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Original Article

Oxypeucedanin and isoimperatorin extracted from *Prangos ferulacea* (L.) Lindl protect PC12 pheochromocytoma cells from oxidative stress and apoptosis induced by doxorubicin

Fereshteh Jalilian[1], Maryam Moieni-Arya[2], Leila Hosseinzadeh[1,*], and Yalda Shokoohinia[3,*]

[1]Pharmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran.
[2]Students Research Committee, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, I.R. Iran.
[3]Ric Scalzo Institute for Botanical Research, Southwest College of Naturopathic Medicine, Tempe, AZ, USA.

Abstract

Background and purpose: Doxorubicin (DOX) as a chemotherapeutic agent has been widely used in the treatment of various types of cancer. However, DOX exerts a toxic effect on normal tissues such as the brain. Furanocoumarins reduce the risk of cardiovascular and brain diseases because of their antioxidant activities. This study has been designed, for the first time, to evaluate the effect of known furanocoumarins oxypeucedanin and isoimperatorin extracted from *Prangos ferulacea* (L.) Lindl on oxidative stress and apoptosis induced by DOX toward pheochromocytoma cell line (PC12).
Experimental approach: NMR and MASS spectrometers were used to characterize the isolated compounds. The protective effects of isolated compounds on DOX-induced cytotoxicity in PC12 cells were examined by MTT assay. PC12 cells were pretreated with oxypeucedanin and isoimperatorin for 2 and 21 h, respectively, subsequently exposure to DOX at IC_{50} concentration. Then, mitochondrial membrane potential (MMP), Bax and Bcl2 mRNA expressions, caspase-3 activation, and the generation of intracellular reactive oxygen species (ROS) were measured after 24 h.
Findings/Results: Pretreatment with oxypeucedanin and isoimperatorin significantly decreased DOX-induced apoptosis through reduction of caspase-3 activity and ROS generation and an increase in MMP. In addition, our finding showed pretreatment with these compounds leads to regulation of Bcl-2.
Conclusion and implications: Taken together our observation indicated that oxypeucedanin and isoimperatorin have a protective effect against apoptosis induced by DOX in PC12 cells by inhibition of ROS production.

Keywords: Apoptosis; Cytotoxicity; Doxorubicin; Isoimperatorin; Oxypeucedanin; *Prangos ferulacea*.

INTRODUCTION

Evidences have shown that due to the high content of polyunsaturated fatty acids, the nervous system has particularly been vulnerable to oxidative stress (1). Also, doxorubicin (DOX) as a highly effective chemotherapy medication for the treatment of a wide variety of tumors, rises levels of reactive oxygen species (ROS) resulting in inducing oxidative stress (2,3). The overproduction of ROS causes damage in organelles' functions such as mitochondria (4). Although the level of penetration of DOX into the brain is low, its penetration ability is increased in presence of mannitol, morphine, dexamethasone, or ondansetron, which cause damage in neurons of the brain (5). However, most of the studies are looked at the well-known adverse effects of DOX treatment on the cardiovascular system and less about its effects on the brain.

*Corresponding authors:
L. Hosseinzadeh, Tel: +98-8338250271, Fax: +98-8334276493
Email: Lhoseinzadeh@kums.ac.ir
Y. Shokoohinia, Tel & Fax: +1-4802229293
Email: yshokoohinia@gmail.com

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Most of the antioxidant compounds used by humans are derived from plant sources. These compounds are divided into various classes with different physical and chemical properties (6). Coumarin and its derivatives consisting of fused benzene and α-pyrone rings represent one of the most important classes of secondary metabolites in seeds, roots, and leaves of many plant species (7). Significant phytochemical studies have been conducted on coumarins and furanocoumarins isolated from the root of *Prangos ferulacea* (L.) Lindl (8-11). Oxypeucedanin (5-[2,3-epoxy-3,3-dimethylpropyloxy]psoralen) (11,12) and isoimperatorin (5-prenyloxypsoralen) (9-13) are two known furanocoumarins extracted from the root of *P. ferulacea*. Some researchers have reported that oxypeucedanin has several biological activities including anti-mutagenic, uterus contraction and blood pressure increase (14) and also isoimperatorin has analgesic, antimicrobial, and vascular relaxing activities (15). In addition, many reports have been published, associating with their toxicity and anti-proliferative activity against cancer cells (16,17). Although it has been proven that the radical scavenging effect of coumarins can be effective in the treatment of brain diseases (18), no data are available on the neuroprotective potential of oxypeucedanin and isoimperatorin. Therefore, in line with our previous studies related to the biological activity of coumarins (19,20), we investigated the effects of oxypeucedanin and isoimperatorin on cytotoxicity induced by DOX in PC12 cells, as a widely-accepted model of neuronal cells (21).

MATERIALS AND METHODS

General instruments and chemicals

The proton nuclear magnetic resonance (^{1}HNMR) spectra were recorded on a Brucker® (400 MHz, Germany) spectrometer. Chemical shift (δ: 7.24) was referenced to the residual signal of CDCl3 as solvent. Agilent 5975C mass spectrometer (Agilent Technologies, Palo Alto, CA) was used for the mass determination. Silica gel (0.04-0.063 mm) was utilized for column chromatography. Thin-layer chromatography (TLC) plates (Silica gel 60 GF254 precoated plates, Merck, Germany) were utilized to investigate separation and were visualized by UV at 254 and 365 nm, and/or staining cerium sulfate/sodium molybdate and heating.3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT). Fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA), rhodamine-123, Triton™ X-100, and fetal bovine serum (FBS) were purchased from Sigma (St Louis, MO, USA). Also, Dulbecco's modified eagle's medium (DMEM-F12) was purchased from Gibco (Grand Island, NY, USA). Express One-Step SYBR GreenER and caspase-3 detection kits were purchased from Invitrogen (Carlsbad, CA) and Sigma, respectively. Other chemicals and all the solvents used for extraction and purification were purchased from Merck (Germany) and Dr. Mojallali (Iran).

