Effect of new antioxidant cysteinyl-flavanol conjugates on skin cancer cells

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Abstract Novel catechin derivatives obtained from grape procyanidins and L-cysteine scavenge free radicals by hydrogen atom donation, rather than electron transfer, and reduce cell viability in A375 and M21 melanoma cells. In particular, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate has a free radical scavenging capacity as strong as that of tea (−)-epigallocatechin gallate and causes a significant S-phase cell-cycle arrest in both cell lines at doses higher than 100 μM. The other cysteinyl compounds do not affect normal cell cycle distribution. The gallate derivative also induces apoptosis in melanoma cells more strongly than the other derivatives and the parent (−)-epicatechin do. The gallate compound seems to trigger nuclear condensation and fragmentation, which is confirmed by DNA laddering. Interestingly, they do not induce apoptosis in keratinocytes (HaCaT).

Keywords: Catechins; Antioxidants; Gallate ester; Melanoma; Cell cycle; Apoptosis

1. Introduction

Cutaneous melanoma ranks fifth in incidence among men and sixth among women in the USA. Its incidence is rising at a rate of about 5% per year [1]. Some studies have shown that UV light exerts its biological effects, at least in part, via the generation of reactive oxygen species (ROS) and free radicals, which play a major role in the induction of skin cancer [2–5]. Chemopreventive approaches utilizing non-toxic agents aimed at both minimizing ROS formation and inducing apoptosis in tumour cells seem attractive. Recent studies on skin cancer prevention show that certain polyphenols cause both actions and appear to be efficient inhibitors of UV radiation-induced skin carcinogenesis [2,6–8].

Of these polyphenols, the gallate-containing flavan-3-ols from green tea extract stand out because of their antioxidant capacity and their induction of apoptosis in tumour cells [9,10]. Grape extract, whose properties are similar to tea extract's, is another important source of flavan-3-ols. While the tea extract includes mainly monomeric flavan-3-ols, mostly gallocatechins [11], grape extracts contain polymeric flavan-3-ols [12,13], mostly procyanidins (see Fig. 1). Interestingly, depolymerisation of procyanidins is an appropriate way of obtaining monomeric derivatives with modified properties [14,15]. A previous study in our Laboratories described a new family of flavan-3-ols obtained by depolymerisation of grape procyanidins in the presence of the natural amino acid cysteine [16]. In the present study, we evaluated the electron transfer capacity of the novel flavan-3-ol derivatives, 4β-(*S*-cysteinyl)epicatechin, 4β-(*S*-cysteinyl)catechin and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Fig. 2), with the new radical chemosensor HNTTM [17]. The study is significant because of the novel compounds, as electron transfer capacity is directly related to pro-oxidant effects in catechins, such as (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG) [18,19]. We also studied the response of two melanoma cell lines, A375 and M21, to treatment with the novel flavan-3-ol derivatives. Melanoma cells were significantly less viable in both cell lines at higher doses. Moreover, the gallate-containing compound showed a significant arrest in the S phase of the cell cycle and a significant activation of the apoptosis mechanism in both tumour cell lines, but not in keratinocytes. The apoptotic induction of the gallate-containing compound was confirmed by flow cytometry and DNA laddering, showing apparent nuclear fragmentation.

2. Materials and methods

4β-(*S*-cysteinyl)epicatechin (Cys-EC) **2**, 4β-(*S*-cysteinyl)catechin (Cys-Cat) **3** and 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Cys-ECG) **4** (Fig. 2) were prepared as described [16] and dissolved in *Dulbecco's* phosphate buffer saline solution (PBS, from Sigma, Steinheim, Germany). (−)-Epicatechin (EC) **1**, staurosporine, ethidium bromide, EDTA, boric acid, isopropanol and α,α,α-Tris(hydroxymethyl)aminomethane were also purchased from Sigma. Ethanol, NaCl, NaOH and $CaCl_2 \cdot 2H_2O$ were from Panreac Química SA (Montcada i Reixac, Spain). Trypsin–EDTA solution C (0.05% trypsin and EDTA 1:5000 in PBS) was from Biological Industries (Beit Haemek, Israel). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), DMSO (dimethyl sulfoxide), Trypan blue solution 0.4%, propidium iodide (PI) and the non-ionic surfactant Igepal CA-630 were from Sigma Chemical Co (Saint Louis, MO, USA). RNase and agarose MP were from Roche Diagnostics (Mannheim, Germany). FITC-Annexin V kit and binding buffer 4× for apoptosis assay were purchased from Bender MedSystems (MedSystems Diagnostics GmbH, Vienna, Austria). The Realpure DNA extraction kit, which included Proteinase K, was purchased from Durviz s.l. (Paterna, Spain). Blue/Orange Loading dye (0.4% orange G, 0.03% bromophenol blue, 0.03% xylene

