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RESEARCH ARTICLES

# Cytotoxic effects of digalloyl dimer procyanidins in human cancer cell lines

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**Abstract**

Flavanols, a class of polyphenols present in certain plant-based foods, have received increasing attention for their putative anticancer activity. In vitro and in vivo studies, which have compared the effectiveness of various monomer flavanols, indicate that the presence of a galloyl residue on the 3 position on the C-ring enhances the cytotoxicity of these compounds. Procyanidins, oligomerized flavanols, have been reported to be more cytotoxic than monomer flavanols in a variety of human cancer cell lines. Given the above, we evaluated the potential anticancer properties of dimer procyanidins that contain galloyl groups. Specifically, the cytotoxicity of synthetic digalloyl dimer B1 and B2 esters {[3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-catechin-3-*O*-gallate (DGB1) and [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-epicatechin-3-*O*-gallate (DGB2), respectively} were tested in a number of in vitro models. DGB1 produced significant cytotoxicity in a number of human cancer cell lines evaluated by three independent methods: ATP content, MTT and MTS assays. For the three most sensitive cell lines, exposure to DGB1 and DGB2 for 24, 48 or 72 h was associated with a reduction in cell number and an inhibition of cell proliferation. Digalloyl dimers exerted significantly higher cytotoxic effects than the structurally related flavanols, (−)-epicatechin, (+)-catechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, (−)-catechin gallate and dimer B1 and B2. These results support the concept that the incorporation of galloyl groups and the oligomerization of flavanols enhances the cytotoxic effects of typical monomer flavanols. The therapeutic value of these compounds and their derivative forms as anticancer agents merits further investigation in whole animal models.


*Keywords:* Flavanols; Digalloyl procyanidins dimer; Anticancer agents

## 1. Introduction

During the last few decades, chemopreventive and chemotherapeutic compounds against various types of cancer have been isolated from a number of plants [1]. Taxol (Paclitaxel) is an outstanding example of an anticancer agent that was obtained from a plant source [1–3]. Taxol and its derivatives have been shown to be beneficial in the treatment of refractory ovarian, breast, non-small cell lung, as well as several other cancers [4]. Similarly, the design of the synthetic compound called flavopiridol, a new anticancer agent, was based on a natural flavonoid rohitukine [5]. Although with dissimilar results, flavopiridol is being evaluated in a number of Phase I and Phase II clinical trials for efficacy against a number of tumors, including lymphomas and leukemia.

Flavanols and flavanol oligomers (procyanidins) present in fruits, vegetables and certain plant-based foods such as green tea, red wine and cocoa have received increasing attention for their putative anticancer and cardiovascular health benefits [6–9]. This interest is largely due to a number of epidemiological studies that suggest the consumption of

*Abbreviations:* DGB1, [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-catechin-3-*O*-gallate; DGB2, [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-epicatechin-3-*O*-gallate; GA, gallic acid; EGCG, (−)-epigallocatechin gallate; EC, (−)-epicatechin; CAT, (+)-catechin; EG, (−)-epicatechin gallate; CG, (−)-catechin gallate; EGC, (−)-epigallocatechin; DB1, (−)-epicatechin-(4β,8)-(+)-catechin; DB2, (−)-epicatechin-(4β,8)-(−)-epicatechin; NCI, National Cancer Institute; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's Modified Eagle Medium; ATP, adenosine 5′-triphosphate; FBS, fetal bovine serum; BrdU, 5-bromo-2′-deoxyuridine; STA, staurosporine; $IC_{50}$, inhibitory concentration 50.

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diets rich in flavonoids, as well as flavanols, is associated with a reduced risk for a number of chronic diseases, including cancer [10–13].

Studies with purified flavanols and a wide range of food-derived procyanidins, using both in vitro and in vivo models, have shown that these compounds can reduce the growth of certain cancer cells as well as induce cell death [14–17]. Interestingly, procyanidins have been suggested to be more effective as anticancer compounds than the monomeric flavanols [18–21]. Among the procyandins, dimers through pentamers have demonstrated promising toxic effects on cancer cells [7,20–22].

The oligomerization of flavanols is not the only structural modification that can influence their anticancer potential. Structure-function studies show that the presence of a galloyl residue on the 3 position on the C-ring can enhance the cytotoxic response of monomeric flavanols [9,23–25]. These findings were based on the particularly high cytotoxic activity of the monomeric flavanol (−)−epigallocatechin gallate (EGCG) when compared with other nongalloyl