Plant material

P. ferulacea roots were collected from Dasht-Room village, Kohkiloye and Boyer Ahmad, Iran, at an altitude of 1800 m above sea level. After identification of the plant by the botany department of Yasouj University, a specimen (Voucher No. 2408) was kept at the Herbarium of School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.

Extraction and isolation of isoimperatorin and oxypeucedanin

The air-dried roots of *P. ferulacea* (850 g) were macerated with acetone (8.5 L × 3) for 3 days at room temperature with mixing. After removing the solvent *in vacuo*, the dark brown viscous residue (85 g) was winterized using MeOH and chromatographed as described previously (11). The defatted extract (77 g) was fractionated by vacuum liquid chromatography (VLC, silica gel column) using a gradient of EtOAc in heptane from 10 to 100% to obtain 4 fractions (F1-F4). Fraction F3 (H: EtOAc, 5:5) was not pure enough which was further purified using recrystallization to give pure compound 1 crystals (732 mg). The second fraction (H: EtOAc, 8:2, 7:3) was separated *via* an open column using silica gel (H: EtOAc, 9:1 to 6:4) to get five fractions (G1-G5). Fraction G2 after evaporation resulted in compound 2 (432 mg).

Cell culture conditions

PC12 cell line possesses intracellular substrates for the synthesis, metabolism, and transportation of dopamine (DA). This makes PC12 cells (originally from rat pheochromocytoma) useful as a model system for neuroprotection study. This cell line was received from the Pasteur Institute of Iran (Tehran, Iran). Cells were cultured under normal conditions including DMEM-F12 with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and incubated in suitable conditions, temperature of 37 °C, 90% humidity, and 5% CO2 (22).

Cell viability assay

MTT assay was performed to examine the cellular toxicities of DOX, oxypeucedanin, and isoimperatorin against PC12 cells. At the beginning of the experiment, cells were cultured in a 96-well microplate (7000 cells/well in 200 µL volume). In the next step, collection of experiments were performed as follows: (1) untreated control cells, (2) cells were treated with different concentrations (0-100 µg/mL) of oxypeucedanin and isoimperatorin for 2 and 21 h, respectively, (3) cells were treated with different concentrations of DOX (0-30 µM) for 24 h, and (4) cells were pretreated with different concentrations of oxypeucedanin (2 h) and isoimperatorin (21 h), then after changing the medium, cells were treated with of DOX at IC_{50} concentration for another 24 h. The concentration of DMSO in the culture medium was kept constant at 0.5% for all the solutions. Briefly, after treatment, the culture medium was replenished by 200 µL of MTT (0.5 mg/mL) in the growth medium and then, the plates were incubated for 2-4h at 37 °C. After the incubation period, to solubilize the composed formazan crystals, 200 µL DMSO was added to each well and shaken. Finally, the absorbance was specified at 570 nm (test wavelength) and 630 nm (reference wavelength) to obtain sample signal (OD_{570}-OD_{630}) by an ELISA plate reader (Biotek, H1 M, USA).

Measurement of mitochondrial membrane potential

There are many different kinds of factors that participated in the induction of apoptosis, among them mitochondria play a crucial role. In this study, mitochondrial membrane potential (MMP) was assessed by rhodamine 123 as a fluorescent cationic dye. For this purpose, PC12 cells were treated with appointed concentrations of oxypeucedanin and isoimperatorin in 6-well tissue culture plates for 2 and 21 h, respectively. After that, DOX (at IC_{50} concentration) was added to the cells and incubated for 24 h. At the end of treatment, cells were incubated with rhodamine 123 (15 µL, 20 µM) for 30 min at 37 ºC. Thereafter, the fluorescence of lysed cells by Triton^{TM} X100 was measured at an exciting wavelength of 488 nm and an emission wavelength of 590 nm using a fluorescence microplate reader (BioTek, H1 M, USA). Also, the Bradford assay was used for the determination of protein content.

Real-time polymerase chain reaction analysis of apoptosis-related gene expression

Total RNA of PC12 cells was extracted using the RNA isolation kit (Roche, Mannheim, Germany) and was assessed qualitatively and quantitatively by spectrophotometer (NanoDrop 2000, USA). Then, samples were stored at -80 °C in order to further investigations. The primers used in this study were selected from previous studies (19). Thermal cycler conditions were 15 min at 50 ºC for cDNA synthesis, 10 min at 95 ºC followed by 40 cycles of 15 s at 95 °C to denature the DNA, and 45 s at 60 ºC to anneal and extend the template. All reactions were carried out in a triplicate using a Corbett system (Australia). The target genes expression was normalized against β-actin and analyzed by the relative gene expression -ΔΔCT method where $-\Delta\Delta CT = (CT_{target} - CT_{\beta\text{-actin}})$ unknown - $(CT_{target} - CT_{\beta\text{-actin}})$ calibrator.

Caspase 3 activation assay

The activity of caspase-3 was determined by a colorimetric caspase-3 kit (Sigma, USA). This assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the enzyme-substrate, Ac-DEVDpNA in an equal amount of cells protein. To this purpose, pretreatment cells with appointed concentrations of oxypeucedanin and

isoimperatorin were treated with DOX, at IC_{50} concentration for 24 h, and then cells were centrifuged at 1200 rpm for 5 min. Thereafter, 50 µL lysis buffer was added to 1×10^6 collected cells and incubated on ice for 10 min. Cell lysates were centrifuged at 14000 rpm for 5 min and 4 °C. After which a 10 µL mixture of cell lysate was combined with an equal amount of substrate reaction buffer, containing a caspase-3 colorimetric substrate, then was incubated for 2 h at 37 °C. The absorbance was then quantified using a microplate reader (BioTek, H1M) at 405 nm. The protein content was determined by the Bradford assay.

