JBUON 2019; 24(3): 997-1002
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Scopoletin exerts anticancer effects on human cervical cancer cell lines by triggering apoptosis, cell cycle arrest, inhibition of cell invasion and PI3K/AKT signalling pathway

Qi Tian , Luying Wang , Xin Sun , Fei Zeng , Qiong Pan , Min Xue

Department of Obstetrics and Gynecology, The Third Xiangya Hospital of Central South University, Changsha, Hunan, 410013, China.

Summary

Purpose: *Cervical cancer causes considerable mortality in women world over and the current treatment options create severe adverse effects. Hence, there is an urgent need to develop novel and efficient treatment regimens for cervical cancer. Herein, we examined the anticancer effects of a natural coumarin, Scopoletin, against a panel of cervical cancer cell lines.*

Methods: *The antiproliferative effect of Scopoletin was examined by cell counting and colony formation assays. Apoptosis was detected by acridine orange (AO) ethidium promide (EB) staining. Cell cycle distribution was determined by flow cytometry. Cell invasion was examined by Boyden chamber assay. Protein expression was checked by western blotting.*

Results: *Scopoletin inhibited the growth of all the cell lines and the IC_{50} ranged between 7.5 to 25 μM. Nonetheless, the cytotoxic effects of Scopoletin were comparatively negligible against the normal cells with an IC_{50} of 90 μM. Investigation of the mechanism of action, revealed that the anticancer effects of Scopoletin against the HeLa cervical cancer cells were due to induction of apoptotic cell death as indicated AO/EB staining. Scopoletin treatment also resulted in enhancement of the Bax, Caspase 3, 8 and 9 expression and decline of the Bcl-2 expression. Scopoletin also blocked the HeLa cells at G2/M checkpoint of the cell cycle. Furthermore, cell invasion assay revealed that Scopoletin inhibited the migration of the HeLa cells concentration-dependently. PI3K/AKT is an imperative pathway involved in theproliferation and tumorigenesis of cancer cells and herein it was found that Scopoletin could inhibit this pathway.*

Conclusion: *Taken together, Scopoletin may prove essential in the development of novel treatment regimes for cervical cancer.*

Key words: *cervical cancer, scopoletin, apoptosis, cell cycle arrest, invasion*

Introduction

Cervical cancer, being one of the common types of cancers in women, ranks second in incidence of cancer world over [1]. Although, cervical cancer is more frequent in underdeveloped countries, it still accounts for 10% of all the cancer in women [2,3]. The treatment of cervical cancer involves radical hysterectomy, chemotherapy and/or radiotherapy [4]. However, the currently available chemotherapeutic agents have adverse effects that negatively impact the patient quality of life. Hence, the identification of novel, effective and safer anticancer agents is required to curb the spread of cervical cancer. Nature has bestowed mankind with an wide array of chemical compounds. These astounding chemical entities stand as an outstanding source of drugs for the management of human diseases [5]. Although usage of herbal extracts dates back to centuries, the usage of pure isolated compounds started only in the 19th century [6]. Since then, a wide array of molecules have been isolated, evalu-

Corresponding author: Min Xue, MD. Department of Obstetrics and Gynecology, the Third Xiangya Hospital of Central South University, 138 Tong Zi Po Rd, Changsha, 410013, Hunan, China.
Tel & Fax: +86 073188618576, Email: MMadalynywa@yahoo.com
Received: 12/09/2018; Accepted: 01/10/2018

ated and used for the treatment of several diseases and disorders [7]. Among plant derived products, coumarins constitute a large and widespread group of secondary metabolites [8]. They have been shown to exhibit a wide array of bioactivities such as anticancer activity [9]. Scopoletin is an important coumarin that is commonly extracted from the plants belonging to *Genus Scopolia* [10]. Although Scopoletin has been shown to inhibit the growth of cancer cells [11], its anticancer effects have not been studied against cervical cancer cells. Herein, the anticancer effects of Scopoletin were examined against a panel of cervical cancer cell lines and an attempt was made to investigate its mechanism of action.

Methods

Cell lines and culture conditions

The normal cell line HCvEpC and the cervical cancer cell lines DoTc2, SiHa, HeLa and C33A were procured from American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Inc., Waltham, MA, USA), antibiotics (100 Uml penicillin and 100 µg/ml streptomycin), and 2 mM glutamine. The cells were cultured in a CO_2 incubator (Thermo Fisher Scientific, Inc.) at 37°C with 98% humidity and 5% CO_2.

