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Eucalyptus globulus Labill. decoction extract inhibits the growth of NCI-H460 cells by increasing the p53 levels and altering the cell cycle profile

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Eucalyptus globulus Labill. is a widespread evergreen plant belonging to the Myrtaceae family. Several species of *Eucalyptus* are known to have a plethora of medicinal properties, particularly anti-tumor activity, which prompts the study of the chemical composition and bioactivity of extracts from this plant. Hereby, the main aims of this work were to (i) profile the phenolic compounds in *E. globulus* extracts prepared by decoction and infusion; (ii) test the cell growth inhibitory activity of *E. globulus* decoction and infusion, in three human tumor cell line models: colorectal, pancreatic and non-small cell lung cancer (HCT-15, PANC-1 and NCI-H460, respectively); and (iii) study the mechanism of action of the most potent extract in the most sensitive cell line. Our work demonstrated that both the decoction and infusion preparations revealed the presence of phenolic acids, flavonoids and gallotannins, the last group being the most abundant polyphenols found, especially two digalloyl-glucosides. Both extracts inhibited the growth of all the tumor cell lines tested. The decoction extract was the most potent in inhibiting the NCI-H460 cell growth (lower GI_{50} determined by sulforhodamine B assay), which could be due to its higher content of phenolic compounds. Hence, the effect of the decoction extract on the NCI-H460 cells was further investigated. For this, cell viability (by Trypan blue exclusion assay), the cell cycle profile and apoptosis (by flow cytometry), cell proliferation (by bromodeoxyuridine assay) and protein expression (by western blot) were analyzed. Two different concentrations of the extract (52 μg mL^{-1} and 104 μg mL^{-1}, corresponding to GI_{50} and 2 × GI_{50} concentration) were tested in these studies. Remarkably, the *E. globulus* decoction extract caused a dose-dependent decrease in the NCI-H460 cell number, which was correlated with a cell cycle arrest in the G0/G1 phase, a decrease in cell proliferation and an increase in the expression of p53, p21 and cyclin D1 proteins. Interestingly, no differences were found in the levels of ds-DNA damage and in the levels of apoptosis. This work highlights the relevance of the *Eucalyptus globulus* Labill. extract as a source of bioactive compounds with potential anti-tumor activity.

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Introduction

Plant extracts have been widely used as a natural source of medicinal compounds for the treatment of several diseases,[1–3] including cancer.[4–6] Cancer has become a global public health problem with an urgent need for novel anti-neoplastic drugs and therapeutic approaches. Given this problematic issue, the screening of natural compounds as potential anti-tumor agents has become an interesting approach worldwide.[7–12] Most notably, nearly half of the small molecules approved for cancer treatment were either actually natural products or derived from such products.[13] Some of the most relevant anti-cancer drugs in this category include frequently used agents such as Taxol, vinblastine and camptothecin.[14–16]

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Eucalyptus globulus Labill. is one of the most widespread evergreen trees, or shrubs, native to Australia and Tasmania regions. *Eucalyptus* belongs to the Myrtaceae family and nowadays it has extensively spread to various countries.[17] This plant has been the object of several studies due to its pronounced potential for several medicinal and pharmacological applications. In addition, *Eucalyptus*' extracts and their essential oils are also currently used in cosmetics and as food additives. Indeed, some of the *Eucalyptus* constituents, such as alkaloids, polyphenols and propanoids display anti-cancer, anti-inflammatory, anti-fungal, anti-bacterial and anti-septic properties.[18–22] For example, *Eucalyptus sideroxylon* and *Eucalyptus torquata* oils and extracts of leaves, stems, and flowers were demonstrated to have anti-microbial and cytotoxic activities in human hepatocellular carcinoma (HEPG2) and in breast adenocarcinoma (MCF7) cell lines.[23] Moreover, compounds isolated from the leaves of *Eucalyptus cinerea* also showed activity against MCF7, HEP2 (laryngeal carcinoma), and CaCo (colonic adenocarcinoma) cell lines, suggesting a wider use of *Eucalyptus* species for pharmaceutical purposes.[24] Furthermore, regarding specifically the *Eucalyptus globulus* Labill. extract, a recent study has shown a promising cytotoxic effect of some phloroglucinol derivatives, isolated from the fruits of this species, in A549 (human lung cancer), 4 T1 (murine breast cancer), and B16F10 (murine myeloma) cell lines.[25]

Therefore, the present study aimed to test the potential anti-cancer cell growth inhibitory effect of *Eucalyptus globulus* Labill. aqueous extracts, prepared by infusion and decoction, in three human tumor cell lines: the human colorectal adenocarcinoma HCT-15, the exocrine pancreatic cancer PANC-1 and the non-small cell lung cancer NCI-H460. Importantly, the effect of the decoction extract of *E. globulus* was further analyzed on the most sensitive cell line (NCI-H460), particularly on cell apoptosis, the cell cycle profile and cell proliferation. Ultimately, the phenolic profile of both aqueous extracts was also tested in order to corroborate the obtained data.

