JBUON 2019; 24(4): 1470-1475
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Zingiberene inhibits *in vitro* and *in vivo* human colon cancer cell growth via autophagy induction, suppression of PI3K/AKT/mTOR Pathway and caspase 2 deactivation

Hai Chen, Xiaocheng Tang, Ting Liu, Liang Jing, Junhui Wu

Department of Gastrointestinal Surgery, The Third Xiangya Hospital of Central South University, Changsha, Hunan, China, 410000

Summary

Purpose: *Colon cancer (CC) is one of the deadly malignancies and the second most frequent cancer in the world. The development of drug resistance and dearth of the viable drug options form a serious obstacle in the treatment of CC. Herein, the anticancer potential of Zingiberene was examined against the CC cells.*

Methods: *The proliferation rate of the CC cells was assessed by cell counting assay. Autophagy was detected by transmission electron microscopy (TEM). The transfected cells were then treated with varied concentrations of Zingiberene (0, 10, 20 and 40 μM) for 24 h and monitored by fluorescent microscopy. Cell cycle analysis was performed by flow cytometry. Protein expression was determined by immunoblotting.*

Results: *Zingiberene could considerably inhibit the proliferation of CC cells. The anticancer activity of Zingiberene against the HT-29 CC cells was found to be due to induction of autophagy. The Zingiberene triggered autophagy was also linked with increase in the expression of LC3-II and decrease in p62 expression. However, no apparent effects were observed on the LC3-I expression. Moreover, it was found that zingiberine also caused activation of autophagy-related caspases in the HT-29 cells. Further, it was found that Zingiberene could inhibit the mTOR/PI3K/AKT signalling pathway in the CC cells. Zingiberene also suppressed the weight and volume of the xenografted tumors concentration-dependently.*

Conclusions: *These results indicate that Zingiberene may inhibit the growth of CC in vitro and in vivo and may be used for the development of systemic therapy against CC.*

Key words: *colon cancer, zingiberene, autophagy, caspases, proliferation*

Introduction

Natural products, especially plants and microbes, have attracted huge attention as anticancer drugs because of their potent activities and lower side effects [1]. Plant-derived natural products such as taxol are being used for the management of cancer [2]. Plants produce a wide array of metabolites which are classified into different groups based on their structure [3]. Sesquiterpenes constitute a large assortment of molecules and are prevalently found across the plant kingdom [4]. Sesquiterpenes from several plants have shown the capacity to inhibit the proliferation of cancer cells, such as lung cancer, breast cancer and gastric cancer to name a few [5]. Sesquiterpenes have shown such potent anticancer activities that even some of the Sesquiterpenes have reached clinical trials [6]. Zingiberene is a monocyclic sesquiterpene prevalently biosynthesized by different plant species. Zingiberene is an important constituent of a medicinally important plant *Zingiber officinale* [7]. *Z. officinale* has been reported to inhibit the growth of different types of cancer cells [8]. However, the anticancer effect of

Corresponding author: Junhui Wu, MD. Department of Gastrointestinal Surgery, the Third Xiangya Hospital of Central South University, Changsha, Hunan 410000, China.
Tel & Fax: +86 0731 88618576, Email: howyver@yahoo.com
Received: 05/01/2019; Accepted: 19/01/2019

Zingiberene has not been thoroughly investigated. Moreover, the underlying mechanisms for the anticancer activity of Zingiberene were also investigated by performing a series of assays. Colon cancer (CC) is the fourth prevalent cause of cancer-related mortality, ranks third in incidence and around 1.4 million new cases of CC are reported every year [9]. In 2013, around 0.7 million deaths were reported to be due to CC worldwide [10]. Although its incidence has declined to some extent, it is believed that it will increase by 60% till 2030 [11]. The late diagnosis and dearth of potent and safe chemotherapeutic drugs and therapeutic targets form a serious obstacle in the treatment of CC [12].The mTOR/PI3K/AKT signalling pathway has been considered as an essential therapeutic target for several cancers [13]. This study was undertaken to investigate for the first time the anticancer effects of Zingiberene in colon cancer along with evaluating its effects on autophagic induction, PI3K/AKT/mTOR signalling pathway and caspase-2.

