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Original article

Hydroxychalcones induce apoptosis in B16-F10 melanoma cells via GSH and ATP depletion

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ABSTRACT

Searching for leading compounds of new drugs for cancer therapy, we studied the toxicity of 13 hydroxychalcones never tested before toward melanoma cell line (B16-F10). The compounds were obtained by aldolic condensation between aldehydes and hydroxylated acetophenones, in alkaline conditions. Three of them showed cytotoxicity to the cell line. Two of them induced mitochondrial GSH and ATP depletion and promoted cell death through apoptosis in melanoma cells. One of the compounds induced cell death through necrosis but did not significantly decrease the intracellular mitochondrial GSH and ATP levels in melanoma cells. The results suggest that the predominant factor for the activity is the molecule shape, and secondarily the number of hydroxyl groups.

1. Introduction

Malignant melanoma is a highly lethal disease, and the incidence and mortality associated to this kind of cancer are increasing worldwide. Currently, between 2 and 3 millions non-melanoma skin cancers and 132.000 melanoma skin cancers occur globally each year [1]. Many factors predispose an individual to skin cancer including personal and family histories of the disease and geographic location [2]. The metastasis and growth of tumors depend on the cell capability to migrate, implant, adhere and penetrate into the membrane, reaching the blood vessel and the tissue [3,4]. Selected B16 murine cell lines (B16-F10 and B16-F1) showed a different metastatic potential: the F10 line colonized the lungs more efficiently than F1; the F10 cell line was the most invasive and also the most virulent in the subcutaneous implantation model [3].

Among the drugs exerting anticancer activity against malignant melanoma, dacarbazine has been extensively used, however, with response rates of 11–25% and with short survival time. Thus, the development of chemotherapy agents against malignant melanoma is justified [5].

Chalcones are essential intermediate compounds in the flavonoid biosynthesis in plants. Naturally occurring chalcones are mainly in hydroxylated form and several reports have documented their biological properties [6]. Many studies have demonstrated antitumoral [7–11], anti-inflammatory [12], antibacterial [13], antiulcerogenic [14], antioxidant [6,15], antimalarial [16] and antileishmaniosis [16,17] activities for hydroxychalcones, aside from other pharmacological effects. Concerning antitumoral activity, Kobori et al. [7] described that the 2′,4′,6,4-tetrahydroxidihydrochalcone (phloretin) induced cell death by apoptosis in B16 mouse melanoma 4A5 and in HL-60 human leukaemia cell lines. Iwashita et al. [9] showed that the isoliquiritigenin (2′,4′,4-trihydroxychalcone) and butein (2′,4′,3,4-tetrahydroxychalcone) inhibited cell proliferation and induced apoptosis in B16 melanoma 4A5 cells; also, Hsu et al. [11] described that isoliquiritigenin induced apoptosis and blocked the cell cycle progression in Hep G2 (human hepatoma cells). For new antitumoral molecules it is very important to demonstrate cell toxicity through apoptosis. The

Abbreviations: DNA, deoxyribonucleic acid; mDNA, mitochondrial DNA; ATP, adenosine triphosphate; GSH, glutathione; GSSG, oxidized gluthathione; DMEM, Dulbecco's modified Eagle's medium; NMR, nuclear magnetic resonance spectroscopy; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NADPH, nicotinamide adenine dinucleotide phosphate.

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death through apoptosis is characterized by nuclear condensation that induces DNA fragmentation caused by endonucleolytic cleavage of genomic DNA [18]. The apoptosis or programmed cell death is a mechanism to control the cell proliferation, thus the resistance of malignant cell to apoptosis is hallmark of cancer and constitutes an important clinical problem [19].

The mitochondria is responsible for the production of 80–90% of ATP needed for cell respiration and survival and play an important role in the pro-and anti-apoptotic stimuli [20]. Skulachev [21] showed the inter-relations between bioenergetic processes and programmed death phenomena, and concluded that ATP is required for certain steps of apoptosis and necrosis. Usually the malfunctioning of mitochondria results in strong decrease in the initial cellular content of ATP, however, in some cases the apoptosis is accompanied by the increase of ATP concentration at early stages followed by a decrease at final stage. The energy production is also affected by mitochondrial glutathione (GSH) depletion. Changes in mitochondrial GSH content have also been related with apoptosis regulation, cell differentiation and growth [22,23].

The objective of this research is based on the fact that melanoma cells have a relatively poor response to the apoptotic stimulus, [24] and that solid tumor cells develop resistance to antineoplasic drugs and that multidrug resistance is a major cause of chemotherapy failures by human malignances [25]. The B16-F10 melanoma cell line has high metastatic potential, and in general does not respond easily to cytotoxic agents. Thus, in this work we studied and characterized the murine B16-F10 melanoma cell death via apoptosis, induced by hydroxychalcones. The results suggest that the principal causes were mitochondrial GSH and ATP depletion. An analysis of the structure was performed to find out a relationship between the cytotoxic effects.

2. Chemistry

Thirteen hydroxychalcones were prepared by aldolic condensation as presented in Fig. 1. Chalcones **1**, **3** and **6** are derived from 1-naphtaldehyde; chalcones **4** and **5** are derived from 2-naphtaldehyde, in order to observe the influence of hydrophobic and voluminous groups in the B-ring in the activity of the hydroxychalcones. Chalcones **2** and **7** are derived from xanthoxyline (2-hydroxy-4,6-dimethoxyacetofenone), to evaluate the influence of two methoxyl groups in the A-ring beyond the presence of a hydroxyl group and to observe the influence of the aldehydes substituted with electron donors or electron acceptor groups; chalcones **8**–**13** are derived from monobromide xanthoxyline (2-hydroxy-3-bromo-4,6-dimethoxychalcone), to verify how much the activity would be affected by the addition of bromide in the A-ring and to observe the influence of the electron donor or electron acceptor substituents in the B-ring on the activity of the compounds. The obtained yields were between 36% and 66%.

The structures of the compounds **5**, **6**, **8**, **9**, **10** and **13**, (new compounds), were confirmed by chemical identification data: ^{1}H NMR, ^{13}C NMR, IR and elemental analysis. For chalcone **1**, the corresponding peak of the hydroxyl group does not appear because probably during analysis the hydroxyl group was exchanged with a deutered hydrogen atom of the chloroform, the solvent used. ^{1}H NMR spectra revealed that all structures were geometrically pure and configured *E* ($J_{H\alpha\text{-}H\beta} = 15.2$–$16.4$ Hz).

