JBUON 2019; 24(4): 1488-1493
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Dentatin exerts anticancer effects on human colon cancer cell lines via cell cycle arrest, autophagy, inhibition of cell migration and JAK/STAT signalling pathway

Wei Xiong[1]*, Jianhua Dong[1]*, Shujia Kong[2]

[1]Department of Colorectal Cancer Surgery, and [2]Department of Pharmacy, Yunnan Cancer Hospital & the Third Affiliated Hospital of Kunming Medical University , Kunming , Yunnan, 650118, China.

*These two authors contributed equally to this work.

Summary

Purpose: *Colon cancer is a malignant disease with significant mortality. In the present study the anticancer effects of a carbazole alkaloid, Dentatin, were examined against colon cancer cells.*

Methods: *The colon cancer HT-29 cell line and the normal CCD-18 CO colon cell line were used in the present study. MTT assay was used to check the proliferation rate of the cancer cells. Autophagy was detected by electron microscopy. DNA damage was checked by alkaline comet assay. Cell cycle analysis was performed by flow cytometry. Cell migration was monitored by wound healing assay. Protein expression was checked by western blot analysis.*

Results: *The results showed that Dentatin inhibited the growth of HT-29 cancer cells in a concentration-dependent manner and with IC_{50} of 25 μM. However, the IC_{50} of Dentatin against the normal CCD-18CO colon cells was four times higher (ie.,100 μM). Dentatin inhibited the proliferation of the HT-29 cancer cells by triggering S-phase arrest. This was also accompanied with increase in the expression of cyclin D1 and decrease in the expression of Cyclin A and B1. Moreover, Dentatin also induced autophagy in the HT-29 cells which was associated with upregulation of LC3 II and downregulation of Beclin-1 expression. Comet assay revealed that Dentatin induced DNA damage in the HT-29 cells. Dentatin also significantly inhibited the migration of the HT-29 cells. Finally the effects of Dentatin were examined on the JAK/STAT signalling pathway and it was found that Dentatin inhibited this pathway.*

Conclusion: *Dentatin may prove to be an essential lead molecule for the management of colon cancer.*

Key words: *colon cancer, dentatin, autophagy, migration, S-phase arrest*

Introduction

Colon cancer, a serious health issue, is considered to be the third most prevalent of cancer in most developed countries and the second in mortality in the western countries [1]. Owing to high rate of relapses, emergence of drug resistance and the adverse effects connected with chemotherapy, colon cancer associated mortality is increasing [2]. Therefore, identification and development of novel anticancer molecules with lower side effects may prove beneficial. Naturally occurring compounds with anticancer activities have attained attention due to their lower toxicity [3]. Dentatin is an important coumarin derivative mainly isolated from *Murraya koenigii* [4]. Coumarins have been shown to exhibit anticancer effects against a wide range of cancers and Dentatin is no exception [5]. Dentatin has been shown to inhibit the growth of prostate cancer cells by inducing apoptotic cell death [6]. Dentatin has also been shown to cause arrest of MCF-7 cancer cells in the G0/G1 phase of the cell

Corresponding author: Dr. Shujia Kong. Department of Pharmacy, Yunnan Cancer Hospital &the Third Affiliated Hospital of Kunming Medical University, Kunming , Yunnan, 650118, China.
Tel: +86 0871 68179481, Email: ShylaFinnecesi@yahoo.com
Received: 17/10/2018; Accepted: 04/11/2018

cycle [7]. However, the anticancer effects of Dentatin have not been fully studied on colon cancer. Henceforth, this study was designed to examine the anticancer effects of Dentatin against colon cancer cells and to explore the underlying mechanism.

Methods

Cell lines and culture conditions

The colon cancer HT-29 and the normal CCD-18CO cell lines were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Mass, USA), supplemeneted with 10% fetal bovine serum (FBS) (Invitrogen), 100µg/ml streptomycin and 100U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37°C with 5% CO_2.

Cell viability assay

Briefly, at around 70% confluence, HT-29 and the CCD-18CO cells were seeded in 96-well plates and treated with 0-200 µM of Dentatin in Dulbecco's modified Eagle's medium. After 24-h incubation, the cells were incubated with MTT for another 4 h. After this, the medium was removed and the colored formazan product was solubilized by 200 µl of dimethyl sulfoxide. The viability of the HT-29 and the CCD-18CO cells was then determined by taking absorbance at 570 nm.