Methods

Cell viability and colony formation assays

The cell counting assay was employed to determine the viability of the normal CCD-18Co and HT-29 CC cells. In brief, 5×10^4 cells/well were seeded in 12-well plates and incubated for 24 h with different concentrations of Zingiberene. The aliquots of cells were then removed and counted in triplicate following Trypan blue staining. The effect of Zingiberene on the formation of HT-29 colonies was investigated as described earlier [14].

Transmission electron microscopy (TEM)

Detection of autophagy was used to assess the induction of autophagy in cancer cells. The cells were fixed in 4% glutaraldehyde solution, 0.05 M sodium cacodylate, postfixed in 1.5% OsO4, and dehydrated in alcohol. Then, they were prepared for flat embedding in Epon 812 and then observed using Zeiss CEM 902 electron microscope.

Transfection assay

To demonstrate the induction of autophagy, the HT-29 cells were grown to 80% confluence and transfected with GFP-LC3 plasmids using Lipofectamine 2000 (Invitrogen) as per manufacturer's guidelines. The transfected cells were then treated with varied concentrations of Zingiberene (0, 10, 20 and 40 µM) for 24 h and monitored by fluorescent microscopy.

In vivo xenograft study

Healthy Swiss Albino mice weighing 25 ± 2.0 g were obtained from the animal house of the Third Xiangya Hospital of Central South University, Changsha, Hunan, China. The mice were randomly divided into four groups- one group was untreated control and the other three groups received Zingiberene dissolved in 0.1% DMSO at 10, 20 and 40 mg/kg. The HT-29 cells were subcutaneously injected into the left flank. The mice were monitored and as the growth of the tumors was apparent, the three groups received Zingiberene dissolved in 0.1% DMSO at 10, 20 and 40 mg/kg, while the fourth group, kept as control, received 0.1% DMSO in normal saline thrice weekly. The mice were euthanized at the end of the study (5 weeks) and the tumors were harvested and used for further experimentation.

Western blotting

Briefly, the HT-29 cells were washed 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 were loaded from each sample and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) before being shifted to polyvinylidenefluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and then 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

All the experiments were performed in triplicate, and the data were presented as mean±standard deviation. SPSS version 17 was used to carry out the statistical

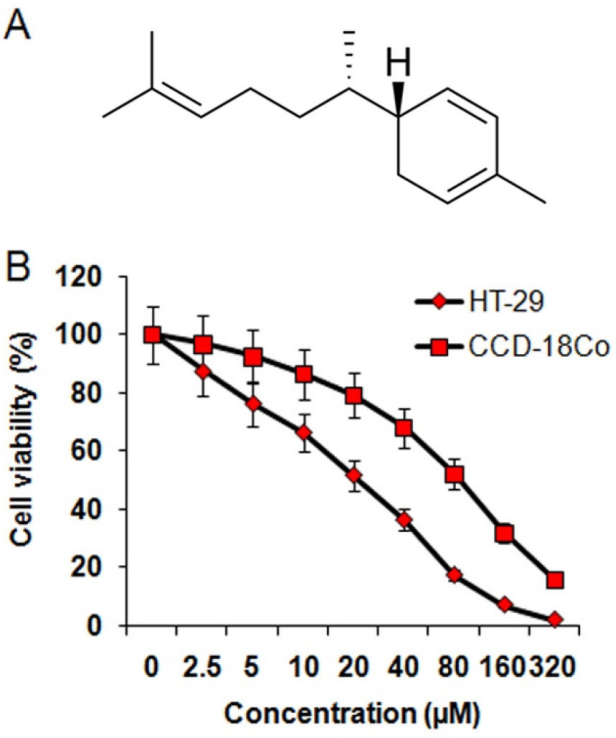

Figure 1. A: Chemical structure of Zingiberene. **B:** Cell counting assay showing the viability of the HT-29 and CDD-18Co cells. The experiments were performed in triplicate and shown as mean±SD ($p<0.05$). Zingiberene led to dose-dependent and selective inhibition of cell proliferation.

analyses, and the results were studied using Student's t-test and one-way ANOVA. P<0.05 was considered statistically significant.

