JBUON 2019; 24(6): 2445-2450
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

# Xanthohumol chalcone acts as a powerful inhibitor of carcinogenesis in drug-resistant human colon carcinoma and these effects are mediated via G2/M phase cell cycle arrest, activation of apoptotic pathways, caspase activation and targeting Ras /MEK/ERK pathway

Xiaokang Liu*, LiJuan An*, Yu Li, Yanwei Wang, Lei Zhao, Xi Lv, Jinyi Guo, Ai Lin Song

Fifth Department of General Surgery, the Second Hospital of Lanzhou University, No.82 Cuiyingmen, Chennguan District, Lanzhou, 730030, Gan Su province, China.

*These authors contributed equally to this article

## Summary

***Purpose:*** *Xanthohumol is a prenylated flavonoid of plant origin and has been reported to exhibit a spectrum of pharmacological properties including anticancer effects. However, the anticancer properties of Xanthohumol have not been thoroughly evaluated against drug-resistant colon cancer cells. This study was undertaken to evaluate the anticancer effects of Xanthohumol against the human colon cancer cell line HT-29 and normal CDD-18Co cell line.*

***Methods:*** *HT-29 cell viability was evaluated by cell counting kit-8 (CCK8) assay. Apoptotic effects were examined by fluorescence microscopy using DAPI staining and flow cytometry using annexin V/propidium iodide (PI) staining. Effects on cell cycle were studied by flow cytometry while western blot analysis was done to study effects on protein expressions.*

***Results:*** *The results showed that Xanthohumol causes a dramatic decrease in the HT-29 cell viability with an $IC_{50}$ of 10 μM. However, an $IC_{50}$ >100 μM for Xanthohumol against the normal CDD-18Co cells suggested cancer cell specific activity. DAPI staining revealed nuclear fragmentation, suggesting xanthohumol induces apoptosis in HT-29 cells. Xanthohumol also caused activation of caspase-3 and 9 and increased the Bax/Bcl-2 ratio. Cell cycle analysis showed that this molecule caused arrest of the HT-29 cells at the G2/M phase of the cell cycle. The induction of G2/M cell cycle was also accompanied with depletion of the expression of cyclin B1. The effects of Xanthohumol were also investigated on the Ras/MEK/ERK signalling pathway which revealed that Xanthohumol also blocks the MEK/ERK signalling pathway in colon cancer cells in a concentration-dependent manner.*

***Conclusions:*** *Xanthohumol may prove an efficient lead molecule for the development of more potent anticancer agents through semi-synthetic approaches.*

***Key words:*** *colon cancer, xanthohumol, cell cycle, apoptosis, migration*

## Introduction

Colon cancer is the fourth cause of cancer-related mortality. It ranks third common type of cancer and around 1.4 million new cases of colon cancer are reported annually [1]. In 2013, around 0.7 million deaths were reported to be due to colon cancer around the world [2]. Although the incidence of colon cancer has declined to some extent, it is believed it will increase by 60% till 2030 [3]. Late

*Corresponding author:* Ai Lin Song, MD. Sixth Department of General Surgery, the Second Hospital of Lanzhou University, no.82 Cuiyingmen, Chengguan District, Lanzhou, Gan Su province, 730030, China.
Tel/Fax: +86 931 894 2262, Email: AngieNguyengmu@yahoo.com
Received: 17/03/2019; Accepted: 11/04/2019

diagnosis, lack of potent and safe chemotherapeutic drugs and therapeutic targets form an obstacle in the treatment of colon cancer [4]. Natural products including plants, microbes and animals have the capability to synthesize a wide array of chemical entities for their own benefits. However, these metabolites have been used by humans across the globe for their health promoting properties [5]. Plants produce a diverse group of metabolites [6,7]. Xanthohumol is a prenylated flavonoid generally extracted from the flowers of *Humulus lupulus,* commonly known as hops [8[. Prenylated flavonoids, such as Xanthohumol, halt the growth of cancer cells via multiple mechanisms such as apoptosis, autophagy and cell cycle arrest and thereby are considered as vital anticancer agents [9] and has been reported to exert antiproliferative effects on human colon cancer cells [10]. Xanthohumol has also been reported to suppress the growth and metastasis of hepatocellular carcinoma cells [11]. In yet another study, Xanthohumol has been shown to exert growth inhibitory effects on the human prostate cancer cells [12]. Given this background the present study was designed to investigate the anticancer effects of Xanthohumol against human colon cancer cells and the underlying mechanism for the anticancer effects together with its effects on the Ras/MEK/ERK signalling pathway were also investigated.

