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Piceatannol Inhibits Akt Activation, Induces G2/M Phase Arrest and Mitochondrial Apoptosis and Augments Cisplatin Efficacy in U2OS Osteosarcoma Cells

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Authors' Contribution
YL designed the study. ZL and RM performed experiments. CL and XC assisted in experimental work. The manuscript was drafted by ZL. MK and YL analyzed the data and proofread the manuscript.

Key words
Piceatannol, CDDP, Osteosarcoma, Apoptosis, Mitochondria

ABSTRACT

Piceatannol (3,3′,4,5′-tetrahydroxy-trans-stilbene), a naturally occurring analog of resveratrol has been shown to exhibit anticancer activity in various human cancer cells. However, the anticancer mechanism of piceatannol is not well studied. In the meantime, cisplatin (CDDP) is the first line treatment for some tumors. The present study was aimed to explore the cellular targets and anticancer mechanism of piceatannol in human osteosarcoma U2OS cells. Cell proliferation was measured using CCK-8 assay and colony forming assay. Flow cytometry was used to determine apoptosis and cell cycle profile. Western blot was used to measure the expression of various proteins. The data demonstrated that piceatannol inhibited growth and induced G2/M phase arrest and apoptosis. Further studies showed that piceatannol induces mitochondrial apoptosis as shown by Bcl-2 family proteins modulation. Finally, piceatannol significantly enhanced apoptotic efficacy of CDDP in U2OS cells. On the basis of our findings, piceatannol is a promising anticancer agent which could be developed into lead for osteosarcoma treatment as a single agent or in combination with CDDP.

INTRODUCTION

Human Osteosarcoma is the most commonly diagnosed malignant bone tumor, which mainly affects adolescents and young adults (Al Qahtani *et al*., 2015; Tan *et al*., 2006). Although the combination of modern surgery and systemic chemotherapy has improved osteosarcoma treatment dramatically, no substantial change in survival has been seen over the past 20 years (Akiyama *et al*., 2008). Therefore, to improve the survival of osteosarcoma patients, it is imperative to explore novel osteosarcoma therapies.

CDDP, a platinum-based alkylating agent is an effective drug for the treatment of various human cancers including osteosarcoma (Lamplot *et al*., 2013; Sasaoka *et al*., 2018). The prominent anticancer effects of CDDP are associated with induction of oxidative stress, DNA damage and apoptosis (Ghosh, 2019). The emergence of drug resistance has limited the use of CDDP in the successful treatment of various cancers including osteosarcoma (Fraser *et al*., 2003; Farrand *et al*., 2013). Therefore, it is important to explore new strategies, such as combination of natural products with clinical cancer drugs to overcome drug resistance.

As the ultimate goal of translational medicine is to develop new treatment strategies and to improve health across populations (Polese and Capunzo., 2013), more and more researchers are focusing to find novel combinations of natural compounds with cancer clinical drugs. Piceatannol, also known as 3-hydroxy resveratrolis, is a naturally occuring analog of the cancer chemopreventive agent resveratrol (trans-3,5,4'-trihydroxystilbene) (Fig. 1A), a natural antioxidant and a naturally occurring polyphenol

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present in the skins of grapes, red wine, and other foods (Ko *et al*., 2012; Kwon *et al*., 2012; Wolter *et al*., 2002). Piceatannol is used in traditional herbal medicine and folk medicine to treat cancer, tumors and warts (Wolter *et al*., 2002).

To date, piceatannol has been known to exhibit anticancer, anti-inflammatory and cardio-protective properties. Piceatannol has been reported to have antitumor effects in various types of human cancer, including breast cancer, bladder cancer, prostate cancer and lung cancer (Dhanapal and Balaraman Ravindrran., 2018; Ko *et al*., 2012; Kwon *et al*., 2012; Kuo and Hsu, 2008). Recently, the anti-angiogenic and anti-metastatic effects of piceatannol have been reported (Kim and Ma, 2019). The effects of piceatannol in osteosarcoma cells are not yet fully understood. In this study, we investigated the anticancer activity of piceatannol and its underlying mechanism in osteosarcoma using U2OS cells. Furthermore, we have evaluated the chemosensitizing effects of piceatannol.

