

## Original Research Article

# Isoxanthohumol exerts anticancer activity against drug-resistant thyroid cancer cells by inhibiting cell migration and invasion, apoptosis induction and targeting PI3K/AKT/m-TOR signaling pathway

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### Abstract

**Purpose:** To investigate the anticancer effect of isoxanthohumol against drug-resistant thyroid cancer cells. Its effect on cellular PI3K/AKT/m-TOR signaling pathway, cell migration and invasion and apoptosis were also analyzed.

**Methods:** The cytotoxicity of isoxanthohumol drug was examined by MTT assay. Cellular morphology was assessed by phase contrast microscopy. Fluorescence microscopy and western blotting were used to evaluate its effect on cellular apoptosis. Further, western blotting was used to monitor the levels of expression of proteins linked to PI3K/AKT/m-TOR signaling pathway.

**Results:** Isoxanthohumol significantly ( $p < 0.05$ ) inhibited the proliferation and progression of drug-resistant human papillary BCPAP cancer cells in a concentration- and time-dependent manner. Further, morphological assessment of BCPAP cells indicate that there were proapoptotic alterations after isoxanthohumol treatment. The results for apoptosis in BCPAP cells showed enhanced number of apoptotic cells, elevated levels of proapoptotic proteins (including Bax), and increased level of expressions of caspase-3 and caspase-9. Isoxanthohumol targeted metastatic features of BCPAP cells by inhibiting both cell migration and cell invasion in a concentration-dependent manner. Western blotting revealed remarkable inhibition of PI3K/AKT/m-TOR signalling pathway through blocking of expression of its allied proteins.

**Conclusion:** Isoxanthohumol exhibits potent anticancer effect in human papillary cancer cells by inducing apoptosis, inhibition of cell migration, as well as invasion and targeting PI3K/AKT/m-TOR signalling pathway. These findings could play a significant role in drug design and discovery for thyroid cancer. However, more conformational investigations are required.

**Keywords:** Thyroid cancer, Drug resistance, Flavonoids, Isoxanthohumol, Apoptosis

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## INTRODUCTION

Global cancer burden is increasing at an alarming rate due to a number of factors

including aging, population growth and some alterations in risks linked to economic and social development. The countries with fast emerging economies have witnessed a shift in poverty

allied cancers and infections to cancers, mainly attributed to adopted lifestyle of representative industrialized countries. The year of 2018 recorded nearly 10 million cancer deaths and above 18 million new cancer cases [1]. Meanwhile, thyroid cancer occurrences are increasing at a rapid pace and almost fastest among all cancer malignancies.

Despite a 2.4-fold increase in the number of thyroid cancer incidences from 1973-2002, the associated mortality rate remained stable [2]. This distressing enhancement in thyroid cancer occurrence is mostly attributed to its primary detection using ultrasound and ultrasound-guided fine needle for investigation of cytology. The exposure to ionizing radiation is only one established major risk factor contributing to thyroid cancer development by epidemiological studies of the disease [3]. Thyroid cancer treatment varies according to tumor stage and type. The currently available treatment approaches include primary radioactive iodine treatment, surgery, TSH suppression therapy and chemotherapy [4]. However, the disease relapse within 3 years of primary treatment remains a big hurdle in thyroid cancer treatment even if the mortality rate is under control.

Potential therapeutics have been isolated from plant based natural products and these products serve as a pool of unknown and unexplored chemical entities that can assist to address current global health issues [5,6]. Flavonoids are a large class of phytochemicals with more than 4,000 known members. They possess diverse structural varieties like flavones, flavonols, flavanones, flavanonols and isoflavones [9-10]. These compounds have been found biologically and medicinally important. They show anti-analgesic, hypolipidemic, hypocholesterolemic, anti-inflammatory, antihypertensive, antiestrogenic and anticancer activities [11]. Isoxanthohumol is an isomeric form of xanthohumol flavanones and it is found in large abundance beer. Likewise its isomeric form isoxanthohumol shows remarkable bioactivities including anticancer activity. It has been reported to induce apoptosis, oxidative stress, reduces mitochondrial membrane potential and adipogenesis [12]. Therefore, this study was designed to explore the anticancer activity of isoxanthohumol against drug-resistant thyroid cancer along with assessing its effects on PI3K/AKT/m-TOR signalling pathway, cell migration and invasion and apoptosis.