Materials and methods

Samples and extract preparations

Eucalyptus globulus Labill. fresh aerial parts were collected randomly, from growing plants in the campus of the Polytechnic Institute of Bragança (Northern Portugal) in June 2016. The collected biomass was separated, cleaned and lyophilized (−49 °C and 0.041 bar, FreeZone 4.5, Labconco, Kansas City, MO, USA). The dried plants were ground to a fine powder (~20 mesh) and stored at 4 °C for further analyses. Decoctions were prepared using 1 g of plant material mixed with 200 mL of distilled water and then allowed to boil. Afterwards, they were left to boil for an additional 5 min in a closed flask, allowed to stand at room temperature for an additional 5 min and then filtered under reduced pressure. Infusions were prepared using 1 g of the plant material with 200 mL of distilled boiling water (100 °C), allowed to infuse for 5 min at room temperature, and then filtered through Whatman no. 4 paper. Both aqueous extracts were then further frozen (−20 °C) and lyophilized to obtain a dry extract. The lyophilized products were then dissolved in water and a stock solution at 25 mg mL^{-1} was prepared and stored at −20 °C.

Phenolic compound characterization by LC-DAD-ESI/MS

The phenolic compounds were determined by HPLC-DAD-ESI/MSn (Dionex Ultimate 3000, Thermo Finnigan, San Jose, CA, USA), following a methodology previously described by the authors.[26] The aqueous extracts were analyzed at a concentration of 5 mg mL^{-1}, and the compounds were detected using a diode array detector (DAD, recording at 280, 330 and 370 nm), and a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA), operating in negative mode. Identification was performed by comparing retention times (RT) and mass spectrum fragments with literature reported data or by comparison with commercially available standards (Extrasynthese, Genay, France). Quantification was made on the basis of the UV-vis signal of each available phenolic standard, where calibration curves were obtained by injecting known concentrations (2.5–100 μg mL^{-1}). The results were processed using the Xcalibur®data system and expressed in mg per g of extract.

Cell culture

Three different human tumor cell lines were studied: PANC-1 (exocrine pancreatic carcinoma), NCI-H460 (non-small cell lung cancer) and HCT-15 (colorectal adenocarcinoma). All the cell lines were maintained in RPMI-1640 medium with Ultraglutamine I and 25 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid, HEPES (Lonza, Basel, Switzerland). This medium was supplemented with 5% fetal bovine serum, FBS (Biowest Nuaillé, France), for the cell growth inhibition assay (sulforhodamine B assay) or with 10% FBS for the remaining experiments. The adherent cells were kept at 37 °C in a humidified incubator containing 5% CO_2. Routinely, cells were observed using an inverted light microscope (Leica DMi1). The cell number and viability were assessed by cell counting with an hemocytometer using the Trypan blue exclusion assay (Trypan Blue reagent – Sigma-Aldrich, St Louis, MO, USA). All the experiments were carried out with cells at the exponential phase of growth and with more than 90% viability.

Cell growth inhibition assay

The sulforhodamine B (SRB) assay was used to assess the cell growth inhibitory potential of the extracts.[27,28] Cells were plated in 96-well plates at a previously determined optimal concentration (5.0×10^4 cells per mL for NCI-H460 and PANC-1 cells and 1.0×10^5 cells per mL for HCT-15 cells). After 24 h incubation, cells were treated with five serial dilutions of *Eucalyptus globulus* L. infusion or decoction, ranging from 200 μg mL^{-1} to 12.5 μg mL^{-1}. Doxorubicin (ranging from 2000 nM to 125 nM for HCT-15 cells and from 100 nM to 6.25 nM for NCI-H460 cells) and gemcitabine (ranging from 5000 to 312.5 nM for PANC-1 cells) were used as positive controls (both from Sigma-Aldrich). The effect of the extracts'

solvent (water) on the growth of the cells was also evaluated, by treating cells with the maximum concentration of water used. In addition, cells were treated with the complete culture medium only, providing a normal cell growth control. Two plates were prepared: one to be analyzed immediately (T0) and another to be analyzed 48 h later (T48). Therefore, following 48 h incubation with treatments for the T48 plate (or immediately for the T0 plate), cells were fixed by adding ice-cold 10% trichloroacetic acid (TCA) (w/v, final concentration; Panreac, Barcelona, Spain), washed with water and then stained with 1% SRB (Sigma Aldrich) in 1% (v/v) acetic acid. The solubilization of the bound dye was done by adding 10 mM Tris base solution (Sigma-Aldrich). The absorbance was measured at 510 nm using a microplate reader (BioTek® Synergy HT, Winooski, VT, USA). The GI_{50} values (concentrations that inhibited cell growth by 50%) were determined for *E. globulus* infusion and decoction extracts in the three cell lines.

Preparation of cells for other analyses

For further analyses of the activity of the extracts – cell cycle profile, apoptosis, cell proliferation and western blot – the NCI-H460 cells were plated at 3.3×10^4 cells per mL in 6-well plates. Following an incubation for 24 h, different treatments of *E. globulus* or controls were applied to the cells: 52 μg mL^{-1} of decoction extract (approximately the GI_{50} concentration), 104 μg mL^{-1} of decoction extract (approximately twice the GI_{50} concentration, $2 \times GI_{50}$), medium (Blank), water (in a concentration equivalent to the one used in the treatments for GI_{50} and $2 \times GI_{50}$ of plant extract, corresponding to Control 1 and Control 2, respectively) and 17 nM doxorubicin (positive control). After 48 h of treatment, cells were trypsinized and centrifuged at 290*g* for 5 min (including medium and detached dead cells). Of note, after trypsinization the cells from each well were collected and counted, using the Trypan blue exclusion assay and the percentage of the viable cell number was determined. Cells were then handled according to the procedures described in the next sections.