MATERIALS AND METHODS

Antibodies and reagents

Piceatannol (Fig. 1B) was purchased from Selleck Chemicals (Cat: S3026, Shanghai, China) with purity >99% as determined by HPLC (Fig. 1C). The antibodies against Bax (sc-493), Bak (sc-832), Bcl-2 (sc-492), Cleaved PARP (sc-56196), P21 (sc-397), P27 (sc-1641), cyclin A (sc-596), cyclin B (sc-166210), CDC2 (sc-54) and cyclin D1 (sc-20044) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against γ-H2AX (9718p), Cleaved caspase-3 (9664), p-AKT (13038p), AKT (4691p), P-GSK-3β (5558p), P-C-Raf (9421p), Lc3B (2775s), p-CDC2 (4539) and P-PTEN (9551p) were purchased from Cell Signaling Technology (Danvers, MA, USA); antibodies against β-actin (66009-1-lg), GAPDH (10494-1-AP), p53(10442-1-AP) and CDK3 (55103-1-AP) were purchased from Proteintech (Wuhan, China).

Dulbecco's Modified Eagle's Medium (DMEM), fatal bovine serum (FBS), cell counting kit-8 (CCK-8) and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Crystal violet solution and Annexin V/PI double staining apoptosis assay kit were obtained from Beyotime institute of Biotechnology (Heimen, Jiangsu, China).

Cell line and culture

Human U2OS Osteosarcoma cells were obtained from ATCC (Manssas, VA, USA) and cultured in DMEM medium supplemented with 10% FBS at 37°C with 5% CO_2 in humidified atmosphere. The medium was replaced for every 2-3 days. Cells were treated with various concentrations (0, 20, 40, 60, 80 μM) of piceatannol in culture medium.

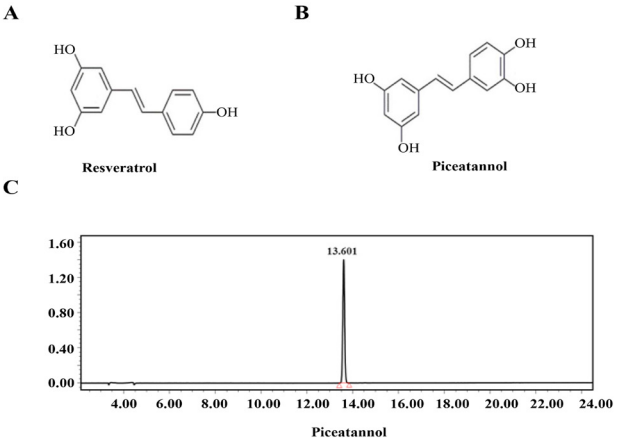

Fig. 1. Chemical structure of medicine. (A) Chemical structure of Resveratrol. (B) Chemical structure of Piceatannol. (C) HPLC purity peak of piceatannol.

Cell counting kit-8 (CCK-8) assay

Cell viability was measured using CCK-8 assay kit according to manufacturer's instructions (Sigma-Aldrich). In this assay, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) is used instead of other tetrazolium dyes such as MTT, XTT or MTS. Live cells convert WST-8 into an orange formazan product which is soluble in cell culture medium. The amount of formazan produced is directly proportional to the number of living cells. Briefly, U2OS cells at 1 x 10^3 cells/well in 100μL complete culture medium were seeded in 96-well plates. After culturing for 24 h, the medium was replaced with fresh DMEM containing various concentration of drug. Then, 10μL of CCK-8 solution was added to each well for another 4 h and the absorbance at 450 nm was measured by Synergy neo HTS multimode microplate reader (Bio Tec).

Cell count

Determination of cell number was carried out by flow cytometry. U2OS cells were plated at a density of 1×10^4 cells/well in 12-well plates. Cells were treated with various concentrations of piceatannol for 24 h. Following drug treatment, the cell numbers was counted by flow cytometry.

Colony forming assay

The anti-proliferative activity of drug was also determined by colony forming assay. Cells were seeded into 6 well cell culture plates and treated with different

concentrations of drug for 24 h. The drug containing medium was replaced with fresh culture medium and cells were allowed to grow for 10 days. On appearance of colonies, cells were fixed with 4% paraformaldehyde (PFA) for 20 min and stained with crystal violet solution for 30 min and photographed.