## EXPERIMENTAL

### Assessment of cellular proliferation

MTT viability test was used to analyze the viability of drug-resistant human papillary BCPAP thyroid cancer cells after treatment with isoxanthohumol. In brief, 10,000 cells/well were cultured 96-well plate for 24 h. Preculturing of BCPAP cells was followed by isoxanthohumol treatment (0, 12, 24, 48 and 96  $\mu$ M for 24 h) by incubation in 5 % CO<sub>2</sub> and 95 % humidified air at 37°C. Post drug exposure cells were washed with phosphate buffered saline. Thereafter, to each well was added MTT stock solution and incubated for 4 h. MTT solution generates formazan crystals with viable cells and those crystals were dissolved with dimethyl sulphoxide for colorimetric assessment. Reading of the optical density was carried out at 490 nm with an ELISA reader (Molecular Devices, Sunnyvale, CA, United States).

### Phase contrast microscopy

Morphological assessment of isoxanthohumol treated human papillary BCPAP thyroid cancer cells was carried via phase contrast microscopy. Briefly,  $2.5 \times 10^5$  BCPAP cells were plated in 12-well plates and incubated overnight at 37 °C. After incubation BCPAP cells were subjected to isoxanthohumol treatment at varying concentrations (0, 12, 48 and 96  $\mu$ M) for 48 h. Afterwards, DMEM was removed and isoxanthohumol treated BCPAP cells were washed with phosphate buffered saline. Finally, morphological assessments were performed under a phase contrast microscope (Leica DMI 3000B, Germany).

### Fluorescence microscopy

Human papillary BCPAP thyroid cancer cells were harvested at 70-80 % of confluence and then seeded on 24-well plates. Afterwards, cells were trypsinized using trypsin over coverslips placed in 24-well plates post treatment with various isoxanthohumol concentrations (0, 12, 48 and 96  $\mu$ M) for 48 h. Thereafter, fixation of the treated cells was performed using formaldehyde (4 %) followed by 10  $\mu$ L of AO/EB staining solution for 3-4 minutes. Cells were finally analyzed under fluorescence microscope (Olympus Co., Tokyo, BX51TRF, Japan) for apoptosis analysis.

## Transwell invasion and migration assays

The effect of isoxanthohumol on cell invasion and migration was analyzed by transwell chambers coated with Matrigel. Target cells were suspended within 2 % FBS containing DMEM and various concentrations of isoxanthohumol (0, 12, 48 and 96  $\mu\text{M}$ ) for 48 h, which was then, loaded onto the upper transwell chambers. These chambers were fitted with membranes of 8  $\mu\text{m}$  pore size. The lower chambers contained FBS (10 %) in addition to DMEM but deprived of target cells. All transwell chambers were incubated for 24 h at 37  $^{\circ}\text{C}$  followed by removal of upper membranes and lower transwell chambers bearing invasive cells which were fixed in ethanol. Post fixation, fixed invasive cells were stained with Giemsa staining solution and numbered under a light microscope.

