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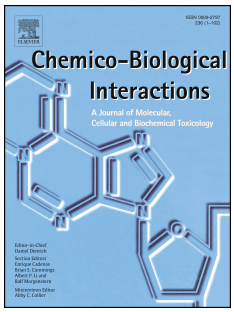

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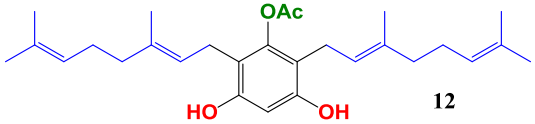

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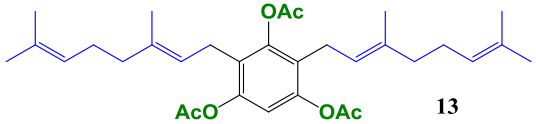

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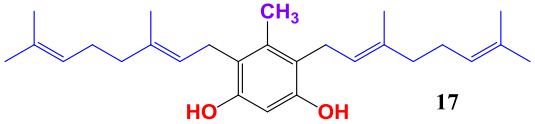

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Antiproliferative effect and Apoptotic activity of linear geranylphenol derivatives from phloroglucinol and orcinol.

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Abstract: Sixteen synthetic linear derivatives geranylphenols, were obtained from phloroglucinol and orcinol, and cytotoxic activity was evaluated *in vitro* against cancer cell lines (HT-29, PC-3, MDA-MB231, DU-145) and one non-tumor cell line, human dermal fibroblast (HDF). IC_{50} values were determined at concentrations of 0–100 µM of each compound for 72 h. Compounds **12**, **13**, **17**, **21**, **22** and **25**, showed cytotoxic activity. To elucidate whether these compounds reduce cell viability by inducing apoptosis, cell lines MCF-7, PC-3 and DHF were treated with each active compound 12, 13, 17, 21, 22 and 25 and were examined after Hoechst 33342 staining. The compounds **12, 13** and **17** induced apoptosis in various cancer cell lines, as shown by nuclear condensation and/or fragmentation. In addition, it was found that compounds **12** and **13**, induced changes in mitochondrial membrane permeability in those cancer cell lines. Such induction was associated with the depletion of mitochondrial membrane potential. These activities led to the cleavage of caspases inducing the cell death process.

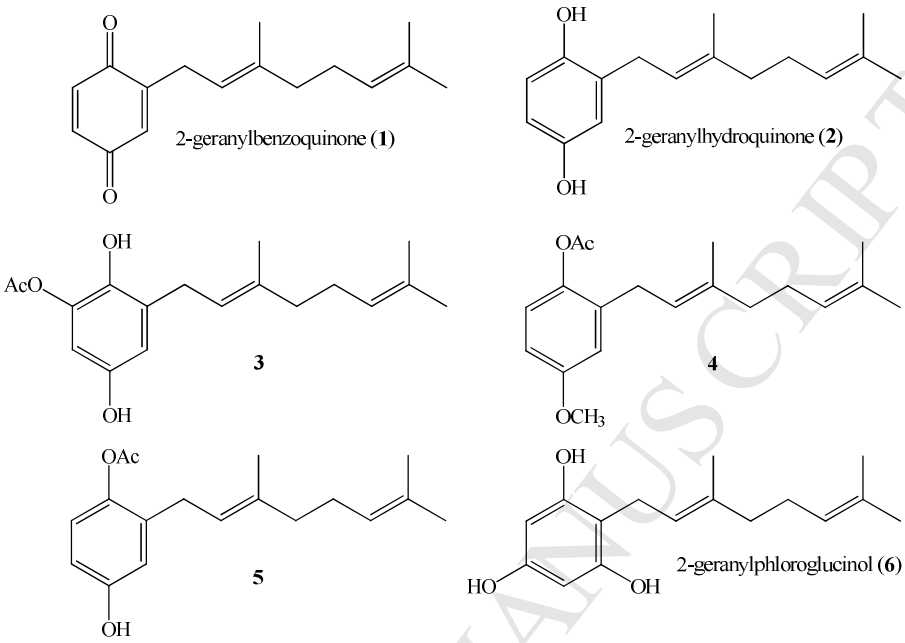
Keywords: cytotoxic activity; cancer cell lines; apoptosis; mitochondrial membrane permeability; caspase-3 activity; linear geranylphenols.

1. Introduction

Prenylated and polyprenylated 1,4-benzoquinones and hydroquinones such as ubiquinones, plastoquinones, and tocopherols are widespread in plants and animals, in which they play important roles in electron transport, photosynthesis, and as antioxidants [1,2]. Prenyl benzoquinones have been also isolated from brown algae of the order Fucales [3-6], sponges [7-10], alcyonaceans [11], gorgonaceans [12], and ascidians belonging to the genus *Aplidium* [13-18]. These substances present a terpenoid portion ranging from one to nine isoprene units.

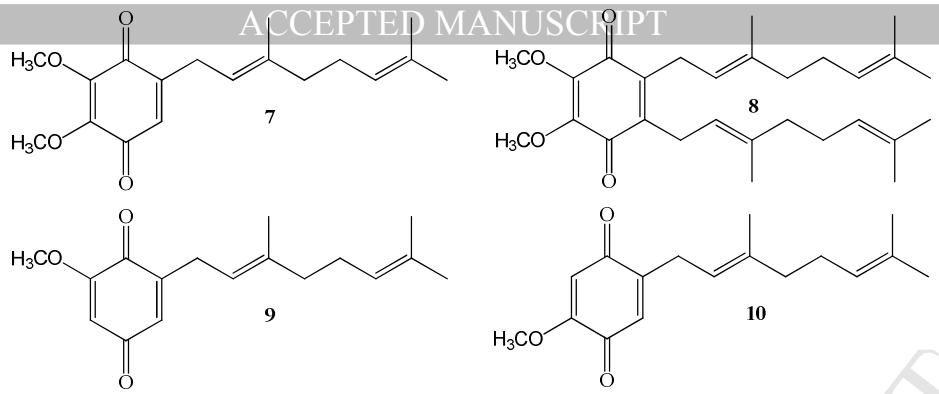
It has been extensively documented that multiple metabolites, obtained from species belonging to the plant kingdom, have special biological properties suitable for controlling several types of animal and plant pathogen. For instance, linear geranylquinones or geranylhydroquinones, present in higher plants and in marine urochordates [19], exhibit cytotoxic activity and inhibit larval growth and development. Some particular compounds such as 2-geranylbenzoquinone (**1**), isolated from *Ascindian Synoicum castellatum* [20], 2-geranylhydroquinone (**2**) isolated from the *Cordia alliodora* tree [21], *Phacelia crenulata* [22-24], *Aplidium antillense* [25] and the tunicate *Amaroucium multiplicatum* [26], have been related to biological activities including toxicity, cytotoxicity, antimicrobial, anti-cancer protective and antioxidant effects, among others [23,26-30]. Additionally, linear geranylmethoxyphenol/acetates (compounds **3**-**5**, see Figure 1), isolated from *Phaceliaixodes* [25], are cytotoxic, allergenic and insecticidal, and topical application of 100 µg of geranylbenzoquinone on pupae of *Tenebrio* caused severe abnormalities and death [31]. However, considering the activity against phytophagous insects and pathogens reported for compounds **3**-**5** (figure 1) [31] and assuming the presence of this property in other geranylphenol analogs, we have recently reported the synthesis, structure determination and their effect on mycelial growth of plant pathogen *Botrytis cinerea* of eight linear geranylphenols molecules, wherein the compounds **2** and 2-geranylphloroglucinol (**6**) were the more active ones, showing an inhibitory effect on the mycelial growth that depends on the applied concentration [32].

Figure 1. Structure of some active natural and synthetic linear geranylphenols.



On the other hand, different studies of the structure activity relation (SAR) in a series of non methoxylated and methoxylated prenylated quinones with side chains containing from one to eight isoprene units reported that the optimum length of the side-chain is two isoprene units and in the para-position relative to the methoxy-group [28,33]. Additionally, these authors informed that all tested quinones (compounds **7-10**, figure 2) have inhibited JB6 Cl41 cell transformation and p53 activity inducing apoptosis and also the activities of AP-1 and NF-κB. Additionally, in previous studies we have reported the synthesis and cytotoxic activity of 2-geranylbenzoquinone (**1**) and 2-geranylhydroquinone (**2**) and some geranylmethoxyphenol/acetate analogs (figure 1) [34-35].

Figure 2. Structure of some active synthetic linear geranylquinones.



Therefore, in this research we report the cytotoxic activity of a series of linear geranylphenol, acetylated and methoxylated derivatives of phloroglucinol (compounds **6**, **11-14**, see Figure 3) and orcinol (compounds **15-25**, see figure 4). These compounds were obtained by traditional methodology [33-37] and by a modification of a previously reported synthetic method [32]. Compounds **6** and **11-25** were evaluated *in vitro* against various human cancer cells lines in order to analyze the cytotoxic activity.

Figure 3. Structure of synthetic linear derivatives geranylphenols acetylated and methoxilated of phloroglucinol.