3. Biological results and discussion

Initially, a selection of the hydroxychalcone toxicity for melanoma B16-F10 cells was done. The compounds **1**, **3** and **13** reduced cell viability by $97.7 \pm 0.7\%$, $75.2 \pm 1.5\%$ and $50 \pm 1.9\%$, respectively, when compared with the control (non-treated cells) (Fig. 2). All other chalcones presented a non-significant effect at the tested concentration. A total of 100 μM was chosen as maximal concentration because much higher doses do not normally reach the blood plasma [26,27] and we are looking for compounds with high activity (low IC_{50}) which could eventually be used in the future as a drug for chemotherapy with very low to no side effects.

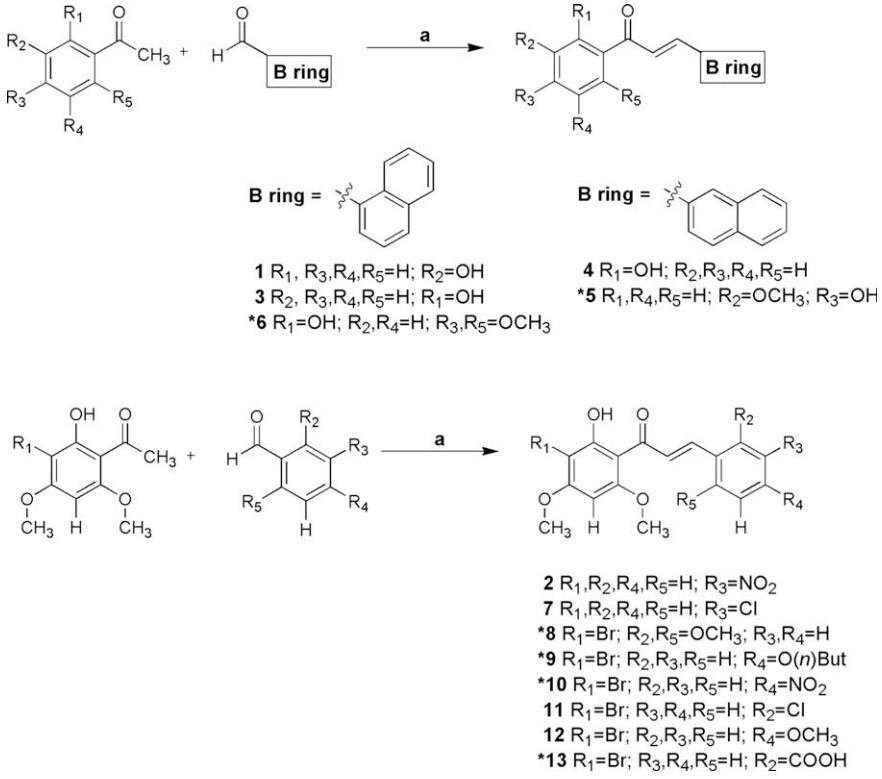

Fig. 1. Synthesis of chalcones. The reaction consists of an aldolic condensation as described in Section 4 (a) methanol, KOH 50%, r.t., 24 h. *New compounds.

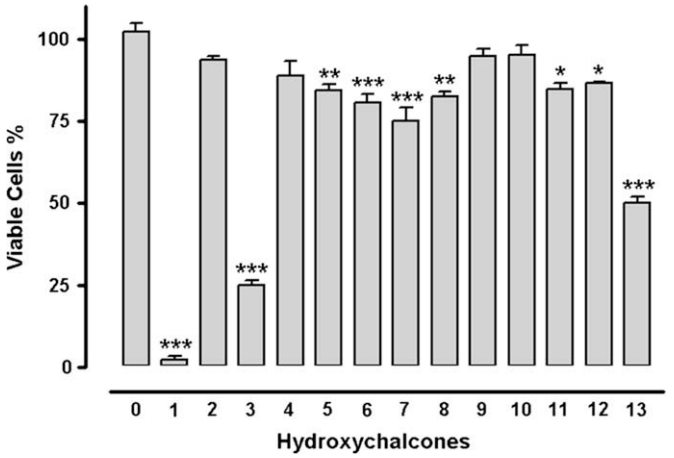

Fig. 2. Toxicity of hydroxychalcones for B16-F10 cell line. The cells (1×10^5) were incubated with 100 μM of the compounds, for 24 h. The cell viability was monitored through MTT assay. Optical density of control groups (C) was taken as 100% of cell viability. Each point represents the mean ± S.E.M. of three experiments in triplicates. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control groups.

Recently, Cabrera et al. [10] have investigated the relation between the structure of some chalcones, flavonones and flavones and their activity against human kidney carcinoma cells TK-40, human mammary adenocarcinoma cells MCF-7 and human colon adenocarcinoma cells HT-29. In general, no cytotoxic selectivity between the different cellular lines was observed. However, in the same substituted series the activity decreased following the sequence chalcones > flavonones > flavones. Some other requirements for activity are the presence of the hydroxyl group that has hydrogen bond donor capability and the presence of the conjugated enone group, since the compounds lose their activity when changed to an aldol structure.

The importance of the presence of a hydroxyl substituent was demonstrated previously by Sabzevari et al. [8] via measurements of the cytotoxic activity of 12 hydroxylated chalcones toward K562 leukaemia or melanoma cells. They observed that an increase in the number of hydroxyl groups on an aromatic ring decreases the pK_a value of the first hydroxyl group suggesting that chalcones with lower pK_a values are less toxic to hepatocytes. However, it should be noted that the arrangement of the hydroxyl groups on the A-ring or B-ring plays a crucial role in determining the mechanism of cytotoxicity toward isolated rat hepatocytes. Thus, if the hydroxyl group is in *ortho* position the chalcone can be metabolized to a quinone intermediate which could react with cell nucleophile and deplete GSH.

In this case, the importance of the hydroxyl group in A-ring for activity is apparent as can be observed in chalcones **1** and **3**. However, this activity is conditioned by the conformation of the molecule as observed with chalcones **4** and **5** where the B-ring consists of 1-naphtyl or 2-naphtyl groups. This is also observed with chalcone **6** where the presence of a methoxyl group in position 6 also changes the conformation of the molecule. This detail may also explain the lack of strong activity of chalcones **2**, **7**–**12**, xanthoxyline derivatives. The presence of a methoxy group in position 6 should give a steric effect forcing the carbonyl group out of the plane of the A-ring and then decrease the hydrogen bond strength with the 2-hydroxyl groups. This conformation change appears to be important to maintain the activity of these compounds. Chalcone **13** with a carboxyl substituent in B-ring exhibits a good activity that may be explained by the probable formation of a nine-membered ring between this group and the carbonyl group. Thus, the planarity and the rigidity of the molecular structure seem to be important factors to the activity in these cases.