Transmission electron microscopy (TEM)

For electron microscopy, the cells were fixed with 4% glutaraldehyde, 0.05 M sodium cacodylate solution, post-fixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and then observed using Zeiss CEM 902 electron microscope.

Cell cycle analysis

The HT-29 colon cancer cells were incubated with varied concentrations of Dentatin (0, 9, 18 and 36 µM) for 24 h. The cells were washed with phosphate buffered saline (PBS). Afterwards, the HT-29 cells were stained with propidium iodide (PI) and the distribution of cells in the cell cycle phases was assessed by FACS flow cytometer.

Cell migration assay

Cell migration was examined by wound healing assay. In brief, the HT-29 colon cancer cells cultured in 6-well plates in Dulbecco's modified Eagle's medium were scratched by 10 µl pipette tip and were treated with 25 µM of Dentatin, photographed under the microscope and then incubated at 37°C and photographed again after 24 h.

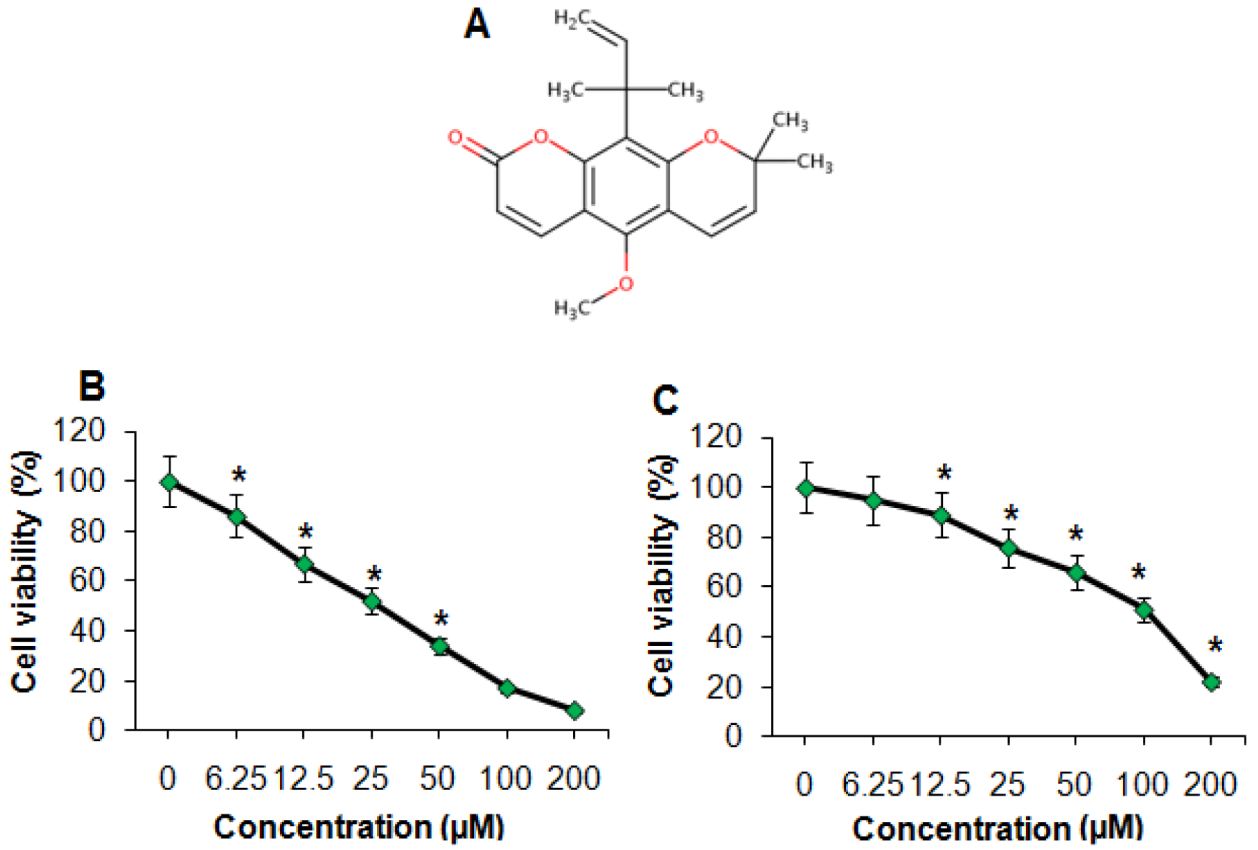

Figure 1. (A): Chemical structure of Dentatin. **(B):** Effect of Dentatin on the cell viability of colon cancer cell line HT-29 and **(C):** of normal cell line CCD-18CO. The experiments were performed in triplicate and the values represent mean ± SD (*p<0.05).

Western blotting

The HT-29 cells were firstly washed with ice-cold PBS and suspended in a lysis buffer at 4°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 antibody at 4°C. Afterwards, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

