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ORIGINAL ARTICLE

Isolation and Anticancer Effect of Brucine in Human Colon Adenocarcinoma Cells HT-29

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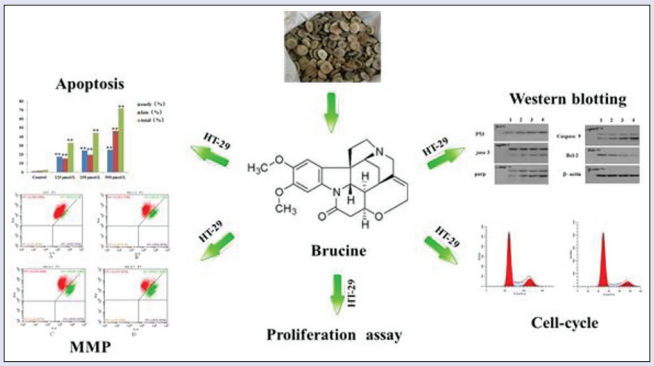
ABSTRACT

Context: Brucine is broadly used in the treatment of numerous types of tumors, and the application of brucine to colon cancer is stated. However, HT-29 cells have established comparatively little consideration, and the mechanism underlying the antitumor activity leftovers largely unknown. **Objectives:** The objective of the study is to isolate and examine the effect of brucine on human colon adenocarcinoma cell line HT-29. **Materials and Methods:** Crude brucine was acquired by the extraction of *Nux vomica* with 80% EtOH. Diatomite chromatography and semipreparative high-performance liquid chromatography were used to obtain brucine in pure form. HT-29 cells were treated with brucine (125, 250, and 500 μM) for 24–72 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to assess the cell proliferation. Annexin V-FITC/propidium iodide (PI) staining was used to identify the activity of apoptotic. Flow cytometry was used to scrutinize the effect of brucine on cell cycle progression and mitochondrial membrane potential (MMP). Bcl-2, p53, caspase-3, PARP, and caspase-9 were spotted by Western blotting test. **Results:** Brucine reduced cell viability with an IC_{50} value of 0.368, 0.226, and 0.168 μmol/L at 24, 48, and 72 h, respectively. The apoptosis of HT-29 was persuaded by 33.06%, 44.47%, and 71.96% at 125, 250, and 1000 μmol/L of brucine, respectively. Brucine at 250 μmol/L led to cell cycle arrest in the G1/S/G2 phase and inhibited the HT-29 cells in the G1 phase. H1-UL/H1-UR was determined to be 1.79, 1.26, and 0.54 at 125, 250, and 1000 μmol/L, respectively. Brucine at 125, 250, and 1000 μmol/L downregulated the expression of Bcl-2 but augmented the expression of p53, caspase-3, PARP, and caspase-9. **Conclusion:** The outcomes displayed that brucine could prevent cell proliferation, arrest the cell cycle, and increase the loss of MMP in the HT-29 cell line. Furthermore, brucine could also persuade cell apoptosis through the expression of proapoptotic and apoptotic proteins comprising p53, caspase-3, caspase-9, and PARP. To sum up, our preclinical data designated that brucine was a probable therapeutic agent for colon cancer.

Key words: Apoptosis, brucine, colon cancer, HT-29, isolation

SUMMARY

- The study exposed that brucine could hinder cell proliferation, arrest the cell cycle, increase the loss of mitochondrial membrane potential, and tempt cell apoptosis through the expression of proapoptotic and apoptotic proteins, comprising p53, caspase-3, PARP, and caspase-9 in the HT-29 cell line. This *in vitro* study delivered important indications that brucine was a probable therapeutic agent for colon cancer.



Abbreviations used: MMP: Mitochondrial membrane potential; HPLC: High-performance liquid chromatography; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS: Fetal bovine serum; DMEM: Dulbecco's modified Eagle medium; ANOVA: One-way analysis of variance.

