

Antiproliferative activity of an angular furanocoumarin-oroselol in human oral cancer cells is mediated *via* autophagy induction, inhibition of cell migration, invasion, and downregulation of PI3K/AKT signalling pathway

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Oral carcinoma is a lethal type of cancer associated with huge morbidity and mortality. Oral cancer patients show a very low survival chances even if diagnosed at early stages. The need for novel naturally occurring chemotherapeutic drugs increases due to high cost and toxicity of currently used clinical drugs. Present study was designed to investigate anticancer property of oroselol, keeping in view the medicinal potential showed by coumarin subclass furanocoumarins. MTT assay and clonogenic assays were implemented for viability assessments. Transmission electron microscopy was used for autophagic studies. The transwell chambers assay was used to investigate the migration and invasion. Western blotting was performed to determine the expressions levels of autophagy and PI3K/AKT signalling related proteins. Results showed that oroselol could potentially inhibit viability of oral cancer SSC-4 cells in time- and dose-reliant fashion. The antiproliferative effects were mediated through autophagy induction as indicated by formation of autophagosomes and enhanced LC3-I expressions and reduced LC3-II and p62 expressions. Cancer cell migration and invasion was potentially suppressed by oroselol cell treatment. The PI3K/AKT signalling pathway was blocked potentially by oroselol in SSC-4 cells which showed reduced phosphorylation of PI3K and AKT. In conclusion, oroselol holds a significant potential to induce autophagy-related antiproliferative effects along with inhibition of cell migration, cell invasion, and PI3K/AKT signalling pathway. Therefore, oroselol may prove to be a lead molecule in oral cancer chemotherapy provided further *in vivo* and toxicological studies are performed on it.

Keywords: Furanocoumarins, oral carcinoma, oroselol, autophagy, cell migration

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Abbreviations: MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; UV, Ultraviolet; DMSO, Dimethyl sulfoxide; PBS, Phosphate-buffered saline; BCA, Bicinchoninic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, Polyvinylidene fluoride or polyvinylidene difluoride

INTRODUCTION

Oral carcinoma is a dangerous type of cancer malignancy located on lips or oral cavity (Barnes 2005). Oral cancer is associated with huge morbidity and mortality globally, with approximately 0.3M new cases and 0.15M

deaths (Lingen *et al.*, 2008). Traditionally, oral cancer because of its occurrence in dental area is defined as an oral squamous cell carcinoma, and out of all oral cancers histologically 90% arise from squamous cells (Warnakulasuriya, 2009; Vitale-Cross *et al.*, 2009). It shows divergent levels towards propensity and differentiation for lymph node metastasis. The incidences of oral cancer remain almost three times higher in males than in females in the maximum ethnic groups (Dissanayaka *et al.*, 2012). Cancers of pharynx and oral cavity are grouped collectively worldwide and ranks 6th among frequently prevailing cancers globally (van der Waal, 2009). It has been reported that about 90% of oral cancer cases potentially originate due to alcohol abuse and smoking, otherwise a preventable disease. A chain smoker and alcohol consumer have the highest chances for the development of oral cancer as these two risk factors pose synergistic effects (Lee *et al.*, 2012; Warnakulasuriya 2011). Other risk factors include UV radiations and HPV (human papillomavirus) infection (initiates carcinogenesis in oropharynx) (Phillips *et al.*, 2013; D'Costa *et al.*, 1998). The presently availed treatment methodologies for oral cancer include radiation therapy, surgery, coadjuvant therapy (therapy involving cisplatin, 5-fluorouracil, carboplatin, docetaxel, and paclitaxel) (Naidu *et al.*, 2004; Stell & Rawson, 1990). These treatments remain highly lethal due to side-effects and being high in cost. Thus, overcoming of these shortcomings of presently availed treatments requires novel therapeutic agents. Furanocoumarins are a rich subclass of chemical entities belonging to naturally occurring coumarin class of compounds mostly found in grapefruit (Piao, 2004). Furanocoumarins from grapefruit have been shown to exhibit numerous biological and medicinal importance including tumor inhibition, anti-inflammation, and antioxidant behaviour (Ho *et al.*, 2001; Guo & Yamazoe, 2004; Wang *et al.*, 2006). These have also shown promising results in the promotion of bone health both *in vivo* and *in vitro*. Furanocoumarins interfere with different molecular pathways thereby potentially inhibiting the proliferation progression in different cancers (Kim *et al.*, 2014). They modulate activator of transcription-3 and signalling transducer, expression of mitogen-stimulated protein kinase, phosphatidylinositol-3-kinase/AKT, and nuclear factor-kB (Hung *et al.*, 2017; Hwang *et al.*, 2010). Therefore, taking under consideration these biological activities, furanocoumarins possess remarkable anticancer potential. Oroselol molecule is an active constituent of *Nardostachys jatamansi* root oil and a member of furanocoumarins (Seshadri & Sood, 1967). The parent plant of oroselol shows significant biological activity like an-