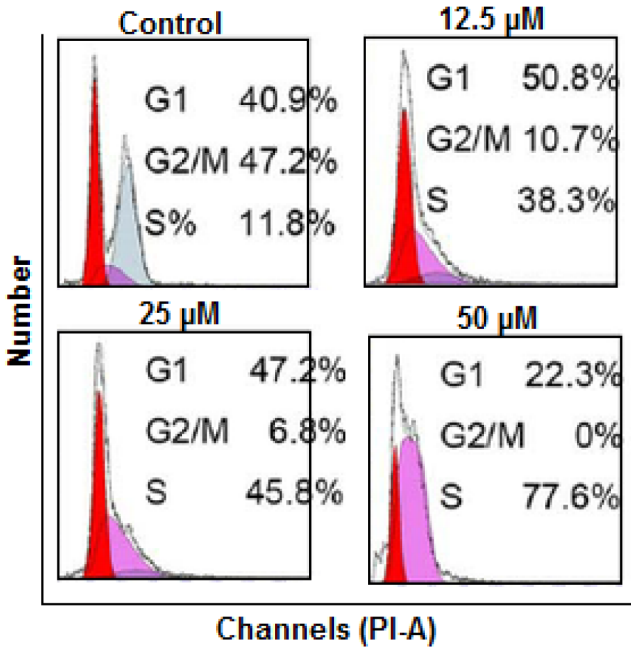

Figure 2. Dentatin triggers G2/M arrest of the HT-29 colon cancer cell line in a concentration-dependent manner, as depicted by flow cytometry. The experiments were performed in triplicate.

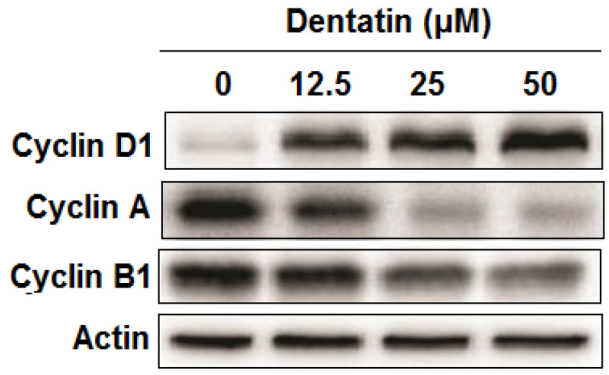

Figure 3. Effect of Dentatin on the cell cycle related proteins in HT-29 cells as depicted by western blot analysis. The Figure shows that dentatin inhibits cyclin A, D1 and B1 in a concentration-dependent manner. The experiments were performed in triplicate.

Statistics

The experiments were performed in triplicate and the data are shown as mean ± SD. Statistical analysis was done using Student's *t*-test, and for graphs the GraphPad prism 7 software was used. Values of $p<0.05$ were taken as indicative of significant difference.

Results

Dentatin inhibited the proliferation of HT-29 colon cancer cells

The anti-proliferative effects of Dentatin (Figure 1A) were examined on HT-29 colon cancer cells and the normal CCD-18CO cells by MTT assay at concentrations ranging from 0 to 200 µM. Dentatin was found to halt the growth of the HT-29 cells concentration-dependently (Figure 1B). The IC_{50} of Dentatin against the HT-29 cells was 25 µM. On the other hand, the effects of Dentatin on the proliferation of the CCD-18CO cells were different. The IC_{50} of Dentatin against the normal CCD-18CO cells was four times higher (ie., 100 µM) (Figure 1B).

