

ANTITUMOR AND APOPTOTIC EFFECTS OF CUCURBITACIN A IN A-549 LUNG CARCINOMA CELLS IS MEDIATED VIA G2/M CELL CYCLE ARREST AND M-TOR/PI3K/AKT SIGNALLING PATHWAY.

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Abstract

Background: The main aim of this study was to demonstrate the antitumor potential of cucurbitacin A on A-549 NSCLC (non-small cell lung cancer cells). The effects of Cucurbitacin A on apoptotic induction, cell physics, cell cycle failure and m-TOR/PI3K/Akt signalling pathway were also investigated in the present study.

Materials and Methods: MTT assay and clonogenic assay were carried out to study effects of this compound on cell cytotoxicity and colony forming tendency in A-549 cells. Moreover, phase and fluorescence microscopic techniques were used to examine the effects on cell morphology and induction of apoptosis. The effects on cell cycle phase distribution were investigated by flow cytometry and effects on m-TOR/PI3K/Akt signalling proteins were assessed by western blot analysis.

Results: Results showed that cucurbitacin A induced dose-dependent cytotoxic effects along with suppressing the colony forming tendency in these cells. Cucurbitacin A also induced morphological changes in these cells featuring chromatin condensation, cell shrinkage and apoptotic body formation. G2/M phase cell cycle collapse was also induced by Cucurbitacin A along with inhibition of expression levels of m-TOR/PI3K/Akt proteins.

Conclusions: In conclusion, cucurbitacin A inhibits cancer growth in A-549 NSCLC cells by inducing apoptosis, targeting m-TOR/PI3K/Akt signalling pathway and G2/M cell cycle.

Key Words: Non-small cell lung cancer, apoptosis, cucurbitacin A, cell cycle, antitumor activity

Introduction

Lung carcinoma is most common form of cancer and is regarded as one of the main causes of cancer-related mortality across the globe (Siegel et al., 2015). Consistent with this, in China most cancer-related deaths are also due to lung cancer (Ni et al., 2014). In about 35-40% of patients, lung cancer advances to the metastatic stage with brain, bone and liver (Ferlay et al., 2010; Herbst et al., 2008). Till date two main types of lung cancers have been identified: non-small cell lung cancer (NSCLC), a more prevalent accounting for about 85% of all lung cancers and small cell lung cancer (SCLC) (Siegel et al., 2015). NSCLC is also relatively resistant to chemotherapy as compared to SCLC. Prior to neoadjuvant and adjuvant chemotherapy NSCLC is primarily treated by surgical resection. Owing to the low survival (<15% patients live up to five years after diagnosis), poor prognosis and a high rate of recurrence of NSCLC makes it more deadly. For patients suffering from NSCLC, the development of resistance against chemotherapy poses another serious problem in their treatment (Doyle., 1993). However, chemotherapy still remains the primary and a main treatment for NSCLC (non-small-cell lung cancer) (Koh et al., 2012). In order to increase the survival rate and response rate, various drug combinations have been used in sequential chemotherapies (Waller et al., 2004). Cucurbitacins are chemically classified as steroids and are produced by some plant species belonging to family cucurbitaceae of angiosperms. Cucurbitacins are usually found in nature in their glycosidic form and these compounds act as defence against herbivores (Chen et al., 2005). It has been reported that many of the cucurbitacins exhibit antitumor activity against a range of cancer cells and *in vivo* tumor models notably lung carcinomas, ovarian cancer cells and nasopharyngeal carcinoma cells (Kapoor., 2013; Ishii et al., 2013; Lui et al., 2009). However, antitumor activity of cucurbitacin A against NSCLC cells (A-549) has not reported so far. Therefore, the objective of the present study was to investigate the apoptotic effects and antitumor activity of cucurbitacin A against

A-549 NSCLC cells along with evaluation of its effects on cell cycle arrest, mitochondrial membrane potential loss and m-TOR/PI3K/Akt signalling pathway.

