Eriodictyol exerts potent anticancer activity against A549 human lung cancer cell line by inducing mitochondrial-mediated apoptosis, G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signalling pathway

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Abstract

Introduction: Eriodictyol is an important flavonoid and is commonly present across the plant kingdom. Flavonoids have been reported to show incredible pharmacological potential. However, the anticancer activity of the important flavonoid eriodictyol has not been well reported. In the present study we determined its anticancer potential against the human lung cancer cell line A549.

Material and methods: The initial cytotoxicity induced by eriodictyol was measured by MTT assay. Flow cytometry was used to study the effects of eriodictyol on apoptosis, cell cycle phase distribution and mitochondrial membrane potential loss. The comet assay was used to measure DNA damage induced by eriodictyol in cancer cells while the western blot assay indicated effects of the compound on Bax/Bcl-2 and PI3K/AKT/m-TOR proteins.

Results: The results showed that eriodictyol has an IC₅₀ value of 50 μM against human lung cancer cells as compared to the IC₅₀ of 95 μM against non-cancerous FR2 cells. The molecule exerted its anticancer activity through induction of apoptosis by regulating the Bcl-2/Bax signalling pathway. It caused cell cycle arrest of human lung cancer A549 cells at G2/M phase. Eriodictyol was also found to cause a reduction of the mitochondrial membrane potential in a dose-dependent manner. Additionally, eriodictyol effectively inhibited the mTOR/PI3K/Akt signalling pathway in a dose-dependent manner.

Conclusions: Based on the above findings, we conclude that eriodictyol exerts its anticancer activity through induction of mitochondrial apoptosis and G2/M cell cycle arrest and inhibition of the TOR/PI3K/Akt cascade, indicating that it may have potential as a lead compound in the treatment of lung cancer, provided further in depth studies are done.

Key words: lung cancer, eriodictyol, apoptosis, cell cycle arrest, flow cytometer.
Introduction

Flavonoids are commonly present across the plant kingdom and have been reported to exhibit tremendous pharmacological potential. A diversity of medicinal plants showing the presence of high amounts of flavonoids have been used in several traditional systems of medicine for the treatment of several diseases and disorders. The medicinal properties of these plants are mainly due to the presence of these secondary metabolites [1, 2]. Among natural products, flavonoids represent an important part of the human diet, and in the United States the estimated regular dietary intake of mixed flavonoids is up to 1 g. This figure may be even higher for people improving their diets with flavonoid-rich herbal preparations [3]. With advances in medical research, flavonoids are being evaluated for their diverse bioactivities. So far they have been reported to exhibit a wide range of activities – anti-inflammatory, estrogenic, enzyme inhibition, antimicrobial, anti-allergic, antioxidant, antitumor and others [4]. Owing to their fairly consistent structure, flavonoids impede the activity of a wide range of eukaryotic enzymes and therefore exhibit diversity of activities. The different parts of the flavonoid molecules have been considered critical for their bioactivities [4]. The present study was therefore designed to evaluate the anticancer potential of eriodictyol (Figure 1) against the human lung cancer cell line A549 and to decipher the underlying mechanism. Lung cancer is the major cause of cancer related deaths in the world and in China [5, 6]. The severe increase in the incidence of cancers, the dearth of suitable cures and the severe side effects accompanying the synthetic drugs have made it necessary to explore new and more effective molecules. With the upsurge in the incidence of cancer related deaths in the world and in China [5, 6], the chemical study of lung cancer A549 cells and it was found to exhibit an IC_{50} of 50 μM. The results of the present study indicated that eriodictyol exhibits significant anticancer activity by inducing apoptosis in human lung cancer A549 cancer cells, altering mitochondrial membrane potential (MMP) and causing cell cycle arrest via downregulation of the expression of Bcl-2 and upregulation of Bax expression. In conclusion, we propose that eriodictyol may prove to be a potential candidate for the development of anticancer chemotherapy for lung cancer.

Material and methods

Chemical reagents, cell lines and culture conditions

All chemicals and reagents including eriodictyol (98% pure by HPLC) were obtained from Sigma-Aldrich. Human lung cancer (A549) and non-cancerous human epithelial FR2 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained in a humidified atmosphere containing 5% CO{sub 2}. All of the reagents were procured from Hyclone (Logan, UT, USA).