## Methods

*Cell counting kit -8 (CCK8) assay for determination of cell viability*

The determination of the drug-resistant cancer cells HT-29 and non-cancer CDD-18Co normal cell viability was done by CCK-8 assay. In brief, the HT-29 cells were seeded in 96-well plates and treated with varied concentrations of Xanthohumol at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% $CO_2$, 95% $O_2$). Optical density $(OD)_{450}$ was taken with the help of a microplate reader to determine the cell viability.

*DAPI staining assay for detection of apoptosis*

The drug-resistant colon HT-29 cells ($0.6\times10^6$) were seeded in 6-well plates and incubated for 24 h at 37°C with varied concentration of Xanthohumol. As the cells cast-off, 10 µl cell culture were put onto glass slides and stained with DAPI. The slides were cover-slipped and examined with a fluorescent microscope.

*Annexin V/PI staining*

ApoScan kit was used to determine the apoptotic HT-29 cell percentage. In brief, Xanthohumol-treated HT-29 cells ($5\times10^5$ cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic HT-29 cells at each concentration was then determined by flow cytometry.

*Cell cycle analysis*

After treating the drug-resistant HT-29 cells with varied concentrations of Xanthohumol for 24 at 37 °C the cells were washed with phosphate buffered saline (PBS). Afterwards, the HT-29 cells were stained with PI and the cell distribution in cell cycle phases was assessed by FACS flow cytometer.

*Western blot analysis*

The determination of the protein expression was estimated by western blot analysis. The drug-resistant HT-29 cells were cultured for 24 h at 37°C. The cultured cells were then treated with 0, 5, 10 and 20 µM concentrations of Xanthohumol for 24 h and then collected by centrifugation. The HT-29 cells were then lysed in lysis buffer containing protease inhibitor. Afterwards, using SDS-PAGE, protein extracts were separated, followed by transferring to PVDF membranes. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated overnight with primary antibodies at 4°C. Subsequently, the membranes were incubated with secondary antibodies. Finally, the bands weres was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalisation.

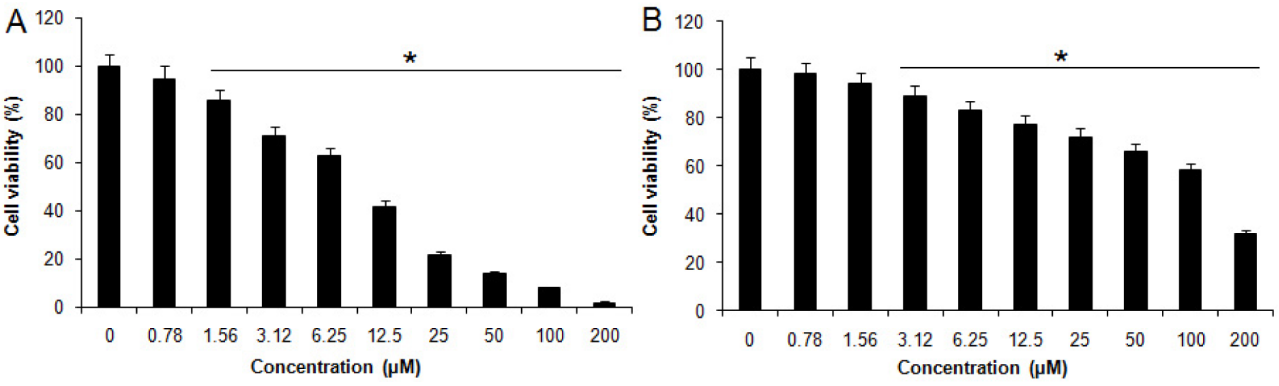


**Figure 1.** Effect of Xanthohumol on the viability of **(A)** HT-29 drug-resistant colon cancer cells and **(B)** normal CDD-18Co cells. The experiments were performed in triplicate and shown as mean ± SD (*p< 0.05).