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INTRODUCTION

Colon cancer, very common cancer in men and women around the world,[1,2] is related with old age, red meat consumption, smoking, obesity, high fat intake, and the lack of physical exercise.[3-6] Despite the accessibility of better drugs and treatments for cancer, colon cancer is still the fourth most common cancer, instigating about 700,000 deaths a year.[7-9] Therefore, finding drugs to treat colon cancer is mainly vital.

Strychni Semen, known as Maqianzi in China, has been a frequently used herbal drug in traditional Chinese medicine for a long time and has important curative properties on rheumatoid arthritis, swelling, trauma, etc.[10,11] The main source of Strychni Semen in China is the dried seed of *Strychnos nux-vomica* L.[12] Studies have exposed that the main active ingredients of Strychni Semen are alkaloids, of which brucine is the most plentiful. Brucine has been extensively used for the treatment of various tumors,[13-17] and this comprises colon cancer.[18-20] There are many cell lines of colon cancer studied, but there have been few reports on the treatment of colon cancer with HT-29 cell line, and the study on its mechanism is not perfect.

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In this study, brucine was isolated from Strychni Semen and recognized by spectroscopic analyses such as MS and NMR. In addition, proliferation assays, cell cycle, apoptosis, and mitochondrial membrane potentials (MMPs) were assessed to regulate the role of brucine in HT-29 cell growth. Western blotting was performed to study the mechanisms involved. This study may deliver a scientific basis for the application of brucine in the treatment of colon cancer.

MATERIALS AND METHODS

Materials

Most of the reagents were acquired from Tianjin Damao Reagent Company (Tianjin, China). Silica gel column chromatography and thin-layer chromatography were all procured from Qindao Ocean Chemical plant (Qindao, China). The separation of alkaloids was conducted by thin-layer chromatography and semipreparative high-performance liquid chromatography. ^{1}H NMR and ^{13}C NMR spectra all with methanol-d_4 as the solvent were recorded on Bruker-400 NMR spectrometers. The human colon adenocarcinoma cell line HT-29 was procured from Chinese Academy of Sciences (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was bought from Sigma-Aldrich (St. Louis, MO, USA). Penicillin-streptomycin, fetal bovine serum (FBS), and calf serum were obtained from Gibco (Grand Island, USA). BCA protein assay reagent kit was acquired from Biyuntian Bioengineering Institute (Shanghai, China). Antibodies for p53, caspase-3, PARP, caspase-9, Bcl-2, and β-actin were obtained from Cell Signaling Technology (Danvers, MA, USA). The kits for testing cell cycle, cell apoptosis, and MMPs were found from Nanjing Kaiji Bioengineering Institute (Nanjing, China).

Plant materials

Nux vomica seeds were obtained from Anhui Bozhou Chinese Herbal Medicine Wholesale Co. Ltd. and was recognized as *Nux vomica* genus *Strychnos nux-vomica* L. dry mature seeds and then processed products for sand iron law by Prof. Pei-Xiangping. The certificate samples were kept in the Herbarium of Shanxi University of Chinese Medicine until use.

Extraction and isolation

The seeds of *Nux vomica* (10.0 kg) were extracted three times with 80% EtOH (60 h and each) after soaked for 12 h under reflux conditions. The combined extracts (about 2.7 L) were concentrated under condensed pressure. The enriched extract was suspended in aqueous hydrochloric acid solution (pH = 2) and partitioned with dichloromethane (3 × 11 L). After removal of the dichloromethane extracts, the residual material was liquefied in a 10% NaOH aqueous solution and partitioned with dichloromethane (4 × 10 L). The dichloromethane fraction (120 g) was fractionated on diatomite chromatography eluted consecutively with petroleum ether/ethyl acetate (2:1, 1:3, and 1:8) and ethyl acetate/methanol (3:1, 1:2, and 0:1) to afford six fractions (A–F). Fraction A was dissolved in H_2O (200 mL) and then loaded onto a Venusil XBP C_{18} column (10 μm, 21.2 mm × 250 mm) and sequentially eluted with mixtures of MeOH and H_2O (0%, 25%, 50%, 75%, and 100% MeOH; 2 L of each solvent mixture) to deliver brucine.[21,22]

Cell culture

HT-29 cells were cultured in MC5A Dulbecco's modified Eagle medium with 10% calf serum, 10% FBS, and 1% penicillin-streptomycin solution, with 5% CO_2 in air at 37°C.

