Anticancer efficacies of persicogenin and homoeriodictyol isolated from Rhus retinorrhoea

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**ABSTRACT**

Herein we examined two flavonones (persicogenin and homoeriodictyol) isolated from Rhus retinorrhoea to elucidate the mechanism of their anticancer effects in MCF-7, HeLa, and HT-29 cells. Based on the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (MTT) cytotoxicity data of persicogenin (500 μg/ml) caused a 58.1 % reduction in HT-29 survival while homoeriodictyol (500 μg/ml) caused a 51.9 %, 66.7 % and 76.2 % reductions in MCF-7, HeLa and HT-29 cell survival, respectively. The neutral red uptake (NRU) assay revealed 53.6 %, 53.9 %, 58.8 % and 83.0 %, 87.7 %, 66.7 % reductions in MCF-7, HeLa, and HT-29 cell survival following persicogenin and homoeriodictyol (500 μg/ml) treatment, respectively. Moreover, the intracellular reactive oxygen species (ROS) was significantly enhanced and dysfunction of mitochondrial membrane potential (ΔΨm) confirmed the mitochondrial injury in all cell types by the flavonones. MCF-7, HeLa, and HT-29 cells exposed to persicogenin and homoeriodictyol (500 μg/ml) had showed 42.5 %, 63.1 %, 62.3 % and 30.7 %, 30.2 %, 23.8 % cells in the sub G1 apoptotic phase. The persicogenin- and homoeriodictyol-treated cell lines had upregulated expressions of p53, caspase-3, caspase-9, bax, and superoxide dismutase 1 (SOD1) genes. Such findings provide novel insight into the comparative anti-cancer efficacy of persicogenin and homoeriodictyol, signifying their promising clinical applications as cancer treatments and their application as bioactive therapeutic agents.

**ARTICLE INFO**

Keywords:
Rhus retinorrhoea
Homoeeriodictyol
Persicogenin
Flavanone
Anti-cancer
Apoptosis

1. Introduction

Despite the technological advancement in medical sciences, cancer is still responsible for millions of deaths globally. Early detection of tumors in cancer using non-invasive imaging by radiomics plays a promising role in distinguishing different tumor types. However, these interventions are expensive; and patients are prone to radiation exposure; and a high rate of false-positive indications is found [1]. The most common treatment regimen for cancer is chemotherapeutic drugs, and they cause many side effects in patients. Consequently, the quest to discover anti-cancer drugs with enhanced efficacy and less side effects continues.

Since ages plants have been utilized as a reliable source of medicines, and they play vital role in healthcare. In particular, the identification of anticancer compounds from natural sources have had a remarkable influence on the medical industry. Among the clinically used cancer drugs, approximately 60 % are derived from natural products or a natural source [2]. Flavonoids, including the subclass of flavonones are low molecular weight secondary metabolites biosynthesized via the phenylpropanoid metabolic pathway [3]. Based on the oxidative status, and the type and number of substituents on the heterocyclic ring flavonoids are subdivided into six classes; isoflavones, flavan-3-ols (or catechins), flavonones, anthocyanidins, flavonols, and flavones. Flavonoids possess several health benefits, including anti-oxidant, anti-inflammatory, anti-bacterial, and anticancer activities. The anticancer effects of flavonoids were previously linked to oncogene regulation, carcinogen metabolism, and tumor suppressor gene expression during cell proliferation and differentiation, which trigger apoptosis [4].

The Anacardiaceae family, which includes the genus Rhus (also...
known as Sumac), has more than 250 species of flowering plants [5]. The geographical distribution of Rhus ranges from temperate to tropical regions worldwide. Many species of the genus Rhus have been traditionally used to treat gonorrhea, syphilis, gangrene, and dysentery [6] and have demonstrated wound curing activity [7]. Furthermore, other species of Rhus were found to exhibit anti-oxidant [8], cytotoxic and hypoglycemic [9,10], anti-microbial [11], and anti-thrombin activities [12]. Previously, the use of Rhus in agricultural application was reported after its insecticidal effect against aphids was demonstrated [13]. Within the Arabian Peninsula, many Rhus retinorrhoea trees are found in the southern parts of Saudi Arabia. In particular, its leaves have been utilized as a traditional medicine in Saudi Arabia to control inflammation of the intestinal cells, such as enterocytes or mucus producing cells [30].

Hence, in this study, persicogenin and homoeriodictyol were examined to elucidate their anticancer effects in MCF-7, HeLa, and HT-29 cells, and unravel their discerning action to induce (i) cytotoxicity (ii) reactive oxygen species (ROS) generation (iii) mitochondrial dysfunction (iv) cell cycle dysregulation and (v) transcriptional regulation of apoptotic and oxidative stress genes.