Determination of intracellular ROS

Intracellular ROS formation was examined by DCF-DA, a non-fluorescent lipophilic ester, which is a known compound for investigating oxidative damage in intact cells. This compound is easily uptake by cells and is de-acetylation by non-specific esterases inside the cytosol, and the oxidation of this molecule with ROS can make it fluorescent (in the form of DCF molecules). Generally, the level of ROS is determined by the intensity of this fluorescence. Briefly, cells were pretreated with appointed concentrations of oxypeucedanin and isoimperatorin, then were treated with DOX (IC_{50}) and after remaining 24 h in an incubator, DCF-DA (20 µL) was added to the whole of wells and were incubated for another 30 min at 37 °C. Thereafter, cells were lysed with Triton™ X100 and the fluorescence was measured at 488 nm (excitation wavelength) and 528 nm (emission wavelength) utilizing a fluorescence microplate reader (BioTek, H1 M, USA).

Statistical analysis

All measurements were repeated in triplicate the results were presented as mean ± SEM. The results were compared by one-way analysis of variance (ANOVA) followed by Tukey tests. A P value < 0.05 was considered to be statistically significant.

RESULTS

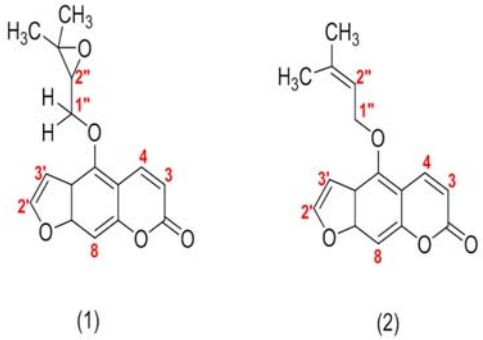
Phytochemical investigation

From the acetone extract of the roots of *P. ferulacea*, two furanocoumarins were isolated by VLC and open column of silica gel; compound 1: oxypeucedanin and compound 2: isoimperatorin (Fig. 1). The structures of these compounds were identified by spectroscopic methods including ^{1}HNMR and mass spectra as well as by comparison of them with related data published in the literature (23,24). The analytical data were as follows:

Oxypeucedanin was isolated as white crystals. The EI-MS: m/z286[M]+, 202 [M-5-subst.]+ and 85 [5-subst.]. C16H14O5 was obtained for oxypeucedanin by the quasimolecular ion at m/z 286[M]+ (25); ^{1}HNMR (400 MHz, CDCl3, J in Hz): 6.34 (1H, d, J = 9.77, H-3), 8.23 (1H, d, J = 9.78, H-4), 7.22 (1H, s, H-8), 7.64 (1H, d, J = 2.32, H-2′), 6.98 (1H, d, J = 1.62, H-3′), 4.61 (1H, dd, J = 6.8, H-1′′), 4.47 (1H, dd, J = 6.8, H-1′′), 3.25 (1H, dd, H-2′′), 1.36(3H, s, CH3), 1.11 (3H, s, CH3).

Isoimperatorin was obtained as a white cubic crystal. The EI-MS gave peaks at m/z 270 [M]+, 255 [M-CH3], 227 [M- (CH3)2CH]+, the molecular ion atm/z 270 [M]+ is in agreement with the C16H14O4 (25); ^{1}HNMR (400 MHz, CDCl3, J in Hz): 6.3 (1H, d, J = 9.6, H-3), 8.19 (1H, d, J = 9.6, H-4), 7.19 (1H, s, H-8), 7.62 (1H, d, J = 2.3, H-2′), 6.99 (1H, d, J = 2.3, H-3′), 5.57 (1H, t, H-2′′), 4.95 (2H, d, J = 6.8, H-1′′), 1.84(3H, s, CH3), 1.73 (3H, s, CH3).

The ^{1}HNMR spectra of oxypeucedanin and isoimperatorin displayed essentially two proton doublets at δH6.3 and δH 8.2 characteristic of α-pyrone protons attributed respectively to H-3 and H-4, and a pair of doublets occurring at δH 6.9 and δH 7.6 typical of furanic protons assignable to H-2′ and H-3′, respectively. These data are characteristic of the furanocoumarin skeleton (23).



(1) (2)

Fig. 1. Chemical structure of compound 1 (oxypeucedanin) and 2 (isoimperatorin).

Cell viability after exposure to DOX, oxypeucedanin and isoimperatorin alone

We examined the effects of different concentrations of oxypeucedanin and isoimperatorin on the viability of PC12 cells by the MTT method to determine the concentrations that are not only non-toxic but also can prevent DOX-induced cytotoxicity. Figure 2A and B clearly revealed that 2-h treatment with oxypeucedanin and 21-h with isoimperatorin with appointed concentrations (0 to 100 µg/mL) had no considerable toxicity on cells. Moreover, PC12 cells were exposed to different concentrations of DOX for 24 h. As it can be seen in Fig. 2C DOX-induced cytotoxicity with IC_{50} value of 5 µM dose-dependently.