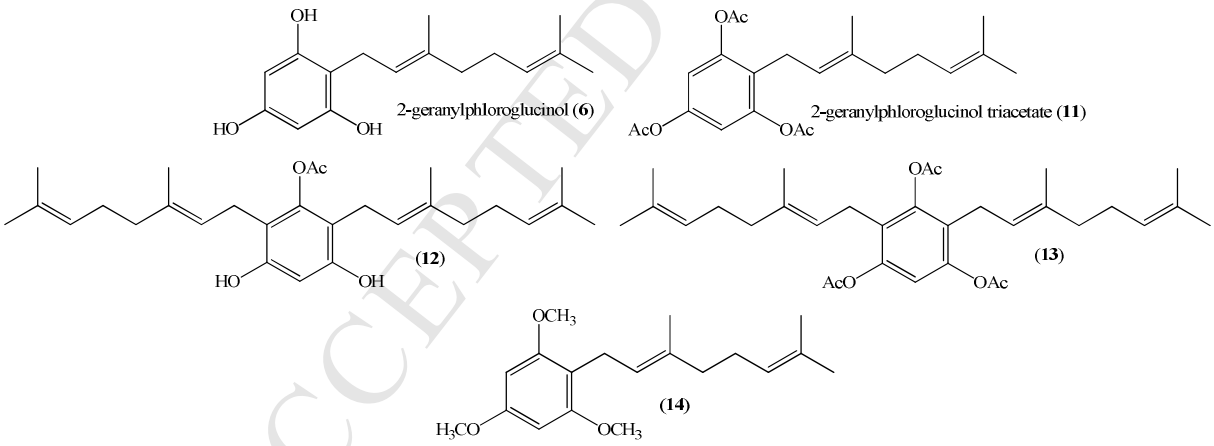
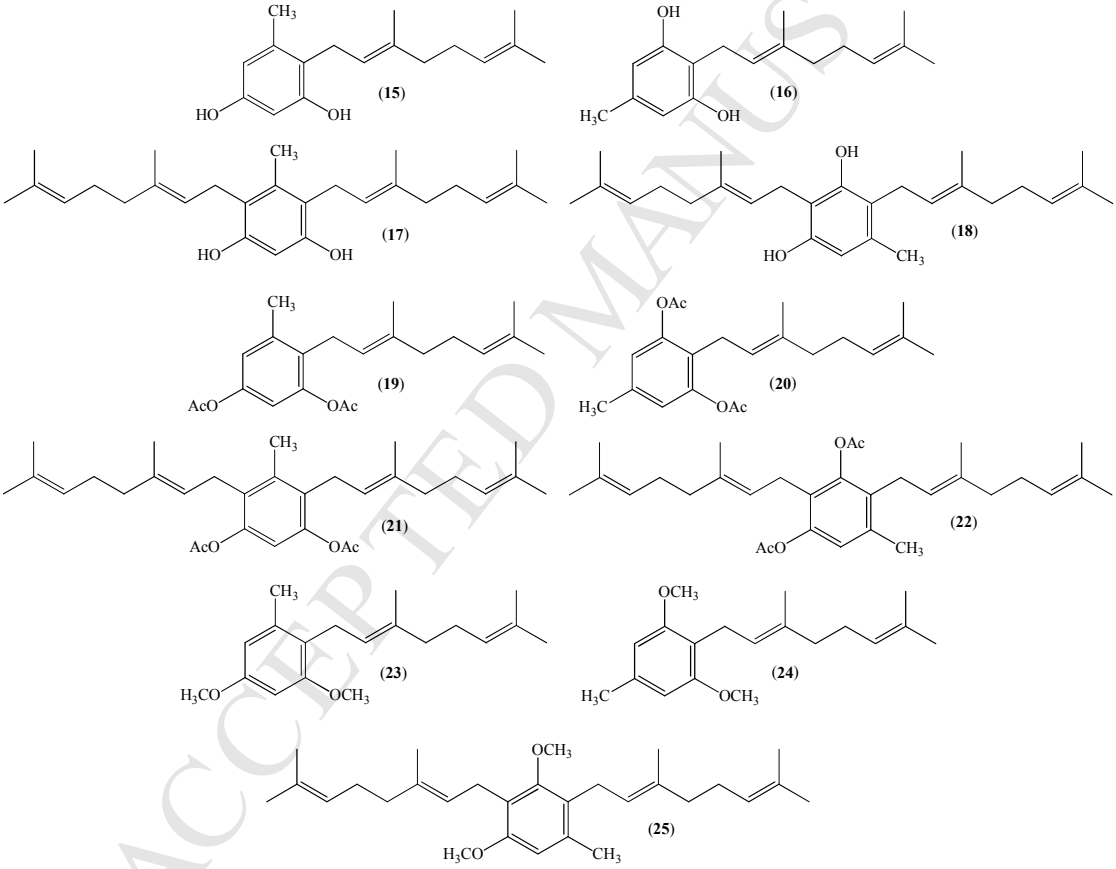


Figure 4. Structure of synthetic linear derivatives geranylphenols acetylated and methoxilated of orcinol.



2. Results and Discussion

2.1. Chemical

2-geranylphloroglucinol (**6**) and derivatives **11-13** were obtained by a previously reported synthetic method [32]. While the trimethoxylated derivative **14** was obtained by methylation reaction of **6**,

(86.8% yield), in dimethyl sulfate/K_2CO_3 system under reflux conditions in acetone. The structure of **14** was mainly established by NMR, where the signals at δ_H =3.92 ppm (s, 3H, OCH_3) and δ_H =3.79 ppm (s, 6H, 2 x OCH_3) confirmed the presence of three methoxyl groups. Additionally, in the ^{13}C NMR spectrum, the signal at δ_C = 55.3 ppm was assigned to 2 x OCH_3 and δ_C = 54.9 ppm was assigned to the third methoxy group.

Compounds **15** and **16** were previously synthesized by means of Electrophilic Aromatic Substitutions reactions, catalyzed by mineral acids, such as orcinol reaction with geraniol in aqueous formic acid solutions [38], condensation reaction of geraniol and orcinol in methylene chloride in the presence of *p*-toluenesulfonic acid [39]. However, the yields obtained with these methodologies were low (2-18% yield). Subsequently we report the preparation of compounds **15** and **16** from orcinol and geraniol with 27.6% and 12.6% yields respectively, using Lewis acid ($BF_3{\cdot}Et_2O$) as catalyst in dioxane [36]. Recently, our research group reported a new synthesis of compounds **15** and **16** with identical yields, by direct geranylation reaction between orcinol and geraniol, using $BF_3{\cdot}OEt_2$ as catalyst and $AgNO_3$ as secondary catalyst but using acetonitrile instead of dioxane as solvent. Additionally in this reaction the compounds **17** and **18** were obtained with 6.1% and 9.9 % yields respectively [40]. Subsequently derivatives **19**-**22** were obtained by standard acetylation reaction (Ac_2O/CH_2Cl_2/DMAP) from phenols **15**-**18** respectively [40].

On the other hand the methoxylated compounds **23**-**25** were obtained by methylation reaction (in dimethyl sulfate/K_2CO_3 system under reflux conditions in acetone) from **15**, **16** and **18**, with 80.1%, 78.7% and 78.1% yield respectively.

The structure of **23**-**25** was mainly established by NMR. For compound **23** in the ^1H NMR spectrum the signals at δ_H =3.81 ppm (s, 3H, OCH_3) and δ_H =3.80 ppm (s, 3H, OCH_3) confirmed the presence of two methoxyl groups. Additionally, in the ^{13}C NMR spectrum, the signals at δ_C =55.5 and 55.1 ppm was assigned to two methoxyl group. For symmetric compound **24**, the signal at δ_H = 3.85 ppm (s, 6H, OCH_3) and the signal at δ_C = 55.5 ppm (2 x OCH_3) in the ^{13}C NMR spectrum, mainly confirm the structure **24**. Similarly compound **25**, showed signals at δ_H =3.82 ppm (s, 3H, OCH_3) and δ_H =3.72 ppm (s, 3H, OCH_3) in the ^1H NMR spectrum. While in the ^{13}C NMR spectrum the signals at δ_C = 61.5 and 55.5 ppm was assigned to two methoxyl group respectively.

2.1.1. *Biological*

2.1.1.1. Cell Viability

2.1.1.1.1 In vitro Growth Inhibition Assay

The cytotoxicity of compounds (**6** and **11**-**25**) was evaluated *in vitro* against different cancer cell

lines: MDA-MB-231 breast cancer, DU-145 and PC-3 prostate cancer, HT-29 colon cancer and one non-tumor cell line, human dermal fibroblast (HDF). A colorimetric assay was set up to estimate the IC_{50} values. The IC_{50} obtained from these assays are shown in Table 1.

Table 1. Cytotoxicity (IC_{50} μM) of compounds **6** and **11-25**.

N° Compound	HDF IC_{50} ± DS	HT-29 IC_{50} ± DS	PC-3 IC_{50} ± DS	MDA-MB231 IC_{50} ± DS	DU-145 IC_{50} ± DS
6	**>100**	**>100**	**>100**	**>100**	**>100**
11	**>100**	**>100**	**>100**	**>100**	**>100**
12	**23.5 ± 2.3**	**21.7 ± 3.5**	**23.7 ± 3.4**	**18.5 ± 3.5**	**29.9 ± 3.5**
13	**27.0 ± 5.6**	**19.4 ± 3.1**	**18.8 ± 2.6**	**66.7 ± 10.4**	**82.3 ± 4.9**
14	**>100**	**>100**	**>100**	**>100**	**>100**
15	**>100**	**>100**	**>100**	**>100**	**>100**
16	**>100**	**>100**	**>100**	**>100**	**>100**
17	**29.9 ± 2.6**	**46.7 ± 4.1**	**41.0 ± 4.6**	**71.1 ± 4.1**	**62.1 ± 5.6**
18	**>100**	**>100**	**>100**	**>100**	**>100**
19	**>100**	**>100**	**>100**	**>100**	**>100**
20	**>100**	**>100**	**>100**	**>100**	**>100**
21	**>100**	**63.2 ± 7.8**	**60.3 ± 7.8**	**>100**	**>100**
22	**68.1 ± 10.5**	**>100**	**44.5 ± 6.5**	**91.8 ± 11.8**	**82.2 ± 7.5**
23	**>100**	**>100**	**>100**	**>100**	**>100**
24	**>100**	**>100**	**>100**	**>100**	**>100**
25	**>100**	**>100**	**>100**	**>100**	**68.1 ± 7.3**

The highest cytotoxicity values were observed for compounds **12** and **13** in all cell lines tested and were more active than those of the rest of compounds. The cytotoxicity of compounds in human dermal fibroblast (HDF) is similar than in the cancer cell lines under study, even so the cytotoxic activity was not previously described for the compounds **12**, **13**, **17**, **21**, **22**, and **25** in the literature.

