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Digital Object Identifier (DOI)
https://doi.org/10.1016/j.jff.2012.01.009

Notes/Citation Information
Published in *Journal of Functional Foods,* v. 4, issue 1.

Available at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Radical scavenging activity of black currant (*Ribes nigrum* L.) extract and its inhibitory effect on gastric cancer cell proliferation via induction of apoptosis

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ARTICLE INFO

Article history:
Received 12 November 2011
Received in revised form
19 January 2012
Accepted 19 January 2012
Available online 12 February 2012

Keywords:
Antioxidant activity
Antiproliferation
Apoptosis
Black currant
Gastric cancer

ABSTRACT

A black currant extract (BCE) was prepared and its antiproliferative activity against gastric cancer SGC-7901 cells was investigated. Strong 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical scavenging activities and a high reducing power were confirmed with BCE. BCE inhibited the proliferation of SGC-7901 cells in a dose- and time-dependent manner, and the IC_{50} were 12.7, 10.2 and 9.0 mg/mL for 12, 24 and 48 h, respectively. Morphologic observations with inverted and fluorescence microscopes yielded vivid evidence of cell shrinkage, formation of cytoplasmic filaments, condensation of nuclear chromatin, and cell apoptosis in the presence of BCE. Flow cytometric analysis also showed that BCE treatment at concentrations of 10–20 mg/mL resulted in marked reductions of viable cells. The high concentration of phenolic compounds present in the BCE (12.2 mg/mL), including six prominent anthocyanins identified by HPLC–ESI-MS^2, appeared to be responsible for BCE's antiradical activity and anticancer effects. These findings of inhibition of SGC-7901 cells and induction of apoptosis suggest that black currant may contribute to the reduction in gastric cancer risk.

1. Introduction

Epidemiological studies have provided strong evidence that diets rich in fruits and vegetables reduce the incidence of cancer (La Vecchia, Altieri, & Tavani, 2001). It is widely believed that phytochemicals in fruits and vegetables are associated with the anticancer effects by affecting the molecular events in the initiation, promotion, and progression stages. Phenolic compounds, in particular, have drawn increasing attention since they possess potent antioxidant potential and are abundantly present in high amounts in fruits and vegetables that exhibit anticancer activity (García-Alonso, Ros, & Periago, 2006; Netzel et al., 2007). However, since more than 5000 phytochemicals have been identified and different antiproliferation mechanisms have been reported, it is believed that synergistic or additive biological effects of multiple phytochemicals, rather than a single compound or a group of compounds, contribute to cancer prevention (Birt, Hendrich, & Wang, 2001; Liu, 2003; Sun & Liu, 2006).

Berry fruits contain a variety of phenolic compounds, such as phenolic acids, flavonoids, and tannins, and are a rich source of anthocyanins, flavonols, and ellagitannins (Määttä-Riihinen, Kamal-Eldin, Mattila, Gonza-les-Paramas, & Torronen, 2004). The beneficial health effects of anthocyanins in berries are associated with their antioxidant, anti-inflammatory, and chemopreventative properties. Some berry extracts also have been shown to inhibit the growth of several cancer cells. Shin, Ryu, Liu, Nock, and Watkins (2008) reported that strawberry extracts were able to inhibit human liver cancer HepG2 cell proliferation by as much as 80%. Additionally,

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1756-4646/$ - see front matter
doi:10.1016/j.jff.2012.01.009

phenolic compounds from blueberries could inhibit liver cancer HepG2 cell growth and induce apoptosis (Yi, Akoh, Fischer, & Krewer, 2006).

Black currant (*Ribes nigrum* L.), a dark-pigmented fruit, native to central and northern Europe and northern Asia and now also cultivated in several states in the US (Oregon, New York, Vermont, and Connecticut), has received much attention in recent years due to its superior antioxidant activity and high anthocyanin content compared with many other fruits. Research on black currant has so far concentrated on the extraction, identification, and characterization of anthocyanins and other phenolic compounds in black currant (Cacace & Mazza, 2003; Slimestad & Solheim, 2002). Amakura, Umino, Tsuji, and Tonogai (2000) showed that black currant extract (BCE) was the second strongest radical scavenger among nine types of berries.

It was not until recently that studies began to examine the potential effects of black currant on cancer cells. BCE has been shown to suppress the growth of colon cancer HT-29/HCT-116 cells, breast cancer MCF-7 cells and HL-60 human promyelocytic leukemia cells (Katsube, Iwashinta, Tsushida, Yamaki, & Kobori, 2003; Olsson, Gustavsson, Andersson, Nilsson, & Duan, 2004; Wu, Koponen, Mykkänen, & Törrönen, 2007). It is not clear whether BCE can be effective in inhibiting gastric cancer cell despite gastric cancer being the second most common cause of cancer-related mortality in the world (Houghton, Fox, & Wang, 2002). In the present study, BCE was prepared and its activity against free radicals and the proliferation of gastric cancer SGC-7901 cells investigated.