We further investigated the cytotoxic effect of the more active compounds which were **1**, **3** and **13** and the IC_{50} values were determined from concentration–response curve, which are 12 ± 0.7 μM, 17 ± 1.5 μM and 30 ± 1.9 μM, respectively (Fig. 3).

In order to elucidate the mechanism of death of B16-F10 cells induced by hydroxychalcones we investigated if these compounds could induce DNA fragmentation, a biochemical marker of apoptosis [28]. The lanes (B and C) that correspond to the DNA of the cells incubated with compounds **1** and **3**, respectively, showed fragmented DNA distributed in bands with defined sizes, characteristic of DNA fragmentation by apoptosis (Fig. 4). The lanes that correspond to the DNA from the cells incubated without hydroxychalcones (A) as well as the lane which corresponds to DNA from cells incubated with hydroxychalcone **13** (D), showed uniform chromatin. Since the hydroxychalcone **13** induced cell death (Fig. 1), it seems to be through another mechanism, not apoptosis.

The mitochondria is a central organelle involved in apoptosis, with the capacity to directly activate the execution pathways [29] a process in which ATP is directly involved. Thus, the compounds were incubated with the cells and the ATP depletion profile was analyzed. The hydroxychalcones **1** and **3** decreased the ATP content of the melanoma cells in a concentration-dependent manner (Fig. 5B); the amount of ATP depletion was higher than the percentage of cell death (Fig. 5A). The cell death through apoptosis can be a consequence of cell energy loss, caused by the compounds [31]. Possibly the hydroxychalcone **13** induced cell death through necrosis, since DNA breakage induced by this compound is not characteristic of apoptosis (Fig. 4). However, it did not significantly alter the ATP intracellular content of melanoma cells as expected for death induced by necrosis. These results are in part, in accordance with the data from the literature by Hu et al. [30] which demonstrated that IN6CPBD induces apoptosis in human melanoma through a mitochondrial dysfunction. Sabzevari et al. [8] suggested that the cytotoxic activity of the phloretin, isoliquiritigenin, and of 10 other hydroxylated chalcones, toward K562 leukaemia or melanoma cells are due to their ability to mitochondrial uncoupling. Cao et al. [29] also detected the induction of apoptosis through mitochondrial hyperpolarization and mtDNA

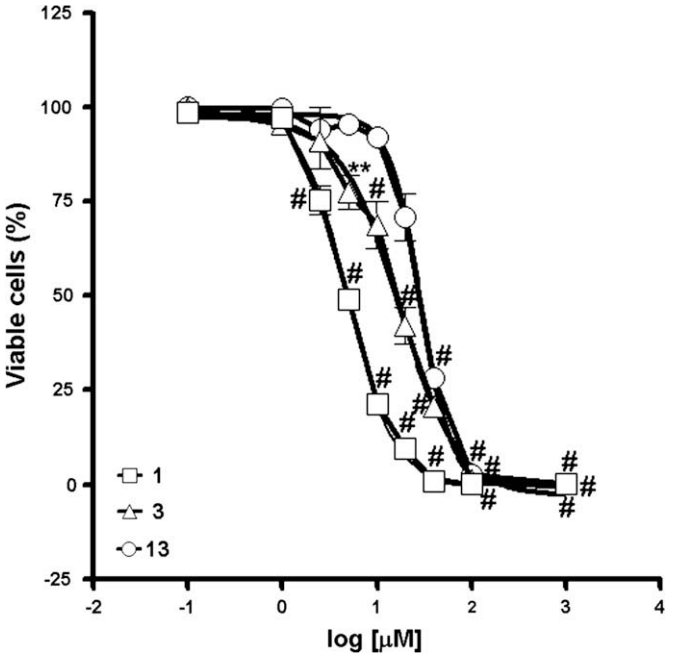

Fig. 3. Concentration–response curves of hydroxychalcones **1**, **3** and **13** on B16-F10 cell line. The cells (1×10^4) were incubated with the compounds (0–40 μM) for 72 h. Optical density of control groups was taken as 100% of cell viability. Each bar represents the mean ± S.E.M. of three experiments. $^{**}P < 0.01$ and $^{\#}P < 0.001$ compared to control groups.

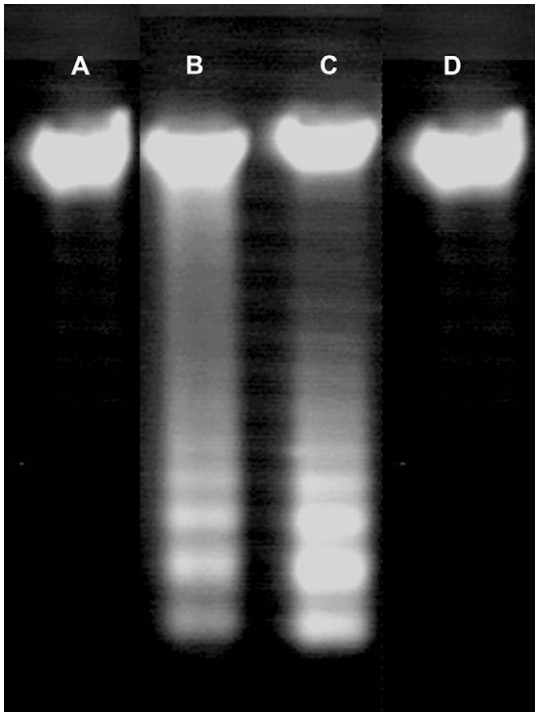

Fig. 4. DNA fragmentation of B16-F10 cells induced by hydroxychalcones. The cells (5×10^5) were incubated for 24 h with: 0 μM, Lane A – Control; Lane B – 12 μM of compound **1**; Lane C – 17 μM of compound **3**; Lane D – 30 μM of compound **13** (IC_{50} concentrations for cell toxicity).

damage in human hepatoma G2 cells by curcumin. Garland and Halestrap [22] also showed that the depletion of intracelular ATP, by ATPase mitochondrial uncoupling capabilities, induced cell death through apoptosis, exhibiting DNA fragmentation in cell lines pro-B interleukin-3 (IL-3)-dependent.