Results

Zingiberene suppresses the growth of CC cells

The growth inhibitory effects of Zingiberene (Figure 1A) were assessed on the HT-29 CC cell line and CCD-18Co non-cancer cell line. Zingiberene treatment caused dose-dependent inhibition on the growth HT-29 CC cells. The IC_{50} of Zingiberene against the HT-29 CC cells was 20 µM (Figure 1B). However, the cytotoxic effects of Zingiberene on the CCD-18Co non-cancer cells were comparatively negligible (IC_{50} 80 µM). Additionally, Zingiberene also induced some morphological changes, such as shrinkage and membrane blebbing of the HT-29 cells (Figure 2). The effects of Zingiberene treatment were also assessed on the colony formation potential of the HT-29 cells, which showed that Zingiberene exerts dose-dependent inhibitory effects on the colony formation of the HT-29 cells (Figure 3).

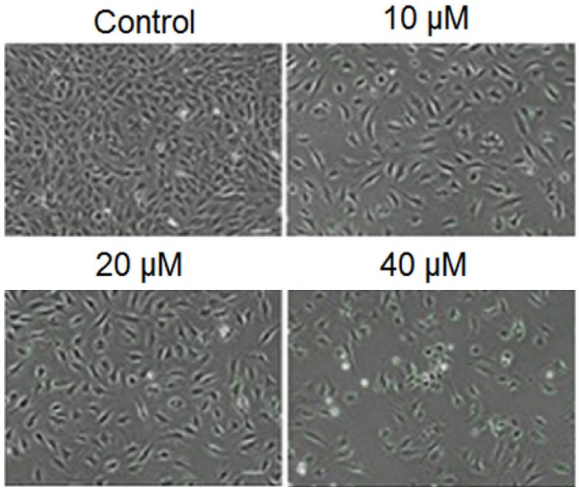

Figure 2. Morphology of the Zingiberene-treated HT-29 cells at indicated concentrations. The experiments were performed in triplicate and shown as mean ± SD (p< 0.05). Zingiberene caused significant changes in cellular morphology.

Zingiberene induces autophagy in the CC cells

The examination of the Zingiberene treated HT-29 cells by electron microscopy showed that Zingiberene treatment of these triggered the development of the autophagic vesicles in the CC cells at IC_{50} (Figure 4A). Fluorescence microscopy showed that Zingiberene enhanced the LC3 expression (Figure 4B). Western blot analysis further confirmed the induction of autophagy in the HT-29 cells as the LC3-II expression increased and of p62 decreased considerably. However, the expression of LC3-I showed no remarkable change (Figure 5). Zingiberene also caused dose-dependent deactivation of caspase 3 in the Zingiberene-treated HT-29 cells (Figure 6).

