

## Original Research Article

# Licoflavanone exerts anticancer effects on human nasopharyngeal cancer cells via caspase activation, suppression of cell migration and invasion, and inhibition of m-TOR/PI3K/AKT pathway

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### Abstract

**Purpose:** To study the anticancer effect of licoflavanone against human nasopharyngeal HK1 carcinoma, and the mechanism involved.

**Methods:** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine the effect of licoflavanone on cell viability, while DAPI staining and western blotting were used to study its proapoptotic effect. Morphological examination was performed under phase contrast microscopy. Transwell chamber assays were used to study cell migration and invasion. The expression levels of mTOR/PI3K/AKT signal pathway-related proteins were assayed by Western blotting.

**Results:** Licoflavanone markedly suppressed the proliferation of nasopharyngeal HK1 cancer cells in a concentration-reliant pattern ( $p < 0.01$ ). The anticancer effects of licoflavanone were mediated via induction of pro-apoptotic effects and blocking of mTOR/PI3K/AKT signal pathway. Licoflavanone enhanced the activities of caspase-3, caspase-8, caspase-9 and cleaved caspase-3, as well as Bax and Bad. Moreover, licoflavanone blocked the migration and invasion of HK1 nasopharyngeal cancer cells.

**Conclusion:** Licoflavanone exerts potent anticancer effects on human nasopharyngeal cancer cells via caspase activation, inhibition of cell migration and cell invasion, and down-regulation of m-TOR/PI3K/AKT signal pathway. Therefore, licoflavanone may be a useful lead drug for the development of a treatment strategy for nasopharyngeal cancer.

**Keywords:** Nasopharyngeal carcinoma, Flavonoids, Apoptosis, Caspase, Cell migration, Cell invasion

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## INTRODUCTION

Nasopharyngeal carcinoma is a type of cancer that originates from nasopharynx epithelium [1]. The epicentre of nasopharyngeal within the boundaries of nasopharynx is located at the

fossa of Rosemmuller from where it migrates and invades neighbouring organs and anatomical spaces. Although there are similarities among nasopharyngeal and other neck or head cancers in terms of tissue of origin or cell lineage, they remain highly distinctive [2].

Nasopharyngeal carcinoma is less prevalent than other cancer types, although it results in considerable mortality and huge morbidity, worldwide. In the year 2012 alone, 86,500 cases of nasopharyngeal carcinoma were reported which accounted for 0.6 % of total cancer cases. More than 70 % of the cases were reported in East and Southeast Asia, while the rest occurred in North and East Africa, and South-Central Asia [3]. Nasopharyngeal carcinoma is classified into three subtypes as per WHO criteria. Type I is highly differentiated and shows surface keratin, whereas type II and type III are undifferentiated and do not have surface keratin [4].

Epstein-Barr virus (EBV) and human papillomavirus (HPV) infections are two major risk factors for nasopharyngeal carcinoma [5]. The incidence of nasopharyngeal carcinoma is on the increase globally. Therefore, research efforts on strategies for managing this global disease are needed in order to lower the mortality and morbidity associated with it. Natural products have played a pivotal role in the treatment of several human malignancies throughout history, with quinine from cinchona and salicylates from willow as well-known examples, in addition to the legendary penicillin [6]. Moreover, throughout the world, plant-based traditional remedies still lead in therapeutic practices. Natural products are leads in current-day medicine, especially in the areas of cancer therapy and antibiotics.

Flavonoids are a large class of phytochemicals with extensive biological and medicinal properties. Studies have demonstrated that flavonoids have potential anti-inflammatory, antioxidant, antimicrobial, cardioprotective, and anticancer properties [7,8]. The anticancer effects of flavonoids have been attributed to mechanisms such as anti-proliferation, apoptosis, cell cycle arrest, inactivation of carcinogens, anti-oxidation and autophagy. Licoflavanone is one of the bioactive flavonoids found in *Glycyrrhiza glabra* L., and it has marked pharmacological potency [9]. Licoflavanone exerts marked anti-inflammatory and antioxidant effects, and it substantially modulates the NF- $\kappa$ B/MAPK signal pathway [10].

The current investigation is the first of its kind on the anticancer effects of licoflavanone on nasopharyngeal carcinoma. In addition, the effect of licoflavanone on caspase-dependent apoptosis, cell migration and invasion, and the mTOR/PI3K/AKT signal pathway were determined.