2. Materials and methods

2.1. Materials

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), neutral red dye, culture-grade dimethylsulfoxide (DMSO), phosphate buffered saline (PBS) (Ca++, Mg++ free), propidium iodide (PI), RNase A, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and rhodamine 123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal Bovine Serum (USA Origin; FBS), antibiotic-antimycotic solution (100x), and trypsin (0.1 %, 0.25 EDTA) were purchased from GIBCO and ThermoFisher Scientific (USA). The RNA isolation kit (iPrep™ PureLink™) was purchased from Invitrogen (USA). Oligo-p(dT)12-18 primer was purchased from GE Health Care (United Kingdom). SYBR Green I Master for real-time PCR was procured from Roche Diagnostics (Rotkreuz, Switzerland). The cell culture wares were purchased from Nunc Pvt. Ltd. (Denmark). All other chemicals were purchased from commercial sources and were of analytical grade or better. The chemicals were used as supplied by the manufacturers without further purification.

2.2. Isolation of flavonones and the cell culture

Rhus retinorrhoea was collected from the Balas Village southern region of Saudi Arabia and identified by Dr. Sultan-ul-Abdeen College of Pharmacy, King Saud University. A voucher specimen No. 15,371 was deposited in the herbarium of the College of Pharmacy King Saud University, Saudi Arabia. Persicogenin and homoeriodictyol were isolated from Rhus retinorrhoea by using a previously reported protocol [19]. 1H-NMR, 13C-NMR and 2D-NMR spectroscopy (refer to supplementary Figs. S1-S10) were used to identify the compounds; the results were found to align with the previously reported values (Fig. 1) [19]. The anticancer activities of persicogenin and homoeriodictyol were assessed in MCF-7, HeLa, and HT-29 cells. Cells were grown in DMEM supplemented with 10 % FBS and 1 ml/100 ml of (100x) antibiotic-free --.

![Fig. 1. Structure of the two flavonones isolated from Rhus retinorrhoea, purified, and chemically characterized in our lab [19].](image-url)
antimycotic solution at 37 °C in a CO₂ incubator (5 %) at a humidity of 95 %. The cell batches were assessed to determine their viability by using trypan blue; batches showing > 95 % of viability were used in the experiments.

2.3. MTT cytotoxicity assay

The MTT cytotoxicity assay was performed to measure the metabolic activity of cells and quantify the amount of NADPH-dependent cellular oxidoreductase enzymes required to reduce the MTT dye to the insoluble formazan product. Cytotoxicity analysis with the MCF-7, HeLa, and HT-29 cells was performed with a previously described method [31]. Briefly, after the confluence of cells grown overnight was recorded, the old medium was replaced with fresh medium supplemented with 100, 250, and 500 μg/ml of persicogenin and homoeriodictyol. Cells were then incubated for 24 h at 37 °C in a CO₂ (5 %) incubator. After the exposure, 10 μl/well of MTT dye (5 mg/ml) were added to 100 μl of cell suspension and incubated at 37 °C for 4 h. After aspirating the aqueous medium from the mixture, we added 200 μl/well of DMSO to the cells. Thereafter, the plates were placed on a rocker shaker for 10 min and their absorbance was measured at 550 nm in a microplate reader (Thermo Scientific, Finland). Control cells were neither treated with persicogenin nor homoeriodictyol. The above concentrations of the flavonones were selected via cytotoxicity screening experiments. As the lower concentrations (10–50 μg/ml) of persicogenin and homoeriodictyol did not exhibit any cytotoxic effects in the three cell types, they were excluded from further experiments.

2.4. Neutral red uptake (NRU) assay

The NRU assay was performed to quantify the live cells that could actively bind to the neutral red dye in lysosomes, and cross the cell membrane by non-ionic diffusion; dead cells cannot uptake the dye owing to alterations in their membrane integrity. The NRU dye in the three cell lines was assessed by the NRU assay according to our previously described method [32]. In brief, the culture medium of MCF-7, HeLa, and HT-29 cells containing persicogenin and homoeriodictyol (100, 250, and 500 μg/ml) for 24 h was discarded. Thereafter, the cells were washed with PBS. Neutral red dye (50 μg/ml) in a fresh culture medium was then added to each well and incubated for 3 h. After incubation, the medium was discarded and washing of the cells was done with a solution containing 0.5 % formaldehyde and 1 % calcium chloride. To extract the neutral red dye, a mixture of ethanol (50 %) and acetic acid (1 %) was poured into each well and incubated for 20 min at 37 °C. Absorbance was then recorded at 540 nm. The control cells (i.e., untreated cells from the three cell types) were run under identical conditions and compared to the treated cells.