Cell cycle analysis

Cell cycle distribution was analyzed using BD Accuri C^6 Flow Cytometer. Treated and untreated cells were collected and fixed in 70% ethanol overnight at 4°C. After that, cells were centrifuged at 1200 rmp for 5 min. The cells pellet was resuspended in 100 µg/mL RNase A and 50 µg/mL PI at 37°C for 30 min in the dark. The samples were analyzed by flow cytometry for analysis of cell cycle profile.

Apoptosis assay

Apoptosis was determined using Annexin V-FITC/PI apoptosis detection kit. Briefly, the cells were harvested and washed with cold PBS and incubated with 500µL binding buffer. After that, 5µL Annexin V-FITC and 10µL PI were added to the cells. In addition, the cells were incubated for 15 min in the dark. Cell apoptosis was analyzed with at least 20,000 cells for each measurement by BD Accuri C^6 flow cytometer.

Western blot analysis

Cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Complete protease inhibitor cocktail (Roche) was added to lysis buffer before use. Protein concentration was determined by detergent-compatible protein assay (Bio-Rad). Protein samples were subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in PBS for 15 min and incubated with primary antibodies Bax (1:500), Bak (1:500), Bcl-2 (1:500), Cleaved PARP (1:500), P^{21} (1:500), P^{27} (1:500), cyclin A (1:500), cyclin B (1:500), CDC2 (1:500), cyclin D1 (1:500), γ-H2AX (1:1000), Cleaved caspase-3 (1:1000), p-AKT (1:1000), AKT (1:1000), P-GSK-3β (1:1000), p-C-Raf (1:1000), Lc3B (1:1000), p-CDC2 (1:1000), p-PTEN (1:1000), β-actin (1:1000), GAPDH (1:1000), p53(1:1000) and CDK3 (1:1000) overnight at 4°C. After washing the membranes with PBST three times for 5 min, the membrane was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for one hour at room temperature. The membranes were washed three times again; signals were developed with enhanced chemiluminescence reagents (Pierce) and exposed to X-ray films. Image digitization and quantification were done with Image Lab software. GAPDH and/or Beta-actin were used as loading control. The bars in graph represent the relative density of the bands normalized to GAPDH and/or β-actin from three repeated experiments.

Statistical analysis

Data are expressed as Mean ± S.D from three independent experiments. Student t-test was used to compare means. *P<0.05, **p<0.01, and ***p<0.001 indicate statistical significant difference compared with control group.

RESULTS

Effects of piceatannol on the inhibition of cell growth

To determine the effect of piceatannol on the growth of U2OS cells, we incubated the U2OS cells with different doses of piceatannol for 72 h and cell proliferation was evaluated by Cell Counting Kit-8 (CCK-8) assay. As shown in Figure 2A, piceatannol inhibited the growth of cells in a dose-dependent manner with IC_{50} value around 46.53 µM. We also counted the number of cells using flow cytometry. As shown in Figure 2B, the number of cells was significantly lower in treatment groups as compared to control group.

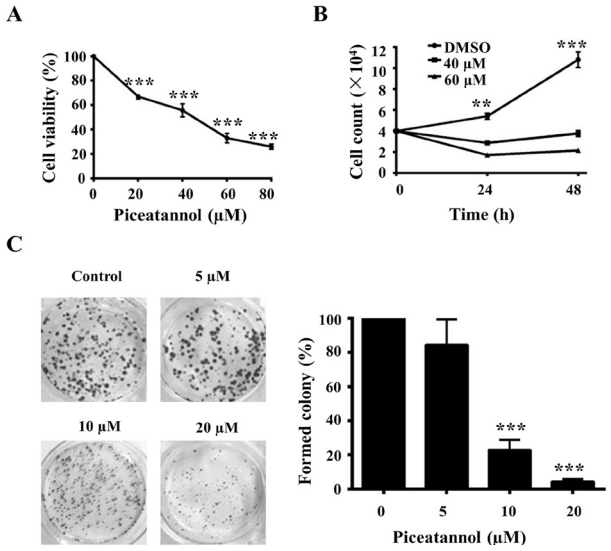

Fig. 2. Piceatannol inhibits cell proliferation. (A) U2OS cells were treated with various concentrations of piceatannol for 72 h and cell proliferation was assessed using CCK-8 assay kit. (B) Cells were incubated with 0, 40 and 60µM for 24 and 48 h and finally $1X10^4$ cells were counted by flow cytometry. (C) Cell proliferation was measured by colony forming assay. The data is expressed as mean ± S.D. from three independent experiments. *p<0.05, **p<0.01, ***p< 0.001 significantly different compared with control.

