethers, monoterpenoid ketones, monoterpernoid alcohols, and monoterpenoid esters, camphor, eucalyptol, α-pinene, borneol [15]. Main components are 1,8-cineol (20-50%), α-pinene (15-26%), camphor (10-25%), bornyl acetate (1-5%), borneol (1-6%), camphene (5-10%) and α-terpineol (12-24%), with variable quantities of limonene, β-pinene, β-cariophilene and myrcene [16]. Pharmacological and clinical studies with *R. officinalis* essential oil show the antimicrobial, fungicide, spasmolytic and relaxation activities, as well as its hypotensive effect [17-21] and support its use for carminative and digestive ailments such as flatulence or sensation of gastrointestinal distension. In this study, aerial parts of samples from spontaneous population of *R. officinalis* were collected in Madrid (Central Spain), in order to assess the *in vitro* antioxidant and neuroprotective potentials of its essential oil, using a H_2O_2-induced oxidative stress model in the astrocytoma U373-MG human cell line.

Methods

Plant material

Aerial parts of samples of *R. officinalis* spontaneously growing in central Spain were harvested during flowering in May, 2014. 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 and kept protected from light and moisture until

Extraction process

The essential oil was obtained by hydrodistillation of the dried ground material in a Clevenger-like apparatus for 2h at atmospheric pressure on about 100g of sample. Time was measured from the falling of the first drop of distillate. For the sample, two replications were done. The essential oil yield was evaluated gravimetrically.

Reagents

Standards of *α-pinene, camphene, β-pinene, myrcene, limonene, 1-8 cineol, γ-terpinene, p-cimene, bornil acetate,* camphor, *borneol, α-terpineol* and *verbenone* were purchased from Extra synthese (Genay, France). Fluorescein (*3', 6'-dihydroxyspiro [isobenzofuran-1[3H], 9' [9H]-xanthen]-3-one*), AAPH (*2, 2'-azobis (2-amidinopropane) dihydrochloride), 2, 2-Diphenyl-1-picrylhydrazyl* (DPPH), Folin-Ciocalteu's phenol reagent, gentamicin, penicillin G and streptomycin were purchased from Sigma-Aldrich (Spain). Dulbecco's modified Eagle's medium (DMEM), RPMI1640 medium, foetal bovine serum (FBS), PBS 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 diacetate* (DCFH-DA) were obtained from Sigma-Aldrich (St Louis, MO, USA).

GC analysis of *R. officinalis* essential oil

The GC analysis was carried out with a Hewlett Packard 6890 Series instrument equipped with an FID and HP-5 capillary columns (30m x 0.25mm, 0.25µm film thickness), working with the following temperature program: 70ºC for ten minutes and then ramp of 3ºC/min to 220ºC; injector and detector temperature, 250ºC; carrier gas nitrogen was adjusted to a flow of 2ml/min. The samples were injected using the split mode (split ratio 1:30) and an injection volume of 0.2µL. Every sample was analyzed in triplicate. Identification of the components was performed by comparison of their retention times with those of pure authentic samples and by means of their linear retention

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indices (LRI) relative to the series of n-hydrocarbons.

Antioxidant capacity and Oxygen scavenging activity

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 (FLUOstar 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.

DPPH assay

The oxygen scavenging activity of the samples was determined by the DPPH assay [22] in a multiwell plate reader (FLUOstar OPTIMA fluorimeter, BMG LABTECH). Briefly, a stock solution of DPPH of 23mg/ 10ml MeOH was kept at 5°C until use; then increasing concentrations of each sample were added and absorbance was recorded at 715 nm. The free radical -scavenging activity of each solution was then calculated as the percentage of inhibition and results are expressed as IC_{50} value, which is defined as the concentration of extract (µg/mL) required to scavenge 50% DPPH radicals. The lower the IC_{50} value is the higher antioxidant activity. Assays were done in triplicate.

Cell culture

Human astrocytoma U373 MG line was obtained from Cell Culture and Biological Resources Unit at Alcala de Henares University [23]. Cells were grown in a humidified incubator at 5% CO_2 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 50 mg/l of each one of the following antibiotics: gentamicin, penicillin and streptomycin.