2. Materials and methods

2.1. Materials and reagents

Freshly harvested and subsequently frozen black currant (*R. nigrum* L.) was purchased from Gaotai Food Corporation (Binxian, Heilongjiang, China) and stored at −20 °C until use. Gastric cancer SGC-7901 cells were obtained from Harbin Medical University (Harbin, Heilongjiang, China). Testing chemicals, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile, water, formic acid, roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), dimethyl sulphoxide (DMSO), and penicillin–streptomycin were purchased from Solabio Corporation (Beijing, China). WST-1 Cell Proliferation and Cytotoxicity Assay Kit and Hoechst Staining Kit were obtained from Beytime Institute of Biotechnology (Hangzhou, Zhejiang, China). Annexin V-FITC-Propidium Iodide Apoptosis Detection Kit was acquired from Biosea Biotechnology Co. Ltd. (Beijing, China). All other chemicals and reagents were of analytical grade.

2.2. Extract and sample preparation

Frozen black currant was thawed at room temperature, and then homogenized by blending for 3 min. An aliquot (100 g) of homogenized black currant was mixed into 900 mL 50% ethanol at 35 °C for 2 h in an enclosed flask with constant agitation. The homogenate was filtered through Whatman No. 2 filter paper, followed by a 0.45 μm filter membrane. The filtrate was subsequently concentrated by rotary vacuum evaporator (Eyela N-1000, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 40 °C. The semi-dried samples were then reconstituted to a final volume of 50 mL with distilled water with the ratio of 2 g of black currant to 1 mL of BCE. The total phenolic content was 12.2 ± 0.1 mg/mL in BCE as determined by the method of Sun et al. (2009). The BCE samples were stored at −20 °C until use.

2.3. HPLC–ESI-MS^2 detection of phenolics in BCE

The phenolics in BCE were separated using high performance liquid chromatography (HPLC) (Waters Corp., Milford, MA, USA) equipped with a UV–Vis detector and identified by mass spectrometry (MS) with electrospray ionization (ESI) (Thermo Fisher Corp., San Jose, USA) according to the method described by Borges, Degeneve, Mullen, and Crozier (2010). Specifically, the BCE was diluted in water and filtered through a 0.22 μm filter paper, and 20 μL of the filtrate was injected into the HPLC column. Chromatographic separation was performed on a C18 column (250 × 4.6 mm, 5.0 μm) with mobile phase A (acetonitrile) and mobile phase B (0.1% formic acid in water, v/v) at a flow rate of 1 mL/min. The gradient elution programme was as follows: 0–30 min, 3–10% A; 30–60 min, 10–25% A. The HPLC column effluent was pumped directly into the MS at a flow rate of 0.2 mL/min. Analyses utilized the positive ion mode. Samples were analyzed using full-scan data-dependent MS^2 scanning from *m/z* 100 to 2000. Capillary temperature was 295 °C, sheath gas and auxiliary gas were 40 and 20 U, respectively, and the source voltage was 5 kV.

2.4. Radical scavenging activity

2.4.1. DPPH scavenging activity

The method described by Wu, Chen, and Shiau (2003) was used to measure the DPPH scavenging activity. Briefly, 0.1 mL of BCE at different concentrations (6.25, 12.5, 25, 50, 75 and 100 mg/mL) was mixed with 3 mL of 0.1 mM DPPH solution (dissolved in 95% ethanol). The mixture was incubated in dark at room temperature for 30 min, and the absorbance at 517 nm was read. DPPH scavenging activity (%) was calculated as (Ac − As) × 100/Ac, where Ac is the absorbance of the control (DPPH solution without BCE), and As is the absorbance of the sample.

2.4.2. ABTS scavenging activity

The ABTS radical scavenging activity was determined according to Ozgen, Reese, Tulio, Scheerens, and Miller (2006). The mixture of 3 mL of diluted $ABTS^{\cdot+}$ solution (absorbance of 0.70 ± 0.01 at 734 nm) and 20 μL of BCE at different concentrations of (6.25, 12.5, 25, 50, 75 and 100 mg/mL) was incubated in dark for 6 min and the absorbance at 734 nm was read. $ABTS^{\cdot+}$ scavenging activity (%) was calculated as (Ac − As) × 100/Ac, where Ac is the absorbance of the control (ABTS solution without BCE), and As is the absorbance of the sample.

2.5. Reducing power

The reducing power of BCE was determined using the procedure of Oyaizu (1986). An aliquot (0.5 mL) of BCE at different concentrations (6.25, 12.5, 25, 50, 75 and 100 mg/mL) was diluted in 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 mL of 1% potassium ferricyanide was then added. After incubation at 50 °C for 20 min, 2.5 mL of 10% trichloroacetic acid (w/v) were added. The mixture was centrifuged at 1000*g* for 10 min. Five milliliters of the supernatant were mixed with an equal volume of distilled water and 1 mL of 0.1% ferric chloride, and the absorbance was measured at 700 nm. The absorbance increase is indicative of an increase in the reducing power.

2.6. Cell culture and cell viability assay

Human gastric cancer SGC-7901 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin–streptomycin in an incubator (95% air and 5% CO_2) at 37 °C. Cells in the logarithmic growth phase were collected for further experiments. The viability of cells treated with BCE was measured using the WST-1 Cell Proliferation and Cytotoxicity Assay Kit in accordance with the manufacturer's instructions. Briefly, suspended cells were seeded at an initial concentration of 5×10^3 cells/well into a 96-well cell culture plate (Costar Corning, Rochester, NY). After incubation for 24 h, the old medium was tapped out and the cells were washed twice with phosphate buffer solution (PBS, 10 mM, pH 7.4).