Materials and methods

Chemicals and other reagents

Cucurbitacin A (>95% purity by HPLC), MTT (3-(4, 5-dimethyl-2-thiazolyl) 2, 5-diphenyl-2H tetrazolium bromide) were possessed from Sigma Aldrich (St. Louis, MO, USA). RPMI-1640 medium, Hoechst and 33258 DMEM (Dulbecco's modified Eagle's medium) were purchased from Wuhan Boster Biological Technology Ltd. (Wuhan, China). Streptomycin, penicillin and Fetal bovine serum (FBS) were purchased from Tianjin HaoYang Biological Manufacture Co., Ltd. (Tianjin, China).

Cell line and culture conditions

A-549 human NSCLC cell line was procured from Cancer Research Institute of Beijing, China, and it was maintained in DMEM (Dulbecco's modified Eagle's medium) and was supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin) in a humidified incubator at 37°C containing 5% CO₂ and 95% air.

MTT assay for evaluating cell proliferation

The anti-proliferation effect of cucurbitacin A on A-549 cells was determined by MTT assay. A-549 cells were grown at 1×10^6 cells per well in 96-well plates for a time period of 12 h and then exposed to 0, 10, 20, 40, 100, 150 and 200 µM cucurbitacin A dose for 24 and 48 h. To each well, MTT solution (20 µl) was added. Prior to the addition of 500 µl of DMSO, the medium was completely removed. To solubilize MTT formazan crystals, 500 µl DMSO was added. ELISA plate reader (Model 550; Bio-Rad, Hercules, CA, USA) was used for the determination of optical density.

Clonogenic assay

For clonogenic assay, A-549 cells at the exponential growth phase were harvested and counted with a hemocytometer. Seeding of the cells was done at 200 cells per well and followed by incubation for a time period of 48 h to allow the cells to settle. Afterwards, different doses (0, 40, 100 and 200 µM) of cucurbitacin A were added to the cell cultures. After treatment, the cells were again kept for incubation for 6 days, washing was done with PBS, methanol was used to fix colonies and then stained with crystal violet for 30 min before being counted under light microscope.

Fluorescence microscopy using Hoechst 33258

The Human NSCLC cells (A-549) were treated with several concentrations (0, 40, 100 and 200 µM) of cucurbitacin A and then these cells were kept in a CO₂ incubator for 48 h at 37 °C. After incubation, the cells were fixed with 2.5 % formaldehyde for 40 min and washed with PBS twice. The solution of Hoechst 33342 was added to the cells and after 20 min of staining, fluorescence microscope at 100x magnification was used to observe the cells (Nikon, Tokyo, Japan).

Phase contrast microscopy

A-549 human non-small cell lung cancer cells were grown in six well plates at 2×10^6 cells/ml and then maintained at favorable conditions for 24 h. Afterwards, the cells were processed with several doses of cucurbitacin A (0, 40, 100 and 200 µM) for 48 h. Inverted light microscope (Nikon Corp., Tokyo, Japan) was used to examine cultural plates following drug treatment and images were captured. DMSO was used as a control.

Cell cycle analysis by flow cytometry

The effect of cucurbitacin A on the phase distribution in cell cycle was assessed by flow cytometry with propidium iodide. Briefly, A-549 cancer cells at 1×10^5 cells per ml were treated with different doses (0, 40, 100 and 200 µM) of cucurbitacin A. After treatment, the cells were harvested, fixed with 70% ice-cold ethanol for 24 h and treated with 30 µg/ml RNase A (Sigma-Aldrich, St. Louis, MO, USA). Propidium iodide (10 µg/ml) was used for staining and then analyzed by flow cytometer (FACS Calibur; BD Biosciences).

Western blot assay

The effect of cucurbitacin A on the protein expressions of PI3K/Akt/m-TOR was examined by western blot assay. Briefly, the A-549 human NSCLC cells were plated in 100 mm culture dish before drug treatment. Thereafter the cells were allowed to fix for 24 h and processed with medium (control) or with different doses (0, 40, 100, 200) of cucurbitacin A for 48 h. Finally, the cells were lysed and protein concentrations were determined by bichinonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Each sample (200 μ g) was loaded on 8–10% SDS-polyacrylamide gels and stained with antibodies probed against p-Akt, Akt, m-TOR and glyceraldehyde phosphate dehydrogenase (GAPDH) overnight at 4 °C and followed by exposure to horseradish peroxidase-conjugated goat anti-mouse antibodies. Finally, blots were developed from West Pico Chemiluminescent substrate (Pierce; Woburn, MA, USA).

