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Ultrasound assisted synthesis and cytotoxicity evaluation of known 2′,4′-dihydroxychalcone derivatives against cancer cell lines

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ABSTRACT

This work reports on the development of an efficient and ecofriendly ultrasound assisted method for the high yield synthesis (70.0–94.0%) of eighteen oxyalkylated derivatives of 2′,4′-dihydroxychalcone. Synthesized compounds were subjected to *in vitro* biological assays against HT-29 (colorectal), MCF-7 (breast), and PC-3 (prostate) human tumor cell lines, these cell lines are among the ten most aggressive malignancies diagnosed in the world. Cytotoxicity evaluations showed that four of the synthesized compounds exhibited moderate to very high toxic activity against MCF-7 (IC_{50} = 8.4–34.3 μM) and PC-3 (IC_{50} = 9.3–29.4 μM) – comparable to 5-fluorouracil (IC_{50} 16.4–22.3 μM). The same compounds only showed moderate activity against HT-29 (IC_{50} 15.3–36.3 μM), closer to daunorubicin (IC_{50} 15.1 μM). Next, although selectivity index (SI) of compounds was weak, compound **18** exhibited a remarkable and selective cytotoxic activity (5.8–10.57) against cancer cells. Outside of these, most compounds significantly reduced cell survival, increased reactive oxygen species (ROS) and caspase activity, and decreased mitochondrial membrane permeability. In this sense, a portion of anti-proliferative activity is due to apoptosis. Notwithstanding, due to its remarkable response, chalcone **18** may be a potential alternative as a chemotherapeutic anti-carcinogen.

1. Introduction

Cancer, currently the leading cause of death worldwide, occurs due to progressive genetic changes to the homeostatic mechanisms of normal cell proliferation (Hahahan and Weinberg, 2011). Although treatments for this pathology range from surgical to chemotherapeutic (Falzone et al., 2018), chemotherapy is the most common – and indeed, effective (Riaz et al., 2019). However, the side effects of systemic chemotherapy used to treat cancer are often severe, and so serious efforts have been made toward the discovery and development of new anticancer drugs (Shim and Liu, 2014).

To this end, a large number of chalcones – open-chain flavonoids, biosynthesized in a variety of plant species (Karthikeyan et al., 2014) – have been studied in the recent decades for their numerous biological properties, e.g., anti-inflammatory, antioxidant, antimicrobial, antimalarial, and anti-cancer activities (Riaz et al., 2019; Shim and Liu, 2014; Karthikeyan et al., 2014; Aichaoui et al., 2009). The anticancer activity of these chalcones has been a topic of interest due to the association of specific functional groups and substituents to a particular activity or lack thereof against a specific cell line (Prabhakar et al., 2014). For instance, the cytotoxicity activity has been firmly associated with the alkoxy or hydroxy groups, which increases the lipophilicity and confer a strong affinity to cancer cells to the molecule (Boumendjel et al., 2008; Epifano et al., 2007).

Despite abundant chalcone literature, little has been dedicated to oxyprenylated chalcones, which are only recently the object of growing demand for bioactive metabolites of natural and synthetic origin (Epifano et al., 2007). Naturally occurring oxyprenylated chalcones, such as

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cordoin and 4-hydroxycordoin, have a remarkable affinity to type-II estrogen receptors and a marked antiproliferative activity against uterus, ovary, and breast tumor cell lines (Bombardelli et al., 1998). In addition, these compounds have shown cytotoxic or cytostatic effects against prostate and colon cancer cells (Bruyère et al., 2011) and have good antimicrobial activity against gram-positive bacteria (Nowakowska et al., 2008). Synthetic oxyprenylated chalcones, such as 2, 4-dimethoxy-4′-butoxychalcone, have been shown to exhibit potent *in vitro* activity against *Plasmodium falciparum* and against *in vivo* rodent parasites *P. berghei* and *P. yoelii* (Chen et al., 1997). Recently, a number of 4-oxyalkyl-isocordoin analogues have been shown to have anticancer potential. These molecules have been described as a potential agent against human melanoma cell line (A2058) (Russo et al., 2019).

In this context, this study sought to develop a rapid and efficient ultrasound assisted synthesis method for a series of oxyalkylated chalcones (**2**–**19**) derived from natural product 2′,4′-dihydroxychalcone (**1**) and to determine the cytotoxicity of these compounds against a panel of cancer cell lines.

2. Materials and methods

2.1. Chemical and reagents

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA), GIBCO BRL Life Technologies (Grand Island, NY, USA), and Santa Cruz Biotechnology (Santa Cruz, CA). 2′,4′-dihydroxychalcone (**1**) was isolated and characterized as previously reported (Chen et al., 1997).