2.5. Quantitation of intracellular ROS

ROS generation in cells upon exposure to both flavanones were quantified according to the peroxide-dependent oxidation of DCFH-DA to the highly fluorescent 2′,7′- dichlorodihlorofluorescein (DCF). ROS generation in MCF-7, HeLa, and HT-29 cells was quantified using our previously described method [32]. After 24 h of exposure to persicogenin and homoeriodictyol (100, 250, and 500 μg/ml), cells were detached and centrifuged (4 °C) at 3000 rpm for 5 min. Cells were washed twice with PBS and resuspended in 500 μl of PBS containing the DCFH-DA dye (5 μM). All tubes were incubated at 37 °C for 1 h in a CO₂ (5 %) incubator and the subsequent analysis of 10,000 cells was performed on a flow cytometer (Beckman Coulter Epics XL/XI-MCL, USA) at a laser excitation of 488 nm. Qualitative analysis of ROS generation due to persicogenin and homoeriodictyol exposure in the above cell lines was carried out. Briefly, MCF-7, HeLa, and HT-29 (1 × 10⁵ cells/well) cells were grown on sterile glass chamber slides at 37 °C for 24 h in a CO₂ (5 %) incubator. Thereafter, the cell lines were separately exposed to 500 μg/ml of persicogenin and homoeriodictyol for 24 h under identical conditions. After exposure, the medium was discarded and cells were stained with 100 μl of PBS containing the DCFH-DA dye (5 μM) for 1 h. The green fluorescence exhibited by the DCF dye was recorded on a fluorescence microscope (Nikon Eclipse 80i, Japan). Images were captured at a magnification of 20x using a color camera attached to the microscope.

2.6. Mitochondrial membrane potential (ΔΨm)

To determine whether persicogenin and homoeriodictyol induced changes in the mitochondrial membrane potential (ΔΨm), staining was performed with Rh123, a cationic fluorescent dye that strongly accumulates electrophoretically in the negatively charged mitochondrial matrix of control cells. Briefly, persicogenin- and homoeriodictyol-(100, 250, and 500 μg/ml) treated MCF-7, HeLa, and HT-29 cells were trypsinized, spun down at 3000 rpm for 5 min, washed twice with PBS, and resuspended in PBS (500 μl) containing the Rh123 dye (5 μg). All tubes were stored in a CO₂ (5 %) incubator at 37 °C for 1 h with intermittent mixing. The fluorescence of 10,000 cells stained with Rh123 was recorded on a flow cytometer at the FL1 log channel, with laser excitation of 488 nm [32]. Qualitative analysis of ΔΨm dysfunction caused by persicogenin and homoeriodictyol exposure in MCF-7, HeLa, and HT-29 cells was assessed. In brief, the three cell lines were grown (1 × 10⁵ cells/well) on sterile glass chamber slides at 37 °C for 24 h in a CO₂ (5 %) incubator. Thereafter, the cell lines were separately exposed to 500 μg/ml of persicogenin and homoeriodictyol for 24 h under identical conditions. After exposure, the medium was discarded and cells were stained with 100 μl of PBS containing Rh123 dye (5 μM) for 1 h. The red fluorescence of the Rh123 dye was then recorded on a fluorescence microscope (Nikon Eclipse 80i, Japan). Images were captured at a magnification of 20x using a color camera attached to the microscope.

2.7. Cell cycle analysis

Cell cycle analysis was performed by staining cells with propidium iodide dye, and measuring the G1, S, and G2/M phases of the normal cell cycle, and quantifying the characteristic apoptotic peak (subG1) upon exposure to persicogenin and homoeriodictyol. The MCF-7, HeLa, and HT-29 cells treated with persicogenin and homoeriodictyol (100, 250, and 500 μg/ml) for 24 h were trypsinized and washed twice with PBS. Thereafter, the cells were fixed with chilled ethanol (70 %) for 1 h, centrifuged (4 °C) at 3000 rpm for 3 min, washed with PBS, and resuspended in PBS (500 μl) containing 0.5 μg/ml RNAase A, propidium iodide (50 μg/ml), and Triton X-100 (0.1 %). Cells were stored in the dark for 1 h at room temperature. Thereafter, the fluorescence of 10,000 cells was recorded on a flow cytometer at a laser excitation of 488 nm and a band-pass filter of 620 nm. Cell debris was excluded and characterized by a low FSC/SSC. Changes in the cell cycle were analyzed using the System II Software, Version 3.0 [33].