Analysis of the cell cycle profile

After the procedure stated above, cells were fixed with ice-cold 70% ethanol and stored at 4 °C for at least 12 h. Cells were then centrifuged at 290*g* for 5 min and pellets were resuspended in a phosphate-buffered saline solution, PBS, containing 0.1 mg mL^{-1} RNase A and 5 μg mL^{-1} propidium iodide (PI) (Sigma-Aldrich). The cellular DNA content was analyzed by flow cytometry. The percentage of cells in the G0/G1, S and G2/M phases of the cell cycle was determined using the BD Accuri™ C6 Flow cytometer (BD Biosciences), after proper exclusion of cell debris and aggregates, and plotting at least 20 000 events per sample.[29–31] Data were analyzed using the FlowJo software (version 7.6.5, Tree Star, Inc., Ashland, OR, USA).

Analysis of apoptosis

Following centrifugation, cells were resuspended in buffer solution from the Annexin V-FITC Apoptosis Detection Kit (eBioscience™), as indicated by the manufacturer's instructions. Then, cells were incubated with Human Annexin V-FICT for 10 min on ice, protected from light. After the addition of PI, cells were immediately analyzed by flow cytometry using the BD Accuri™ C6 Flow cytometer and its corresponding software, plotting at least 10 000 events per sample.[30,31] The autofluorescence of each sample and of the Annexin and PI was also measured, in order to make proper fluorescence compensation.

Analysis of protein expression

Cell pellets were washed with PBS (200*g*, 5 min) and then lysed in Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA), complemented with protease inhibitor cocktail (Roche, Basel, Switzerland). The total protein content was quantified using the DC™ Protein Assay kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Protein lysates corresponding to each treatment were loaded (in equal amounts) on 12% SDS-PAGE gel and transferred into a nitrocellulose membrane (Amersham Protran 0.45 NC, GE Healthcare, Buckinghamshire, UK). After blocking with 5% (w/v) non-fat dry milk (Molico), membranes were incubated with the following primary antibodies: rabbit anti-PARP-1 (1 : 2000; sc-7150, Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-p53 (1 : 5000; sc-126, Santa Cruz Biotechnology), mouse anti-p21 (1 : 250; OP64, Calbiochem, San Diego, CA, USA), rabbit anti-γ-H2A·X (1 : 200; sc-101696, Santa Cruz Biotechnology), mouse anti-Cyclin D1 (1 : 400; sc-8396, Santa Cruz Biotechnology) and goat anti-Actin (1 : 2000, sc-1616, Santa Cruz Biotechnology). The corresponding secondary antibodies were goat anti-rabbit IgG-HRP (sc-2004), goat anti-mouse IgG-HRP (sc-2031) and donkey anti-goat IgG-HRP (sc-2020) (all at a dilution of 1 : 2000 from Santa Cruz Biotechnology). For signal detection, the Amersham™ ECL Western Blotting Detection Reagents (GE Healthcare), the High Performance Chemiluminescence Film (GE Healthcare) and the Kodak GBX developer and fixer (Sigma-Aldrich) were used.[32,33] The intensity of the bands was analyzed using the software Quantity One – ID Analysis (Bio-Rad).

Analysis of cell proliferation

Cell proliferation was assessed using the bromodeoxyuridine (BrdU) incorporation assay, according to the previously described protocol.[34] Briefly, 3 h before harvesting the cells (during the 48 h treatment), cells were incubated with 10 μM BrdU (Sigma-Aldrich). Cells were washed and fixed with 4% paraformaldehyde, PFA (Panreac) in PBS. After the preparation of cytospins, cells were treated with 2 M HCl for 20 min (DNA denaturation). Cells were then incubated with a mouse anti-BrdU antibody (1 : 10; Dako), followed by the incubation with anti-mouse-Ig-FITC (1 : 100; Dako). After that, slides were prepared with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole, DAPI (Vector Laboratories Inc, Burlingame, CA, USA). The detection of BrdU incorporation (green nuclei) was possible using a DM2000 microscope

(LEICA; Wetzlar, Germany) and a semi-quantitative evaluation was performed by counting a minimum of 500 cells per slide.

Statistical analysis

Experimental data are shown as the mean ± standard error of the mean (S.E.M.), from at least three independent experiments. Statistical analysis was performed using the two-tailed paired Student's *t*-test. A p value < 0.05 was established to consider statistically significant data.

Results and discussion

Phenolic compound composition in *Eucalyptus globulus Labill.* aqueous extracts

The chromatographic characteristics of the tentatively identified phenolic compounds in *E. globulus* extracts, prepared by decoctions and infusions, are presented in Table 1. Eighteen polyphenols were detected, seven of which were flavonoids (quercetin, isorhamnetin, and myricetin derivatives), three phenolic acids (chlorogenic acid and ellagic acid derivatives), and eight gallotannin derivatives. The identified compounds in the aqueous extracts were identified taking into account the previous findings found by some of the authors in a hydromethanolic extract of *E. globulus.*[35] Nevertheless, these extracts did not reveal the presence of the pentagalloyl-glucoside and presented a slightly lower amount of the identified compounds, probably due to the different solvents and extraction methodology applied.

