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Original Article

Antioxidant activity, neuroprotective properties and bioactive constituents analysis of varying polarity extracts from *Eucalyptus globulus* leaves

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ABSTRACT

Eucalyptus globulus is employed as herbal tea and therapeutical purposes. In this work, it is investigated for first time the neuroprotective activities, based on antioxidant properties, of varying polarity extracts (acetone, ethanol and methanol) from *E. globulus* leaves and elucidate their main bioactive constituents. Methanol and acetone extracts contained the highest phenolic compounds amount and chlorogenic acid was the major compound identified by UPLC-ESI-MS/MS. Moreover, the three tested extracts showed significant antioxidant properties, varying their potency depending on the *in vitro* technique used. Furthermore, *E. globulus* extracts were effective in ameliorating H_2O_2-induced oxidative stress by increasing cell viability, GSH levels and antioxidant enzymes activity and, by decreasing ROS production and lipid peroxidation levels in SH-SY5Y cells. Taken together, *E. globulus* leaves extracts could be used as raw material for food and pharmaceutical supplements for their high content in antioxidant compounds with health benefits properties against oxidative stress.

1. Introduction

Eucalyptus globulus Labill. (Lan An) is an evergreen tree native to Australia and extensively cultivated in Mediterranean and subtropical areas for firewood and pulp [1]. *E. globulus* leaves are traditionally employed for asthma and bronchitis treatment. Moreover, they are used to make herbal tea. Furthermore, recent studies have demonstrated antimicrobial, antifungal, anthelmintic and anti-diabetic properties for leaves extracts and essential oils [2].

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Oxidative stress is the result of a cell redox imbalance, leading to biomolecules structure and function disturbance. The brain is vulnerable to reactive oxygen species (ROS) injury because of its high concentrations of polyunsaturated fatty acids, high oxygen consumption and lack of antioxidant defense mechanisms. Particularly, neurons are especially susceptible to oxidative damage owing to its reduced glutathione low levels and its minimal regenerative capacity. Extensive evidence points to oxidative stress as major contributor in the pathogenesis of Alzheimer's disease (AD) and Parkinson's disease (PD). Central nervous system pathologies account for 12% of all deaths worldwide and this number continues to rise due to aging-associated increased incidence [3].

Exogenous antioxidants, which prevent from redox imbalance by scavenging ROS, chelating metals and up-regulating enzymatic and non-enzymatic system, constitute the most promising therapeutic strategy for oxidative stress-mediated pathologies. Hence, due to the increasing number of people suffering from neurodegenerative diseases and the lack of effective treatments, there is a serious need to find new antioxidant sources that slow CNS pathologies progression. Polyphenols are presently getting great attention for their antioxidant properties and their potential role as neuroprotective agents. They act as effective electron and hydrogen donors, singlet oxygen quenchers and reducing agents, preventing from ROS harmful effects [4].

The aim of this study was to investigate for first time the neuroprotective effect, based on its antioxidant properties, of acetone, ethanol and methanol extracts of *E. globulus* leaves against H_2O_2-induced oxidative stress in neuroblastoma cells.

2. Methods

2.1. Reagents

Acetonitrile, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA), dimethyl sulphoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's modified Eagle's medium (DMEM), hydrogen peroxide and 2,4,6-tripyridyl-s-triazine (TPTZ) were acquired from Sigma–Aldrich (St. Louis, MO, USA). All standards were also purchased from Sigma–Aldrich (St. Louis, MO, USA) except for luteolin-4-glucoside and isorhamnetin 3-O-glucoside which were from Extrasynthese (Genay, France). Fetal bovine serum (FBS), phosphate-buffered saline (PBS) and gentamicin were from Gibco (Invitrogen, Paisley, UK).

2.2. Plant material

E. globulus leaves (manufacturer JSC "Acorus Calamus", Švenčionys, Lithuania) were ground to a powder using a mill (IKA® A11 basic, Staufen im Breisgau, Germany). Loss on drying before analysis was determined by drying about 1 g of milled leaves in a moisture analyzer (Precisa HA 300, Precisa Instruments AG, Dietikon, Switzerland) to complete evaporation of water and volatile compounds (drying temperature: 105 °C).

2.3. Extraction

Raw material (125 g) was soaked for 3 h in 70% (v/v) methanol, ethanol or acetone. Soaked raw material was transferred to a percolator, covered with extractant (250 mL) and left to macerate (48 h). Then it was percolated (rate 0.3 mL min^{-1}) and high concentration extract (85% of total extract amount) was obtained. Low concentration extract was decanted and it was evaporated using rotary evaporator (IKA® HB 10, Staufen im Breisgau, Germany) up to 15% of total liquid extract amount. Remaining part of low concentration extract was transferred to a single container with high concentration extract. Organic phase of liquid extract was evaporated using rotary evaporator and remaining aqueous phase was lyophilised using lyophilisator Zirbus (Zirbus technology GmbH, Germany) at 0.01 mbar pressure and, condenser temperature of −85 °C.

2.4. UPLC-ESI-MS/MS conditions

Separation of phenolic compounds was performed with Acquity H-class UPLC system (Waters, USA) equipped with triple quadrupole tandem mass spectrometer (Xevo, Waters, USA) with an electrospay ionization source (ESI) to obtain MS/MS data. YMC Triart C18 (100 × 2.0 mm; 1.9 μm) column (YMC Europe Gmbh, Dislanken, Germany) was used for analysis. Column temperature was maintained at 40 °C. Gradient elution was performed with mobile phase consisting of 0.1% formic acid water solution (solvent A) and acetonitrile (solvent B) with flow rate set to 0.5 mL min^{-1}. Linear gradient profile was applied as follows for solvent A: initially 95% for 1 min; to 70% over 4 min; 50% over 7 min; 95% over 2 min. Negative electrospray ionization was applied for analysis: capillary voltage −2 kV, source temperature 150 °C, desolvation temperature 400 °C, desolvation gas flow 700 L h^{-1}, cone gas flow 20 L h^{-1}. Collision energy and cone voltage were optimized for each compound separately.