Thereafter, 100 μL of a fresh culture medium containing BCE (final concentrations 0, 2.5, 5, 10, 15 and 20 mg/mL) were added into the plate, and the cells were further incubated for 12, 24, or 48 h to monitor activity or apoptosis. Blank wells contained 100 μL growth medium without cells. At the end of the incubation time, the treated medium was removed and the cells were washed twice with PBS. An aliquot (110 μL) of medium containing 10 μL of WST-1 reagent was added into each well, and cells were cultured for additional 4 h. The plate was shaken thoroughly for 1 min, and the absorbance (450 nm) of the samples was measured using a Bio-Rad 680 ELISA reader (Bio-Rad Laboratories, Hercules, CA). Cell viability (%) was expressed as the optical density ratio of the treatment to control.

2.7. Apoptosis and cell morphology

SGC-7901 cells were seeded at a density of 5×10^5 cells/well onto a cover slip loaded in 6-well plate (Costar Corning, Rochester, NY). After incubation for 24 h, the old medium was tapped out and the cells were washed twice with PBS. Fresh medium containing BCE (final concentrations 0, 2.5, 5, 10, 15 and 20 mg/mL) were added into the plate, and the cells were incubated for additional 12 or 24 h. At the end of each treatment time, cells were photographed with an inverted microscope (AE 31 model, Motic China Group Co. Ltd., Xiamen, China) under 200× magnification to observe morphological changes.

Following photographing, the cells were stained with Hoechst 33258 to observe features of cell apoptosis. Briefly, the treatment medium was removed and the cells were washed twice with PBS. This was followed by fixation with 0.5 mL fixing solution for 10 min and two times of washing with PBS. Subsequently, 0.5 mL Hoechst 33258 was added and the staining continued for 5 min. Stained cells were then washed twice with PBS. The cover slip was placed on a glass slide, and a drop of fluorescence quenching solution was added. Cell morphology were observed with a fluorescence microscope (DM 4000, Leica Optical Co. Ltd., Wetzlar, Germany) under 400× magnification.

2.8. Assays of apoptotic cells by flow cytometry

Apoptotic and necrotic cells were evaluated by annexin V (AV) binding and propidium iodide (PI) uptake using an AV-FITC-PI Apoptosis Detection Kit. Briefly, cells were placed into 6-well plate at a density of 5×10^5 cells/well and cultured for 24 h. The old medium was replaced by fresh medium containing BCE (final concentrations 0, 2.5, 5, 10, 15 and 20 mg/mL). After 24 h treatment with BCE, all adhering and floating cells were collected and centrifuged at 4 °C (1000*g*, 10 min). The cell pellet was washed twice with PBS under the same centrifugal condition. Subsequently, the cell pellet was suspended in 200 μL binding buffer, and the suspended cells were stained with 10 μL AV-FITC in dark at room temperature for 15 min. Then, 300 μL binding buffer and 5 μL PI were added to the AV-FITC-stained cells to distinguish the necrotic cells. The fluorescence of the cells was determined by flow cytometry (FACSCalibur, Becton Dickson, USA) within 1 h.

2.9. Statistical analysis

All experiments were carried out with triplicate sample analysis. Three independent experimental trials (replications) were conducted. Data were analyzed using the General Linear Models procedure of Statistix 8.1 software package (Analytical Software, St. Paul, MN) for microcomputer. Analysis of variance (ANOVA) was conducted to determine the significance of the main effects. Significant differences ($P < 0.05$) between means were identified using Tukey procedure.

3. Results and discussion

3.1. Identification of phenolics in BCE

Major UV peaks of the BCE HPLC spectrum at 365 nm are shown in Fig. 1, and the main compounds are presented in Table 1. These compounds were tentatively identified based on parent molecular ions, retention times, and the fragments reported in literature (Anttonen & Karjalainen, 2006; Borges et al., 2010; Määttä, Kamal-Eldin, & Törrönen, 2003; Slimestad & Solheim, 2002). Six particular anthocyanins, including delphinidin 3-*O*-glucoside, delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, cyanidin 3-*O*-rutinoside, peonidin 3-*O*-rutinoside, and pelargonidin 3-*O*-rutinoside, accounted for 52.1% of total peak area. This result was in good agreement with that reported by Slimestad and Solheim (2002), who also identified the first four anthocyanin compounds as listed above. In addition to anthocyanins, some other phenolics were

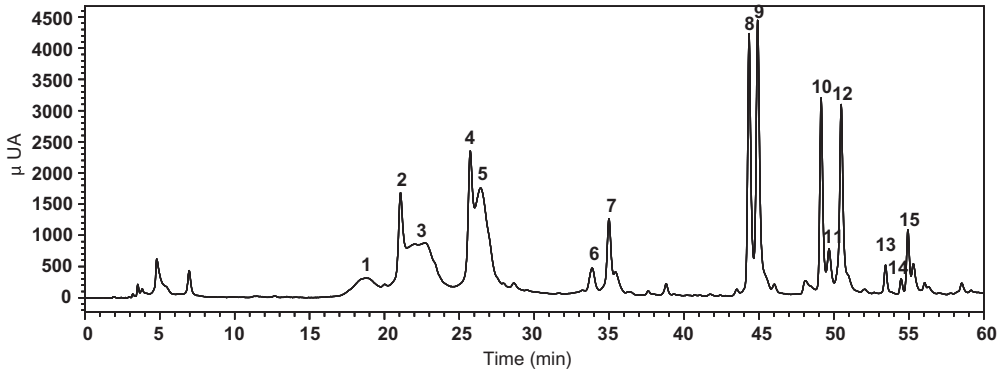

Fig. 1 – HPLC spectrum of the phenolic compounds in black currant extract (BCE) at 365 nm. For identification of numbered peaks see Table 1.