2.2. Synthesis

A mixture of 2′,4′-dihydroxychalcone (**1**), alkyl bromide and anhydrous potassium carbonate on a ratio of 1:1.2:1 in 2 mL of acetone and a ratio of 1:2:4 in 2 mL of acetonitrile for monoalkylated and dialkylated chalcones, respectively. The reaction mixture was irradiated in water bath of an ultrasonic cleaner (Elmasonic S 10 H, Elma Schmidbauer GmbH, Sigen, Germany) with frequency of 37 KHz and a nominal power of 240 W at 25–30 °C for 20–30 min. After the reaction time the extraction, separation and identification protocols was performed according to reported procedures (Flores et al., 2016; Montenegro et al., 2018).

2.3. Cell culture and proliferation assays

Cell lines were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium, containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in humidified incubators with 5% CO_2. Cytotoxic activities of the compounds against HT-29 (human colorectal adenocarcinoma), MCF-7 (human mammary gland adenocarcinoma) and PC-3 (human prostate adenocarcinoma) cancer cell lines and CCD 841 CoN (colon epithelial) normal cells line were determined using a 48 h sulforhodamine B (SRB) cellular viability assay as described previously (Madrid Villegas et al., 2011). Briefly, cells were treated with different concentrations of molecules **1**–**19** (5, 10, 25, 50 and 100 μM) and incubated for 48 h under the same conditions. Daunorubicin and 5-fluorouracil were used as positive controls.

2.4. Nuclear morphology

Hoechst 33342 was used to observe nuclear morphologic aspects by the procedure previously described in reference (Said et al., 2018). Briefly, on 24-well chamber slides, 1×10^4 cells/mL HT-29, MCF-7 and PC-3 were cultured and exposed to 50 μM compounds for 48 h. The control group was also exposed to ethanol 1%. After treatments 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, the cells were incubated in a dark room at room temperature for 30 min. After being washed, the cells were examined under an immunofluorescence microscope (Olympus IX 81 model inverted microscope, Olympus, Tokyo, Japan).

2.5. Cell treatment

Due to the observation of significant cytotoxicity of selected compounds towards cancer cell lines, the following analyses were done on them only. Cells were treated with compound **3**, **12**, **18** and **19** (25–50 μM).

2.5.1. Flow cytometric determination of reactive oxygen species (ROS) in cells

Reactive oxygen species (ROS) determination was performed by using a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). The fluorescent dye was assessed by the procedure previously described in references (Rhote and Valet, 1990; Wang and Joseph, 1999). Briefly, cancer cells were treated with the different compounds for 24 h. After treatment, cells were further incubated with 10 μM DCFH-DA at 37 °C for 30 min. Subsequently, cells were harvested, rinsed, re-suspended in PBS and analyzed for 2′,7′-dichlorofluorescein (DCF) fluorescence by flow cytometry (FacScalibur, Beckton Dickinson).

2.5.2. Mitochondrial membrane permeability assay

The changes in mitochondrial membrane permeability was performed by using a cationic voltage-sensitive probe Rhodamine 123, as previously described (Montenegro et al., 2014) with minor modifications. Briefly, exponentially growing cells were incubated with ent-labdanes as indicated in the figure legends. Cells were labeled 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, the samples were analyzed by flow cytometry. Data is expressed in percentage of cells stained with rhodamine 123.

2.5.3. Caspase assay

Caspase activity was determined by using the CaspACE™ FITC-VAD-FMK (Promega, Santiago, Chile) as described previously (Said et al., 2018). Briefly, cells were treated with the analyzed compounds (0 and 25 μM) for 48 h. The cells were incubated with CaspACE™ FITC-VAD-FMK in darkness for 20 min at room temperature. Then, the medium was removed and cells were washed twice with PBS. Exposed cells were collected by tripsinization and centrifugation (10 min at 1500×*g*). The supernatant was discarded and the cells were re-suspended in PBS and analyzed by flow cytometry using the filter FL3. Results are expressed as percentage of cells stained with CaspACE™ FITC-VAD-FMK.

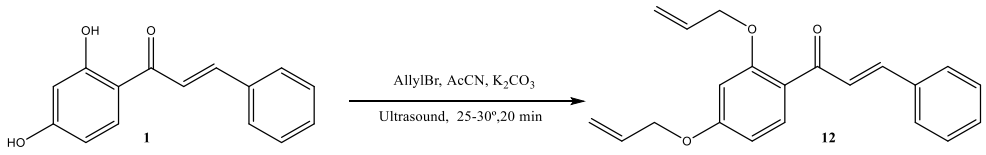
2.6. Statistical analysis

All *in vitro* assays were performed in triplicate and the results expressed as mean values ± SD. Statistical significance was defined as $p < 0.05$. The results were analyzed using the standard method (Said et al., 2018; Montenegro et al., 2014).