Cell treatment

Different concentrations of rosemary essential oil (0.5-250 µM) were dissolved in DMEM and added to the cell plates for 24h. In order to evaluate the protective effect against an oxidative insult, culture medium was removed and cells were treated with 1 mM H_2O_2 for 45 min. For all the experiments, every sample was analyzed in triplicate, with four plates for each condition.

MTT assay

Cell viability (cell growth inhibition) was determined by MTT assay (Mosmann, 1983) 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 rosemary essential oil for another 24 h. 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 formazan 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 [23] with some modification, This assay is based on the oxidation of the nonfluorescent 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 2 h in a microplate fluorescence reader (FLx800, Bio-Tek Instrumentation) with excitation at 480 nm and emission at 510 nm.

Glutathione levels and antioxidant enzymes activity

The content of reduced glutathione (GSH) and the activity of the antioxidant enzyme catalase (CAT) were determined and results expressed in units (U) or mU/mg protein. The GSH levels were determined according to Hissin and Hilf [24]. Determination of GSH was performed by adding 50 µL of the sample to a mixture of 150 µL of 0.1 M sodium phosphate buffer (pH 8.0), and 20 µL of *o-phthaldehyde* (1 mg/mL methanol). 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).

Determination of Caspase 3

The fluorogenic caspase 3 was used for a fluorimetric assay [25]. After the hydrolytic cleavage of the coumarin derivative by thecytosolic caspase, the fluorescent product is determined using a multiwell-fluorescence reader (Bio-Tek Instruments, USA). Fluorescence was measured at 360 nm excitation and 480 nm emission within a microplate reader Bio-Tek FL 800.

Statistical analysis

Each experiment was carried out in triplicate. Data are expressed as Mean±SD values of at least three experiments. Linear correlation analysis was used to explore the relationships between the studied variables. Correlation coefficients (R) and p values were evaluated to judge the fit of the correlation; two-sided $p< 0.05$ and $p< 0.01$ values of correlations were considered significant and highly significant, respectively. The statistical analyses were conducted using the statistical package SPSS version 22.0 for Windows (SPSS, Chicago; IL, USA).

Results and Discussion

Extraction yield and CG analysis

The yield in essential oil with respect to dry plant is high,

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with a value of 1.86%. Chromatographic analysis allows the identification and quantification of thirteen compounds in one single analysis, which correspond to nearly 70% of the total components of the essential oil. The most abundant components are camphor, *1,8-cineol* and *myrcene* [Table 1], this composition reflecting the camphor-borneol type which is predominant in the Iberian Peninsule.

Table 1: Chemical composition of essential oil of *R. officinalis L.* collected in Spain, by GC, according to their retention time and by means of their linear retention indices (LRI) relative to the series of n-hydrocarbons. In brief, analysis was carried out with a HP-5 capillary columns (30m x 0.25mm, 0.25µm film thickness) with the following temperature program: 70ºC for ten minutes and then ramp of 3ºC/min to 220ºC; injector and detector temperature, 250ºC; carrier gas nitrogen was adjusted to a flow of 2ml/min. Every sample was analyzed in triplicate.

Compound	Percentage
Essential oil yield	1.86
α-pinene	6.44
Camphene	5.06
β- pinene	7.07
Myrcene	10.15
Limonene	0.78
1,8-cineol	16.39
γ-terpinene	0.94
p-cimene	0.52
Bornylacetate	0.26
Camphor	18.15
Borneol	1.7
α-terpineol	0.23
Verbenone	0.49

In vitro antioxidant activity

In this study, the antioxidant activity was first evaluated in *R. officinalis* essential oil by the oxygen radical absorbance capacity (ORAC) method [26]. Trolox as 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 [27,28]. Results show an ORAC value for rosemary essential oil of 1.20 ± 0.13 µmol TE/mg (value is mean ± SD, n=3), this indicating a moderate antioxidant capacity. Then, the oxygen scavenging activity of the samples was determined by the DPPH assay

Cell viability

The direct effect of Rosemary extract on cell viability (MTT) showed no statistically significant differences on cell survival with respect to the control group (untreated cells) for concentrations between 1.0 and 12.5 µg/mL; the lowest (0.5 µg/mL) and the highest (25-250 µg/ml) concentrations induced a decrease in cell survival (Figure 1). Thus, concentrations ranging from 1.0to 12.5 µg/mL were chosen for the following assays. Pretreatment of cells with doses of 1.0, 2.5, 5 and 12.5 µg/mL of essential oil for 24h before H_2O_2 exposure was able to significantly recover cell viability when compared to the toxic alone (Figure 1).