Effect of oxypeucedanin and isoimperatorin pretreatment on DOX-induced cell death

For evaluation of cytoprotective effects of isolated compounds on DOX, PC12 cells were incubated with oxypeucedanin and isoimperatorin for 2 and 21 h, respectively, then IC_{50} concentration of DOX (5 µM) was added and cells incubated for another 24 h. A glance at Fig. 3A and B demonstrated that the presence of oxypeucedanin and isoimperatorin caused increasing the cell survival and it can be concluded that these compounds could protect cells from DOX-induced cytotoxicity. The results showed that the most protective effect is related to the concentration of 80 µg/mL of oxypeucedanin and 80 and 100 µg/mL of isoimperatorin.

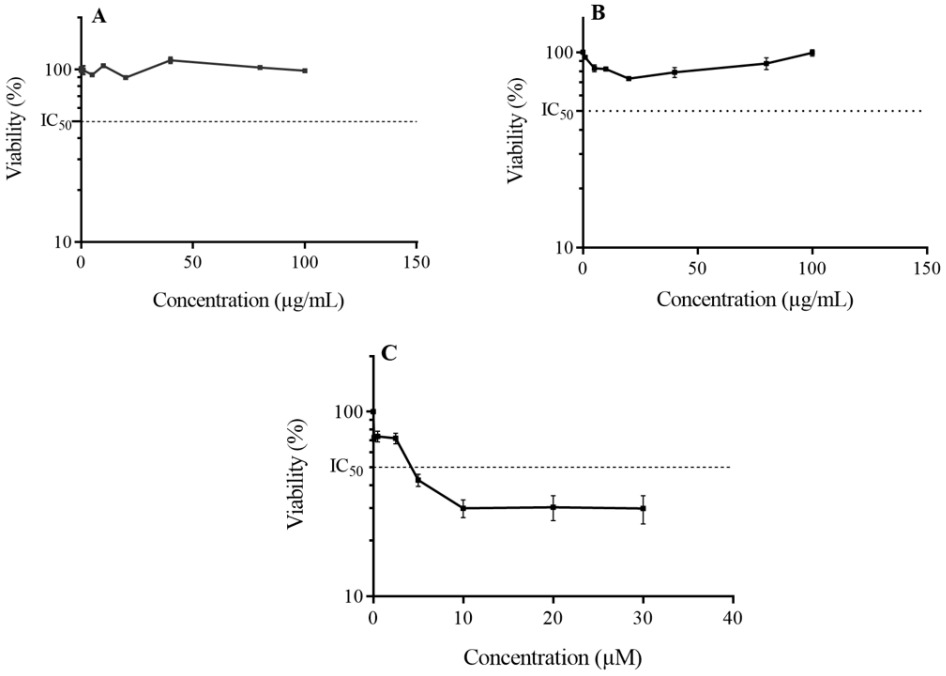


Fig. 2. The effect of (A) oxypeucedanin, (B) isoimperatorin, and (C) doxorubicin on PC12 cells viability. The cell viability was determined by MTT assay. Data are expressed as the mean ± SEM of three separate experiments.

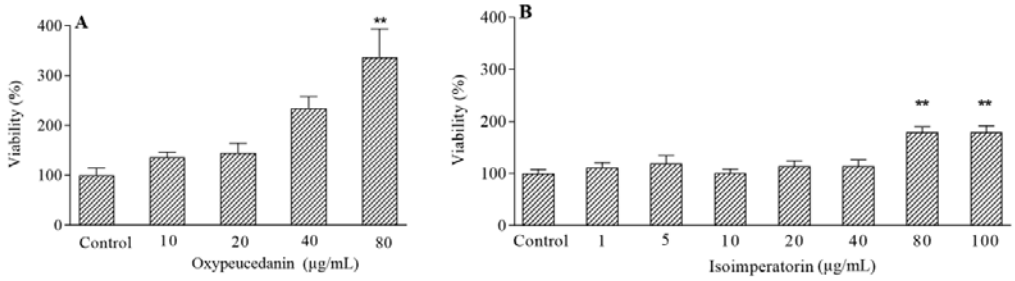


Fig. 3. The effect of oxypeucedanin and isoimperatorin on doxorubicin-induced cytotoxicity in PC12 cells. Cells were pretreated with different concentrations of (A) oxypeucedanin for 2 h and (B) isoimperatorin for 21 h before exposure to 5 µM of doxorubicin. Data are expressed as the mean ± SEM of three separate experiments. $^{**}P < 0.01$ Indicates significant differences compared to the control.

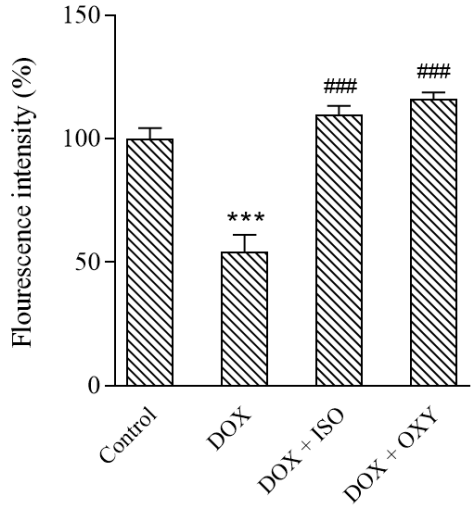


Fig. 4. Effect of oxypeucedanin and isoimperatorin on DOX-induced mitochondrial membrane potential collapse. Cells were pretreated with oxypeucedanin and isoimperatorin at the same concentration of 80 μg/mL for 2 h and 21 h, respectively, before exposure to DOX at 5 μM. The graph is showing the changes in MMP as represented by the mean fluorescence intensity of rhodamine 123. ***P < 0.001 Indicates significant difference compared to the control group and $^{###}P$ < 0.001 versus DOX-treated group. DOX, Doxorubicin; ISO, isoimperatorin; OXY, oxypeucedanin.