*Statistics*

The experiments were carried in triplicate and the values are shown as means±SD. One-way ANOVA was used to evaluate significant intergroup differences. P<0.05 was considered as statistically significant.

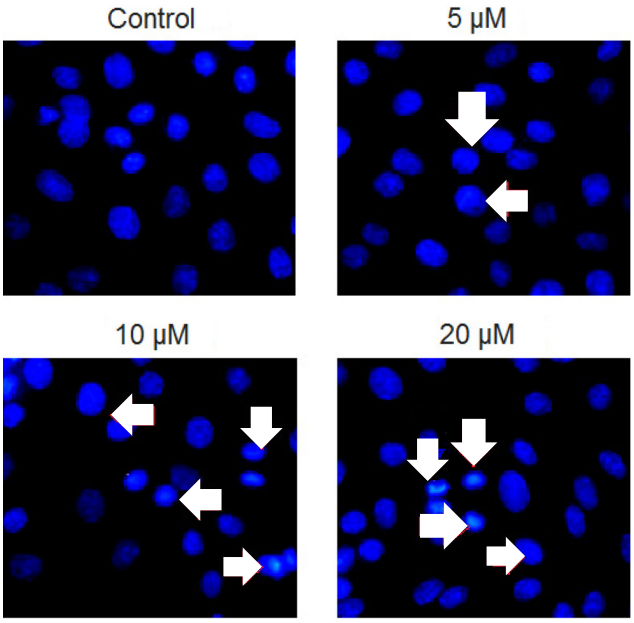


**Figure 2.** DAPI staining showing induction of apoptosis in HT-29 cells. The experiments were performed in triplicate. The Figure shows that Xanthohumol induces apoptosis as shown by arrows (chromatin condensation and nuclear fragmentation).

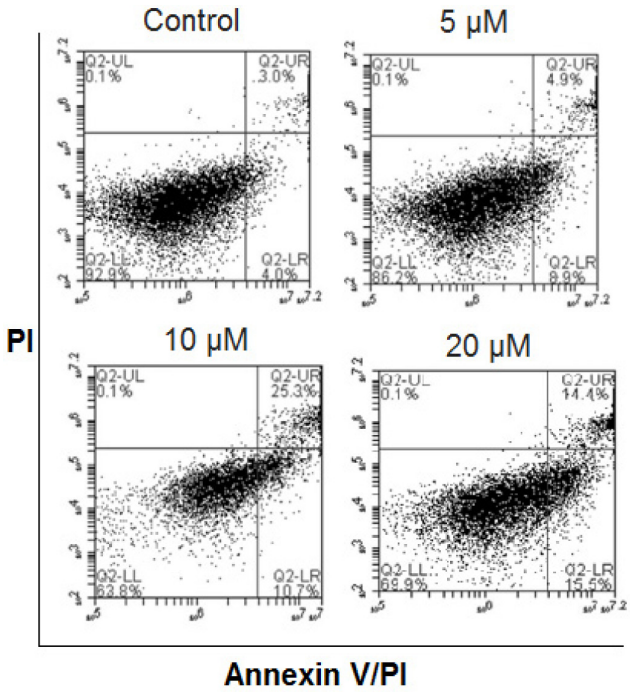


**Figure 3.** Annexin V/PI staining showing the percentage of the apoptotic HT-29 cells. The experiments were performed in triplicate. The Figure shows increase in the percentage of apoptotic cells with increasing dose of Xanthohumol.

## Results

*Xanthohumol suppresses colon cancer cell growth*

The CCK-8 assay was used to assess the impact of Xanthohumol on the viability of the drug-resistant HT-29 colon cancer and CDD-18Co normal cells. Xanthohumol caused a significant reduction in the viability of the HT-29 cells which was dose-dependent and $IC_{50}$ of 10 μM was observed for Xanthohumol against the HT-29 cells (Figure 1A). Nonetheless, the effects of Xanthohumol on the normal CDD-18Co cells showed an $IC_{50}$ > 100 μM (Figure 1B).