tifungal, cardioprotective, and hepatoprotective (Purnima & Kothiyal, 2015). Therefore, the current study was designed to explore the anticancer activity of oroselol molecule against oral cancer. Moreover, its effects on cellular autophagy, migration and invasion, and PI3K/AKT signalling pathway were also investigated.

EXPERIMENTAL

Viability assay

The effects of oroselol drug on oral cancer SSC-4 cell proliferation were assessed via MTT assay. In brief, SSC-4 cells were plated onto 96-well plate at 37°C in CO₂ (5%) incubator with each well carrying 1.5×10⁴ cells. Then SSC-4 cells in each well were exposed to oroselol drug for 48 h and 72 h at variant doses viz 15, 30, 60 and 120 µM, controls were left untreated at normaxia. Consequently, treated cells were added with MTT stock solution (50 µL with concentration 5 mg/mL) for 20 minutes with incubation. 200 µL of DMSO were used to dissolve then evolved formazan crystals. Finally, determination of optical density was performed with absorbance measurements at 570 nm with a microplate reader (OLYMPUS, Japan). The experiments for each concentration, including controls, were executed thrice.

Colony formation assay

To determine the impact of oroselol drug on colony formation of SSC-4 cells it was evaluated by clonogenic assay. The SSC-4 cells were loaded into 6-well plate with 200 cells per well and cultured for 48 h with variable doses of oroselol drug viz 15, 60 and 120 µM, and controls were left within oroselol deprived complete cultural medium. Cells were harvested at 80% confluence and then washed two times using PBS. Thereafter, oroselol treated SSC-4 cells were left untouched during an incubation for 10 consecutive days at 37°C. Afterwards, SSC-4 cells were washed three times within PBS and then fixed in alcohol. Fixed cells were stained with crystal violet for visualization under a stereomicroscope (Carl Zeiss, Germany). Colonies bearing minimum of 50 cells were considered as significant for calculation.

Transwell migration and invasion assay

To monitor migration and invasion in SSC-4 cells, after being treated with oroselol drug, were investigated with transwell chambers assay. SSC-4 cells were transferred to upper transwell chambers post harvesting at a logarithmic phase of growth. The upper chambers contained cultural medium holding a 10% fetal bovine serum and variant doses of oroselol drug viz 15, 60 and 120 µM and controls were left at normaxia. Lower wells of the transwell chambers were filled with complete cultural media deprived of oroselol drug. After 48 h the non-migrated cells were rinsed with a cotton swab, and migrated cells were washed in PBS and fixed in ethanol. Thereafter, migrated cells were stained for visualization with crystal violet and numbered under a light microscope. Similar method was performed to evaluate the invasion of SSC-4 cells, except the walls of transwell chambers were coated with Matrigel.