The effect of oxypeucedanin and isoimperatorin pretreatment on MMP induced by DOX

To further elucidate how DOX can alter the mitochondrial events, the collapse of MMP in the cell line was evaluated with rhodamine 123. Fig. 4 clearly shows DOX at its IC_{50} (5 μM) concentration considerably reduced the MMP by 50% in PC12 cells. Pretreatment of cells with oxypeucedanin and isoimperatorin attenuated the MMP changes caused by DOX. Fluorescence intensity increased 25% and 34% in cells pretreated with oxypeucedanin and isoimperatorin at the same concentration (80 μg/mL) in comparison to the DOX-treated group.

The effects of oxypeucedanin and isoimperatorin pretreatment on mRNA expression of Bax and Bcl-2

To approve our results and also to investigate the effect of oxypeucedanin and isoimperatorin on the decrease of DOX-induced apoptosis, the mRNA expression of Bax and Bcl2 were examined in cells. DOX significantly increased the expression level of Bax (2.5 fold) while decreasing Bcl-2 expression (0.3 fold) in comparison to the control, indicating 2.32-fold increase in the ratio of Bax to Bcl2 (Fig. 5). However, our results revealed the fact that pretreatment of PC12 cells with a concentration of 80 μg/mL of both compounds oxypeucedanin and isoimperatorin dramatically decreased the Bax/Bcl2 ratio to 1.1 and 0.57, respectively.

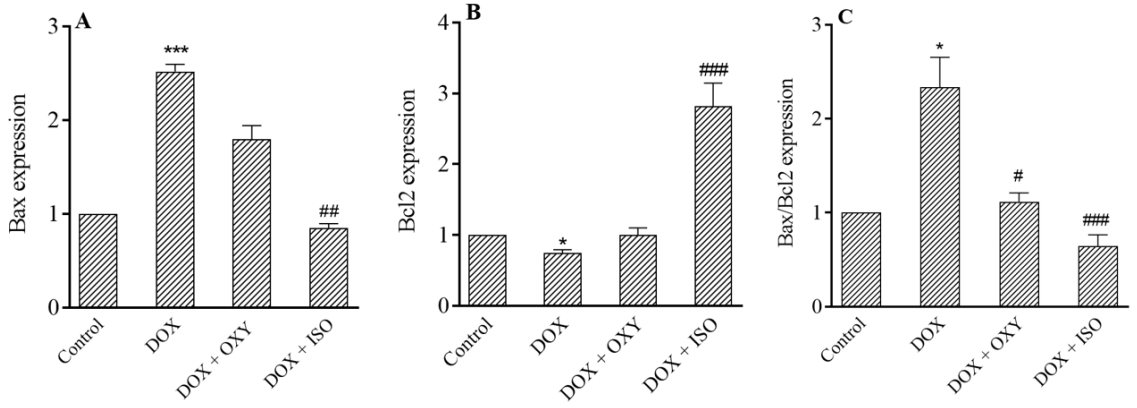


Fig. 5. The effect of oxypeucedanin and isoimperatorin on (A) Bax, (B) Bcl-2 mRNA expression, and (C) Bax/Bcl-2 in PC12 cells. Cells were pretreated with oxypeucedanin and isoimperatorin (80 μg/mL) for 2 h and 21 h, respectively, before exposure to DOX at 5 μM of. Normalization relative to B-actin was performed. Levels of mRNA are expressed relative to the control cells as the mean ± SEM values derived from three independent experiments. *P < 0.05 and ***P < 0.001 indicate significant differences compared to the control, $^{##}P$ < 0.01 and $^{###}P$ < 0.001 versus DOX-treated group. DOX, Doxorubicin; ISO, isoimperatorin; OXY, oxypeucedanin.

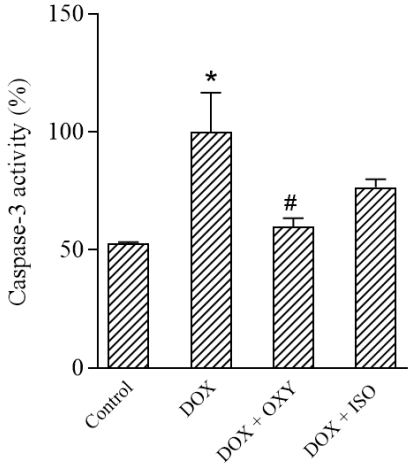


Fig. 6. The effect of oxypeucedanin and isoimperatorinon on caspase-3 activity in PC12 cells. Cells were pretreated with oxypeucedanin and isoimperatorin (80 µg/mL) for 2 h and 21 h, respectively, before exposure to DOX at 5 µM. Data are expressed as mean ± SEM of three separate experiments. $^{*}P < 0.05$ Indicates significant difference compared to the control group and $^{\#}P < 0.05$ versus DOX-treated cells. DOX, Doxorubicin; ISO, isoimperatorin; OXY, oxypeucedanin.

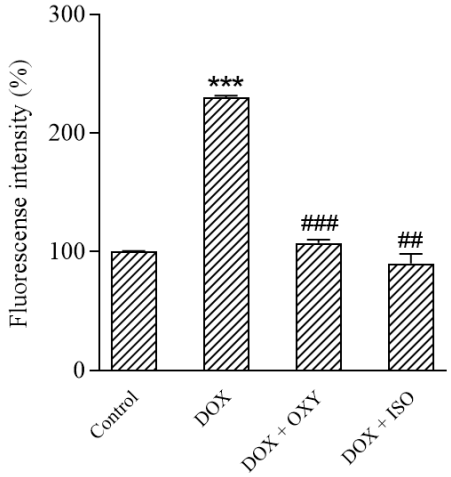


Fig. 7. Effect of oxypeucedanin and isoimperatorinon on DOX-induced reactive oxygen species overproduction. Cells pretreated with oxypeucedanin and isoimperatorin (80 µg/mL) 2 h and 21 h, respectively, before exposure to DOX 5 µM. Data are represented as mean ± SEM for three independent experiments. $^{***}P < 0.001$ Indicates significant difference in comparison to the control group, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus DOX-treated cells. DOX, Doxorubicin; ISO, isoimperatorin; OXY, oxypeucedanin.