2.5. Validation of the method

The validation of the UPLC-ESI-MS/MS method was performed on the basis of the US Food and Drug Administration [5].

The evaluation of the selectivity of the method for peak identification and purity were based on the comparison of the retention times and MS spectra of the analytes with those of the standard compounds.

The limit of detection (LOD) and limit of quantification (LOQ) of the analytes were assessed by comparing the peak height to the baseline noise. The signal-to-noise ratio was 3:1 for a limit of detection and it was 10:1 for a limit of quantification. The determined LOD varied from 0.4 ng/mL to 32.51 ng/mL and the determined LOQ varied from 1.2 ng/mL to 100.25 ng/mL. Calibration curves were obtained by plotting the peak areas of analytical standards. The estimated determination coefficients (R^2) of calibration curves ≥0.96 (Supporting information).

The precision and accuracy were analyzed by evaluating quality control standard mixture at twice the level of LOQ. Accuracy and precision were determined by analyzing samples on three consecutive days (6 × 3 injections). Accuracy and

precision were estimated using relative standard deviation (RSD). The calculated % RSD for the peak area varied from 2.7% (p-coumaric acid) to 9.16% ((−)-Epicatechin gallate). The mean values of accuracy and precision were below acceptable 15% at two times the LLOQ level.

2.6. Antioxidant activity evaluation

DPPH method. DPPH solution in 96.3% v/v ethanol (3 mL, 6×10^{-5} M) was mixed with 10 μL of extracts. A decrease in absorbance was determined at λ = 515 nm in a double beam UV/VIS spectrophotometer M550 (Spectronic Camspec, Garforth, UK) [6]. This spectrophotometer was employed for all antioxidant experiments. Results are expressed as mmol Trolox equivalent (TE) per gram of absolutely dry weight (DW).

ABTS• + method. 3 mL of ABTS•+ solution was mixed with 10 μL of extracts. A decrease in absorbance was measured at λ = 734 nm [7].

TFPH• + method. 3 mL of TFPH•+ solution was mixed with 10 μL of extracts and absorbance was measured at λ = 502 nm [8].

FRAP Assay. FRAP solution included TPTZ (0.01 M dissolved in 0.04 M HCl), $FeCl_3 \times 6H_2O$ (0.02 M in water), and acetate buffer (0.3 M, pH 3.6) (ratio 1:1:10). 3 mL of a freshly prepared FRAP reagent was mixed with 10 μL of extracts. An increase in absorbance was recorded at λ = 593 nm [9].

CUPRAC Assay. CUPRAC solution included copper (II) chloride (0.01 M in water), ammonium acetate buffer solution (0.001 M, pH = 7), and neocuproine (0.0075 M in ethanol) (ratio 1:1:1). 3 mL of CUPRAC reagent was mixed with 10 μL of extracts. An increase in absorbance was recorded at λ = 450 nm [10].

2.7. Cell culture

Human neuroblastoma SH-SY5Y cells were grown in DMEM, FBS (10%) and gentamicin (50 mg mL^{-1}) at 37 °C in humidified atmosphere of 5% CO_2. The SH-SY5Y cell line was provided by the University of Alcalá (Madrid, Spain), CAI Medicine and Biology, Cell Culture Unity.

Extracts were first dissolved in DMSO and then diluted to the desired concentrations in PBS (final concentration of ≤0.1% DMSO). Cells were pre-treated with different concentrations (from 5 μg mL^{-1} to 1000 μg mL^{-1}) prior to H_2O_2 (0.1 mM, 30 min).

2.8. MTT cytotoxic assay

MTT dye solution (2 mg mL^{-1}) was added and after 1 h of incubation, formazan crystals were dissolved with DMSO. Absorbance was measured spectroscopically at 570 nm (LT-4000, Microplate Reader, Labtech International Ltd, UK). Results were expressed as relative percentages, considering absorbance readings of untreated cells as 100% [11].

2.9. Intracellular ROS production

Cells were incubated with DCFH-DA (10 mM) in PBS-glucose for 30 min in darkness at 37 °C. Fluorescence was recorded at λexc = 480 nm and at λem = 510 nm in a microplate reader FLx800, Bio-Tek Instrumentation [12].

2.10. Reduced and oxidized glutathione content

To measure GSH concentration, supernatants of cell lysate samples (50 μL) were incubated with ortho-phthalaldehyde (20 μL, 1 mg mL^{-1} metanol) for 15 min. To measure GSSG concentration, supernatants of cell lysate samples (50 μL) were first incubated with *N*-ethylmaleimide (3 μL) for 5 min and then with ortho-phthalaldehyde (20 μL, 1 mg mL^{-1} metanol) for 15 min. Fluorescence values were measured at λexc = 350 nm and λem = 420 nm. Results were calculated using GSH and GSSG calibration curves and expressed as GSH/GSSG ratio [13].

2.11. TBARS assay

Lipid peroxidation was determined by thiobarbituric acid reactive substances (TBARS) assay [14]. Briefly, cellular lysates (50 μL) were incubated with thiobarbituric acid (TBA), trichloroacetic acid (TCA) and HCl reagent in boiling water bath for 10 min. Then, the mixture was centrifuged (3000 rpm, 10 min, 4 °C). Absorbance was measured at 535 nm in a microplate reader Digiscan 340 (Asys Hitech GmbH, Eugendorf, Austria). Total TBARS content was expressed as % of control.

2.12. Antioxidant enzymes activities

For total lysates, cell pellets were resuspended in a lysis buffer pH 7.4 (Tris 25 mM, EDTA 1 mM NaCl 150 mM, and 0.1% Triton X-100) contained proteinase inhibitors (20 μL mL^{-1}, leupeptin, 35 μL mL^{-1} PMSF and 10 μL mL^{-1} pepstatin) for 20 min at 4 °C. Then, cells were centrifuged at 2500 g for 10 min at 4 °C and supernatants were employed for enzymatic assays.