2.8. Real-time PCR (qPCR) transcriptional analysis

Cells subjected to adverse circumstances can experience alterations in their transcriptional process, regulating gene expression and deregulating different pathways related to cell signaling or reinstating a checkpoint. Thus, we sought to determine whether persicogenin and homoeriodictyol could induce changes in the transcriptional expression of apoptotic and oxidative stress genes in MCF-7, HeLa, and HT-29 cells [33]. Briefly, a commercially available kit (iPrep™ PureLink™, Invitrogen, USA) was used to isolate total RNA from MCF-7, HeLa, and HT-29 (3 × 10⁵ cells/well) following exposure to 100 μg/ml of persicogenin and homoeriodictyol for 24 h. Thereafter, total RNA was purified on an automated system (Invitrogen*, USA). The concentration and purity of RNA were determined with a Nanodrop 8000
2.9. Statistical analysis

Statistical analysis was performed using one-way ANOVA (Dunnett’s multiple comparisons test) and Sigma Plot 11.0, USA. p value < 0.05 was considered to indicate statistical significance, unless otherwise stated.

3. Results

3.1. Cytotoxicity assay

In the MTT assay, MCF-7 and HeLa cells did not exhibit cytotoxicity, at the highest persicogenin concentration (500 μg/ml). Although, a visible detachment and irregular cell morphology of MCF-7 and HeLa cells were observed after persicogenin exposure (Fig. 2), HT-29 cells exposed to persicogenin (500 μg/ml) displayed a 58.1 % reduction in the cell survival (Fig. 3). Comparatively, MCF-7, HeLa, and HT-29 cells at the highest concentration of homoeriodictyol (500 μg/ml) had respective reductions of 51.9 %, 66.7 % and 76.2 % in cell survival. At 250 μg/ml, homoeriodictyol significantly reduced (44.7 %) cell survival in HT-29 alone; such effects were not observed in MCF-7 and HeLa cells (Fig. 3). Exposure to the lower concentrations (10 – 50 μg/ml) of persicogenin and homoeriodictyol did not induced cytotoxic effects in the three cell types (data not shown).

3.2. Lysosomal toxicity analysis by the NRU assay

In the NRU assay, the MCF-7, HeLa, and HT-29 cells treated with persicogenin and homoeriodictyol exhibited a concentration-dependent lysosomal toxicity. Compared to the untreated control, the highest concentration of persicogenin (500 μg/ml) induced a 53.6 %, 53.9 %, and 58.8 % reduction (p < 0.01) in the survival of MCF-7, HeLa, and HT-29 cells, respectively (Fig. 4). Comparatively, MCF-7 and HeLa cells treated with the highest concentration (500 μg/ml) of homoeriodictyol displayed greater toxicity, with 83.0 % and 87.7 % reduction in the cell survival. In addition, HT-29 cells treated with a similar concentration had a 66.7 % (p < 0.01) reduction in cell survival (Fig. 4).

3.3. Intracellular ROS generation

ROS quantification by flow cytometry showed that persicogenin and homoeriodictyol exposure enhanced intracellular ROS generation. At the highest persicogenin concentration (500 μg/ml), MCF-7, HeLa, and HT-29 cells displayed a 1.82-, 2.44- and 2.37-fold greater generation of ROS. Similarly, homoeriodictyol (500 μg/ml) treatment caused a 1.55-, 1.76-, and 1.30-fold greater ROS generation in MCF-7, HeLa, and HT-29 cells, respectively (Fig. 5).

Qualitative analysis revealed an increase in the green fluorescence of DCF in persicogenin- (500 μg/ml) treated MCF-7, HeLa, and HT-29 cells (Fig. 5). In addition, homoeriodictyol (500 μg/ml) treatment caused an increase in the green fluorescence of DCF in MCF-7, HeLa, and HT-29 cells (Fig. 5). Overall, the fluorescence data validated the flow cytometric data of intracellular ROS generation.

3.4. Mitochondrial dysfunction by persicogenin and homoeriodictyol

MCF-7, HeLa, and HT-29 cells treated with persicogenin and homoeriodictyol displayed an altered mitochondrial function (ΔΨm). Compared to the 100 % fluorescence of Rh123 in control cells, persicogenin exposure (500 μg/ml) resulted in a 2.36-, 2.66-, and 2.58-fold higher Rh123 fluorescence in MCF-7, HeLa, and HT-29 cells (Fig. 6). In addition, reasonable but significant increases of 1.57-, 1.50-, and 1.60-fold were recorded for the fluorescence in MCF-7, HeLa, and HT-29 cells exposed to the highest concentration (500 μg/ml) of homoeriodictyol (Fig. 6).