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Abbreviations: Cys-Cat, 4β-(*S*-cysteinyl)catechin; Cys-EC, 4β-(*S*-cysteinyl)epicatechin; Cys-ECG, 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; EC, (−)-epicatechin; HNTTM, tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl radical

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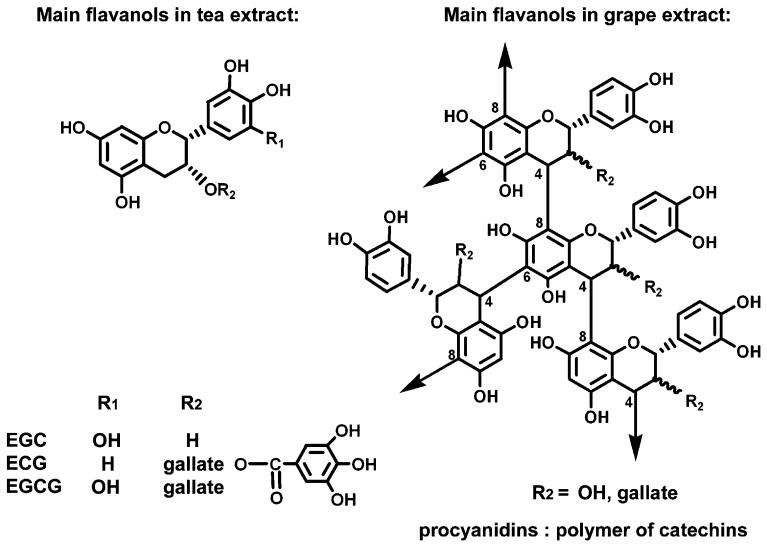

Fig. 1. Molecular structure of the main components in tea extract (monomers of gallocatechins) and grape extract (polymer of catechins).

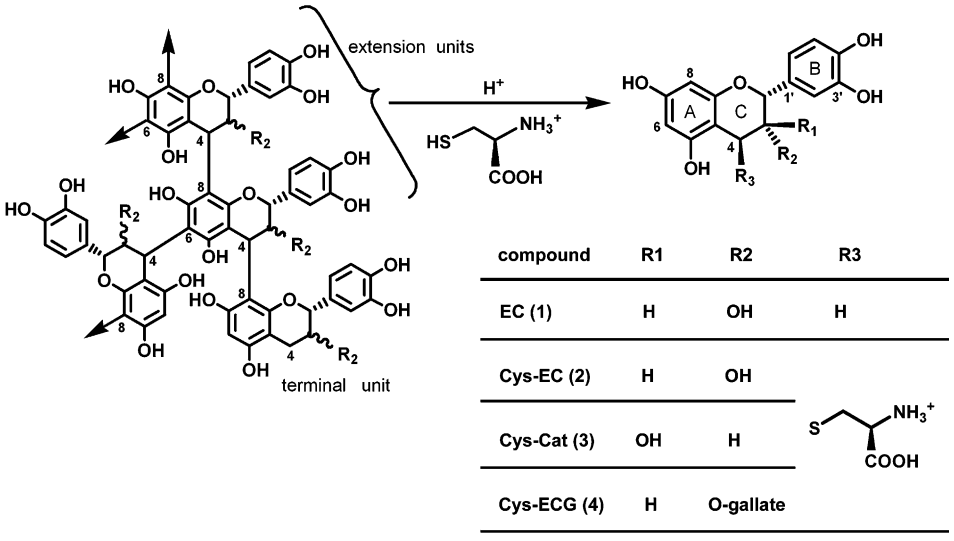

Fig. 2. Molecular structures of (−)-epicatechin and the novel thio-derivatives of flavan-3-ols obtained by depolymerization of grape procyanidins in the presence of cysteine.

cyanol FF, 15% Ficoll 400, 10 mM Tris–HCl, pH 7.5 and 50 mM EDTA, pH 8) and 1 kb DNA ladder were from Promega (Madison, WI, USA).