Cell viability and colony formation assays

The viability of the normal cell line HCvEpC and the cervical cancer cell lines DoTc2, SiHa, HeLa and C33A was examined by cell counting assay. In brief, 5×10^4 cells/well were seeded in 12-well plates and incubated for 24 h with different concentrations of Scopoletin. The aliquots of cells were then removed and counted 3 times following Tryphan blue staining. The effect of Scopoletin on the formation of HeLa colonies was investigated as described earlier [12].

AO/EB staining and comet assay for apoptosis

For AO/EB staining, the cervical cancer HeLa cells (0.6×10^6) were grown in 96-well plates. Following an incubation of around 12h, the HeLa cells were subjected to Scopoletin treatment for 24 h at 37°C. As the cells sloughed off, 25 µl of cell culture were put onto glass slides and subjected to staining with 1 µl of AO and EB. The slides were cover-slipped and examined with fluorescent microscope. Comet assay was performed as described previously [13].

Cell cycle analysis

The HeLa cells were incubated with varied concentrations Scopoletin (0, 7.5, 15 and 30 µM) for 24 h. The cells were then subjected to washing with phosphate buffered saline (PBS) and stained with propidium iodide (PI). The distribution of the cells in the cell cycle phases was assessed by FACS flow cytometer.

Table 1. Anticancer effects of Scopoletin on the cervical cancer and normal cell lines expressed as IC_{50}

S. no.	*Cell line*	*IC_{50}*
1	DoTc2	25
2	SiHa	15
3	HeLa	7.5
4	C33A	25
5	HCvEpC	90

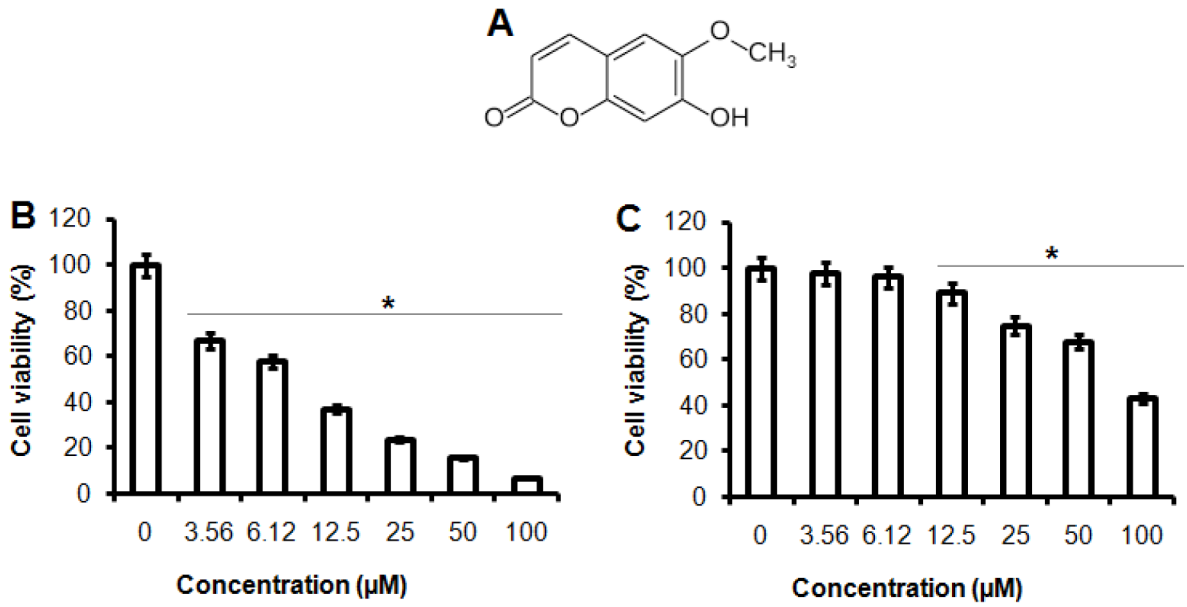

Figure 1. A: Structure of Scopoletin. **B:** Scopoletin decreases the viability of the HeLa cervical cancer cells. **C:** normal HCvEpC cervical cells. The values represent the mean of three independent experiments ± SD (*p< 0.05).