Both extracts, decoctions and infusions, revealed a high concentration of gallotannins, followed by phenolic acids and flavonoids, respectively. The digalloyl-glucoside (compound **1**) was the main gallotannin present in the decoctions and infusions, with 5-*O*-caffeoylquinic acid being the second major compound. The decoctions were the aqueous extracts that revealed the highest concentration in all the mentioned polyphenol groups.

E. globulus extracts inhibited the growth of various human tumor cell lines

In order to determine the *in vitro* cytotoxicity of the *E. globulus* aqueous extracts in human tumor cell lines, the sulforhodamine B (SRB) assay was performed. The extracts prepared by infusion and decoction were tested in three different human tumor cell line models: non-small cell lung cancer (NCI-H460 cell line), colorectal adenocarcinoma (HCT-15 cell line) and pancreatic ductal adenocarcinoma (PANC-1 cell line). Indeed, the first two cell line models represent two of the most frequent and fatal types of oncologic malignancies (lung and colorectal cancers), while the latter remains as one of the most lethal types of cancers worldwide.[5,36,37]

Table 2 and Fig. 1 present the results of the SRB assay, showing that both extracts have cell growth inhibitory potential in all the cell lines tested. Doxorubicin and gemcitabine, two clinically used drugs to treat these types of cancers, were used as positive controls.

Importantly, the *in vitro* cytotoxicity screening of *E. globulus* extracts in these distinct tumor cell models revealed that NCI-H460 was the most sensitive cell line, followed by PANC-1 and HCT-15, respectively. Interestingly, we have also observed enhanced cytotoxic potency of the decoction *E. globulus* extracts on the NCI-H460 cell line compared to the infusion preparation. Indeed, the decoction preparation was the most potent one, presenting the lowest observed GI_{50} (51.7 ± 2.0 μg mL^{-1}). This could be related to its higher concentration in phenolic compounds, particularly in gallotannins (digalloyl-glucoside, eucaglobulin/globulusin B, trigalloyl-glucoside, and tetragalloyl-glucose).

Taken together, the observed anti-tumor effect of *E. globulus* seems to be heavily reliant on not only the type of tumor cell line but also the type of extract. In order to understand the putative anti-tumor mechanism of action of the *E. globulus* extracts, additional experiments were conducted using the most potent *E. globulus* (decoction) preparation, in the most sensitive (NCI-H460) tumor cell line.

E. globulus decoction extract reduced the NCI-H460 cellular viability

The effect of two concentrations of the decoction extracts [52 μg mL^{-1} and 104 μg mL^{-1}, corresponding to approximately the GI_{50} and twice the GI_{50} ($2 \times GI_{50}$) concentrations, respectively] was studied in the most sensitive cell line (NCI-H460).

Consistently, the *E. globulus* decoction extract, at both concentrations, significantly decreased the number of NCI-H460 viable cells, corroborating the results obtained previously with the SRB assay. Indeed, the results presented in Fig. 2 show that treatment with 52 μg mL^{-1} of *E. globulus* decoction extract significantly reduced the percentage of the viable cell number by 33%, while treatment with a higher concentration (104 μg mL^{-1}) caused a reduction of 62%, when compared to the number of viable cells in the Blank control (cells without any treatment). These results confirm that the *E. globulus* decoction extract induced a dose-dependent decrease in the NCI-H460 viable cell number. It is noteworthy that no differences were found between the Blank control and the additional negative controls (Controls I and II). Interestingly, no prominent morphological alterations were noticed on the cells treated with either concentrations of the extract, when compared to the controls (images not shown).

E. globulus decoction extract altered the cell cycle profile of NCI-H460 cells

Given the anti-tumor cell growth inhibitory potential of the *E. globulus* decoction extract, we have further investigated whether this extract is able to influence the NCI-H460 cell cycle profile. Importantly, we have observed by flow cytometry that NCI-H460 cells treated with the $2 \times GI_{50}$ concentration of the *E. globulus* decoction extract (104 μg mL^{-1}) had a significant increase in the % of cells in the G0/G1 phase, in relation to the Blank, together with a statistically significant decrease in the percentage of cells in the S phase (Fig. 3).

Table 1 Chromatographic characteristics, tentative identification and quantification (mean value ± standard deviation) of the phenolic compounds present in the aqueous extracts of *Eucalyptus globulus* Labill