To better understand the inhibitory effect of piceatannol on cell proliferation, anti -proliferative nature of piceatannol was investigated by colony forming assay. We found that piceatannol inhibited colony formation in a dose-dependent manner as shown in Figure 2C. Overall, these results indicated that piceatannol has an anti-proliferative activity against U2OS cells.

Piceatannol induces morphological changes and cell death in U2OS cells

To determine whether piceatannol exerts any cytotoxic effects, we observed cell morphological changes. U2OS cells were incubated with 0, 20, 40 and 80 µM piceatannol for 24 h and cellular morphology was observed under phase-contrast microscope. Piceatannol induces severe morphological changes including the reduction in the total number of cells and loss of cellular geometry (Fig. 3A). In addition to cell morphological changes, we also examined nuclear morphological changes using Hoechst 33258 staining. Piceatannol treatment induced DNA fragmentation in U2OS cells in a dose-dependent manner, as shown in Figure 3B.

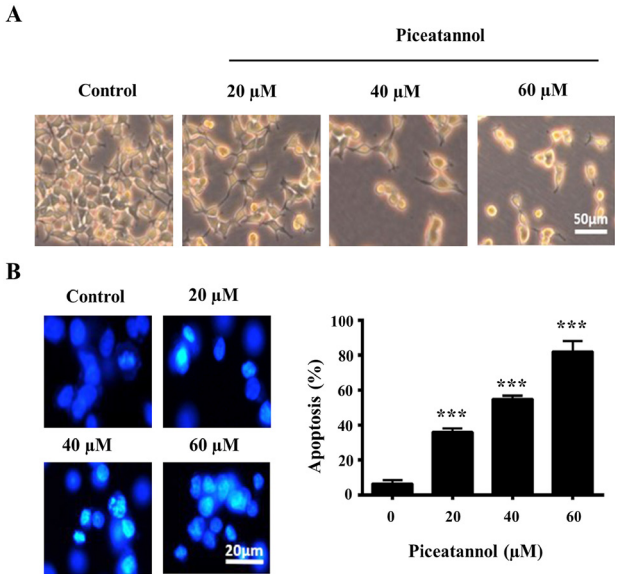

Fig. 3. Piceatannol induces morphological changes and cell death in U2OS cells. (A) U2OS cells were incubated with various concentrations of piceatannol for 24 h and cellular morphology was observed under microscope. (B) U2OS cells were treated with different concentrations of Piceatannol for 24 h. Cells were stained with Hoechst 33258 and nuclear morphological changes were examined by fluorescence microscopy.