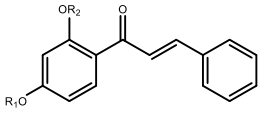
3. Results and discussion

3.1. Synthesis of chalcone derivatives 2-19

Because chalcones exhibit a variety of biological activities for both *in vitro* and *in vivo* models (Wang and Joseph, 1999), their synthesis has experienced explosive growth in recent years. A large quantity of literature is dedicated to synthesis protocols; indeed, this research group recently reported on the synthesis of oxyalkylchalcones by alkylation of the corresponding alkyl bromide in the presence of a slightly basic medium (Flores et al., 2016; Montenegro et al., 2018). In this work,



Scheme 1. Ultrasound assisted synthesis of chalcone derivatives 2-19.



1 R_1=R_2= H

2 R_1=methyl; R_2= H; 94.0%
3 R_1=allyl; R_2= H; 91.2%
4 R_1=2-methyl-1-propenyl; R_2= H; 89.3%
5 R_1=crotyl; R_2= H; 86.8%
6 R_1=1-butenyl; R_2= H; 87.5%
7 R_1=1-pentenyl; R_2= H; 88.0%
8 R_1=prenyl; R_2= H; 84.7%
9 R_1=geranyl; R_2= H; 78.4%
10 R_1=farnesyl; R_2= H; 74.0%
11 R_1=R_2=methyl; 93.8
12 R_1=R_2=allyl; 92.6%
13 R_1=R_2=2-methyl-1-propenyl; 88.8%
14 R_1=R_2=crotyl; 86.4%
15 R_1=R_2=1-butenyl; 85.6%
16 R_1=R_2=1-pentenyl; 83.9%
17 R_1=R_2=prenyl; 82.1%
18 R_1=R_2=geranyl; 79.0%
19 R_1=R_2=farnesyl; 70.0%

Fig. 1. Structure of 2′,4′-dihydroxychalcone **1** and derivatives **2**–**19**.

however, hydroxyl groups of the natural chalcone are substituted with different halides, performed under ultrasound irradiation to shorten the reaction time and improve yield, e.g., that of compound **12** (Scheme 1), whose yield was increased by 12% in a reaction time 3.5h quicker.

Oxyalkylated chalcones **2**–**19** were obtained in good to high yields (70.0%–94.0%) (Fig. 1). NMR data of **2**–**19** were consistent with what we had previously reported (Flores et al., 2016; Montenegro et al., 2018).

3.2. Biological assays

The *in vitro* cytotoxic activity of natural chalcone **1** and its oxyalkylated derivatives **2**–**19** were tested against three human malignant cell lines (HT-29, MCF-7, and PC-3) using the *in vitro* sulforhodamine B (SRB) assay. Daunorubicin and 5-fluorouracil (5-FU) were employed as the positive controls. Recorded IC_{50} values are summarized in Table 1. Interestingly, at the tested concentrations, derivatives showed no cytotoxic effect against normal human cells.

As observed in Table 1, compound **18** showed a strong cytotoxic effect against MCF-7 and PC-3 cells, as is evident from the IC_{50} values obtained, which were below 10 μM, the value established by the US National Cancer Institute plant screening program for considering a pure compound as cytotoxic (Wang et al., 2019). Their cytotoxic effects were superior to that of 5-FU. It is interesting to note that **18** also showed a moderate inhibitory effect against HT-29 with IC_{50} values lower than 20 μM, with a value comparable to daunorubicin. It should be noted that also compounds **12** and **19** showed moderate cytotoxicity values in the three tumor cells. In contrast, the cytotoxic assay demonstrated that the 4′-mono-*O*-alkylated chalcones had no effects, with IC_{50} values higher than 100 μM (Table 1). Among them, compounds **9** and **10** presented values of IC_{50} with weak cytotoxicity values, however, compound **3** exhibited moderate cytotoxic effects with IC_{50} values lower than 30 μM against PC-3 cells. In addition, it is appreciated that the dialkylated molecules presented values of IC_{50} of 2–3 times less in comparison to the monoalkylated molecules.

Moreover, the selectivity index (SI) of the compounds to cancer cells was lower than that of daunorubicin and 5FU (Table 1). However, compound **18** presents selectivity indexes higher than daunorubicin in HT-29 cells, and presents selectivity values higher and comparable to 5FU against MCF-7 and PC-3 cells, respectively. These results suggested that compound **18** could be used as a potential antiproliferative agent against tumor cells, supporting the bibliographic data that the higher SI values the compound is more effective and generates less in healthy cells (Wang et al., 2019).

Table 1
In vitro cytotoxic activity of 2′,4′-dihydroxychalcone 1 and derivatives 2–19.