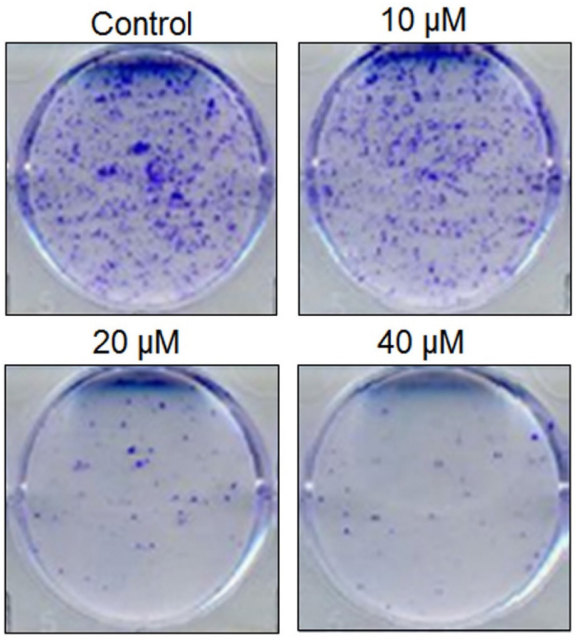

Figure 3. Colony formation assay of the Zingiberene-treated HT-29 cells at indicated concentrations. The experiments were performed in triplicate. The results show that zingiberene led to inhibition of cancer cell colony formation in a dose-dependent manner.

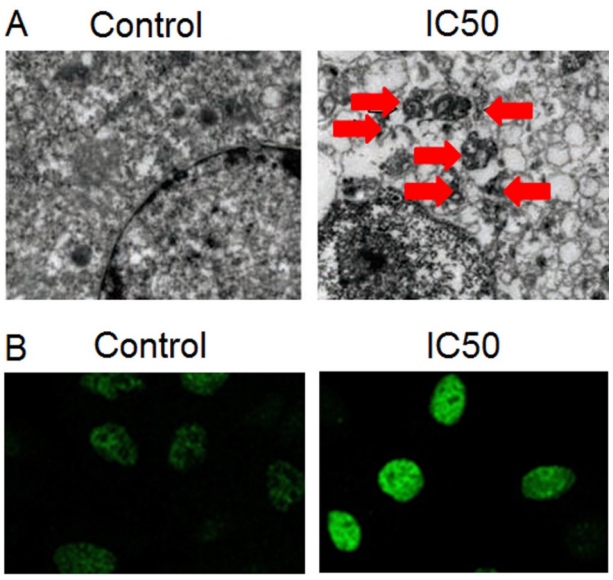

Figure 4. A: Electron microscopic analysis of Zingiberene-treated HT-29 cells. Arrows indicate the formation of autophagic vesicles in the CC cells at IC_{50} value. **B:** Effect of Zingiberene on the expression of the LC3 II proteins at IC_{50}; fluorescence microscopy showed that Zingiberene enhanced the LC3 expression. The experiments were performed in triplicate.

Zingiberene blocks the PI3K/mTOR/AKT signalling pathway

We also examined the effects of Zingiberene on the PI3K/AKT signalling pathway of HT-29 cancer cells. It was revealed that Zingiberene caused concentration-dependent decline in the phosphorylation of mTOR, PI3K and AKT, while no apparent effect was observed on the expression of total mTOR, PI3K and AKT (Figure 8).

Zingiberene inhibited xenografted tumor growth in vivo

The effects of Zingiberene were investigated on the xenografted tumor growth *in vivo*. The results showed that the administration of Zingiberene at 0, 10, 20 and 40 mg/kg caused significant decrease in the tumor volume and weight of the xenografted tumors, indicative of the *in vivo* anticancer activity of Zingiberene.

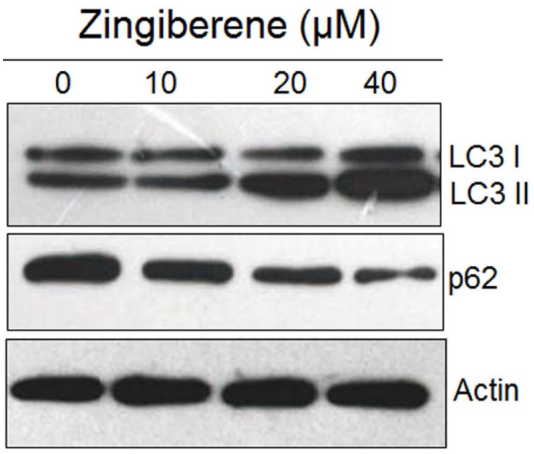

Figure 5. Effect of Zingiberene on the expression of autophagy-related proteins as depicted by western blotting. The LC3-II expression increased and of p62 decreased considerably while LC3-I did not show any significant change. The experiments were performed in triplicate.