Qualitative analysis of ΔΨm revealed greater red fluorescence of the Rh123 dye in persicogenin- (500 μg/ml) treated MCF-7, HeLa, and HT-29 cells (Fig. 6). Although lower than persicogenin, homoeriodictyol (500 μg/ml) caused an increase in fluorescence in the Rh123-stained cells (MCF-7, HeLa, and HT-29) (Fig. 6). The fluorescence microscopic analysis of the flavanone-treated cells confirmed the flow data obtained for ΔΨm.

3.5. Effect of persicogenin and homoeriodictyol on cell cycle

Persicogenin exposure induced concentration-dependent apoptosis in MCF-7, HeLa, and HT-29 cells. The representative cell cycle images of the highest concentration of the persicogenin- (500 μg/ml) treated MCF-7, HeLa, and HT-29 cells showed 42.5 %, 63.1 %, and 62.3 % of the apoptotic subG1 peak, respectively (Fig. 7). The average data analysis of the subG1 peaks from 500 μg/ml of persicogenin treatment revealed a 42.4 ± 0.6 %, 63.5 ± 4.0 %, and 62.5 ± 1.5 % (p < 0.01) increase in subG1 peaks in the MCF-7, HeLa, and HT-29 cells versus the untreated controls, which showed 1.3 ± 0.1 %, 4.2 ± 0.2 %, and 6.1 ± 0.4 % of the background subG1 peak (Table 1). The typical cell cycle images obtained from homoeriodictyol (500 μg/ml) exposure also displayed 30.7 %, 30.2 %, and 23.8 % of the subG1 apoptotic phase in MCF-7, HeLa, and HT-29 cells (Fig. 7). The average data for the subG1 peaks from homoeriodictyol (500 μg/ml) exposure displayed a 30.2 ± 0.5 %, 30.6 ± 2.9 %, and 23.6 ± 0.9 % increase in MCF-7, HeLa, and HT-29 cells compared to the untreated controls, which showed 2.5 ± 0.3 %, 2.6 ± 0.2, and 8.6 ± 0.1 % of the background subG1 peak (Table 1).

3.6. Transcriptional changes in the apoptotic and oxidative stress genes

Treating MCF-7, HeLa, and HT-29 cells with persicogenin and homoeriodictyol resulted in the upregulation of apoptotic and antioxidant genes. MCF-7 cells exposed to the lowest concentration (100 μg/ml) of persicogenin displayed a 2.6-, 5.4-, 2.9-, 11.7-, and 6.7-fold increase in the expressions of p53, caspase 3, caspase 9, bax, and SOD1 genes. In addition, HeLa and HT-29 cells exposed to the same concentration showed 2.2-, 2.6-, 2.0-, 3.7-, and 3.1-fold and 1.5-, 0.7-, 1.4-, 1.4-, and 1.1-fold upregulations of p53, caspase 3, caspase 9, bax,
and SOD1, respectively (Fig. 8). When, HeLa and HT-29 cells were exposed to the lowest concentration (100 μg/ml) of homoeriodictyol, the p53, caspase 3, caspase 9, bax, and SOD1 genes were upregulated by 2.1-, 2.5-, 1.7-, 3.7-, and 3.4-fold and 3.3-, 0.8-, 1.5-, 0.3-, and 1.0-fold, respectively. However, in MCF-7 cells, the upregulated expressions of these genes were found to be very marginal, except that of SOD1, which displayed a 2.1-fold greater expression than the untreated control (Fig. 8).

4. Discussion

Flavonoids containing the polyphenolic structure are broadly present in nature and serve as a milestone in the discovery of medicines. The bioactive benefits of flavonoids as anticancer, anti-aging, anti-diabetic, anti-viral/bacterial, anti-inflammatory, and cardioprotective agents have attracted meticulous attention for many years, as demonstrated by the availability of several research publications [34]. Many flavonoids, including flavones, protect normal cells by acting as antioxidants to neutralize the toxic ROS, which occurs via the donation of hydrogen ions, ultimately providing short- and long-term protection [35]. Flavonoids/flavones within normal/healthy cells can modulate the cell-signaling pathways to augment the expression of phase-2 proteins, which enhance the glutathione and heme-oxygenase 1 generation, boosting the natural defense of cells against oxidative stress [36,37]. Compared to normal cells, cancer cells have a higher level of oxidative stress, enabling their greater susceptibility to killing by drugs that enhance ROS levels, including flavonoids/flavones [38]. Flavonoids also interfere with multiple signal transduction pathways of cancer cells, thereby limiting their proliferation, angiogenesis, metastasis, or increase in apoptosis [38].