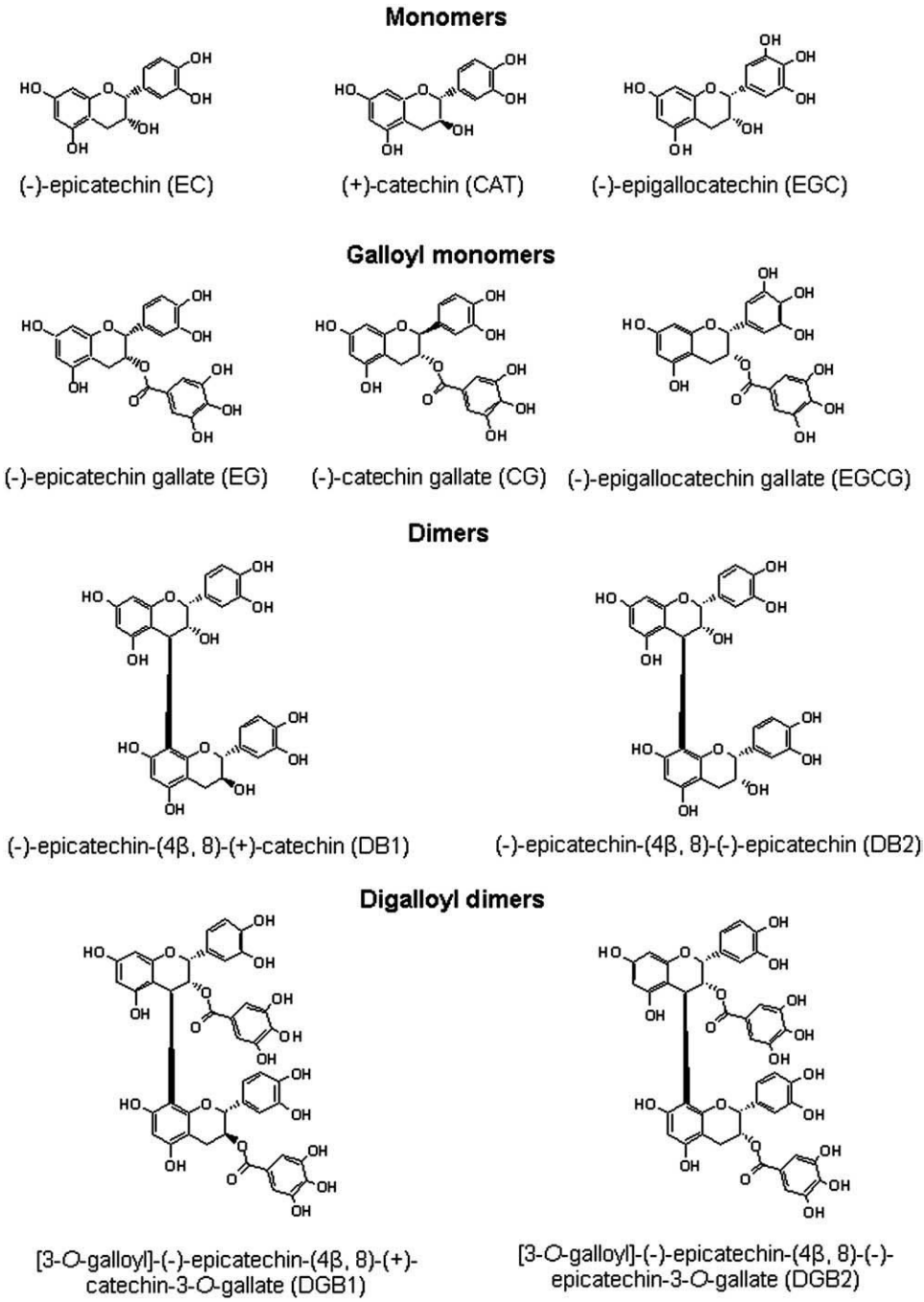


Fig. 1. Chemical structures of EC, CAT, EGC, EG, CG, EGCG, DB1, DB2, DGB1 and DGB2.

flavanols [i.e., (−)-epicatechin (EC), (+)-catechin (CAT) and (−)-epigallocatechin (EGC)] [25–27].

By extension, we hypothesized that galloyl procyanidin dimers are toxic compounds against cancer cells, and they have a higher cytotoxic potential than their structurally related nongalloyl dimers and galloyl monomeric species.

To test whether galloyl procyanidin dimers are toxic compounds against cancer cells, we evaluated the effects of synthetic flavanol digalloyl dimer B1 and B2 esters {[3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-catechin-3-*O*-gallate (DGB1) and [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-epicatechin-3-*O*-gallate (DGB2)} in several human cancer cell lines. Then, and in order to evaluate the effect of the galloyl groups addition and the oligomerization of flavanols on the cytotoxic potential, the results obtained with DGB1 and DGB2 were compared to galloyl monomers, nongalloyl monomers and nongalloyl dimers.

The results presented in the current article provide evidence that digalloyl flavanol dimers produce toxic effects in multiple cancer cell lines and that they are more cytotoxic than nongalloyl or galloyl monomers and nongalloyl dimers.

## 2. Materials and methods

### 2.1. Test compounds

Synthetic [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(+)-catechin-3-*O*-gallate (DGB1), [3-*O*-galloyl]-(−)-epicatechin-(4β,8)-(−)-epicatechin-3-*O*-gallate (DGB2), (−)-epicatechin-(4β,8)-(+)-catechin (DB1) and (−)-epicatechin-(4β,8)-(−)-epicatechin (DB2) were provided by Mars Inc. (Hackettstown, NJ, USA). EC, CAT, (−)-epicatechin gallate (EG), (−)-catechin gallate (CG), EGC, EGCG and gallic acid (GA) (Fig. 1) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Cell culture and treatment conditions

Cell culture and treatment conditions were in accordance with the protocol for compound screening recommended by the National Cancer Institute (NCI) [28,29]. Fourteen cancer cell lines were selected to test DGB1 (Table 1). Seven of these were chosen from the NCI cell lines panel for drug screening, and the remaining were included to incorporate cancer cell lines derived from other tissues (e.g., gastrointestinal tract and central nervous system). Briefly, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) without phenol red, 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37°C, 5% $CO_2$ and 90% humidity. Cells were seeded in 96-well plates (100 μl per well) at densities ranging from 5000 to 20,000 cells per well depending on the doubling time of each cell line (Table 1). After 24 h of incubation, cells were treated with the test compounds. Test compounds were diluted in DMEM with 5% FBS, and different dilutions were added to each well at a final volume of 200 μl. The final concentrations tested were 12.5, 25, 50, 75 and 100 μM.

Given that flavanols and procyanidins in certain cell culture conditions can generate cytotoxic levels of hydrogen peroxide [30], which, in turn, may generate cell death, cytotoxicity was determined in the presence, as well as the absence of 500 U/ml bovine liver catalase (Calbiochem, San Diego, CA, USA).

### 2.3. Cell metabolic activity assays

Cytotoxicity was assessed by measuring cellular metabolic activities using three methods:

#### 2.3.1. Quantification of adenosine 5′-triphosphate

Total adenosine 5′-triphosphate (ATP) content was determined by a luminescent based assay according to the manufacturer's protocol (CellTiter Glo, Promega, Madison, WI, USA). Briefly, following the incubation of cells with the test compounds, 100 μL of media was replaced by 100 μL of luminescent reagent. The luminescence was measured using a Wallac Victor2 Plate Reader (Perkin Elmer Life Sciences, Waltham, MA, USA). Standard curves were prepared using ATP disodium salt hydrate (Sigma-Aldrich). No change in the luminescence of cell culture media with or without flavanols was detected.

#### 2.3.2. Reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was determined according to the manufacture's protocol (CellTiter 96, Promega). Briefly, following the incubation of cells with the test compounds, MTT reagent (10 μL) was added to each well and incubated for an additional 4 h. After that, 100 μL of solubilization/stop solution was added and the plate was incubated at room temperature overnight to completely solubilize the formazan crystals. The reduction of MTT was monitored at 570 nm, and the amount of reduced MTT was calculated as a percentage of the absorbance determined for the vehicle control treatment. Interactions between MTT and natural compounds with intrinsic

Table 1
Name, tissue and number seeded per well of the cell lines used in the study

| Cell line | Tissue | Cells per well |
|---|---|---|
| MDA-MB-231 | Breast | 20,000 |
| MCF-7 | Breast | 10,000 |
| BT-20 | Breast | 20,000 |
| MDA-MB-435 | Breast | 15,000 |
| SK-MEL-5 | Melanoma | 10,000 |
| HT-29 | Colon | 5000 |
| A549 | Non-small cell lung | 7500 |
| U-87 | Central nervous system | 7,500 |
| LNCaP | Prostate | 10,000 |
| DU-145 | Prostate | 10,000 |
| HuTu-80 | Duodenum | 7500 |
| SW-1463 | Rectum | 10,000 |
| SNU-1 | Stomach | 10,000 |
| U2O2 | Bone | 10,000 |

reductive potential (ascorbic acid, vitamin E and *N*-acetylcysteine) have been described by Bruggisser et al. [31]. We determined that the media containing DGB1 and DGB2 gave false readings due to their interference with the MTT reagent (data not shown). To prevent this artifact, after 48 h of incubation with all the test compounds, the treatment media was replaced by 100 μL of DMEM with 5% FBS.