## Western blotting

BCPAP cells were cultured in 24-well plates bearing  $2.5 \times 10^4$  cells in each well and harvested after 90 % of growth confluence. Afterwards, BCPAP cells were treated with isoxanthohumol drug at various concentrations (0, 12, 48 and 96  $\mu\text{M}$ ) for 48 h. The treated cells were then lysed using lysis buffer and the proteins within each lysate was measured by bicinchoninic acid assay. Equal amounts of 45  $\mu\text{g}$  proteins were loaded in SDS-PAGE (10 %) followed by transfer to polyvinylidene difluoride membranes electrophoretically. These membranes were blocked with skimmed milk (5 %) in TBST followed by incubation with 1:1, 000 dilutions of primary antibodies (anti-Bax, anti-Bcl-2, anti-Bcl-XL) (Cell Signaling Technology, Inc., Danvers, MA, United States)) overnight at 4  $^{\circ}\text{C}$ . Thereafter, incubation with horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA, United States) was performed for 2 h at  $25 \pm 1^{\circ}\text{C}$ . Finally, protein signals were detected using iBright Western Blot Imaging Systems (ThermoFisher Scientific, Inc., Waltham, MA, United States).

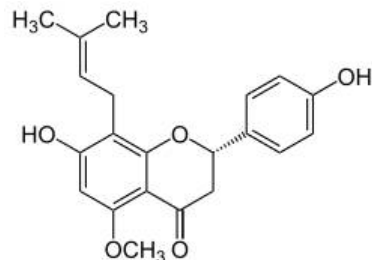
## Statistical analysis

The experiments were repeated thrice for each drug concentrations in all assays. Data was shown as mean of three experiments  $\pm$  SEM (standard error of the mean).  $P < 0.01$  was considered statistically significant and statistical analyses were carried via Student's Newman t-test or Keul's test.

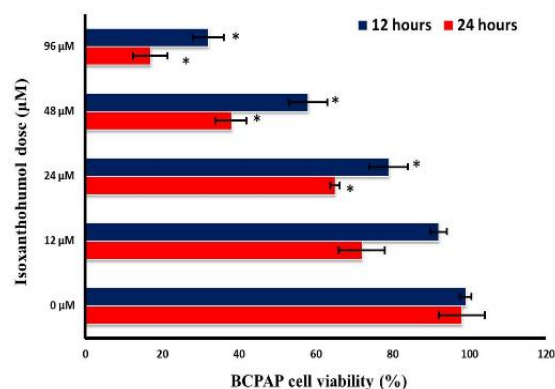
## RESULTS

### Isoxanthohumol induced cytotoxicity in BCPAP cells

Flavonoids have been reported with substantial cytotoxic effects against several human cancer cell lines both *in vitro* and *in vivo*. Herein, the cytotoxic effects of isoxanthohumol were estimated via MTT assay. Isoxanthohumol (Figure 1) was employed at different concentrations (0, 12, 24, 48 and 96  $\mu\text{M}$  for 24 h) against BCPAP cells and cell viability was determined using colorimetric analysis. The results revealed that isoxanthohumol induced remarkable cytotoxicity in BCPAP cells in concentration as well as time-dependent manner. Considering control cells as 100 % viable, the viability was reduced to 20 % at 96  $\mu\text{M}$  (Figure 2).



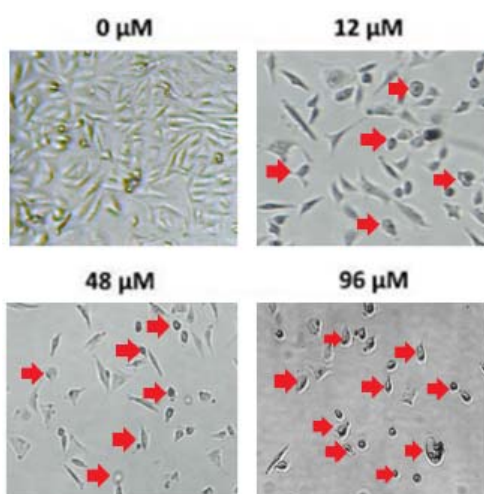
**Figure 1:** Chemical structure of isoxanthohumol molecule



**Figure 2:** Effect of isoxanthohumol on drug-resistant human papillary BCPAP thyroid cancer cells. BCPAP cells were treated with the indicated concentrations of isoxanthohumol for the indicated time intervals. The figure shows reduced viability of BCPAP cells on isoxanthohumol exposure in comparison to the control group. The experiments were repeated thrice. Data was shown as mean of three experiments  $\pm$  SEM (standard error of the mean).  $P < 0.01$  was considered as statistically significant