Compound	IC_{50} (μM)[a]				SI[b]		
	HT-29	MCF-7	PC-3	CCD 841 CoN	HT-29	MCF-7	PC-3
1	100.8 ± 0.6	103 ± 0.7	102.6 ± 0.2	130.8 + 0.2	<1	<1	<1
2	>200	>200	>200	>200	nd	nd-	nd
3	36.3 ± 0.4	34.3 ± 0.9	23.1 ± 0.5	39.7 + 0.4	1.09	1.16	1.72
4	>200	>200	>200	>200	nd	nd-	nd
5	>200	>200	>200	>200	nd	nd	nd
6	>200	>200	>200	>200	nd	nd	nd
7	>200	>200	>200	>200	nd	nd	nd
8	188.9 ± 0.4	188.7 ± 0.8	187.8 ± 0.2	186.6 + 0.4	<1	<1	<1
9	51.3 ± 0.2	55.7 ± 0.4	50.5 ± 0.1	60.8 + 0.2	1.18	1.09	1.20
10	70.2 ± 0.6	84.3 ± 0.1	86.7 ± 0.5	78.9 + 0.2	1.12	<1	<1
11	110.2 ± 0.7	112.1 ± 0.4	100.6 ± 0.5	100.2 + 0.7	<1	<1	<1
12	18.5 ± 0.2	20.3 ± 0.1	25.0 ± 0.2	29.7 + 0.6	1.6	1.46	1.19
13	152.0 ± 0.3	151.9 ± 0.7	148.1 ± 0.9	140.5 + 0.8	<1	<1	<1
14	167.2 ± 0.6	166.9 ± 0.2	162.8 ± 0.6	160.6 + 0.4	<1	<1	<1
15	>200	>200	>200	>200	nd	nd	nd
16	>200	>200	>200	>200	nd	nd	nd
17	102 ± 0.5	103.0 ± 0.4	109.1 ± 0.1	100.6 + 0.7	<1	<1	<1
18	15.3 ± 0.4	8.4 ± 0.5	9.3 ± 0.1	88.8 + 0.2	5.80	10.57	9.54
19	24.1 ± 0.3	29.3 ± 0.2	29.4 ± 0.8	27.9 + 0.1	1.16	<1	<1
Daunorubicin	15.1 ± 0.5	0.33 ± 0.02	0.41 ± 0.04	14.2 + 0.1	<1	43.03	34.63
5-FU	2.9 ± 0.7	22.3 ± 0.2	16.4 ± 0.6	55.8 + 0.3	19.24	2.50	3.40

[a] IC_{50} (μM) was evaluated using SRB assay and ± is the standard deviation from three independent experiments.
[b] SI = Selectivity Index. nd: not determined.