#### *2.3.3. Reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt dye*

Cell metabolic activity was also determined by monitoring the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium inner salt (MTS) using a commercial assay (CellTiter 96 AQueous, Promega). Briefly, MTS reagent (15 μL) was added to each well and incubated for 2 h at room temperature. The reduction of MTS was monitored by recording the change in absorbance at 490 nm. The amount of reduced MTS was calculated as a percentage of the absorbance determined for the vehicle control treatment. As observed in the MTT assays, there was an interaction between MTS and the digalloyl dimers. Therefore, following 48 h of incubation after addition of the test compounds, the treatment media was replaced by 100 μL of DMEM with 5% FBS.

### *2.4. Cell number estimation*

Cell number was estimated by quantification of cellular DNA content using a fluorescence-based assay (Cyquant NF, Molecular Probes, Eugene, OR, USA). Total DNA content was quantified using standard curves made with bacterio-phage λ DNA. Then, cell number was estimated by comparing the results of total DNA content with calibration curves made with different dilutions of the cell lines tested. Cellular DNA content was determined according to the manufacturer's protocol. Briefly, following the incubation of cells with the test compounds, cell culture media was replaced by 100 μL of dye solution and incubated at 37°C for 30 min. The amount of DNA was determined by measuring the fluorescent intensity (excitation/emission 485/530 nm) in a plate reader. Staurosporine (STA) (Sigma-Aldrich) at 1 μM was used as a positive control.

### *2.5. Proliferation assay*

The measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesized cellular DNA was used to determine cell proliferation. BrdU content was determined according to the manufacture's protocol (Roche Applied Science, Indianapolis, IN, USA). Briefly, following the incubation of cells with test compounds, 20 μL of BrdU was added to the cell culture media. After 2 h of incubation with BrdU, cells were fixed and incubated with anti-BrdU conjugated with peroxidase. Subsequent to substrate addition, chemiluminescence was measured using a plate reader. STA at 1 μM was used as a positive control.

### *2.6. Cytotoxicity assays with other structurally related flavanols*

With the objective to compare the cytotoxic effects of the digalloyl dimer esters with other flavanols, total cellular ATP content after the addition of 50 μM EC, CAT, EG, CG, EGCG, DB1 or DB2 for 48 h in the cell culture media was determined in HuTu-80, A549 and HT-29 cell lines. In another experiment, and with the aim to evaluate whether flavanol galloyl dimer esters could produce higher cytotoxic effects than their individual nonoligomerized monomer components, total cellular ATP content after the addition of 25 μM DGB1 for 48 h in the cell culture media was compared to (A) 50 μM GA plus 25 μM EC plus 25 μM CAT and (B) 25 μM EG plus 25 μM CG. Finally, the ATP content in cells incubated with 25 μM DGB2 in the cell culture media was compared to (C) 50 μM GA plus 50 μM EC and (D) 50 μM EG. All experiments were carried out in the presence of 500 U/ml catalase in the media.

### *2.7. Data analysis*

Cytotoxicity results are expressed as a percentage of control (values obtained without the addition of test compounds). Statistical evaluation of the data was performed by one-factor analysis of variance followed by Tukey test for multiple comparisons. *P* values lower than .05 were regarded as statistically significant. All analyses were performed with StatView 5.0.1 software (SAS Institute, Mountain View, CA, USA). Data presented were obtained from at least 3 independent experiments ($n$=3) and are expressed as the mean ± S.E.M. Inhibitory concentrations 50 ($IC_{50}$) were calculated by adjusting curves to the equation for a sigmoid curve with 4 parameters using routines available in Sigma Plot 9.0 (Systat Software, San Jose, CA, USA).

## 3. Results

### *3.1. DGB1 and DGB2 produce cytotoxic effects in multiple cancer cell lines*

Evaluation of total cellular ATP content demonstrated that the incubation of the cancer cell lines with 50 μM DGB1 resulted in clear evidence of toxicity (Fig. 2). Furthermore, when the cytotoxicity of DGB1 was evaluated by MTT and MTS reduction, 10 and 13 cancer cell lines, respectively, showed significantly lower metabolic activity compared to untreated control cells.

The addition of procyanidins and galloyl flavanols to cell culture media has been shown to result in the production of hydrogen peroxide, which may itself lead to cytotoxicity. In order to eliminate these potentially artifactual cytotoxic effects, 500 U/ml of catalase were added to the media. The addition of catalase did not significantly influence the ATP levels in untreated control