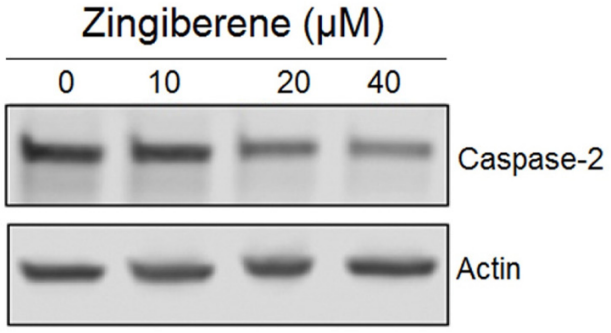

Figure 6. Effect of Zingiberene on the Caspase-2 expression as depicted by western blotting. The results showed that the expression of caspase-2 decreased significantly in a dose-dependent manner. The experiments were performed in triplicate.

Discussion

Ranked as the fourth most prevalent type of cancer, colon cancer causes significant morbidity and mortality across the globe [15]. The clinical outcomes with the current treatment strategies are far from descent owing to the side effects and low efficacy of the drugs [11]. Therefore, the identification of novel anticancer molecules and subsequent development of efficient and safer treatment regimes for clinical cancer are required. Natural products possess impressive potential acting as drugs for the alleviation of human disorders and several natural products are already being used as drugs throughout the world [16]. Herein, we examined the anticancer potential of Zingiberene. It was found that Zingiberene exerted growth inhibitory effects on the HT-29 CC cell line. Nonetheless, the cytotoxic effects of Zingiberene were comparatively negligible against the CCD-18Co cells, indicative of the cancer cell specific activity of Zingiberene. The antiproliferative effects of Zingiberene were also confirmed by alteration in the morphology and colony formation of the HT-29 CC cells. Previous studies have also shown that *Z. officinale* of which Zingiberene is an important constituent inhibits the growth of cancer cells [17]. Next, to investigate

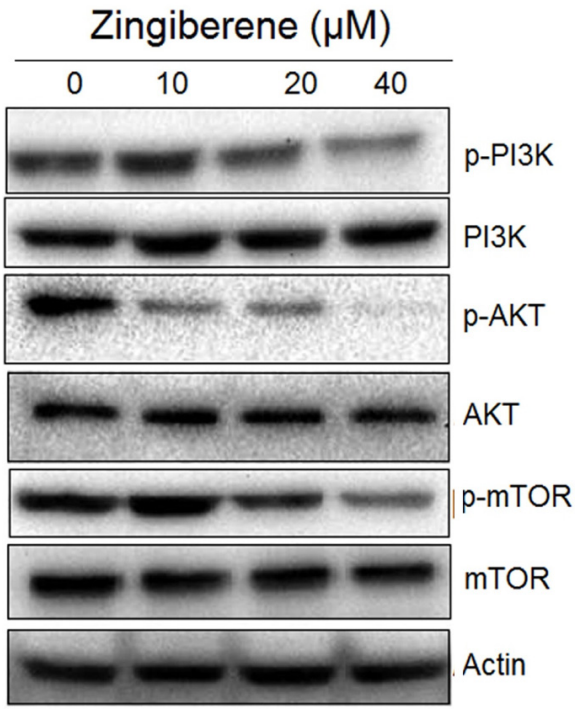

Figure 7. Western blots showing the effect of Zingiberene on the phosphorylation of PI3K/AKT/mTOR signalling pathway at indicated concentrations. Zingiberene caused concentration-dependent decline in the phosphorylation of mTOR, PI3K and AKT, while no apparent effect was observed on the expression of total mTOR, PI3K and AKT. The experiments were performed in triplicate.