Statistical analysis

All experiments were carried out in triplicates and expressed in the form of mean \pm standard error (S.E.). One-way ANOVA was used for analyzing the differences between groups, differences were considered statistically significant at * $p < 0.05$, ** $p < 0.01$.

Results**Cucurbitacin A inhibited cancer cell proliferation in A-549 NSCLC cells**

The antiproliferative effects of cucurbitacin A (Figure 1) in A-549 cells were determined by using MTT assay. MTT assay results are shown in Figure 2 and indicate that cucurbitacin A exhibits a potent cytotoxic effect in these cells. The assay was carried out at different concentrations of the compound (0, 10, 20, 40, 100, 150 and 200 μ M) and incubated for 24 h and 48 h. It was observed that cucurbitacin A showed inhibitory effects on the cell proliferation in a dose and time dependent manner. However, the effect of incubation time was more pronounced at higher doses of the compound.

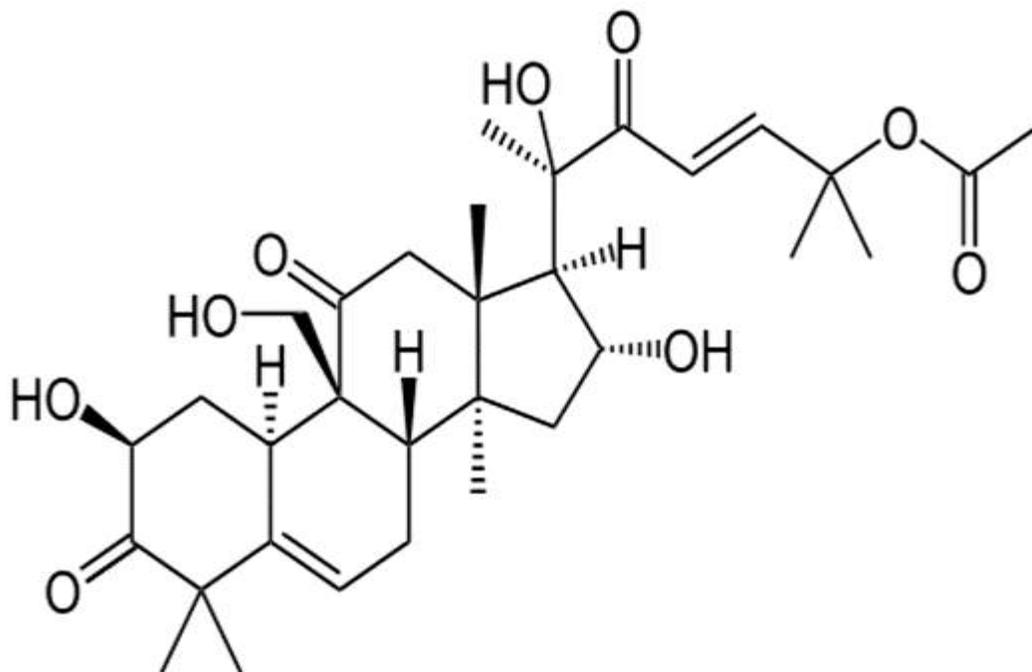


Figure 1: Cucurbitacin A: Chemical structure.