### Isoxanthohumol disturbed normal morphology of BCPAP cells

Normal morphology of cell membrane, cell organelles and nucleus maintain the integrity of cells. Changes, if any, induced in normal morphology of isoxanthohumol treated BCPAP cells were assessed through phase contrast microscopy. Results indicated that isoxanthohumol brought about significant morphological modifications which indicate that antiproliferative effects of isoxanthohumol could be mediated via alterations in cell morphology. Isoxanthohumol disturbed the normal morphology of plasma membranes, caused membrane blebbing and nuclear disintegration (Figure 3).

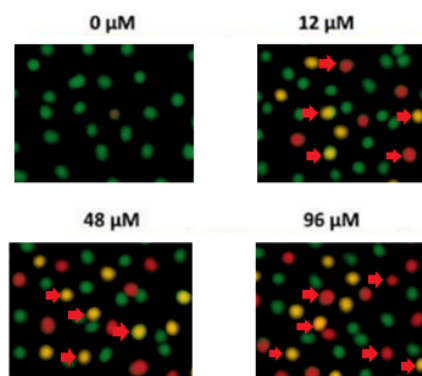


**Figure 3:** Morphological assessment of BCPAP cells after being exposed to indicated concentrations of isoxanthohumol. The figure depicts remarkable morphological modifications indicating cell death of these target cells. Arrows point towards the structural deformity induced by isoxanthohumol like membrane blebbing, rupture, disintegration, circular cells and loss in density. The experiments were repeated thrice. Data was shown as mean of three experiments

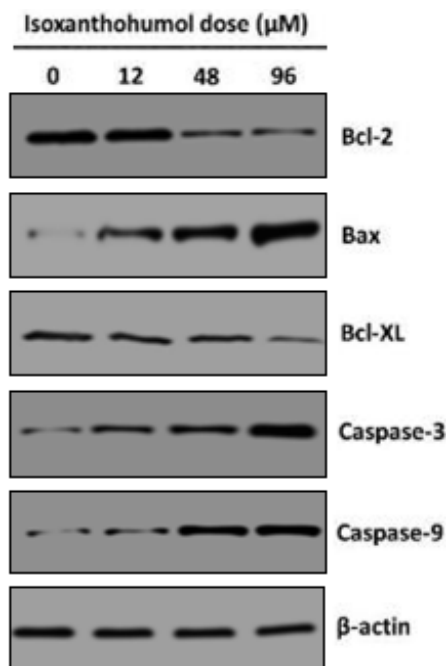
### Isoxanthohumol induced apoptosis in BCPAP cells

Apoptosis is one of the leading and potential targets often selected by chemopreventive drugs. It is a natural process activated under stressful conditions and injury. BCPAP cells were treated with different isoxanthohumol concentration (0, 12, 48 and 96  $\mu\text{M}$ ) for 24 h and apoptotic investigations were carried out via fluorescence microscopy. Results showed that isoxanthohumol potentially stimulated apoptosis in BCPAP cells. Yellow-green (early apoptotic), orange-red (late apoptotic) and red (necrotic cells) fluorescence indicate different stage

apoptotic cells (Figure 4). The fact that apoptosis was induced by isoxanthohumol in BCPAP cells was further supported by western blotting assay. Results showed enhancement in proapoptotic (Bax and caspases) protein levels and reduction in the anti-apoptotic (Bcl-2 and Bcl-XL) protein levels (Figure 5). Therefore, it may be concluded that antiproliferative property of isoxanthohumol against BCPAP cells was due its apoptosis stimulation propensity.