Catalase (CAT) activity. Briefly, total lysates (30 μL) were dissolved in 150 mM of H_2O_2 (670 μL) dissolved in 50 mM of phosphate buffer (pH 7.4). Hydrogen peroxide decomposition was recorded spectrophotometrically (UVIKON 930, Kontron Instruments, UK) for 1 min at 240 nm [15].

Superoxide dismutase (SOD). Briefly, the assay mixture consisted of 1 mM EDTA (20 μL), 96 mM NBT (20 μL), 50 mM hydroxylamine hydrochloride (5 μL) and cellular lysate (25 μL). Absorbance was measured at 560 nm using a LT-4000, Microplate Reader, Labtech International Ltd, UK [16].

Glutathione reductase (GR). Briefly, the assay mixture consisted of 20 mM EDTA (20 μL), 50 mM phosphate buffer (40 μL, pH 7.4), 2 mg mL^{-1} NADPH (20 μL), 5 mM GSSG (20 μL) and cellular lysate (25 μL). Absorbance was measured at 340 nm using a microplate reader [17].

Glutathione peroxidase (GPx). Briefly, the assay mixture consisted of 50 mM phosphate buffer (100 μL, pH 7.4), cellular lysate (20 μL), 0.048 U of GR, 10 mM GSH (20 μL), 2 mg mL^{-1} NADPH (20 μL) and 63.5 mM H_2O_2 (20 μL). Absorbance was measured for at 340 nm using a microplate reader [18].

Table 1 – Qualitative and quantitative analysis of bioactive constituents of varying polarity extracts from *Eucalyptus globulus* leaves.

	Compound	*m/z*	Daughter ion	Cone voltage V	Collision energy eV	Acetone extract C, μg g^{-1} DW	Ethanol extract C, μg g^{-1} DW	Methanol extract C, μg g^{-1} DW
1	3,4-Dihydroxybenzoic acid (Protocatechuic acid)	153	81	32	20	207.88 ± 8.22^{b}	90.59 ± 3.26^{a,c}	199.96 ± 6.20^{b}
2	p-coumaric acid	163	93	28	22	34.23 ± 0.92^{b,c}	20.10 ± 0.06^{a}	22.05 ± 0.78^{a}
3	Vanillic acid	167	152	26	12	8.13 ± 0.38^{b,c}	0,19 ± 0.01^{a,c}	6.30 ± 0.27^{a,b}
4	Gallic acid	169	51	36	30	415.42 ± 19.32^{c}	334.70 ± 12.36	270.19 ± 10.31^{a}
5	Caffeic acid	179	107	36	22	6.99 ± 0.27^{c}	6.42 ± 0.27^{c}	3.53 ± 0.15^{a,b}
6	Quinic acid	191	85	40	26	1801.68 ± 87.32	2159.58 ± 0.93	1994.00 ± 80.36
7	Ferulic acid	193	134	32	18	35.71 ± 1.65^{b}	17.55 ± 0.79^{a,c}	41.53 ± 1.26^{b}
8	Sinapic acid	223	208	32	14	2.42 ± 0.08^{b}	ND	0.21 ± 0.01^{a}
9	Chrysin	253	63	56	30	ND	0.54 ± 0.02	0.65 ± 0.02
10	Apigenin	269	117	54	36	25.00 ± 1.13^{b}	459.00 ± 19.32^{a,c}	2.79 ± 0.09^{b}
11	Naringenin	271	151	46	18	19.17 ± 0.87^{b}	26.13 ± 1.12^{a,c}	14.39 ± 0.65^{b}
12	Phloretin	273	167	42	16	31.72 ± 1.30	36.92 ± 1.56^{c}	25.59 ± 0.16^{b}
13	Luteolin	285	133	58	36	157.21 ± 6.29^{b,c}	84.07 ± 3.30^{a}	69.70 ± 2.73^{a}
14	Scutellarein	285	117	58	36	0.77 ± 0.03	ND	1.16 ± 0.04
15	Eriodictyol	287	151	96	14	3.49 ± 0.14^{c}	1.90 ± 0.07^{c}	11.15 ± 0.47^{a,b}
16	(−)-Epicatechin	289	123	60	34	1.72 ± 0.05^{c}	ND	12.23 ± 0.57^{a}
17	(+)-Catechin	289	123	60	34	54.34 ± 2.50	ND	70.98 ± 3.46
18	Diosmetin	299	284	46	20	32.24 ± 0.85^{b,c}	9.13 ± 0.37^{a}	8.97 ± 0.41^{a}
19	Quercetin	301	151	48	20	685.05 ± 33.47^{c}	828.68 ± 35.63^{c}	420.95 ± 17.36^{a,b}
20	Ellagic acid	301	273	64	38	28.06 ± 1.02^{b}	7.73 ± 0.31^{a}	ND
21	Taxifoline	303	151	38	18	ND	1.50 ± 0.06	1.23 ± 0.04
22	Isorhamnetin	315	300	44	22	13.42 ± 0.48^{c}	15.28 ± 0.65^{c}	8.28 ± 0.35^{a,b}
23	Chlorogenic acid	353	191	32	14	15,377.21 ± 589.31^{b}	7476.25 ± 303.26^{a,c}	18,768.28 ± 803.32^{b}
24	Neochlorogenic acid	353	191	32	14	292.41 ± 26.36^{c}	338.58 ± 15.63^{c}	826.57 ± 30.26^{a,b}
25	Rosmarinic acid	359	161	36	16	667.31 ± 29.32^{b,c}	30.06 ± 1.38^{a,c}	297.86 ± 11.32^{a,b}
26	Apigenin 7-glucoside	431	268	66	32	9.42 ± 0.33^{b}	6.39 ± 0.23^{a}	8.15 ± 0.32^{b}
27	Vitexin	431.1	311	50	22	409.17 ± 18.01^{b}	211.77 ± 7.03^{a,c}	410.96 ± 18.96^{b}
28	Quercetin 3-O-α-L-arabinofuranoside (Avicularin)	433	301	50	20	131.34 ± 5.64^{b}	51.49 ± 2.01^{a,c}	122.37 ± 4.65^{b}
29	Quercetin 3-O-α-L-arabinopyranoside (Guaijaverin)	433	300	56	26	41.04 ± 1.29	ND	43,60 ± 1.36
30	Phloretin-2′–O-glucoside (Phlorizin)	435	273	42	14	4666.50 ± 203.69^{b}	3382.28 ± 130.60^{a,c}	5515.93 ± 252.36^{b}
31	(−)-Epicatechin gallate	441	169	40	16	25.58 ± 0.85	ND	18.63 ± 0.75
32	Homoorientin (Isoorientin)	447	327	50	24	423,09 ± 19.21^{b}	124.80 ± 5.32^{a,c}	411.89 ± 19.32^{b}
33	Kaempherol-3-O-glucoside (Astragalin)	447	284	54	28	224.83 ± 8.63^{b}	109.15 ± 4.74^{a,c}	213.63 ± 8.36^{b}
34	Luteolin-4-glucoside	447	285	36	16	127.39 ± 5.21	ND	89.57 ± 3.72
35	Luteolin-7-O-glucoside (Cynaroside)	447	285	66	26	8.84 ± 0.32^{b,c}	2.88 ± 0.11^{a,c}	14.88 ± 0.63^{a,b}
36	Orientin	447	327	50	20	407.37 ± 16.30^{b}	106.32 ± 4.96^{a,c}	425.90 ± 19.26^{b}
37	Quercetin 3-O-rhamnoside (Quercitrin)	447	300	50	26	268.80 ± 11.23^{b,c}	60.93 ± 2.36^{a,c}	178.86 ± 7.53^{a,b}
38	Kaempherol-3-O-glucuronide	461	285	36	20	6007.94 ± 265.32^{b}	226.87 ± 8.56^{a,c}	5543.54 ± 203.69^{b}
39	Quercetin 3-O-glucoside (Isoquercitrin)	463	301	52	28	1143.85 ± 49.63^{b}	101.99 ± 4.03^{a,c}	1408.52 ± 69.31^{b}
40	Isorhamnetin -3-O-glucoside	477	314	60	28	39.01 ± 1.82	ND	48.90 ± 2.06
41	Quercetin malonylglycoside	549	300	20	38	3.24 ± 0.14	ND	1.17 ± 0.04