We further analyzed a possible effect of hydroxychalcones on mitochondria function measuring mitochondrial GSH content in melanoma cells after incubation with the compounds. Fig. 6 shows that the compounds **1** and **3** reduced the amount of mitochondrial GSH after 24 h of incubation, this reduction may be related to ATP depletion induced by these compounds. Kachadouriam and Day [32] showed that low GSH content in the mitochondria of human lung epithelial cells A549, treated with 2′,5′-hydroxychalcone was related to mitochondrial dysfunction. The GSH, present in mitochondria, plays an important role in the control of reactive oxygen species generation, in the integrity of mitochondrial membranes, release of proapoptotic factors and in the modulation of cell death by several pathways [33]. Moreover, the GSH in mitochondria protects the integrity of mitochondrial components, thus playing a determinant role in response to apoptosis.

The hydroxychalcones **1** and **3** may be considered promising molecules, based on the fact that mitochondrial GSH and ATP depletion cause mitochondrial dysfunction and the rapid growth of tumor cells is highly energy dependent.

In summary, 13 hydroxychalcones were synthesized, and **1**, **3** and **13** exhibited effective cytotoxicity against melanoma B16-F10 cells. Compound **1** was the most active, with an IC_{50} value of 12 μM. Compounds **1** and **3** induced cell apoptosis, suggested to occur through mitochondrial GSH and ATP depletion. Compound **13** did not induce cell death through apoptosis nor did it induce ATP depletion. The main similarity between chalcones **1** and **3** is the presence of a unit of 1-naphtyl at B-ring. The higher activity of compound **1** seems to be due to the free hydroxyl group at A-ring. This is an interesting compound since it occurs naturally on the chalcones in this same position [34].

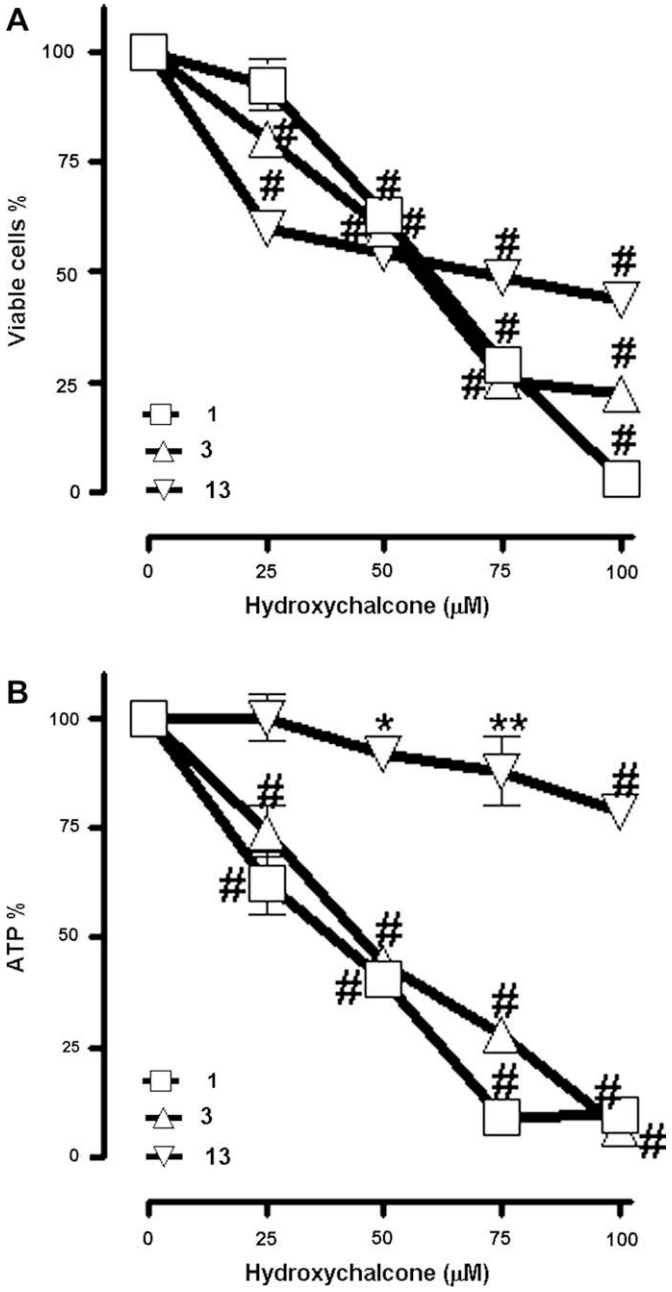

Fig. 5. Effect of hydroxychalcones **1**, **3** and **13** on ATP amounts, compared to cytotoxic effect, in B16-F10 cells. The cells (1×10^5) were incubated with the compounds (0–100 μM) for 24 h to analyze the cell viability (A). 3×10^5 cells were incubated with the compounds at the same concentrations for 24 h, for ATP concentration measurements (B). Each bar represents the mean ± S.E.M. of three experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.001$ compared to control groups.

Compounds **1** and **3** were selected to continue the research. Further studies are being developed by our group to elucidate the mechanism of action. The analyses of the molecule structures suggest that the predominant factor for activity is the molecule shape, and secondarily, the number of hydroxyl groups in the chalcone.

4. Experimental

4.1. Preparation of the compounds

The compounds were prepared by aldolic condensation. All reagents used were obtained commercially (Merck, Sigma–Aldrich),

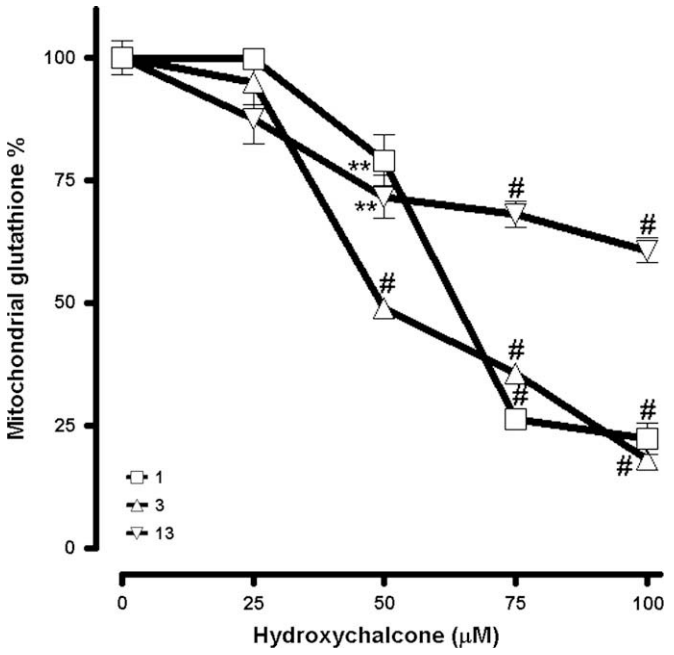

Fig. 6. Effect of hydroxychalcones **1**, **3** and **13** on mitochondrial GSH content. The cells (3×10^5) were incubated with the compounds (0–100 μM) for 24 h. Mitochondrial glutathione (GSH + GSSG) was measured using the glutathione reductase method, as described in Section 4. Each bar represents the mean ± S.E.M. of three experiments in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.001$ compared to control groups.