Table 1 – Separation and identification of phenolics in black currant extract (BCE) by HPLC–ESI-MS^2.

Peak	RT[a]	MS (*m*/z)	MS/MS (*m*/z)	Tentative compounds[b]	RPA[c] (%)
1	18.65	465	303	Delphinidin 3-O-glucoside	3.08
2	21.01	611	303, 465	Delphinidin 3-O-rutinoside	6.13
3	22.53	449	287	Cyanidin 3-O-glucoside	13.41
4/5	25.80/26.41	595	287, 449	Cyanidin 3-O-rutinoside	8.81/14.44
6	33.86	609	301, 463	Peonidin 3-O-rutinoside	1.44
7	34.85	579	271	Pelargonidin 3-O-rutinoside	4.78
8	44.43	627	319, 481	Myricetin rutinoside	11.48
9	44.90	481	319	Myricetin glucoside	13.90
10/11	49.16/49.61	611	303, 465	Quercetin 3-O-rutinoside	7.87/1.82
12	50.37	465	303	Quercetin glucoside	8.81
13	53.49	595	287, 449	Kaempferol rutinoside	1.01
14	54.48	625	317, 479	Isorhamnetin rutinoside	0.51
15	54.93	449	287	Kaempferol glucoside	2.50

[a] RT, retention time (min).
[b] Compound identification was based on parent molecular ions, retention times, and the fragments according to Fig. 1 and described in the literature (Anttonen & Karjalainen, 2006; Borges et al., 2010; Slimestad & Solheim, 2002; Määttä-Riihinen et al., 2004).
[c] Relative peak area percentage (peak area relative to the total peak area %).

identified, for example, several myricetin, quercetin, kaempferol, and isorhamnetin conjugates, which contributed to the rest 47.9% total peak area. Thus, the main components in BCE were anthocyanins and other phenolics. The total concentration of phenolic compounds present in the BCE tested in the present study was 12.2 mg/mL.

3.2. *Radical scavenging activity and reducing power*

BCE exhibited strong inhibition against DPPH and ABTS radicals in a concentration-dependent manner (Fig. 2). The DPPH$^{\cdot}$ scavenging activity ranged from 24 (6.25 mg/mL BCE) to 96% (100 mg/mL BCE). The ABTS$^{\cdot+}$ scavenging activity of BCE correlated well with its DPPH$^{\cdot}$ scavenging activity, yielding 15 (6.25 mg/mL BCE) to 99% (100 mg/mL BCE) activity. The inhibition of DPPH and ABTS radicals was almost completed at 75 mg/mL BCE. Similarly, the reducing power (absorbance at 700 nm) increased from 0.40 at 6.25 mg/mL BCE to 2.69 at 75 mg/mL BCE. The above activity assays involved the acquisition of free electrons by reagent radicals (DPPH$^{\cdot}$, ABTS$^{\cdot+}$) which were donated by BCE, or the conversion of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) as in the case of the iron-ferricyanide reducing power test. Hence, the strong response of BCE in these tests indicated that BCE had excellent potential to donate electrons or hydrogen to free radicals and convert them into stable diamagnetic molecules. The high concentrations of phenolic compounds present in the BCE in the present study (12.2 mg/mL) appeared to be a major contributing factor to the observed radical scavenging and reducing capability, consistent with the report of Borges et al. (2010) that phenolics were the main contributor to total antioxidant capacity of black currant. Lapornik, Prošek, and Wondra (2005) noted that black currant marc extracts exhibited the highest antioxidant activity compared with grape and red currant marc. Many other fruit extracts have also been shown to have strong radical scavenging activity, for example, extracts from mulberry (Bae & Suh, 2007), honey pineapple, banana, and Thai seedless guava (Alothman, Bhat, & Karim, 2009).

3.3. *Cell viability*

It is known that cumulative production of reactive oxygen species (ROS), including various free radicals, can lead to damage of critical macromolecules resulting in chromosome