Dentatin arrested the HT-29 cells at S phase

To explore the underlying mechanism for the anti-proliferative effects of Dentatin, the cycle phase distribution of HT-29 cells was examined by flow cytometry at 0, 12.5, 25 and 50 µM concentration of Dentatin. It was found that the percentage

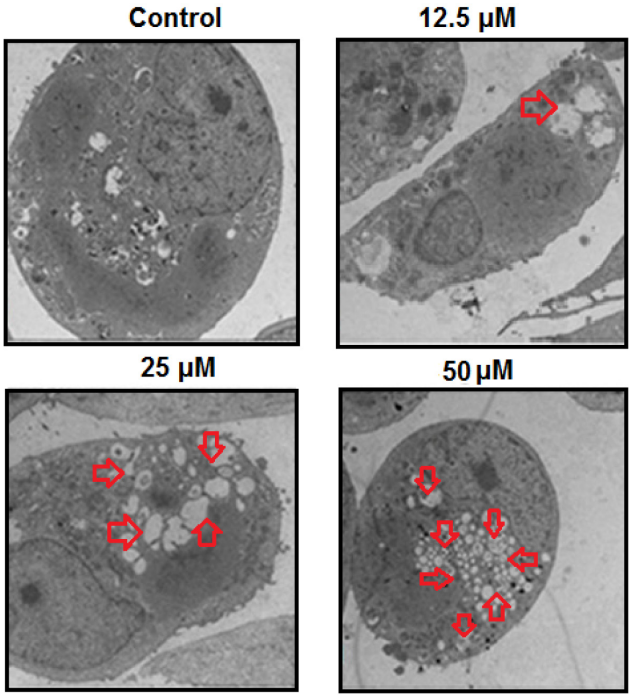

Figure 4. Dentatin induces autophagy in HT-29 colon cancer cells as evidenced from electron microscopy. The arrows depict autophagic vesicles. The experiments were performed in triplicate.

of S phase cells increased significantly with 11.8% in the control to 77.6% at 50 μM dose of Dentatin (Figure 2). The arrest of the HT-29 cells at the S phase was associated with changes in the expression of several of the cell cycle related proteins. The protein expression of cyclin D1 considerably increased upon Dentatin treatment while the expression of A and B1 was significantly increased (Figure 3).

Dentatin induced autophagy in HT-29 cells

Electron microscopic analysis of the Dentatin-treated cells was performed to examine if Dentatin induces autophagy in the HT-29 colon cancer cells (Figure 4). It was found that Dentatin treatment induced the formation of autophagic vacuoles in HT-29 cells dose-dependently. The induction of autophagy was further confirmed by western blot analysis. It was found that Dentatin treatment of the HT-29 colon cancer cells caused significant upregulation of the LC3 II protein expression. However, the expression of the LC3 I remained unaltered. Moreover, the expression the Beclin-1 was considerably decreased (Figure 5). Comet assay was also performed and showed that Dentatin triggered dose-dependent damage in the HT-29 cells (Figure 6).

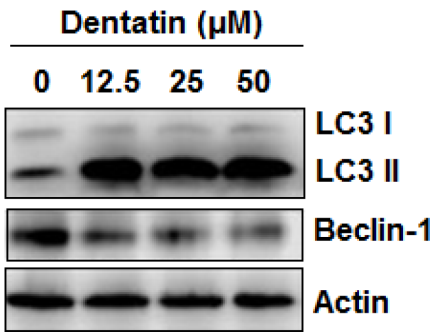

Figure 5. Effect of Dentatin on the autophagy related proteins in HT-29 cells as depicted by western blot analysis. The Figure shows that dentatin enhances the expression of LC3II expression in a dose-dependent manner. The experiments were performed in triplicate.