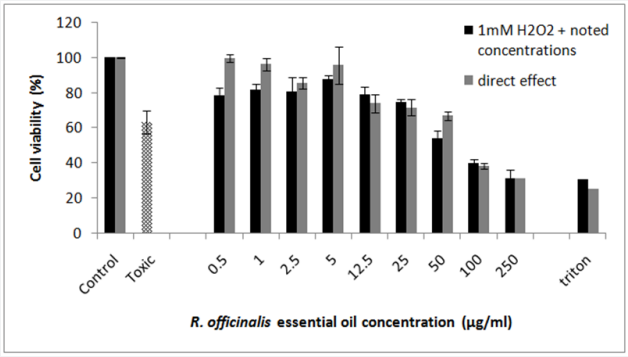

Figure 1: Effect of *R. officinalis* essential oil on cell viability compared to positive control (0.5mM Trolox). Direct effect: U-373MG cells were treated with different concentrations of the compounds or essential oil for 24h. Protective effect: U-373MG cells were treated with different concentrations of the essential oil for 24h. Then cells were washed and 1mM H_2O_2 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.

Effect on antioxidant defenses

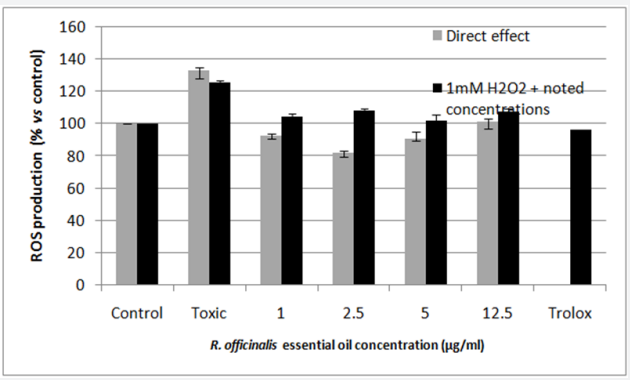

Figure 2: Effect of *R. officinalis* essential oil on intracellular ROS production on U373 MG cells. For the Direct effect: U-373MG cells were treated with different concentrations of the compounds or essential oil for 24h. For the Protective effect: U-373MG cells were treated with different concentrations of the essential oil for 24h. Then cells were washed and 1mM H_2O_2 was added to all the cultures except for controls for 3h. Values are means ± SD (n=3, 3 replicates).

To test the effect of different concentrations on intracellular ROS levels, doses of 1.0, 2.5, 5 and 12.5 µg/mL of the essential oil were added and evaluated by the DCFH-DA assay (Figure 2). H_2O_2 as the oxidant insult caused an increase in ROS levels by 35% when compared to control cells. Rosemary essential oil alone did not increase ROS concentration, this indicating no cellular stress or oxidative damage which could influence the functional

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conditions of cells. Pretreatment of the cells with the essential oil previous to oxidative insult allowed ROS levels inferior to those achieved by untreated cells, although no statistically significant differences were found (Figure 2). Then, the protective effect of rosemary on GSH concentration was determined in cells treated with 1 mM H_2O_2, 1 mM H_2O_2 plus noted concentrations of essential oil, or Trolox as a positive standard (Table 2). Untreated cells served as control. A 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 Troloxpartially recovered the depletion of GSH. Cotreatment with 12.5µg/mL rosemary essential oil completely prevented the depletion of GSH levels, although the GSH recover was no statistically significant for every tested concentration.

Table 2: Protective effect of *R. officinalis* essential oilon the antioxidant defenses of U373 MG cells. GSH levels 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* essential oil.Values are means ± SD, n=3. Values are expressed as redox index (RI). Different letters indicate statistically significant differences ($p<0.05$) among groups.