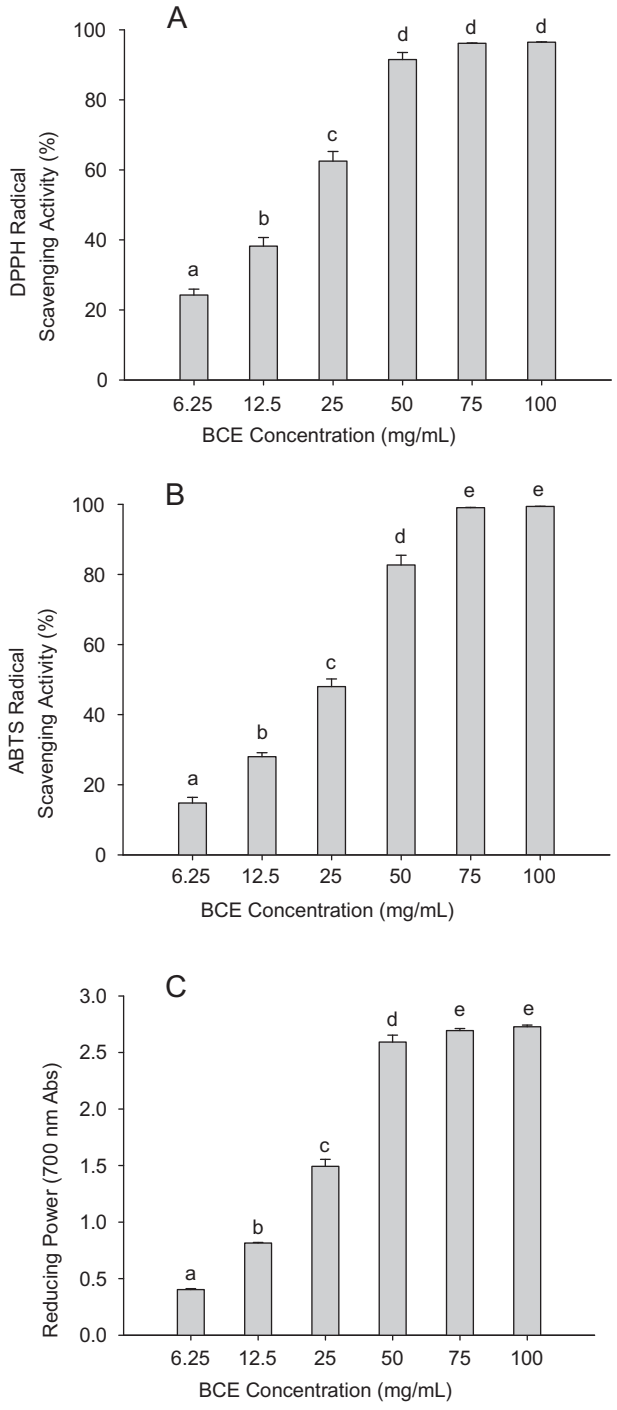

Fig. 2 – Scavenging activity of black currant extract (BCE) at different concentrations against DPPH (A) and ABTS (B) radicals, and the reducing power of BCE (C). Error bars refer to the standard deviations obtained from triplicate sample analysis. Means with different letters (a–e) differ significantly ($P < 0.05$).

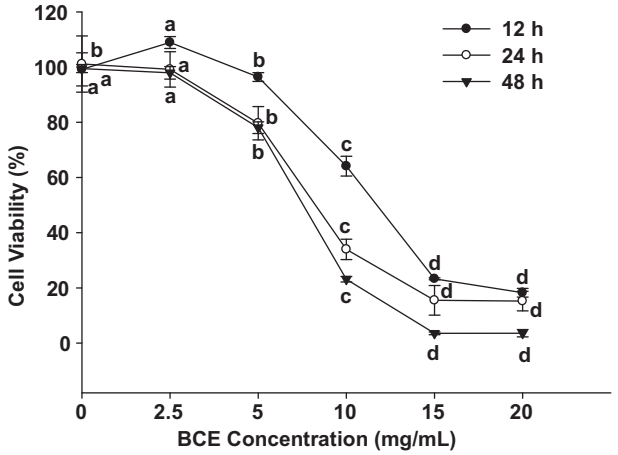

Fig. 3 – Influence of black currant extract (BCE) at different concentrations on the SGC-7901 cell viability. Error bars refer to the standard deviations obtained from triplicate sample analysis. Means with different letters (a–d) differ significantly ($P < 0.05$).

instability, genetic mutation, and modulation of cell growth as related to cancer (Klaunig, Wang, Pu, & Zhou, 2011). Antioxidative phytochemicals, including phenolic compounds, have been shown to be absorbed into the plasma, therefore, may exert protection of cells (Birt et al., 2001). The strong radical scavenging capability demonstrated by BCE suggested that it might offer potential chemopreventive property, including the inhibition of gastric cancer cell proliferation. To test this hypothesis, BCE was applied to cancer cell cultures and its in vitro efficacy was measured.

The WST-1 assay for the viability of SGC-7901 cells provided solid evidence that indeed, BCE was able to modulate the growth of this particular cell line. As shown in Fig. 3, the cell viability was not affected by 2.5 mg/mL BCE, but with increasing BCE concentrations and a prolonged incubation time, the population of live cells dropped dramatically ($P < 0.05$). The concentrations required for 50% inhibition of the gastric cell proliferation (IC_{50}) were 12.7, 10.2 and 9.0 mg/mL corresponding to 12, 24 and 48 h of incubations, respectively.

Wu et al. (2007) reported that cell viability of colon cancer HT-29 cells decreased 20% when exposed to 20 mg/mL BCE (containing 4.8 mg/mL anthocyanins and 290 μg/mL flavonols) for 24 h. In our tests, the same BCE concentration and incubation time resulted in 84.8% viability decrease for SGC-7901 cancer cells, indicating that gastric cancer cells were more sensitive to BCE. BCE has also been found to inhibit the growth of colon cancer HCT-116 cells and HL-60 human promyelocytic leukemia cells (Katsube et al., 2003). Olsson et al. (2004) reported that BCE decreased the proliferation of both colon cancer HT-29 cells and breast cancer MCF-7 cells.