Western blotting

To investigate the impact of oroselol molecules on PI3K/AKT signalling in SSC-4 cells, western blotting

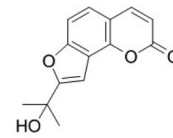


Figure 1. Chemical structure of oroselol molecule.

was implemented. After harvesting the SSC-4 cells at a logarithmic phase of growth, treatment with oroselol drug (0–120 µM) was instigated with 24-well plates for 48 h. Then cells were lysed in lysis buffer followed by protein content quantification with BCA assay within each lysate. Equal amounts of proteins were separated via SDS-PAGE then loaded over PVDF membranes through electrophoresis. These membranes were doped to eliminate non-specific binding using Tween 20 (pH of 7.5) and tris-buffer-saline holding 5% BSA, overnight. Thereafter, membranes were blotted with specific primary antibodies against LC3 I and II, p62, PI3K and AKT (1:1000 dilution) at 4°C for 12 h in the dark. Post primary antibody treatment, membranes were subjected to horseradish peroxidase conjugated secondary antibody (Cell Signalling Technology, MA, United States) for 1h in the dark. Finally, visualization of the protein bands was performed using luminol reagent (Bio-Rad) by obeying manufacturer's guiding principle and chemiluminescence was investigated through Image Lab Software (Bio-Rad, Chem-doc).

Statistical analysis

All the experimental data collected by accomplishment of individual experiments in triplicates were subjected to regression and correlation analysis. Whole data was analysed by one-way ANOVA ($p < 0.05$, post hoc: tukey test) and represented as mean ± S.D.

RESULTS

Oroselol inhibits viability of oral cancer SSC-4 cells

To assess the effects of oroselol (Fig. 1) on viability of oral cancer SSC-4 cells, MTT assay was performed after the treatment at various concentrations (0–120 µM). It was observed that oroselol drug could potentially retard the proliferation in SSC-4 cells. Oroselol induced mild

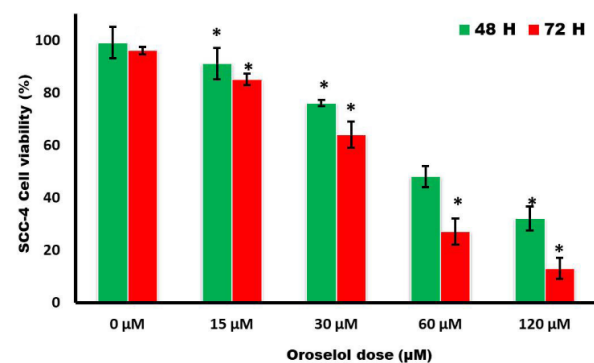


Figure 2. The oral cancer SSC-4 cells were subjected to MTT assay to determine the cellular viability after oroselol treatment at indicated doses.

Individual experiments were given three repetitions for each concentration and data was shown as mean ± S.D. considering $p < 0.02$ as a significant figure.

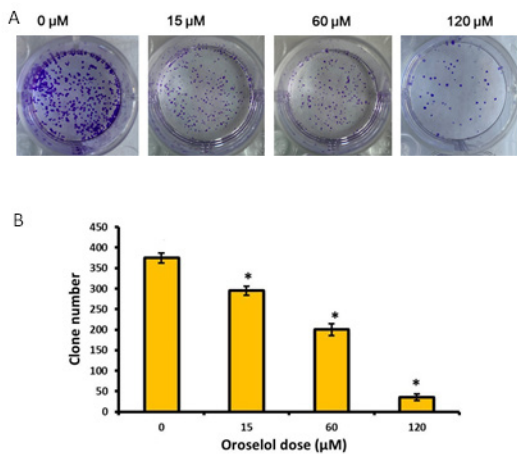


Figure 3. One title

(A) The SSC-4 cells were subjected to clonogenic analysis to determine the effects of oroselol on their colony generation. Cells were exposed for 10 days to indicated doses of oroselol drug and finally numbered under microscopy. (B) Graphical presentation of number of colonies generated after oroselol exposure of SSC-4 cells. Individual experiments were given three repetitions for each concentration and data was shown as mean \pm S.D. considering $p < 0.02$ as a significant figure. Colonies with minimum number of 50 cells were considered for calculations.

anti-viability effects at low exposure time and significant results were observed after 72 h of treatment. Viability was suppressed to almost 10% in comparison to controls (considered 100% viable) after 72 h long treatment (Fig. 2). The study of SSC-4 colony formation was done *via* clonogenic assay and colonies were numbered after 10 days of treatment. Results showed that oroselol could possess remarkable anti-clonogenic activity against SSC-4 cells (Fig. 3A). The number of SSC-4 cell colonies were reduced to almost 20 at 120 μ M in comparison to controls, where the number stood at almost 370 (Fig. 3B).