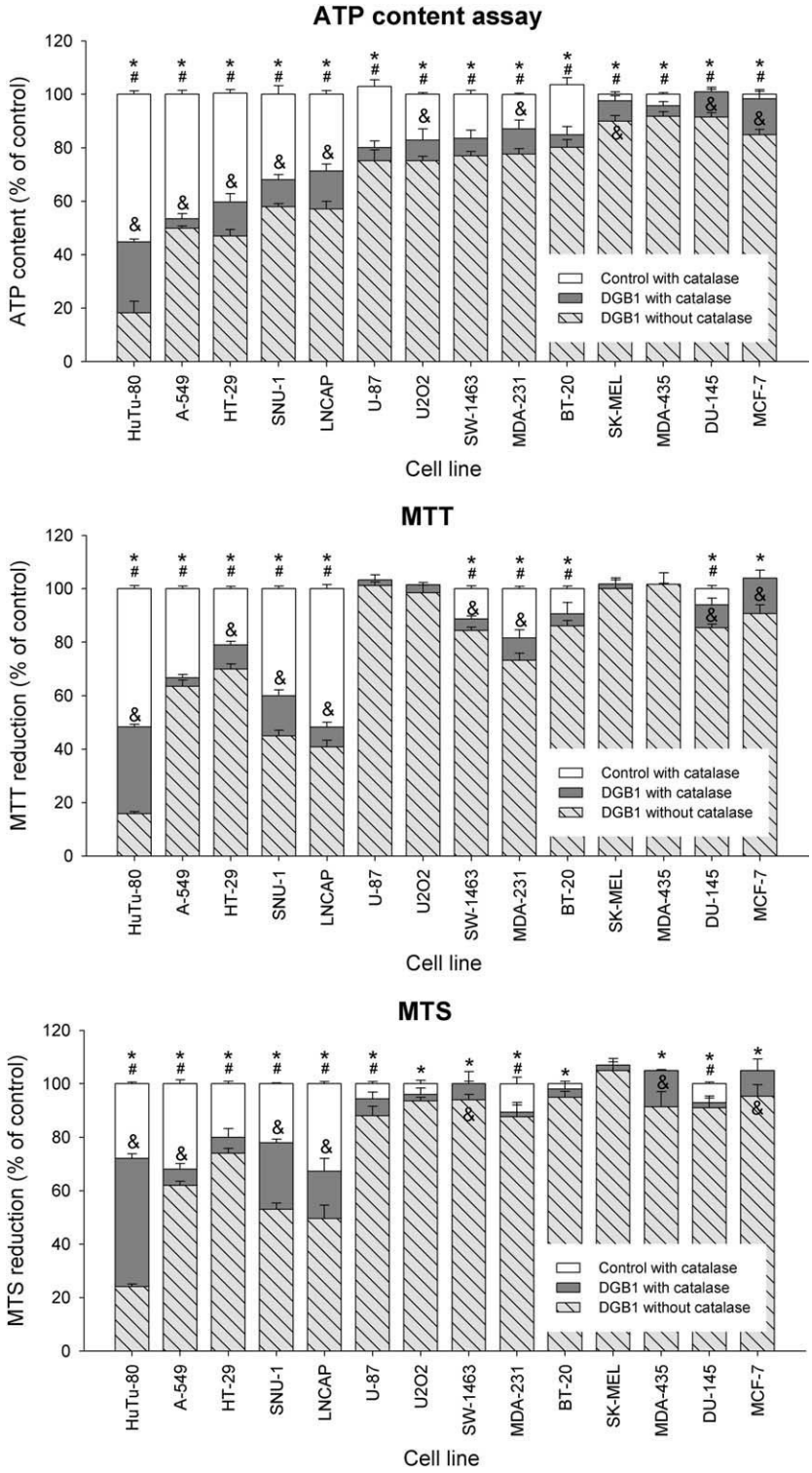


Fig. 2. Cytotoxicity following incubation for 48 h in the media with 50 μM DGB1. Cytotoxic effects were measured by ATP content, MTS and MTT assay in the absence or presence of 500 U/ml of catalase in the cell culture media. Values are mean ± S.E.M. of at least three independent experiments. ∗Statistically significant cytotoxic effect of 50 μM DGB1 in the media without catalase compared to control. [#]Statistically significant cytotoxic effect of 50 μM DGB1 in the media with catalase compared to control. [&]Statistically significant cytotoxic effect of 50 μM DGB1 in the media with catalase compared to 50 μM DGB1 in the media without catalase.

cells; however, the cytotoxicity of DGB1 was attenuated in 10 cell lines.

To exclude the possibility that the toxic effects observed in cells treated with 500 U/ml of catalase were due to remaining hydrogen peroxide in the cell culture media, the catalase concentration was increased to 1000 U/ml. The cytotoxicity determined in the sensitive cell lines exposed to catalase at this higher concentration was not significantly different from the results obtained with 500 U/ml of catalase (data not shown).

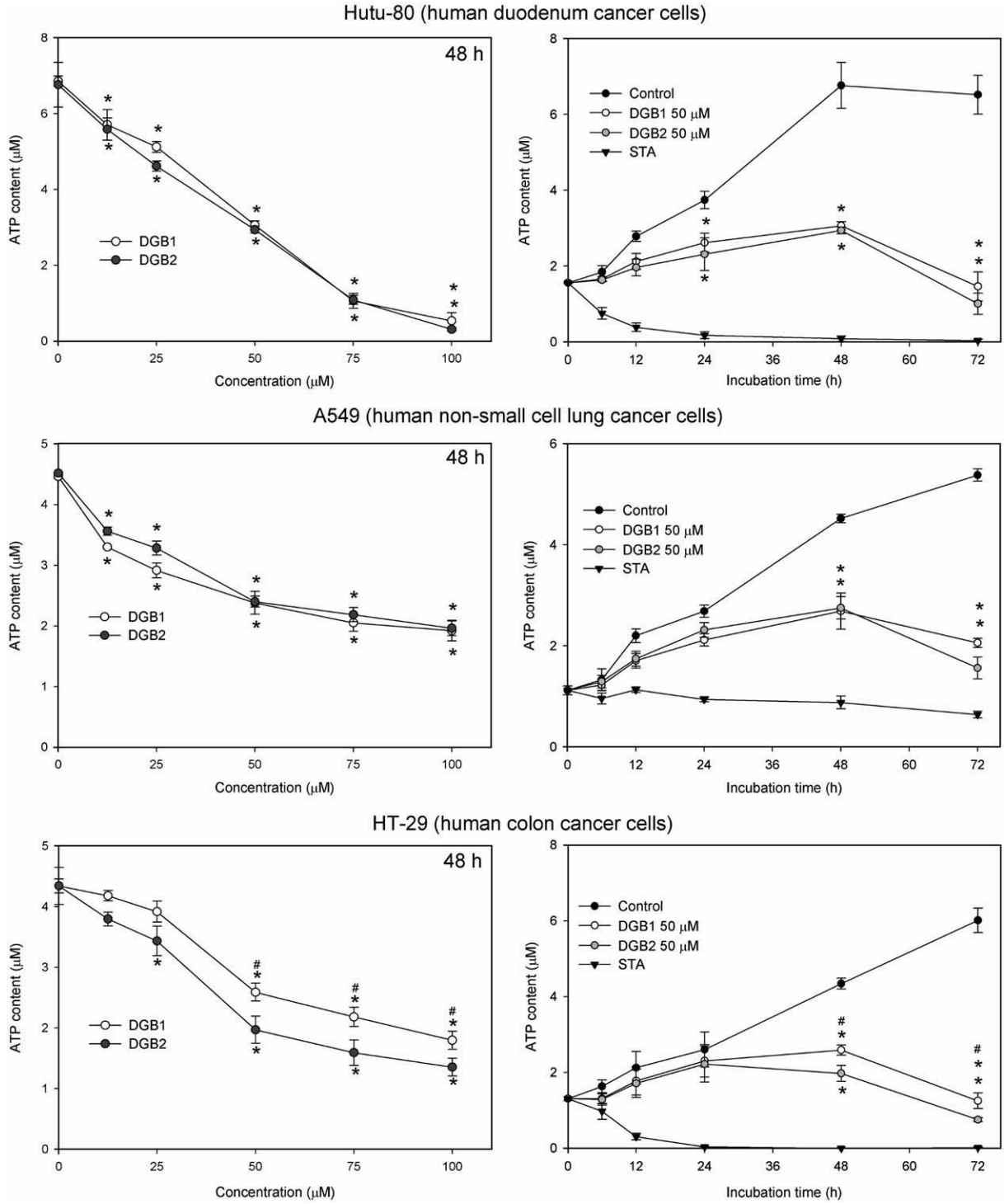


Fig. 3. ATP content at different concentrations and incubation times of DGB1 and DGB2 in HuTu-80, A549 and HT-29 cells. ATP content following incubation for 48 h in the media with 12.5, 25, 50, 75 and 100 μM DGB1 or DGB2 (left panel) and ATP content after 6, 12, 24, 48 and 72 h of incubation in the media with 50 μM of DGB1 or DGB2 (right panel) in HuTu-80, A549 and HT-29 cell lines. Values are mean ± S.E.M. of three independent experiments. * Statistically significant cytotoxic effect compared to control treatment. # Statistically significant cytotoxic effect compared to DGB2 treatment.