Homoeriodictyol, a dihydroflavone that is widely found in plants, demonstrated its diverse biological actions, such as the inhibition of human CYP1B1 enzyme, vasorelaxation, and anti-inflammatory [39–41]. Previously, homoeriodictyol was reported to protect the vascular cells from oxidative insult [42]. Despite the potential biological activities of persicogenin and homoeriodictyol, studies describing their mode of action in the inhibition of cancerous cells of different origins have been seldom revealed. Consequently, both flavanones were studied to fill the data gap in their anticancer application. The MTT cytotoxicity data indicated that persicogenin and homoeriodictyol reduced the survival of MCF-7, HeLa, and HT-29 cells. In addition, persicogenin was found to decrease the survival of HT-29 cells; however, these effects were not found in MCF-7 and HeLa cells. The cytotoxicity data obtained herein align with those of earlier studies, which indicate the cytotoxic potential of persicogenin in mouse tsFT210 and HepG2 cells [21,43]. The MTT assay relies on the intracellular dehydrogenases, which are mainly localized in the mitochondria for the reduction of the dye to form the strong light-absorbing product, formazan. Differences in the survival of MCF-7 and HeLa cells could be due to their mitochondria depolarization effects, which have been reported to prevent the accumulation of dye in the mitochondria [44]. The MTT assay revealed a significant cytotoxic effect only at the highest concentration (500 μg/ml). In contrast, growth enhancement was exhibited by both flavanones at the lower concentrations (100 and 200 μg/ml). Such biphasic effects indicate the hormesis nature of homoeriodictyol and persicogenin, as previously reported for polyphenols, flavonoids, and synthetic and natural molecules [45–47]. The lysosomal toxicity analysis by the NRU assay revealed the prominent effects of homoeriodictyol and persicogenin on all three cell lines. In the NRU assay, both flavanones exhibited pronounced toxicity, even at the lowest concentration (100 μg/ml). Such findings, could be attributed to the explicit lysosomal damage. Based on these findings, persicogenin and homoeriodictyol-mediated alterations in the delicate lysosomal membrane may lead to lysosomal fragility, resulting in a decrease in the binding and uptake of neutral red dye, possibly via a decrease in the action of cellular lysosomal acid phosphatase [48]. Furthermore, lysosomal destabilization is considered to be an earlier event in mitochondrial injury [49].