Anti-viability effects of oroselol on SSC-4 cells mediated *via* autophagic cell death

It has also been reported that several furanocoumarins introduce their antiproliferative effects *via* stimulation of autophagy. Therefore, oroselol treated SSC-4 cells was further supported with western blotting at molecular levels. It indicated that the levels of activity of LC-I significantly augmented on application of oroselol in comparison to controls (Fig. 4A). Further, the levels of expressions of LC3-II and p62 decreased on enhancing oroselol doses. Thus, autophagy assessments indicated that anti-viability potency of oroselol drug in SSC-4 cells could potentially be due its autophagy induction.

Oroselol inhibited invasion and migration in SSC-4 cells

Furanocoumarins have been shown with great tendency to overcome cancer metastasis. Thus, in this study

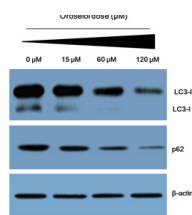


Figure 4. Western blotting showing the expression levels of autophagy allied proteins at indicated doses of oroselol in SSC-4 cells.

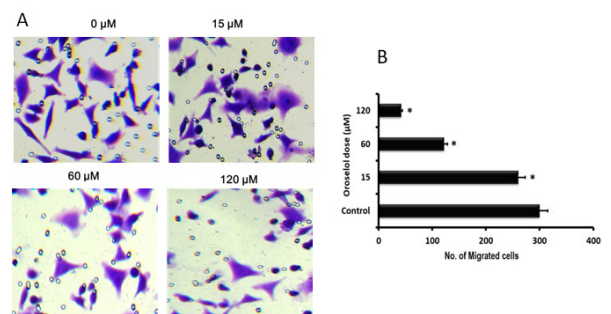


Figure 5. One title

(A) Transwell chambers migration assay. Results showing the decrease in migration of SSC-4 cells after exposure to oroselol drug at indicated doses. (B) Graphical representation of number of migrated cells after oroselol treatment of SSC-cells in transwell chambers. Individual experiments were given three repetitions for each concentration and data was shown as mean \pm S.D. considering $p < 0.02$ as a significant figure.

oroselol was investigated for its anti-migration and anti-invasion capability of SSC-4 cells to inhibit metastasis in them. For that transwell migration and invasion assays were performed. SSC-4 cells were exposed to variant oroselol drug doses ranging from 0–120 μ M for 48 h and numbered under microscopy. Oroselol drug showed significant suppressive effects on migration of SSC-4 cells (Fig. 5A). The number of migrated SSC-4 cells declined to almost 40 in comparison to controls where migrated cell number stood at 300 (Fig. 5B). Likewise, oroselol could potentially inhibit invasiveness of SSC-4 cells as indicated in Fig. 6A. The number of invasive cells at 120 μ M of oroselol concentration was recorded to be nearly 60 in comparison to almost 380 at

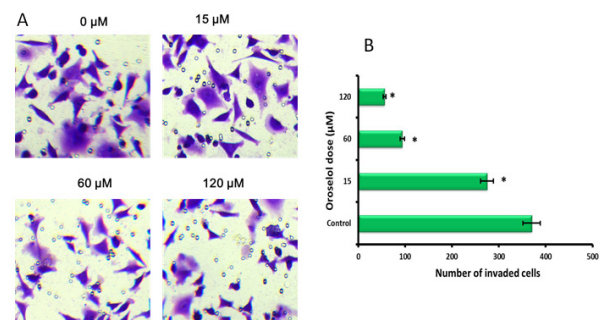


Figure 6. One title

(A) Transwell chambers invasion assay. Results showing the decrease in invasion of SSC-4 cells after exposure to oroselol drug at indicated doses. (B) Graphical representation of number of invasive cells after oroselol treatment of SSC-cells in transwell chambers. Individual experiments were given three repetitions for each concentration and data was shown as mean \pm S.D. considering $p < 0.02$ as a significant figure.

the controls (Fig. 6B). Therefore, transwell migration and invasion analysis of oroselol treated SSC-4 cells showed that oroselol could inhibit metastasis in oral cancer.

