JBUON 2019; 24(3): 936-942
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

In vitro and *in vivo* cytotoxic effects of chrysoeriol in human lung carcinoma are facilitated through activation of autophagy, sub-G1 cell cycle arrest, cell migration and invasion inhibition and modulation of MAPK/ERK signalling pathway

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Summary

Purpose: *Lung cancer is a malignancy that imposes huge health, psychological and financial burden on patients and their families. Owing to lack of viable treatment options and late diagnosis, there is need for the development of new candidate drugs. In the current study the anticancer potential of Chrysoeriol was examined against lung cancer cells.*

Methods: *The proliferation rate of the lung cancer cells was checked by WST-1 assay. Autophagy was detected by electron microscopy and propidium iodide (PI) staining. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by immuno blotting. Xenografted mice models were used for in vivo evaluation of Chrysoeriol.*

Results: *The results revealed that Chrysoeriol could significantly inhibit the proliferation of the A549 lung cancer cells with lower cytotoxicity against the normal MRC-5 cells. The anticancer activity of Chrysoeriol against the A549 cells was due to induction of autophagy. The Chrysoeriol-prompted autophagy was also associated with alteration in the autophagy-related protein expression. The expression of LC3II and Beclin-1 was significantly upregulated upon chrysoeriol treatment. Chrysoeriol could also induce sub-G1/G0 cell cycle arrest. Furthermore, it could also inhibit the migration and invasion of the A549 cells. In addition, it was observed that Chrysoeriol could inhibit the MAPK/ERK signalling pathway in the A549 lung cancer cells. The effects of the Chrysoeriol were also examined in vivo in xenografted mice models which revealed that Chrysoeriol inhibited the growth of xenografted tumors.*

Conclusion: *Chrysoeriol considerably and selectively suppresses the growth of lung cancer in vitro and in vivo and may prove beneficial in the management of this disease.*

Key words: *chrysoeriol, autophagy, apoptosis, cell cycle, lung cancer*

Introduction

Accounting for about 25% of all the cancers, lung cancer is the most prevalently detected malignancy across the globe and is responsible for about 20% of cancer-related mortality [1]. Despite the progress been made in the field of cancer, the overall survival is still far from descent. Additionally, late diagnosis and the development of drug resistance of cancer cells form another major hurdle in the treatment of lung cancer [2]. The presently used drugs exhibit a number of side effects and, hence, the identification of new candidate drugs is a need looking for answer. Plants and their sec-

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Received: 30/08/2018; Accepted: 17/09/2018

ondary metabolites have provided humans with a wide array of drugs for the treatment of deadly conditions and they are likely to serve as source of more important drugs in the future [3]. Plants are specialised to produce metabolites to overcome biotic and abiotic stresses. Such metabolites, commonly referred to as secondary metabolites, have been employed for the treatment of diseases such as cancer [4]. For example, podophyllotoxins belong to common anticancer agents of plant origin [5]. Although these secondary metabolites have been chemically classified into different groups, flavones constitute an important group of plant secondary metabolites with enormous pharmacological potential [6]. They have been found to exhibit a wide range of bioactivities such as anticancer and antimicrobial to name a few [7]. Chrysoeriol is an important plant-derived metabolite commonly found across the plant kingdom [8,9]. However, the antiproliferative effects of Chrysoeriol have not been examined against lung cancer. Herein, we report for the first time the anticancer activity of Chrysoeriol against lung cancer cells along with its mode of action by studying its effects on autophagy, cell cycle, cell migration and invasion and MAPK/ERK signalling pathway.

Methods

Cell lines and culturing conditions

The lung cancer cell line A5495 and the normal lung cell line MRC-5 were procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in CO_2 incubator (Thermo Fisher Scientific, Shanghai, China) at 37°C with 98% humidity and 5% CO_2.