The percentage of attenuation in cytotoxicity ranged from 3% in the A-549 cell line to 32% in the HuTu-80 cell line. In SK-MEL, MDA-435, DU-145 and MCF-7 cell lines, the cytotoxicity observed with 50 μM of DGB1 was effectively eliminated by the addition of catalase in the media. Similar attenuation of the cytotoxicity was observed when MTT or MTS reduction were evaluated in the presence of catalase.

Despite the attenuation of the cytotoxicity by catalase, the presence of 50 μM DGB1 in the cell culture media

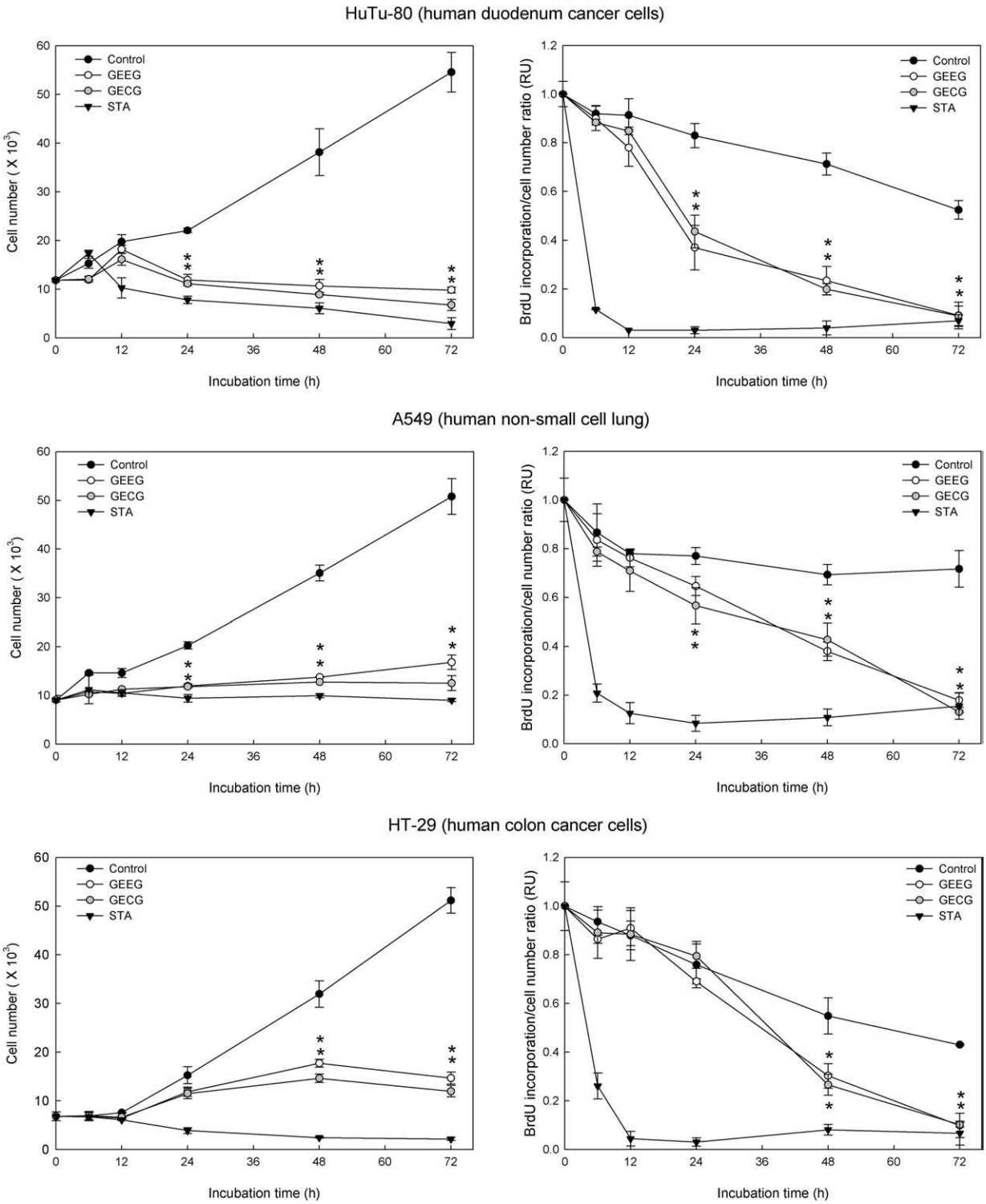


Fig. 4. Cell number and BrdU incorporation by cell number following incubation from 0 to 72 h in the media with 50 μM DGB1 or DGB2 in HuTu-80, A549 and HT-29 cell lines. Values are mean ± S.E.M. of at least three independent experiments.

showed toxic effects in 10, 9 and 8 cell lines when total cellular ATP content, MTT or MTS assays were used, respectively.

Having demonstrated the cytotoxicity of DGB1, additional dose- and time-dependent experiments in the three most sensitive cell lines were carried out to compare the cytotoxic potential of DGB1 to the structurally related DGB2. Hutu-80 and A549 cells exposed to 12.5 μM DGB1 and DGB2 in the presence of catalase exhibited a statistically significant reduction in total cellular ATP content at 48 h compared to values in untreated control cells (Fig. 3, left panels). Interestingly, a significantly higher toxic effect was measured when incubating HT-29 cells with 50, 75 and 100 μM of DGB2 compared to DGB1. Moreover, a significantly lower $IC_{50}$ was determined for DGB2 than for DGB1 in this cell line (53±4 and 67±4, respectively, $P<.001$).

For the time-dependent experiments, we selected the lowest concentration of digalloyl dimers that significantly reduced the ATP content at 48 h in the three cell lines tested (50 μM). When DGB1 and DGB2 were added to the cell culture media of the HuTu-80 cells, the ATP content was significantly lower than that in control cells after 24 h (62% and 70% relative to control cells, respectively) (Fig. 3, right panels). The addition of 50 μM DGB1 and DGB2 to A549 and HT-29 cells produced a significant reduction in the total cellular ATP content only after 48 h of incubation. STA, a well known pro-apoptotic compound, was used as a positive control.