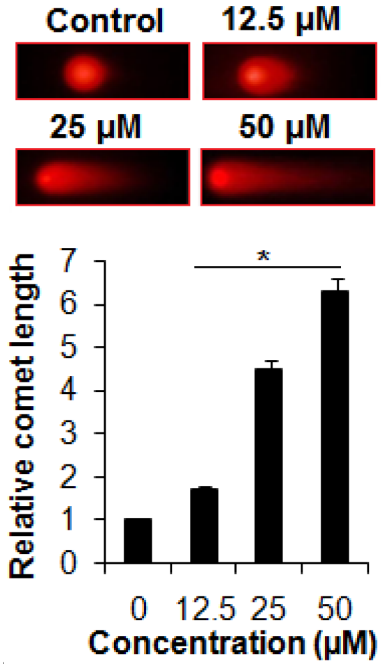

Figure 6. Comet assay showing the effect of Dentatin on the DNA damage. The experiments were performed in triplicate and the values are mean ± SD (*p<0.05).

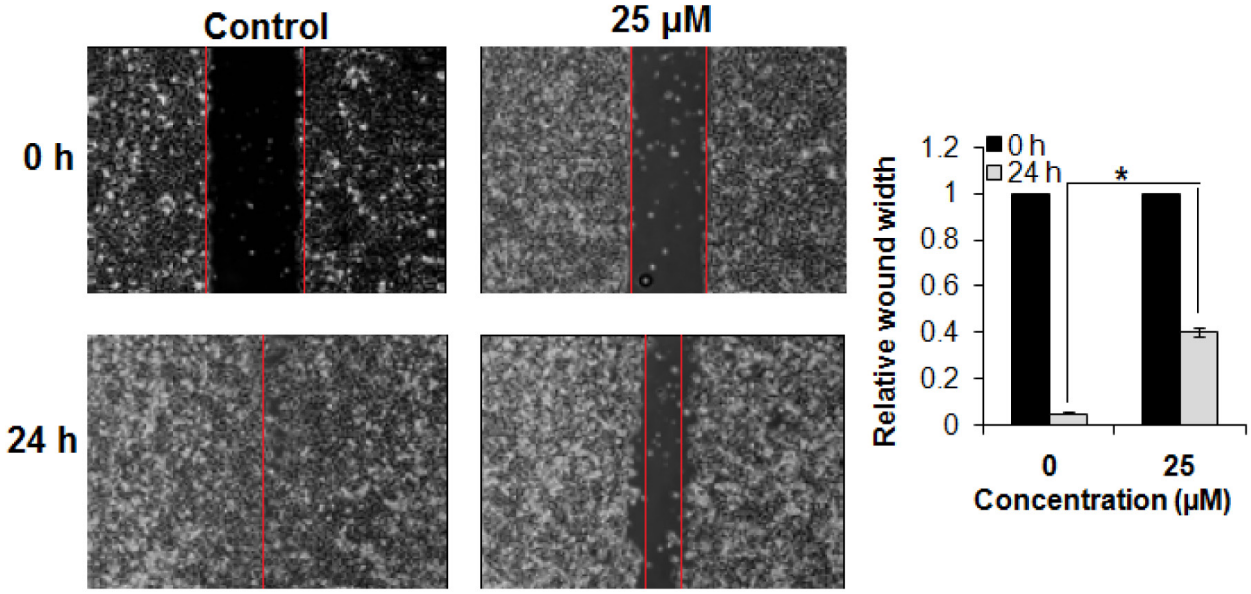

Figure 7. Dentatin inhibits the migration of the HT-29 colon cancer cells as depicted by wound healing assay. The experiments were performed in triplicate and the values represent mean ± SD (*p<0.05).

