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ORIGINAL ARTICLE

# Kutkoside -an iridoid glycoside- exerts anti-proliferative effects in drug-resistant human oral carcinoma cells by targeting PI3K/AKT signalling pathway, inducing apoptosis and suppressing cell migration and invasion

Jun-Chi Hou, Xiao-Nan Xu

Department of Stomatology, Jining No.1 People's Hospital, Jining 272000 , Shandong, China



## Summary

***Purpose:*** *The main purpose of the current research work was to investigate the anticancer potential of Kutkoside -a naturally occurring iridoid glycoside, against drug-resistant human oral carcinoma cells along with evaluating its effects on PI3K/AKT signalling pathway, cellular apoptosis, cell migration and cell invasion.*

***Methods:*** *Cell viability was assessed by using MTT colorimetric assay, while effects on cellular apoptosis were evaluated by acridine orange (AO)/ethidium bromide (EB) as well as by using flow cytometry employing annexin V-FITC. Effects on cell migration and cell invasion were evaluated by in vitro wound healing assay and transwell Matrigel assay. Effects on PI3K/AKT signalling pathway were evaluated by western blot method.*

***Results:*** *Kutkoside led to significant and dose-dependent inhibition of HSC-2 human oral cancer cells using doses of 0, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM. Fluorescence microscopy revealed that on increasing the dose of kutkoside, the number of both apoptotic and necrotic cells increased, showing that Kutkoside induces apoptotic cell death in HSC-2 oral cancer cell line in dose-dependent manner. Flow cytometry indicated that the percentage of apoptotic cells at different dose exposures of Kutkoside, namely 0, 8, 16 and 32 μM was 4.9%, 12.18%, 22.18% and 34.10%, respectively. Kutkoside treatment also led to cell migration inhibition and cell invasion inhibition. This molecule upregulated the expression of AKT and PI3K, simultaneously downregulating the expressions of p-AKT and p-PI3K in a dose-dependent manner.*

***Conclusion:*** *Kutkoside shows potential anticancer and pro-apoptotic effects in HSC-2 oral carcinoma cells and as such may be developed as a possible anticancer agent provided further studies are performed.*

***Key words:*** *Kutkoside, flow cytometry, oral cancer, apoptosis, cell migration*

## Introduction

Oral cancer, conventionally defined as a squamous cell carcinoma (OSCC), is a common neoplasia associated with lips or oral cavity, worldwide. Histologically in the dental area about 90% of cancers grow from the squamous cells [1]. With the diagnosis of around 264 000 new cases of oral cavity cancer each year, oral cancer remains an important health problem [2,3]. Global cancer analysis revealed that oral cancer is the 8$^{th}$ lethal malignancy, with higher frequency in men than in women [4]. Smoking and alcohol intake have been recognised as the main risk factors, and studies have revealed that about 65% of oral cancer are attributed mainly to smoking alone [5]. Despite overall advancements in cancer chemotherapy, the survival rate of oral cancer has not improved significantly. Moreover, the toxicity typically related to current chemotherapy provides further motivation for exploring novel cancer tackling strategies. Recent cancer research has been focusing on di-

*Corresponding author:* Xiao-Nan Xu, MD. Department of Stomatology, Jining No.1 People's Hospital, No.6 Jiankang Rd, Jining 272000, Shandong, China
Tel/Fax: +86 0537 2253104, Email: TrevonCooleyhul@yahoo.com
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etary habits that show a significant involvement in the prevention and progression of oral cavity carcinoma [6]. These studies found that poor intake of vegetables and fruits results in increase of oral cancer occurrence and high consumption of these results in its reduction [7,8]. Natural products, mainly due to nontoxic profiles and various pharmacological activities, serve as a pool of natural therapeutic agents [9-13]. Iridoids belong to such natural products, containing cyclopenta[c]pyranoid skeleton with a monoterpene structure, and are present in various medicinal plants including *Picrorhiza kurroa* and *Harpagophytum procumbens*. Iridoid glycosides have shown different pharmacological activities like in the treatment of diabetes, mental stress, snake bite and rheumatoid diseases, in addition to antibacterial, antiviral and anti-inflammatory properties too [14-18].