Proliferation assay

The MTT assay was used to identify the effect of brucine on cell proliferation. HT-29 cells with a concentration of 3 × 10^4 cells/mL were inoculated in 96-well plates for 24 h and treated with brucine with the concentrations of 31.25, 62.50, 125, 250, 500, and 1000 μmol/L for 24, 48, and 72 h at 37°C in a 5% CO_2 incubator, respectively. Then, each well was added by 10 μL of MTT and cultured for 4 h at 37°C, 490 nm was used to quantity the wavelength, and GraphPad Prism 6 software package (GraphPad, Inc., La Jolla, CA, USA) was employed to calculate IC_{50} values.

Induction of apoptosis

HT-29 cells were diluted to 2.5 × 10^4 cells/mL, inoculated into a 6-well plate for 24 h, and then treated with brucine with the concentrations of 125, 250, and 500 μmol/L for 24 h, respectively. Cells were extracted with trypsin-free EDTA, washed twice with frozen PBS, and then stained for 15 min at room temperature darkness with 5 μL annexin V-FITC and 5 μL PI. Cell apoptosis was distinguished by flow cytometry.

Cell cycle analyses

HT-29 cells were diluted to 2.5 × 10^4 cells/mL, inoculated into a 6-well plate for 24 h, and treated with brucine with the concentrations of 250 μmol/L for 48 h. Then, treated cells were harvested by trypsinization, washed twice with frozen PBS and fixed with precooled 70% ethanol at 4°C for 4 h, and washed with ice-cold PBS twice and centrifuged at 1000 rpm for 5 min. The cells were washed twice with frozen PBS, treated with 50 μg/mL RNAse (100 μL) at 37°C for 30 min, and stained with 50 μg/mL (PI, 400 μL) at 37°C for 30 min in the dark. Cell cycle outlines were closely analyzed with flow cytometry.

Mitochondrial membrane potential (Δψm) analysis

HT-29 cells were inoculated with 3 × 10^4 cells/mL in a 96-well plates for 24 h and then cultured with brucine (125, 250, and 500 μmol/L) in a 5% CO_2 incubator at 37°C for 48 h, respectively. The intracellular MMP was evaluated using JC-1 dye. Briefly, the cells were incubated with a JC-1 staining buffer for 20 min in the dark and washed with 1 × incubation buffer. The fluorescence intensity was examined by fluorescence microscopy, the excitation wavelength of green fluorescence at 514 nm and the emission wavelength of 529 nm were observed, while the excitation wavelength of red fluorescence at 585 nm and the emission wavelength of 590 nm were also scrutinized.

Western blotting

HT-29 cells were cultured in 6-well plates, treated with 125, 250, and 500 μmol/L solutions of brucine, respectively, and then washed with frozen PBS and lysed using a lysis buffer (PMSF). BCA protein assay kits were employed to measure proteins. The proteins were separated by 8%–10% SDS-PAGE, electrophoresed at 60 V, and then moved to PVDF membranes. After blocking with 5% nonfat dry milk in TBS and 0.05% Tween 20 for 2 h at room temperature, the blots were probed with the corresponding primary antibodies overnight at 4°C and then followed by secondary antibody (diluted 1:2000) at room temperature for 2 h. Enhanced chemiluminescence was employed to identify specific bands.

Statistical analysis

SPSS 19.0 (International Business Machines Corporation, NewYork, USA) was used for data analysis. Data were articulated as the mean ± standard deviation. One-way analysis of variance was used to compare the difference between groups, and $P < 0.05$ was measured to be statistically significant.

RESULTS

Identification of brucine

Brucine was found by extraction from *Nux vomica* and separation as described earlier and categorized by MS, ^{1}H NMR, and ^{13}C NMR spectroscopy.