Based on the obtained results, the cytotoxic action against studied cell lines would be explained by the structure-activity relationship analysis of the results showed in table 1. It is clearly seen, that the presence of both geranyl chains on the aromatic ring is critical for apoptotic activity, because

monogeranyl compounds have not activity on any of the studied cell lines. In the same way, by comparing phloroglucinol with orcinol derivatives it can be seen that introduction of a methyl group (orcinol derivatives) in replace of acetyl group (phloroglucinol derivatives) decreases the activity, as it can be seen by comparing the cytotoxicity of compounds **12** and **13** with that exhibited by compounds **17**, **21**, **22** and **25**.

On the other hand, when comparing only orcinol derivatives it can be seen that the presence of a methyl group between two geranyl chains increases the cytotoxicity on the non-tumor line cells (HDF) compared with cancer cell lines (compares **17** and **18** compounds). Finally, the differences on cytotoxic activities between compounds **12** and **13** and the others compounds may be related to the presence of acetyl and the absence of methyl groups in their structure.

The more elevated log P value for compounds **12**, **13 17**, **21**, **22** and **25** (data not shown) suggests that these compounds could penetrate membranes more easily. The log P corresponding to partition coefficient and is the ratio of the equilibrium concentration of a substance between two immiscible liquids (octanol/water), to major value of log P more affinity with to lipid phase [41].

Additionally, the data in Table 1 show that compound 12 exhibits the highest inhibitory activity against studied cell lines including non-tumor cells. This results render this compound useless as anticancer agents. Interestingly, the other active compounds affect selectively some cell lines. For example, compounds 13 inhibit more efficiently HT-29 and PC-3 cells, whereas compound 22 acts selectively on PC-3 cell and compound 25 inhibits only DU-145 cells. The reason of these effects is not clear, and as the active compounds are just a few it is difficult to find a relation with the chemical structure.

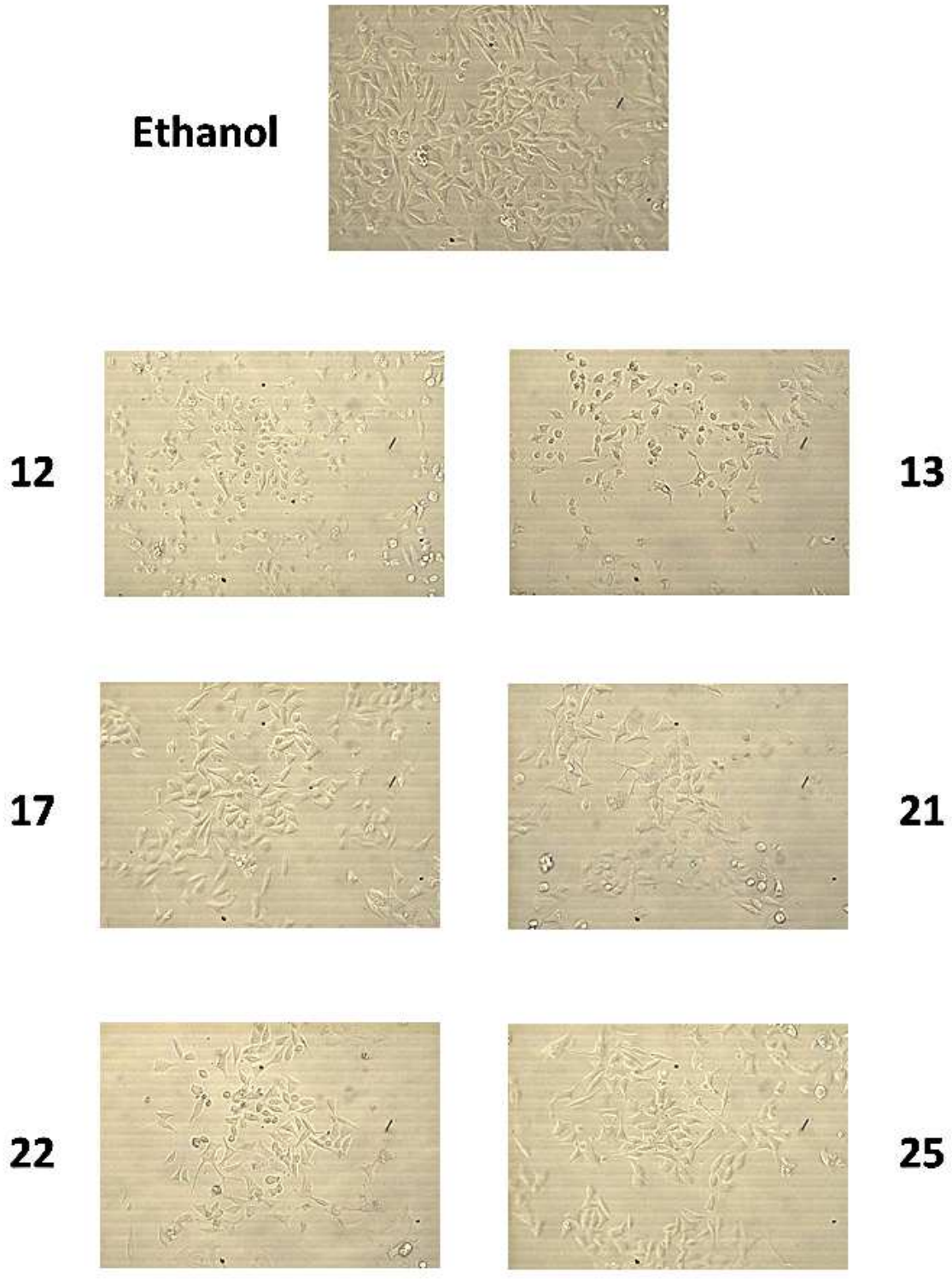
2.1.1.1.2 Morphological Assessment of Cell Apoptosis

In view of the above and given that compounds **12, 13**, **17, 21, 22** and **25** had inhibitory effects on the viability of the cancer cell lines tested, the effect of these compounds deserve to be studied in greater detail. Moreover, the biological cytotoxic activity was not previously described for the compounds **12**, **13**, **17**, **21**, **22**, and **25** in the literature.

First, the appearance of morphological changes in the cells treated with 50 μM compound for 24 h was analyzed. Direct observation, using a phase contrast microscope, revealed that the morphologies of PC-3 (Figure 5), MDA-MB231, DU-145, HT-29 and HDF (data not shown) cells were distorted and cells became rounded after treatment with compounds **12, 13**, **17, 21, 22** and **25**. Moreover, the cells showed a reduction in number, indicating an increasing progression toward cell death. The control-treated cells (1% ethanol) displayed normal and healthy shapes (Figure 5).

Figure 5. Effect of tested compounds on the morphologies of PC-3 cells. Images obtained with an inverted phase contrast microscope (200×) after 24 h exposure of the cells to 50 μM of compounds **12,**

13, **17, 21,** **22** and **25** (1% ethanol).



To elucidate whether the compounds reduced cell viability in the cell lines tested by inducing apoptosis (MCF-7, PC-3 and HDF cells), cells treated with each active compound **12, 13**, **17, 21, 22** and **25** were examined after Hoechst 33342 staining. Nuclear changes in PC-3, HT-29, MDA-MB231, DU-145 and HDF cells were observed under a fluorescence microscope (200×) and chromatin condensation and/or fragmentation was quantified (Table 2).

Table 2 Percentage of condensed and/or fragmented nuclei after treatment with compounds **12, 13**, **17, 21, 22**, **25** or ethanol (* $p < 0.01$).

Compound	PC-3	HT-29	MDA-MB 231	DU-145	HDF
12	23.7 ± 3.2*	20.1 ± 2.0*	23.3 ± 2.1*	22.7 ± 3.5*	19.1 ± 2.0*
13	22.3 ± 2.3*	21.8 ± 2.1*	13.9 ± 1.3*	14.4 ± 3.3*	18.7 ± 1.1*
17	16.3 ± 1.1*	15.3 ± 0.9*	12.9 ± 2.0*	14.5 ± 1.4*	18.4 ± 1.3*
21	13.7 ± 1.0*	14.3 ± 1.5*	5.2 ± 0.8	6.6 ± 0.9	6.1 ± 0.9
22	15.1 ± 1.4*	9.3 ± 1.3	8.7 ± 1.2	11.0 ± 1.3	12.9 ± 1.0*
25	7.4 ± 0.9	8.9 ± 1.3	8.0 ± 1.0	13.8 ± 0.9*	6.7 ± 1.1
EtOH	6.9 ± 1.1	7.8 ± 1.0	7.3 ± 1.1	7.6 ± 1.2	6.4 ± 1.0

As shown in Table 2 treatment with compounds **12, 13** and **17** significantly increased the number of cells with condensed and/or fragmented nuclei *versus* control-treated cells (* $p < 0.01$). By other hand compounds **21, 22** and **25** increased nuclear fragmentation only in some cell lines coinciding with cellular viability results (Table 1 and 2).