2.1. Electron transfer assay

EPR measurements were performed on a Varian (Palo Alto, CA) E-109 spectrometer working in the X-band (microwave power, 20 mW; modulation amplitude, 3.2 G). The radical scavengers were tested at various concentrations in chloroform-methanol (2:1). Aliquots (1 mL) were reacted with a solution (1 mL) of HNTTM (tris(2,4,6-trichloro-3,5-dinitrophenyl)methyl) radical (120 μM in chloroform–methanol (2:1)) [17] and the mixture was incubated for 30 min. The initial concentration of the compounds tested ranged from 1 to 30 μM. Each point was acquired in triplicate. A dose–response curve was obtained for each product. The results were expressed as the efficient dose ED_{50} given as μmoles of compound able to consume half the amount of free radical divided by μmoles of initial HNTTM radical. The stoichiometric value (theoretical concentration of antioxidant to reduce 100% of the radical) was obtained by multiplying the ED_{50} by two. The inverse of this value represents the moles of radical reduced by one mole of antioxidant and gives an estimate of the number of electrons involved in the process.

2.2. Cell culture

A375 cell line (human malignant melanoma) was purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco modified Eagle's medium (DMEM) from Sigma. The medium was supplemented with 10% (v/v) heat-inactivated FCS (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and antibiotics: 100 U/mL penicillin and 100 μg/mL streptomycin (Biological Industries, Beit Haemek, Israel). M21 cell line (human malignant melanoma), generously provided by Prof. A. Mazo (UB, Barcelona, Spain), was cultured in RPMI-1640 medium supplemented as before. The spontaneously immortalised human keratinocyte cell line HaCaT was provided by Advancell (Barcelona, Spain), cultured in DMEM medium from Biowhittaker/Cambrex (Milan, Italy) and supplemented with 10% FCS, 2 mM L-glutamine, 1% HEPES buffer (Invitrogen) and 0.1% antibiotics. Cells were grown in an isolated 37 °C, 5% CO_2 tissue incubator compartment and their medium was changed every 3 days.

2.3. Growth inhibition assay

Cell growth was determined by means of a variation of the MTT assay described by Mosmann [20]. A375 and M21 cells were seeded into 96-well plates at a density of 1.5×10^3 cells/well and 1.2×10^3 cells/well, respectively. Adherent cell lines were incubated for 24 h prior to addition of the compounds. After 3 days culture, the supernatant was aspirated and 100 μL of filtered MTT (0.5 mg/mL in cell culture medium) was added. The cell plates were incubated for 1 h and metabolically active cells reduced the dye to purple formazan. The supernatant was removed, and the dark blue MTT formazan precipitated was dissolved in DMSO (100 μL). Optical density (OD) was measured at 550 nm on a multi-well reader (Merck ELISA System MIOS®).

The IC_{50} or compound concentration causing a 50% reduction in the mean OD value relative to the control was calculated using a GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd. Microsoft Corp., Surrey, UK) curve option: IC_{50}curve – start at 0.

2.4. Cell cycle analysis

Flow cytometry was used to analyse cell cycles and quantify apoptosis. A375 and M21 cells were seeded into 6-well plates at a density of 3.5×10^4 cells/well and incubated for 24 h prior to addition of the compounds. The IC_{50} and $2 \times IC_{50}$ concentration values of each sample were measured after 3 days subculture. Both cell lines were resuspended in ice-cold TBS 1× buffer (1 mL of 10 mM Tris and 150 mM NaCl, pH 7.4). PI (50 μL, 50 μg) and 1 mL Vindelov buffer at pH 7.4, containing 10 mM Tris, 10 mM NaCl, PI (50 μL, 50 μg), Rnasa (1 μL, 10 μg) and Igepal CA-630 (1 μL), were added to each sample. Cells were incubated for 1 h at 4 °C in the dark [21]. Cell cycle distribution was analysed by flow cytometry using the fluorescence-activated cell sorting (FACS) system. DNA histograms were collected with an Epics XL flow cytometer (Coulter Corporation, Miami, FL) and analysed by the Multicycle program (Phoenix Flow Systems, San Diego, CA).