## EXPERIMENTAL

The suppressive effects of licoflavanone on nasopharyngeal HK1 cancer cells were determined using MTT assay. The HK1 cells were cultured overnight at 37 °C in RPMI-1640 in 96-well plates at a density of  $2.5 \times 10^4$  cells/well. Thereafter, each well was incubated with various concentrations of licoflavanone viz 12.5, 25, 50 and 100  $\mu$ M for 24 h. Cells in the control group were not treated with licoflavanone. Thereafter, MTT solution was added to each well and the cells were further incubated for 4 h at 37 °C. The formazan crystals formed were dissolved in dimethyl sulfoxide (DMSO). The absorbance of formazan solution in each well was read at 490 nm in an enzyme-linked immunosorbent assay reader (Molecular Devices, Sunnyvale, United States), and the absorbance readings were used for estimation of cell viability.

### Morphological analysis

The HK1 cells were harvested at logarithmic phase of growth and plated in 24-well microtiter plates at a density of  $2 \times 10^5$  cells/well. Each well was allowed to incubate at 37°C for 24 h for cell attachment. Thereafter, the cells were treated with various concentrations of licoflavanone viz 12.5, 25 and 100  $\mu$ M, for 72 h (cells in control group were not treated). The morphologies of the licoflavanone-treated cells were examined under a phase contrast microscope (Leica, Wetzlar, Germany).

### Determination of effect of licoflavanone on HK1 cell apoptosis

The pro-apoptotic effect of licoflavanone on nasopharyngeal HK1 cells was studied via fluorescence microscopy using DAPI staining. The nasopharyngeal cancer cells were seeded in 6-well plates at a density of  $0.8 \times 10^5$  cells/well and incubated overnight at 37 °C. Then, the cells were treated with various licoflavanone concentrations viz 12.5, 25 and 100  $\mu$ M, at 37 °C for 24 h. Untreated cells served as control. Post-sloughing off of licoflavanone-treated HK1 cells was followed by loading of 20  $\mu$ L of cell culture onto glass slides, and cell staining using 1  $\mu$ L of DAPI solution. Thereafter, the slides were covered with coverslips and examined for apoptotic changes under a fluorescent microscope (OLYMPUS, Japan).

### Determination of the effect of licoflavanone on cell migration and invasion

The effect of licoflavanone on the migration and invasion of nasopharyngeal cells was

investigated using Transwell chambers assays. The cells were placed in the upper chambers of the Transwell containing RPMI-1640 medium, while the lower chambers were loaded with medium alone. The upper chambers contained various concentration of licoflavanone viz 12.5, 25 and 100  $\mu\text{M}$ . The cells were incubated for 24 h. Finally, cell migration and invasion analyses were performed as described previously [11].

### Western blotting assay

The expression levels of apoptosis and mTOR/PI3K/AKT signal pathway-related proteins in licoflavanone-treated HK1 cells were estimated via western blotting assay. The cells were harvested at >90 % growth confluence, followed by 24 h treatment with various licoflavanone concentrations viz 12.5, 25 and 100  $\mu\text{M}$ . Thereafter, the cells were lysed using RIPA lysis buffer, and the protein concentrations were determined with bicinchoninic acid method. The proteins were then resolved with SDS-PAGE, followed by electrophoretic transfer to nitrocellulose membranes. The membranes were blocked with fat-free skimmed milk prior to incubation overnight in the dark at 4° C with primary antibodies against caspases, Bax, Bcl-2, XIAP, Bcl2-xL, Bad, pmTOR, p-PI3K and p-AKT. Then, the membranes were incubated with HRP-conjugated secondary antibodies (1:1,000 dilution) for 1 h in dark at room temperature. Finally, enhanced chemiluminescence reagent was used to develop the protein signals.

### Statistical analysis

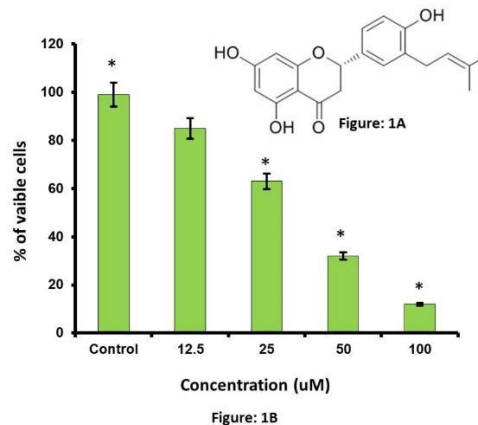
All experiments were done in triplicate. Data are presented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using SPSS software (version 21, Armonk, NY, United States). Differences amongst groups were calculated using GraphPad Prism 7 software and one-way ANOVA. Values of  $p < 0.01$  were taken as indicative of significant differences.