42	Luteolin-7-rutinoside (Scolymoside)	593	285	82	36	14.28 ± 0.63^{c}	ND	1.88 ± 0.07^{a}
43	Quercetin-3-O-rutinoside (Rutin)	609	300	70	38	1986.30 ± 92.35^{b}	30.49 ± 1.32^{a,c}	2010.50 ± 87.31^{b}
44	Isorhamnetin-3-O-rutinoside	623	315	70	32	26.06 ± 1.02^{b}	4.57 ± 0.18^{a,c}	25.37 ± 0.98^{b}
	Total (μg g^{-1} DW)					**35,865.62 ± 1456.32^{b}**	**16,364.81 ± 625.32^{a,c}**	**39,572.78 ± 1563.32^{b}**

Statistical significances of quantitative composition are shown in superscripts. a: Statistically significant differences *versus* acetone extract values. b: *versus* ethanol extract values. c: *versus* methanol extract values.

2.13. Statistical analysis

Experiments were performed in triplicate and results were expressed as mean ± SD. One-way ANOVA, followed by Fisher's least-significant-difference (LSD) test ($P \leq 0.05$) was performed using GraphPad Prism v.5 software.

3. Results and discussion

3.1. Evaluation of phenolic compounds using UPLC-ESI-MS/MS

Qualitative and quantitative phenolic composition of *E. globulus* extracts was investigated using UPLC-MS/MS (Table 1). Total phenolic compounds amount in acetone and methanol extracts was similar, being 35,865.6 μg g^{-1} and 39,572.8 μg g^{-1}, respectively. Total phenol content in ethanol extract (16,364.8 ± 625.3 μg g^{-1}) was 2.2 and 2.4 times less than total amount of these compounds in acetone and methanol extracts, respectively. These results confirm previous works regarding the extraction solvent effect on biological active compounds composition [19].

UPLC-ESI-MS/MS analysis showed that the highest proportion of phenolic compounds in extracts were compounds of flavonoid group (i.e. quercetin, luteolin, kaempferol, isorhamnetin, phloretin). The maximum total amount of flavonoids was determined in methanol extract (17,142.3 ± 702.2 μg g^{-1}) and the lowest in ethanol extract (5883.1 ± 203.6 μg g^{-1}). The largest group of flavonoids was quercetin glycosides with antioxidant, anti-cancer, antimicrobial and cardiovascular protective properties [20].

Other flavonoids in lesser amounts were isorhamnetin and its 3 glycosides, luteolin and its 2 C-glycosides, luteolin-4′-methyl ether, apigenin and its 2 glycosides, 2 kaempherol glycosides, phloretin and phlorezin. Particularly, kaempherol-3-O glucoside was predominant in methanol and acetone samples while phlorizin was in ethanol extract (Table 1). Moreover, the monomeric flavan-3-ol compounds (+)-catechin, (−)-epicathechin and (−)-epicatechin gallate were quantified; they have antioxidant, anti-inflammatory, hypocholesterolemic and vasodilatory actions [21].