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M\text{-}H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg g^{-1} extract)		Student's t-test
						Decoction	Infusion	
1	5.6	276	483	331(31), 313(27), 271(100), 211(12), 169(5)	Digalloyl-glucoside[(A)]	27.8 ± 0.1	22.9 ± 0.7	<0.001
2	6.3	272	483	331(22), 313(21), 271(100), 211(16), 169(5)	Digalloyl-glucoside[(A)]	15.7 ± 0.5	11.7 ± 0.2	<0.001
3	7.2	328	353	191(100), 179(32), 161(5), 135(5)	5-*O*-Caffeoylquinic acid[(B)]	22.5 ± 0.1	18.5 ± 0.8	<0.001
4	9.4	277	635	483(14), 465(100), 313(16), 211(5), 169(3)	Trigalloyl-glucoside[(A)]	9.33 ± 0.03	7.1 ± 0.3	<0.001
5	11.0	340	493	317(100)	Myricetin-*O*-glucuronide[(C)]	5.4 ± 0.7	4.62 ± 0.08	0.052
6	11.7	277	635	483(100), 465(9), 331(5), 313(7), 271(5), 211(5), 169(3)	Trigalloyl-glucoside[(A)]	6.7 ± 0.3	5.47 ± 0.03	<0.001
7	12.5	278	635	483(100), 465(41), 313(14), 211(6), 169(3)	Trigalloyl-glucoside[(A)]	6.3 ± 0.2	5.4 ± 0.1	0.001
8	13.2	253, sh360	463	301(100)	Ellagic acid glucoside[(D)]	18.5 ± 0.2	19.0 ± 0.4	0.044
9	14.5	276	787	635(22), 617(27), 483(80), 465(100), 447(20), 423(78), 313(13), 271(8), 169(5)	Tetragalloyl-glucose[(A)]	3.1 ± 0.1	2.3 ± 0.1	<0.001
10	15.4	277	787	635(27), 617(22), 483(58), 465(100), 447(52), 423(19), 313(7), 271(5), 169(3)	Tetragalloyl-glucose[(A)]	5.3 ± 0.1	3.1 ± 0.1	<0.001
11	18.2	355	477	301(100)	Quercetin-3-*O*-glucuronide[(B)]	4.95 ± 0.03	4.1 ± 0.1	<0.001
12	18.5	350	463	301(100)	Quercetin-3-*O*-glucoside[(B)]	4.19 ± 0.04	4.48 ± 0.05	<0.001
13	18.6	283	497	313(55), 169(100)	Eucaglobulin/Globulusin B[(A)]	17.69 ± 0.05	19.2 ± 0.8	0.011
14	21.4	354	433	301(100)	Quercetin-*O*-pentoside[(B)]	1.31 ± 0.03	1.33 ± 0.01	0.149
15	22.5	354	447	301(100)	Quercetin-*O*-rhamnoside[(B)]	2.7 ± 0.1	2.80 ± 0.03	0.306
16	23.3	358	461	315(100)	Isorhametin-*O*-rhamnoside[(B)]	1.77 ± 0.04	1.82 ± 0.04	0.093
17	24.2	250, sh364	447	315(100), 301(38)	Methylellagic acid pentoside[(D)]	2.94 ± 0.01	3.0 ± 0.1	0.186
18	33.5	355	547	463(67), 301(100)	Quercetin derivative[(B)]	1.18 ± 0.01	1.16 ± 0.02	0.073
					Total phenolic acids	**43.91 ± 0.05**	**41 ± 1**	**0.002**
					Total gallotannins	**92.0 ± 0.4**	**77 ± 1**	**<0.001**
					Total flavonoids	**21.5 ± 0.5**	**20.3 ± 0.1**	**0.004**
					Total phenolic compounds	**157.4 ± 0.2**	**138 ± 3**	**<0.001**

Standard calibration curves: A – gallic acid ($y = 131538x + 292163$; $R^2 = 0.997$); B – 5-*O*-caffeoylquinic acid ($y = 168823x - 161172$; $R^2 = 0.999$); C – quercetin-3-*O*-glucoside ($y = 34843x - 160173$; $R^2 = 0.999$); D – ellagic acid ($y = 26719x - 317255$; $R^2 = 0.999$).

Table 2 GI_{50} concentrations of *E. globulus* extracts in three distinct human tumor cell lines

	GI_{50} concentrations (μg mL^{-1}) in different cell lines		
Extracts	PANC-1	HCT-15	NCI-H460
Infusion	70.4 ± 6.0	109.7 ± 17.2	68.0 ± 4.6
Decoction	74.0 ± 8.5	117.0 ± 22.5	51.7 ± 2.0

GI_{50} concentrations, determined with the SRB assay, correspond to the mean ± S.E.M. of at least three independent experiments. Doxorubicin (311.6 ± 26.5 nM in HCT-15 cells and 17.0 ± 2.0 nM in NCI-H460 cells) and Gemcitabine (0.9 ± 0.2 μM in PANC-1 cells) GI_{50} concentrations were used as positive controls.