## RESULTS

### Effect of licoflavanone on HK1 cell viability

The effect of licoflavanone on the viability of HK1 cells is shown in Figure 1 A. Several flavonoids have been previously reported with strong cytotoxicity against diverse human cancer cell lines including CNE1, CNE2 and HK1 nasopharyngeal carcinoma cells. The results obtained in this study showed that licoflavanone significantly induced cytotoxicity in HK1 cancer cells in a concentration-dependent manner. Cell viability in control was considered as 100 %.

Licoflavanone-treated group showed dose-dependent decreases in viability (Figure 1 B).



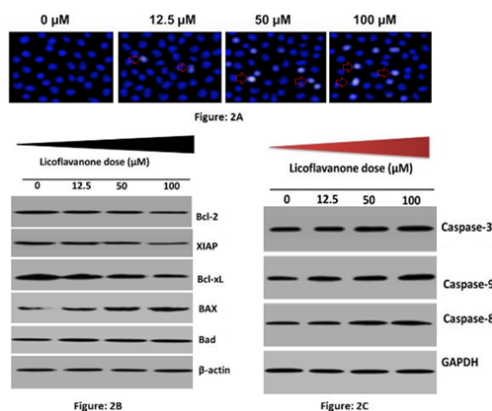
**Figure 1:** (A). Chemical structure of licoflavanone molecule. (B) Effect of licoflavanone on viability of nasopharyngeal carcinoma HK1 cells, as determined via MTT assay. After exposure to indicated concentrations of licoflavanone, results showed that the percentage viability decreased markedly from 100 % in control to almost 10 % in licoflavanone-treated group. Each experiment was repeated in triplicate. Data are presented as mean  $\pm$  SD;  $p < 0.05$  was taken as statistically significant

### Licoflavanone-induced apoptotic cell death in HK1 cells

Apoptosis has been a dominant target of chemopreventive drugs in several studies. The mechanism of apoptosis involves several molecular cascades and proteins. Apoptosis is stimulated by both intrinsic and extrinsic factors (mitochondria-mediated or caspase-dependent, and extracellular signal-mediated apoptosis, respectively). Apoptosis is regulated by two key types of proteins considered as proapoptotic (Bax and Bad) and antiapoptotic (Bcl-2 family). In this study, the apoptotic effect of licoflavanone was determined via DAPI staining and western blotting assay. Results of DAPI staining revealed that licoflavanone induced pro-apoptotic changes in HK1 cells, indicating apoptotic cell death. In contrast, the control group showed normal cell structure and visible intact nuclear membrane.

The licoflavanone-treated group showed disturbances in cell morphology such as membrane damage, chromatin condensation, nuclear disintegration, formation of apoptotic crops and loss of normal cell morphology. These lesions are shown in Figure 2 A. Results of Western blotting assay indicated that the protein expression levels of proapoptotic caspase-3, caspase-8, caspase-9 and cleaved caspase-3; Bax and Bad were significantly up-regulated,

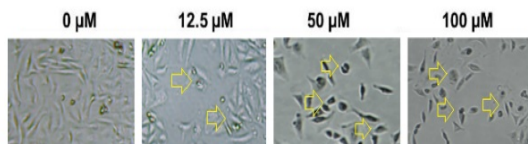
while the protein expression levels of anti-apoptotic Bcl-2, XIAP and Bcl-2-xL were markedly down-regulated on exposure to licoflavanone. These results are presented in Figure 2 B and C.



**Figure 2:** Apoptotic effect of licoflavanone on HK1 cells. (A) DAPI staining showing apoptotic cell morphology with nuclear condensation and formation of apoptotic crops (as indicated with arrows). (B) Western blotting results showing the protein expression levels of pro-apoptotic (Bad and Bax) and anti-apoptotic (Bcl-2, Bcl-xL and XIAP) factors. (C) Western blotting results showing the protein expression levels of caspase-3, caspase-8, caspase-9 and cleaved caspase-3. All experiments were done in triplicate

### Effect of licoflavanone on HK1 cell morphology

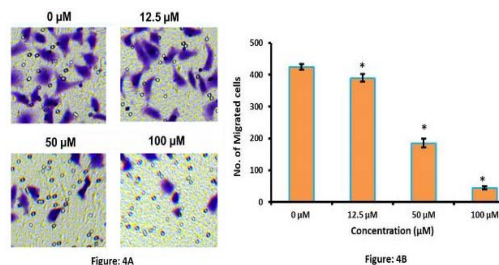
The cellular morphology of licoflavanone-treated HK1 cells was examined under phase contrast microscopy. Results showed that licoflavanone treatment damaged the morphology of the HK1 cells, when compared to cells in the control group. The licoflavanone-induced morphological alterations were membrane blebbing, nuclear disintegration, plasma membrane rupture, cell deformity, and nuclear condensation. These lesions are shown in Figure 3.