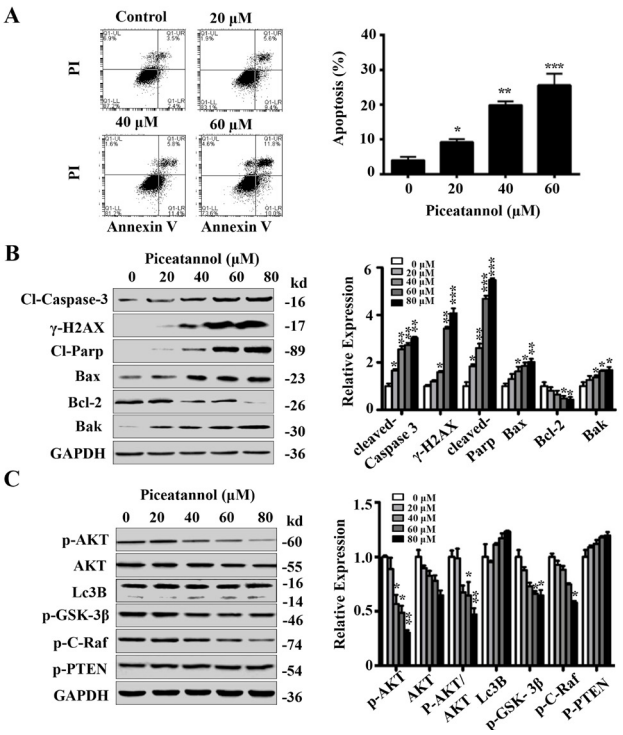

Fig. 4. Piceatannol effectively induces apoptosis and suppresses PI3K/AKT pathway in U2OS cells. (A) Cells were stained with Annexin V/PI and apoptosis was determined by flow cytometry. (B) U2OS cells were treated with different concentrations of piceatannol for 24 h and expression of apoptosis regulating proteins was measured by Western blotting. (C) The inhibitory effect of piceatannol on PI3K/AKT pathway was assessed by Western blot. The bars in graph (B & C) represent the relative density of the bands normalized to GAPDH from three repeated experiments.

Piceatannol effectively induces apoptosis and suppresses PI3K/AKT pathway in U2OS cells.

The effects of piceatannol on U2OS cells apoptosis was evaluated by Hoechst 33258 staining and Annexin V/PI double staining kit. As shown in Figure 3B, piceatannol induces DNA fragmentation in U2OS cells in a dose-dependent manner. The apoptotic effect of piceatannol was further verified by flow cytometry analysis. U2OS cells were treated with piceatannol and stained with Annexin V-FITC and PI for the analysis of apoptosis. The data showed that piceatannol induces apoptosis in U2OS cells in a dose-dependent manner (Fig. 4A). To further confirm the induction of apoptosis by piceatannol, we measured the expression of cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase (PARP) which are considered the markers of apoptotic cell death. The Western blot analysis showed that the expressions of cleaved caspase-3

and cleaved-PARP were increased in piceatannol-treated groups (Fig. 4B). Since piceatannol induced DNA damage in U2OS cells in a dose-dependent manner, we measured the expression of Phosphorylated histone H2AX (ɣH2AX) which is an early marker of DNA damage (Kuo *et al*., 2008). We found an increase in the expression of ɣH2AX in piceatannol-treated cells (Fig. 4B). Next, we measured the expression of Bcl-2 family proteins. The data indicated that piceatannol up-regulated the expression of pro-apoptotic proteins bax and bak while down-regulated the expression of anti-apoptotic bcl-2 protein (Fig. 4B). Taken together, the data indicated that piceatannol triggered cell death in U2OS cells by inducing apoptosis, which was associated with the up-regulation of pro-apoptotic proteins and down-regulation of anti-apoptotic proteins. We also evaluated the effect of piceatannol on phosphoinositide 3-kinase (PI3K)/ protein kinase B (AKT) signaling pathway. Our Western blot data showed that piceatannol effectively inhibits PI3K/AKT pathway as evident from decreased expression of p-AKT and phosphorylated Glycogen synthase kinase-3 beta (p-GSK-3β) in U2OS cell (Fig. 4C).

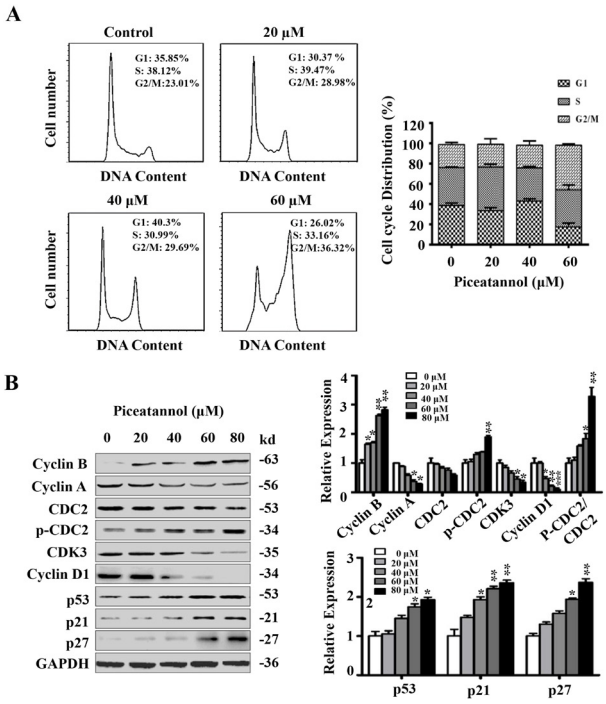

Fig. 5. Piceatannol induces G2/M phase arrest in U2OS cells. (A) U2OS cells were treated with various concentrations of Piceatannol for 24 h. Flow cytometry was used to assess cell cycle profile. (B) U2OS cells were treated with Piceatannol for 24 h. The expression of cell cycle regulators was analyzed by Western blotting. The bars in graph represent the relative density of the bands normalized to GAPDH from three repeated experiments.

