Taraxerol exerts potent anticancer effects via induction of apoptosis and inhibition of Nf-κB signalling pathway in human middle ear epithelial cholesteatoma cells

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Abstract

Purpose: To investigate the effect of taraxerol on the proliferation of middle ear epithelial cholesteatoma cells.

Methods: The anti-proliferative effect of taraxerol was investigated by cell counting kit-8 (CCK8) and clonogenic assays. Apoptosis was measured using DAPI, while mitochondrial membrane potential was determined with the aid of rhodamine 123 staining. Protein expression was studied by western blotting.

Results: Taraxerol induced concentration-dependent anti-proliferative effects on the middle ear epithelial cholesteatoma cells, and also inhibited their colony formation potential. The drug induced apoptosis in the middle ear epithelial cholesteatoma cells by reducing mitochondrial membrane potential, and also triggered sub-G1 cell cycle arrest in these cells. Moreover, taraxerol inhibited the expression of Nf-κB.

Conclusion: These findings reveal that taraxerol may be a potential lead compound for the treatment of middle ear cholesteatoma.

Keywords: Cholesteatoma, Epithelial tissues, Taraxerol, Apoptosis, Cell cycle arrest

INTRODUCTION

Middle ear cholesteatoma is a very serious pathology which results from proliferation of epithelial tissues. The reasons for the extension into the middle ear and aetiology of cholesteatotoma are yet to be fully understood [1]. However, it is believed that the epithelium proliferates due to some growth stimulus, leading to chronic inflammation [2]. A few studies reported the involvement of infections in the onset of cholesteatoma. However, many other studies have suggested the involvement of inflammatory cytokines [3]. Cholesteatoma causes serious bone destruction during its proliferation, and the current treatment strategies are not only insufficiently effective, but are also associated with several side effects [4].

Natural products are considered important sources of compounds that can be utilized for the management cholesteatoma. A number of
Currently used drugs are derived from natural sources and new drugs from natural sources are being frequently discovered [5]. Moreover, compounds derived from natural sources are thought to be comparatively less toxic than their synthetic counterparts [6]. Plants are important sources of drugs. Indeed, a number of drugs currently used in clinics have their origins in plants [5].

_Clitotiaternatea_ L. (butterfly pea) is an important medicinal plant and a source of some important bioactive compounds. It has been utilised in the management of numerous diseases and disorders such as chronic bronchitis, goitre, leprosy, mucous and dropsy [7,8]. The roots of this plant are important sources of two important compounds taraxerol and taraxerone. Taraxerol is a pharmacologically important compound that has been reported to exhibit tremendous pharmacological activities which include anti-tumour, antimicrobial and anti-inflammatory properties [9].

This investigation was performed to find out the impact of taraxerol on cultured human middle ear epithelial cholesteatoma cells.

**EXPERIMENTAL**

**Cell cultures, chemicals and regents**

Cultured human middle ear epithelial cholesteatoma cells were obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The study was approved the Ethical Committee of Quanzhou First Hospital Affiliated to Fujian Medical University (approval number: QFH/0987C/2016). All the procedures used were as per standard guidelines [10]. Taraxerol (98 % HPLC-pure) and all other chemicals were obtained from Sigma-Aldrich Ltd, US.

**Anti-proliferative and colony formation assays**

The impact of taraxerol on the proliferation of human middle ear epithelial cholesteatoma cells was investigated by CCK8 assay. The cells (5×10^5) were grown in a ninety-six well plate and subjected to incubation at 37 °C in an atmosphere of 5% CO₂. Following this, the cells were administrated with various doses of taraxerol (0, 7.5, 15, and 30 μM) for overnight. Thereafter, the addition of CCK8 (10 μL) was done and incubated at 37 °C for 50 min. The optical density of each well’s content was read at 450 nm using a microplate spectrophotometer.

Cell proliferation calculated as percentage of the control.

**Colony formation assay**

Cultured human middle ear epithelial cholesteatoma cells were seeded at 200 cells/well. Thereafter, the cells were incubated for 48 h to allow for attachment, after which they were exposed to different doses (0, 7.5, 15 and 30 μM) of taraxerol. The cells were then subjected to incubation for 6 days, and thereafter washed with PBS, and treated with methanol for colony fixation. The crystal violet staining of the colonies was performed for about 30 min, and counted under the light microscope as described previously [10].