Cell invasion assay

For cell invasion analysis, the HeLa cells were subjected to treatment with 0, 7.5, 15 and 30 µM concentrations of Scopoletin. The cell invasion assay was then performed as described previously [14].

Western blotting

The HeLa cells were firstly subjected to washing with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About, 40 µg of protein was loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris buffered saline (TBS) and exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

Data are shown as mean ± standard error of the mean (SEM). Statistical analysis was done using Student's *t*-test with GraphPad prism 7 software. P values <0.05 were taken as indicative of significant difference.

Results

Scopoletin suppressed the growth of cervical cancer cells

The anti-proliferative effects of Scopoletin (Figure 1A) were assessed on the normal cell line HCvEpC and the cervical cancer cell lines DoTc2, SiHa, HeLa and C33A. Scopoletin treatment caused dose-dependent inhibition on the growth of all the cervical cancer cell lines. The IC_{50} of Scopoletin against the different cervical cancer cell lines ranged from 7.5 µM to 25 µM (Table 1). The lowest IC_{50} of 7.5 µM was reported against the HeLa cells (Figure 1B). Nonetheless, the cytotoxicity of Scopoletin on the HCvEpC non-cancer cells was comparatively negligible (IC_{50}; 90 µM) (Figure 1C).

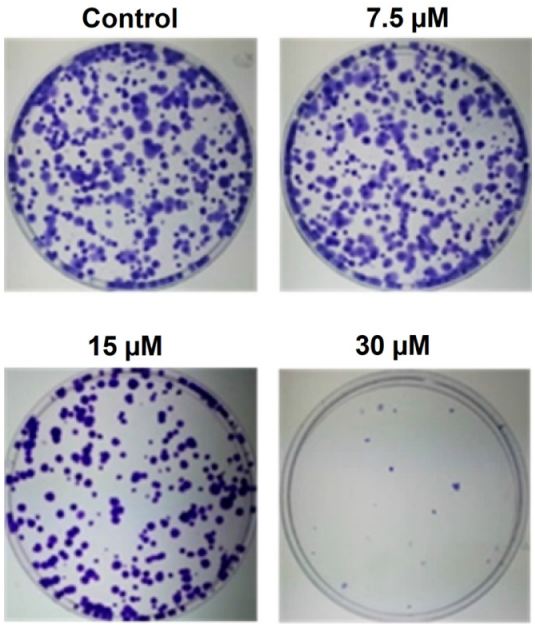

Figure 2. Scopoletin affects the colony formation of the HeLa cervical cancer cells. The Figure shows that Scopoletin inhibits the colony formation of HeLa cells concentration-dependently. The experiments were performed in triplicate.

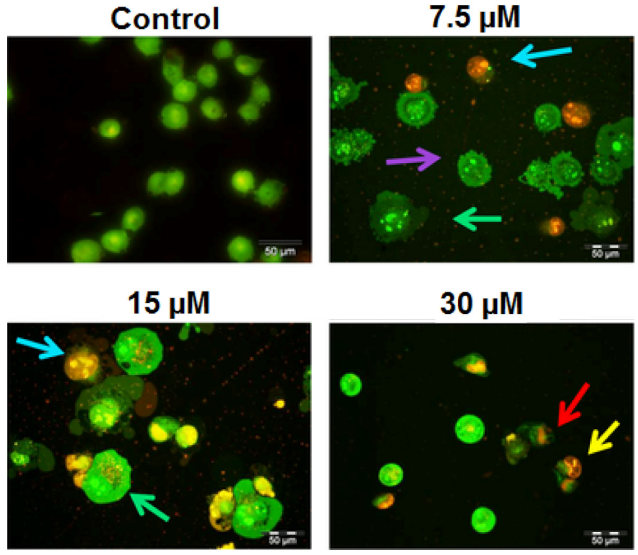

Figure 3. Scopoletin induces apoptotic cell death in HeLa cervical cancer cells as depicted by AO/EB staining. Green arrows indicate early apoptotic cells, blue arrows depict late apoptotic cells, purple arrows depict membrane blebbing, yellow arrows depict cells which appear shrunken and red arrows depict loss of membrane shape. The experiments were performed in triplicate.