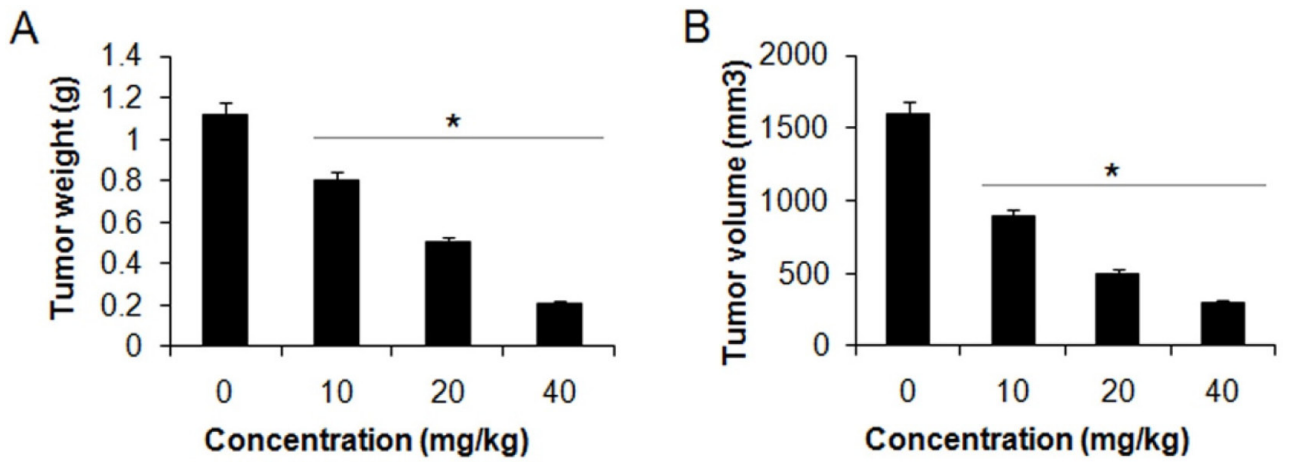

Figure 8. Zingiberene decreases the tumor weight **(A)** and tumor volume **(B)** in xenografted mice models concentration-dependently. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

the underlying mechanism of anticancer action of zingiberene, we carried out electron microscopic analysis and the results showed that Zingiberene leads to development of autophagic vesicles in the HT-29 CC cells and the Zingiberene-induced autophagy was also accompanied with upregulation of LC3 II and downregulation of p62. Autophagy is a vital process that helps in the elimination of dysfunctional and abnormal cells from the body [18]. Several of the sesquiterpenes have been shown to induce autophagy in cancer cells, for example, Zerumbone, a natural sesquiterpene, induces autophagy in prostate cancer cells via oxidative stress [19]. Similarly, sesquiterpene Bigelovin induces autophagy in liver cancer cells [20]. The mTOR/PI3K/AKT signal transduction pathway is considered an important pathway that regulates the proliferation and tumorigenesis of several types of cancers [21]. Herein, we found that Zingiberene blocks this pathway by inhibiting the phosphorylation of mTOR, PI3K and AKT proteins. The evaluation of the Zingiberene *in vivo* in xenografted mice revealed that this molecule could suppress the growth of xenografted tumors *in vivo*, suggestive of its potent anticancer effects.

In conclusion Zingiberene is an important sesquiterpene with anticancer potential. Zingiberene inhibits the growth of the colon cancer cells by inducing autophagy and it also suppresses the growth of xenografted tumors *in vivo*. Hence, it may prove beneficial in treating colon cancer.

Conflict of interests

The authors declare no conflict of interests.