Cell viability assay

The effect of Chrysoeriol on the viability of lung cancer cell line by WST-1 assay was assessed as described previously [10]. In brief, the lung cancer cells were cultured at a density of 2.5×10^5 cells/well in 96-well plates and subjected to treatment with varied concentrations of Chrysoeriol. This was followed by incubation of the lung cancer cells with WST-1 for 3 h at 37°C and the proliferation rate was determined by taking the absorbance at 450 nm.

Electron microscopy

The electron microscopic analysis of Chrysoeriol-treated A549 lung cancer cells was used to detect the induction of autophagic cell death. Briefly, the lung cancer cells were exposed to 0, 20, 40 and 80 μM Chrysoeriol for 24 h. The cells were then collected by trypsinization and washed with phosphate buffered saline (PBS), followed

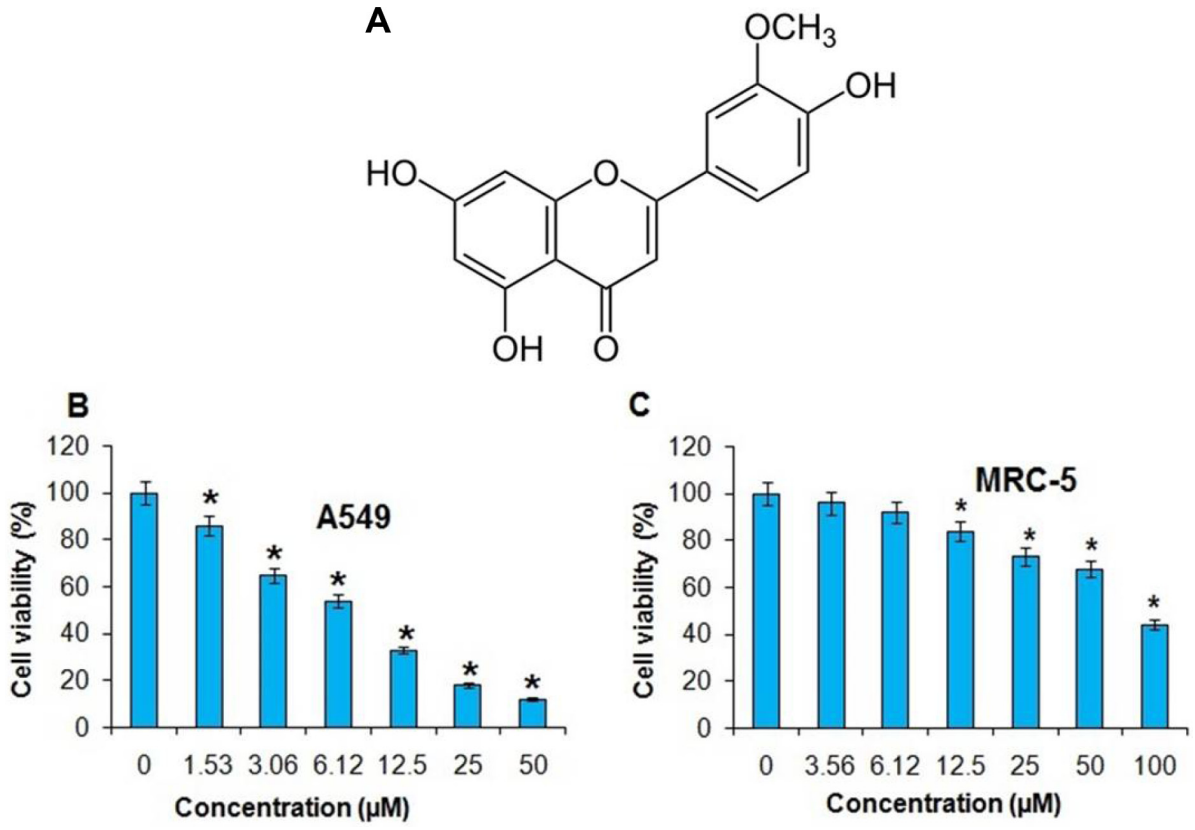

Figure 1. A: Chemical structure of Chrysoeriol. **B:** Effect of Chrysoeriol on the viability of A549 lung cancer cells. **C:** MRC-5 cells as determined by MTT assay. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by treatment of the cells with ethanol and embedding in resin. Thin sections (70 nm) were then cut with the help of an ultramicrotome and subjected to electron microscopy.