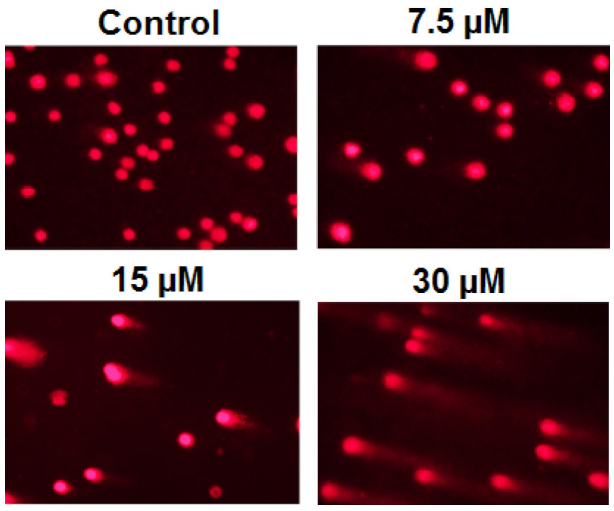

Figure 4. Scopoletin causes DNA damage in the HeLa cervical cancer cells as depicted by Comet assay. The Figure shows that Scopoletin induces DNA damage in the HeLa cells concentration-dependently. The experiments were performed in triplicate.

The effects of Scopoletin were also assessed on the colony formation potential of HeLa cells and it was revealed that this molecule exerted dose-dependent inhibitory effects (Figure 2).

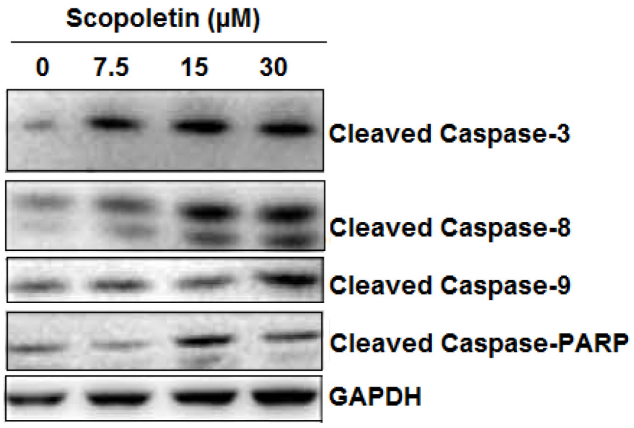

Figure 5. Scopoletin alters the expression of the cleaved caspase 3, 8, 9 and PARP expression in HeLa cancer cells as depicted by western blotting. The Figure shows that Scopoletin increases the cleavage of the caspase-3, 8, 9 and PARP in a dose-dependent manner. The experiments were performed in triplicate.

Scopoletin activated apoptotic cell death of cervical cancer cells

Since MTT assay showed more potent effects of Scopoletin on the HeLa cells, only this cell line was taken forward for further experimentation. To investigate the mechanism of the anticancer effects of Scopoletin, AO/EB staining was performed and remarkable changes were observed in the nuclear morphology and membrane blebbing of HeLa cells (Figure 3). Comet assay also showed that Scopoletin induced DNA damage in HeLa cells (Figure 4). Scopoletin also caused considerable increase in the expression of Caspase 3, 8, 9 and PARP and also activate their cleavage in HeLa cells (Figure 5).

Scopoletin caused the G2/M cell cycle arrest of cervical cancer cells

The impact of Scopoletin on the distribution of HeLa cells in various cell cycle phases was assessed by flow cytometry. It was found that Scopoletin caused remarkable increase in the percentage of the HeLa cells in the G2 phase of the cell cycle. The percentage of HeLa cells in the G2 phase