2.1.1.1.3 Analysis of Mitochondrial Membrane Permeability

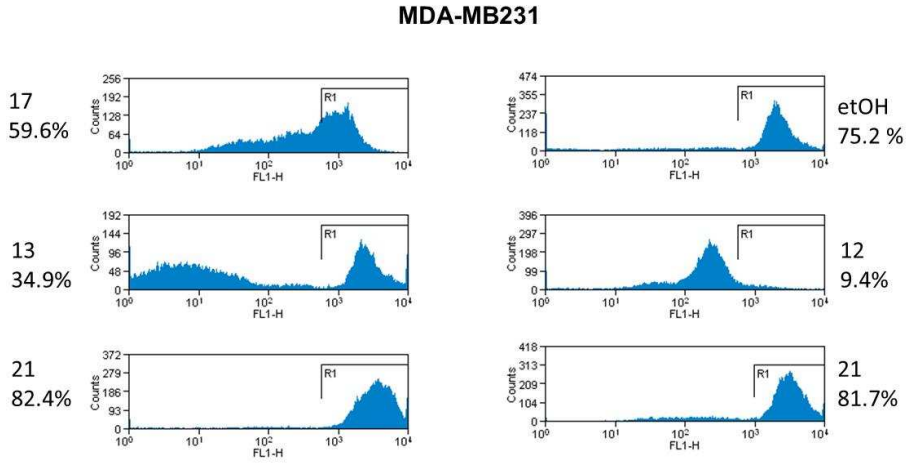
Mitochondria play a crucial role in the cell death decision, participating in apoptotic cascade by serving as a convergent center of apoptotic signals originating from both the extrinsic and intrinsic pathways [42]. Changes induced in the mitochondrial membrane potential (MMP) have been reported previously to represent a determinant in the execution of cell death [43]. This effect may be determinant in cell death, we have analyzed the effect of compounds **12, 13**, **17, 21,** and **22** (50 μM) on the mitochondrial membrane potential using flow cytometry with rhodamine 123 stain [44]. As shown in Figure 6B, the percentage of rhodamine 123 stained-cells were decreased significantly after treatments with compounds **12** and **13** in all cell lines studied. However, compounds **17**, **21** and **22** diminished the rhodamine-stained cells only in one or two cell lines, as compared to 85–90% in the control cells (1% ethanol).

Figure 6 shows that compounds **12** and **13** increased mitochondrial membrane permeability in cancer cells with a greater effect than compounds **17, 21** and **22**. The compounds have not differences in the effect on mitochondrial membrane potential between HDF and cancer cell lines. Thus, the effect of compounds **12** and **13** on the decrease of mitochondrial membrane potential correlated with diminished viability (see Table 1) and increased nuclear condensation and/or fragmentation (see Table 2). Moreover, compounds **17, 21** and **22** only changed the mitochondrial membrane potential when compared to control cells in PC-3 and HDF, MDA-MB231 and PC-3 cell lines, respectively. Probably,

the difference in the observed activity of these compounds to mitochondrial level, could be associated with the polarity of these derivatives, since those having lower polarity, corresponding to phloroglucinol derivatives **12** and **13**, exhibit a greater effect and increased mitochondrial membrane permeability. The reason for this increase in activity is probably due to that the derivatives of minor polarity might cross cell membranes more easily and lead to an increase in the bioavailability and activity of these compounds. Specifically, we considered that the passage through the mitochondrial membrane should lead to an alteration of membrane potential. This change in mitochondrial membrane potential would cause the release of apoptogenic factors. The precise mechanism by which compounds **12** and **13** are decreasing mitochondrial membrane potential has not been established, though the structural similarity to diacylphloroglucinol (DAPG) may suggest that the compounds act as uncouplers in the mitochondrion [45].

Figure 6. A) Compounds **12, 13**, **17, 21** and **22** treatment-induced changes in the mitochondrial membrane permeability's for MDA-MB231 cells. The cells were stained with

rhodamine 123, and then analyzed by flow cytometry. B) The table shows the percentage values of rhodamine 123 stained cells treated without or with compounds **12, 13**, **17, 21** and **22** (50 µM) for the different cell lines MDA-MB231, PC-3, DU-145, HT-29 and HDF (* $p < 0.05$ *vs.* control treated cells).



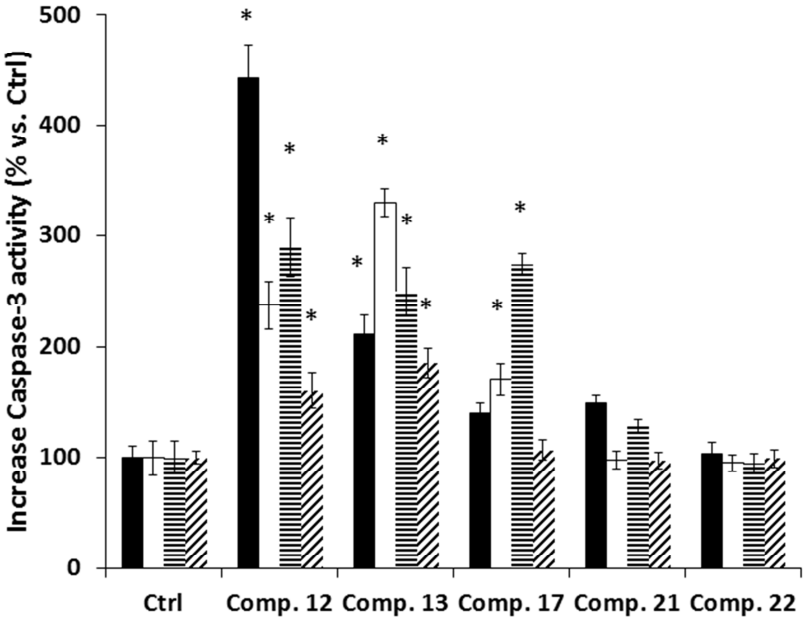
	MDA-MB231	PC-3	DU-145	HT-29	HDF
	50 µM	50 µM	50 µM	50 µM	50 µM
Comp. 12	9.6 ± 1.1*	36.8 ± 4.7*	60.4 ± 3.9*	66.3 ± 2.8*	31.3 ± 2.4*
Comp. 13	34.9 ± 3.6*	32.3 ± 2.4*	50.3 ± 4.1*	41.3 ± 6.4*	15.2 ± 3.5*
Comp. 17	59.8 ± 3.7*	51.8 ± 5.0*	79.9 ± 5.9	81.3 ± 6.5	51.6 ± 4.9*
Comp. 21	82.4 ± 6.6	77.8 ± 6.1	77.3 ± 5.5	84.9 ± 6.5	79.3 ± 7.1
Comp. 22	81.7 ± 6.8	65.2 ± 4.9*	81.9 ± 6.5	87.1 ± 8.8	80.8 ± 5.3
Control	80.5 ± 8.4	86.1 ± 8.5	85.8 ± 8.2	82.4 ± 6.9	81.5 ± 10.5

2.1.1.1.4 Caspase 3 Activity Assay

Depletion of mitochondrial membrane potential leads to the release of apoptogenic factors, such as cytochrome c in the apoptotic cascade, and activation of caspases [45, 46]. Then we investigated the effects of our compounds on caspase-3 activity, the main executor of apoptosis playing a central role in its biological processing. We analyzed the effect of treatment with compound **12, 13**, **17, 21** and **22** on caspase-3 activation in non-tumor and cancer cell lines (Figure 7).

Figure 7. Effect of compounds **12, 13**, **17, 21** and **22** on caspase-3 activity of MDA-MB231 (black

bar), HDF (white bar), PC-3 (striped bar) and HT-29 (slashed bar) cells. Cells were exposed to compounds at 50 μM for 48 h. Values are mean ± S.D. (n = 3). All data are reported as the percentage change in comparison with the vehicle-treated cells (1% ethanol), which were arbitrarily assigned 100%. * $p < 0.05$, significantly different from the vehicle-treated cells.



As shown in Figure 7, the activation of caspase-3 in cells exposed to compounds **12, 13** and **17** is increased *versus* control-treated cells (1% ethanol). Compound **12** increased the activity of caspase-3 by 4.43 ± 0.11, 2.38 ± 0.16, 2.90 ± 0.15 and 1.61 ± 0.06 times *versus* control cells in MDA-MB231 , HDF , PC-3 and HT-29 cells, respectively. Compound **13** increased the activity of caspase 3 by 2.12 ± 0.30, 3.30 ± 0.21, 2.50 ± 0.26, and 1.86 ± 0.16 times *versus* control cells in MDA-MB231 (black bar), HDF, PC-3 and HT-29 cells, respectively. Compound **17** only changed significantly the activity of caspase-3 in HDF and PC-3 cells. Finally, compounds **21** and **22** did not change the activity of caspase-3 when compared with control cells.