except the xanthoxyline (2-hydroxy-4,6-dimethoxyacetofenone) and the monobromide xanthoxyline (2-hydroxy-3-bromo-4,6-dimethoxychalcone), that were prepared as previously described [35]. All chalcones were prepared by magnetic agitation of hydroxylated acetophenone (2 mmol), methanol (30 mL), KOH 50% w/v (5 mL) and the corresponding aldehyde (2 mmol), at room temperature for 24 h. Distilled water and chloric acid 10% were added in the reaction for total precipitation of the compounds. The compounds were then obtained by vacuum filtration and later recrystallized in dichloromethane and hexane. The chalcones **1**, **3**, **4**, **7** and **12** were previously cited, respectively, by Hollinshead [36], Subbanwad and Vibhute [37], Liu et al. [16], Hsu et al. [38] and by Bora et al. [39]. The compounds **2** and **11** were described by Boeck and co-workers [17] and the structures **5**, **6**, **8**–**10** and **13** are new chalcones.

4.2. Physico-chemical data of the compounds

The purified chalcones were obtained in yields between 36% and 66%. The structures were identified using melting points (m.p.), infrared spectroscopy (IR), ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR), and to the unpublished ones, also elementary analyses. Melting points were determined with a Microquímica MGAPF-301 apparatus and are uncorrected. IR spectra were recorded with an Abb Bomen FTLA 2000 spectrometer on KBr disks. NMR (^{1}H and ^{13}C NMR) were recorded on Varian Oxford AS-400 (400 MHz), using tetramethylsilane as an internal standard. Elementary analyses were obtained with a CHNS EA 1110. Percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values to C). The purity of the synthesized chalcones was analyzed by thin-layer chromatography (TLC) using Merck silica pre-coated aluminum plates 200 μm in thickness with several solvent systems of different polarities. Compounds were visualized with ultraviolet light ($\lambda = 254$ and 360 nm) and using sulfuric anisaldehyde solution followed by heat as developing agent and purified by recrystallization from hexane and dichloromethane. ^{1}H NMR spectra revealed that all the structures were geometrically pure and configured *E* ($J_{H\alpha\text{-}H\beta} = 15.2$–16,4 Hz).

4.3. Physico-chemical data of synthesized compounds

4.3.1. (2E)-1-(3′-Hydroxyphenyl)-3-(1-naphtyl)-2-propen-1-one

Yellow solid, m.p. 174–175. ^{1}H NMR ($CDCl_3$) δ 7.10 (d, 1H, $J = 8.0$ Hz, H4′), 7.40 (dd, 1H, H5′), 7.51–7.65 (m, 5H, H3, H4, H6, H7, H2′), 7.60 (d, 1H, $J = 15.6$ Hz, Hα), 7.90 (d, 2H, H5, H8), 7.94 (d, 1H, $J = 8.4$ Hz, H6′), 8.26 (d, 1H, $J = 8.0$ Hz, H2), 8.67 (d, 1H, $J = 15.6$ Hz, Hβ). ^{13}C NMR ($CDCl_3$) δ 115.07 (C2′), 120.22 (C4′), 120.32 (C6′), 123.41 (Cα), 124.97 (C2), 125.56 (C3), 125.89 (C8), 126.52 (C6), 127.30 (C7), 129.07 (C4), 130.08 (C5), 130.93 (C5′), 132.01 (C9), 132.34 (C10), 134.16 (C1), 139.91 (C1′), 140.39 (Cβ), 158.06 (C3′), 189.09 (C=O). IR ν_{max}/cm^{-1} 3176, 1642, 1351, 1571, 1273, 1475, 1172, 962, 772, 707 (KBr). Anal. Calcd for $C_{19}H_{14}O_2$: C 83.19, H 5.14. Found: C 83.42, H 5.21. Yield: 51%.

4.3.2. (2E)-1-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-(3-nitrophenyl)-2-propen-1-one

Orange solid, m.p. 171–172 °C. ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 3.94 (s, 3H, OCH_3), 5.98 (s, 1H, H5′), 6.12 (s, 1H, H3′), 7.74 (d, 1H, $J = 15.6$ Hz, Hα), 7.84–7.88 (m, 2H, H4, H5), 7.98 (d, 1H, $J = 15.6$ Hz, Hβ), 8.22 (d, 1H, $J = 7.75$ Hz, H6), 8.46 (s, 1H, H2), 14.09 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 55.56 (OCH_3), 55.82 (OCH_3), 91.37 (C5′), 93.84 (C3′), 106.20 (C1′), 122.08 (C2), 123.99 (C4), 129.78 (Cα), 130.51 (C5), 134.01 (C6), 137.43 (C1), 138.68 (Cβ), 148.71 (C3), 162.45 (C2′), 166.45 (C6′), 168.46 (C4′), 191.78 (C=O). IR ν_{max}/cm^{-1} 1640, 4580 (KBr). Anal. Calcd for $C_{17}H_{15}NO_6$: C 62.00, H 4.59, N 4.59. Found: C 62.08, H 4.26. Yield: 51%.

4.3.3. (2E)-1-(2′-Hydroxyphenyl)-3-(1-naphtyl)-2-propen-1-one

Yellow solid, m.p. 106–108 °C. ^{1}H NMR ($CDCl_3$) δ 6.97 (dd, 1H, $J = 7.4$ Hz, H5′), 7.06 (d, 1H, $J = 8.4$ Hz, H3′), 7.51–7.62 (m, 4H, H3, H6, H7, H4′), 7.76 (d, 1H, $J = 15.2$ Hz, Hα), 7.90–7.99 (m, 4H, H6′, H4, H5, H8), 8.29 (d, 1H, $J = 8.4$ Hz, H2), 8.79 (d, 1H, $J = 15.2$ Hz, Hβ), 12.88 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 118.93 (C3′), 119.17 (C5′), 122.99 (Cα), 123.67 (C2), 125.59 (C8), 125.68 (C1′), 126.67 (C3), 127.41 (C6), 129.07 (C4, C5), 130.00 (C9, C6′), 131.48 (C10), 136.74 (C1, C4′), 142.66 (Cβ), 163.93 (C2′), 194.12 (C=O). IR ν_{max}/cm^{-1} 3451, 1635, 1351, 1576, 1203, 1015, 3047, 1435, 1162, 972, 760 (KBr). Anal. Calcd for $C_{19}H_{14}O_2$: C 83.19, H 5.14. Found: C 83.88, H 5.18. Yield: 57%.