Piceatannol induces G2/M phase arrest in U2OS cells

To determine whether piceatannol exerts any effect on the cell cycle progression of U2OS cells, the DNA contents at different phases of the cell cycle were analyzed by flow cytometry. Piceatannol increased cell number at G2/M phase in a dose-dependent manner accompanied by a decreased cell number at S and G0/G1 phases in U2OS cells (Fig. 5A). Furthermore, we detected the expression of various cell cycle-regulators including P^{53}, P^{21}, P^{27}, cyclin A, cyclin B, cyclin D, cyclin-dependent kinase 1(cdc2), p-cdc2 and cyclin-dependent kinase-3 (cdk3). We found that piceatannol increased the expressions of p^{53}, p^{21}, p^{27}, cyclin B and p-cdc2 while decreased the expressions of cyclin A, cyclin D1 and cdk3 (Fig. 5B).

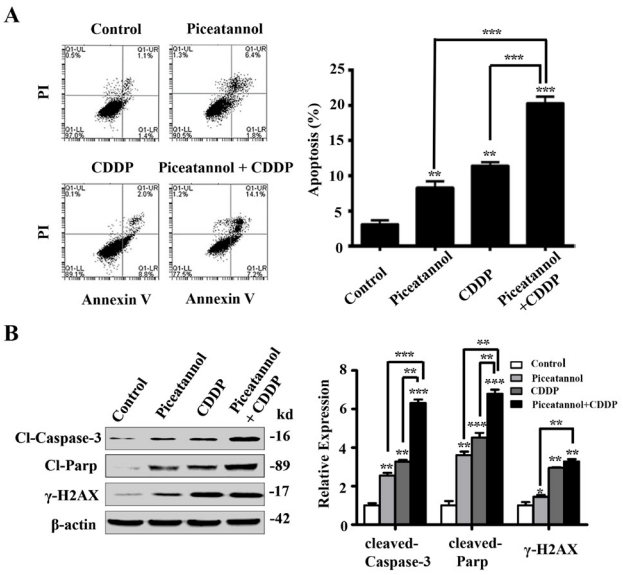

Fig. 6. Combine treatment of piceatannol and CDDP induces apoptosis in U2OS cells with additive effect. (A) U2OS cell were treated with piceatannol (40μM), and CDDP (20μg/ml) either alone or in combination for 24 h. The apoptosis rates were measured by staining the cells with Annexin V/PI using flow cytometry. (B) The protein levels of cleaved-caspase-3, cleaved-PARP, and γH2AX were determined using Western blot in U2OS cell. The bars in graph represent the relative density of the bands normalized to β-actin from three repeated experiments.

Piceatannol enhances CDDP –induced apoptosis in U2OS cell

Next, we asked if piceatannol could enhance the efficacy of CDDP in U2OS cells. For this, we treated the cells with piceatannol (40 μM) and CDDP (20 μg/ml) individually or a combination of both for 24 h and measured apoptosis rates using flow cytometry analysis. As shown in Figure 6A, the rate of apoptosis in combine

treatment of cells with piceatannol and CDDP was higher compared with either drug alone. These results were further confirmed by measuring the expressions of cleaved caspase-3, cleaved PARP and γH2AX (Fig. 6B). Taken together, these results indicated that piceatannol enhanced the effect of CDDP on apoptosis in U2OS cells.