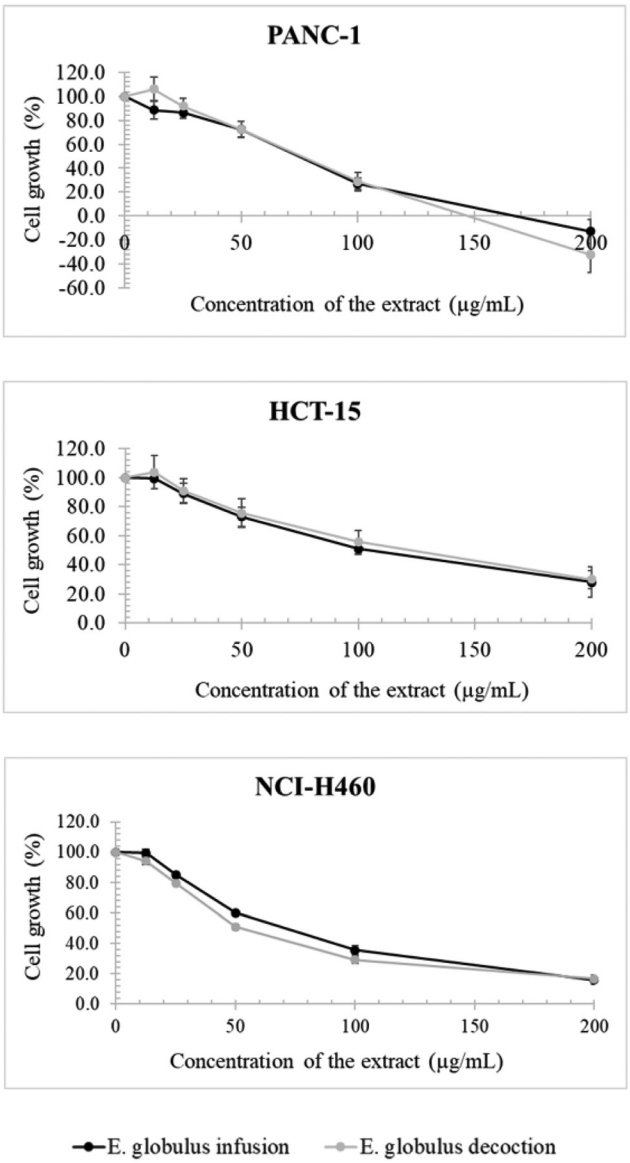

Fig. 1 Dose–response curves of *E. globulus* infusion and decoction extract treatments on PANC-1, HCT-15 and NCI-H460 tumor cells. The results were determined with the SRB assay following 48 h incubation with the extracts. Five concentrations of the extracts were tested, ranging from 200 μg mL^{-1} to 12.5 μg mL^{-1}. The results are presented as the percentage (%) of cell growth when compared to Blank cells. The results are the mean ± S.E.M. of at least three independent experiments.

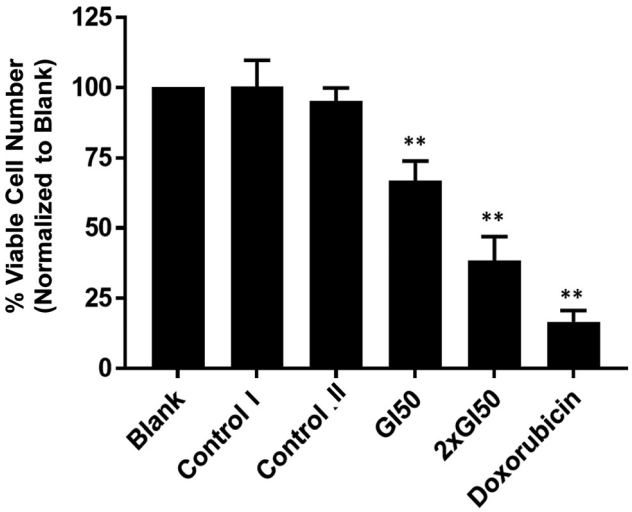

Fig. 2 Effect of the *E. globulus* decoction extract on the NCI-H460 viable cell number. The viable cell number was analyzed 48 h following incubation with the complete medium (Blank), 52 μg mL^{-1} (GI_{50}) or 104 μg mL^{-1} (2 × GI_{50}) of the *E. globulus* decoction extract, or with the corresponding vehicle concentrations alone (Controls I and II). The results are presented in relation to the Blank and are the mean ± S.E.M. of eight independent experiments. $^{**}p \leq 0.01$, Blank *vs.* Treatment.

Moreover, in order to verify whether the *E. globulus* decoction extract is also able to cause programmed cell death in NCI-H460 cells, we have performed the Annexin V-FITC/PI assay by flow cytometry. Interestingly, the treatment of NCI-H460 cells with either the GI_{50} or 2 × GI_{50} concentrations of the *E. globulus* decoction extract only caused a very modest increase (not statistically significant) in the % of apoptotic cells (GI_{50}: 5%, 2 × GI_{50}: 8%), when compared to the Blank condition (data not shown). Altogether, these findings indicate that the decoction extract of *E. globulus* may limit the growth of NCI-H460 cells mainly by interfering with the cell cycle in the G0/G1 and S phases, rather than by causing cellular apoptosis.

E. globulus decoction extract decreased NCI-H460 cellular proliferation

Given the alterations in the NCI-H460 cell cycle, we have further investigated its repercussion on cell proliferation, by analyzing the levels of BrdU incorporation following 48 h treatment (Fig. 4a and b). Concordantly with the cell cycle profile data, we have observed a decrease (although not statistically significant) in the percentage of proliferating cells treated with the 2 × GI_{50} concentration of the *E. globulus* extract, in relation to the Blank control.