Effect of oxypeucedanin and isoimperatorin pretreatment on caspase-3 activation

Activation of caspases is known as an important factor that drives apoptosis (26). Caspase-3 activity is one of the key markers of cell apoptosis, which can be evaluated to improve the MTT results and can also help understand cellular death mechanisms (apoptosis or necrosis). As expected, the caspase-3 activity in PC12 cells treated with DOX at IC_{50} concentration increased dramatically about 2 folds (Fig. 6). However, pretreatment with the same concentration (80 µg/mL) of oxypeucedanin and isoimperatorin effectively attenuated the activity of caspase-3 induced by DOX. Oxypeucedanin caused a 40% decrease in caspase-3 activity, while this value was 25% for isoimperatorin compared to DOX-treated PC12 cells.

Effect of oxypeucedanin and isoimperatorin pretreatment on ROS induced by DOX

As expected, the presence of DOX (IC_{50} concentration) for 24 h led to an increase in DCF fluorescence. Our results indicated a significant increase (2.3-fold) in the level of intracellular ROS in comparison with the control group (Fig. 7). However, we investigated the inhibitory effect of oxypeucedanin and isoimperatorin (80 µg/mL for both) on ROS production by DOX. As expected, both compounds were able to significantly decrease ROS, indicating their protective potential toward DOX-induced oxidation

DISCUSSION

Active oxygen species in brain and nerve tissues act as a source of oxidative stress and damage glial cells and neurons. Various types of natural antioxidants such as coumarins are potent therapeutics, which can be used to prevent neurons from oxidative stress (6,24). Our former studies were focused on the activity of osthol as a coumarin compound, against oxidative stress-induced in PC12 cells (19,20). In the current study, we investigated the protective and inhibitory effects of oxypeucedanin and isoimperatorin, as two main furanocoumarin compounds constituent of the *P. ferulacea* roots, on cytotoxic and apoptotic effects of DOX in PC12 cells.

Because of the significant cytoprotective effect of oxypeucedanin and isoimperatorin against DOX-induced toxicity, we evaluated key parameters involved in apoptosis, to achieve complete information about possible involved mechanisms. Irreversible and widespread mitochondrial outer membrane permeabilization has a pivotal role in apoptosis (27), which is controlled by the apoptosis regulator (Bcl-2) protein family (28). Bcl-2 family regulates the mitochondrial apoptotic pathway. The members of this protein family classify into pro- and anti-apoptotic proteins. Bcl2, an anti-apoptotic protein, binds to pro-apoptotic members such as Bax leading to the inhibition of pore formation and cytochrome c release (from the outer membrane of mitochondria) and the induction of apoptosis (28). As mentioned in the results section, treatment with DOX significantly caused enhancement of Bax/Bcl-2 and lowered the MMP in PC12 cells. Pal *et al.* reported that treatment with DOX leads to MMP reduction, disturbs the balance of Bcl-2 family protein, cytochrome c release, and finally induces apoptosis in the cells of the brain tissue (29). It is demonstrated that the amount of success in the progress of apoptosis depends on caspases, a family of cysteine-dependent proteases. Caspases play a critical role in execution of apoptosis by or the proteolysis-mediated activation of other apoptotic proteins and or directly by the cleavage of nuclear proteins (30). Following the interaction, activation of caspase 9, an initiator caspase, causes initiating a caspase cascade and activates executioner caspase-3, leading to the destruction of the cell (31). Therefore, to get more insights into cell death pathway that occurred in response to the pretreatment with oxypeucedanin and isoimperatorin, we evaluated the activation of caspase-3. Similar to the previous studies, DOX significantly increased caspase-3 activation (30). However, when the protective effect of oxypeucedanin and isoimperatorin on DOX-induced cytotoxicity was examined, we observed the growth of MMP and reduction in Bax/Bcl-2 ratio with pretreatment of PC12 cells at a sub-toxic concentration of these compounds, which represents protective effect of oxypeucedanin and isoimperatorin against DOX-induced cytotoxicity. Inhibition of rapid collapse of MMP by oxypeucedanin and isoimperatorin and suppression of caspase-3 activity indicates that these compounds prevented apoptosis in PC12 cells *via* the intrinsic mitochondrial pathway, which can be the possible mechanism for inhibition of apoptosis. Caspases *via* the death receptor and the mitochondrial pathway have a vital role in the apoptotic process (30). This result was in line with the findings of previous studies (30,31).

One of the most important factors in the destruction of cell brain, cardiac, hepatic, lung and kidney is the production of ROS by DOX (32). Previous studies have shown that active species of oxygen can lead to cell death and apoptosis in neurons, eventually leading to neurodegenerative diseases (33). Klaassen and Rozman suggested that free radical-mediated oxidative stress can be the main mechanism by which DOX induces apoptosis in the brain (34). Another study reported that osthole, a coumarin compound extracted from the plant-derived medicine *Cnidium monnieri*, ameliorates MPP+-induced apoptosis in PC12 cells *via* inhibition of ROS generation (22). Moreover, Liu *et al.* realized that the production of ROS in PC12 cells declines in the presence of osthole (22). Piao *et al.* showed furanocoumarins isolated from *Angelicae dahuricae* have a potent antioxidant effect against renal epithelial cell injury induced by o 2, 2′-azobis (2-aminodinopropane) dihydrochloride (AAPH) (35). In line with the previously published studies, our *in vitro* experiment revealed the neuroprotective effect of oxypeucedanin and isoimperatorin due to the reduction of ROS levels.