2.5. Assessment of apoptosis

In the assessment of apoptosis, after 3 days subculture of the cell plates in the same way as for the cell cycle treatment, both melanoma cell lines were washed once in ice-cold binding buffer (10 mM HEPES sodium hydroxide pH 7.4, 140 mM sodium chloride, 2.5 mM calcium chloride) and resuspended in the same buffer (95 μL) at a maximum of 0.8×10^6 cells/mL in the presence of FITC-Annexin V binding (3 μL). After 30 min incubation at room temperature in the dark, PI (20 μL, 20 μg) was added [22]. Cells, double-stained with PI and annexin V-FITC, were processed by flow cytometry and laser-scanning cytometry (LSC), which collected green (525 nm) fluorescence for FITC conjugated antibody and red (675 nm) fluorescence for PI, under 488 nm excitation. HaCaT cells (human keratinocytes) were seeded into 6-well plates at 8.5×10^4 cells/well density and treated in the same way as the melanoma cell lines described above.

2.6. DNA fragmentation assay

Cells were treated with compound **4** and stausporine for 120 h. After treatment, cells were scraped off the plates and collected by centrifugation at 400 g for 5 min. Cells were lysed in a lysis buffer (1 mL, Realpure kit) and incubated with Proteinase K (6 μL, 120 μg) at 55 °C for 1 h. RNA was digested by incubation with RNase (7 μL, 70 μg) at 37 °C for 1 h. After treatment, protein precipitation buffer (800 μL, Realpure kit) was added and lysates were vortexed and centrifuged at $16\,000 \times g$ for 10 min. The DNA in the aqueous supernatant was extracted with isopropanol (600 μL) and centrifuged at $14\,000 \times g$ for 3 min. The supernatant was removed and the pellet was rinsed with 70% ethanol (600 μL), dried at room temperature for 15 min and resuspended in DNA hydratation solution (100 μL, Realpure kit).

Hydrated DNA was diluted 1/50 in MilliQ water for DNA quantification by UV spectrophotometer at 260 nm. Loading dye (3 μL) was added to 20 μg of DNA for each treatment, and the samples were resolved over 1% agarose gel (75 min at 80 V), containing ethidium bromide (2 μL) in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA; pH 8.0). The DNA bands were viewed under a UV transilluminator and (Vilber Lourmat, Marne-la-Vallée, France), followed by a video copy processor (Mitsubishi P91 system, Kyoto, Japan).

2.7. Statistics

The Student's *t* test was used for statistical analysis. For each compound, a minimum of four independent experiments with duplicate values to measure growth inhibition and a minimum of three independent experiments for cell cycle analysis, assessment of apoptosis and DNA fragmentation were conducted. Data are given as the mean ± SD.

3. Results and discussion

3.1. Free radical scavenging activity. Hydrogen donation versus electron transfer

We had previously measured the hydrogen donation capacity of **1**–**4** in the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) assay [16]. The thio-derivatives **2**–**4** donated 4.2, 3.8 and 10.0 hydrogen atoms per molecule, respectively, whereas the underivatised (−)-epicatechin **1** gave 2.8 hydrogen atoms. Using the new radical chemosensor HNTTM [17], we measured the electron transfer capacity of **1**–**4**. The epicatechin and catechin conjugates **2** and **3** transferred only 2.4 and 2.3 electrons per molecule to HNTTM, respectively, and compound **1** had similar results (2.4). The most potent compound, Cys-ECG **4**, transferred 6.7 electrons per molecule, which was still less than the 10.0 hydrogen atoms donated to DPPH (Table 1). The hydrogen donation capacity of **4** was similar to that of EGCG and superior to the vitamin E analogue (Trolox) and another galloylated compound, propyl-gallate. The results prove that the thio-derivatives showed greater scavenging capacity by hydrogen atom donation than by electron transfer (high H/e values, Table 1). The order of efficiency in both scavenging

Table 1
Free radical scavenging power and stoichiometry

Compound	DPPH (hydrogen donation)[a]			HNTTM (electron transfer)			
	ARP ($1/ED_{50}$)	Stoichiometric value	H atoms per molecule[b]	ARP ($1/ED_{50}$)	Stoichiometric value	Electrons per molecule[c]	H/e^- ratio
1	5.5	0.36	2.8	4.8	0.42	2.4	1.2
2	8.3	0.24	4.2	4.8	0.42	2.4	**1.7**
3	7.7	0.26	3.8	4.7	0.43	2.3	1.7
4	20.0	0.10	**10.0**	13.3	0.15	**6.7**	**1.5**
Trolox	3.9	0.52	1.9	5.4	0.37	2.7	0.7
EGCG	21.3	0.09	10.6	11.3	0.17	5.9	**1.8**
Propyl-gallate	9.5	0.21	4.7	6.1	0.33	3.1	**1.5**