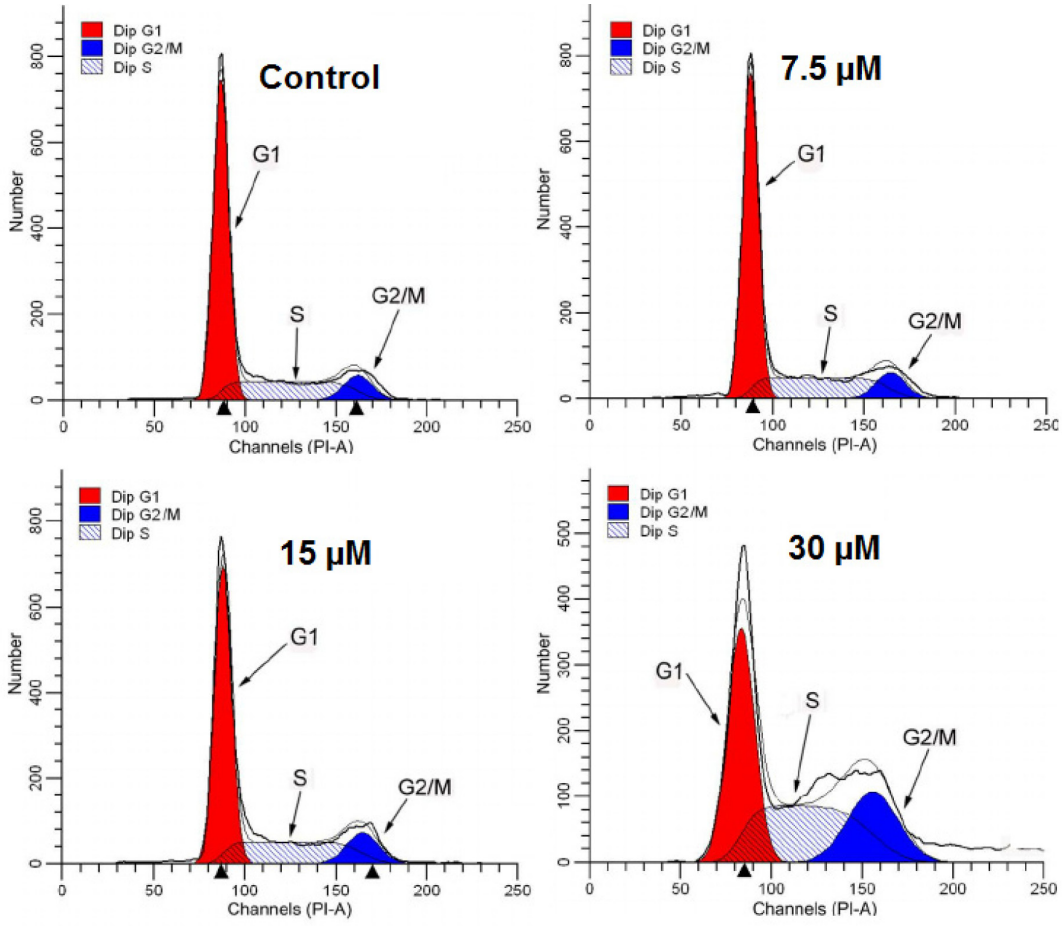

Figure 6. Effect of Scopoletin on the distribution of the cell cycle arrest of the HeLa cells as depicted by flow cytometry. The Figure shows that Scopoletin induces G2/M arrest of the HeLa cells in a concentration-dependent manner. The experiments were performed in triplicate.

increased from 5.12% to 28.17% upon treatment with Scopoletin (Figure 6). These results clearly indicated that Scopoletin induced G2/M cell cycle arrest of the cervical cancer cells.

Scopoletin inhibited the migration of cervical cancer cells

The anti-metastatic effects of Scopoletin were investigated by cell invasion assay. It was found that Scopoletin could significantly inhibit the invasion of the cancer cells in a dose-dependent manner (Figure 7).

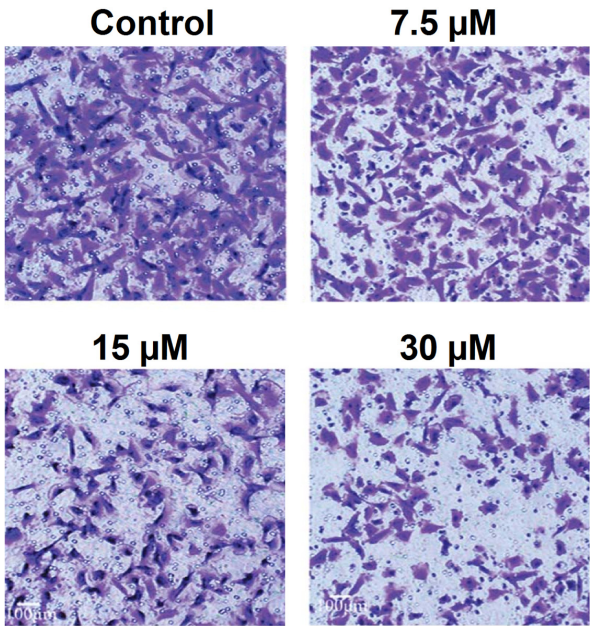

Figure 7. Scopoletin inhibits the invasion of the HeLa cancer cells as depicted by cell invasion assay. The Figure shows that Scopoletin inhibits the invasion of the HeLa cells in a concentration-dependent manner. The experiments were performed in triplicate.