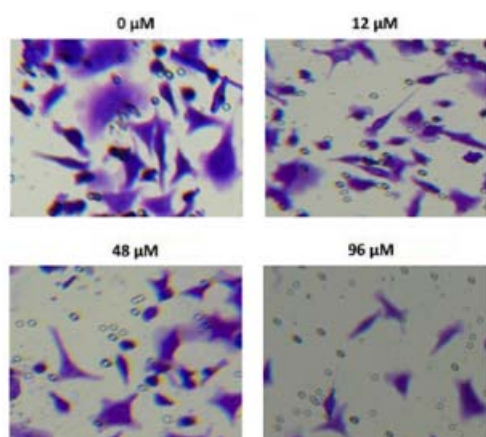
**Figure 4:** Apoptosis-allied antiproliferative effect of isoxanthohumol. After treatment of BCPAP cells with the indicated concentrations of test drug, AO/EB staining was performed. The arrows point at early apoptotic (yellow-green), late apoptotic (orange red) and necrotic (red) BCPAP cells in isoxanthohumol treated group and in case of control group no such fluorescence was observed



**Figure 5:** Enhanced expressions of proapoptotic proteins and decreased expressions of anti-apoptotic proteins. Each individual experiment was executed in triplicates.  $\beta$ -Actin was used as normalization control

### Isoxanthohumol targeted cell migration and invasion of BCPAP cells

We all know cancer is a dangerous malignancy, but it is more dangerous when exists in metastatic form which often leads to poor diagnosis and complicates chemotherapy. Herein, isoxanthohumol was investigated for its anti-metastatic effect against BCPAP cells. After exposure to different isoxanthohumol concentrations (0, 12, 48 and 96  $\mu\text{M}$ ) cells were analyzed for invasion and migration via transwell chambers invasion and migration assay. Isoxanthohumol was observed to remarkably reduce the number of invasive BCPAP cells in a concentration-dependent manner (Figure 6). It was also observed that the number of migrated BCPAP cells reduced on isoxanthohumol treatment and upon increasing its concentrations (Figure 7). Therefore, it may be concluded that isoxanthohumol could inhibit the metastatic nature of the human thyroid cancer cells by inhibiting cell invasion and migration.

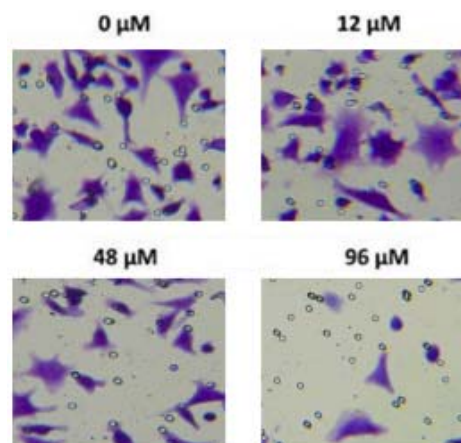


**Figure 6:** BCPAP cell invasion after exposure to isoxanthohumol at indicated concentrations. The molecule led to decreased cell invasion in a concentration -dependent manner. Each individual experiment was repeated in triplicates

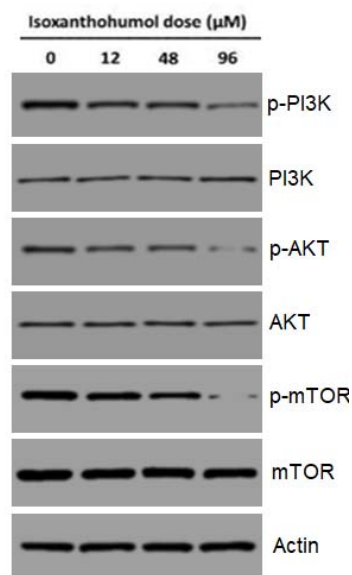
### Isoxanthohumol inhibited PI3K/AKT/m-TOR signalling pathway in BCPAP cells

The PI3K/AKT/m-TOR signalling pathway has been reported to play a key role in a number of survival processes of a cell. This pathway hence serves as a leading target for chemopreventive drugs. Isoxanthohumol was investigated for its effects on PI3K/AKT/m-TOR signalling pathway through western blotting assay. Results showed that the expression levels of phosphorylated PI3K, AKT and m-TOR reduced remarkably on isoxanthohumol drug exposure, in comparison to controls (Figure 8). The expression levels of

PI3K, AKT and m-TOR remained almost intact. Therefore, it may be concluded that isoxanthohumol inhibited the progression of PI3K/AKT/m-TOR signalling pathway.