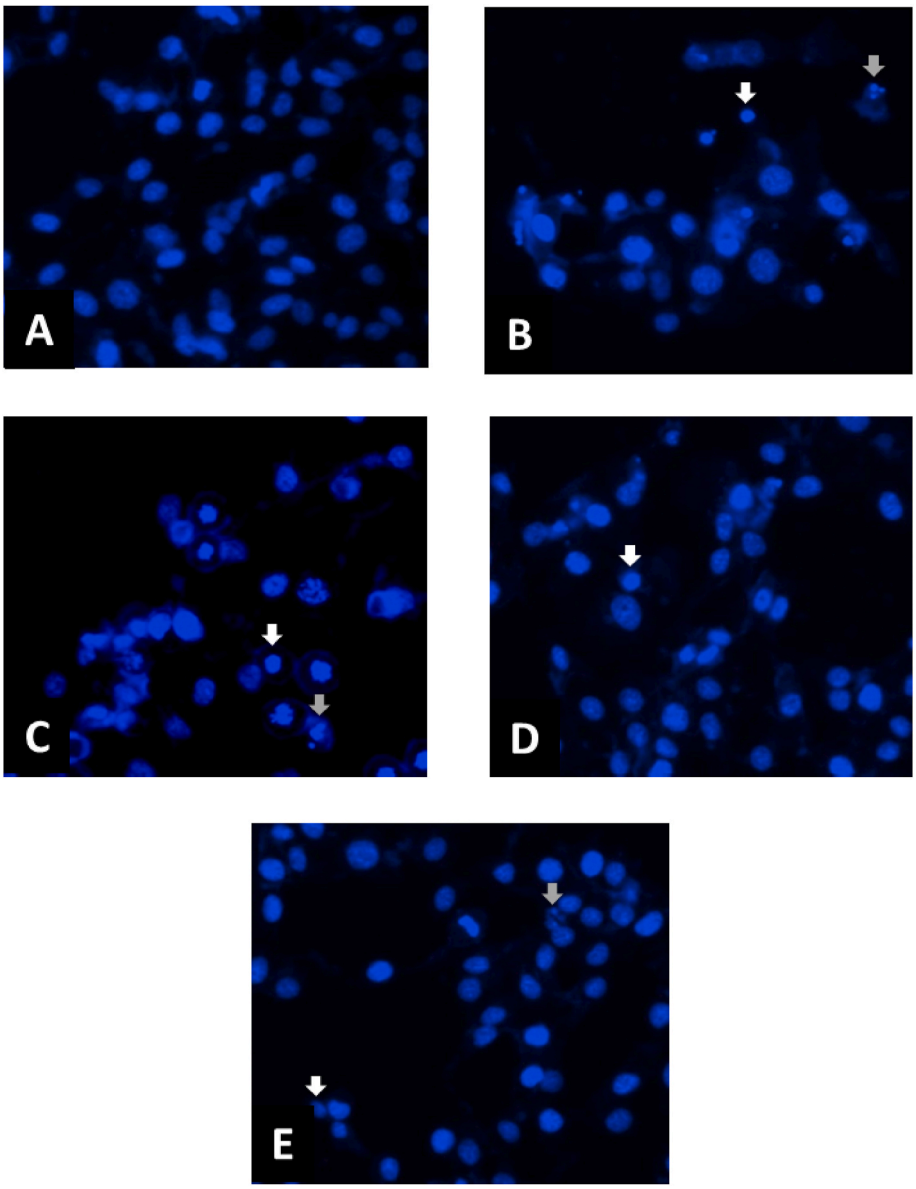


Fig. 2. Effect of compounds on nuclear morphology in MCF-7 cells. Representative photographs presented here show nuclear morphologic changes observed by fluorescent microscopy of the treated cells after Hoechst 33342 staining: (**A**) control (ethanol); (**B**) compound **3**; (**C**) compound **12**; (**D**) compound **18**; (E) compound **19**. Arrow indicates condensed and/or fragmented nuclei.

Previous works have shown the importance of obtaining different oxyalkylchalcones for both medicine and the agro-industrial sector (Rammohan et al., 2020; Ngaini et al., 2012). In another studies, the introduction of an *O*-alkyl chain on the A-ring of chalcone lead to a loss of the cytotoxic activity, except for those molecules which have free hydroxyl groups and one or two alkyl units (Cabrera et al., 2007). Interestingly, the derivative **3**, with an *O*-allyl unit in the aromatic ring and one free hydroxyl group, is active in the three cellular lines. However, dialkylated derivatives **12**, **18** and **19** were shown to be more active against tumor cells. Moreover, it has been shown that the presence of *O*-alkylated chains in the A-ring of chalcones enhances the cytotoxic activity of this group of compounds due to an increase in the lipophilicity of the molecules (Ngaini et al., 2012; Cabrera et al., 2007; Da Silva et al., 2012).

Since compounds **3**, **12**, **18** and **19** had inhibitory effects on the growth of the cancer cell types tested, the effect of these compounds required study in greater detail. To elucidate whether the compounds reduced cell viability in the cell lines tested (HT-29, MCF-7 and PC-3 cells) by inducing apoptosis, cells treated with each active compound **3**, **12**, **18** and **19** were examined after Hoechst 33342 staining. Nuclear changes in all the cell lines were observed under fluorescence microscope (200 ×), Fig. 2 shows MCF-7 cells.

Moreover, condensed and/or fragmented nuclei, as an apoptotic characteristic, were quantified in HT-29, MCF-7 and PC-3 cells, as shown

Table 2
Percentage of condensed and/or fragmented nuclei after treatment with compounds **3**, **12**, **18** and**19**.

Compounds	HT-29	MCF-7	PC-3
3	27.4 ± 3.2*	26.1 ± 3.0*	26.4 ± 4.4*
12	23.0 ± 2.9*	24.6 ± 1.7*	28.6 ± 3.3*
18	17.9 ± 2.3*	21.4 ± 4.7*	19.9 ± 3.5*
19	15.9 ± 2.3*	18.4 ± 2.2*	16.3 ± 2.8*
Control	7.4 ± 0.7	6.8 ± 0.6	6.2 ± 0.8

Values are mean ± S.D. (n = 3); *$p < 0.05$; significantly different from the control-(ethanol) treated cells.

Table 3
Percentage of ROS-positive cells after treatment with compounds **3, 12**, **18** and **19**.

Compound	Concentration (μM)	HT-29	MCF-7	PC-3
3	**25**	30.8 ± 3.2*	25.1 ± 3.3*	16.1 ± 3.3*
	50	39.1 ± 4.4*	46.9 ± 4.9*	27.1 ± 2.2*
12	**25**	39.9 ± 3.3*	35.1 ± 4.0*	21.0 ± 2.9*
	50	53.7 ± 4.4*	53.2 ± 5.7*	37.4 ± 5.1*
18	**25**	29.9 ± 5.5*	53.7 ± 6.1*	17.5 ± 8.3*
	50	36.3 ± 4.4*	60.8 ± 7.3*	29.4 ± 4.2*
19	**25**	34.3 ± 2.8*	16.4 ± 4.2*	15.9 ± 2.3*
	50	36.3 ± 4.4*	26.1 ± 3.0*	27.4 ± 3.2*
Control	-	15.2 ± 0.8	6.1 ± 0.6	7.4 ± 0.7

Values are mean ± S.D. (n = 3); *$p < 0.05$; significantly different from the control-(ethanol) treated cells.

in Table 2.