Anthocyanins are commonly believed to be the main contributor to antiproliferative property of some berries (Borges et al., 2010). However, McDougall, Ross, Ikeji, and Stewart (2008) found the anthocyanin-rich fraction of lingonberry was considerably less effective in inhibiting the growth of Hela cells than the original extract. Olsson et al. (2004) noted that the anthocyanin fraction of blueberry had a lower inhibition activity against HT29 cells than total ethanol extract but a greater inhibition against MCF-7 cells. They proposed that it was difficult to evaluate the relative importance of individual compounds for the anticancer effects of fruits and vegetables and the protective effect might be due to additive or

synergistic actions of several compounds. Sun and Liu (2006) supported the view of additive and synergistic effects of multiple phytochemicals rather than a single compound or a class of compounds, by showing that cranberry phytochemical extracts exhibited remarkable inhibitions against human breast cancer MCF-7 cells. Hogan et al. (2010) found açai extract remarkably suppressed the proliferation of C-6 rat brain glioma cells and five phenolic compounds were identified in the extract. Moon et al. (2010) showed that the chloroform fraction of guava leaf extract, abundant in phenolics, inhibited human gastric cancer SNU-16 cell proliferation. Therefore, crude BCE was selected in our study, despite anthocyanins being the main phenolics in BCE, to achieve a maximum efficacy.

3.4. *Cell morphology*

Cell morphological changes were examined to observe the effect of BCE on SGC-7901 cells by inverted microscope and fluorescence microscopes. As shown in Fig. 4A, the cells in control had healthy growth pattern with a high cell density. After the treatment with 2.5 mg/mL BCE for 12 h, the cells showed nearly no change. When the BCE concentration was raised to 5 mg/mL, there was a slight reduction in cell numbers. However, with the incubation at a dose of 10 mg/mL BCE for 12 h, the characteristic features of the SGC-7901 cells changed remarkably: shrinkage, cytoplasm condensation, and formation of cytoplasmic filaments were noted. At the higher doses (15 and 20 mg/mL) of BCE, these characteristics were even more pronounced and the cells displayed partial detachment. When the incubation time was prolonged to 24 h, as shown in Fig. 4C, the cells exhibited more dire features consistent with apoptosis as compared with the same concentration of BCE at 12 h, especially at the dose above 10 mg/mL. Here, some substances in the cytosol were apparently released into the media and aggregation of rounding dead cells became salient.

In order to further evaluate whether the cell growth inhibition was caused by apoptosis, SGC-7901 cells were stained with Hoechst 33258 dye after treatment with various concentrations of BCE for 12 and 24 h. As presented in Fig. 4B and D, control cells showed homogenous DNA fluorescence and an intact nuclear structure. Whereas, after being treated with BCE, the cells exhibited typical characteristics of apoptosis, such as increased fluorescent intensity, nuclear chromatin condensation, and the formation of apoptotic bodies. Overall, such apoptotic features became intensified with increasing BCE concentrations and the incubation time. The cell morphology results suggested that BCE inhibited cell growth in a dose- and time-dependent manner, in accordance with the results of WST-1 assay, and the decreased cell viability was related to apoptosis.