Phenolic acids including hydroxybenzoic acids (protocolatechic acid and vanillinic acid) and hydroxycinnamines acids (caffeic acid, ferulic acid, synapic acid, p-coumaric acid and chlorogenic acid) were also identified. The methanol extracts had the highest phenolic acid content (20,436.5 ± 808.4 μg g^{-1}) whereas the lowest concentration was for ethanol extract (8314.4 ± 352.2 μg g^{-1}). The chlorogenic acid was the most abundant analyte; it has antioxidant, anti-inflammatory, renoprotective, hepatoprotective, anti-diabetic and anti-lipidemic properties [22].

3.2. Antioxidant activity

Scientific studies have proven that antioxidants have the capacity to slow down PD and AD progression. There is growing interest in antioxidants to pursue opportunities to apply them in medicine, pharmacy, cosmetic and food industry [23].

We then investigated *in vitro* antiradical and reductive activity of acetone, ethanol and methanol *E. globulus* extracts (Table 2). Herbal extracts have a variety of compounds which act as antioxidant through different reaction mechanisms. Therefore, performing a single test of antioxidant activity is not adequate, it is recommended to use at least two different methods [24].

Herbal products can exert their antioxidant action through two main mechanisms: hydrogen atom abstraction (HAT) and single electron transfer (SET). Antioxidant effects depend on chemical antioxidant structure, physico-chemical properties (solubility, partition coefficient) and medium properties (type of solvent, pH) [25]. The *in vitro* antioxidant properties of extracts should be determined using different modelled systems with different reaction mechanisms with the aim to predict *in vivo* antioxidant acitivity. Particularly, we assessed 5 different antioxidant capacity assays (ABTS, CUPRAC, DPPH, FRAP, and TFPH). ABTS, DPPH and TFPH assays are based on the ability of antioxidants to scavenge $ABTS^{\cdot+}$, $DPPH^{\cdot}$ or $TFPH^{\cdot+}$ free radicals [6–8] whereas FRAP and CUPRAC assays measure the capability of antioxidants to reduce Fe(III) to Fe(II) or Cu(II) to Cu(I) [10]. The knowledge of the antioxidant activity could be useful for the standardization of the raw materials of *E. globulus* leaves extracts.

The present study showed that the highest *in vitro* antioxidant activity by ABTS and CUPRAC methods was for acetone extracts (10.1 and 3.65 mmol g^{-1}, respectively). Moreover, the highest antioxidant activity determined by DPPH method was

Table 2 – Antioxidant activity of extracts of *Eucalyptus globulus* leaves assessed by ABTS, CUPRAC, DPPH, FRAP and TFPH assays.

	ABTS TE, mmol g^{-1} DW	CUPRAC TE, mmol g^{-1} DW	DPPH TE, mmol g^{-1} DW	FRAP TE, mmol g^{-1} DW	TFPH TE, mmol g^{-1} DW
Acetone extract	10.06 ± 0.44^{b}	$3.65 \pm 0.10^{b,c}$	0.97 ± 0.01^{c}	1.31 ± 0.19^{b}	1.13 ± 0.10^{b}
Ethanol extract	$1.69 \pm 0.07^{a,c}$	$0.48 \pm 0.03^{a,c}$	0.96 ± 0.04^{c}	$9.79 \pm 0.24^{a,c}$	$1.78 \pm 0.07^{a,c}$
Methanol extract	9.91 ± 0.32^{b}	$2.69 \pm 0.08^{a,b}$	$1.56 \pm 0.03^{a,b}$	1.10 ± 0.08^{b}	0.91 ± 0.13^{b}

Statistical significances of antioxidant capacities values are shown in superscripts letters: a statistically significant differences *versus* values of acetone extract; b *versus* values of ethanol extract and c *versus* values of methanol extract ($p < 0.05$).