The current study was undertaken to explore the anticancer activity of Kutkoside, an iridoid glycoside, along with examining its pro-apoptotic effects in drug-resistant human oral carcinoma cells and checking its effects on PI3K/AKT signalling pathway, cell migration and invasion.

## Methods

*Cell viability assay*

The cell proliferation/viability of Kutkoside-treated OSCC line HSC-2 and normal human oral mucosal epithelial cell line hTERT-OME were determined by MTT assay. These cell lines were placed into 96-well plates and incubated for 24 h in a 95% oxygen / 5% $CO_2$ incubator at 37oC. Later, each concentration of Kutkoside, namely 0, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 µM was added to each well. After Kutkoside treatment, these treated cells were incubated for two days with the same conditions. After two days of incubation, MTT solution was added to each well and cells were further incubated for 4 h. Finally, DMSO (dimethyl sulfoxide) was used to dissolve blue formazan crystals and absorbance was determined at 630nm using EL-311 BioTek Microelisa Reader (BioTek Winooski, VT, USA). Percent cell viability was measured in comparison to negative control cells.

*Apoptosis detection assays*

AO/EB staining assay was performed on OSCC line HSC-2 in exponential growth phase. These cells were cultured on sterile coverslips for 12 h and consequently treated with 0, 8, 16 and 32 µM Kutkoside for 24 h. Washing was performed twice with phosphate-buffered saline (PBS) followed by mixing these treated cells with 1 ml dye mixture comprising of 100 mg/ml EB and 100 mg/ml AO in PBS. Further, cellular morphological changes were examined under fluorescence microscope (magnification×200).

To establish the rates of Kutkoside-induced apoptosis, OSCC line HSC-2 cells were treated with Annexin V-FITC and propidium iodide (PI) and were then investigated by flow cytometry. The cells were seeded in 6-well plates at $2\times10^4$ cells in each well and were incubated overnight. After incubation, cells were exposed to kutkoside at varying doses (0, 8, 16 and 32 µM) for 48 h. Negative control cells were treated only with DMSO (vehicle) for same time period. After treatments, harvesting of cells was performed with trypsin, which was followed by washing twice with PBS. These washed cells were then suspended in 500 µL of binding buffer and finally stained with Annexin V-FITC apoptosis detection kits (Enzo Life Sciences AG, France) in accordance with manufacturer's instructions in the dark for 5 min at room temperature. FACS Scan flow cytometer (BD Biosciences, Heidelberg, Germany) was used to examine the stained cells, and the data was developed through FACSCanto II software.

*In vitro wound healing assay*

To establish cell motility of OSCC line, HSC-2 cells were cultured at a density of $1\times10^5$ cells per ml in 6-well culture plates and developed up to 80-90% confluence. After decanting medium, cell monolayer was worn out with a sterile micropipette tip to generate a gap/stripped zone of persistent width. Next, cellular debris was cleared with PBS washing and HSC-2 cells were then subjected to Kutkoside exposure at 0, 8, 16, 32 µM with serum-free medium. Olympus CKX-41 inverted mi-