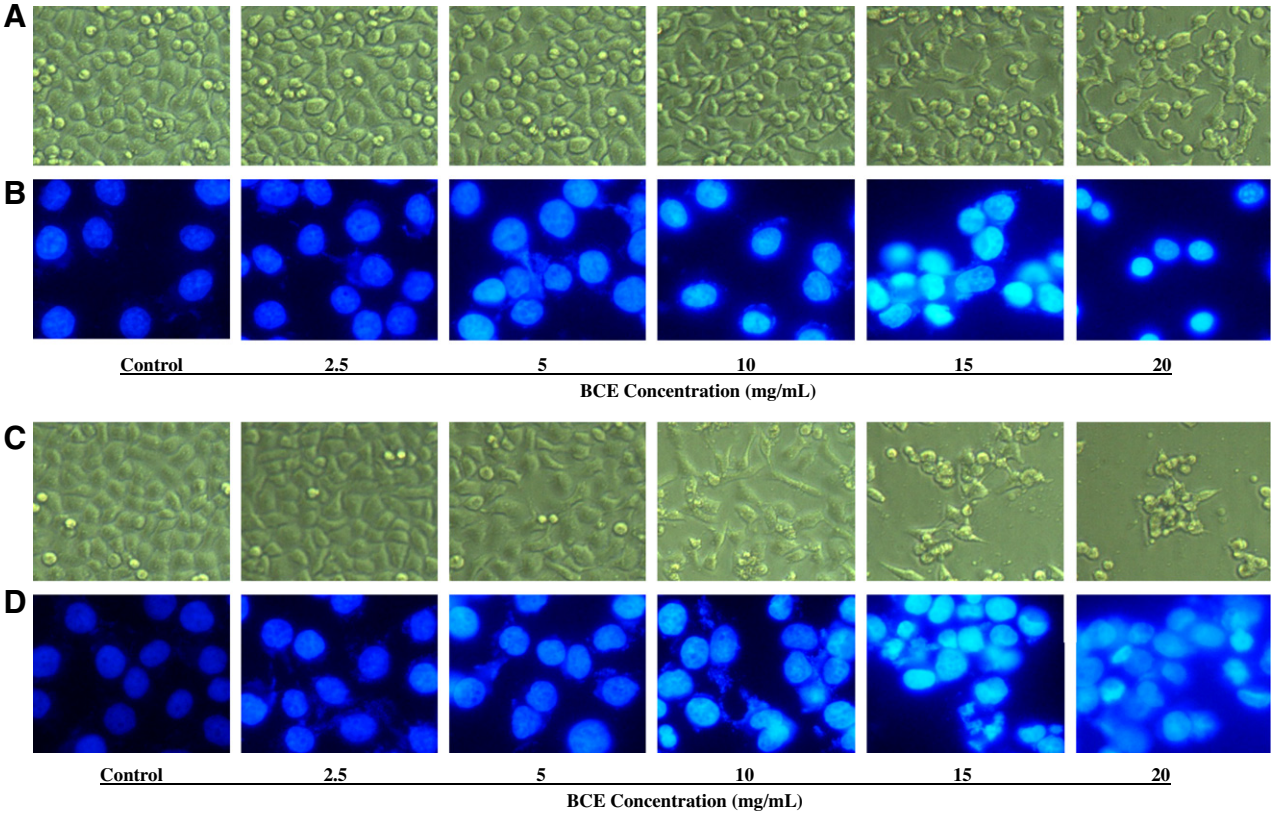

Fig. 4 – Induction of apoptosis of SGC-7901 cells following the treatment with black currant extract (BCE). After incubation with various concentrations of BCE for 12 h (A and B) and 24 h (C and D), the cells were examined using an inverted microscope (a and c, magnification, 200×) or stained with Hoechst 33258 dye then observed under a fluorescent microscope using a blue filter (b and d, magnification, 400×).

3.5. *Flow cytometric analysis of apoptosis*

The induced apoptotic effect of BCE was further confirmed by the determination of the percentage of apoptotic cells using flow cytometric analysis with the AV/PI double staining (Fig. 5). The AV+/PI− staining represents the early apoptotic cells due to the strong affinity of AV-FITC with phosphatidylserine, which transports from the inner leaflet of the plasma membrane to the outer surface of the membrane during early apoptosis. On the other hand, AV−/PI+ staining represents the necrotic cells, since PI, which could not cross through intact cell membrane, penetrates the compromised membrane of dead cells or late apoptotic cells and binds to nucleic acid (Tripathi, Singh, Mishra, Raisuddin, & Kakkar, 2010). Meanwhile, viable cells can be marked by AV−/PI−, and AV+/PI+ staining is indicative of late apoptotic cells.

The representative dot plots of flow cytometric analysis of apoptosis showed that the percentage of viable cells in control was 96.9%, while the cells in early apoptosis and late apoptosis were 0.6% and 2.3%, respectively; and 0.2% cells showed necrosis (Fig. 5A). As compared with control, the 2.5 mg/mL BCE treatment failed to induce cell apoptosis, while the 5 mg/mL BCE treatment produced a slight decrease in viable cells and an increase in apoptotic cells. However, at 10 mg/mL BCE, percent viable cells declined to 72.9% and the proportion of cells in early and late apoptosis increased to