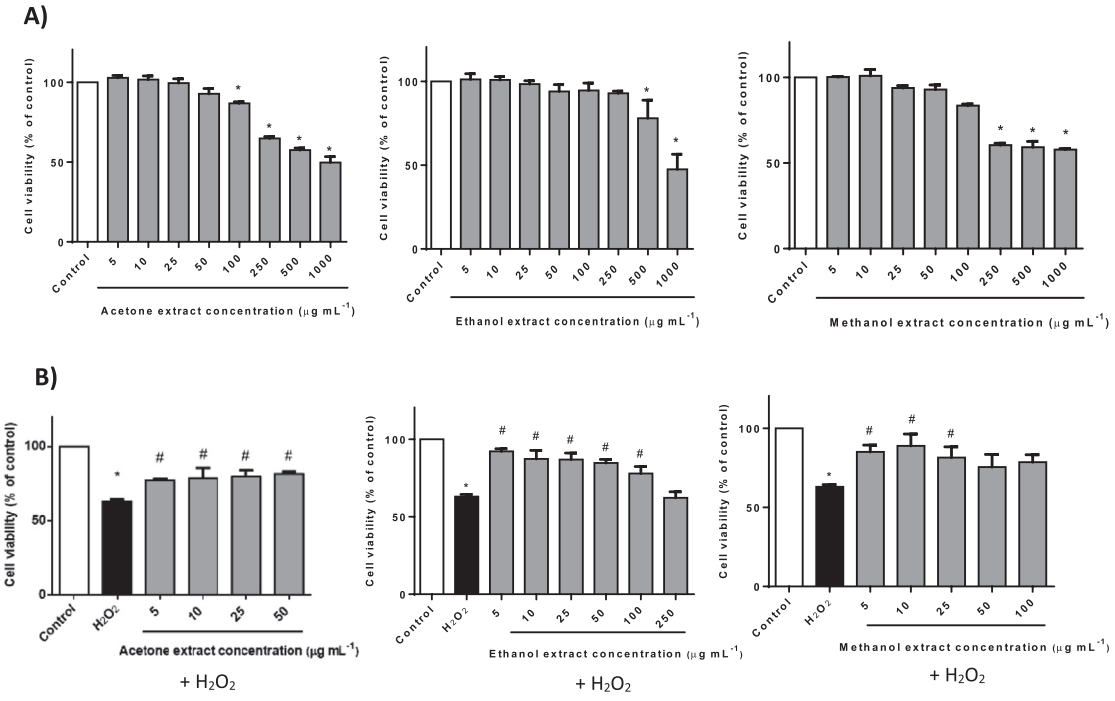

Fig. 1 – (A) Effects of *Eucalyptus globulus* extracts on cell viability. Human neuroblastoma SH-SY5Y cells were treated with various concentrations (from 5 μg mL^{-1} to 1000 μg mL^{-1}) of acetone extract, ethanol extract and methanol extract for 24 h. (B) Protective effects of *Eucalyptus globulus* extracts against hydrogen peroxide toxicity. Human neuroblastoma SH-SY5Y cells were pretreated with non-cytotoxic concentrations of acetone extract, ethanol extract and methanol extract for 24 h, prior to the toxic agent hydrogen peroxide (0.1 mM, 30 min). The viable number of cells was determined by using MTT assay. Results were expressed as percentage of untreated cells. Each point represents mean values ± SEM of three independent experiments. $^{*}p < 0.05$ compared with control and $^{\#}p < 0.05$ compared with hydrogen peroxide.

found for methanol extract (1.6 mmol g^{-1}). Finally, the highest values for FRAP and TFPH methods were 9.8 and 1.8 mmol g^{-1}, respectively, in ethanol extracts (Table 2). Such results may have been due to the characteristics of these methods and antioxidants physicochemical properties: different environment pH, hydrophilic–lipophilic properties, unequal different antioxidant distribution between lipophilic and hydrophilic media, differences in reaction mechanisms and in the qualitative and quantitative composition of antioxidant active compounds. The pH of the environment is one of the most affecting factors for antioxidant activity. Since the degree of solubility and stability of different antioxidant forms is highly dependent on the pH of the environment and different human body fluids have different pH, each exogenous antioxidant has a specific antioxidant capacity *in vivo*, which may differ from only one *in vitro* method used. In our study, the presence of low pH (FRAP and TFPH methods), the strongest antioxidant activity was found in ethanol *E. globulus* extracts, while when the pH value was close to the human body fluids (ABTS and CUPRAC methods), the strongest antioxidant activity was found for acetone extracts. Many of phenolic compounds antioxidative activity are higher when reaction medium pH is close to neutral or weakly alkaline [6–10, 26], therefore pH of medium is very important factor deciding biologically active compounds effect potency. Yet different other results were obtained by evaluating the antiradical activity of extracts in non-aqueous organic environment using DPPH method. The strongest antiradical activity was found in methanol extracts, which, when tested by other methods, did not showed such strong antioxidant activity. Such unequal results could derive because of applied methods deficiencies limiting totally adequate and precise evaluation of extracts biologically active compounds antioxidative activity *in vitro*: application of DPPH method for evaluation of hydrophilic antioxidants activity is very complicated [27], FRAP method can not determine antioxidants which action is based on hydrogen transference reactions [28], by ABTS method, any compound which may be even not real antioxidant like sugars and citric acid but having redox potential lower than ABTS radical-cation can react [29] Depending on the obtained results, it is very difficult to make an unequivocal and undisputable conclusion which of the extraction solvent is the best to obtain as many natural antioxidants from *E. globulus* leaves as possible and to provide an extract rich in antioxidants for manufacture of dietary supplements and medicinal products.

3.3. *Neuroprotective activity*

Since SH-SY5Y cells remain properties of innate neurons [30], we used this cell model to evaluate the potential neuroprotective effect of *E. globulus* extracts.

Initially, we investigated whether 24 h of treatment with any concentration from 5 μg mL^{-1} to 1000 μg mL^{-1} of extracts affected SH-SY5Y cell viability (Fig. 1A). MTT assay showed significantly cytotoxic effects when cells were treated with acetone extract at concentrations above 100 μg mL^{-1}, methanol extract above 250 μg mL^{-1} concentration and ethanol extract above 500 μg mL^{-1} concentration. Therefore, non-cytotoxic concentrations [from 5 μg mL^{-1} to 50 μg mL^{-1} for acetone extract; from 5 μg mL^{-1} to 100 μg mL^{-1} methanol extract, and from 5 μg mL^{-1} to 250 μg mL^{-1} for ethanol extract] were chosen to study the potential protective effect of *E. globulus* extracts against H_2O_2 toxicity.

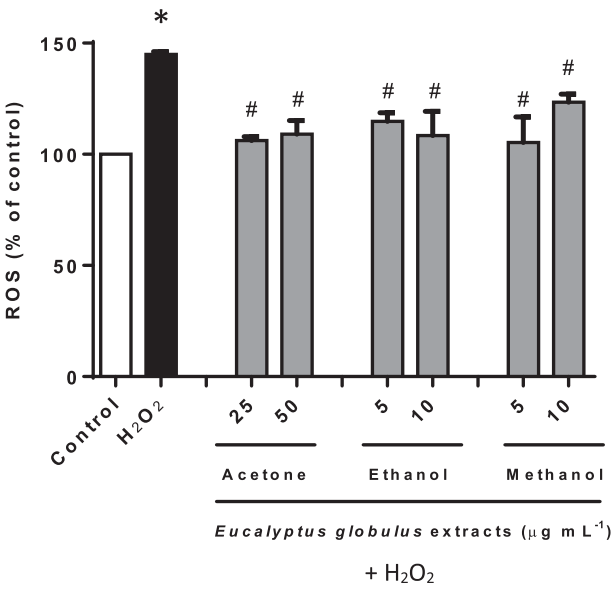

Fig. 2 – Effects of *Eucalyptus globulus* extracts on ROS production. Human neuroblastoma SH-SY5Y cells were pretreated with non-cytotoxic concentrations of extracts for 24 h, prior to the toxic agent hydrogen peroxide (0.1 mM, 30 min). The intracellular ROS generation was measured by 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. Each point represents mean values ± SEM of three independent experiments. *$p < 0.05$ compared with control; #$p < 0.05$ compared with hydrogen peroxide.