### *3.2. DGB1 and DGB2 reduce total cell number and inhibit cell proliferation in cancer cell lines*

The lower ATP content observed in cells treated with DGB1 or DGB2 could be caused by a decrease in the rate of ATP production or by a decrease in cell number. In order to determine the cause of the lower ATP content, total cell number was evaluated after the addition of 50 μM of DGB1 or DGB2 in the cell culture media containing 500 U/ml catalase. A significantly lower cell number compared to control untreated cells was determined in HuTu-80 and A549 cell lines at 24 h. In HT-29 cells, cell number was also affected by incubation with DGB1 and DGB2; however, significant reductions emerged later in this cell line, occurring after 48 h (Fig. 4, left panel).

The observed decrease in total cell number could be the result of an inhibition of cell proliferation and/or by an increase in cell death. To examine these mechanisms, cell proliferation was determined as the ratio of BrdU incorporated to cell number. In HuTu-80 and A549, cell proliferation rates were reduced after the addition of either 50 μM of DGB1 or DGB2, compared to vehicle control treated cells after 24 h (Fig. 4, right panel). Similar results were obtained for the HT-29 cell line, although the effects were not statistically different until the 48 h time point.

### *3.3. DGB1 and DGB2 are more cytotoxic than other tested flavanols*

The cytotoxic effects of the digalloyl dimer esters were compared with other structurally related flavanols. The cytotoxicity of DGB1 and DGB2, in nearly all cases, was significantly higher compared to monomers and galloyl monomers; the only exception to this being CG treatment in HuTu-80 cells (Fig. 5, left panel). In addition, the ATP level determined in the presence of galloyl dimers was approximately 40% lower when compared to non-galloyl B1 and B2 dimers. Thus, the incorporation of galloyl groups into dimer B1 and B2 markedly enhanced the cytotoxic effects of these compounds.

To determine whether or not flavanol galloyl dimer esters could produce greater cytotoxic effects than their individual nonoligomerized monomer components, the ATP content was measured in cells incubated with different mixtures of the individual components of DGB1 and DGB2. Different outcomes were observed for the cell lines tested. In HuTu-80 cells, the incubation of cells with the digalloyl dimers, as well as the incubation with its structurally related galloyl monomeric components (EG and CG) resulted in near equal reductions in cellular ATP content. In marked contrast, the addition of GA in combination with the nongalloyl flavanol monomers did not reduce significantly cellular ATP content. Interestingly, these findings were not observed in either the A549 or HT-29 cell lines. In A549 cell lines, neither incubation with the galloyl monomers nor the combination of GA with nongalloyl monomers resulted in increased reduction in ATP content, relative to what was observed with DGB1 or DGB2. In HT-29 cells, the incubation of DGB2 significantly reduced the cellular ATP content compared to galloyl monomers or the combination of GA with nongalloyl monomers. However, the incubation of DGB1 did not result in any difference in the cellular ATP content with respect to the individual nonoligomerized components. Hence, while the oligomerization of galloyl flavanol monomers can enhance the toxic effects of these compounds, these effects are cell line-specific.

## 4. Discussion

The data obtained in this investigation demonstrate that (i) synthetic digalloyl dimer esters produced significant cytotoxicity in most of the cancer cell lines tested; (ii) at least part of the toxic effects observed were due to the inhibition of cell proliferation by these compounds; (iii) digalloyl dimer esters were more cytotoxic than the other structurally related flavanols evaluated; and (iv) the incorporation of galloyl groups or, for some of the cell lines tested, the oligomerization of monomer flavanols enhanced the cytotoxic effects of these compounds.

Besides other limitations, the generation of artifactual events is a common problem in in vitro models. It is now well established that the incubation of flavonoids in cell culture media can generate cytotoxic levels of hydrogen