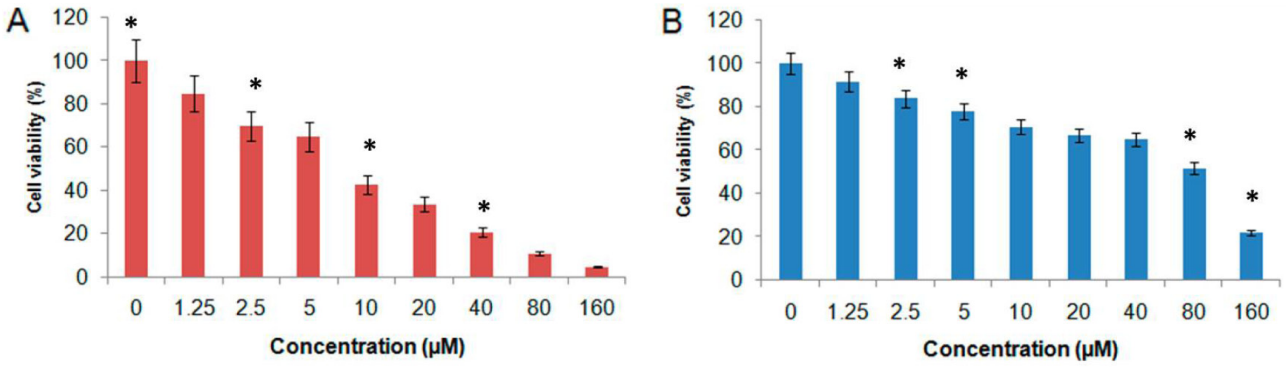


**Figure 1.** Effect of Kutkoside on the viability of the human oral carcinoma cells as evaluated by MTT assay. **Figure 1A** shows the effect on HSC-2 oral cancer cells, while **Figure 1B** shows the effect on oral normal mucosal epithelial cell line hTERT-OME. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

croscope was used to examine the healing of the wound and pictures were captured by Olympus E410 camera (Olympus, Tokyo, Japan) at 0, 12, 24 and 48 h after treatment. All the experiments were performed in triplicate.

*In vitro cell transwell invasion assay*

Cell invasion was determined by transwell invasion assay, which was performed through Biocoat Matrigel Invasion Chamber (BD Biosciences), in accordance with the manufacture's guidelines. In brief, $4\times10^4$ cells were plated at the upper chambers with Matrigel (BD Biosciences). RMPI-1640 medium filled the bottom chamber with 10% fetal bovine serum (FBS) as invasion inducer. After one day, cells of the upper surface were detached and those attached to the inferior side of the membrane were fixed and crystal violet was used for staining prior to calculation under a microscope in four randomly selected fields.

*Western blotting analysis*

For western blotting analysis, OSCC HSC-2 cells were assembled and lysed with complete cell lysis (Beyotime Institute of Biotechnology, Inc., Haimen, China) and with protease inhibitor cocktail (Roche, Basel, Switzerland). About 40 μg of proteins were loaded to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes. Those membranes were blocked by using TBS containing TBST (tween) with 5% non-fat dry milk for 24 h at 4°C. Next, primary antibody treatment of HSC-2 cells was done overnight at 4°C. In continuation, cells were subjected to secondary antibody treatment followed by incubation. Lastly, standardisation was done by Actin and Odyssey Infrared Imaging System used for signal recognition.

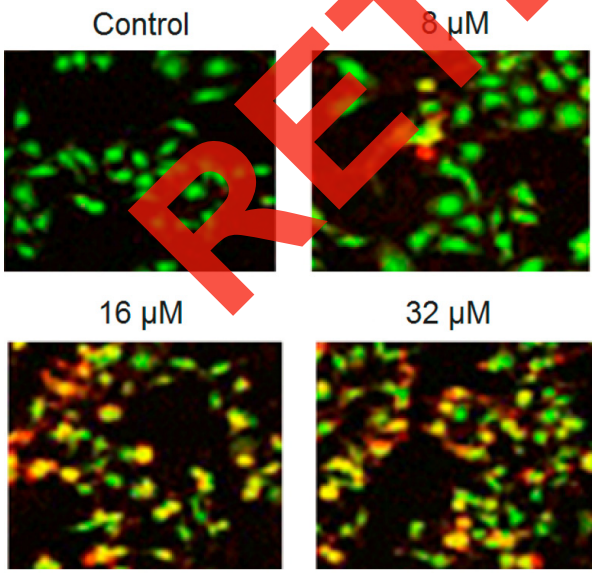


**Figure 2.** Stimulation of apoptosis in the HSC-2 oral cancer cells by Kutkoside as shown by fluorescence microscopy using AO/EB staining. The experiments were performed in triplicate. The Figure shows that increasing doses of the molecule lead to increasing percentage of apoptotic cells shown by yellow (early apoptosis) and orange (late apoptosis) fluorescence.

*Statistics*

The data is shown as mean ± standard error of the mean (SEM) of three independent experiments. Statistical investigations were performed by one-way ANOVA, by Dunnett's multiple comparison test and also by two-tailed, paired Student's *t*-test. The SPSS software was used for statisticalanalyses. P<0.01 showed significant difference compared with the controls.