Standard deviation ($n = 3$): $\leqslant 0.3$ (ARP), $\leqslant 0.04$ (stoichiometric value), $\leqslant 0.2$ (H or e^- per molecule).
[a]Results for compounds **1**–**4** from Ref. [16].
[b]Moles reduced DPPH per mole antioxidant.
[c]Moles of reduced HNTTM per mole antioxidant.

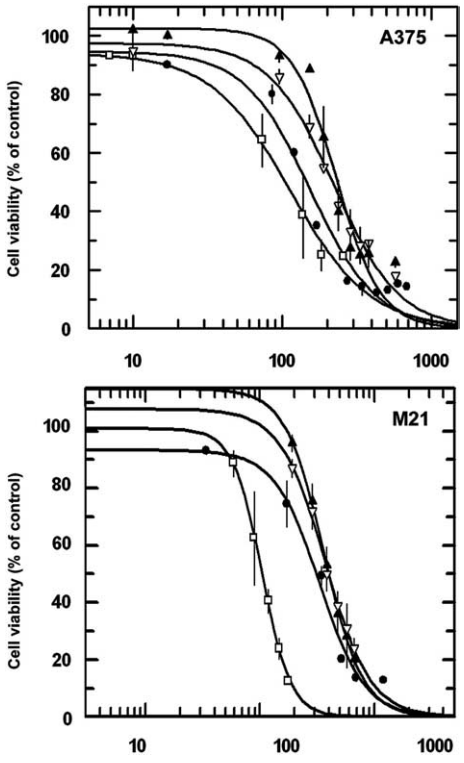

Fig. 3. Proliferation of A375 cells and M21 cells in response to EC (●), Cys-EC (▲), Cys-Cat (▽) and Cys-ECG (□). Cell cultures were treated with increasing doses of these compounds, as indicated on the *x* axis. Mean ± S.D., $n \leqslant 8$.

assays was **4** > **3** ∼ **2** ∼ **1**. This may be of biological significance because the capacity to transfer electrons is regarded sometimes as an undesired effect [23] related to the pro-oxidant action of gallocatechins such as (−)-epigallocatechin (EGC) and (−)-epigallocatechin-gallate (EGCG), and is mainly associated with the pyrogallol group on ring B [18,19]. Potent compounds such as Cys-ECG **4**, which do not include this group, might be safer than equipotent gallocatechins such as EGCG.

3.2. Growth inhibition capacity

Treatment of melanoma cells with the thio-conjugates for 72 h resulted in a reduction in cell viability in a dose-dependent fashion (Fig. 3), with the order of cell growth inhibition capacity being **4** > **1** ∼ **2** ∼ **3** for both A375 and M21 cells, according to the IC_{50} values obtained (Table 2). The cysteinyl conjugates were equally or slightly less efficient than the underivatised (−)-epicatechin, **1**, in inhibiting the growth of melanoma cells. Interestingly, the gallate-containing derivative **4**, which was the most effective compound as a free radical scavenger, was also the most efficient molecule in inhibiting cell growth in both melanoma cell lines.

Table 2
Antiproliferative potency against human melanoma cell lines

Cell line	Compound	*n*[a]	Mean IC_{50} (μM)	S.D.
A375	**1**	4	154	15
	2	4	231	14
	3	4	223	10
	4	4	113	6
M21	**1**	7	332	39
	2	5	363	18
	3	5	365	14
	4	5	104	4

S.D., standard deviation.
[a]*n*, number of experiments performed.