DISCUSSION

Piceatannol, a naturally occurring polyphenol is an analog of resveratrol, a potent natural chemopreventive agent isolated from grapes (Wolter *et al*., 2002). Piceatannol has been reported to exhibit broad-spectrum anticancer activity in various human cancer cell lines (Seyed *et al*., 2016). However, till now no report is available on anticancer activity of piceatannol on osteosarcoma. The present study was conducted to evaluate the anticancer effects of piceatannol in osteosarcoma using U2OS cell line. Piceatannol inhibited the growth of U2OS osteosarcoma cells as evident from CCK-8 and colony formation assays. Apoptosis and cell cycle arrest are the main factors associated with cancer cell growth inhibition in response to chemotherapy (Khan *et al*., 2012a,b). Therefore, we determined apoptosis rate in U2OS cells in response to piceatannol treatment. The data demonstrated that piceatannol remarkably induced apoptotic cell death in a dose-dependent manner as evident from Annexin V/PI staining, Hoechst 33258 staining and cleavage of caspase-3 and PARP. The data is in agreement with other research reports indicating that piceatannol induces apoptosis in various other cancer cell lines (Seyed *et al*., 2016; Du *et al*., 2017). Bcl-2 family proteins are considered one of the most important players of apoptosis induction in a large number of human cancer cell lines (Khan *et al*., 2013; Khan *et al*., 2016). Therefore, we examined if piceatannol could modulate the expression of bcl-2 family proteins. We found that piceatannol up-regulated the expression of pro-apoptotic bax and bak while down-regulated the expression of anti-apoptotic bcl-2 protein. Similar results of piceatannol on apoptotic machinery have been reported by Kim *et al*., in human U937 leukemia cells (Kim *et al*., 2008).

Next we asked if picatannol could induce cell cycle arrest at a specific checkpoint. Therefore, we analyzed the cell cycle phase distribution profile of U2OS cells. The data showed that piceatannol arrested the cell cycle at G2/M phase. P^{53} being the major tumor suppressor protein plays vital role in G2/M phase arrest by activating its downstream target genes p^{21} and p^{27} (CDKs inhibitors). Cell cycle progression from G2 to M phase is mediated by cyclinB1/cdc2 complex. Cells with suppressed activity of this complex are arrested at G2 phase (Khan *et al*., 2011; Khan *et al*., 2012b). Here in this study, piceatannol increased the expression of p^{53}, p^{21} and p^{27} and decreased the expression of cyclin A, cyclin D1 and cdk3. Piceatannol have been previously reported to induce cell cycle arrest at S and G1 phase in colorectal and prostate cancer cells, respectively (Lee *et al*., 2009; Wolter *et al*., 2002). This discrepancy in data might be the result of different cancer types.

PI3k/Akt is a survival pathway and is implicated in key cellular processes including cell proliferation, survival, migration and metabolism. This pathway is frequently overexpresses in various cancer cells and promotes cell survival by inhibiting apoptosis and is involved in cell cycle regulation by inhibiting GSK-3β through phosphorylation. Inhibition of PI3k/Akt signaling pathway plays vital role in cell growth inhibition, induction of apoptosis and cell cycle arrest (El-Deiry, 2001; Khan *et al*., 2015). To further explore the anticancer role of piceatannol, we examined its effects on PI3k/Akt signaling pathway. Piceatannol effectively inhibited this signaling pathway as could be seen from decreased phosphorylation of AKT and GSK-3β.

Development of drug resistance has limited the scope of chemotherapy in the successful treatment of cancer (Maryam *et al*., 2017). Exploring novel combination of natural bioactive compounds with clinical drugs is a rational approach to enhance the efficacy of existing clinical drugs. In the present study, we investigated the chemo-sensitizing effect of piceatannol. The data provided strong support for the additive effects of piceatannol in combination with CDDP in U2OS cells.

CONCLUSION

In conclusion, we have shown that piceatannol inhibits cells proliferation, induces intrinsic apoptosis and G2/M phase cell cycle arrest in U2OS osteosarcoma cells. Furthermore, we revealed that piceatannol increased the sensitivity of U2OS cells to CDDP via inducing apoptosis. Taken together, these results suggest that piceatannol is a promising natural compound for the treatment of human Osteosarcoma tumor. Further study is needed to explore the in-depth anticancer mechanism and validate its activity using *in vivo* animal models in order to develop it into a lead for osteosarcoma therapy.

ACKNOWLEDGEMENTS

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Statement of conflict of interest

The authors declare no conflict of interests.

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