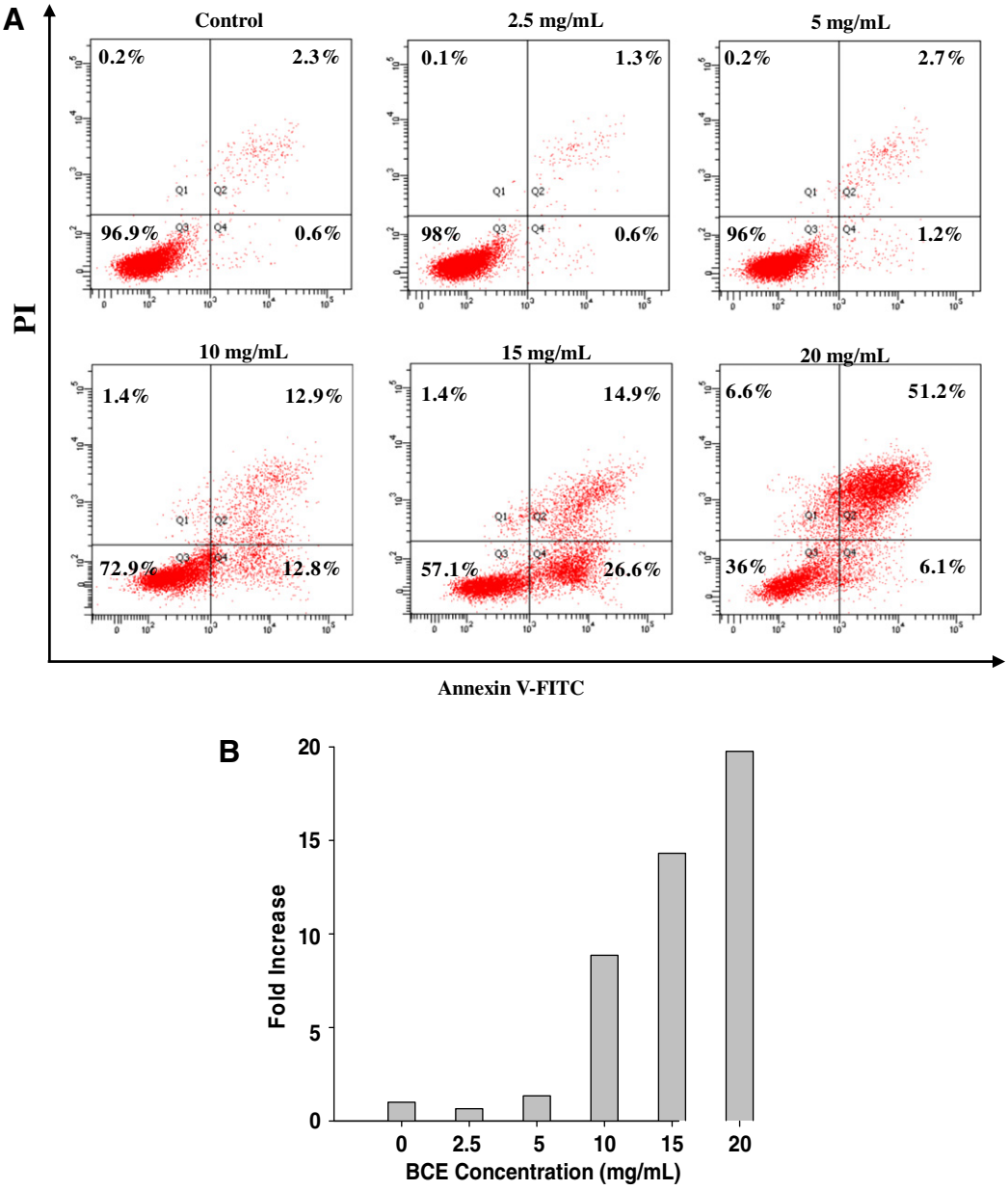

Fig. 5 – Representative dot plots of flow cytometric analysis of annexin V-FITC/propidium iodide (PI) double stained cells treated with various concentrations of black currant extract (BCE) for 24 h (A), and fold increase in the percentage of apoptotic populations compared to control (B). Q_1 represents the necrotic cells, Q_2 represents the late apoptotic cells, Q_3 represents the viable cells, and Q_4 represents the early apoptotic cells.

12.8% and 12.9%, respectively. When the cells were exposed to 15 mg/mL BCE, total apoptosis rose to 41.5% (26.6% early apoptosis and 14.9% late apoptosis), whereas the percent viable cells was only 57.1%. At the final assay dose of 20 mg/mL BCE, 6.6% cells underwent necrosis and a strong shift from early apoptotic cells to late apoptotic cells occurred (now 6.1% and 51.2%, respectively). It was noted that the percentage of apoptotic cells was 19.8-fold higher after the treatment with 20 mg/mL BCE when compared with control (Fig. 5B). The results further suggested that the antiproliferative effect of BCE against SGC-7901 cells was caused by inducing cell apoptosis.

Apoptosis plays a key role in the development and growth regulation of normal cells, and is often deregulated in cancer cells. Anthocyanin-rich extracts have exhibited pro-apoptotic effects in multiple cell types in vitro (Martin, Giannone, Andriantsitohaina, & Martinez, 2003; Reddivari, Vanamala, Chintharlapalli, Safe, & Miller, 2007). They induce apoptosis through both extrinsic (Fas) and intrinsic (mitochondrial) pathways (Chang, Huang, Hsu, Yang, & Wang, 2005; Reddivari et al., 2007). In the extrinsic pathway, anthocyanins modulate the expression of Fas and Fas ligands in cancer cells resulting in apoptosis; in the intrinsic pathway, they are able to increase the mitochondrial membrane potential, promote the release of cytochrome *c*, and modulate caspase-dependent pro- and anti-apoptotic proteins (Wang & Stoner, 2008).

BCE was reported to inhibit colon cancer HT-29 cell proliferation mainly via the $p21^{WAF1}$ pathway, an inhibitor of cell proliferation and a member of the cyclin kinase inhibitors (Wu et al., 2007). The pro-apoptotic marker, Bax, showed no significantly change while the anti-apoptotic marker, Bcl-2, was probably down-regulated since it was only detected in control with low levels of expression. Moon et al. (2010) reported that human gastric cancer SNU-16 cell apoptosis induced by the chloroform fraction of guava leaf extract was associated with an increase in Bax/Bcl-2 ratio, activation of caspase-3 and caspase-8, and degradation of poly ADP-ribose polymerase (PARP). Jang et al. (2010) found the extract of *Ganoderma lucidum* (EGL) induced human gastric cancer AGS cells apoptosis through activation of the intrinsic caspase pathway along with the death receptor-mediated extrinsic pathway. Moreover, the inactivation of Akt may play an important role in EGL-induced apoptosis. Furthermore, Dai, Gupte, Gates, and Mumper (2009) reviewed that the prooxidant action of phenolics may be an important mechanism for their anticancer and apoptosis-inducing properties. Therefore, the induced apoptotic effect of BCE on SGC-7901 cells as demonstrated in the present study may be related to one or several different pathways. It would be very interesting to investigate which specific signaling pathways were involved in the induction of SGC-7901 cell apoptosis by BCE and explain the underlying mechanisms in further studies.

4. Conclusion

The extract of black currant displayed strong radical scavenging activity and a high reducing power, and also effectively inhibited gastric cancer SGC-7901 cell proliferations through inducing cell apoptosis. These results indicated that BCE could be an effective chemotherapeutic agent in gastric cancer treatment. Further research is needed to elucidate BCE induced gastric cancer cell apoptotic signaling pathways and possible mechanisms. This should be done both in vivo and in animal models because it is almost certain that not all biologically active phenolic compounds present in BCE are equally absorbable through the gastrointestinal system or are able to penetrate through the membrane of the gastric cancer SGC-7901 cells.

Acknowledgment

This study, a collaborative project between Northeast Agricultural University (BK) and University of Kentucky (YLX), was supported by Heilongjiang Natural Science Youth Fund (Grant No. QC2011C001), and the Program for Innovative Research Team of Northeast Agricultural University (Grant No. CXZ011).

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