## Results

*Kutkoside caused inhibition of cell proliferation HSC-2 cells*

The influence of Kutkoside on the viability/proliferation of HSC-2 oral cancer cell line was done

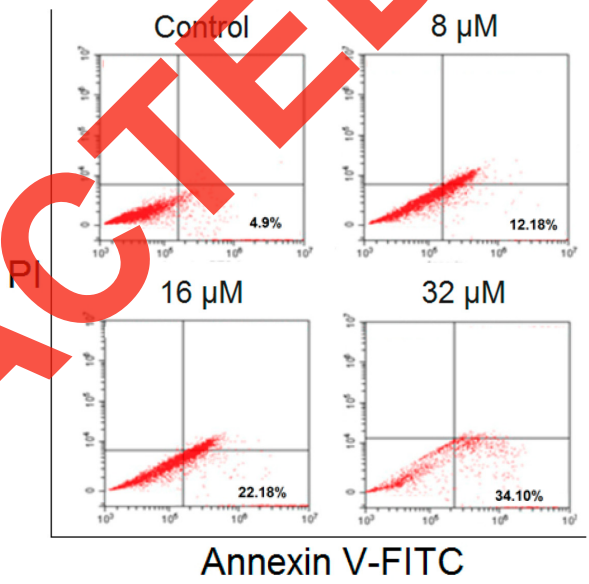


**Figure 3.** Annexin-V FITC assay using flow cytometry to evaluate and quantify the apoptotic effects of Kutkoside on HSC-2 oral cancer cells. The Figure shows a dramatic concentration-dependent increase in the percentage of apoptotic cells. The experiments were performed in triplicate.

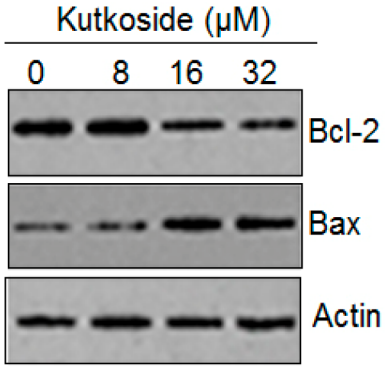


**Figure 4.** Pro-apoptotic effects of Kutkoside on the expression of apoptosis-associated proteins (Bcl-2 and Bax) as revealed by the western blot analysis. The experiments were performed in triplicate. Bax expression increased, while Bcl-2 expression decreased dose-dependently.

through MTT assay which revealed significant effects of the given molecule on inhibiting the viability of cancer cells in a dose-dependent manner (Figure 1A). On exposure to Kutkoside at varying concentrations, namely 0, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM, the viability of HSC-2 cells decreased significantly from 100 to near about 5%. The effect on normal hTERT-OME cell viability by Kutkoside was less significant than in cancer cells (Figure 1B). Thus it was concluded that the tested molecule showed significant selectivity on inhibiting cancer cell proliferation in a dose-dependent manner.

*Kutkoside led to induction of apoptosis in HSC-2 cells*

AO/EB staining was performed to evaluate the apoptosis induction effects of Kutkoside on HSC-2 oral cancer cell line. The results revealed significant morphological changes, indicative of apoptosis. Thus, on increasing the dose of the drug, the number of both apoptotic cells as well as necrotic cells increased, clearly showing that this molecule induces apoptotic cell death in HSC-2 oral cancer cell line in a dose-dependent manner (Figure 2). Further, to confirm apoptotic cell death induction by Kutkoside, annexin V-FITC and PI were used (Figure 3) and it was seen that this molecule resulted in increasing the number of apoptotic cells in a dose-dependent manner. The percentage of apoptotic cells at different dose exposures (0, 8, 16 and 32 μM) was 4.9, 12.18, 22.18 and 34.10%, respectively, showing thus the potential of the drug in inducing apoptotic cell death. Apoptosis was further confirmed by western blotting analysis through examination of apoptosis-related protein expressions. It was observed that the expression of BCL-2 decreased as compared to BAX, which increased in a dose-dependent manner (Figure 4).