Based on the above findings, we performed flow cytometric and fluorescence microscopic analyses to reaffirm the disturbances in the ΔΨm. The persicogenin- and homoeriodictyol-treated MCF-7, HeLa, and HT-29 cells had a steady increase in the fluorescence of the Rh123 dye. Fluorescence enhancement of Rh123 is associated with the intrinsic behavior of the mitochondria, which swells under irresistible conditions.
structure. The presence of the \(+\text{CH}_3\) group in molecules has been re-
methylate A during apoptosis and higher ROS generation in cells [50,51]. Conse-
sequently, we quantitatively and qualitatively determined the in-
tracellular ROS level in MCF-7, HeLa, and HT-29 cells exposed to per-
sicogenin and homoeriodictyol. Compared to the control, persicogenin and homoeriodictyol induced a greater level of ROS generation in the treated cells, which unequivocally validated the role of ROS in the in-
duction of \(\Delta \Psi_m\) dysfunction in MCF-7, HeLa, and HT-29 cells. Depo-
larization of the mitochondrial membrane is reported to be related to apo-
ptosis [52]. Owing to the plausible link between ROS and \(\Delta \Psi_m\) for the promotion of cell death, we performed a further evaluation of apo-
ptosis in the three cell types. The cell cycle data revealed a dose-
dependent increase in the apoptotic peak (subG1) in all cell types ex-
posed to homoeriodictyol. Comparatively, persicogenin exposure in-
duced a higher apoptotic response, as demonstrated by the marked increase in the subG1 peak in MCF-7, HeLa, and HT-29 cells. The be-
avioral differences between the two flavonanes (persicogenin and homoeriodictyol) could be related to the presence of two methyl groups \(\text{\textsuperscript{13}CH}_3\) in persicogenin and only one \(\text{\textsuperscript{13}CH}_3\) in the homoeriodictyol structure. The presence of the \(\text{\textsuperscript{13}CH}_3\) group in molecules has been re-
ported to play a crucial role in their binding to nucleotides, resulting in DNA methylation, which can promote DNA damage and cell death. In this study, however, DNA methylation was not examined. Nonetheless, based on our previous mechanistic work on the pesticide, which could methylate A–T nucleotides and preferentially bind to the minor groove of DNA [53], we can postulate that the greater cell death observed in MCF-7, HeLa, and HT-29 cells treated with persicogenin could be due to a higher methylation of the DNA nucleotide. Based on this implication, homoeriodictyol would induce low cell death because of less DNA methylation. Ultimately, the observed apoptotic events, thus confirmed the influence of functional alterations in the mitochondria and lysosomes on the triggering of cell death in treated cells [54,55]. Mitoch-
tonia-mediated apoptosis and necrosis involve the opening of membrane permeability transition pores (MPTP), which assist in the dissipation of \(\Delta \Psi_m\). The opening of MPTP aids in the swelling of the mitochondria, leading to the rupture of its outer membrane, and the ultimate release of apoptogens to trigger the apoptotic process [56]. Membrane damage in lysosomes, as depicted in the NRU assay, is attributed to the release of lysosome protease into the intracellular spaces, which conclusively affects adjacent cells and initiates the pro-
cess of cell death [57]. The differential toxic effects of homoeriodictyol at higher concentrations (100 – 500 \(\mu\)g/ml) in treated cells was sup-
ported by a recent report where homoeriodictyol was found to bind to the mouse cerebellum GABA \(_A\) receptor complex at an IC50 > 100 \(\mu\)M [58]. Additionally, the low to high concentration effects of persicogenin aligned with that found in a previous study where an IC50 > 100 \(\mu\)g/ml of persicogenin was required to inhibit mouse tsT210 cancer cells [21]. Owing to anomalies and toxicological consequences, cells amend their gene expression to deregulate the cell signalling pathways or re-
store checkpoints [59]. Herein, we found that homoeriodictyol sig-
nificantly enhanced the expression of the p53 gene in HeLa and HT-29 cells. Persicogenin exposure also led to a significant increase in the expression of p53 gene in MCF-7, HeLa, and HT-29 cells. Typically, the activation of p53 occurs because of DNA damage or cellular stress; this is because it is translocated to the nucleus where it induces pro-
apoptotic gene expression on the mitochondrial membrane to activate the effector caspases and augment cell death [60,61]. Concurrently, the collapse of \(\Delta \Psi_m\), as observed in our experiments exhibits significant importance in the early onset of the apoptotic event. Stimulation of the caspase-3 and caspase-9 genes in homoeriodictyol-
treated HeLa and HT-29 cells, and the persicogenin-exposed MCF-7, HeLa, HT-29 cells imply the involvement of mitochondrial impairment in their mechanism. Caspases are considered to be critical for apoptosis. In particular, \(\Delta \Psi_m\) dysfunction has been regarded as the key-factor for the mitochondrial-dependent intrinsic pathway in cell death, which subsequently assists in the release of cytochrome \(c\), thereby activating caspase-9 followed by caspase-3 proteins [62]. Caspase-3 is considered to be the “point-of-no-return” in the multi-steps signalling cascades of apoptosis [63]. Bax was found to be maximally upregulated in the homoeriodictyol-treated HeLa cells, and persicogenin-treated MCF-7, HeLa, and HT-29 cells, respectively. Previously, the upregulation of bax was reported to be caused by p53 [64]. Consequently, we presumed that p53 plays a decisive role in bax upregulation by incorporating bax into the mitochondrial membrane, and thus aggravates apoptosis following exposure to homoeriodictyol and persicogenin. Interestingly, despite the observed lysosomal toxicity, mitochondrial injury, and cell death observed in homoeriodictyol-treated MCF-7 cells, the hallmark genes of apoptosis, especially p53, were neither upregulated nor downregulated, while a marginal upregulation of caspase-3 and 9 was observed. These responses can be attributed to the possible induction of p53-independent apoptosis in MCF-7 cells, which is reported to act parallel to the canonical DNA and chromosome damage response pathways for the elimination of cells harboring altered genomes [65]. p53-independent apoptosis is suggested to be related to the functional impairment of the cell cycle checkpoints (ATM, ATR, Chk1) and p38-MAPK/MK2 regu-
lators, which leads to the activation of caspase-3 [66]. Although we did not examine the DNA damage and cell cycle checkpoints in the present study, we postulated that a similar mode of repair impairment may have been activated in MCF-7 upon homoeriodictyol exposure. A mechanistic translational study of the apoptotic proteins and DNA da-
mage is thus warranted to establish a positive connection with the p53-
independent mechanism of apoptosis in MCF-7 cells. Based on our findings, the oxidative stress gene (SOD1) in MCF-7 cells was highly upregulated. As ROS generation can be increased by homoeriodictyol, cytoplasmic SOD1 might be involved in the scavenging of free radicals. However, as observed in the apoptotic process in MCF-7 cells, the excess oxidative stress was beyond the attenuation capacity of SOD1 gene to curtail cellular damage.