Brucine

ESI-MS *m/z*: 394.1887 (M + H)+, calculated value ($C_{23}H_{26}N_2O_4$): *m/z*: 394.1893. ^{1}H NMR (400 MHz, methanol-d_4) δ_H 7.76 (s, 1H), 6.69 (s, 1H), 5.96 (s, 1H), 4.26 (d, *J* = 8.4 Hz, 1H), 4.13 (dd, *J* = 13.9, 6.9 Hz, 1H), 4.04 (d, *J* = 5.8 Hz, 1H), 3.86 (s, 3H), 3.82 (s, 4H), 3.81 (s, 1H), 3.77 (s, 1H), 3.73 (s, 1H), 3.33–3.26 (m, 1H), 3.15 (s, 1H), 3.07 (dd, *J* = 17.5, 8.4 Hz, 1H), 2.83 (s, 1H), 2.78 (s, 1H), 2.61 (dd, *J* = 17.5, 3.0 Hz, 1H), 2.35 (dt, *J* = 14.3, 3.9 Hz, 1H), 1.93–1.88 (m, 1H), 1.85 (dd, *J* = 12.5, 6.2 Hz, 1H), 1.48 (d, *J* = 14.5 Hz, 1H), 1.27 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ_C: 168.96, 149.55, 146.52, 138.73, 135.91, 129.37, 122.46, 105.58, 101.12, 77.16, 64.52, 60.18, 60.10, 56.57, 56.27, 52.60, 52.06, 50.64, 50.21, 48.07, 42.31, 42.05, 31.39, 26.48. In combination with the ^{1}H and ^{13}C spectral analysis, the data of the compound were essentially reliable with that of the brucine by comparison with the literature.[23-25] Therefore, the compound was recognized as brucine.

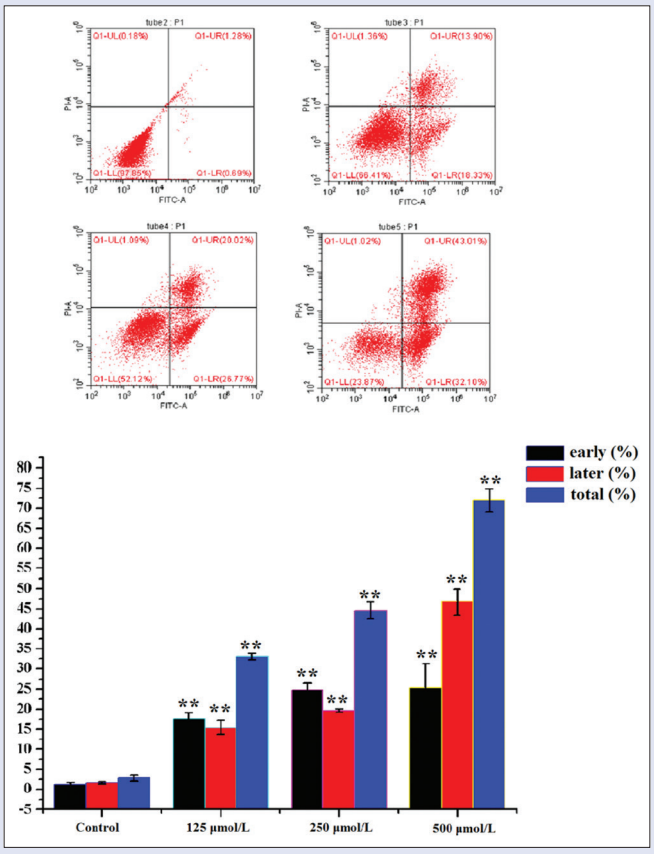

Figure 1: Flow cytometric analysis of the early, late, and total number of apoptotic cells in HT-29 cells treated with brucine at different concentrations (125, 250, and 500 μmol/L) for 24 h. The values are expressed as the means ± standard deviation (*n* = 3). ***P* < 0.01 was considered as significant versus the control