Hydrogen peroxide is a byproduct of aerobic metabolism involved in many relevant biological and physiological processes, but at high concentrations it also plays a role as inductor of oxidative stress by producing ROS. Consistent studies determined that H_2O_2 is formed during early stages of aggregation of amyloid-beta in AD and of α-synuclein in PD. Substantial accumulation of H_2O_2 and its oxygen-derived free radicals leads to oxidative stress situation [31].

To examine the protective effects of *E. globulus* extracts against H_2O_2 toxicity, SH-SY5Y cells were pretreated with non-cytotoxic concentrations of extracts for 24 h and then H_2O_2 was added at 0.1 mM for 30 min. As shown in Fig. 1B, incubation with H_2O_2 significantly reduced cell viability to 63% compared to control cells. However, pretreatments with extracts led to an increase in cell viability compared to H_2O_2 cells. Particularly, exposure to acetone extract markedly restored cell viability to 77.1% (5 μg mL^{-1}), 78.7% (10 μg mL^{-1}), 79.8% (25 μg mL^{-1}) and 81.6% (50 μg mL^{-1}). Moreover, ethanol extract significantly increased cell viability to 92.1% (5 μg mL^{-1}), 89.1% (10 μg mL^{-1}), 88.5% (25 μg mL^{-1}), 85.8% (50 μg mL^{-1}) and 79.7% (100 μg mL^{-1}). Furthermore, pretreatments with methanol extract exerted significant protective effect at 5 μg mL^{-1}, 10 μg mL^{-1} and 25 μg mL^{-1} concentrations by increasing cell viability to 85.1%, 88.9% and 81.5%, respectively. Therefore for

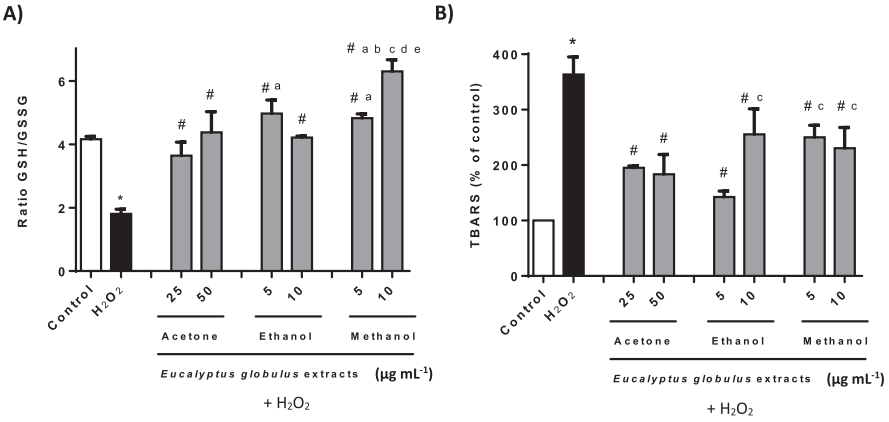

Fig. 3 – Effects of *Eucalyptus globulus* extracts on oxidative stress markers. Human neuroblastoma SH-SY5Y cells were pretreated with non-cytotoxic concentrations of extracts for 24 h, prior to the toxic agent hydrogen peroxide (0.1 mM, 30 min). (A) Effects on glutathione levels, as expressed by ratio GSH/GSSG ratio. (B) Effects on lipid peroxidation, as expressed by thiobarbituric acid reactive substances percentage (TBARS). Each point represents mean values ± SEM of three independent experiments. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with hydrogen peroxide; $^{a}p < 0.05$ compare with acetone 25 µg mL^{-1}; $^{b}p < 0.05$ compare with acetone 50 µg mL^{-1}; $^{c}p < 0.05$ compare with ethanol 5 µg mL^{-1}; $^{d}p < 0.05$ compare with ethanol 10 µg mL-1; $^{e}p < 0.05$ compare with methanol 5 µg mL^{-1}.

further studies, we have selected the concentrations 25 and 50 µg mL^{-1} for acetone extract and, 5 and 10 µg mL^{-1} for ethanol and methanol extracts.

Since intracellular ROS overproduction contributes to cell damage and lead to oxidative stress situation, we investigated whether *E. globulus* extracts could protect from H_2O_2-induced ROS generation. Compared with control cells, H_2O_2 generated significant ROS increase (145%). However, all concentrations of extracts markedly decreased ROS generation in H_2O_2-treated SH-SY5Y cells. Particularly, acetone extracts (25 and 50 µg mL^{-1}) were the most active, balancing intracellular ROS production by scavenging action (Fig. 2). These results support previous works which revealed that *E. globulus* leaves water extract possess significant ability to ROS and RNS scavenge [32].

Following, we studied the effect of *E. globulus* extracts on cellular redox status markers GSH/GSSG ratio and TBARS level. GSH is the most effective and abundant antioxidant defense mechanism in human body. Previous studies have shown that patients with AD and PD present decreased GSH levels [33]. As shown in Fig. 3A, when SH-SY5Y cells were treated with H_2O_2, GSH/GSSG ratio was declined compared to control cells (GSH/GSSG = 4.1 control cells *versus* GSH/GSSG = 1.80H_2O_2-treated neurons) due to low GSH levels and high GSSG levels. However, pretreatments with extracts reversed such drop in GSH/GSSG ratio. Particularly, it highlights methanol extracts (GSH/GSSG = 4.8 at 5 µg/mL and GSH/GSSG = 6.3 at 10 µg mL^{-1}) and ethanol extract at 5 µg mL^{-1} (GSH/GSSG = 4.9).