E. globulus decoction extract altered the NCI-H460 cell expression levels of cell cycle-related proteins

Considering the previous results, we have further confirmed the effect of the *E. globulus* decoction extract on the NCI-H460 cell cycle profile and apoptosis, by assessing the expression

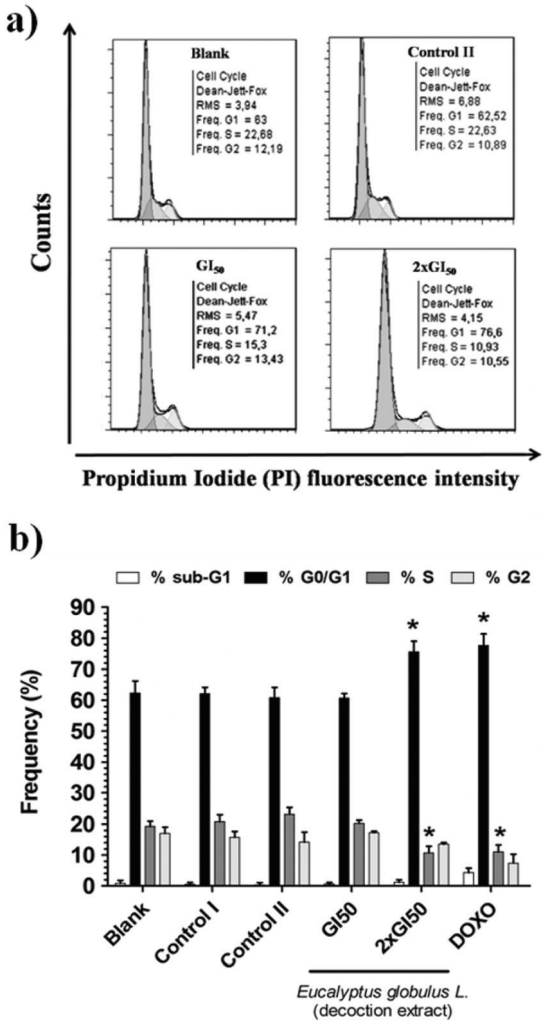

Fig. 3 Cell cycle distribution of NCI-H460 cells treated with the *E. globulus* decoction extract, analyzed by flow cytometry. (a) Representative histograms of the NCI-H460 cell cycle profile. (b) Percentage of NCI-H460 cells in the different phases of the cell cycle. The cell cycle profile was analyzed 48 h following incubation with the complete medium (Blank), 52 μg mL^{-1} (GI_{50}) or 104 μg mL^{-1} (2 × GI_{50}) of the *E. globulus* decoction extract, or with the corresponding vehicle concentrations alone (Controls I and II). The results are presented as the mean ± S.E.M. of at least three independent experiments. *$p \leq 0.05$, Blank *vs.* Treatment.

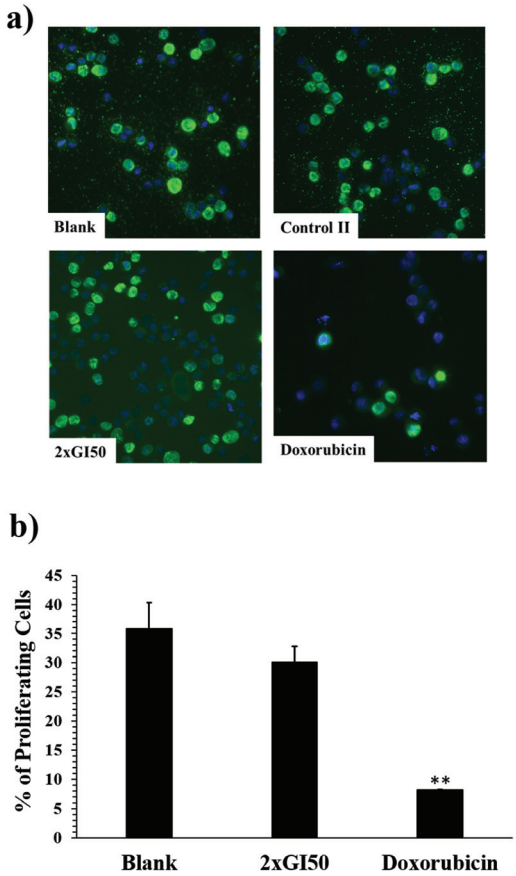

Fig. 4 Effect of the *E. globulus* decoction extract on NCI-H460 cellular proliferation. (a) Representative fluorescence microscopy images of BrdU incorporation (green) and DAPI stained nuclei (blue). (b) Percentage of BrdU-incorporating cells. The results are presented as the mean ± S.E.M. of 3 independent experiments. **$p \leq 0.01$, Blank *vs.* Treatment. Amplification = 200×.

levels of a panel of the cell cycle and apoptotic-related proteins by western blot.

Interestingly, we have observed an increased expression in the NCI-H460 cell line levels of p53 upon treatment (Fig. 5a). Indeed, cell treatment with the GI_{50} and 2 × GI_{50} concentrations of the extract caused nearly 4- and 12-fold increases in the p53 levels, respectively, when compared to the Blank control. Importantly, these findings are aligned with the results obtained in the cell cycle analysis. Indeed, higher levels of p53 expression may trigger alterations in the cell cycle profile by causing an activation of p21, an inhibitor of the cyclin-dependent kinase at the G1-checkpoint.[38–41] A cyclin D1 protein is required for the cell cycle progression through the G1 phase, during which it is synthesized and accumulated in the nucleus, following degradation when the cell enters in the S phase.[42] Indeed, it has been shown that cells with increased levels of p53 arrest in the G1 phase, characterized by the accumulation of p21, which in turn induces cyclin D1 production.[43] Importantly, and consistently with this line of thought, we also found an increase in the NCI-H460 levels of both p21 (with a 3- and 7-fold increase for GI_{50} and 2 × GI_{50}, respectively) and cyclin D1 (for 2 × GI_{50}, with a 4-fold increase) upon treatment with the *E. globulus* decoction extract (Fig. 5a). Therefore, the observed increased levels of both p21 and cyclin D1 in NCI-H460 cells upon treatment with the *E. globulus* decoction extract strongly support the hypothesis that the *E. globulus* extract induces cell cycle arrest in G0/G1 *via* p53 activation.