*Kutkoside resulted in suppression of cell migration and invasion in HSC-2 oral cancer cells*

Wound healing assay and transwell cell invasion assay by Kutkoside resulted in inhibition of these two functions. The wound scratched through micropipette tip was observed 24 h after Kutkoside treatment and it was seen that the migrated cell number was very low/ negligible in the case of treated cells, while no significant impact was observed in the case of control untreated cells (Figure 5). Furthermore, the number of invaded cells decreased significantly with treatment with varying

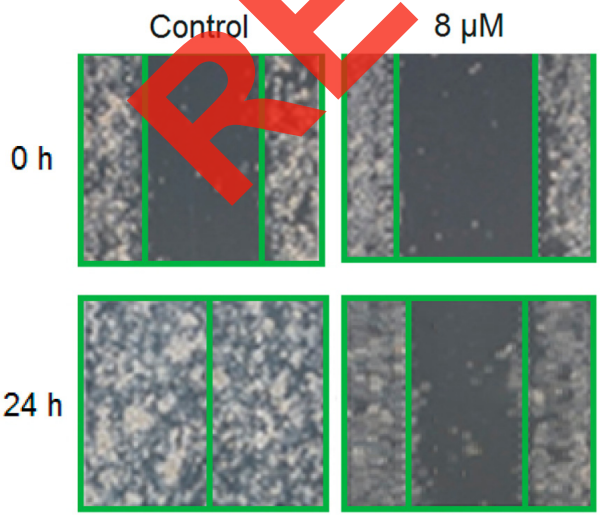


**Figure 5.** Anti-migratory effects of Kutkoside on the HSC-2 oral cancer cells as shown by *in vitro* wound healing assay. Kutkoside led to significant inhibition of cell migration at 8μM dose of the molecule for 24 h. The experiments were performed in triplicate.

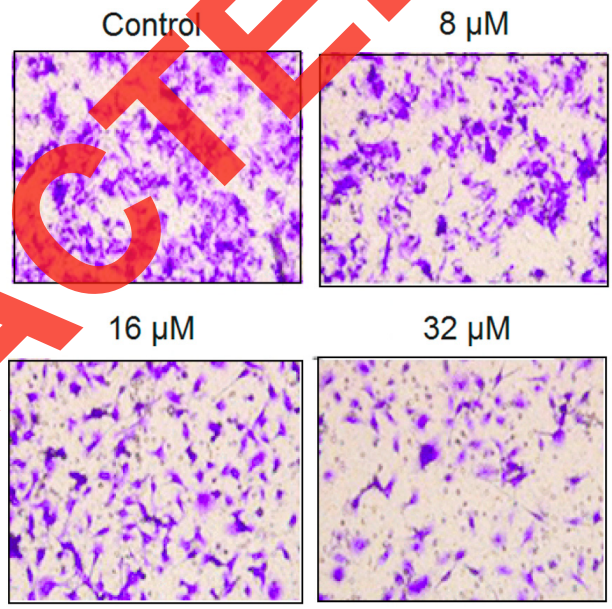


**Figure 6.** Matrigel assay indicating the effect of Kutkoside on the HSC-2 oral cancer cells exhibiting dose-dependent suppression of cell invasion. The experiments were performed in triplicate.

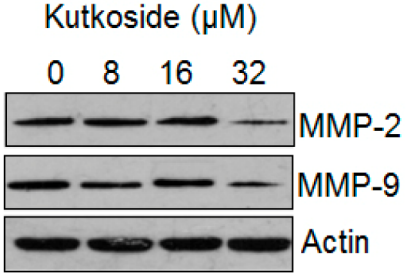


**Figure 7.** Effect of Kutkoside on the expression of cell migration-related protein expressions including MMP-9 and MMP-2 matrix metalloproteinases. The experiments were performed in triplicate. Increasing doses of Kutkoside led to significant and dose-dependent inhibition of MMP-2 and MMP-9 expressions which in turn indicate effects on cell migration and invasion.