**Apoptosis assay**

The cholesteatoma epithelial cells were grown at 2x10⁵ cell density per well in ninety-six well plates and subjected to treatment with varied doses of taraxerol (0, 7.5, 15, and 30 μM), followed by incubation for 24h. They were then stained with DAPI, and there after subjected to PBS washing and then fixed in formaldehyde (10 %). The DAPI-stained cells were then observed and photography under a fluorescent microscope as described previously [11].

**Determination of mitochondrial membrane potential**

Mitochondrial membrane potential of the middle ear epithelial cholesteatoma cells was studied using confocal microscopy, following staining with rhodamine123 (Rh123). Taraxerol-treated and cultured cells at a density of 2 × 10^5/mL/well were grown in six-well plates and incubated for 24 h. This was followed by trypsinization and washing with PBS and then re-suspended in the medium and stained with rhodamine 123, followed by incubation for 25 min at 37 °C. Thereafter the cells were collected and washed with PBS, and finally examined using a laser scanning confocal microscope as described previously [12].

**Cell cycle analysis**

To investigate the distribution of middle ear epithelial cholesteatoma cells in various cell cycle phases, the taraxerol-treated (0, 7.5, 15, and 30 μM) cells were collected and twice subjected to PBS washing. This was followed by fixation of the cells in ethanol (70 %) for 1 h. The cells were finally re-suspended in propidium iodide solution (50 μl/mL) and RNase1 (250μg/mL) and then subjected to incubation for
30 min at 25 °C. Finally, the dissemination of the cells in various phases of cell cycle was estimated using fluorescence-activated cell sorting cater-plus cytometer at 10,000 cells/group as described previously [13].

**Western blotting**

Taraxerol-treated ear epithelial cholesteatoma cells were lysed in lysis buffer, and proteins were harvested. The concentration of proteins in each sample was determined by BCA assay, and Western blotting was carried out as described previously [1].

**Statistical analysis**

Data were shown as mean (of three biological replicates) ± SD. Statistical analysis was performed using student’s t test by GraphPad prism 7 software. Values of \( p<0.01 \) were taken as indicative of statistically significant differences.

**RESULTS**

**Taraxerol exerted anti-proliferative effects on epithelial cholesteatoma cells**

The results obtained showed that taraxerol inhibited the proliferation of middle ear epithelial cholesteatoma cells in a concentration-dependent fashion (Figures 1 B - C).

**Taraxerol inhibited colony formation of epithelial cholesteatoma cells**

The colony formation potential of cultured middle ear epithelial cholesteatoma cells were significantly and dose-dependently inhibited by taraxerol (\( p<0.01 \), Figure 2 A and B).

**Taraxerol induced apoptosis in epithelial cholesteatoma cells**

Taraxerol induced apoptosis in epithelial cholesteatoma cells as obvious from the development of apoptotic crops and cell blebbing (Figure 3A). Moreover, the apoptosis-inducing potential of taraxerol was concentration-dependent, and the apoptotic cell percentage cells significantly (\( p<0.01 \)) enhanced with increase in taraxerol concentration (Figure 3 B).

**Taraxerol reduced mitochondrial membrane potential of epithelial cholesteatoma cells**

Taraxerol reduced mitochondrial membrane potential in a concentration-dependent manner (Figure 4 A - B).

**Taraxerol triggered cell cycle arrest in epithelial cholesteatoma cells**

It was observed that taraxerol caused significant enhancement in the sub-G1 cell populations of the cells, ultimately leading to sub-G1 cell cycle arrest. These effects of taraxerol were also concentration-dependent (Figure 5).

**Taraxerol suppressed Nf-kB expression in epithelial cholesteatoma cells**

The effect of taraxerol on the expression of Nf-kB was investigated by western blotting. The results showed that taraxerol down-regulated the expression of Nf-kB in middle ear cholesteatoma epithelial cells in a concentration-dependent manner (Figure 6).

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Figure 1: (A) Chemical structure of taraxerol. (B) Effect of taraxerol on the proliferation of human middle ear epithelial cholesteatoma cells at the indicated doses. (n = 3), *\( p<0.01 \).
Figure 2: Effect of taraxerol on the colony formation potential. (A) Plates showing effect of taraxerol on colony formation in human middle ear epithelial cholesteatoma cells. (B) Quantification of the colonies \( (n = 3) \), \(* p < 0.01\).