Propidium iodide staining for autophagy

The effects of Chrysoeriol on the initiation of the programmed cell death was assessed by propidium iodide (PI) staining [11]. The lung cancer cells (0.6×10^6) were grown in 6-well plates and after 12-h incubation they were subjected to Chrysoeriol treatment for 24 h at 37°C. Then, they were immediately centrifuged and the pellets were washed with phosphate buffered saline (PBS). Afterwards, the cells were PI-stained, centrifuged and PBS-washed. Finally, the PI stained cells were examined by fluorescence microscopy.

Wound healing and Boyden chamber assay

The potential of the A549 cells transfected to migrate was examined by wound healing assay. A549 cells at a density of 5×10^4 cells per well were cultured in 96-well plates. The plates were then incubated at 37°C for 24 h to allow the cells to adhere. A scratch was done on the plate with a sterile pipette tip. The cells were then washed with PBS, monitored after 48-h interval and photographed. Cell invasion was examined by Boyden chamber assay as described previously [12].

Western blotting

To determine the expression of the selected proteins in the Chrysoeriol-treated lung cancer cells, the cells were subjected to lysis with RIPA buffer and the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for a period of 24 h. After this, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

In vivo study

The anticancer effects of Chrysoeriol were also examined in vivo in xenografted mice models. Three-week old male BALB/c nude mice were kept in the animal facility as per the National Institutes of Health standards for the care and use of laboratory animals. The mice were injected then with 5×106 A549 cells subcutaneously at the left flank. The mice (n=5) for each group were injected intraperitoneally with DMSO (0.1%) dissolved Chrysoeriol and diluted with 100 µL normal saline at 10, 20 and 40 mg/kg body weight and taken as the day one of the experiment. Chrysoeriol was given subcutaneously to the rats thrice a week, while the control mice were given DMSO (0.1%) in normal saline only. At the end of 6 weeks, the mice were euthanized and the tumors were removed and used for analysis.

Statistics

Statistical analyses were performed using SPSS software package (SPSS Inc., Chicago, Ill, USA). The results were presented as mean ± SD from 3 independent experiments. Differences between groups were examined by Student's t-test and $p<0.05$ was considered to indicate a statistically significant difference.

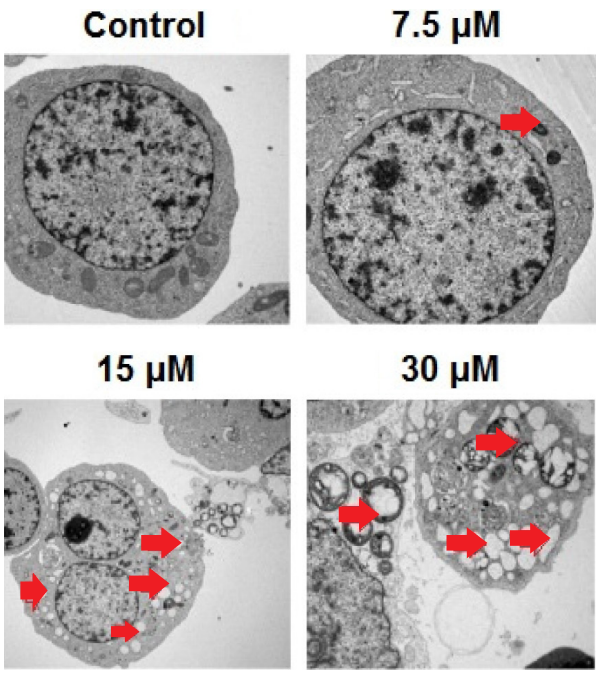

Figure 2. Electron microscopy images of Chrysoeriol-treated A549 cells showing induction of autophagy in a dose-dependent manner. Arrows show autophagosomes. The experiments were performed in triplicate.