3.3. Cell cycle analysis and apoptosis induction

To examine the effects of the flavan-3-ols **1**-**4** on the cell cycle pattern at concentrations equal to their IC_{50} and twice their IC_{50}, A375 and M21 cells were treated with each compound for 72 h, and then analysed by FACS (Fig. 4). The galloylated cysteinyl compound **4** induced the most significant increase in the S cycle phase, at $2 \times IC_{50}$ over the untreated cells. The rest of the flavanols, **1**-**3**, showed a cell cycle distribution similar to the untreated cells in A375 and M21. The fact that Cys-ECG (**4**) treatment caused a major arrest in S phase in both cell cycles, whereas Cys-EC (**2**) did not cause any significant effect on either A375 or M21 at twice its IC_{50}, demonstrated that the gallate structure was, at least in part, responsible for this effect on the cell cycle (Fig. 4).

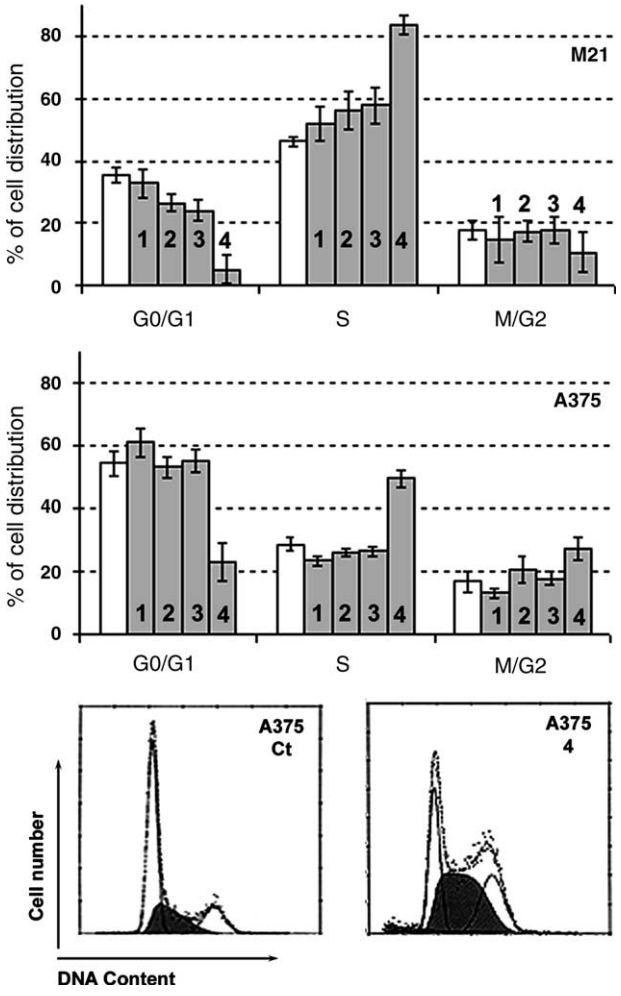

Fig. 4. Influence of the flavanols on M21 and A375 cell cycles after 72 h of treatment. Non-treated cells are indicated by open bars. Doses ($2 \times IC_{50}$) in M21 cells: EC (**1**), 660 μM; Cys-EC (**2**) and Cys-Cat (**3**), 720 μM; Cys-ECG (**4**), 208 μM. Doses ($2 \times IC_{50}$) in A375 cells: **1**, 310 μM; **2** and **3**, 420 μM; **4**, 237 μM. Cell cycle distribution of untreated A375 cells (Ct) and Cys-ECG (**4**) in A375 cells.

Since cell cycle arrest may lead to apoptosis, in the next series of experiments we used FITC-FACS analysis to establish whether necrosis or apoptosis was involved. A375 and M21 cells treated with the compounds **1**-**4** at their IC_{50} slightly increased the number of early ($PI^-/FITC^+$) and late ($PI^+/FITC^+$) apoptotic cells from the control cell figure. With increasing doses ($2 \times IC_{50}$) of compounds **1**–**3**, apoptosis induction doubled (Fig. 5, *right bottom* $PI^-/FITC^+$ *and upper* $PI^+/FITC^+$ *quadrants*). The gallate-containing Cys-ECG (**4**, at $2 \times IC_{50}$) showed an apoptotic effect four times greater than in control cells in both melanoma cell lines (Fig. 5 *right quadrants*, where 21% of cells in A375 and 31% of cells in M21 were considered apoptotic cells). This result corroborates findings of other authors, who described how gallate compounds induced apoptosis effectively, whereas induction was weaker in compounds that lacked a gallate ester [10,24–28]. To discriminate between late apoptotic and necrotic cells after treatment with compound **4**, we investigated PI and annexin V-FITC positive cells using LSC analyses with microscopic observations. A375 cells treated with compound **4** showed 8% of cells in the *right bottom quadrant* ($PI^-/FITC^+$ region). These, viewed by LSC microscope, showed the limited nuclear fragmentation and the typical green appearance, which is a distinctive morphology of early apoptosis caused by the labelling of annexin V by FITC. Late apoptosis/necrosis was present in 13% (*right upper quadrant*, $PI^+/FITC^+$ region) of A375 cells, which showed advanced nuclear fragmentation and limited staining with pycnotic nuclei, which is a definitive sign of the formation of the apoptotic cell. Similarly in M21, compound **4** had 5% of cells in early apoptosis and 26% of cells in late apoptosis/necrosis (Fig. 5).