References

1. Butler MS. Natural products to drugs: natural product-derived compounds in clinical trials. NAT Prod Rep 2008;25:475-516.
2. Cragg GM, Newman DJ. Antineoplastic agents from natural sources: Achievements and future directions. Expert Opin Investig Drugs 2000;9:2783-97.
3. Balandrin MF, Klocke JA, Wurtele ES, Bollinger WH. Natural plant chemicals: sources of industrial and medicinal materials. Science 1985 ;228:1154-60.
4. Zhang S, Won YK, Ong CN, Shen HM. Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. Cur Med Chem Anticancer Agents 2005;5:239-49.
5. Kreuger MR, Grootjans S, Biavatti MW, Vandenabeele P, D'Herde K. Sesquiterpene lactones as drugs with multiple targets in cancer treatment: focus on parthenolide. Anticancer Drugs 2012;23:883-96.
6. Ghantous A, Gali-Muhtasib H, Vuorela H, Saliba NA, Darwiche N. What made sesquiterpene lactones reach cancer clinical trials?. Drug Discov Today 2010;15:668-78.
7. Millar JG. Rapid and simple isolation of zingiberene from ginger essential oil. J Nat Prod 1998;61:1025-6.
8. Habib SH, Makpol S, Hamid NA, Das S, Ngah WZ, Yusof YA. Ginger extract (Zingiber officinale) has anti-cancer and anti-inflammatory effects on ethionine-induced hepatoma rats. Clinics 2008;63:807-13.
9. Siegel R, DeSantis C, Jemal A. Colorectal cancer statistics, 2014. CA: Cancer J Clin 2014;64:104-17.

10. 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.
11. Guinney J, Dienstmann R, Wang X et al. The consensus molecular subtypes of colorectal cancer. Nat Med 2015;21:1350.
12. Van Cutsem E, Cervantes A, Adam R et al. ESMO consensus guidelines for the management of patients with metastatic colorectal cancer. Ann Oncol 2016;27:1386-422.
13. Morgensztern D, McLeod HL. PI3K/Akt/mTOR pathway as a target for cancer therapy. Anticancer Drugs 2005;16:797-803.
14. Bruce WR, Meeker BE, Valeriote FA. Comparison of the sensitivity of normal hematopoietic and transplanted lymphoma colony-forming cells to chemotherapeutic agents administered in vivo. J Natl Cancer Inst 1966;37:233-45.
15. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106.
16. Newman DJ, Cragg GM. Natural products as sources of new drugs over the last 25 years. J Nat Prod 2007;70:461-77.
17. Ramakrishnan RA. Anticancer properties of Zingiber officinale–Ginger: A review. Int J Med Pharm Sci 2013;3:11-20.
18. Cudjoe EK, Kyte SL, Saleh T, Landry JW, Gewirtz DA (Eds). Autophagy Inhibition and Chemosensitization in Cancer Therapy. In: Targeting Cell Survival Pathways to Enhance Response to Chemotherapy, 2019, pp 259-73.
19. Chan ML, Liang JW, Hsu LC, Chang WL, Lee SS, Guh JH. Zerumbone, a ginger sesquiterpene, induces apoptosis and autophagy in human hormone-refractory prostate cancers through tubulin binding and crosstalk between endoplasmic reticulum stress and mitochondrial insult. Naunyn-Schmiedeberg's Arch Pharmacol 2015;388:1223-36.
20. Wang B, Zhou TY, Nie CH, Wan DL, Zheng SS. Bigelovin, a sesquiterpene lactone, suppresses tumor growth through inducing apoptosis and autophagy via the inhibition of mTOR pathway regulated by ROS generation in liver cancer. Biochem Biophys Res Commun 2018;499:156-63.
21. Chen M, Yin X, Lu C et al. Mahanine induces apoptosis, cell cycle arrest, inhibition of cell migration, invasion and PI3K/AKT/mTOR signalling pathway in glioma cells and inhibits tumor growth in vivo. Chemicobiol Interactions 2019;299:1-7.