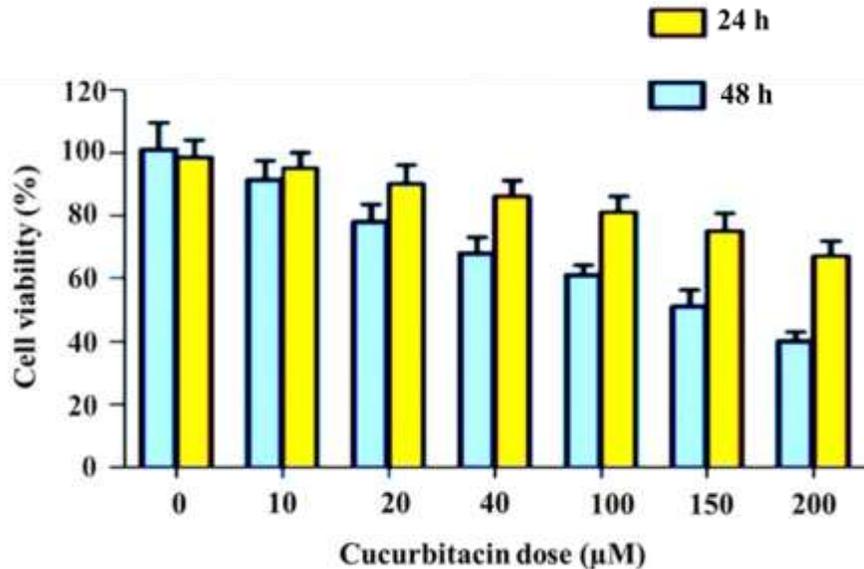


Figure 2: Evaluation of cell cytotoxicity induced by cucurbitacin B in A-549 human lung cancer cells by MTT assay. The cells were processed with 0, 10, 20, 40, 100, 150 and 200 µM dose of the drug and then incubated for 24 and 48 time intervals. The data of all the three independent experiments are shown as the mean ± SD. *, P < 0.05, **, P < 0.01, vs 0 µM (control).

Cucurbitacin A inhibits colony formation efficacy of A-549 NSCLC cells

Clonogenic assay was carried out in order to examine the effect of cucurbitacin on the colony formation tendency in A-549 NSCLC cells. The results shown in Fig.3 A-D indicate that cucurbitacin A was able to suppress the capability of these cancer cells to form colonies. In comparison to the untreated control cells, cucurbitacin A-treated cells exhibited its effect in a dose-dependent manner and significantly suppressed the number of cancer cell colonies. These results suggest that cucurbitacin A has not only anchorage-dependent but also anchorage-independent growth inhibitory effects in A-549 human NSCLC cells (non-small cell lung cancer cells).

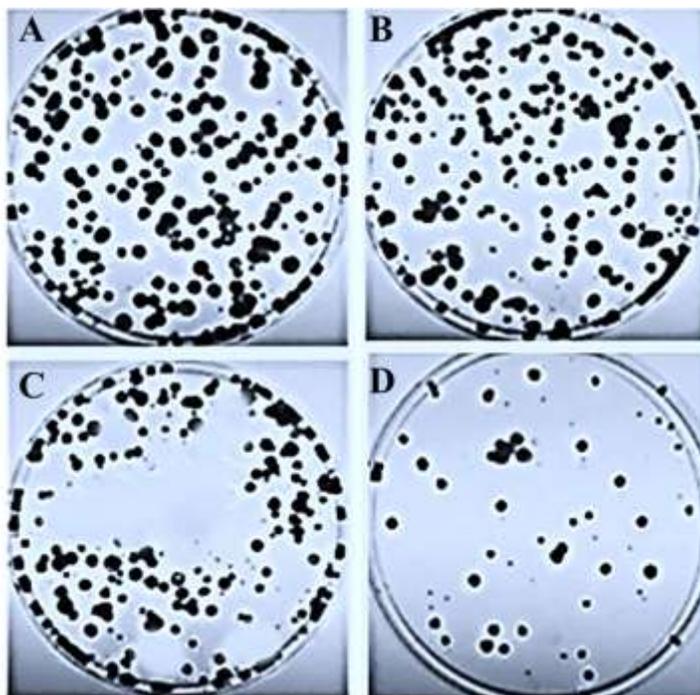


Figure 3: Cucurbitacin A decreases the potential of colony formation of A-549 human lung cancer cells. These cells were treated with 0 (A), 40 (B), 100 (C) and 200 (D) μM dose of cucurbitacin A for 48 h and then analyzed using a light microscope.