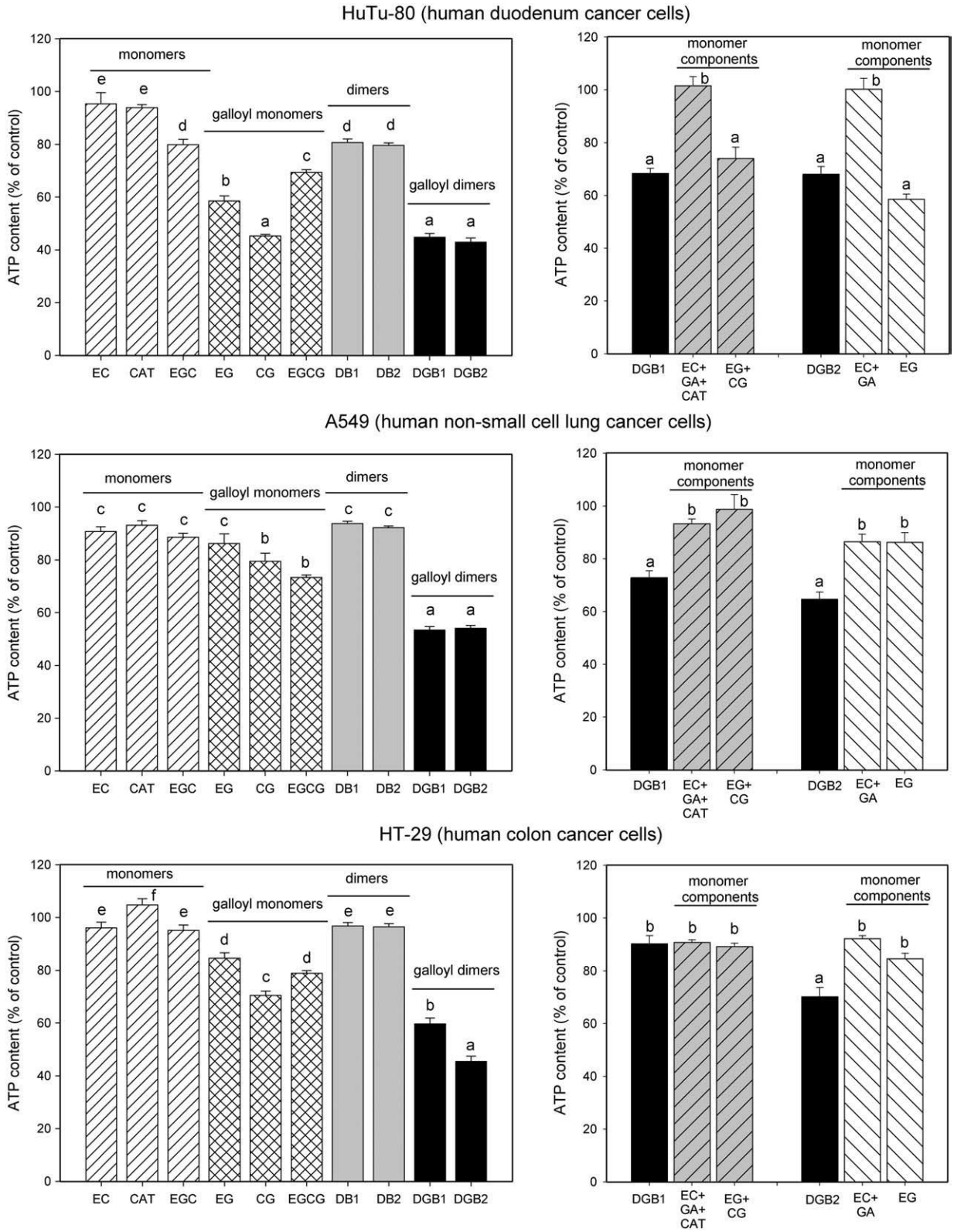


Fig. 5. Cytotoxicity following incubation for 48 h in the media with 50 μM of DGB1, DGB2, CAT, EC, EGC, EG, CG, EGCG, DB1 and DB2 in HuTu-80, A549 and HT-29 cells (left panel). Cytotoxicity following incubation for 48 h in the media with 25 μM DGB1, 25 μM DGB2, 50 μM EG, a mix of polyphenols containing 50 μM GA plus 25 μM EC plus 25 μM CAT or 25 μM EG plus 25 μM CG, or 50 μM GA plus 50 μM EC. Values are mean ± S.E.M. of at least three independent experiments. Different letters represent statistically significant cytotoxic effect compared to the other letters.

peroxide [30,32]. In our experiments, the enzymatic decomposition of the hydrogen peroxide by catalase significantly attenuated the cytotoxicity of DGB1 in most of the cell lines tested. While the effects of diverse flavonoids on a number of cancer cell lines has been extensively investigated during the last few years, the addition of catalase to prevent the events triggered by hydrogen peroxide, such as inhibition of cell proliferation [33–35] and the promotion of apoptosis [36,37], has been often overlooked. Therefore, the results presented in the current paper, as well as those of others [30,38], strongly support the concept that the cytotoxic effects that have been reported for a number of flavonoids in cell culture models are in part artifactual. For that reason, the addition of catalase in cell culture media containing flavonoids is essential to prevent erroneous conclusions. However, in our experiments, the cytotoxic effects of the digalloyl dimer esters were still observed even in the presence of catalase, indicating that these compounds can exert cytotoxic effects independent of their ability to generate hydrogen peroxide.

Differential toxic effects were observed in the variety of cancer cells studied. DGB1 showed no, or low, cytotoxicity in some cell lines, while marked effects were noted in others. Interestingly, three of the five most sensitive lines to DGB1 were cells derived from gastrointestinal tract cancers (duodenum, colon and stomach). On the other hand, significantly different cytotoxic effects were observed in the two prostate cell lines tested (LNCaP and DU-145). While DGB1 produced cytotoxicity in the LNCaP cell line, it had no effect on the DU-145 cell line. The most important distinction between these two cell lines is the expression of androgen receptor that regulates cell growth in LNCaP, while in DU-145 cells, growth is androgen independent. A differential effect of flavonoids and procyanidins on androgen-dependent, and -independent, prostate cancer cell lines has been described by others [39–41].

A variety of purified galloyl monomer flavanols [27,42] and plant-derived procyanidin fractions [15–17,43] have been suggested as anticancer agents due to their ability to inhibit proliferation and to produce cell death by apoptosis or necrosis. In our experimental conditions, an inhibition of cell proliferation was observed when digalloyl dimer esters were added to the cell culture media. Therefore, a decrease in cell proliferation can explain in part the reduction in the cancer cell number observed. However, the increase in cell death initiated by a proapoptotic action of digalloyl dimer esters might be another possible mechanism for their cytotoxicity.

A significantly higher toxicity was observed when the digalloyl dimers were compared to nongalloyl dimers and when galloyl monomers were compared to nongalloyl monomers. This observation demonstrated that the incorporation of galloyl groups into monomer and dimer flavanols can markedly alter the cytotoxic potential of these molecules, indicating that this is an important structural improvement with regard to the toxic response. Interestingly, despite the similar chemical structures between DGB1 and DGB2 (stereoisomers), these compounds showed markedly different cytotoxic effects in the HT-29 cell line. The same dissimilarity was observed when other stereoisomers, CG with EG and CAT with EC, were compared in this cell line. The differential effects produced by these stereoisomers indicate that small changes in the chemical structure, even the spatial orientation of certain chemical groups, influence their cytotoxicity. Distinct effects produced by flavanol stereoisomers have been reported in other biological systems [44–46].

That procyanidins can be more cytotoxic than the monomer flavanols has been previously reported [18–21,47]. However, it remains uncertain to what extent the oligomerization of flavanols can change the cytotoxic effect from that observed with their monomer components. In the current study, the comparison between the galloyl dimer esters, with a mix of their individual components, suggests that the oligomerization of nongalloyl monomers enhanced the toxicity of these compounds, but the oligomerization of galloyl monomers did not always enhance this effect.

In order to understand the significance of these results, it is especially important to identify the limits of our experiments. First, the entire work presented here was done in cancer cells cultured in vitro. Therefore, the inferences from them are very restricted. Second, we verified that digalloyl dimer esters are not totally stable in the cell culture conditions evaluated. In a control experiment, 50 μM DGB1 decreased in total amount to 3 μM at 48 h. Although the concentration of these compounds is decreasing in the cell culture media because of their degradation or metabolism, this situation does not always represent the stability of the compounds in vivo. Hence, further investigation regarding the stability of the digalloyl dimers should be done to elucidate their comportment in other experimental conditions.

In summary, the present results support the concept that digalloyl dimer B1 and B2 esters are cytotoxic compounds in a number of cancer cell lines. These toxic effects occurred in part through an inhibition of cell proliferation. Moreover, these results indicate that the addition of hydroxyl groups from a galloyl structure and the coupling of monomers can have profound effects on the cytotoxicity of flavanols. The value of these compounds and their derivative forms as anticancer agents merits further investigation.

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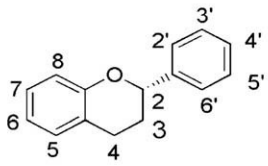
## Appendix A. Supplemental information

*Procyanidins*

The flavanols are polyphenolic compounds which fall under the major classification of proanthocyanidins which are defined as the condensed family of polyphenols. Proanthocyanidins comprise 11 subsets of compounds which are based on the hydroxylation pattern about the aglycone as shown below. The procyanidin and prodelphinidin subsets are the most common occurring proanthocyanidins which enter into consumable foods and beverages. The include items such as a variety of cereal grains, nuts, tea, cocoa, various fruits, wines, and spices. The difference between these two is an extra hydroxyl group for the prodelphinidin types. The flavanol oligomers studied in this paper (DB1, DB2, DGB1, and DGB2) are procyanidin-type proanthocyanidins.