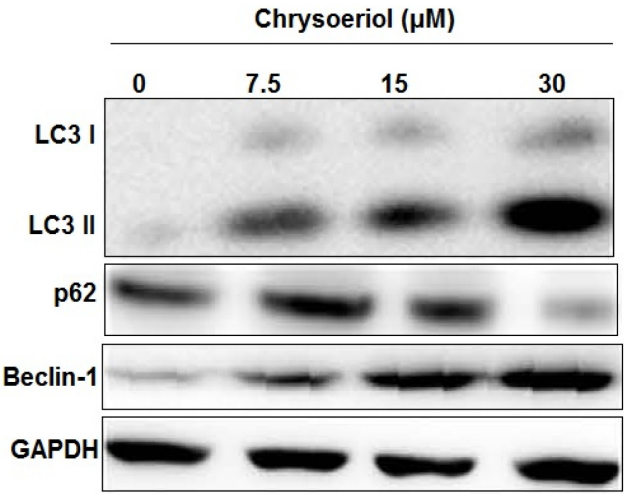

Figure 3. Effect of Chrysoeriol on the autophagy-related protein expression as revealed by western blot analysis. The Figure shows that Chrysoeriol treatment led to increased expression of LC3-I, LC3-II and Beclin-1. The experiments were performed in triplicate.

Results

Chrysoeriol inhibited the growth of lung cancer cells

The effects of Chrysoeriol (Figure 1A) on the proliferation of lung cancer cells were examined by WST-1 assay. It was found that Chrysoeriol exerted antiproliferative effects on the A549 lung cancer cells with an IC_{50} of 15 μM (Figure 1B). On the contrary, anticancer effects were also observed against the normal MRC-5 cells but on a significantly higher IC_{50} of 93 μM (Figure 1C). Additionally, it was found that the anticancer effects of Chrysoeriol on the A549 lung cancer cells were concentration-dependent.

Chrysoeriol induced autophagy in lung cancer cells

The impact of Chrysoeriol on the A549 lung cancer cells was investigated by electron microscopy. The results revealed that Chrysoeriol caused production of autophagosomes in the A549 lung cancer cells indicating that this molecule induced autophagy (Figure 2). Furthermore, for the confirmation of autophagy, the expression of autophagy-related proteins was investigated and it was revealed that Chrysoeriol caused increase of Beclin-1 and LC3-II and suppression of p62 expression. On the contrary, no effects were found on the expression of LC3-I (Figure 3). Development of autophagic vacuoles was observed with drug treatment (Figure 4).

Chrysoeriol caused sub-G1/G0 arrest of lung cancer cells

The effects of Chrysoeriol on the distribution of A549 lung cancer cells in various cell cycle phases was assessed by flow cytometry. It was found that this molecule caused remarkable increase in the percentage of the A549 lung cancer cells in the sub-G1/G0 phase of the cell cycle. The percentage of A549 lung cancer cells in the sub-G1/G0 phase increased from 0.79% to 78.54% upon Chrysoeriol

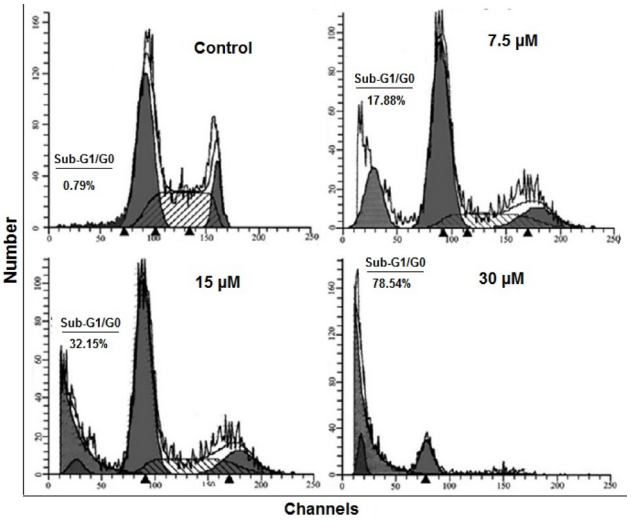

Figure 5. Effect of Chrysoeriol on the distribution of the A549 lung cancer cells as determined by flow cytometry. The Figure shows that Chrysoeriol induced sub-G1/G0 cell cycle arrest dose-dependently. The experiments were performed in triplicate.