Oroselol blocked PI3K/AKT signalling pathway in SSC-4 cells

The PI3K/AKT signalling pathway shows crucial role in activation of m-TOR, which is an inhibitor of autophagy. Therefore, targeting this pathway leads to a smooth progression of autophagy. Western blotting showed that PI3K/AKT signalling pathway was potentially blocked by oroselol application in SSC-4 cells. The

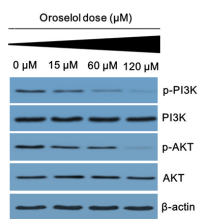


Figure 7. Western blotting results representing the activity of PI3K/AKT signalling pathway allied phosphorylated and non-phosphorylated proteins. SSC-4 cells were treated for 48 h with indicated oroselol doses.

levels of activity of phosphorylated PI3K and AKT declined remarkably in positive control groups than in negative controls. Moreover, the expressions of non-phosphorylated AKT and PIK remained almost the same all the time (Fig. 7).

DISCUSSION

Unfortunately, oral cancer treatment remains one of the major challenges for scientists and researchers. Alcohol consumption and smoking are the two main risk factors leading to oral cancer development, otherwise a preventable disease (Ko *et al.*, 1995; Marshall *et al.*, 1992). There is a pressing need for novel methodologies and chemopreventive drugs that can overcome the hazards of currently accessible management options. Therefore, the current study was undertaken to investigate oroselol furanocoumarin for its anticancer effects against oral cancer. Oroselol molecule's anticancer effects were searched for autophagy stimulation, migration inhibition, invasion inhibition, and targeting of PI3K/AKT signalling pathway. Oroselol drug significantly targeted the proliferation progression in oral cancer SSC-4 cells. Moreover, the colony formation was also retarded by oroselol drug in a concentration dependent manner. Therefore, studies were carried out to unleash the mechanism beneath the antiproliferative effects of oroselol. Autophagy is one of the imperative defensive mechanisms in multicellular organisms and remains a potential target for chemopreventives (Mizushima 2007; Mathew *et al.*, 2007; Hu *et al.*, 2017). Autophagy is a mechanism in which impaired organelles, accumulated proteins and macromolecules are degenerated within autophagosomes (Lu *et al.*, 2016). Autophagy is governed by a number of pro-autophagy and anti-autophagy proteins including LC3's, p62, and Beclin-1. Western blotting analysis indicated that oroselol could significantly target autophagy in oral cancer cells. Autophagy induction was recorded with enhanced LC3-I activity levels and reduced p62 and LC3-II activity levels. The migration and invasion in cancer cells cause metastatic disease which moves to distant places and proliferates (Kempen *et al.*, 2008). Oroselol was investigated for effects on migration and invasion of SSC-4 cells through transwell chambers. It induced significant retardation of both migration and invasion and hence revealed potential as a significant anti-metastatic agent against oral cancer. Further, PI3K/AKT signalling pathway plays important role in autophagy inhibition (Heras-Sandoval *et al.*, 2014; Zhai *et al.*, 2014). PI3K/AKT is the upstream signalling pathway which stimulates m-TOR and m-TOR results in autophagy inhibition via modulation of transcription and translation of allied genes. Oroselol was examined for its effects on PI3K/AKT signalling pathway and showed remarkable potential of inhibition in PI3K/AKT

signalling pathway related proteins. The phosphorylation of PI3K/AKT signalling pathway proteins remarkably declined in comparison to non-phosphorylated proteins whose activity levels remained almost intact.

CONCLUSION

Taking altogether, the results of this research indicate that oroselol furanocoumarin could potentially block the proliferation of oral carcinoma cells. These effects were mediated *via* stimulation of autophagy, migration and invasion inhibition and blockade of PI3K/AKT signalling pathway. Therefore, oroselol could suppress the growth and proliferation of oral cancer and hence may prove a lead molecule in management of oral carcinoma.

Conflict of interest

The authors declare that there is no conflict of interest to report.

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