**Figure 7:** BCPAP cell migration after exposure to isoxanthohumol at indicated concentrations. The figure shows reduced migrated cells after test drug exposure in comparison to the control group. Each individual experiment was repeated in triplicate



**Figure 8:** Effect of isoxanthohumol on PI3K/AKT/m-TOR signalling pathway proteins. The figure shows that the expression levels of phosphorylated PI3K, AKT and m-TOR reduced remarkably on isoxanthohumol exposure. Each individual experiment was repeated in triplicates.  $\beta$ -Actin was used as normalization control

## DISCUSSION

Thyroid cancer cases increased rapidly in the past 2-3 decades despite being associated with low mortality [13]. Till date, different histological

sub-types of thyroid cancer have been identified including follicular, papillary, and weakly differentiated cancers (medullary and anaplastic) [14]. Thyroid cancer acquires resistance to applied therapeutic methods such as low intake of radioactive iodine from blood in radioactive iodine therapy [15]. Several genetic mutations have been linked to decreased iodine uptake of tumorous thyroid cells most importantly B-RAF (gene encoding B-Raf protein) mutations [16].

Acquired resistance, disease relapse and scarcity of efficient therapeutic drugs create a need of novel chemopreventives. Therefore, this research is aimed at exploring the anti-thyroid cancer potency of isoxanthohumol. The effects of the test drug on PI3K/AKT/m-TOR signalling pathway, cell migration and invasion and apoptosis against BCPAP thyroid cancer cells was also determined. It was observed that isoxanthohumol was cytotoxic in tumorous BCPAP cells. This cytotoxicity was estimated with MTT assay which revealed its concentration- and time-dependence. Afterwards, an attempt was made to search for the mechanism underlying the antiproliferative potency of isoxanthohumol in BCPAP cells.

Previously, isoxanthohumol had been reported to induce proapoptotic effects in adipocytes (3T3-L1) [12]. Apoptosis is a highly regulated and conserved mechanism in multicellular organisms and mammals. It is controlled by different intrinsic and extrinsic signals. Apoptosis in a normal cell remains dormant and operates in case of aging, malfunctioning and damage [17]. Herein, isoxanthohumol was reported to have proapoptotic effects against tumorous BCPAP cells as well. The apoptotic cell death was supported by enhanced levels of Bax, caspase-3 and caspase-9 and downregulation of Bcl-2, Bcl-XL protein expressions. Isoxanthohumol produced remarkable suppressive effects on migration and invasion of tumorous BCPAP cells. Therefore, isoxanthohumol significantly inhibited thyroid cancer metastasis. The PI3K/AKT/m-TOR signalling pathway regulates a number of survival functions of normal/cancerous cells [18]. It regulates cell growth, differentiation and development and in cancer cells due to uncontrolled multiplication of cells its role remains very important. For chemopreventives, the PI3K/AKT/m-TOR signalling pathway serves as a major target in a cancer cell to initiate cell death mechanism. Herein, isoxanthohumol drug remarkably targeted the expression of PI3K/AKT/m-TOR signalling pathway allied proteins in BCPAP cells thereby downregulating their expressions in concentration-dependent manner.

## CONCLUSION

The results of this investigation indicate the remarkable anticancer activity of isoxanthohumol against drug-resistant thyroid cancer. The anticancer effect of isoxanthohumol is exerted by targeting PI3K/AKT/m-TOR signalling pathway, inhibition of cell migration and invasion, and apoptosis induction. Thus, these findings indicate that isoxanthohumol is a potential lead candidate for the development of an anti-thyroid cancer drug. Further investigations are, however, required.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with 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.

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