3. Experimental Section

3.1. Chemical

Unless otherwise stated, all chemical reagents purchased (Merck, Darmstadt, Germany or Aldrich, St. Louis, MO, USA) were of the highest commercially available purity and were used without previous purification. IR spectra were recorded as thin films in a FT-IR Nicolet 6700 spectrometer (Thermo Scientific, San Jose, CA, USA) and frequencies are reported in cm^{-1}. Low resolution mass spectra were recorded on an Agilent 5973 spectrometer (Agilent Technologies, Santa Clara, CA, USA) at 70eV ionizing voltage, in a GC 6890N DB-5 m, 30 m × 0.25 mm × 0.25 μm column, and data are given as *m/z* (% rel. int.). High resolution mass spectra were recorded on an LTQ Orbitrap XL spectrometer (Thermo Scientific, San Jose, CA, USA) by applying a voltage of 1.8 kV in the positive and 1.9 kV in the negative, ionization mode. The spectra were recorded using full scan mode, covering a mass range from *m/z* 100–1,300. The resolution was set to 50,000 and maximum loading time for the ICR cell was set to 250ms. ^{1}H, ^{13}C, ^{13}C DEPT-135, sel. gs-1D ^{1}H NOESY, gs-2D HSQC and gs-2D HMBC spectra were recorded in $CDCl_3$ solutions and are referenced to the residual peaks of $CHCl_3$ at δ = 7.26 ppm and δ = 77.0 ppm for ^{1}H and ^{13}C, respectively, on a Bruker Avance 400 Digital NMR spectrometer (Bruker, Rheinstetten, Germany), operating at 400.1 MHz for ^{1}H and 100.6 MHz for ^{13}C. Chemical shifts are reported in δ ppm and coupling constants (*J*) are given in Hz. TLC spots were detected by heating after spraying with 25% H_2SO_4 in H_2O.

3.1.1 Synthesis of Geranylmethoxy Derivatives

(E)-2-(3,7-dimethylocta-2,6-dienyl)-1,3,5-trimethoxybenzene(**14**). To a solution of 2-geranyl phloroglucinol (**6**) (360 mg; 1.4 mmol), dimethyl sulfate (0.58 g; 4.6 mmol) and K_2CO_3 (0.58 g; 4.2 mmol) in acetone (50 mL), was refluxed for 6 hours. The end of the reaction was verified by TLC, and then the mixture was filtered and the solvent was evaporated under reduced pressure. The crude was re-dissolved in EtOAc (30 mL) then the organic layer was washed with 5% NaOH (30 mL) and water (2 x 20 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The compound **14** was obtained as a viscous yellow oil, 370 mg (86.8% yield). IR (cm^{-1}): 2935, 2836, 1608, 1596, 1496, 1455, 1437, 1417, 1377, 1322. ^{1}H-NMR ($CDCl_3$, 400.1 MHz) : δ 6.14 (2H, s, H-4 and H-6); 5.19 (1H, t, *J* = 6.9 Hz, H-2'); 5.09 (1H, t, *J* = 6.3 Hz, H-6'); 3.92 (3H, s, OCH_3); 3.79 (6H, s, OCH_3); 3.29 (2H, d, *J* = 6.8 Hz, H-1'); 2.08-2.05 (2H, m, H-5'); 1.99-1.95 (2H, m, H-4'); 1.78 (3H, s, CH_3-C3'); 1.66 (3H, s, H-8'); 1.59 (3H, s, CH_3-C7'). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 159.0 (C-5); 158.4 (C-1 and C-3); 133.6 (C-3'); 130.6 (C-7'); 124.3 (C-6');123.2 (C-2'); 110.5 (C-2); 90.4 (C-4 and C-6); 55.3 (2 x OCH_3); 54.9 (OCH_3); 39.6 (C-4'); 26.5 (C-5'); 25.3 (C-8'); 21.4 (C-1'); 17.3 (CH_3-C7'); 15.6 (CH_3-C3'). MS

(m/z, %): 304 $[M]^+$ (11%); 235 (24); 181 (100); 167 (15); 69 (13). Spectroscopic data of compound **14** was consistent with those in the literature [36].

(E)-2-(3,7-dimethylocta-2,6-dienyl)-1,5-dimethoxy-3-methylbenzene (**23**). To a solution of **15** (226 mg; 0.784 mmol), dimethyl sulfate (0.58 g; 4.6 mmol) and K_2CO_3 (0.58 g; 4.2 mmol) in acetone (50 mL), was refluxed for 6 hours. The end of the reaction was verified by TLC, and then the mixture was filtered and the solvent was evaporated under reduced pressure. The crude was re-dissolved in EtOAc (30 mL) then the organic layer was washed with 5% NaOH (30 mL) and water (2 x 20 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The compound **23** was obtained as a viscous dark yellow oil, 201 mg (80.1% yield). ^{1}H-NMR ($CDCl_3$, 400.1 MHz): 6.36 (2H, s, H-4 and H-6); 5.10-5.08 (2H, m, H-6'and H-2'); 3.81 (3H, s, OCH_3); 3.80 (3H, s, OCH_3); 3.32 (2H, d, *J* = 6.5 Hz, H-1'); 2.29 (3H, s, CH_3-Ar); 2.11-2.06 (2H, m, H-5'); 2.02-1.98 (2H, m, H-4'); 1.79 (3H, s, CH_3-C3'); 1.68 (3H, s, H-8'); 1.61 (3H, s, CH_3-C7'). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 158.2 ($^+$C-5); 158.1 ($^+$C-1); 138.1 (C-3); 134.3 (C-3'); 131.1 (C-7'); 124.4 (C-6'); 123.0 (C-2'); 121.1 (C-2); 106.5 (C-4); 96.1 (C-6); 55.5 (OCH_3); 55.1 (OCH_3); 39.7 (C-4'); 26.7 (C-5'); 25.6 (C-8'); 24.6 (C-1'); 19.9 (CH_3-Ar); 17.6 (CH_3-C7'); 16.0 (CH_3-C3'). $^+$ Interchangeable signals. HRMS: (M + 1) calcd. for $C_{19}H_{28}O_2$: 289.2089, found: 289.2097.

(E)-2-(3,7-dimethylocta-2,6-dienyl)-1,3-dimethoxy-5-methylbenzene (**24**). To a solution of **16** (243 mg; 0.843 mmol), dimethyl sulfate (0.58 g; 4.6 mmol) and K_2CO_3 (0.58 g; 4.2 mmol) in acetone (50 mL), was refluxed for 6 hours. The end of the reaction was verified by TLC, and then the mixture was filtered and the solvent was evaporated under reduced pressure. The crude was re-dissolved in EtOAc (30 mL) then the organic layer was washed with 5% NaOH (30 mL) and water (2 x 20 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The compound **24** was obtained as a viscous dark yellow oil, 206 mg (78.7% yield). ^{1}H-NMR ($CDCl_3$, 400.1 MHz): δ 6.43 (2H, s, H-4 and H-6); 5.27 (1H, t, *J* = 7.0 Hz, H-2'); 5.15 (1H, t, *J* = 6.5 Hz, H-6'); 3.85 (6H, s, OCH_3); 3.40 (2H, d, *J* = 7.0 Hz, H-1'); 2.40 (3H, s, CH_3-Ar); 2.14-2.10 (2H, m, H-5'); 2.04-2.01 (2H, m, H-4'); 1.84 (3H, s, CH_3-C3'); 1.72 (3H, s, H-8'); 1.65 (3H, s, CH_3-C7'). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 157.9 (C-1 and C-3); 136.5 (C-5); 134.1 (C-3'); 130.8 (C-7'); 124.6 (C-6'); 123.1 (C-2'); 115.4 (C-2); 104.7 (C-4 and C-6); 55.6 (OCH_3); 39.8 (C-4'); 26.7 (C-5'); 25.6 (C-8'); 21.9 (CH_3-Ar and C-1'); 17.5 (CH_3-C7'); 15.9 (CH_3-C3'). HRMS: (M + 1) calcd. for $C_{19}H_{28}O_2$: 289.2089, found: 289.2094.

2,4-bis((E)-3,7-dimethylocta-2,6-dienyl)-1,3-dimethoxy-5-methylbenzene (**25**). To a solution of **18** (205.6 mg; 0.484 mmol), dimethyl sulfate (0.58 g; 4.6 mmol) and K_2CO_3 (0.58 g; 4.2 mmol) in acetone (50 mL), was refluxed for 6 hours. The end of the reaction was verified by TLC, and then the mixture was filtered and the solvent was evaporated under reduced pressure. The crude was re-

dissolved in EtOAc (30 mL) then the organic layer was washed with 5% NaOH (30 mL) and water (2 x 20 mL), dried over Na_2SO_4, filtered and evaporated to dryness. The compound **25** was obtained as a viscous dark yellow oil, 178 mg (78.1% yield). ^{1}H-NMR ($CDCl_3$, 400.1 MHz): 6.54 (1H, s, H-6); 5.25 (1H, t, *J* = 7.0 Hz, H-2'); 5.11-5.10 (3H, m, H-2'', H-6' and H-6''); 3.82 (3H, s, OCH_3); 3.72 (3H, s, OCH_3); 3.38 (2H, d, *J* = 7.0 Hz, H-1'); 3.36 (2H, d, *J* = 7.4 Hz, H-1''); 2.29 (3H, s, CH_3-Ar); 2.10-2.08 (4H, m, H-5' and H-5''); 2.04-2.00 (4H, m, H-4' and H-4''); 1.80 (3H, s, CH_3-C3'); 1.79 (3H, s, CH_3-C3''); 1.68 (6H, s, H-8' and H-8''); 1.61 (6H, s, CH_3-C7' and CH_3-C7''). ^{13}C-NMR ($CDCl_3$, 100.6 MHz): δ 157.1 (C-3); 156.3 (C-1); 135.8 (C-5); 134.7 (C-3''); 134.3 (C-3'); 131.2 ($^+$C-7'); 131.0 ($^+$C-7''); 125.6 (C-4); 124.5 ($^{++}$C-6'); 124.3 ($^{++}$C-6''); 123.8 ($^{+++}$C-2'); 123.7 ($^{+++}$C-2''); 120.9 (C-2); 108.8 (C-6); 61.5 (OCH_3); 55.5 (OCH_3); 39.8 (C-4'); 39.6 (C-4''); 26.7 ($^{++++}$C-5'); 26.6 ($^{++++}$C-5''); 25.6 (C-8' and C-8''); 25.5 (C-1''); 23.2 (C-1'); 19.8 (CH_3-Ar); 17.6 (CH_3-C7' and CH_3-C7''); 16.1 (CH_3-C3''); 16.1 (CH_3-C3'). $^+$ Interchangeable signals. HRMS: (M + 1) calcd. for $C_{29}H_{44}O_2$: 425.3341, found: 425.3349.