Figure 3: Taraxerol-induced apoptosis in human middle ear epithelial cholesteatoma cells. (A) DAPI staining at indicated concentrations. (B) Concentration-dependent effect of taraxerol on cell apoptosis. \((n = 3)\), \(* p < 0.01\).

Figure 4: Effect of taraxerol on mitochondrial membrane potential. (A) Rhodamine-123 staining of human middle ear epithelial cholesteatoma cells at indicated doses. (B) Concentration-dependent taraxerol on mitochondrial membrane potential. \((n = 3)\), \(* p < 0.01\).
Figure 6: Effect of taraxerol on the expression of NF-kB in human middle ear epithelial cholesteatoma cells at indicated doses (n = 3)

DISCUSSION

Middle ear cholesteatoma is caused by proliferation of epithelial tissues, resulting in serious complications. The aetiology of this pathological condition is not well understood [1,2]. Moreover, the current treatments are not satisfactory, and are associated with side-effects [3]. Therefore, it is believed that development of new effective treatment options with minimal side-effects may prove beneficial. Natural products have a long history of being used for the treatment of several diseases and disorders [5]. In different systems of medicine including Chinese system of traditional medicine, Ayurveda and Unani medicine, different plant extracts and decoctions are used to treat a diversity of pathological conditions [6]. In most of the cases, treatments based on these traditional remedies have produced very effective outcomes. Indeed, researchers across the globe have used the knowledge from these traditional systems to isolate compounds that are effective in the treatment of several diseases and disorders [5].

Several compounds that are currently used as drugs were isolated from plants. For instance, podophyllotoxin, artemisinin and several other drugs were isolated from plants, and have shown tremendous therapeutic potential [5,6]. As part of the search program for natural bioactive compounds that can inhibit the proliferation of middle ear epithelial cholesteatoma cells, the present study was focussed on the anti-proliferative effects of taraxerol. Preliminarily, the anti-proliferative effect of taraxerol on ear epithelial cholesteatoma cells was evaluated by CCK8 and clonogenic assays. The results clearly showed that taraxerol suppressed the proliferation of these cells dose-dependently. Furthermore, results from colony formation assay complemented the results of CCK8 assay, and showed that taraxerol inhibited the colony-forming potential of the epithelial cholesteatoma cells. These results are in agreement with the results reported in a previous study wherein taraxerol was shown to inhibit the proliferation of several cancerous cells [14,15].

It has been reported that anti-proliferative agents inhibit the growth of the cells through apoptosis and cell cycle arrest, which are two important mechanisms that have been found effective in the elimination of the malignant and harmful cells [16,17]. Therefore, in the present study, investigation was carried out to see if taraxerol could induce apoptosis in epithelial cholesteatoma cells. Interestingly, it was shown that taraxerol triggered apoptosis in...
cholesteatoma epithelial cells in a dose dependent manner.

To investigate if this apoptosis follows mitochondrial pathway, mitochondrial membrane potential levels were also evaluated. The results showed that taraxerol reduced mitochondrial membrane potential dose-dependently. Previous investigations have also indicated that taraxerol prompts apoptosis in human gastric epithelial cells [18]. In the present study, it was observed that treatment of the epithelial cholesteatoma cells with taraxerol caused considerable accumulation of the cells in sub-G1 phase of the cell cycle, resulting in sub-G1 cell cycle arrest. Several anti-proliferative agents have been reported to induce cell cycle arrest in human cells [16,19].

In addition, it has been observed that NF-κB signalling pathway is upregulated in cholesteatoma epithelial cells and could prove to be an important therapeutic target [20]. Therefore the effect of taraxerol on the expression of NF-κB was investigated by western blot analysis, and the results showed that taraxerol suppressed the expression of NF-κB in a concentration-dependent manner.

**CONCLUSION**

The results obtained in this study indicate that taraxerol triggers apoptosis and cell cycle arrest in middle ear epithelial cholesteatoma by inhibiting the expression of NF-κB. Hence, taraxerol may be beneficial in the management of middle ear cholesteatoma.

**DECLARATIONS**

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**Conflict of interest**

There is no conflict of interest with regard to this work.

**Contribution of authors**

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. This paper was written and the whole study was designed by Jun Liao. Fengfang Wu, Wen Lin, Zhiwei Huang offer assistance for performing experiments under the supervision of Jun Liao.

**REFERENCES**