*Apoptotic effects of Xanthohumol on colon cancer cells*

To investigate the mechanism behind the antiproliferative effects of Xanthohumol, the drug-resistant HT-29 cells were treated with different doses of Xanthohumol and then stained with DAPI. The results of DAPI showed that Xanthohumol caused nuclear fragmentation of the HT-29 cells in a dose-dependent manner which indicated the induction of apoptosis (Figure 2). Annexin/PI staining showed that the apoptotic cell percentage was 4.0%, 8.9%, 10.7% and 15.5% at concentrations of 0, 5, 10 and 20 μM (Figure 3). The Xanthohumol-induced apoptosis was also accompanied with increase in the Bax and decrease in the Bcl-2 expression in a dose-dependent manner (Figure 4).

*Xanthohumol causes activation of caspases*

The effects of Xanthohumol were investigated on the expression of caspase-3, caspase-8 and caspase-9 in drug-resistant HT-29 colon cancer cells at concentrations of 0, 5, 10 and 20 μM. The western blot analysis showed that the expression of caspase-3, caspase-8 as well as the expression of caspase-9 was increased in a dose-dependent manner (Figure 5). These results indicate Xanthohumol

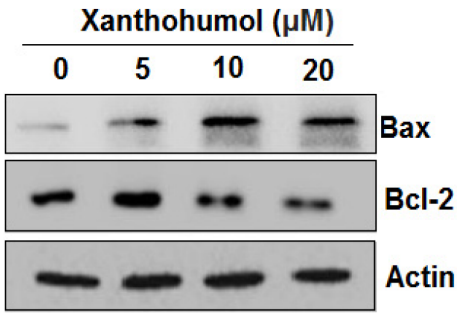


**Figure 4.** Effect of Xanthohumol on the expression of Bax and Bcl-2 in HT-29 cells as depicted by Western blot analysis. Xanthohumol led to increase in the expression of Bax and decrease in the expression of Bcl-2 in a dose-dependent manner.

increases the expression of caspases in HT-29 colon cancer cells.

*Xanthohumol causes arrest of the HT-29 cells at the G2/M checkpoint*

The effects of Xanthohumol were also evaluated on the cell cycle distribution of the drug-resistant HT-29 cells. The results indicated that Xanthohumol treatment caused a significant increase in the G2/M phase of the cell cycle, indicative of G2/M arrest (Figure 6). This was also accompanied by downregulation of Cyclin A and B1 expression in a dose-dependent manner (Figure 7).

*Effect of Xanthohumol on the MEK/ERK pathway in colon cancer cells*

Xanthohumol's effects on the Ras/MEK/ERK signalling pathway was assessed by western blot analysis after treating the drug-resistant HT-29 cells with 0, 5, 10 and 20 μM (Figure 8). It was found that the expression of Ras was decreased concentration-dependently in the HT-29 cells. Moreover the phosphorylation of the MEK and ERK was also suppressed by Xanthohumol in HT-29 cells in a concentration-dependent manner (Figure 8).

## Discussion

The clinical outcomes of colon cancer with the current drugs are unsatisfactory and there exists an obvious need for discovery of new drug candidates. The development of drug resistance in colon cancer cells makes it even difficult to manage this disease. The currently available chemotherapeutic agents have a number of adverse effects [13]. This study was carried out to evaluate the anticancer effects of a pharmacologically important prenylated flavonoid Xanthohumol against human colon cancer cells. The findings of the present study showed that the HT-29 cell viability was decreased significantly upon treatment with Xanthohumol. Additionally, the findings indicated selective anticancer effects this molecule against the drug-resistant HT-29 colon cancer cells as a very high $IC_{50}$ of xanthohumol was observed against the normal cells. Xanthohu-

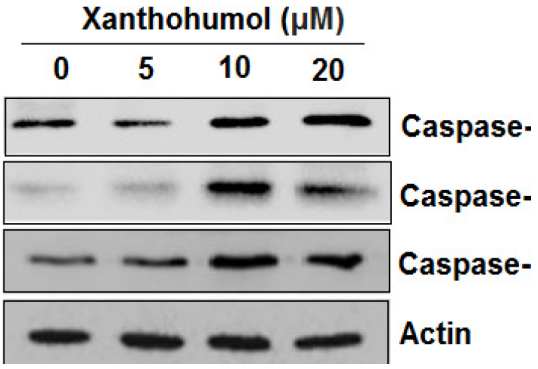


**Figure 5.** Effect of Xanthohumol on the expression of caspases in HT-29 cells as depicted by Western blot analysis. Xanthohumol led to increase in the expression of caspase-3 and 9, indicating onset of apoptosis.