Cucurbitacin A induces apoptosis in A-549 NSCLS cells

The effects of cucurbitacin A on the cellular and nuclear morphology in A-549 NSCLC cells (non-small cell lung cancer cells) was evaluated by fluorescence microscopy using hoechst 33258 as a staining dye. The results indicated that in comparison to the untreated control cells showing normal morphology with no signs of apoptosis (Fig.4 A), the cucurbitacin A-treated cells exhibited clear and visible changes in cell morphology including nuclear condensation, membrane blebbing and apoptotic body formation (Fig.4 B-D). These morphological changes are characteristic of the process of apoptosis. The dose of cucurbitacin A was positively correlated to the appearance of these apoptotic bodies.

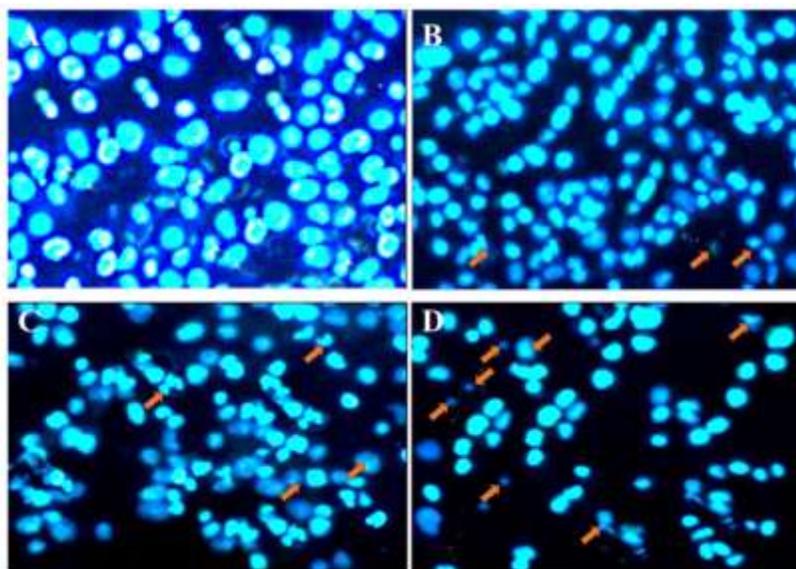


Figure 4: Effects of cucurbitacin A on the cellular morphology of A-549 human lung cancer cells (NSCLC). The cells were treated with 0 (A), 40 (B), 100 (C) and 200 (D) μM (unreacted control cells) dose of cucurbitacin A for 48 h and then stained with hoechst 33258. Fluorescence microscopy was then used to visualize the changes in cellular and nuclear morphology and apoptotic body formation. Arrows indicate apoptotic cells.

Effect of cucurbitacin A on the cellular morphology of A-549 human NSCLC cells

Phase contrast microscopy was used to observe the effect of cucurbitacin A on the cell physic or morphology in A-549 NSCLC cells. Results of this assay are given in Fig. 5 A-D and indicate that increasing doses of cucurbitacin A result in detachment of cells from each other leading to the reduction in the number of cell clusters suspended in the medium. The cell developed a rough shape rough and were unable to stabilize their undamaged membranes. However, control untreated cells showed normal cellular morphology and were attached together to form firm clusters.