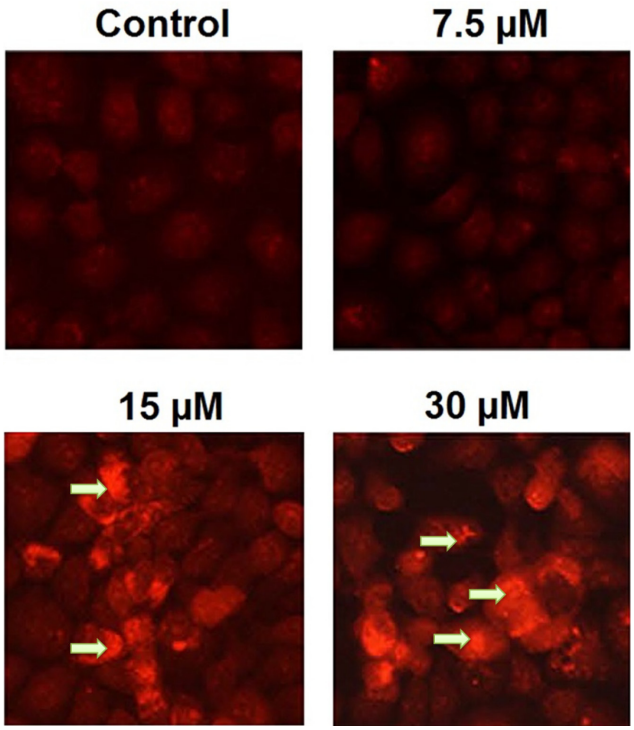

Figure 4. Propidium iodide staining images showing induction of autophagic vacuoles (arrows) by Chrysoeriol on the A549 images.

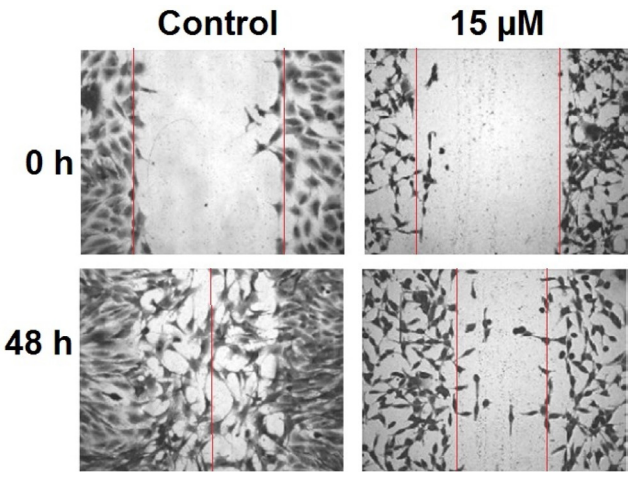

Figure 6. Effect of Chrysoeriol on the migration of the A549 cells as determined by wound healing assay. The Figure shows that Chrysoeriol inhibited the trend for migration of cancer cells. The experiments were performed in triplicate.

treatment (Figure 5). These results clearly indicated that Chrysoeriol induced sub-G1/G0 cell cycle arrest of the A549 lung cancer cells.

Chrysoeriol inhibited the cancer cells'migration and invasion

The effect of Chrysoeriol on the migration of A549 cells was investigated by wound healing assay. This effect was checked at IC_{50} (15 μM) and it was found that this compound considerably inhibited the migration of A549 cells (Figure 6). The effects of Chrysoeriol on the invasion of the A549 cells were investigated by the Boyden Chamber assay and the results showed that Chrysoeriol inhibited the invasion of the A549 cells in a dose-dependent manner (Figure 7).