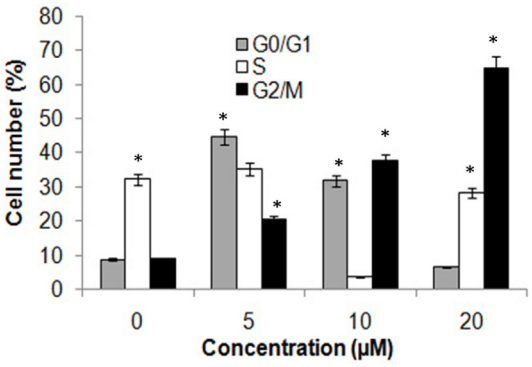


**Figure 6.** Effect of Xanthohumol on the cell cycle distribution of the HT-29 cells. Xanthohumol treatment led to G2/M phase cell cycle arrest. The experiments were performed in triplicate and shown as mean±SD (*p<0.05).

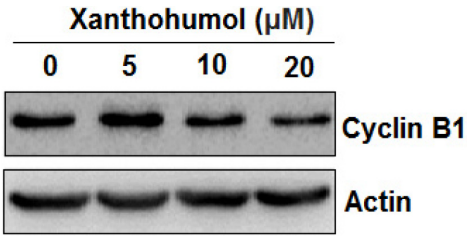


**Figure 7.** Effect of Xanthohumol on the expression of cyclin B1 in HT-29 cells as depicted by Western blot analysis. Xanthohumol treatment led to decrease in the expression of cyclin B1.

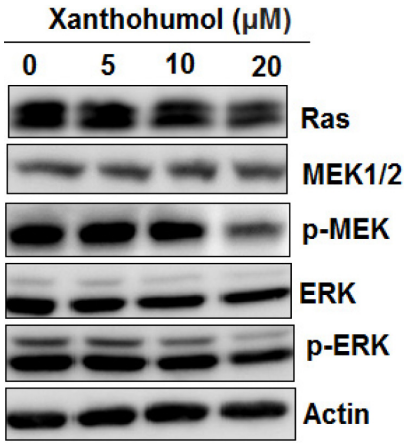


**Figure 8.** Effect of Xanthohumol on the Ras/MEK/ERK signalling pathway in HT-29 cells as depicted by Western blot analysis. It was found that the expression of Ras decreased concentration-dependently in the HT-29 cells. Moreover, the phosphorylation of MEK and ERK was also suppressed by Xanthohumol.

mol has also been reported to inhibit the growth of epithelial ovarian cancer cells [14]. The antiangiogenic effects of Xanthohumol have also been reported to inhibit the growth of HUVEC, human unbilical vascular endothelial cells [15]. In yet another study Xanthohumol has been found to interact with histones to exert antiproliferative effects [16]. Given these studies, we sought to know if Xanthohumol causes apoptosis of the colon HT-29 cancer cells and the DAPI staining showed that this molecule induces apoptosis in these cells. This was also associated with activation of caspases 3 and 9 as well as increase in the Bax/Bcl-2 ratio. This is in agreement with previous studies wherein xanthohumol has been shown to trigger apoptotic cell death cancer cells [17]. Moreover, Xanthohumol has also been shown to induce apoptotic cell death in many human cancer cells [18]. Many of the flavonoids with anticancer activity have been reported to induce cell cycle arrest of cancer cells and herein we observed that Xanthohumol caused arrest of the cancer cells at the G2/M phase of the cell cycle, which was also accompanied with suppression of cyclin B1 expression. Owing to the therapeutic importance of MEK/ERK signalling in the treatment of human cancer [19], studies are being directed to identify the molecules that could block this pathway. Herein, we found that Xanthohumol blocked this pathway in the HT-29 cells in a concentration-dependent manner, indicating that Xanthohumol may prove a beneficial lead molecule for colon cancer treatment.