To explore the selectivity of the effect on tumour cells, we tested compounds **1**-**4** in a non-malignant cell line. HaCaT cells were treated with the compounds at the highest doses used for A375 melanoma, and the apoptosis mechanism was activated in none of them (Fig. 5).

The order of efficiency in both the antiradical and apoptotic induction assays coincided. The gallate-containing compound **4** was the most scavenging agent and compounds **2** and **3** were little more efficient than underivatized **1** as free radical scavengers, with the order of efficiency being $\mathbf{4} > \mathbf{3} \sim \mathbf{2} \sim \mathbf{1}$ (Table 1). Moreover, compound **4** was also the major apoptotic inducer in melanoma cells and compounds **2** and **3** were slightly less efficient than **1**, with the order of induction being $\mathbf{4} > \mathbf{1} \sim \mathbf{2} \sim \mathbf{3}$. The gallate group provides, first, great scavenging power through its three contiguous hydroxyl groups [16,29] and, second, pro-apoptotic action probably through inhibition of kinase activities [30,31]. Although electronic distribution within the gallate moiety might play a role in both cases, the two effects may, in the final analysis, be unrelated.

3.4. Detection of fragmented DNA by agarose gel electrophoresis

The induction of apoptosis will stimulate endonuclease that involves double-strand DNA breaks into oligonucleosome length fragments, resulting in a characteristic ladder of DNA electrophoresis [32]. DNA fragmentation was observed in melanoma cells A375, but not in HaCaT cells at Cys-ECG (**4**) concentrations of 200 μM. Fragmentation is a late event in apoptosis mechanism and DNA fragments (DNA ladders), showing varying sizes between 250 and 2000 bp, were clearly visible after agarose gel electrophoresis (Fig. 6). No specific DNA fragments were detected when control cell cultures were analysed. As a positive control, an apoptotic inducer (staurosporine, 1.6 μg/mL) was incubated in A375 and HaCaT cell cultures 5 h before DNA extraction. Staurosporine treatment