Proliferation assay

The cytotoxic effect on HT-29 cells treated with brucine at 31.25, 62.5, 125, 250, 500, and 1000 μmol/L was achieved using the MTT assay after 24, 48, and 72 h of treatment. Brucine has time-dependent and concentration-dependent antiproliferation effects on HT-29 cells. The inhibition rate augmented with increasing time and concentration. HT-29 cells were treated with brucine with the concentrations of 31.25-1000 μmol/L, and the inhibitory rate was found to be <50% in the concentration range of 31.25–125 μmol/L. The inhibitory rate rose to more than 50% at 250 μmol/L for 72 h. Finally, the inhibitory rate of brucine in HT-29 reached to 83.31% ± 0.63%, 90.84% ± 2.47%, and 98.54% ± 0.68% at 1000 μmol/L of brucine, for 24, 48, and 72 h, respectively (The results were shown in Table 1).

Induction of apoptosis

Induction of apoptosis is the major consequence of many cytotoxic drugs on tumor cell death.[26] The results presented that the number of late, early, and total apoptotic cells (17.59%, 15.47%, and 33.06% in 125 μmol/L of brucine; 24.84%, 19.63%, and 44.47% in 250 μmol/L of brucine; and 25.31%, 46.65%, and 71.96% in 500 μmol/L of brucine, respectively) was assessed in comparison with untreated controls. The numbers of late, early, and total apoptotic cells all suggestively increased upon treatment with brucine at all concentrations [Figure 1]. Hence, the results exhibited that brucine inhibited the apoptosis and augmented the apoptosis of HT-29 cells in a dose-dependent manner.

Cell cycle analyses

To clarify the anticancer properties and apoptotic effects of brucine on HT-29 cells, the cell cycle profile of brucine after 48 h was designed [Figure 2]. Compared with the untreated control group,

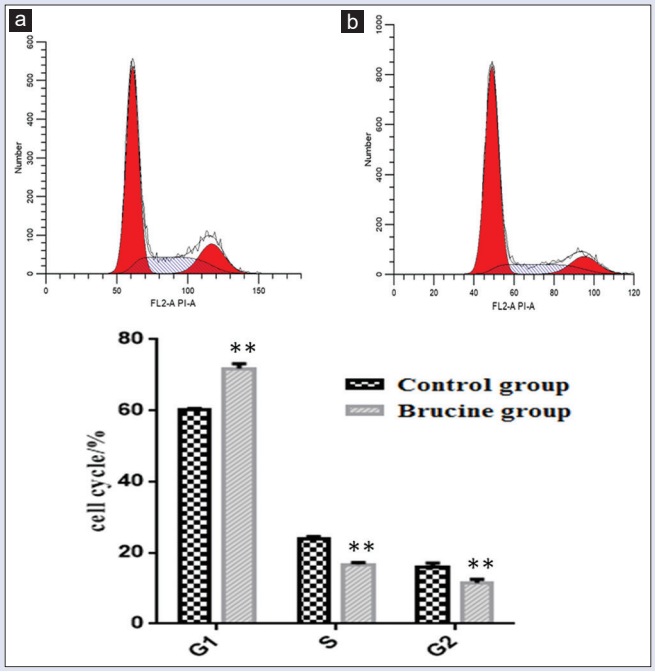

Figure 2: Schematic representation of cell cycle distribution in cells treated with brucine for 48 h. (a) Control group; (b) brucine group (250 μmol/L); ***P* < 0.01 was considered as significant versus the control