5. Conclusions

In conclusion, persicogenin and homoeriodictyol have exhibited anticancer effects in MCF-7, HeLa, and HT-29 cells, and significantly reduced their survival according to the MTT assay. Comparatively, however, a greater lysosomal toxicity was observed in the NRU assay, indicating their preferential toxicity of cells owing to the degeneration of the lysosomal membrane. Both flavanones induced a significant level of oxidative stress and altered the mitochondrial function, which lead to

Fig. 4. The NRU lysosomal toxicity assay showing the reduction in the cell survival of three different cell lines as an effect of persicogenin and homoeriodictyol treatments for 24 h. Each histogram represents the mean ± SD of three experiments performed in triplicate wells. **p < 0.01 versus the control.
the onset of apoptosis in the MCF-7, HeLa, and HT-29 cells. The homoeriodictyol-treated HeLa and HT-29 cells, and the persicogenin-treated MCF-7, HeLa, HT-29 cells showed transcriptional upregulation of the $p53$, $\text{caspase-3}$, $\text{caspase-9}$, and $\text{bax}$ genes, thereby validating the involvement of the $p53$-dependent cell death mechanism. The lack of response of the apoptotic genes in homoeriodictyol-treated MCF-7 cells indicates the putative involvement of a $p53$-independent mechanism in cell death. Based on the overall findings, the selectivity of persicogenin

**Fig. 5.** Flow cytometric quantitation of persicogenin and homoeriodictyol induced intracellular ROS generation after 24 h of exposure. Each histogram represents the mean ± SD of three experiments performed in triplicate wells. $^*p < 0.05$ and $^{**}p < 0.01$ versus the control. Qualitative analysis showing the increase in DCF fluorescence after persicogenin and homoeriodictyol exposure for 24 h. Images were captured at a magnification of 20x on a fluorescence microscope.

**Fig. 6.** Flow cytometric analysis of the mitochondrial membrane potential ($\Delta\Psi_{m}$) in cells treated with persicogenin and homoeriodictyol for 24 h. Each histogram represents the mean ± SD of three experiments performed in triplicate wells. $^*p < 0.05$ and $^{**}p < 0.01$ versus the control. Qualitative analysis showing the increase in the fluorescence of Rh123 fluorescence after persicogenin and homoeriodictyol exposure for 24 h. Images were captured at a magnification of 20x on a fluorescence microscope.
Fig. 7. Representative flow cytometric images demonstrating the increase in the apoptotic (subG1) peak after 24 h of persicogenin and homoeriodictyol treatments. Values in the inset represent the percentage of cells that appeared in different phases of the cell cycle and analyzed using individual markers.
Table 1
Persicogenin and homoeriodictyol induced an increase in the subG1 apoptotic peak in cells after 24 h of exposure.

<table>
<thead>
<tr>
<th>Concentrations (μg/ml)</th>
<th>Persicogenin SubG1 Apoptotic Peak (%)</th>
<th>Homoeriodictyol SubG1 Apoptotic Peak (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCF-7 HeLa HT-29</td>
<td>MCF-7 HeLa HT-29</td>
</tr>
<tr>
<td>Control</td>
<td>1.3 ± 0.1 4.2 ± 0.2 6.1 ± 0.4</td>
<td>2.5 ± 0.3 2.6 ± 0.2 8.6 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>4.4 ± 0.3* 15.1 ± 0.5** 10.4 ± 0.3</td>
<td>9.8 ± 0.3** 20.4 ± 1.5** 12.0 ± 0.8*</td>
</tr>
<tr>
<td>250</td>
<td>24.6 ± 2.3** 40.6 ± 1.5** 35.5 ± 1.8**</td>
<td>18.3 ± 0.8** 30.1 ± 1.0** 17.0 ± 2.8**</td>
</tr>
<tr>
<td>500</td>
<td>42.4 ± 0.6** 63.5 ± 4.0** 62.5 ± 1.5**</td>
<td>30.2 ± 0.5** 30.6 ± 2.9** 23.6 ± 0.9**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of three independent experiments run in triplicate wells.

...versus the untreated control.

Fig. 8. Real-time quantitative PCR of the apoptotic and oxidative stress genes in cells treated with persicogenin and homoeriodictyol for 24 h.

and homoeriodictyol to inhibit cancer cells can be hierarchized as follows: HeLa > HT-29 > MCF-7, and MCF-7 > HeLa > HT-29. The varying effects of the two flavonones can also be attributed to their substituents, which should be examined in-depth in future transcrip-
tional and translational studies on structure-activity-relationship. Collectively, our data unequivocally imply that persicogenin and homoeriodictyol can be prospectively applied as putative bioactive pharmaceutical or therapeutic agents against human breast carcinoma, human cervical cancer, and human colon cancer.

CRediT authorship contribution statement


Declaration of Competing Interest

None.

Acknowledgement

The authors are thankful to the Researchers Supporting Project number (RSP-2019/86), King Saud University, Riyadh, Saudi Arabia.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2020.02.008.

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