Anti-proliferative assay

MTT was used to determine the anti-proliferative activity of eriodictyol against the human lung cancer A549 cells [7]. The A549 cells in 100 μl of culture medium were seeded in a 96-well plate at a density of 3 × 10{sup 3} cells/ml and kept at 37°C in 5% CO{sub 2} for 48 h. After 24 h, an additional 100 μl of complete medium with either no additions or different concentrations (0–100 μM) of eriodictyol was added. Thereafter, the cells were incubated for 12, 24, and 48 h. This was followed by the addition of 20 μl of MTT solution (5 mg/ml) and incubation for 4 h. Afterwards the medium was removed and 150 μl of DMSO added. The absorbance (OD) of each well was measured at 490 nm using a Tunable Mi-185 microplate Reader (EL-x 800, BioTek Instruments, USA).

Determination of apoptotic populations and DNA damage

Human A549 cells at a density of 2 × 10{sup 5} cells/well were seeded in 6-well plates and then administered with 0, 25, 50 and 100 μM eriodictyol for 48 h. For estimation of apoptotic cell populations an FITC-Annexin V/PI Apoptosis detection kit was used following the manufacturer’s instructions (Beijing Biosese Biotechnology, China). The alkaline comet assay was carried out to evaluate DNA damage efficacy of eriodictyol as described previously [8, 9].

Cell cycle analysis

For cell cycle analysis the A549 cells were treated with 0, 25, 50 and 100 μM eriodictyol concentration of KAM and the percentage of cells in each of the cell cycle phases was estimated using

![Figure 1. Chemical structure of eriodictyol](image-url)

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the Muse Cell Analyzer and Muse Cell Cycle Kit according to the manufacturer's protocol (Merck Millipore).

Mitochondrial membrane potential (MMP)

Human lung cancer A549 cells were seeded at a density of $2 \times 10^5$ cells/well in a 6-well plate and kept for 24 h and treated with 0, 25, 50 and 100 μM of the test compound for 48 h at 37°C in 5% CO₂ and 95% air. Afterwards, the cells from all treatments were collected, washed twice with PBS and re-suspended in 500 μl of DOC (1 μmol/l) for MMP at 37°C in a dark room for 30 min. The samples were then analyzed instantly using flow cytometry.

Western blotting analysis

After administration with various concentrations of eriodictyol, cells were harvested and lysed in lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Out of the total protein samples a 20 μg aliquot was separated on 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% BSA and probed with a primary antibody. This was followed by probing with the required secondary antibody. Finally, the signal was perceived with WESTSAVE Up luminal-based ECL reagent (ABFrontier, Korea).

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean ± standard deviation (SD). The values were considered significant at $p^* < 0.05$ and $p^{**} < 0.01$. The statistical analysis was carried out by Student’s t test using GraphPad prism 7 software.

Results

Anticancer activity of eriodictyol on A549 human lung cancer cells

Anticancer activity of eriodictyol (Figure 1) was evaluated against human A549 cancer and non-cancerous FR2 cells. The MTT assay at 0–100 μM concentration showed that eriodictyol exhibited a concentration-dependent activity. The IC₅₀ of eriodictyol against human A549 cells was found to be IC₅₀ 50 μM (Figure 2 A) as against the IC₅₀ of 95 μM against the noncancerous FR2 cells (Figure 2 B).

Eriodictyol caused apoptosis and DNA damage in human A549 lung cancer cells

In order to confirm apoptotic cell death induced by eriodictyol annexin V/PI staining was carried out at the concentrations of 0, 25, 50 and 100 μM. Flow cytometric results showed that the percentage of apoptotic cell population increased to 9.7%, 27.4% and 39.5% in human A549 cancer cells after 48 h at the concentrations of 25, 50 and 100 μM, respectively, as compared to the untreated control (Figure 3). Thus the results indicate that eriodictyol caused apoptotic cell death of human A549 cancer in a concentration-dependent manner. Furthermore, the results of the comet assay showed that eriodictyol caused DNA damage in A549 human lung cancer cells dose dependably (Figure 4) and the DNA damage increased with the increasing concentration of the drug.