3.2 Biological

3.2.1 Cell Lines

The experimental cell cultures were obtained from the American Type Culture Collection (Rockville, MD, USA). MDA-MB-231 (breast cancer cell line), HT-29 (colon cancer cell line), PC-3 and DU-145 (prostate cancer cell lines), and human dermal fibroblast (HDF, non-tumor cell line) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM glutamine. Cells were seeded into 96 well microtiter plates in 100 μL at a plating density of 5×10^3 cells/well. After 24 h incubation at 37 °C under a humidified 5% CO_2 atmosphere to allow cell attachment, the cells were treated with different concentrations of drugs and incubated for 72 h under the same conditions. Stock solutions of compounds were prepared in ethanol and the final concentration of this solvent was kept constant at 1%. Control cultures received 1% ethanol alone.

3.2.2 Cell Viability

3.2.2.1. *In vitro* Growth Inhibition Assay

The sulforhodamine B assay was used according to the method of Skehan *et al.* [47] with some modifications [48]. Briefly, the cells were set up at 3×10^3 cells per well of a 96 200 μL well, flat-bottomed, microplate. Cells were incubated at 37°C in a humidified 5% CO_2/95% air mixture and treated with the compounds at different concentrations for 72 h. At the end of drug exposure, cells were fixed with 50% trichloroacetic acid at 4°C. After washing with water, cells were stained with 0.1%

sulforhodamine B (Sigma-Aldrich, St. Louis, MO, USA), dissolved in 1% acetic acid (50 μL/well) for 30 min, and subsequently washed with 1% acetic acid to remove unbound stain. Protein-bound stain was solubilized with 100 μL of 10mM unbuffered Tris base, and the cell density was determined using a fluorescence plate reader (wavelength 540 nm). Values shown are the mean ± SD of three independent experiments in triplicate. The software used to calculate the IC_{50} values was GraphPad (GraphPad Software, San Diego, CA, USA).

3.2.3. Morphological Assessment of Cell Apoptosis

Morphological changes in the nuclear chromatin of cells undergoing apoptosis were revealed by a nuclear fluorescent dye, Hoechst 33342. Briefly, on 24-well chamber slides, 1×10^4 cells/mL were cultured and exposed to compounds for 24 h. The control group was also exposed to 1% ethanol. The cells were washed twice with phosphate buffer solution, fixed with 3.7% formaldehyde, and washed again with phosphate buffer solution. Following the addition of 1 μM Hoechst 33342 (Sigma-Aldrich, Santiago, Chile), they were reacted in a dark room at room temperature for 30 min. After washing, they were examined under an immunofluorescence microscope (IX 81 model inverted microscope, Olympus, Santiago, Chile).

3.2.4. Analysis of Mitochondrial Membrane Permeability

Rhodamine 123, a cationic, voltage-sensitive probe that reversibly accumulates in mitochondria, was used to detect changes in transmembrane mitochondrial membrane potentials. Exponentially growing cells were incubated with the compound as indicated in the figure legends. Cells were labelled with 1 μM rhodamine 123 at 37 °C in cell medium for 60 min before terminating the experiment. Cells were detached from the plate, after washing with ice cold PBS, and the samples were analyzed by flow cytometry. Data are expressed in percentage of cells with rhodamine 123 [44].

3.2.5. Caspase 3 Activity Assay

Caspase activity was measured using a colorimetric assay [49]. Briefly, the cells exposed to compounds were collected by centrifugation at 1000 rpm and lysed with lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris–HCl, pH 8.0, 2 mM dithiothreitol, 1 mM PMSF, 1 g/mL aprotinin, 1 mg/mL leupeptin). Thereafter, the lysates were transferred to wells in a 96-well microplate and were incubated with DEVD-pNA (final concentration 200 μM) specific for caspase-3/7, at 37°C for 1 h. The intensity of the developed color was read at 405 nm in a microplate reader (SpectraMax, Winooski, VT, USA). The results are expressed as percentages of the control level.

3.2.6. Statistical Analysis

Data shown in tables are average results obtained by means of three or 10 replicates and are presented as average ± standard errors of the mean (SEM). Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by GLM procedures. Results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with a Student–Newman–Keuls (SNK) test. The EC_{50} values for each activity were calculated by PROBIT analysis based on percentage of mortality obtained at each concentration of the samples. EC_{50} is half maximal effective concentration. Complete statistical analysis was performed by means of the MicroCal Origin 6.0 statistics and graphs PC program.

4. Conclusions

Different compounds derived from phloroglucinol and orcinol were tested as potential cytotoxic agents against various cancer cell lines: PC-3 and DU-145 (prostate cancer), MDA-MB 231 (breast cancer), HT-29 (colon cancer) and one non-tumoral cell line human dermal fibroblasts (HDF). Initial primary screens indicated IC_{50} concentration between 0 and 100 μM for compounds **12**, **13**, **17**, **21**, **22** and **25**. Among the six, three were found to be effective against most of the cancer cell lines. Compounds **12**, **13**, **17**, **21**, **22** and **25**, which showed cytotoxicity, were evaluated in an apoptosis assay. Compounds **12, 13** and **17** showed a good apoptotic response against all cell lines studied, whereas the compounds **21**, **22**, and **25** had minor impact on induction of chromatin nuclear condensation and/or fragmentation, mitochondrial membrane potential and caspase-3 activity. Moreover the compounds **12, 13** and **17** also depleted the mitochondrial membrane potential and increased caspase-3 activity. According these results, the analysis of the relation between the activity and the structure of compounds indicate that the biological activity depend strongly on, first: mono or disubstitution geranyl chains on the aromatic (disubstituted compounds geranyl chains are more active than those monosubstituted) second: the increasing order in biological activity of the oxygen function in the aromatic ring, could be established as OH > OAc >> OMe. Finally we note that the disubstituted compounds with two geranyl chains and symmetrical structure (i.e. compounds **12**, **13** and **17**) are more active than those with asymmetrical disubstitution (compound **18**, **22** and **25**). While it is true, compounds **12** and **13** have higher activity against tumor cells than against normal, this difference is not significant, which it makes little useful as anticancer drugs. However, these compounds could be used as leads for the search of new derivatives that would have significantly higher activity against cancer cells.

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Author Contributions

LE, LT and JV designed research; AM, LE, LT, JV, MC and HC performed research and analyzed the data; LE, LT, HC and JV wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Figure 1. Structure of some active natural and synthetic linear geranylphenols.

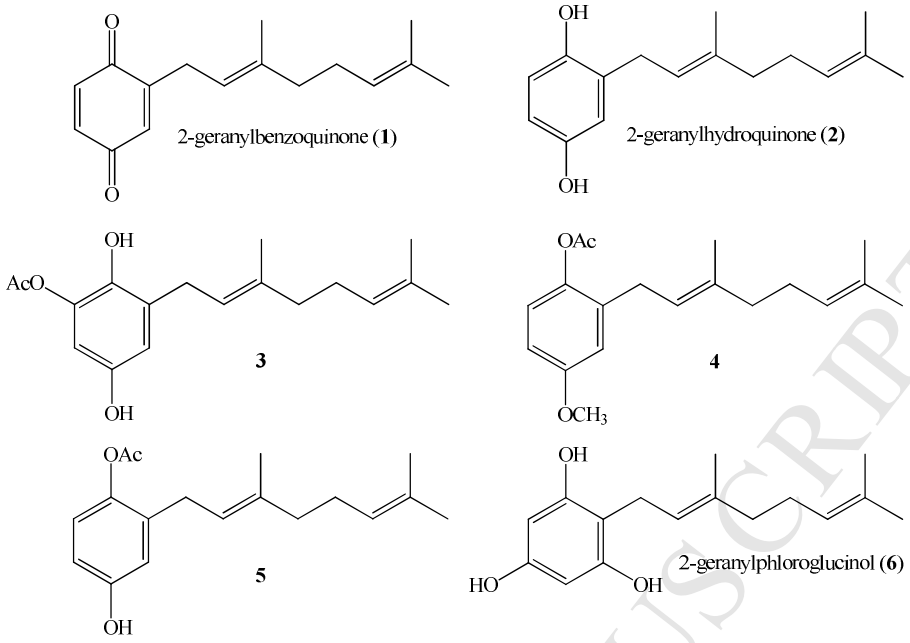


Figure 2. Structure of some active synthetic linear geranylquinones.