The data indicates that compounds **3**, **12**, **18** and **19** induce significantly changes in the morphology of the nuclei, which are related to an apoptotic cell death (Basabe et al., 2010). These results are in accord with previous literature data; where, cytotoxicity of chalcones arises its ability to interact with proteins, DNA via several functional groups by ionic interaction (Galluzzi et al., 2009) or by DNA intercalation (Berning et al., 2009).

Numerous studies have indicated that increased caspase-dependent apoptosis, reactive oxygen species (ROS) generation and mitochondrial damage are phenomena, which can be frequently observed altogether in cells subjected to anticancer drugs treatment, that is, the excessive generation or accumulation of ROS can induce oxidative stress, resulting in significant damage to cell structures, loss of cell functioning and cell death by a variety of stimuli (Zarate et al., 2016; Loo, 2003; Wochna et al., 2007). The changes in intracellular ROS levels were made by using the fluorescent probe DCFH-DA. Changes in the percentage of DCF-positive cells in treated cells versus ethanol-treated cells was interpreted as increase of the amount of internal ROS. Compounds **3**, **12**, **18** and **19** caused a significant increase of intracellular ROS in all cell lines used (Table 3).

In addition, a representative histogram showing ROS generation in MCF-7 cells is presented in Fig. 3.

The active compounds **3**, **12**, **18** and **19** significantly increased the production of ROS in MCF-7 cells compared to untreated (ethanol) cells (Fig. 3). Compound **18** showed the highest ROS level (8.8-fold higher) ($p < 0.05$) at the lowest concentration used (25 μM). Which shows that chalcones induce an apoptotic cell death mechanism by producing ROS, which coincides with what was previously reported for other alkyl-chalcones as cordoin, flavokawain B and xanthohumol (Boumendjel et al., 2008; Brodská and Holoubek, 2011; Kuo et al., 2010).

It has been described that ROS can also be involved the change of the mitochondrial membrane permeability (Festa et al., 2011; Lee et al., 2007), because they can cause oxidative damage and tissue dysfunction which can cause irreversible damage to macromolecules with

Table 4
Percentage of rhodamine 123 stained-cells after treatment with compounds **3, 12**, **18** and **19** on HT-29, MCF-7 and PC-3-treated cells.

Compound	Concentration (μM)	HT-29	MCF-7	PC-3
3	**25**	55.7 + 5.4*	44.9 ± 6.1*	57.5 ± 6.7*
	50	81.3 ± 7.3*	74.6 ± 8.2*	82.3 ± 6.9*
12	**25**	59.3 ± 6.6*	41.9 ± 5.0*	47.4 ± 6.2*
	50	73.3 ± 5.9*	44.6 ± 5.1*	53.2 ± 5.4*
18	**25**	73.3 ± 4.4*	63.5 ± 5.0*	66.2 ± 4.3*
	50	81.7 ± 8.7*	76.3 ± 9.1*	69.9 ± 8.3*
19	**25**	62.4 ± 6.7*	74.4 ± 4.1*	64.3 ± 3.2*
	50	83.3 ± 8.2*	74.9 ± 4.3*	75.9 ± 7.2*
Control	-	92.0 ± 10.2	81.4 ± 8.3	84.5 ± 7.5

Values are mean ± S.D. (n = 3); *$p < 0.05$; significantly different from the control-(ethanol) treated cells.