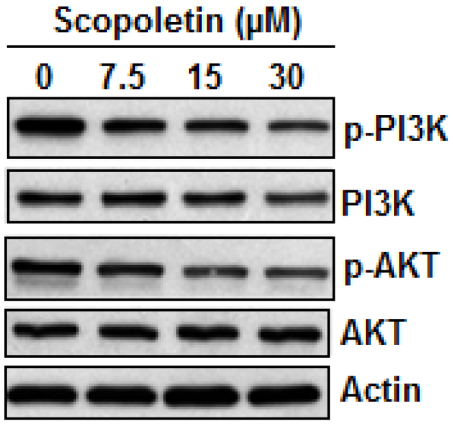

Figure 8. Scopoletin inhibits PI3K/AKT signalling pathways as shown by western blot analysis. The Figure shows that Scopoletin inhibits the phosphorylation of PI3K and AKT in a concentration-dependent manner. The experiments were performed in triplicate.

Scopoletin inhibited the PI3K/AKT signalling pathway

Next, we sought to know the effects of Scopoletin on the PI3K/AKT signalling pathway of HeLa cancer cells. The results showed that Scopoletin caused concentration-dependent decline in the phosphorylation of p-PI3K and p-AKT while no apparent effect was observed on the expression of total PI3K and AKT (Figure 8).

Discussion

Cervical cancer is considered as the second most common type of cancer in females across the world [15]. Since the clinical outcomes are far from satisfactory and the treatment options have a number of side effects, the identification of novel anticancer molecules and subsequent development of efficient and safer treatment regimens for clinical cancer are required [1-3]. In this study, we examined the anticancer properties of Scopoletin. It was found that Scopoletin exerted growth inhibitory effects on all the cervical cancer cell lines. Nonetheless, the cytotoxic effects of Scopoletin were comparatively lower against the normal HCvEpC cells, indicative of the selective activity of Scopoletin on cancer cells. The antiproliferative effects of Scopoletin were also confirmed by the colony formation assay. Previous studies have also shown that Scopoletin inhibits the growth of melanoma cells and prostate cancer cells [16,17]. In order to understand the mechanism for the anticancer activity of Scopoletin, we performed the AO/EB staining which clearly showed membrane blebbing and induction of apoptotic cell death. The apoptotic cell death of the HeLa cervical cancer cells was confirmed by examining the expression of apoptosis marker proteins. It was found that Scopoletin treatment prompted increase in the expression of Bax and cleavage of caspase 3, 8 and 9. Moreover, the expression of Bcl-2 was considerably downregulated. Furthermore, comet assay also showed that Scopoletin treatment causes DNA damage in the HeLa cells. These results are also supported by previous investigations wherein Scopoletin has been shown to induce apoptosis in melanoma and promyeloleukemic cells [16,18]. Also Scopoletin derivatives have been reported induce DNA damage in hepatocellular carcinoma [19]. Apoptosis is a vital process that removes the defective cells from the body and maintains tissue homeostasis. It also prevents the development of chemoresistance in cancer cells [20]. Scopoletin was also found to block the HeLa cells at the G2/M check point. Previously, several plant-derived coumarins have been shown to cause cell cycle arrest of cancer cells. For exam-

ple, 7-hydroxy-coumarin causes cell cycle arrest of lung cancer cells [21]. Next, the anti-metastatic potential of Scopoletin was examined by the cell invasion assays and the results showed that this molecule inhibits the migration of HeLa cells concentration-dependently, indicating that Scopoletin may prove beneficial against metastatic cancers. PI3K/AKT signal transduction pathway is considered important because it regulates the proliferation and tumorigenesis of several types of cancers [22]. Herein, we found that Scopoletin blocks this pathway by inhibiting the phosphorylation of PI3K and AKT proteins.

Conclusion

In conclusion, Scopoletin is an important coumarin with anticancer properties. Scopoletin inhibits the growth of cervical cancer cells by induction of apoptosis and cell cycle arrest. Hence, it may prove beneficial in treating cervical cancer and therefore warrants *in vivo* evaluation.

Conflict of interests

The authors declare no conflict of interests.