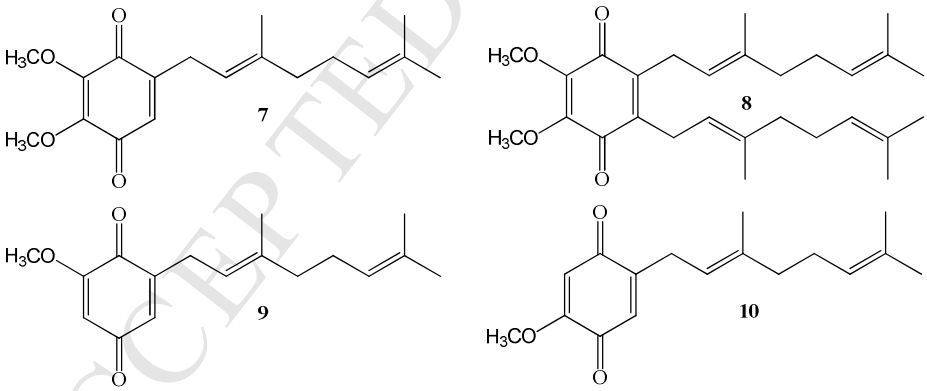


Figure 3. Structure of synthetic linear derivatives geranylphenols acetylated and methoxilated of phloroglucinol.

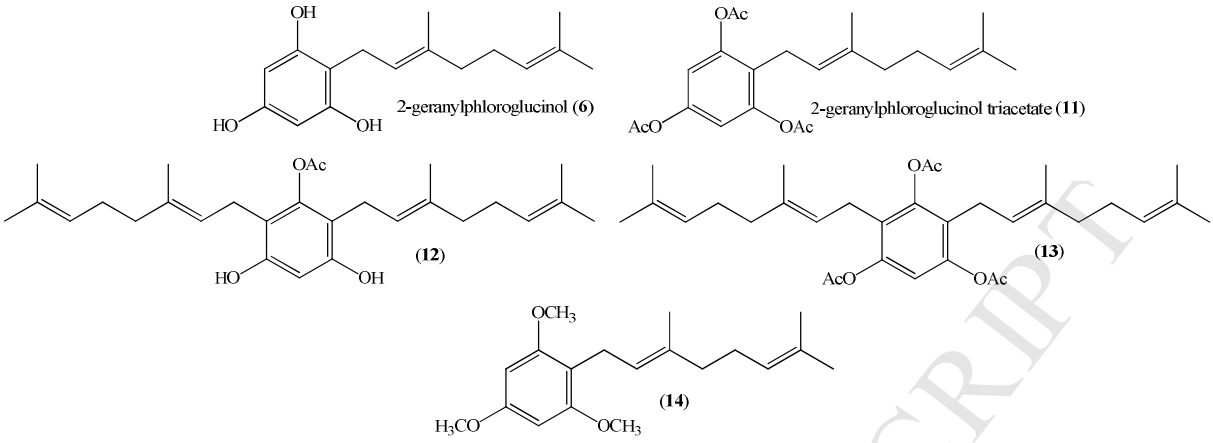


Figure 4. Structure of synthetic linear derivatives geranylphenols acetylated and methoxilated of orcinol.

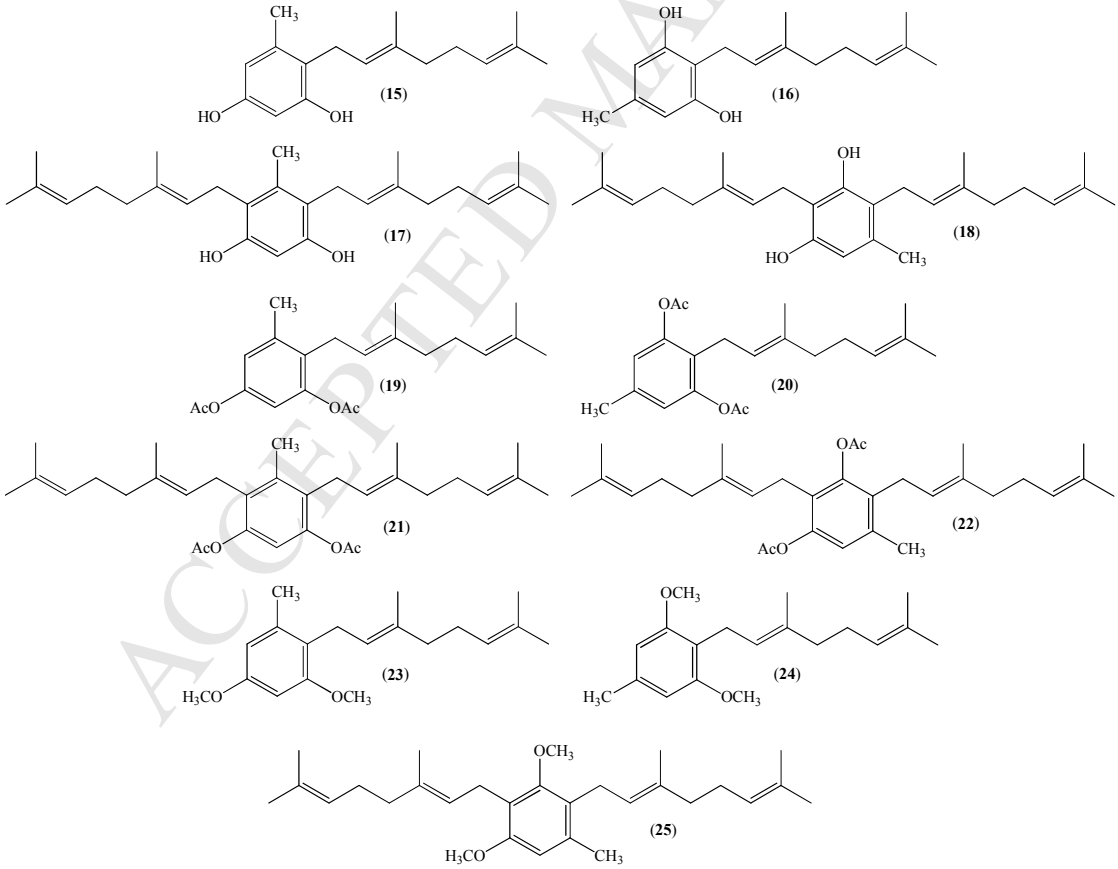


Figure 5. Effect of tested compounds on the morphologies of PC-3 cells. Images obtained with an inverted phase contrast microscope (200×) after 24 h exposure of the cells to 50 μM of compounds **12, 13**, **17, 21, 22** and **25** (1% ethanol).

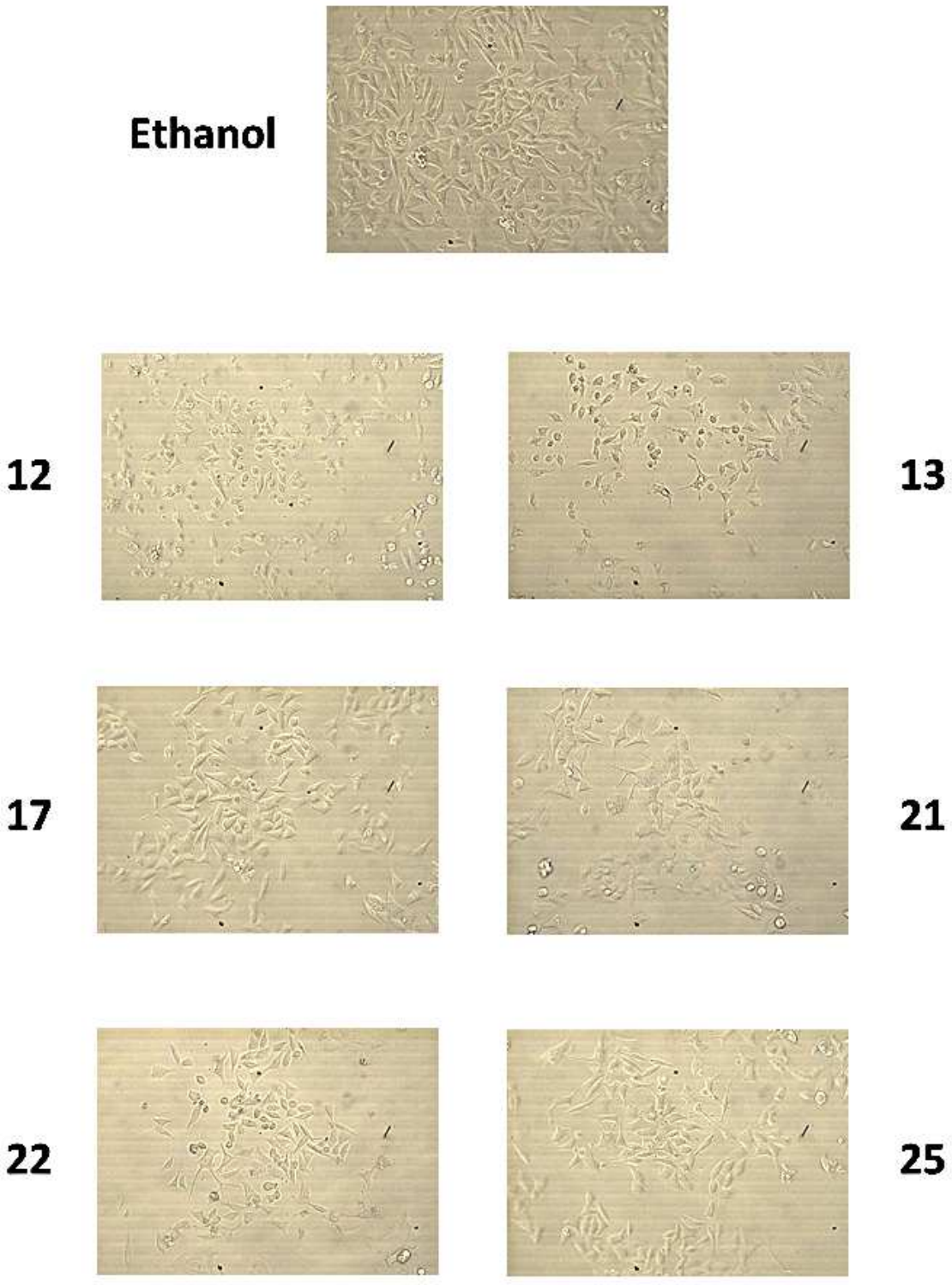
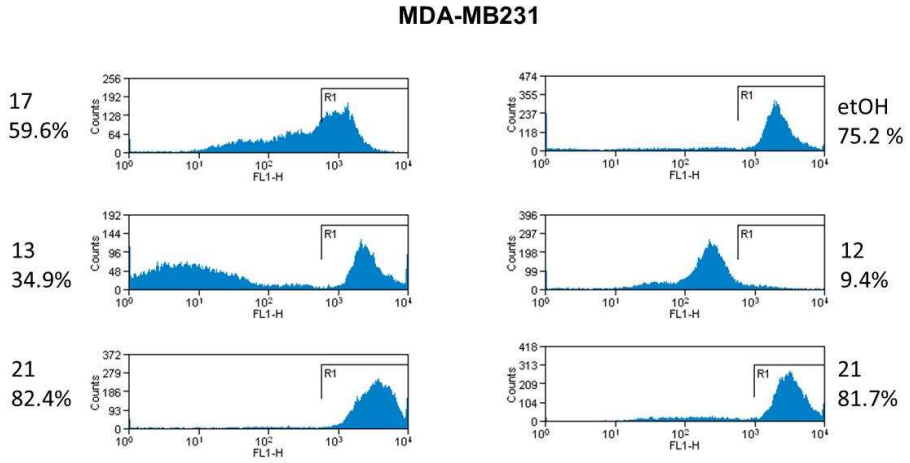