Lipid peroxidation is an early event in oxidative stress-related neurodegenerative diseases [34]. Then, we investigated the effect of extracts on lipid peroxidation by measuring TBARS content. As shown in Fig. 3B, H_2O_2 treatment significantly increased TBARS levels up to 391.9% fold. When SH-SY5Y cells were pretreated with extracts, lipid peroxidation was significantly decreased compared to H_2O_2-treated cells. The maximum protective effect against lipid peroxidation was observed for ethanol extract at 5 µg mL^{-1} concentration (151.4%). These findings are in accordance with previous works that demonstrated that *E. globulus* notably decreased plasma and liver malondialdehyde, another product of lipid peroxidation, in streptozotocin-induced diabetic rats [35].

CAT, SOD, GR and GPx antioxidant enzymes play a key role in ROS detoxification and therefore, in prevention and protection against the resulting oxidative stress damage. Previous studies have reported that the neuroprotective mechanism of action of plant phenolic extracts could result from the upregulation of these antioxidant enzymes. As shown in Fig. 4A–D, H_2O_2 significantly reduced CAT, SOD, GPx and GR enzymes activity in SH-SY5Y cells by 59.9%, 33.5%, 23.2% and 26.3%, respectively. On the contrary, pretreatments with extracts increased antioxidants enzymes activities compared to H_2O_2-treated cells. The highest protective effect was observed for all extracts at their higher concentration assayed. Previous works have shown that the aqueous-ethanol leaves extract of *E. globulus* avoid the loss of CAT, SOD and GPx activities in the kidney of rats treated with acetaminophen [36].

4. Conclusions

E. globulus leaves extracts are an important source of antioxidant compounds. The antioxidant potency of acetone, ethanol and methanol extracts depends on the *in vitro* techniques. Moreover, UPLC-ESI-MS/MS analysis profile revealed that these three extracts are rich in phenolic compounds

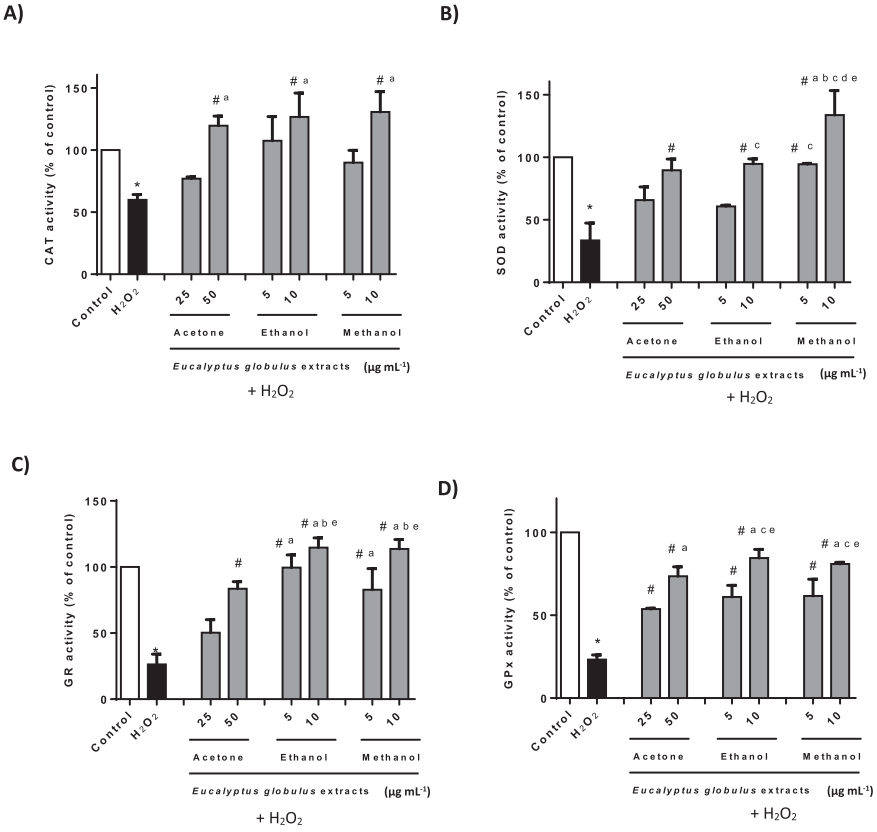

Fig. 4 – Effects of *Eucalyptus globulus* extracts on antioxidant enzymes. Human neuroblastoma SH-SY5Y cells were pretreated with non-cytotoxic concentrations of extracts for 24 h, prior to the toxic agent hydrogen peroxide (0.1 mM, 30 min). (A) Effects on catalase (CAT) activity; (B) Effects on superoxide dismutase (SOD) activity; (C) Effects on glutathione reductase (GR) activity and (D) Effects on glutathione peroxidase (GPx) activity. Each point represents mean values ± SEM of three independent experiments. $^{*}p < 0.05$ compared with control; $^{\#}p < 0.05$ compared with hydrogen peroxide; $^{a}p < 0.05$ compare with acetone 25 μg mL^{-1}; $^{b}p < 0.05$ compare with acetone 50 μg mL^{-1}; $^{c}p < 0.05$ compare with ethanol 5 μg mL^{-1}l; $^{d}p < 0.05$ compare with ethanol 10 μg mL^{-1}; $^{e}p < 0.05$ compare with methanol 5 μg mL^{-1}.

(methanol > acetone > ethanol), being chlorogenic acid the major compound identified. Furthermore, *E. globulus* extracts have been demonstrated to prevent from H_2O_2-induced oxidative stress damage by increasing cell viability, GSH levels and antioxidant enzymes activity and, by decreasing ROS production and lipid peroxidation in SH-SY5Y cells. Therefore, these extracts are a potential source of antioxidant compounds with phenolic nature that possess health benefits against oxidative stress, being able to use as raw material for nutritional, food and pharmaceutical supplements.

Conflicts of interest

Authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.05.010.

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