## Conclusion

Xanthohumol selectively supresses the growth of the colon cancer cells via induction of apoptosis and cell cycle arrest. Additionally, Xanthohumol also blocks the MEK/ERK pathway in colon cancer cells. To sum up, Xanthohumol may prove beneficial in colon cancer treatment provided further *in vivo* studies are performed.

## Conflict of interests

The authors declare no conflict of interests.

## References


1. Siegel R, DeSantis C, Jemal A. Colorectal cancer statistics, 2014. CA: Cancer J Clin 2014;64:104-17.
2. Arnold M, Sierra MS, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global patterns and trends in colorectal cancer incidence and mortality. Gut 2017;66:683-91.
3. Guinney J, Dienstmann R, Wang X et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350.
4. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. Epidemiol Rev 1993;15:499-545.
5. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Products 2007;70:461-77.
6. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. Medicinal Res Rev 2003;23:519-34.
7. Chahar MK, Sharma N, Dobhal MP, Joshi YC. Flavonoids: A versatile source of anticancer drugs. Pharmacognosy Rev 2011;5:1.
8. Stevens JF, Page JE. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry 2004;65:1317-30.
9. Miranda CL, Stevens JF, Helmrich A et al. Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines. Food Chem Toxicol 1999;37:271-85.
10. Pan L, Becker H, Gerhäuser C. Xanthohumol induces apoptosis in cultured 40-16 human colon cancer cells by activation of the death receptor-and mitochondrial pathway. Mol Nutrition Food Res 2005;49:837-43.
11. Dorn C, Weiss TS, Heilmann J, Hellerbrand C. Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. Int J Oncol 2010;36:435-41.
12. Deeb D, Gao X, Jiang H, Arbab AS, Dulchavsky SA, Gautam SC. Growth inhibitory and apoptosis-inducing effects of xanthohumol, a prenylated chalcone present in hops, in human prostate cancer cells. Anticancer Res 2010;30:3333-9.
13. Tauriello DV, Palomo-Ponce S, Stork D et al. TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis. Nature 2018;554:538.
14. Drenzek JG, Seiler NL, Jaskula-Sztul R, Rausch MM, Rose SL. Xanthohumol decreases Notch1 expression and cell growth by cell cycle arrest and induction of apoptosis in epithelial ovarian cancer cell lines. Gynecol Oncol 2011;122:396-401.
15. Albini A, Dell'Eva R, Vené R et al. Mechanisms of the antiangiogenic activity by the hop flavonoid xanthohumol: NF-κB and Akt as targets. FASEB J 2006;20:527-9.
16. Wyns C, Van Steendam K, Vanhoecke B, Deforce D, Bracke M, Heyerick A. Prenylated chalcone xanthohumol associates with histones in breast cancer cells–a novel target identified by a monoclonal antibody. Mol Nutrition Food Res 2012;56:1688-96.

17. Nishimura R, Tabata K, Arakawa M et al. Isobavachalcone, a chalcone constituent of Angelica keiskei, induces apoptosis in neuroblastoma. Biol Pharmaceut Bull 2007;30:1878-83.
18. Strathmann J, Klimo K, Sauer SW, Okun JG, Prehn JH, Gerhäuser C. Xanthohumol-induced transient superoxide anion radical formation triggers cancer cells into apoptosis via a mitochondria-mediated mechanism. FASEB J 2010;24:2938-50.
19. Priyadarsini RV, Murugan RS, Maitreyi S, Ramalingam K, Karunagaran D, Nagini S. The flavonoid quercetin induces cell cycle arrest and mitochondria-mediated apoptosis in human cervical cancer (HeLa) cells through p53 induction and NF-κB inhibition. Eur J Pharmacol 2010;649:84-91.
20. Montagut C, Settleman J. Targeting the RAF–MEK–ERK pathway in cancer therapy. Cancer Lett 2009;283:125-34.