**Figure 3:** Morphological changes in HK1 cells after exposure to licoflavanone at indicated concentrations. Results showed complete disruption of cell morphology due to with membrane blebbing, nuclear condensation, plasma membrane rupture and loss of cell integrity in licoflavanone-treated groups, relative to control group. All experiments were done in triplicate

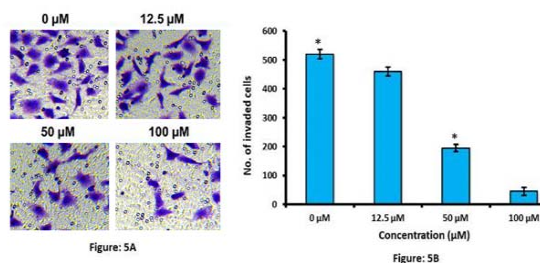
### Licoflavanone suppressed the migration and invasion of HK1 cells

Transwell chamber assay was used to determine the anti-migratory and anti-invasive potency of licoflavanone in HK1 cells. The results of Transwell migration assay indicated that licoflavanone inhibited the migration of HK1 cells in a concentration-dependent fashion. The number of migrated cells in licoflavanone-treated groups reduced from >400 cells to almost 50 cells at higher drug concentrations, when compared to control group. These results are presented in Figure 4 A and B).



**Figure 4:** Effect of licoflavanone on migration of HK1 cells. (A) Cell migration was markedly suppressed in a concentration-dependent manner by licoflavanone. (B) The number of migrated cells reduced from >400 cells to almost 50 cells in licoflavanone-treated group. Each experiment was carried out in triplicate. Data are mean  $\pm$  SD; \* $p < 0.05$

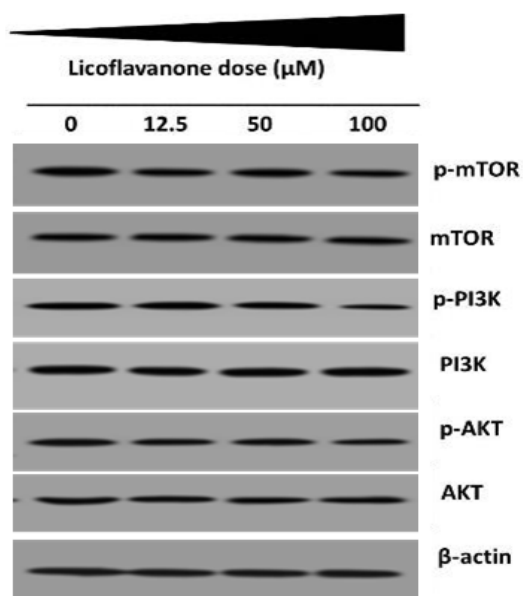
Transwell chambers coated with Matrigel showed that licoflavanone inhibited the invasion of HK1 cells in a concentration-dependent fashion (Figure 5 A). The number of invaded HK1 cells reduced from > 500 cells to almost 40 cells in licoflavanone-treated groups, relative to control group (Figure 5 B). Therefore, it may be concluded that licoflavanone substantially targeted HK1 cell migration and invasion.



**Figure 5:** Cell invasion in licoflavanone-treated HK1 cells. (A) After treatment with indicated concentrations of licoflavanone, cell invasion was potentially suppressed in a concentration-dependent manner. (B) The number of invaded cells reduced from >500 cells to almost 40 cells in treated groups. Each experiment was done in triplicate. Data are presented as mean  $\pm$  SD; \* $p < 0.05$

### Effect of licoflavanone on mTOR/PI3K/AKT signal pathway in HK1 cells

The mTOR/PI3K/AKT signal pathway plays a vital role in carcinogenesis, cancer cell growth and differentiation. Therefore, this pathway is a high-value target of chemo-preventive drugs in cancer suppression. In this study, Western blotting assay was used to measure the expression levels of mTOR/PI3K/AKT signal pathway-allied proteins. Results indicated that the expressions of m-TOR, PI3K and AKT remained almost constant, while the levels of p-mTOR, p-PI3K and p-AKT decreased markedly on licoflavanone exposure (Figure 6). Therefore, it may be concluded that the anti-proliferative effect of licoflavanone on HK1 cells was mediated via inhibition of mTOR/PI3K/AKT signal pathway.