**Representative Proanthocyanidins**




| Proanthocyanidins | | Substitution Pattern | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Class | Monomer | 3 | 5 | 7 | 8 | 3' | 4' | 5' |
| Proapigeninidin | Apigeniflavan | H | OH | OH | H | H | OH | H |
| Proluteolinidin | Luteoliflavan | H | OH | OH | H | OH | OH | H |
| Protricetinidin | Tricetiflavan | H | OH | OH | H | OH | OH | OH |
| Propelargonidin | Afzelechin | OH | OH | OH | H | H | OH | H |
| Procyanidin | Catechin | OH | OH | OH | H | OH | OH | H |
| Prodelphinidin | Gallocatechin | OH | OH | OH | H | OH | OH | OH |
| Proguibourtinidin | Guibourtinidol | OH | H | OH | H | H | OH | H |
| Profisetinidin | Fisetinidol | OH | H | OH | H | OH | OH | H |
| Prorobinetinidin | Robinetinidol | OH | H | OH | H | OH | OH | OH |
| Proteracacinidin | Oritin | OH | H | OH | OH | H | OH | H |
| Promelacacinidin | Prosopin | OH | H | OH | OH | OH | OH | H |

## Supplemental data

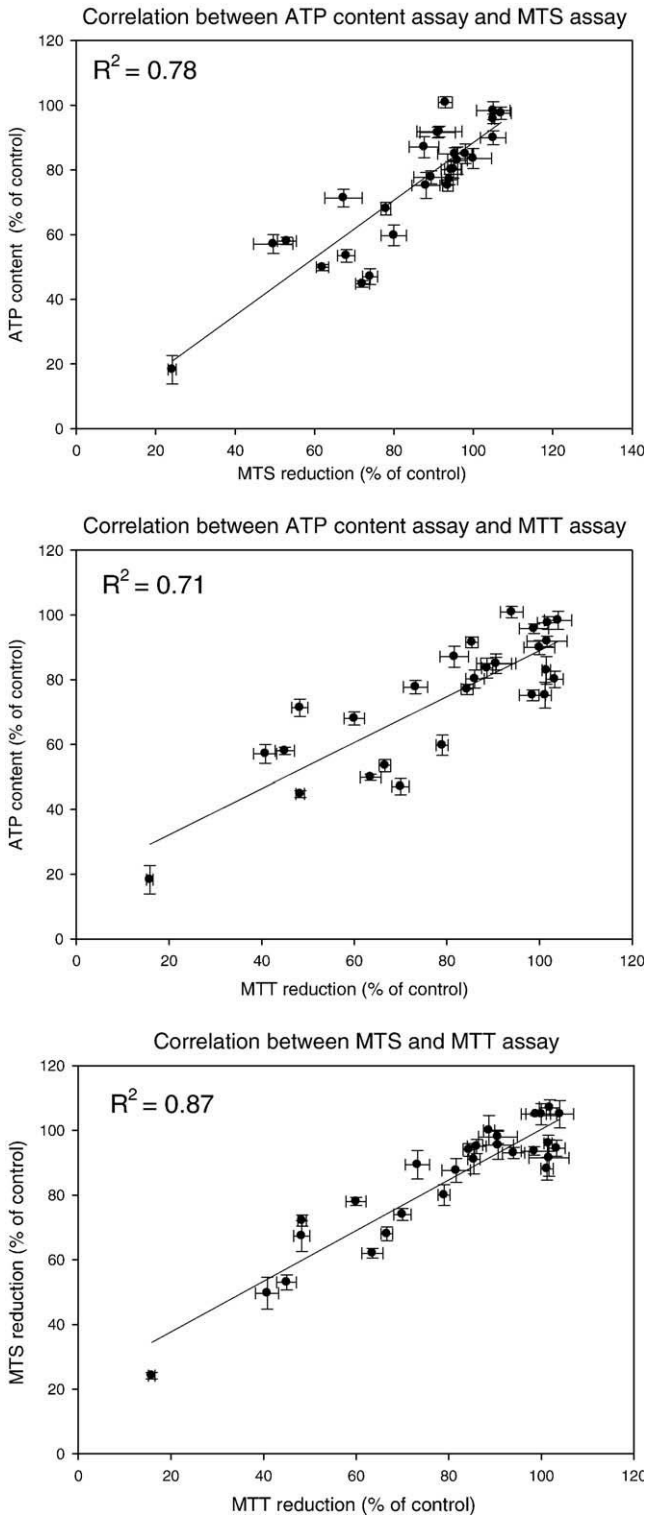



Fig. A. Correlation between the results obtained using the three methods to evaluate cytotoxicity. ATP content assay vs MTS assay (A). ATP content assay vs MTT assay (B), and MTS assay vs MTT assay (C).

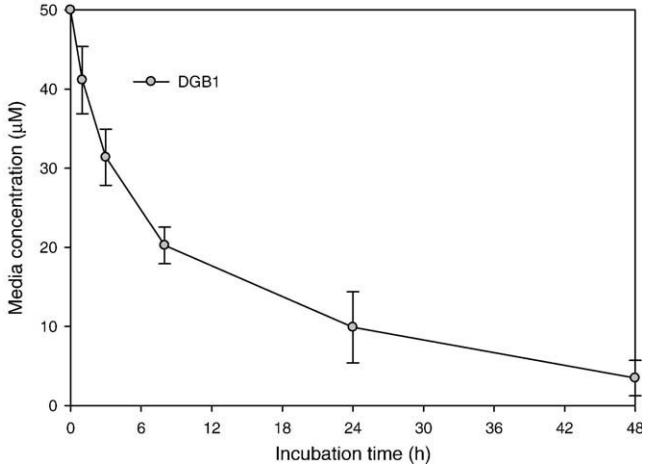



Fig. B. Concentration of DGB1 in cell culture media after incubation with 50 UM DGB1 during 48 h in the experimental conditions.

Table 2

| | $IC_{50}$ | |
|---|---|---|
| Cell line | DGB1 (μM) | DGB2 (μM) |
| HuTu-80 | 44.7 ± 1.7 | 37.6 ± 3.2 |
| A549 | 56.6 ± 3.1 | 61.8 ± 1.0 |
| HT-29 | 67.4 ± 4.3 | 53.1 ± 4.1* |

Inhibitory concentration 50 ($IC_{50}$) for DGB1 and DGB2 in the three most sensitive cell lines. *$P<.05$.