Chrysoeriol blocked the MAPK/ERK signalling pathway

The effects of Chrysoeriol were also examined on the MAPK/ERK signalling pathway of A549 lung cancer cells. The experiment showed that Chrysoeriol caused dose-dependent decrease in the expression of p-p38 and p-ERK1/2, while no visible effect was found on the expression of total p38 and MAPK/ERK (Figure 8). To sum up, the results indicate that Chrysoeriol blocked the m-TOR/PI3K/AKT signalling pathway in A549 cells.

Chrysoeriol inhibited tumor growth in vivo

Since Chrysoeriol was found to exert considerable anticancer effects on lung cancer cell lines *in vitro*, we also investigated its anticancer effects *in vivo* in xenografted mice models. The results indicated that Chrysoeriol at the dosage of 50 mg/kg

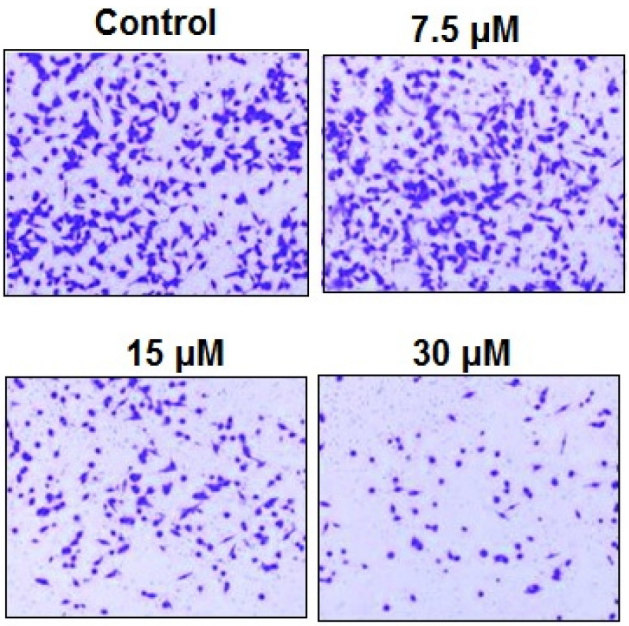

Figure 7. Effect of Chrysoeriol on the invasion of the A549 cells as determined by Boyden chamber assay. The Figure shows that Chrysoeriol inhibited the tendency for invasion. The experiments were performed in triplicate.

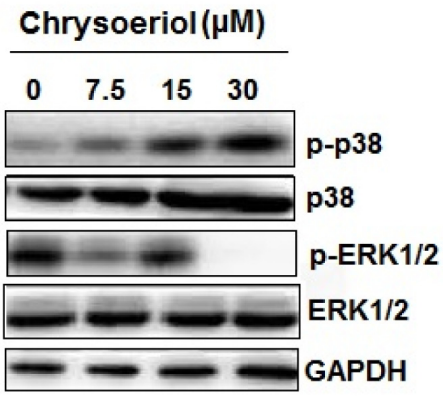

Figure 8. Effect of Chrysoeriol on the MAPK/ERK signalling pathway as depicted by western blot analysis. Chrysoeriol led to dose-dependent inhibition of MAPK-ERK signalling pathway. The experiments were performed in triplicate.