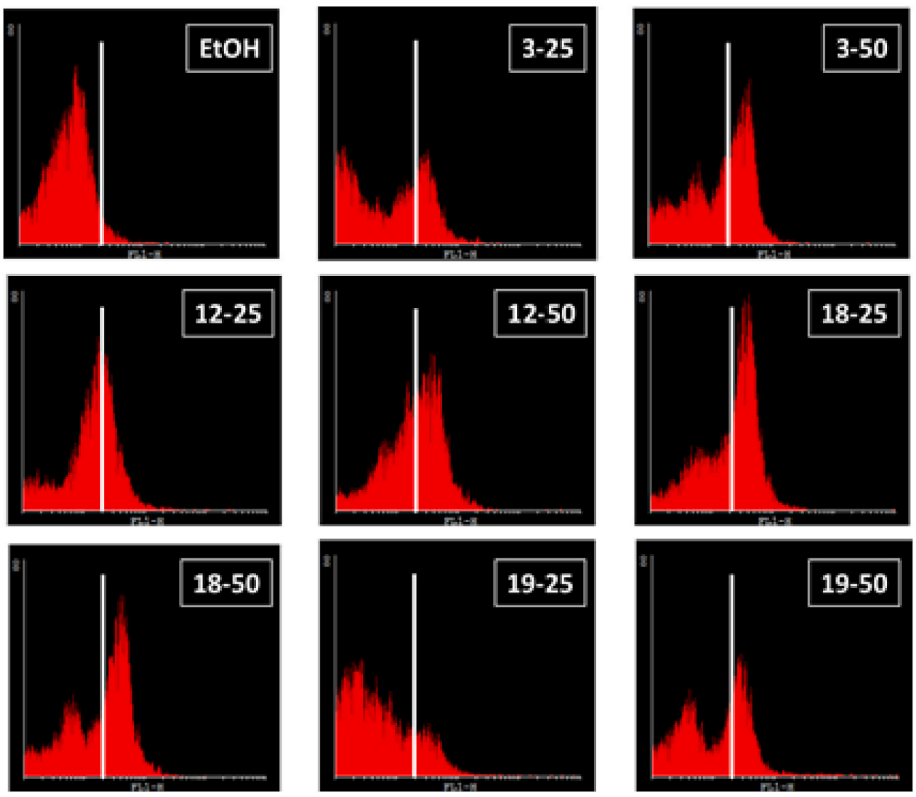


Fig. 3. Histogram showing ROS generation after treatment with different concentration of the compounds **3**, **12**, **18** and **19** in MCF-7 cells. Compounds-treated (25 and 50 μM) MCF-7 cells were stained with DCFDA and subjected to flow cytometry analysis.

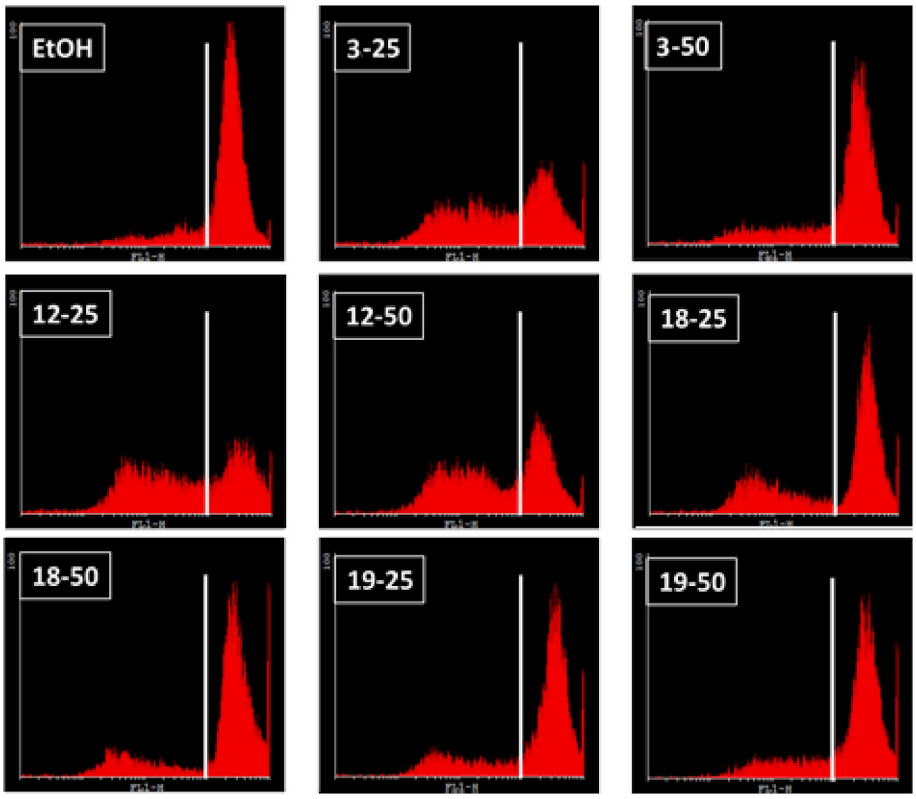


Fig. 4. Histogram showing mitochondrial membrane permeability after treatment with different concentration of the compounds **3**, **12**, **18** and **19** in MCF-7 cells. Compounds-treated (25 and 50 μM) MCF-7 cells were stained with rhodamine 123 and subjected to flow cytometry analysis.

consequent loss of their function and cell damage (Venditti and Di Meo, 2020). To check the effect of compounds **3**, **12**, **18** and **19** on mitochondrial membrane potential, rhodamine 123 staining was carried out in compounds **3**, **12**, **18** and **19** treated HT-29, MCF-7, and PC-3 cells (25 and 50 μM). The percentage of rhodamine 123 stained-cells are summarized in Table 4. Clearly, mitochondrial membrane potential plays important role in activation of cellular apoptosis (Vercesi et al., 1997).

Additionally, a representative histogram showing changes of mitochondrial membrane permeability in MCF-7 cells is presented in Fig. 4.