**Figure 6:** Effect of licoflavanone on expressions of mTOR/PI3K/AKT signal pathway proteins in HK1 cells. Licoflavanone markedly and concentration-dependently down-regulated the expression levels of phosphorylated m-TOR, AKT and PI3K, while the protein expression levels of their non-phosphorylated counterparts were almost constant ( $p < 0.01$ ). All experiments were carried out in triplicate

### DISCUSSION

Cancer remains an ailment that has continued to affect millions of people for decades [12]. Nasopharyngeal carcinoma is among the less frequent cancers, but its incidence is also on the increase. Although radiation therapy is a potential treatment strategy for nasopharyngeal carcinoma, there is a need for chemo-preventive

drugs that can overcome the lethal side-effects posed by radiation [13].

Natural products provide a wide range of chemical entities which are beneficial in the management of nasopharyngeal carcinoma. The current investigation was carried out to determine the anticancer effect of licoflavanone on nasopharyngeal carcinoma, and mechanism involved. Results from MTT viability assay revealed that licoflavanone induced marked cytotoxicity in nasopharyngeal cancer cells in a concentration-dependent fashion. Therefore, further investigations were performed to elucidate the mechanism through which licoflavanone inhibited proliferation of HK1 cells. Morphological analysis of HK1 cells under a phase contrast microscope showed marked licoflavanone-induced cellular lesions such as membrane rupture, membrane blebbing, nuclear condensation and cellular disintegration. These results indicate that the antiproliferative effect of licoflavanone could be due to its pro-apoptotic effect. Apoptosis plays a vital role in regular maintenance of tissue health, and in the elimination of damaged and malfunctioning cells [14]. Flavonoids have been reported to exert pro-apoptotic effects against a variety of human cancer cells. In the present study, licoflavanone produced anti-proliferative effect on HK1 cells via induction of apoptosis. The expressions of pro-apoptotic proteins (caspase-3, caspase-8, caspase-9 and cleaved caspase-3), as well as Bax and Bad proteins were up-regulated on exposure of HK1 cells to licoflavanone. In contrast, the protein expression levels of Bcl-2, XIAP and Bcl-2-xL were markedly down-regulated on licoflavanone exposure. Migration and invasion are the two main mechanisms which lead to cancer metastasis. In previous studies, it was reported that flavonoids induced anti-migratory and anti-invasive effects on several human cancer cell lines. This is in agreement with the results obtained in the present study which showed that licoflavanone substantially inhibited the migration and invasion potential of nasopharyngeal carcinoma cells [15].

The mammalian target of rapamycin (mTOR)/phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT) pathway is an important pathway that modulates a number of essential physiological functions in cells. Moreover, this pathway is associated with malignant makeover of human cancers and actively participates in their metastasis, proliferation, growth and metabolism [16]. It has been reported that the PI3K/AKT/mTOR signaling pathway is stimulated by a number of genes encoding key components and somatic mutations [17]. In addition, the

PI3K/AKT/mTOR signaling pathway is a poor prognostic factor in different cancers, and it is associated with resistance to conventional therapies [18]. Therefore, the PI3K/AKT/mTOR signaling pathway serves as a central target for many chemo-preventive drugs. In this study, results from Western blotting showed that on exposure of HK1 cells to licoflavanone, the levels of PI3K/AKT/mTOR signaling pathway-allied proteins (p-mTOR, p-AKT and pPI3K) dropped markedly. These lower levels of activity indicated that licoflavanone induced significant inhibitory effects on the PI3K/AKT/mTOR signal pathway.

## CONCLUSION

The results obtained in this investigation show that licoflavanone exhibits potential anticancer effect against human nasopharyngeal carcinoma cells. The anticancer effect is mediated via induction of caspase-dependent apoptosis, inhibition of migration, suppression of invasion, and blocking of mTOR/PI3K/AKT signal pathway. Therefore, licoflavanone is a potential lead drug in the development of a novel therapy for nasopharyngeal carcinoma.

## 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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