G2/M phase arrest of A549 cancer cells triggered by eriodictyol

Our results indicated that eriodictyol caused G2/M cell cycle arrest in a dose. It was observed that the percentage of cells was considerably increased in G2/M at the concentrations of 0 to 100 μM of eriodictyol, causing G2/M arrest (Figure 5). Additionally, the populations of A549 cells

![Figure 2](image-url)  

**Results.** Effect of eriodictyol on viability of (A) human A549 lung cancer and (B) non-cancerous FR2 cell lines at 48 h of incubation. All experiments are representatives of three biological replicates ± SD

Results were considered significant at $p^* < 0.05$ and $p^{**} < 0.01$. 

Figure 2
Eriodictyol exerts potent anticancer activity against A549 human lung cancer cell line by inducing mitochondrial-mediated apoptosis, G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signalling pathway.

In G2/M phase were only slightly elevated at a dose of 25 μM. However, the apoptotic cell populations significantly increased at G2/M phase at the concentration of 50 μM. Moreover, the eriodictyol induced G2/M phase increase of A549 cancer cells showed a dose-dependent trend.

**Figure 3.** Induction of apoptosis by eriodictyol at the indicated doses by Annexin V/PI staining observed by flow cytometry. All experiments are representatives of three biological replicates.

**Figure 4.** Induction of DNA damage at the indicated doses of eriodictyol by comet assay. All experiments are representatives of three biological replicates.
MMP loss in human A549 lung cancer cells

Cells were administered with 0, 25, 50 and 100 μM eriodictyol for various time periods and the levels MMP were evaluated. A significant reduction of MMP level (Figure 6 A) was observed in the treated A549 cells as compared to the control. At concentrations of 0, 25, 50 and 100 μM, the MMP was found to be 81, 52 and 33% as compared to the untreated human A549 cancer cells.

Effect of eriodictyol on Bcl-2/Bax signalling pathway

To evaluate whether eriodictyol could induce apoptosis, the expression of pro-apoptotic proteins Bcl-2/Bax was evaluated using western blot assay. The findings are shown in Figure 6 B and indicate an interesting outcome. The increased Bax/Bcl-2 ratio causes activation of caspase 3 and hence apoptosis. In our results, compared

![Graph showing MMP loss in human A549 cells](image)

![Graph showing Bcl-2/Bax expression](image)

Figure 5. Effect of indicated doses of eriodictyol on cell cycle phase distribution of A549 cancer cells. All experiments are representatives of three biological replicates

Figure 6. Effect of indicated doses eriodictyol on (A) mitochondrial membrane potential of A549 cancer cells, (B) expression of Bax/Bcl-2 by western blotting. All experiments are replicates of three biological replicates

The values were considered significant at p* < 0.05 and p** < 0.01.
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Figure 7. Effect of indicated doses of eriodictyol on key proteins of PI3K/AKT/mTOR signalling pathway. All experiments were carried out in triplicate.
The results showed eriodictyol acid-treated cells showed concentration-dependent downregulation of Bcl-2 and upregulation of Bax proteins, ultimately inducing apoptosis. Members of the Bcl-2 family proteins, such as Bcl-2, Bax and Bak, are believed to play key controlling roles in the execution of cell apoptosis. However, several studies have shown that Bcl-2 family proteins also aim at the apoptotic pathway [19, 20]. Finally, the effect of eriodictyol on the PI3/AKT/mToR signalling pathway was investigated and it was observed that it caused inhibition of a key protein of the pathway. These results are interesting since this target is considered important cancer chemotherapy.

Taken together, our results have shown that eriodictyol is a potent anti-cancerous molecule. Furthermore, flavonoids are generally non-toxic and can hence be used at higher concentrations in humans. The results of the present study pave the way for further evaluation of this molecule against more cell lines and under in vivo conditions.

In conclusion, in the present study, our data provide a framework for the construction of cell death pathways in A549 cells in response to eriodictyol by inducing apoptosis, regulating the Bcl-2/Bax signalling pathway and mainly by inhibition of the PI3/AKT/mToR signalling pathway. All of these contribute to the inhibition of growth of cancer cells, and eriodictyol might potentially serve as a potential candidate for cancer therapy.

Acknowledgments
Yong Zhang and Rui Zhang contributed to this work equally.

Conflict of interest
The authors declare no conflict of interest.

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