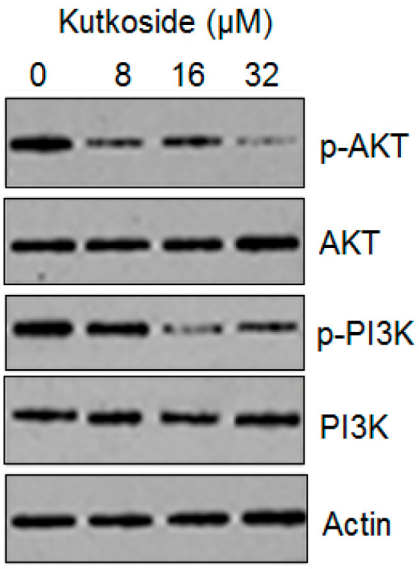


**Figure 8.** Effect of Kutkoside on the PI3K/AKT signalling pathway as evaluated by western blot assay. Kutkoside led to downregulation of the expressions of p-AKT and p-PI3K in a dose-dependent manner. The expression of PI3K and AKT remained unchanged. The experiments were performed in triplicate.

doses of the molecule (0, 8, 16 and 32 μM) (Figure 6). It was also revealed by western blotting that the expressions of proteins related to cell invasion and migration were also altered like MMP-2 and MMP-9 expression which were shown to decrease (Figure 7), clearly indicating that Kutkoside inhibits both cell migration and cell invasion of HSC-2 oral cancer cells.

*Kutkoside targeted PI3K/AKT signalling pathway in drug-resistant HSC-2 cells*

PI3K/AKT signalling pathway was tested in drug-resistant HSC-2 carcinoma cells through western blot analysis. The results revealed that Kutkoside treatment upregulated the expression of AKT and PI3K, simultaneously downregulating the expressions of p-AKT and p-PI3K in a dose-dependent manner (Figure 8).

## Discussion

Oral cavity carcinoma is the eighth most frequent neoplasm worldwide. Despite chemo and radio-therapeutic improvements, its overall survival rate (OSR) has not improved significantly over the past few decades. Oral cavity cancer has a 5-year survival rate marginally above 50%. Thus, to improve overall survival there is a pressing need to search for novel therapeutic strategies. Natural products have immensely larger pharmacological potential [19,20]. Iridoid glycosides (natural products) have revealed different pharmacological activities like antibacterial, antiviral, anti-inflammatory, antidiabetic, mental stress, snake bite etc. Kutkoside belongs to the family of iridoid glycosides. The current study was performed to analyze the anticancer potential of Kutkoside on OSCC line HSC-2. For that we performed different assays to determine its anticancer properties as well as its mode of action. Cell viability was determined through MTT assay, which revealed the anticancer effect of the given test molecule was selective against the HSC-2 cancer cells without causing toxicity to the normal cells and this antiproliferative effect was dose-dependent. Furthermore, AO/EB staining and Annexin V-FITC assay with PI-double staining was performed to detect apoptosis induction by our test molecule. The results showed that Kutkoside induced apoptosis in a dose-dependent manner. The percentage of apoptotic cells at different dose exposures (0, 8, 16 and 32 μM) was 4.9, 12.18, 22.18 and 34.10%, respectively. Furthermore, the effect of Kutkoside on cell migration and invasion in oral carcinoma cells revealed that both functions were inhibited, which was confirmed by western blotting analysis, which showed that the expression of cell migration and cell invasion-related proteins MMP-2 and MMP-9 were downregulated in a dose-dependent manner. Also, performing western blotting analysis of the treated cells (with 0, 8, 16 and 32 μM of Kutkoside) showed that this molecule altered the expressions of the PI3K/AKT signalling pathway-related proteins, resulting in upregulation of expression of AKT and PI3K, simultaneously downregulating the expressions of p-AKT and p-PI3K in a dose-dependent manner.

## Conclusion

From the above results it may be concluded that Kutkoside, an iridoid glycoside, is a potent anticancer agent exerting anti-proliferative effects in drug-resistant human oral HSC-2 carcinoma cells by targeting PI3K/AKT signalling pathway, inducing apoptosis and suppressing cell migration and invasion. Further investigations are needed to determine its molecular mechanism and mode of actions on different human carcinoma cell lines.

## Conflict of interests

The authors declare no conflict of interests.

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