CONCLUSION

It can be concluded that oxypeucedanin and isoimperatorin isolated from acetone-extract of *P. ferulacea* roots are able to inhibit oxidative stress and apoptosis induced by DOX in the PC12 cells. However, further studies are necessary to indicate explicit neuroprotective mechanisms before definite conclusions can be drawn.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

M. Moeeni-Arya designed and performed experiments, and analyzed data. F. Jalilian, wrote the manuscript. L. Hosseinzadeh and Y. Shokoohinia supervised the work, designed the study, corrected the manuscript, and provided the facilities for the study. The final version of the manuscript has been approved by all authors.

REFERENCES

1. Xu P, Xu J, Liu S, Ren G, Yang Z. *In vitro* toxicity of nanosized copper particles in PC12 cells induced by oxidative stress. J Nanopart Res. 2012;14:906-1004. DOI: 10.1007/s11051-012-0906-5.
2. Park ES, Kim SD, Lee MH, Lee HSS, Lee IS, Sung JK, *et al*. Protective effects of N-acetylcysteine and selenium against doxorubicin toxicity in rats. J Vet Sci. 2003;4(2):129-136. DOI: 10.4142/jvs.2003.4.2.129.
3. Abbasi A, Hajialyani M, Hosseinzadeh L, Jalilian F, Yaghmaei P, Navid SJ, *et al*. Evaluation of the cytotoxic and apoptogenic effects of cinnamaldehyde on U87MG cells alone and in combination with doxorubicin. Res Pharm Sci. 2020;15(1):26-35. DOI: 10.4103/1735-5362.278712.
4. Farzaei MH, Bahramsoltani R, Rahimi R, Abbasabadi F, Abdollahi M. A systematic review of plant-derived natural compounds for anxiety disorders. Curr Top Med Chem. 2016;16(17):1924-1942. DOI: 10.2174/1568026616666160204121039.
5. Karlsson M, Kurz T, Brunk UT, Nilsson SE, Frennesson CI. What does the commonly used DCF test for oxidative stress really show. Biochem J. 2010;428(2):183-190. DOI: 10.1042/BJ20100208.
6. Al-Majedy Y, Al-Amiery A, Kadhum AA, Bakar Mohamad A. Antioxidant activity of coumarins. Sys Rev Pharm. 2017;8(1):24-30. DOI: 10.5530/srp.2017.1.6.
7. Al-Majedy YK, Al-Duhaidahawi DL, Al-Azawi KF, Al-Amiery AA, Kadhum AAH, Mohamad AB. Coumarins as potential antioxidant agents complemented with suggested mechanisms and approved by molecular modeling studies. Molecules. 2016;21(2):135-145. DOI: 10.3390/molecules21020135.
8. Abyshev AZ, Denisenko PP, Kostyuchenko NP, Ermakov AI, Sheinker YN. Gosferol-a new furocoumarin from the roots of *Prangos ferulacea*. Chem Nat Compd. 1972;8(1):43-47. DOI: 10.1007/BF00564434.
9. Abyshev A. Structure of some components of the roots of *Prangos ferulacea*. Chem Nat Compd. 1974;10(5):581-585. DOI: 10.1007/BF00567845.
10. Eshbakova K, Saidkhodzhaev A, Baser K, Duman H, Vdovin A, Abdullaev N. Furocoumarins from *Prangos ferulacea*. Chem Nat Compd. 2006;42(1):102-103. DOI: 10.1007/s10600-006-0047-0.
11. Shokoohinia Y, Sajjadi SE, Gholamzadeh S, Fattahi A, Behbahani M. Antiviral and cytotoxic evaluation of coumarins from *Prangos ferulacea*. Pharm Biol. 2014;52(12):1543-1549. DOI: 10.3109/13880209.2014.907322.
12. Gholivand MB, Yamini Y, Dayeni M, Shokoohinia Y. The influence of the extraction mode on three coumarin compounds yield from *Prangos ferulacea* (L.) Lindl roots. J Iran Chem Soc. 2015;12:707-714. DOI: 10.1007/s13738-014-0529-0.
13. Delnavazi MR, Soleimani M, Hadjiakhoondi A, Yass N. Isolation of phenolic derivatives and essential oil analysis of *Prangos ferulacea* (L.) Lindl. aerial parts. Iran J Pharm Res. 2017;16(Suppl):207-215. PMID: 29844792.
14. Cai Y, Baer-Dubowska W, Ashwood-Smith M, DiGiovanni J. Inhibitory effects of naturally occurring coumarins on the metabolic activation of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene in cultured mouse keratinocytes. Carcinogenesis. 1997;18(1):215-222. DOI: 10.1093/carcin/18.1.215.
15. Wang M, Jia M, Ma Y, Li X. Pharmacological effect of four linear furocoumarins in Radix *Angelicae dahuricae*. Nat Prod Res Dev. 2010;22(3):485-489.
16. Tong K, Xin C, Chen W. Isoimperatorin induces apoptosis of the SGC-7901 human gastric cancer cell line *via* the mitochondria-mediated pathway. Oncol Lett. 2017;13(1):518-524. DOI: 10.3892/ol.2016.5387.
17. Park SH, Hong JY, Park HJ, Lee SK. The Antiproliferative activity of oxypeucedanin *via* induction of G2/M phase cell cycle arrest and p53-dependent MDM2/p21 expression in human hepatoma cells. Molecules. 2020;25(3):501-515. DOI: 10.3390/molecules25030501.
18. Kahkeshani N, Farahanikia B, Mahdaviani P, Abdolghaffari A, Hassanzadeh G, Abdollahi M, *et al*. Antioxidant and burn healing potential of *Galium odoratum* extracts. Res Pharm Sci. 2013;8(3):197-203. PMID: 24019829.
19. Shokoohinia Y, Hosseinzadeh L, Moieni-Arya M, Mostafaie A, Mohammadi-Motlagh HR. Osthole attenuates doxorubicin-induced apoptosis in PC12 cells through inhibition of mitochondrial dysfunction and ROS production. Biomed Res Int. 2014;2014:156848,1-7. DOI: 10.1155/2014/156848.