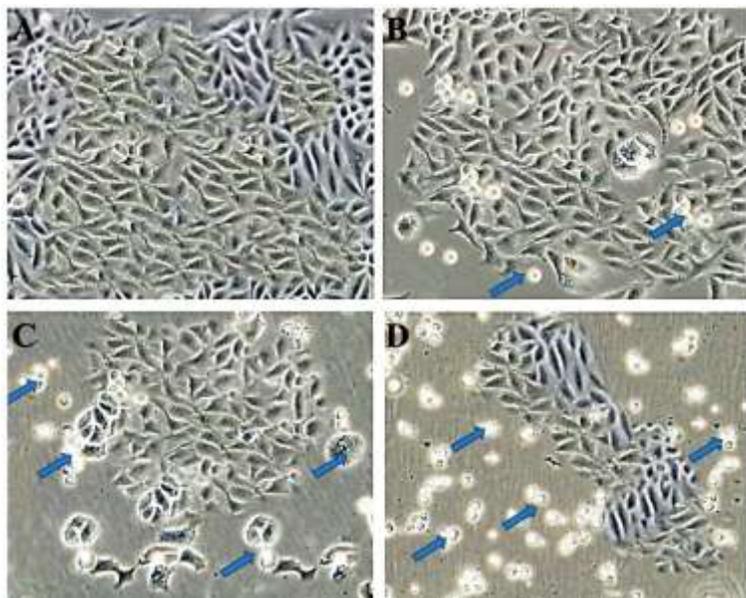


Figure 5: Impact of cucurbitacin A on the cell physic or morphology of A-549 NSCLC cells. Physical (Morphological) changes were examined under phase-contrast microscope after the cells were treated with 0 (A), 40 (B), 100 (C) and 200 (D) μ M of cucurbitacin A. Arrows indicate changes in cell morphology.

Cucurbitacin A induces G2/M phase cell cycle arrest

To assess the effect of cucurbitacin A on the phase distribution of cell cycle, A-549 NSCLC cells were examined by flow cytometry using propidium iodide as the probe. It was observed that in untreated control cells the G2/M phase of the cell cycle was represented by 7.2% cells. However cells administrated with varied concentrates of cucurbitacin A (40, 100 and 200 μ M) 14.4%, 33.5% and 64.2% cells were arrested in the G2/M phase of the cell cycle (Fig.6). Thus cucurbitacin A led to G2/M phase cell cycle arrest in a dose dependent dose-dependent manner.

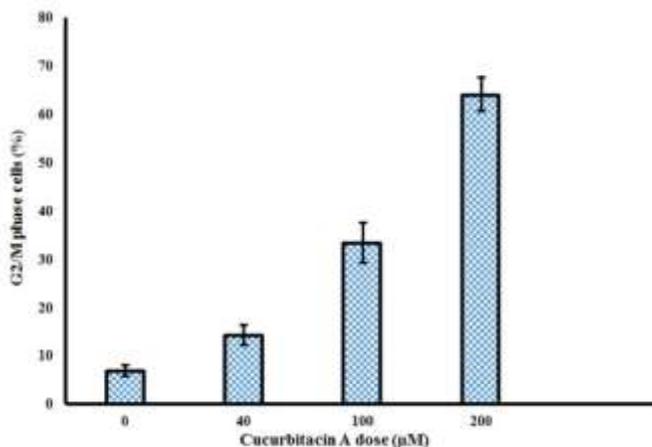


Figure 6: Cucurbitacin A induces G2/M cell cycle arrest in A-549 human non-small cell lung cancer cells. The cells were reacted with 0, 40, 100 and 200 μ M dose of cucurbitacin A and then analyzed by flow cytometry. There was a significant increase in the number of G2/M phase cells as the dose of cucurbitacin A increased.

Cucurbitacin A led to downregulation of m-TOR/PI3K/Akt signalling pathway

The effects of cucurbitacin on the expression levels of m-TOR/PI3K/Akt signalling proteins are shown in Fig.7. The results indicate that cucurbitacin A led to significant and dose-dependent downregulation of m-TOR/PI3K/Akt proteins. As compared to the untreated control which had highest expression levels of these m-TOR, PI3K and Akt proteins, cucurbitacin A-treated groups showed considerable downregulation of all these proteins.