Regarding cell apoptosis, the *E. globulus* extract treatment did not alter the NCI-H460 cellular levels of total poly (ADP-ribose) polymerase (PARP), whose reduction is associated with cellular apoptosis[44,45] (Fig. 5b). Importantly, this result is in accordance with the previous Annexin V-FITC/PI results,

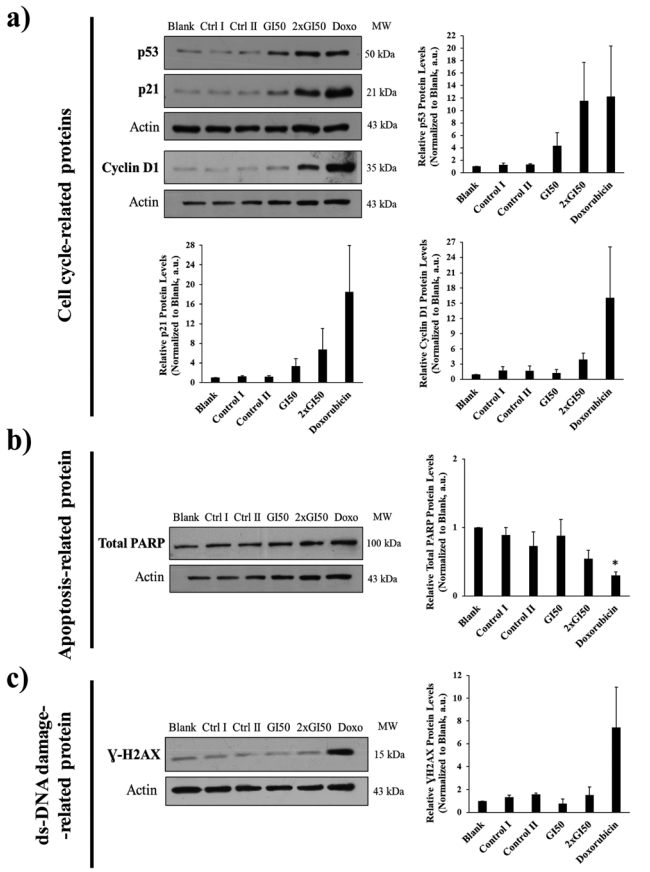

Fig. 5 Expression levels of (a) cell cycle-related proteins – p53, p21 and Cyclin D – of (b) the apoptosis-related protein – total PARP, and (c) the ds-DNA damage related protein – γ-H2AX, in NCI-H460 cells following treatment with the *E. globulus* decoction extract, analyzed by western blot. Cells were treated for 48 h with the complete medium (Blank), 52 μg mL^{-1} or 104 μg mL^{-1} of extract or with the highest vehicle concentration (H_2O). Actin was used as loading control. The representative images and densitometry analysis of the western blots are from at least three independent experiments. The results are presented as the mean ± S.E.M. from at least three independent experiments and were expressed after the normalization of the values obtained for each protein with the values obtained for actin. a.u. refers to arbitrary units. *$p \leq 0.05$, Blank *vs.* Treatment.

showing that the *E. globulus* decoction extract does not have an impact on NCI-H460 cell death, at least at the concentrations tested.

We have then investigated whether the *E. globulus* treatment was able to induce double-stranded DNA damage in NCI-H460 cells. For that, the levels of the γ-H2A·X protein – a molecular marker of DNA double-strand breaks[46] – were assessed. Importantly, NCI-H460 cells treated with both concentrations of the extract had no alteration in γ-H2A·X protein levels (Fig. 5c). However, this apparent lack of ds-DNA damage in NCI-H460 cells upon treatment does not exclude the possibility that other forms of DNA damage may be present (for instance single-strand DNA damage), which could also explain the activation of p53 and consequent cell cycle arrest in the G0/G1 phase with a slower NCI-H460 cell growth.[47,48]

Conclusions

Herein we demonstrated that the *E. globulus* aqueous extracts prepared by two different procedures – decoction and infusion – are able to hinder the cell growth of three different human tumor cell lines. Both extracts are a rich source of phenolic compounds, especially gallotannins, and in particular digalloyl-glucoside. Importantly, this anti-tumor effect was more pronounced in the non-small cell lung cancer cell line, NCI-H460, with the decoction formulation, which could be related to its higher content in polyphenols. Mechanistically, the *E. globulus* decoction extract seems to primarily impact the NCI-H460 cell cycle profile, causing an increase in the population of cells in the G0/G1 phase *via* activation of the p53 pathway, with a consequent decrease in cell proliferation (observed as an S phase decrease and also a BrdU incorporation decrease). Interestingly, this extract does not seem to cause ds-DNA damage and did not induce programmed cell death.

To the best of our knowledge, we present one of the first reports on the mechanism of action of the *E. globulus* extract on the NCI-H460 cells. Therefore, this work underlines the potential of the *E. globulus* decoction extract as a source of bioactive compounds with anti-tumor activity.

Conflicts of interest

The authors declare no conflicts of interest.

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