4.3.4. (2E)-1-(2′-Hydroxyphenyl)-3-(2-naphtyl)-2-propen-1-one

Yellow solid, m.p.: 135–137 °C (lit. p.f.: 146–148 °C)[132]. ^{1}H NMR ($CDCl_3$) δ 6.96 (d, 1H, $J = 8.0$ Hz, H3′), 7.03–7.10 (m, 1H, H5′), 7.48–7.54 (m, 3H, H3, H4, H4′), 7.75 (d, 1H, $J = 16.0$ Hz, Hα), 7.78–7.89 (m, 5H, H5, H6, H7, H8, H6′), 7.93 (d, 1H, $J = 16.0$ Hz, Hβ), 8.04 (s, 1H, H1), 12.89 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 118.89 (C3′), 120.41 (C5′), 121.93 (C3), 123.89 (Cα), 125.65 (C1′), 126.80 (C6), 127.13 (C7), 127.33 (C1), 128.40 (C5), 129.08 (C8), 129.94 (C4), 131.39 (C6′), 132.31 (C10), 133.57 (C9), 134.78 (C2), 136.65 (C4′), 145.79 (Cβ), 163.86 (C2′), 193.91 (C=O). IR ν_{max}/cm^{-1} 3195 (OH), 1689 (C=O), 1568 (C=C), 3046, 1482, 1432, 1021, 985, 819, 752 (Ar) (KBr). Anal. Calcd for $C_{19}H_{14}O_2$: C 83.19, H 5.14. Found: C 82.04, H 5.18. Yield: 66%.

4.3.5. (2E)-1-(3′-Methoxy-4′-hydroxyphenyl)-3-(2-naphtyl)-2-propen-1-one

Light yellow solid, m.p.: 166–168 °C. ^{1}H NMR ($CDCl_3$) δ 4.01 (s, 3H, OCH_3), 6.10 (s, 1H, OH), 7.02 (d, 1H, $J = 8.0$ Hz, H5′), 7.29 (s, 1H, H2′), 7.52–7.54 (m, 1H, H6′), 7.66–7.72 (m, 3H, H3, H6, H7), 7.83 (d, 1H, $J = 15.6$ Hz, Hα), 7.80–7.87 (m, 3H, H4, H5, H8), 7.97 (d, 1H, $J = 15.6$ Hz, Hβ), 8.05 (s, 1H, H1). ^{13}C NMR (DMSO-d_6) δ 56.37 (*m*-OCH_3), 110.74 (C2′), 114.05 (C5′), 121.99 (C3), 123.96 (Cα), 126.97

(C6′), 127.51 (C6), 128.03 (C7), 128.85 (C1), 128.91 (C8), 130.68 (C4, C5), 131.33 (C1′), 132.81 (C10), 133.63 (C9), 134.54 (C2), 144.30 (Cβ), 147.15 (C4′), 150.64 (C3′), 188.74 (C=O). IR ν_{max}/cm^{-1} 3265 (OH), 1643, 1202 (C=O), 1280, 1025 (C–O), 1563 (C=C), 2950, 2835, 1522, 1445, 970, 844, 816, 779 (Ar) (KBr). Anal. Calcd for $C_{20}H_{16}O_3$: C 78.93, H 5.30. Found: C 78.86, H 5.76. Yield: 39%.

4.3.6. (2E)-1-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-(1-naphtyl)-2-propen-1-one

Yellow solid, m.p. 116–117 °C. ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 5.98 (s, 1H, H3′), 6.14 (s, 1H, H5′), 7.50–7.61 (m, 3H, H3, H6, H7), 7.83–7.92 (m, 3H, H4, H5, H8), 7.97 (d, 1H, $J = 15.2$ Hz, Hα), 8.31 (d, 1H, H2), 8.61 (d, 1H, $J = 15.2$ Hz, Hβ), 12.83 (OH). ^{13}C NMR ($CDCl_3$) δ 55.87 (OCH_3), 56.15 (OCH_3), 91.54 (C5′), 94.03 (C3′), 101.63 (C1′), 124.00 (Cα), 125.39 (C2), 125.71 (C8), 126.46 (C3), 127.03 (C6), 128.93 (C7), 130.46 (C4), 130.55 (C5), 132.02 (C9), 133.32 (C10), 133.99 (C1), 139.49 (Cβ), 162.79 (C2′), 166.55 (C6′), 168.68 (C4′), 192.79 (C=O). IR ν_{max}/cm^{-1} 3450, 1627, 1341, 1571, 1215, 1109, 1440, 1153, 975, 810, 768 (KBr). Anal. Calcd for $C_{21}H_{18}O_4$: C 75.43, H 5.43. Found: C 75.66, H 5.94. Yield: 58%.

4.3.7. (2E)-1-(2′-Hydroxy-4′,6′-dimethoxyphenyl)-3-(3-chlorophenyl)-2-propen-1-one

Yellow solid, m.p.: 104–106 °C. ^{1}H NMR ($CDCl_3$) δ 3.84 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 5.97 (s, 1H, H3′), 6.11 (s, 1H, H5′), 7.33–7.35 (m, 3H, H4, H5, H6), 7.57 (s, 1H, H2), 7.68 (d, 1H, $J = 15.6$ Hz, Hα), 7.87 (d, 1H, $J = 15.6$ Hz, Hβ), 13.95 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 55.89 (OCH_3), 56.19 (OCH_3), 91.58 (C5′), 94.02 (C3′), 106.50 (C1′), 126.88 (Cα), 128.09 (C6), 129.10 (C2), 130.07 (C4), 130.35 (C5), 135.06 (C3), 137.68 (C1), 140.70 (Cβ), 162.73 (C2′), 166.68 (C6′), 168.68 (C4′), 192.51 (C=O). IR ν_{max}/cm^{-1} 3451 (OH), 1624, 1215 (C=O), 1029 (C–O), 1576 (C=C), 2936, 1439, 977, 909, 822 (Ar) (KBr). Anal. Calcd for $C_{17}H_{15}ClO_4$: C 64.06, H 4.74. Found: C 64.61, H 5.39. Yield: 36%.