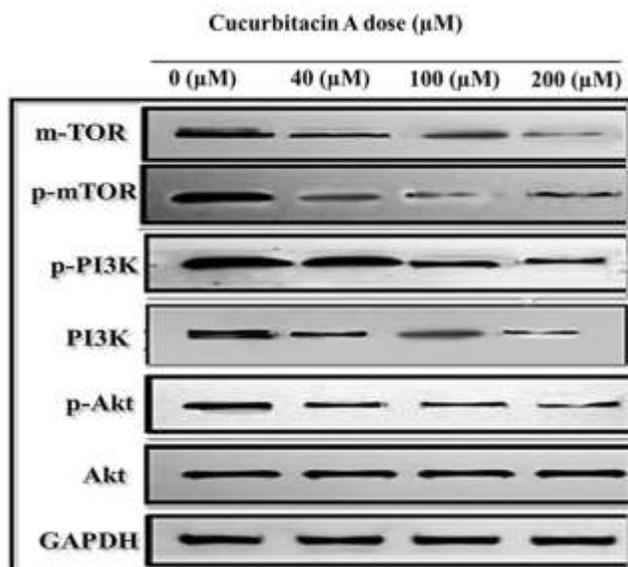


Figure 7: Effect of cucurbitacin A on the m-TOR, PI3K/ Akt pathways in human A-549 NSCLC cells (non-small cell lung). Western blot analysis was used evaluate the expressions of these proteins. GAPDH- served as a control.

Discussion

Despite the recent advancements in cancer therapies, the resistance and the lack of effective treatments for different types of lung cancer, especially NSCLC, has led to copious research endeavours to investigate new anti-cancer drugs from natural sources. The family *Cucurbitaceae* has been a treasured target in this field, demonstrating a wide array of in vitro and in vivo pharmacological activities, including anti-tumor activity (Chen et al., 2012, Rios et al., 2012). In the current study, effects of cucurbitacin A on antitumor and apoptotic effects on A-549 human NSCLC cells were investigated. Further, the effects on cell cycle and m-TOR/PI3K/Akt signalling pathways were also investigated. MTT and clonogenic assays revealed that cucurbitacin A induced cytotoxicity and inhibited colony formation tendency of these A-549 lung cancer cells respectively. These results indicate that cucurbitacin A caused growth inhibited the growth of the cells in time and concentration dependent on anchorage. Phase contrast microscopy and fluorescence microscopy techniques revealed that cucurbitacin A induced morphological changes in these cells, characteristic of apoptosis process. Flow cytometry using propidium iodide indicated that cucurbitacin A led to a G2/M phase cell cycle arrest in these cells in a dose-dependent nature. The increasing knowledge of cell signaling has led to strategies to target the signaling pathways, which are altered in tumor cells (Neuzillet et al., 2014). In the present study, western blot assay interpreted that the anticancer effects of cucurbitacin A are actually mediated via the downregulation of key signalling proteins including m-TOR/PI3K/Akt.

The chemotherapeutic or chemopreventive compounds from plants are effective alternative sources of anticancer drugs. Several bioactive natural product based compounds inhibit cancer cell growth by targeting the cell cycle, which is in turn regulated and controlled by a series of cell cycle regulators and check points (Pietenpol & Stewart., 2002). Phosphoinositide 3-kinase has been observed to play vital roles in various cellular processes like cell differentiation, cell proliferation and cell survival. Most of the compounds which inhibit PI3K/Akt pathway are promising anticancer agents. Our results indicate that cucurbitacin A could inhibit m-TOR/PI3K/Akt signalling pathway in a dose-dependent manner. Thus, provided cucurbitacin A is further evaluated for *in vivo* studies, it can be a promising anticancer drug (Engelman et al., 2006; Bader et al., 2005; Vivanco et al., 2002; Hennessy et al., 2005).

Conclusion

In summary, the current results reveal that cucurbitacin A exhibits potent antitumor effects in human A-549 NSCLC (non-small cell lung cancer cells) and these antitumor effects are mediated via apoptosis induction, downregulation of m-TOR/PI3K/Akt signalling pathway and G2/M phase cell cycle arrest.