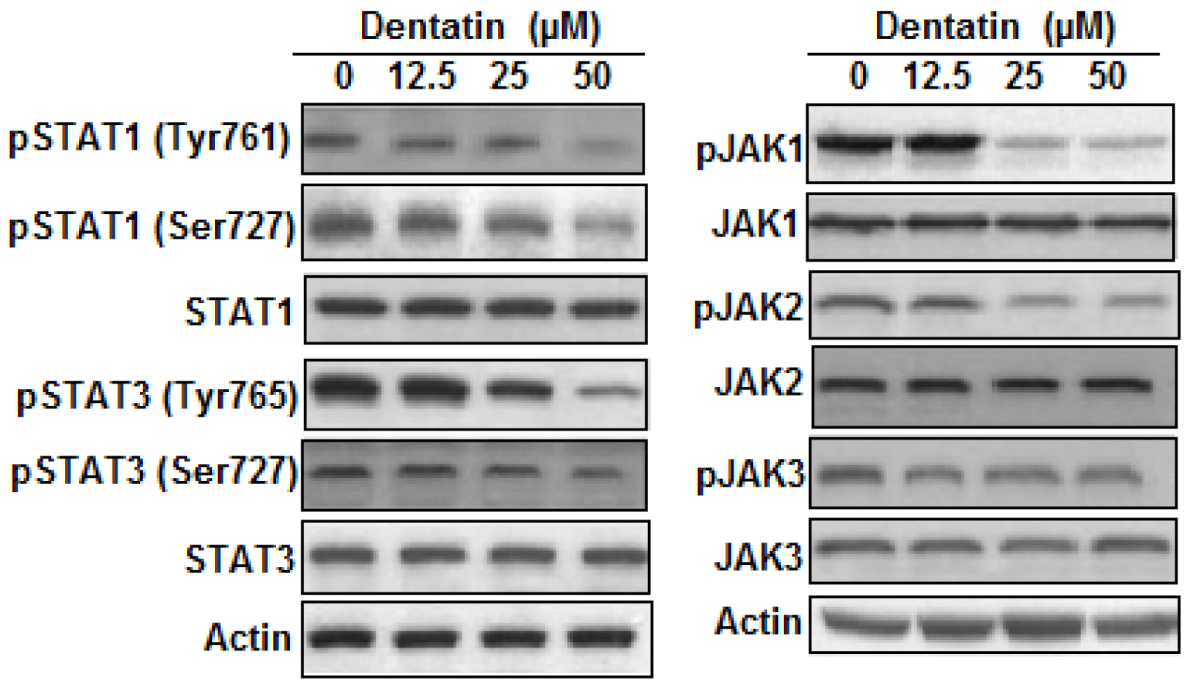

Figure 8. Dentatin inhibits the JAK/STAT signalling pathway in HT-29 cells as depicted by western blot analysis. The Figure shows that dentatin inhibits the JAK/STAT signalling pathway in HT-29 cells in a concentration-dependent manner. The experiments were performed in triplicate.

Dentatin inhibited the migration of HT-29 cells

The effect of Dentatin was also investigated on the migration of the HT-29 cells by transwell assay. It was found that Dentatin inhibited the migration of the HT-29 cells in a concentration-dependent manner. The wound width decreased considerably in the control in comparison to Dentatin-treated cells (Figure 7).

Dentatin inhibited the JAK/STAT

The effects of Dentatin were also examined on the JAK/STAT signalling pathway by western blot analysis. It was found that Dentatin inhibited the phosphorylation of STAT1 (Tyr761), STAT1 (Ser727), STAT3 (Tyr765), STAT3 (Ser727), pJAK1, JAK2 and JAK3 dose-dependently (Figure 8). However, the levels of STAT1, STAT3, JAK1, 2 and 3 remained constant.

Discussion

Colon cancer is a malignancy with high mortality worldwide. Over the years the incidence of colon cancer has increased significantly [10]. To curb the increasing incidence of colon cancer, new treatment strategies need to be explored. Identification of the safer plant-derived anticancer agents may prove essential to manage the growing incidence of the cancers around the world [11]. Herein, the anticancer effects of the carbazole alkaloid Dentatin were investigated on the HT-29 colon cancer cells as well as the CCD-18CO normal colon cells. Dentatin caused significant decrease in the viability of the HT-29 cells with minimal effects on the viability of the normal CCD-18CO cells. Previous studies had indicated that Dentatin causes arrest of the cancer cells [7]. Hence, the effect of Dentatin was also examined on the distribution of the HT-29 cells in different cell cycle phases and it was found that this molecule caused arrest of the HT-29 cells in the S-phase of the cell cycle. Studies have also shown that many of the anticancer drugs halt the growth of cancer cells by causing cell cycle arrest [12]. Cycle cell arrest is associated with alteration in the expression of several of the marker proteins [13]. Herein, it was found that the expression of cyclin D was increased while that of cyclin A and B1 was considerably decreased. Next, the electron microscopy analysis of the Dentatin-treated colon cancer cells was also performed and the results revealed that Dentatin triggers the formation of autophagic vesicles in the HT-29 cells, indicative of autophagy. Autophagy is a vital mechanism that helps eliminate the harmful cells [14]. The induction of autophagy was also connected with upregulation of LC3 II and downregulation of Beclin-1 expression, which is characteristic of autophagy [15]. Cell migration is considered important for the development of metastasis of cancer cells [16]