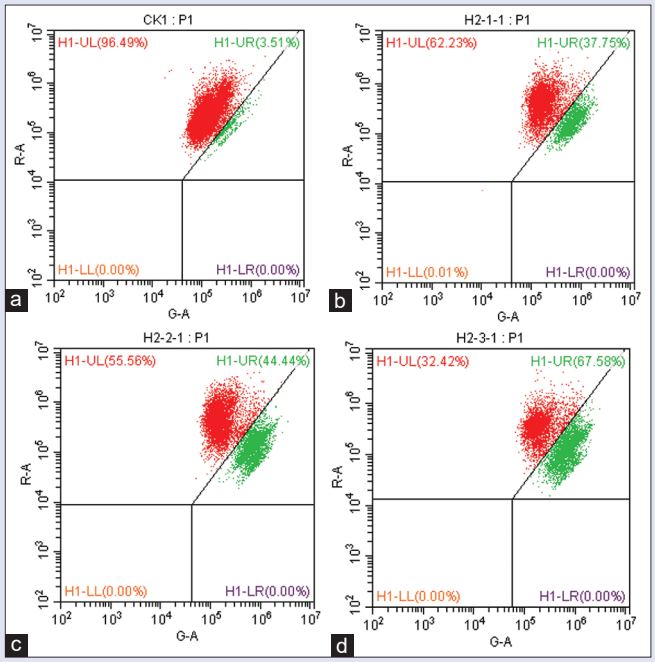

Figure 3: Mitochondrial membrane potential analysis in HT-29 cells. (a) Control group; (b) brucine group (125 μmol/L); (c) brucine group (250 μmol/L); (d) brucine group (500 μmol/L)

Table 1: Inhibitory rate and IC_{50} values of brucine on HT-29 cells at 24, 48, 72h

Concentration (μmol/L)	Inhibitory rate (%)		
	24 h	48 h	72 h
31.25	2.33±0.13	5.01±0.98	9.84±1.06
62.5	8.53±1.95	12.68±2.24	19.80±1.55
125	16.58±2.26	35.18±1.07	39.12±1.86
250	30.79±1.30	45.82±1.24	54.49±1.60
500	62.63±1.89	78.03±1.93	84.86±2.32
1000	83.31±0.63	90.84±2.47	98.54±0.68
IC_{50} (μmol/mL)	0.368	0.226	0.168

IC_{50}: 50% Inhibitory Concentration

250 μmol/L brucine knowingly augmented the proportion of G1 phase cells and significantly abridged the proportion of S phase and G2 phase cells. Furthermore, it exposed that knockdown of brucine at 250 μmol/L could lead to cell cycle arrest in G1/S/G2 phase and inhibit the HT-29 cells in the G1 phase.

Mitochondrial membrane potential (Δψm) analysis

The green fluorescence of the brucine-treated group augmented expressively compared with the control group, along with the reduction of red fluorescence, which designates the loss of MMP [Figure 3]. These results establish that brucine effectively augmented the loss of MMP, which contributes to apoptosis in HT-29.

Western blotting

The results revealed that 125 μmol/L brucine knockdown improved the levels of p53 and PARP. Higher concentrations of brucine (250 and 500 μmol/L) knockdown boosted the levels of p53, caspase-3, PARP, and caspase-9, while the expressions of Bcl-2 were expressively downregulated at all concentrations of brucine [Figure 4].

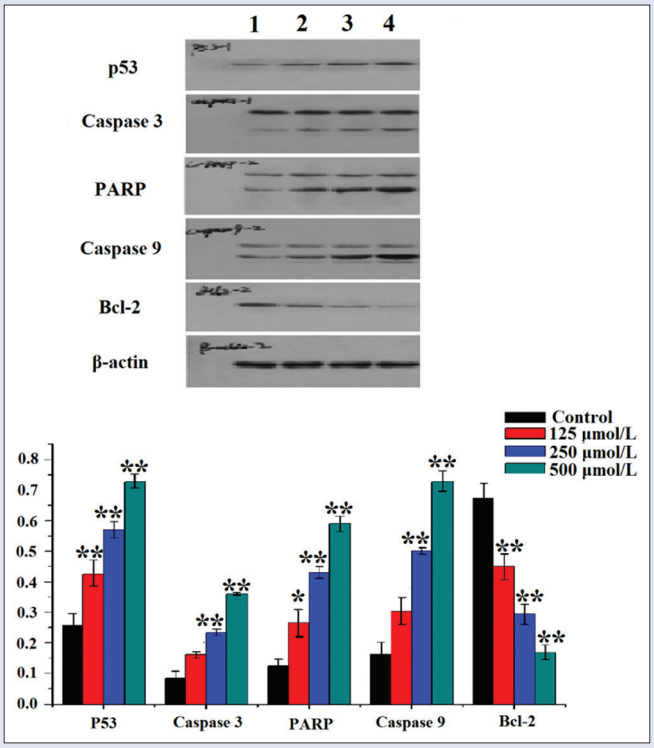

Figure 4: The expression of p53, caspase-3, PARP, caspase-9, Bcl-2, and β-actin was assessed by western blotting and the ratio of the expression was quantified and compared with the control group. 1: Control group; 2: Brucine group (125 μmol/L); 3: Brucine group (250 μmol/L); 4: Brucine group (500 μmol/L). *$P < 0.05$, **$P < 0.01$ considered as significant versus the control