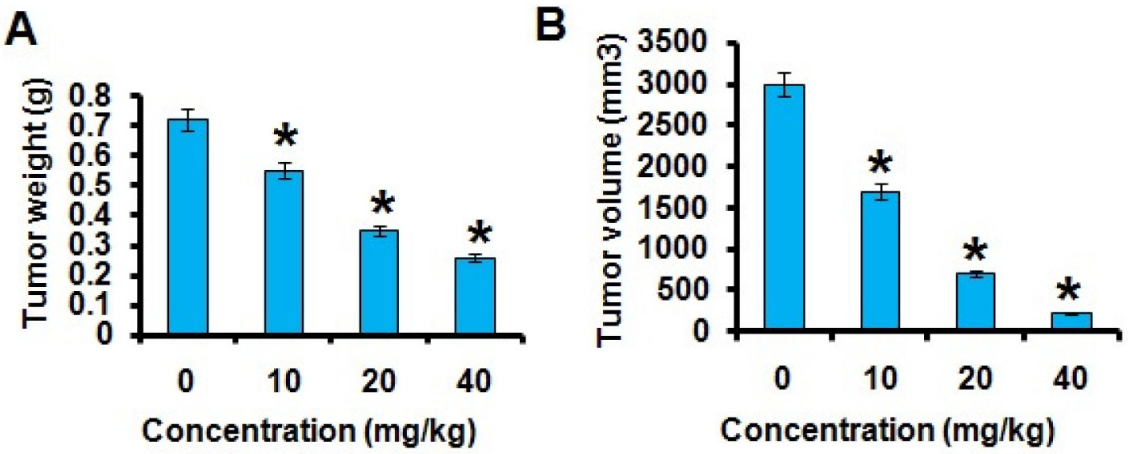

Figure 9. Effect of Chrysoeriol on xenografted tumor growth. **A:** Tumor weight and **B:** Tumor volume. The experiments were performed in triplicate and shown as mean ± SD (*p<0.01).

considerably suppressed the growth of the xenografted tumors. In addition, Chrysoeriol inhibited the tumor weight and volume concentration-dependently (Figure 9A and B).

Discussion

Lung cancer causes considerable mortality and accounts for about 25% of all cancer-related deaths [13]. Late diagnosis and the unavailability of efficient chemotherapeutic drugs form a major obstacle in the treatment of lung cancer [14]. Additionally, the chemotherapeutic agents used show a number of adverse effects, deteriorating the overall health of the patients [2]. Compounds isolated from plants have attained extraordinary attention in the recent past due to their comparatively lower toxic effects. Therefore, researchers are evaluating natural products against cancer cells to develop efficient systemic therapies for cancer in general and lung cancer in particular [3]. In this investigation, the antiproliferative effects of Chrysoeriol were investigated against A549 lung cancer cells and what was found showed that Chrysoeriol led to significant decline in the proliferation rate of lung cancer cells. In the studies carried out previously, many flavonoids have been reported to inhibit the growth of cancer cells [15].

Autophagy is a vital process leading to death of harmful cells and promoting the survival of normal cells [16]. Herein, the investigation of mechanism of action of Chrysoeriol showed that this compound induced autophagy A549 lung cancer cells. This was also associated with changes in the expression of autophagy-related protein expression. Previous studies have indicated that several of the anticancer molecules induce autophagy and apoptosis of cancer cells [17]. Furthermore, arrest of the cells at different phases of the cell cycle is an important route by which anticancer agents halt the growth of cancer cells [18]. Herein, we found that Chrysoeriol triggers sub-G1/G0 cell cycle arrest concentration-dependently. Cell migration and invasion capacity of cancer cells determines their metastatic potential [19]. In this study it was found that Chrysoeriol suppressed both migration and invasion of the A549 lung cancer cells and that the MAPK/ERK signalling pathway was activated in many cancer types and this play an important role in their proliferation [20-22].

In this study we found that Chrysoeriol could inhibit the expression of p-p38 and p-ERK1/2 in A549 cells concentration-dependently. Because of the interesting results the *in vitro* study, the antiproliferative effects of Chrysoeriol were also investigated *in vivo* which revealed that Chrysoeriol inhibited the growth of xenografted tumors, suggestive of its potential in the management of lung cancer.

Conclusion

In conclusion, Chrysoeriol suppresses the proliferation of lung cancer cells by autophagy and apoptotic cell death, along with sub-G1/G0 cell cycle arrest. In addition it also inhibited the tumor growth *in vivo*. Therefore, Chrysoeriol may prove a vital therapeutic agent and merits further investigation.

Conflict of interests

The authors declare no conflict of interests.

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