References

1. Schiffman M. Cervical cancer screening: epidemiology as the necessary but not sufficient basis of public health practice. Prev Med 2017;98:3.
2. Arbyn M, Castle PE. Offering self-sampling kits for HPV testing to reach women who do not attend in the regular cervical cancer screening program. Cancer EpidemiolPrev Biomarkers 2015:cebp-1417.
3. Di Felice E, Caroli S, Paterlini L, Campari C, Prandi S, Rossi PG. Cervical cancer epidemiology in foreign women in Northern Italy: role of human papillomavirus prevalence in country of origin. Eur J Cancer Prev 2015;24:223-30.
4. Motoki Y, Mizushima S, Taguri M et al. Increasing trends in cervical cancer mortality among young Japanese women below the age of 50 years: an analysis using the Kanagawa population-based Cancer Registry, 1975-2012. Cancer Epidemiol 2015;39:700-6.
5. Harvey AL, Edrada-Ebel R, Quinn RJ. The re-emergence of natural products for drug discovery in the genomics era. Nat Rev Drug Discov 2015;14:111.
6. Felice MR, Giuffre L, El LA et al. Looking for new antifungal drugs from flavonoids: impact of the genetic diversity of Candida albicans on the in-vitro response. Cur Med Chem 2017; 4; 7-11.
7. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 2016;79:629-61.
8. Thakur A, Singla R, Jaitak V. Coumarins as anticancer agents: A review on synthetic strategies, mechanism of action and SAR studies. Eur J Med Chem 2015;101:476-95.
9. Klenkar J, Molnar M. Natural and synthetic coumarins as potential anticancer agents. J Chem Pharm Res 2015;7:1223-38.
10. Seo EJ, Saeed M, Law BY et al. Pharmacogenomics of scopoletin in tumor cells. Molecules 2016;21:496.
11. Asgar MA, Senawong G, Sripa B, Senawong T. Scopoletin potentiates the anticancer effects of cisplatin against cholangiocarcinoma cell lines. Bangladesh J Pharmacol2015;10:69-77.
12. Nakamura N, Kusunoki Y, Akiyama M. Radiosensitivity of CD4 or CD8 positive human T-lymphocytes by an in vitro colony formation assay. Radiat Res 1990;123:224-7.
13. Pu X, Wang Z, Klaunig JE. Alkaline comet assay for assessing DNA damage in individual cells. Curr Protocols Toxicol 2015;65:3-12.
14. Wang Y, Zhao W, Fu Q. miR-335 suppresses migration and invasion by targeting ROCK1 in osteosarcoma cells. Mol Cell Biochem 2013;384:105-11.
15. Piver MS, Rutledge F, Smith JP. Five classes of extended hysterectomy for women with cervical cancer. Obstet Gynecol 1974;44:265-72.
16. Khuda-Bukhsh AR, Bhattacharyya SS, Paul S, Boujedaini N. Polymeric nanoparticle encapsulation of a naturally occurring plant scopoletin and its effects on human melanoma cell A375. Zhong Xi Yi Jie He Xue Bao 2010;8:853-62.
17. Li CL, Han XC, Zhang H, Wu JS, Li B. Effect of scopoletin on apoptosis and cell cycle arrest in human prostate cancer cells in vitro. Tropical JPharmaceut Res 2015;14:611-7.
18. Kim EK, Kwon KB, Shin BCet al. Scopoletin induces apoptosis in human promyeloleukemic cells, accompanied by activations of nuclear factor κB and caspase-3. Life Sci 2005;77:824-36.
19. Zhao P, Chen L, Li LH et al. SC-III3, a novel scopoletin derivative, induces cytotoxicity in hepatocellular cancer cells through oxidative DNA damage and ataxia telangiectasia-mutated nuclear protein kinase activation. BMC Cancer 2014;14:987.
20. Lowe SW, Lin AW. Apoptosis in cancer. Carcinogenesis 2000;21:485-95.
21. Lopez-Gonzalez JS, Prado-Garcia H, Aguilar-Cazares D, Molina-Guarneros JA, Morales-Fuentes J, Mandoki JJ. Apoptosis and cell cycle disturbances induced by coumarin and 7-hydroxycoumarin on human lung carcinoma cell lines. Lung Cancer 2004;43:275-83.
22. Luo J, Manning BD, Cantley LC. Targeting the PI3K-Akt pathway in human cancer. Cancer Cell 2003;4:257-62.