DISCUSSION

Brucine is a natural product and has established significant attention in recent years as a potent antitumor agent. A number of reports have publicized that brucine displays cytotoxic activity against a range of diverse types of cancer, containing colon cancer. Among the various colon cancer cell lines, the effects of brucine on HT-29 cells have received a great deal of attention. Therefore, in this study, broad research was put forth toward the studying the effect and mechanism of brucine on HT-29 cells. Cell proliferation is a key feature in the development of cancer.[27] Our study displayed that brucine with 250, 500, and 1000 μmol/L all repressed HT-29 cell proliferation. The ability of brucine to hinder the growth of HT-29 cell may also be related to encouraging apoptosis and blocking cell cycle progression. Apoptosis plays a noteworthy role in normal physiological and homeostatic processes and is also a defense mechanism against systemic prearranged attacks that lead to the death of senescent or damaged cells.[28,29] Our results designated an inhibitory effect of brucine on HT-29 cells in a dose-dependent way, and it could hinder HT-29 cells in the G1 phase. MMP is an early pointer of cell apoptosis and mitochondrial function initiation. A large body of evidence recommends that MMP testing can be employed as a more precise measure to evaluate early mitochondrial damage.

In the present study, brucine effectually augmented the loss of MMP. In addition, the collapse of MMP was found to persuade the mitochondrial permeability and lead to the release of cytochrome C into the cytoplasm of mitochondria, thus indorsing the formation of apoptotic complex. Caspases are vital for cancer suppression and play an important role

in the apoptosis of cancer cells.[30] Caspase-3 and caspase-9 are central constituents of apoptotic machinery in cells. The activation of caspase-3 is a hallmark in apoptotic process,[31] and caspase-9 can persuade caspase-3 to enter the apoptotic complex,[32] which can produce many cells and biochemical events related to apoptosis.[33,34] PARP is measured to be an important pointer of apoptosis and is typically measured as an indicator of caspase-3 activation.[9]

In the present study, brucine stimulated damaged HT-29 cells to undergo apoptosis and prohibited the proliferation of HT-29 cells by upregulation of PARP, caspase-3, and caspase-9. Furthermore, the Bcl-2 family of proteins is an important mediator of cell death and existence and plays a key role in the regulation of mitochondrial apoptosis pathway. Bcl-2 is a well-known antiapoptotic factor.[35,36] p53 is a multifunctional protein involved in the activation of the transcription factors that control the expression of apoptotic genes.[9] It had been reported that the cell apoptosis was promoted by activating the mitochondria-mediated apoptosis pathway, accumulating the apoptotic promoters and constraining the expression of antiapoptotic Bcl-2 family.[37] In our work, brucine augmented the expression of p53 protein and diminished the expression of Bcl-2 protein in a dose-dependent manner in HT-29 cells. These results recommended that brucine may contribute in the apoptotic pathway by inducing the expression of p53 gene and Bcl-2 protein.

CONCLUSION

This study confirmed that brucine could hinder cell proliferation, persuade apoptosis, arrest the cell cycle, and upsurge the loss of MMP in HT-29 cells. Underlying mechanisms may include augmented the expression of p53, caspase-3, PARP, and caspase-9 and reduced the expression of Bcl-2. The consequence of this study involves a possible clinical application of brucine for colon cancer treatment. However, more animal and human trials are desirable to check the efficacy of brucine on averting and treating colon cancer.

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Conflicts of interest

There are no conflicts of interest.

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