4.3.8. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(2,6-imethoxyphenyl)-2-propen-1-one

Orange solid, m.p. 193–194 °C. ^{1}H NMR ($CDCl_3$) δ 3.91 (s, 6H, OCH_3), 3.96 (s, 6H, OCH_3), 6.04 (s, 1H, H5′), 6.57 (d, 2H, $J = 8.4$ Hz, H3, H5), 7.28 (t, 1H, H4), 8.29 (d, 1H, $J = 15.6$ Hz, Hα), 8.34 (d, 1H, $J = 15.6$ Hz, Hβ), 14.65 (s, 1H, OH). ^{13}C NMR (acetone-d_6) δ 55.72 ($2CH_3$), 56.06 (OCH_3), 56.32 (OCH_3), 88.35 (C5′), 94.76 (C3′), 104.21 (C1′, C3, C5), 112.81 (C1), 129.11 (Cα), 132.35 (C4), 134.85 (Cβ), 160.81 (C2, C6), 162.35 (C6′), 162.91 (C2′), 166.31 (C4′), 205.56 (C=O). IR ν_{max}/cm^{-1} 3442, 2941, 1616, 1552, 1325, 1253, 1193, 916, 782 (KBr). Anal. Calcd for $C_{19}H_{19}BrO_6$: C 53.92, H 4.52. Found: C 53.67, H 4.44. Yield: 42%.

4.3.9. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(4-buthoxyphenyl)-2-propen-1-one

Yellow solid, m.p. 172–173 °C. ^{1}H NMR ($CDCl_3$) δ 0.98 (t, 3H, –CH_3), 1.47–1.60 (m, 2H, –CH_2CH_3), 1.75–1.81 (m, 2H, –$CH_2CH_2CH_3$), 3.98 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 4.01 (t, 2H, –OCH_2–), 6.06 (s, 1H, H5′), 6.91 (d, 2H, $J = 8.4$ Hz, H3, H5), 7.54 (d, 2H, $J = 8.4$ Hz, H2, H6), 7.75 (d, 1H, $J = 15.6$ Hz, Hα), 7.83 (d, 1H, $J = 15.6$ Hz, Hβ), 14.96 (OH). ^{13}C NMR (acetone-d_6) δ 13.42 (CH_3), 19.18 (–CH_2CH_3), 31.28 (–$CH_2CH_2CH_3$), 56.25 (OCH_3), 67.86 (–OCH_2–), 88.37 (C5′), 94.61 (C3′), 104.43 (C1′), 115.19 (C3, C5), 124.46 (Cα), 128.04 (C1), 130.71 (C2, C6), 143.72 (Cβ), 160.02 (C4), 168.46 (C2′), 168.82 (C6′), 176.12 (C4′), 198.07 (C=O). IR ν_{max}/cm^{-1} 3450, 2945, 1615, 1554, 1221, 963 (KBr). Anal. Calcd for $C_{21}H_{23}BrO_5$: C 57.94, H 5.33. Found: C 57.27, H 5.27. Yield: 47%.

4.3.10. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(4-nitrophenyl)-2-propen-1-one

Yellow solid, m.p. 230–231 °C. ^{1}H NMR ($CDCl_3$) δ 3.94 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 6.03 (s, 1H, H5′), 7.61 (d, 2H, $J = 8.0$ Hz, H2, H6), 7.74 (d, 1H, $J = 16.0$ Hz, Hα), 7.93 (d, 1H, $J = 160$ Hz, Hβ), 8.24 (d, 2H, $J = 8.0$ Hz, H3, H5), 14.73 (s, 1H, OH). ^{13}C NMR ($CDCl_3$) δ 55.74 (OCH_3), 56.32 (OCH_3), 86.69 (C5′), 91.63 (C3′), 106.55 (C1′), 109.77 (C3, C5), 123.78 (Cα), 126.66 (C2, C6), 128.82 (C1), 131.03 (Cβ), 150.47 (C4), 161.92 (C6′), 162.62 (C2′), 162.68 (C4′), 203.27 (C=O). IR ν_{max}/cm^{-1} 3450, 2943, 1600, 1509, 1345, 1093, 976, 852 (KBr). Anal. Calcd for $C_{17}H_{14}BrNO_6$: C 50.02, H 3.46, N 3.43. Found: C 49.57, H 3.02, N 4.53. Yield: 42%.

4.3.11. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(2-chlorophenyl)-2-propen-1-one

Light yellow solid, m.p. 210–212 °C. ^{1}H NMR ($CDCl_3$) δ 3.98 (s, 3H, OCH_3), 4.04 (s, 3H, OCH_3), 6.21 (s, 1H, H5′), 7.29–7.32 (m, 2H, H4, H5), 7.43 (d, 1H, H3), 7.67 (d, 1H, H6), 7.83 (d, 1H, $J = 15.2$ Hz, Hα), 8.18 (d, 1H, $J = 15.2$ Hz, Hβ), 14.67 (s, 1H, OH). ^{13}C NMR (acetone-d_6) δ 56.85 (OCH_3), 57.08 (OCH_3), 87.80 (C5′), 92.80 (C3′), 106.00 (C1′), 127.72 (Cα), 128.60 (C5), 130.22 (C6), 131.06 (C3), 131.62 (C4), 134.33 (C2), 136.21 (C1), 139.49 (Cβ), 162.90 (C2′), 163.00 (C6′), 163.93 (C4′), 193.25 (C=O). IR ν_{max}/cm^{-1} 3450, 2934, 1618, 1557, 1216, 1125, 965, 788 (KBr). Anal. Calcd for $C_{17}H_{14}BrClO_4$: C 51.35, H 3.55. Found: C 51.45, H 3.58. Yield: 62%.

4.3.12. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(4-methoxyphenyl)-2-propen-1-one

Yellow solid, m.p. 144–146 °C. ^{1}H NMR ($CDCl_3$) δ 3.86 (s, 3H, OCH_3), 3.97 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 6.07 (s, 1H, H5′), 6.93 (d, 2H, $J = 8.8$ Hz, H3, H5), 7.56 (d, 2H, $J = 8.8$ Hz, H2, H6), 7.79 (d, 1H, $J = 16.4$ Hz, Hα), 7.89 (d, 1H, $J = 16.4$ Hz, Hβ), 14.81 (OH). ^{13}C NMR (acetone-d_6) δ 55.17 (OCH_3), 56.25 (OCH_3), 56.37 (OCH_3), 88.34 (C5′), 94.76 (C3′), 106.66 (C1′), 114.70 (C3, C5), 124.61 (Cα), 128.10 (C1), 130.68 (C2, C6), 143.59 (Cβ), 162.17 (C4), 162.92 (C2′, C6′), 163.28 (C4′), 205.72 (C=O). IR ν_{max}/cm^{-1} 3443, 1615, 1557, 1220, 1177, 824 (KBr). Anal. Calcd for $C_{18}H_{17}BrO_5$: C 54.98, H 4.36. Found: C 51.95, H 4.46. Yield: 40%.