Figure 6. A) Compounds **12, 13**, **17, 21** and **22** treatment-induced changes in the mitochondrial membrane permeability's for MDA-MB231 cells. The cells were stained with rhodamine 123, and then analyzed by flow cytometry. B) The table shows the percentage values of rhodamine 123 stained cells treated without or with compounds **12, 13**, **17, 21** and **22** (50 µM) for the different cell lines MDA-MB231, PC-3, DU-145, HT-29 and HDF (* $p < 0.05$ *vs.* control treated cells).



	MDA-MB231	PC-3	DU-145	HT-29	HDF
	50 µM	50 µM	50 µM	50 µM	50 µM
Comp. 12	9.6 ± 1.1*	36.8 ± 4.7*	60.4 ± 3.9*	66.3 ± 2.8*	31.3 ± 2.4*
Comp. 13	34.9 ± 3.6*	32.3 ± 2.4*	50.3 ± 4.1*	41.3 ± 6.4*	15.2 ± 3.5*
Comp. 17	59.8 ± 3.7*	51.8 ± 5.0*	79.9 ± 5.9	81.3 ± 6.5	51.6 ± 4.9*
Comp. 21	82.4 ± 6.6	77.8 ± 6.1	77.3 ± 5.5	84.9 ± 6.5	79.3 ± 7.1
Comp. 22	81.7 ± 6.8	65.2 ± 4.9*	81.9 ± 6.5	87.1 ± 8.8	80.8 ± 5.3
Control	80.5 ± 8.4	86.1 ± 8.5	85.8 ± 8.2	82.4 ± 6.9	81.5 ± 10.5

Figure 7. Effect of compounds **12, 13, 17, 21 and 22** on caspase-3 activity of MDA-MB231 (black bar), HDF (white bar), PC-3 (striped bar) and HT-29 (slashed bar) cells. Cells were exposed to compounds at 50 μM for 48 h. Values are mean ± S.D. (n = 3). All data are reported as the percentage change in comparison with the vehicle-treated cells (1% ethanol), which were arbitrarily assigned 100%. * $p < 0.05$, significantly different from the vehicle-treated cells.

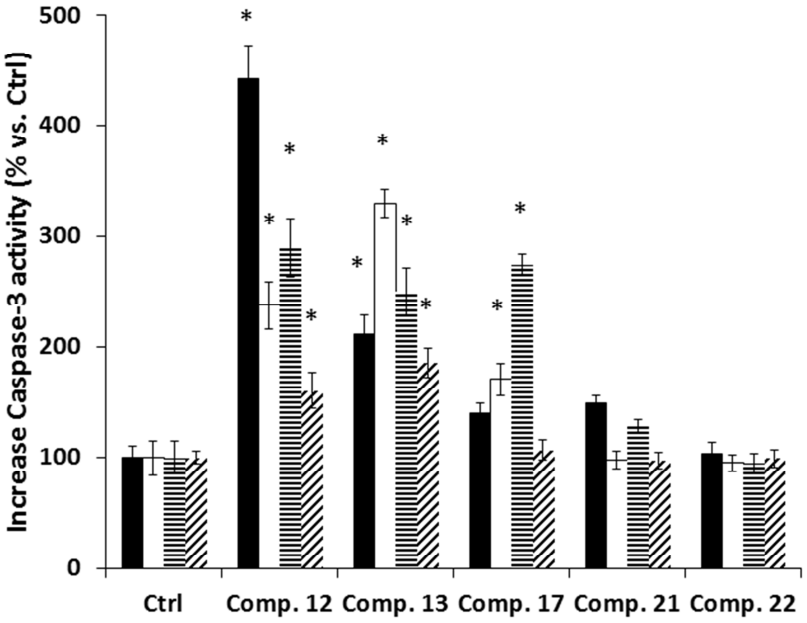


Table 1. Cytotoxicity (IC_{50} µM) of compounds **6** and **11-25**.

N° Compound	HDF IC_{50} ± DS	HT-29 IC_{50} ± DS	PC-3 IC_{50} ± DS	MDA-MB231 IC_{50} ± DS	DU-145 IC_{50} ± DS
6	**>100**	**>100**	**>100**	**>100**	**>100**
11	**>100**	**>100**	**>100**	**>100**	**>100**
12	**23.5 ± 2.3**	**21.7 ± 3.5**	**23.7 ± 3.4**	**18.5 ± 3.5**	**29.9 ± 3.5**
13	**27.0 ± 5.6**	**19.4 ± 3.1**	**18.8 ± 2.6**	**66.7 ± 10.4**	**82.3 ± 4.9**
14	**>100**	**>100**	**>100**	**>100**	**>100**
15	**>100**	**>100**	**>100**	**>100**	**>100**
16	**>100**	**>100**	**>100**	**>100**	**>100**
17	**29.9 ± 2.6**	**46.7 ± 4.1**	**41.0 ± 4.6**	**71.1 ± 4.1**	**62.1 ± 5.6**
18	**>100**	**>100**	**>100**	**>100**	**>100**
19	**>100**	**>100**	**>100**	**>100**	**>100**
20	**>100**	**>100**	**>100**	**>100**	**>100**
21	**>100**	**63.2 ± 7.8**	**60.3 ± 7.8**	**>100**	**>100**
22	**68.1 ± 10.5**	**>100**	**44.5 ± 6.5**	**91.8 ± 11.8**	**82.2 ± 7.5**
23	**>100**	**>100**	**>100**	**>100**	**>100**
24	**>100**	**>100**	**>100**	**>100**	**>100**
25	**>100**	**>100**	**>100**	**>100**	**68.1 ± 7.3**

Table 2 Percentage of condensed and/or fragmented nuclei after treatment with compounds **12, 13**, **17, 21, 22**, **25** or ethanol (* $p < 0.01$).

Compound	PC-3	HT-29	MDA-MB 231	DU-145	HDF
12	23.7 ± 3.2*	20.1 ± 2.0*	23.3 ± 2.1*	22.7 ± 3.5*	19.1 ± 2.0*
13	22.3 ± 2.3*	21.8 ± 2.1*	13.9 ± 1.3*	14.4 ± 3.3*	18.7 ± 1.1*
17	16.3 ± 1.1*	15.3 ± 0.9*	12.9 ± 2.0*	14.5 ± 1.4*	18.4 ± 1.3*
21	13.7 ± 1.0*	14.3 ± 1.5*	5.2 ± 0.8	6.6 ± 0.9	6.1 ± 0.9
22	15.1 ± 1.4*	9.3 ± 1.3	8.7 ± 1.2	11.0 ± 1.3	12.9 ± 1.0*
25	7.4 ± 0.9	8.9 ± 1.3	8.0 ± 1.0	13.8 ± 0.9*	6.7 ± 1.1
EtOH	6.9 ± 1.1	7.8 ± 1.0	7.3 ± 1.1	7.6 ± 1.2	6.4 ± 1.0

Highlights

The phloroglucinol & orcinol derivatives show cytotoxicity against cancer cell lines.

These compounds may induce apoptosis through activation of the mitochondrial pathway.

The activity of these compounds depend of the amount of chains geranyl presents.

The activity depend of the symmetry of substitution in the aromatic ring.