As shown in Fig. 4, all the compounds decreased the mitochondrial membrane permeability, however, we observed that MCF-7 cells when treated with compound **12** showed a biggest loss of mitochondrial permeability effect. These data indicate that the mitochondrial function is affected by the treatments with the compounds. Moreover, compounds that dissipate the mitochondrial membrane potential, such as valinomycin (for potassium ions) or dinitrophenol (DNP, for protons), prevent rhodamine 123 uptake (Villena et al., 2013). Similar results are triggered by electron transport inhibitors such as, antimycin A (Chen, 1988), or rotenone (Han et al., 2008). These data correlate with the results shown in the viability and ROS essays. In addition, results of the present study are in accord with previous studies demonstrating that chalcones were good to candidates in producing apoptosis in cancer cell lines through inhibition of mitochondrial functionality (Li et al., 2003; Hsu et al., 2006).

In an attempt to assess whether the inhibitory effects on cell growth associated with effects on caspase-mediated apoptosis pathway, caspases activity was measured. As shown in Fig. 5 the activation of caspases is higher in cells exposed to compounds **3**, **12**, **18** and **19** than in ethanol-treated cells except for HT-29 cells. In other words, compounds **3**, **12**, **18** and **19** induce the activation of caspases suggesting a cell death by apoptosis.

On the whole, all the four compounds showed increase in caspases activity with increase in concentration suggesting that there is the

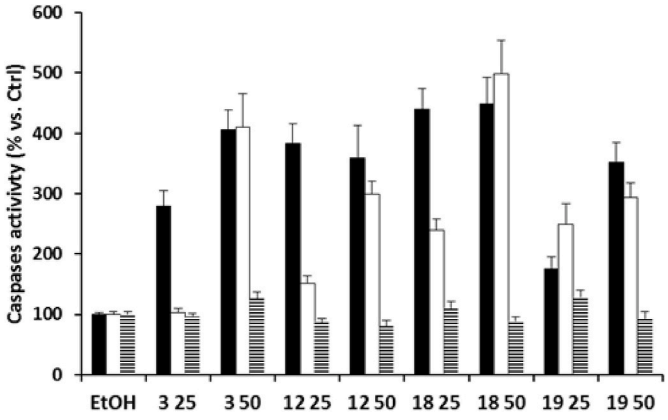


Fig. 5. Effect of compounds on caspases activation of different cell lines. Activation of caspases in MCF-7 (black bars), PC-3 (white bars) and HT-29 (dashed bars) cells treated with compounds. Data is reported as ratio of activities of treated cells and ethanol-treated cells, which were arbitrarily assigned a unitary value. Data are reported as mean values ± SD of three different experiments with samples in triplicate.

activation of apoptotic pathways (Hsu et al., 2006; Syam et al., 2012; Singh et al., 2001). These data suggest that the introduction of oxyalkyl chains on the A-ring of chalcone may be a contributory factor in caspases activation, bearing in mind factors such as the size of the substituent, position in the ring and its lipophilicity (Abdullah and Ravanan, 2018; Khoshneviszadeh et al., 2012). On the other hand, to explain why caspases activation of compounds **18** and **19** on HT-29 decrease when the doses increase is a challenge, because always we assume a relationship between caspases activation and apoptosis, however, it could be possible that at a higher dose, a different cell death mechanism appears like a

necrotic process, characterized by the rapid loss of plasma membrane integrity cell death (Green and Llambi, 2015). Elucidating the precise mechanism or combination of these is a new challenge that requires further study.

Finally, we think changes in membrane permeability produced by these chalcones would be due to a non-ionic surfactant effect, altering the mitochondrial functionality dissipating the proton gradient or altering electronic transport. For that reason continuation of this research is to elucidate the mechanism of action that produces in mitochondrial membrane this type compounds, including the *in vivo* studies.

4. Conclusion

In conclusion, a rapid and convenient approach for the introduction of *O*-alkyl into the structure of 2′,4′-dihydroxychalcone by ultrasound irradiation have been developed and the alkyl chain characteristics contribute to an increase in cytotoxic activity and are able to trigger apoptotic death in cancer cell lines tested. Among all the compounds tested, compound **18** display strong growth inhibitory effects in cancer cells *in vitro*. Therefore, these promising results strongly encourage further *in vivo* studies with the purpose of producing new molecules with anti-proliferative activities in the near future.

CRediT authorship contribution statement

Joan Villena: Methodology, Formal analysis, Writing - review & editing. **Iván Montenegro:** Writing - review & editing, Software. **Bastian Said:** Investigation. **Enrique Werner:** Formal analysis, Writing - review & editing. **Susana Flores:** Investigation. **Alejandro Madrid:** Conceptualization, Writing - original draft, Methodology, Writing - review & editing, Resources.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2021.111969.

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