20. Shokoohinia Y, Khajouei S, Ahmadi F, Ghiasvand N, Hosseinzadeh L. Protective effect of bioactive compounds from *Echinophora cinerea* against cisplatin-induced oxidative stress and apoptosis in the PC12 cell line. Iran J Basic Med Sci. 2017;20(4):438-445.
DOI: 10.22038/IJBMS.2017.8587.
21. Rebois RV, Reynolds EE, Toll L, Howard BD. Storage of dopamine and acetylcholine in granules of PC12, a clonal pheochromocytoma cell line. Biochemistry. 1980;19(6):1240-1248.
DOI: 10.1021/bi00547a031.
22. Liu WB, Zhou J, Qu Y, Li X, Lu CT, Xie KL, *et al*. Neuroprotective effect of osthole on MPP+-induced cytotoxicity in PC12 cells *via* inhibition of mitochondrial dysfunction and ROS production. Neurochem Int. 2010;57(3):206-215.
DOI: 10.1016/j.neuint.2010.05.011.
23. Aloui L, Kossentini M, Geffroy-Rodier C, Guillard J, Zouari S. Phytochemical investigation, isolation and characterization of coumarins from aerial parts and roots of *Tunisian Pituranthos chloranthus* (*Apiaceae*). Pharmacogn Commun. 2015;5(4):237-243.
DOI: 10.5530/pc.2015.4.4.
24. Bai Y, Li D, Zhou T, Qin N, Li Z, Yu Z, *et al*. Coumarins from the roots of *Angelica dahurica* with antioxidant and antiproliferative activities. J Funct Foods. 2016;20:453-462.
DOI: 10.1016/j.jff.2015.11.018.
25. Wei Y, Ito Y. Preparative isolation of imperatorin, oxypeucedanin and isoimperatorin from traditional Chinese herb "bai zhi" *Angelica dahurica* (Fisch. ex Hoffm) Benth. et Hook using multidimensional high-speed counter-current chromatography. J Chromatogr A. 2006;1115(1-2):112-117.
DOI: 10.1016/j.chroma.2006.02.081.
26. Grütter MG. Caspases: key players in programmed cell death. Curr Opin Struct Biol. 2000;10(6):649-655.
DOI: 10.1016/s0959-440x(00)00146-9.
27. Galluzzi L, Kepp O, Kroemer G. Mitochondrial regulation of cell death: a phylogenetically conserved control. Microb Cell. 2016;3(3):101-108.
DOI: 10.15698/mic2016.03.483.
28. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol. 2014;15(1):49-63.
DOI: 10.1038/nrm3722.
29. Pal S, Ahir M, Sil PC. Doxorubicin-induced neurotoxicity is attenuated by a 43-kD protein from the leaves of *Cajanus indicus* L. *via* NF-κB and mitochondria dependent pathways. Free Radic Res. 2012;46(6):785-798.
DOI: 10.3109/10715762.2012.678841.
30. Mojarrab M, Mehrabi M, Ahmadi F, Hosseinzadeh L. Protective effects of fractions from *Artemisia biennis* hydro-ethanolic extract against doxorubicin-induced oxidative stress and apoptosis in PC12 cells. Iran J Basic Med Sci. 2016;19(5):503-510.
PMID: 27403257.
31. Hosseinzadeh L, Mirzaei S, Hajialyani M, Ahmadi F, Emami S, Mojarrab M. The protective effect of different extracts of aerial parts of *Artemisia ciniformis* against H2O2-induced oxidative stress and apoptosis in PC12 pheochromocytoma cells. J App Pharm Sci. 2019;9(4):16-23.
DOI: 10.7324/JAPS.2019.90403.
32. Al Seyedan A, Dezfoulian O, Alirezaei M. *Satureja khuzistanica Jamzad* essential oil prevents doxorubicin-induced apoptosis *via* extrinsic and intrinsic mitochondrial pathways. Res Pharm Sci. 2020;15(5):481-490.
DOI: 10.4103%2F1735-5362.297851.
33. Arabsolghar R, Saberzadeh J, Khodaei F, Borojeni RA, Khorsand M, Rashedinia M. The protective effect of sodium benzoate on aluminum toxicity in PC12 cell line. Res Pharm Sci. 2017;12(5):391-400.
DOI: 10.4103/1735-5362.213984.
34. Rozman KK, Klaassen CD. Casarett and Doull's Toxicology: The Basic Science of Poisons. 9th ed. New York: McGraw-Hill; 2007. pp: 705.
35. Piao XL, Park IH, Baek SH, Kim HY, Park MK, Park JH. Antioxidative activity of furanocoumarins isolated from *Angelicae dahuricae*. J Ethnopharmacol. 2004;93(2-3):243-246.
DOI: 10.1016/j.jep.2004.03.054.