and herein we observed the effects Dentatin on the migration of the HT-29 cells by transwell assay. The results showed that this molecule inhibits significantly the migration of the HT-29 colon cancer cells. Several of the signalling pathways are dysregulated in cancer cells and JAK/STAT signalling is one such pathway. It plays an important role in development and tumorigenesis of cancer cells [8]. Studies have reported that JAK/STAT pathway may prove a beneficial target for the anticancer drugs [9] and in this study we found that Dentatin blocks this pathway, indicative of its anticancer potential.

Conclusion

It is concluded that Dentatin inhibits the growth of colon cancer cells by S-phase cell cycle arrest and autophagy. It also inhibits the migration and modulates the expression of the JAK/STAT pathway in colon cancer cells. As such, Dentatin may prove a beneficial lead molecule and warrants further investigations.

Conflict of interests

The authors declare no conflict of interests.

References

1. Ricci-Vitiani L, Lombardi DG, Pilozzi E et al. Identification and expansion of human colon cancer initiating cells. Nature 2007;445:111.
2. Petersen GM, Roth MP. Genetic epidemiology of colon cancer. A primate model for the study of colitis and colonic carcinoma. The Cotton-top tamarin (Saguinus Oedipus) 2018, Jan 18:187.
3. 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.
4. Ng RC, Kassim NK, Yeap YS, Ee GC, Yazan SL, Musa KH. Isolation of Carbazole Alkaloids and Coumarins from Aegle marmelos and Murraya koenigii and Their Antioxidant Properties. Sains Malaysiana 2018;47:1749-56.
5. 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.
6. Arbab IA, Looi CY, Abdul AB et al. Dentatin induces apoptosis in prostate cancer cells via Bcl-2, Bcl-xL, survivin downregulation, caspase-9,-3/7 activation, and NF-κB inhibition. Evidence-Based Compl Altern Med 2012;7:21-6.
7. Taha MM, Abdelwahab SI, Ali HM, Mohan S. Dentatin isolated from Clausena excavata induces apoptosis in MCF-7 cells through the intrinsic pathway with involvement of NF-κB signalling and G0/G1 cell cycle arrest: a bioassay-guided approach. J Ethnopharmacol 2013;145:343-54.
8. Thomas SJ, Snowden JA, Zeidler MP, Danson SJ. The role of JAK/STAT signalling in the pathogenesis, prognosis and treatment of solid tumours. Br J Cancer 2015;113:365.
9. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB, Laurence A. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Ann Rev Medicine 2015;66:311-28.
10. Reddy BS. Diet and colon cancer: evidence from human and animal model studies. In:Diet, nutrition and cancer: a critical evaluation. 2018 Jan 18, pp 47-66. CRC Press.
11. Yarla NS, Bishayee A, Sethi G et al. Targeting arachidonic acid pathway by natural products for cancer prevention and therapy. Semin Cancer Biol 2016;40: 48-81
12. Hua F, Li CH, Chen XG, Liu XP. Daidzein exerts anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting the Raf/MEK/ERK cascade. Int J Mol Med 2018;41:3485-92.
13. Otto T, Sicinski P. Cell cycle proteins as promising targets in cancer therapy. Nat Rev Cancer 2017 Feb;17: 93.
14. Karakaş HE, Gözüaçik D. Autophagy and cancer. Turk J Biol 2014;38:720-39.
15. Schläfli AM, Berezowska S, Adams O, Langer R, Tschan MP. Reliable LC3 and p62 autophagy marker detection in formalin fixed paraffin embedded human tissue by immunohistochemistry. Eur J Histochem 2015;59: 6-9.
16. Lawson CD, Ridley AJ. Rho GTPase signaling complexes in cell migration and invasion. J Cell Biol 2018;217;447-57.