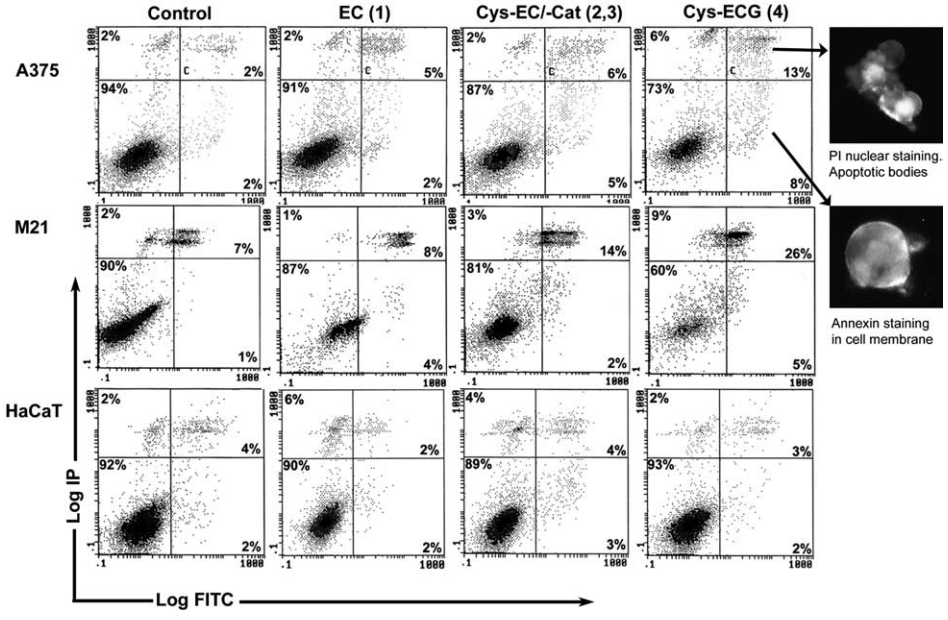

Fig. 5. Flavanols induced apoptosis of A375 and M21 cells, but not of HaCaT cells. Doses in A375 cells: EC (**1**), 310 μM; Cys-EC (**2**) and Cys-Cat (**3**), 420 μM; Cys-ECG (**4**), 237 μM. Doses in M21 cells: **1**, 660 μM; **2** and**3**, 720 μM; **4**, 208 μM. In HaCaT cells: **1**, **2** and **3**, 400 μM; **4**, 200 μM. The percentage of apoptotic cells was determined using PI (represented on the *y* axis) and annexin V-FITC staining (on the *x* axis). After 72 h treatment, early apoptosis ($PI^-/FITC^+$, right bottom quadrant) and late apoptosis ($PI^+/FITC^+$, right upper quadrant) are shown as % of apoptotic cells. The LSC photomicrographs showed single cells as representative of A375 cell population treated with **4**.

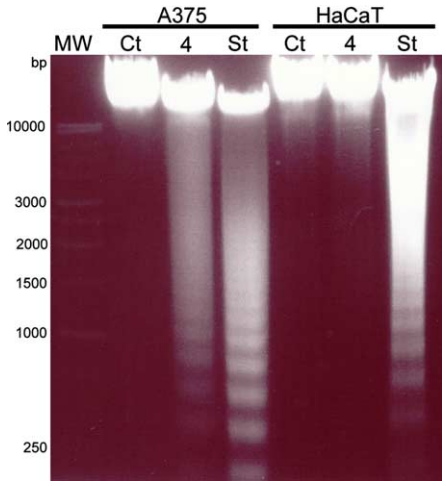

Fig. 6. Compound **4** causes DNA laddering in A375 melanoma cells, but not in HaCaT cells. Staurosporine (ST) is used as a positive control for both cell lines. Cells were incubated with compound **4** (200 μM) or staurosporine (2 μM) for 120 h. Cell DNA was extracted and analysed by agarose gel electrophoresis. Data shown are representative of three independent experiments.

showed specific DNA fragmentation in both cell lines (Fig. 6). At 72 h of treatment, FACS analysis showed less than 25% of cells in apoptotic mechanism (Fig. 5), so apoptotic bodies were not noticeable in DNA fragmentation assay. To increase the rate of apoptotic cells, fragmentation was measured after 120 h of treatment. In this case, the later events in programmed cell death were clearer and the results confirmed those obtained in FACS analysis, showing compound **4** as an apoptotic inducer in melanoma cells but not in keratinocytes.

Most chemotherapeutic drugs currently used in cancer therapy kill cancer cells by indirectly activating checkpoint-mediated apoptosis after creating non-selective damage to DNA or microtubules, which accounts for their toxicity toward normal cells. A promising example that might avoid such damage is lapachone, which acts by activating checkpoints in cancer cells resulting in cell cycle arrest in S phase and selective induction of apoptosis in cancer cells, but not in proliferating normal cells [33]. The new flavanol derivative 4β-(*S*-cysteinyl)epicatechin 3-*O*-gallate (Cys-ECG, **4**), obtained from grape procyanidins, showed an antioxidant capacity equal to the well-known EGCG and with the advantage that compound **4** enhanced the scavenging capacity by hydrogen atom donation rather than by electron transfer, which is related to the pro-oxidant effects of catechins such as EGC and EGCG [18,19]. Moreover, compound **4** triggers cell cycle arrest in S phase and selective induction of apoptosis in melanoma cells, but not in proliferating keratinocytes. Interestingly, several authors describe EGCG as an inducer of apoptosis in HaCaT cells [34]. All these findings make compound **4** a promising molecule to be considered in new strategies seeking to target cancer cells by directly activating checkpoint regulators of the cell cycle without creating non-selective DNA damage. Furthermore, as it lacks the pyrogallol group on the condensed flavanic structure, this compound might be safer than other potent polyphenols of the gallocatechin type.

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