	GSH (nmol/mg protein)
control	$286.61^{c} \pm 15.31$
1mM H_2O_2	$142.80^{a} \pm 9.21$
1µg/ml + 1mM H_2O_2	$145.49^{a} \pm 7.32$
2.5 µg/ml + 1mM H_2O_2	$217.85^{c} \pm 10.30$
5 µg/ml + 1mM H_2O_2	$152.47^{a} \pm 4.61$
12.5 µg/ml + 1mM H_2O_2	$276.51^{c} \pm 11.30$
0.5 mM Trolox	$202.54^{a,b} \pm 8.27$

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 [29]. Therefore, maintaining GSH concentration above a critical threshold while facing a stressful situation represents a crucial advantage for cell survival. In order to determine whether rosemary essential oil may influence cell apoptosis, the activity of the executioner caspase 3 was tested. The study on the protective effect after oxidative injury showed a decrease in caspase 3 mainly at 5 µg/mL with no statistically significant differences among the assayed concentrations (Figure 3). Previous studies conducted with *R. officinalis* show strong antioxidant ability on different disorders related to oxidative stress [4,13,30]. Our results show that the antioxidant ability of this species may be weakly related to its essential oil, although it is not the key factor of the positive effect which could be mainly due to polyphenolic compounds. Astrocytes were chosen as cell model due to their role in neurons protection. Astrocytes are less sensitive to ischemic injury and metabolic inhibition than neurons; moreover, these cells are more resistant than neurons towards mitochondrial damage, probably due to their higher intracellular GSH and other antioxidant defenses levels.

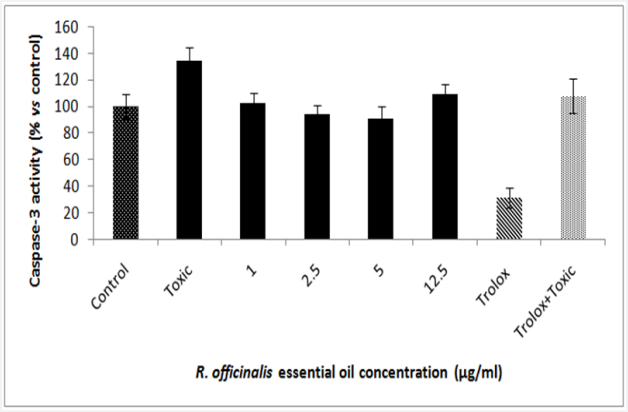

Figure 3: Effect of *R. officinalis* essential oil on caspase-3 activity compared to positive control (0.5mM Trolox). U-373MG cells were treated with different concentrations of the compounds or essential oil for 24h. Results were expressed as % versus control. Results were expressed as mean ± SD of at least three independent experiments (n=3, 4 replicates).

Moreover, astrocytes are able to release cystein-glycin (CysGly) as the forefather of GSH. Due to the consequences of oxidative stress in central nervous system function such as aging, brain ischemia or neurodegenerative disorders, the study of potential antioxidant agents acting at this level is one of the main research lines. In our study, *R. officinalis* essential oil effect on cell viability 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. *R. officinalis L.* essential oil shows *in vitro* antioxidant activity through the ORAC and DPPH assays. It is not toxic through the MTT assay at concentrations ranging from 1.0 to 12.5µg/ml and is able to protect astrocytes simultaneously treated with 1mM H_2O_2. Again, oxidative damage by 1mM H_2O_2 was shown as a significant decrease in GSH concentration which was completely reverted by 12.5µg/ml rosemary essential oil. The study on the protective effect after oxidative injury showed a decrease in the executioner caspase 3 activity, mainly at 5 µg/mL [31].

Conclusion

In conclusion, the results obtained in this work show a moderate *in vitro* antioxidant effect of *R. officinalis* essential oil, with a better profile within cell culture assays: rosemary essential oil is not toxic on the assayed cell line and exerts moderate antiradical and antioxidant activities by partially recovering GSH levels and decreasing caspase-3 activity. These results may contribute to the knowledge of the mechanism effect, although further experiments are needed to assess and

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define the molecular mechanism of action involved in such antioxidant effect.

Conflict of interest

The authors report no declarations of interest.

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