Conflict of interest: The authors declare no conflict of interest

References

1. Bader, A.G., Kang, S., Zhao, L., Vogt, P.K. (2005). Oncogenic PI3K deregulates transcription and translation. *Nat. Rev. Cancer*, 5:921–9.
2. Chen X, Bao J, Guo J, Ding Q, Lu J, Huang M, Wang Y (2012) Biological activities and potential molecular targets of cucurbitacins: a focus on cancer. *Anti-Cancer Drugs* 23(8):777–787.
3. Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A. and Qiu, S.X. (2005). Cucurbitacins and cucurbitane glycosides: structures and biological activities. *Nat. Prod. Rep.*, 22: 386-399.
4. Doyle, L.A. (1993). Mechanisms of drug resistance in human lung cancer cells. *Semin. Oncol.*, 20:326-37.
5. Engelman, J.A., Luo, J. and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.*, 7:606–19.
6. Ferlay, J., Shin, H.R., Bray, F. and Forman, D., Mathers, C. and Parkin, D.M. (2010). GLOBOCAN 2008, Cancer incidence and mortality worldwide: IARC Cancer Base No. 10. Lyon, France: Int. Agency Res. Cancer. <http://globocan.iarc.fr>.
7. Hennessy, B.T., Smith, D.L., Ram, P.T., Lu, Y. and Mills, G.B. (2005). Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.*, 4:988–1004.
8. Herbst, R.S., Heymach, J.V. and Lippman, S.M. (2008). Lung cancer. *N. Engl. J. Med.*, 359: 1367-1380.
9. Ishii, T., Kira, N., Yoshida, T. and Narahara, H. (2013). Cucurbitacin D induces growth inhibition, cell cycle arrest, and apoptosis in human endometrial and ovarian cancer cells. *Tumour Biol.*, 34: 285–91.
10. Kapoor, S. (2013). Cucurbitacin B and Its Rapidly Emerging Role in the Management of Systemic Malignancies Besides Lung Carcinomas. *Cancer Biother. Radiopharm.*, 28: 359.
11. Koh, P.K, Faivre-Finn, C., Blackhall F.H., De Ruyscher D. (2012) Targeted agents in non-small cell lung cancer (NSCLC): clinical developments and rationale for the combination with thoracic radiotherapy. *Cancer Treat Rev* 38(6):626–640.
12. Lui, V.W., Yau, D.M., Wong, E.Y., Ng, Y.K., Lau, C.P., Ho, Y., Chan, J.P., Hong, B., Ho, K., Cheung, C.S., Tsang, C.M., Tsao, S.W. and Chan, A.T. (2009). Cucurbitacin I elicits anoikis sensitization, inhibits cellular invasion and in vivo tumor formation ability of nasopharyngeal carcinoma cells. *Carcinogenesis*, 30: 2085–94.
13. Neuzillet C, Tijeras-Raballand A, de Mestier L, Cros J, Faivre S, Raymond E (2014) MEK in cancer and cancer therapy. *Pharmacol Ther* 141(2):160–171.
14. Ni C.H., Yu C.S., Lu, H.F., Yang J.S., Huang H.Y., Chen, P.Y., Wu, S.H., Ip, Sw., Chiang, Y.S., Lin, J.G., Chung, J.G., 2014. Chrysophanol-induced cell death (necrosis) in human lung cancer A549 cells is mediated through increasing reactive oxygen species and decreasing the level of mitochondrial membrane potential. *Environ Toxicol.* 29(7):740-9.
15. Pietenpol, J.A. and Stewart, Z.A. (2002). Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. *Toxicology*, 181–182:475–481.
16. Rios JL, Andujar I, Escandell JM, Giner RM, Recio MC (2012) Cucurbitacins as inducers of cell death and a rich source of potential anticancer compounds. *Curr Pharm Des* 18(12):1663–1676.
17. Siegel, R.L, Miller, K.D., Jemal, A. (2015) Cancer statistics, 2015. *CA Cancer J Clin* 65(1):5–29.
18. Vivanco, I., Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer*, 2:489–501.
19. Waller, D., Peake, M.D., Stephens, R.J., Gower, N.H., Milroy, R., Parmar, M.K., Rudd, R. M. and Spiro S.G. (2004). Chemotherapy for patients with non-small cell lung cancer: the surgical setting of the Big Lung Trial. *Eur. J. Cardiothorac. Surg.*, 26:173-82.