4.3.13. (2E)-1-(2′-Hydroxy-3′-bromo-4′,6′-dimethoxyphenyl)-3-(2-carboxyphenyl)-2-propen-1-one

Yellow solid, m.p. 230 °C. ^{1}H NMR (acetone-d_6) δ 3.79 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 6.00 (s, 1H, H5′), 7.64 (t, 1H, $J = 8.0$ Hz, H4), 7.64 (d, 1H, $J = 16.0$ Hz, Hα), 7.79 (d, 1H, $J = 8.0$ Hz, H6), 7.87 (t, 1H, $J = 8.0$ Hz, H5), 7.87 (d, 1H, $J = 16.0$ Hz, Hβ), 7.92 (d, 1H, $J = 8.0$ Hz, H3), 14.23 (COOH), 14.57 (OH). ^{13}C NMR (acetone-d_6) δ 57.25 (OCH_3), 57.74 (OCH_3), 88.12 (C5′), 94.78 (C3′), 106.14 (C1′), 124.40 (Cα), 126.49 (C6), 130.09 (C4), 130.73 (C2), 132.61 (C3), 134.88 (C5), 135.66 (C1), 136.76 (Cβ), 156.72 (C6′), 165.62 (C2′), 166.90 (C4′), 180.67 (COOH), 206.97 (C=O). IR ν_{max}/cm^{-1} 3441, 2942, 2640, 1627, 1559, 1216, 1125, 964, 781 (KBr). Anal. Calcd for $C_{18}H_{15}BrO_6$: C 53.09, H 3.71. Found: C 49.08, H 3.71. Yield: 59%.

4.4. Biological assays

4.4.1. Reagents

Tissue culture media and fetal bovine serum were purchased from CULTILAB (São Paulo, Brazil), penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA), isopropanol was purchased from Merck (Darmstadt, Germany), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma (St. Louis, MO, USA). The luciferin–luciferase kit was purchased from Bio-Orbit® (Turku, Finland).

4.4.2. Cell culture

Murine B16-F10 melanoma cell lines were obtained from American Type Culture Cell (ATCC). The cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM

HEPES. The cell cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere and pH 7.4. Once the cells reached 90% of confluence, a cell suspension was obtained by trypsinization. In all experiments, viable cells were checked in the beginning of the experiment by Trypan Blue exclusion.

4.4.3. Cytotoxicity

The cytotoxic effect of synthetic hydroxychalcones was evaluated using an MTT assay. Briefly, 1×10^4 cells/well were incubated for 72 h at 37 °C, in triplicate, with compounds dissolved in DMSO (0.1% final concentration) and diluted with culture medium to their final concentrations, in 96-well microplates. After the incubation time, the cells were washed with new culture medium and 10 μL MTT (5 mg/mL) were added followed by 3 h of incubation at 37 °C. The precipitated formazan was dissolved in 100 μL of acid isopropanol solution (isopropanol containing HCl 0.04 M) and the absorbance was measured at 540 nm using microwell system reader (Organon Teknika, Belgium). The cells were incubated with the hydroxychalcones in a concentration range of 0–40 μM, following with MTT assay. The IC_{50} values (a concentration that produces 50% reduction of viable cell number) were calculated from the concentration–response curves. The cell viability was checked in the beginning of experiment by Trypan Blue exclusion.

4.4.4. DNA fragmentation analysis by gel electrophoresis

Briefly, 5×10^5 cells/well were incubated for 24 h with compounds dissolved in DMSO (0.1% final concentration) and diluted with culture medium to final concentrations (the IC_{50} for cell viability of each compound). To isolate DNA fragments, the cells were harvested and washed twice with cold phosphate-buffered saline (PBS). Cell pellets were then incubated in a lyses buffer containing EDTA 10 mM, Tris–HCl 50 mM, at pH 8.0, NP-40 – 0.25%, proteinase K 0.5 g/L at 50 °C for 2 h. The DNA was then precipitated with 2.5 vol of ethanol–NaCl at −25 °C overnight and dried in air. After washing with ice-cold 70% ethanol, the pellets were then dissolved in TE buffer containing Tris-HCl 10 mM, at pH 8.0, EDTA 1 mM and RNase A 0.6 mg/mL and incubated at 37 °C for 1 h. DNA solutions were then separated in agarose gels 1.5%, stained with ethidium bromide and visualized by 2UV Transilluminator (MacroVue UV-20 Hoefer) for ladder formation.

4.4.5. Bioluminescent assay for ATP

The amount of intracellular ATP was determined by bioluminescent assay measuring the light output of the luciferin–luciferase reaction. Approximately 3×10^5 cells were incubated in the presence of hydroxychalcones at different concentrations then pelleted and extracted with 40 μL of trichloroacetic acid 1.25%. The cell extracts were kept on ice for 30 min and neutralized with 20 μL of Tris-acetate 1 M, at pH 7.5. After centrifugation, the supernatants were diluted with the same buffer (×4) and used for ATP quantification.

4.4.6. Mitochondrial glutathione measurement

Mitochondria enriched preparation was obtained by centrifugation [40]. Total glutathione (GSH + GSSG, reduced and disulphide forms, respectively) was measured through the glutathione reductase method [41]. Approximately 3×10^5 cells were washed in PBS and homogenized in a buffer containing Tris-sacarose 10 mM/250 mM, resuspended in 100 μL chilled Milli-Q water containing EDTA 1 mM and sonified for 10 s. Then, 20 μL of each homogenate were transferred to a 96-well plate followed by addition of 180 μL of the reaction medium containing DTNB 75 μM; NADPH 120 μM, glutathione reductase 1 U/mL and EDTA 10 mM in phosphate buffer 100 mM, at pH 7.4. A standard curve was made with GSH (0.001–1 μM). The absorbance was measured immediately and then every minute during 5 min at 412 nm using a Microwell Systems (Organon Teknika). Values were expressed as percentage of control GSH values.

4.4.7. Statistical analysis

The statistical program GraphPad Prism® 4.0 Windows (GraphPad software, Inc., San Diego, CA, 2003) [42] was used to calculate the IC_{50} and the statistical significance. The results were presented as mean ± S.E.M. of triplicates from three independent experiments. Statistical significance was assessed by ANOVA followed by Bonferroni's *t*-test, p values <0.05 were taken as statistically significant.

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