Separation, Purification and Bioactivity of Broccolini Leaves Flavonoids

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Broccolini (*Brassica oleracea Italica* × *Alboglabra*) is a hybrid of broccoli and Chinese kale. To date, no information is available on the separation and purification of flavonoids in broccolini (not broccoli). The objectives of this work were the separation and purification of broccolini leaves flavonoids using solvent extraction and polyamide resin chromatography. By this method, three purified fractions (BLF1, BLF2 and BLF3) were obtained and the flavonoids content of the purified products increased from 10.2 % to 41.6 %. HPLC and LC-MS/MS analyses indicated that BLF1 primarily consisted of quercetin (content up to 85.4 %), BLF2 was primarily composed of kaempferol (content up to 78.5 %), and BLF3 contained two major constituents kaempferol and apigenin (contents up to 82.6 %). Furthermore, the anti-cancer activity of the extract before and after purification was compared using a human colon cancer cell line SW480. The results showed that the crude extract BLF0 and the purified extracts (BLF1, BLF2 and BLF3) all exhibited an inhibitory effect on the growth of SW480 cells, and the IC₅₀ values were estimated to be 88.14, 65.06, 72.62 and 79.42 µg/mL, respectively, i.e. the anti-cancer activities on SW480 cells were increased by 10-26 % after purification.

1. Introduction

Flavonoids are a large group of naturally occurring phenolic compounds ubiquitously distributed across the plant kingdom, and have many positive impacts on human health including antioxidation, anti-inflammatory, and anti-cancer properties, prevention of atherosclerosis and anti-microbial activity [1]. Flavonoids can be obtained by different extraction and separation techniques, such as liquid-liquid extraction and column chromatographic procedures including silica gel, Sephadex, polyamide, macroporous resin, etc. [2,3]. In order for a successful investigation of bioactive plant constituents, it is important to perform efficient separation and fast identification of the chemical constituents. HPLC is the most widely used technique for the separation of complex mixtures of polyphenolic compounds in plant extracts [4,5]. Recently, the application of LC-MS to the analysis of flavonoids in plants has increased with the advent of “soft” ionization techniques such as electrospray ionization (ESI) [6-9]. ESI generates mainly protonated and deprotonated ions from relatively small plant metabolites like flavonoids [10]. LC-ESI-MS/MS has proved to be a powerful tool for the rapid characterization and identification of the chemical constituents [11].

Broccolini (*Brassica oleracea Italica* × *Alboglabra*) is a green vegetable similar to broccoli but with small florets and long, thin stalks. It is sweeter than broccoli and the delicate stalks do not need to be peeled. Although often misidentified as young broccoli, it is a cross between broccoli and kailan (Chinese broccoli, Chinese kale or Cabbage mustard). It is also called baby broccoli or asparation. Nutritionally, broccolini is high in some active ingredients such as vitamins (A, C), folate, flavonoids and glucosinolates.

To date, no information is available on the separation and purification of flavonoids in hybrid broccolini (not broccoli). The objectives of this work were: (1) the separation and purification of flavonoids from the leaves of broccolini using a polyamide resin-based column chromatography approach, (2) the identification of the flavonoids from broccolini leaves using a high-performance liquid chromatography method coupled with electrospray ionization mass spectrometry (HPLC-ESI-MS/MS), and (3) the anti-cancer activities of various fractions in human colon cancer cells using MTT assay.

2. Experimental

2.1 Materials and chemicals

Broccolini was bought from Guangzhou Research Institute of Vegetables (Guangzhou, China). The dried leaf was ground in a cutting mill to pass through an 80-mesh sieve to obtain fine powder, which was stored in a sealed container for further use. Rutin and quercetin were purchased from Sigma Chemicals Co. Other chemicals were purchased from China National Medicine Group Shanghai Corporation (Shanghai, China). All chemicals and solvents used were of analytical grade.

2.2 Extraction of broccolini leaves flavonoids

Flavonoids were extracted from the dried leaf powder samples (2000 g) by ultrasonic-assistance under the designed conditions: ethanol concentration 70 %, solid to liquid ratio 1:30 (w/v, ethanol), ultrasonic power 180 W (25 Hz), water bath temperature 60 °C and extraction time 60 min. The crude extract was filtered and kept for further use.

2.3 Determination of total flavonoids

The total flavonoids content in the extracts was determined using a colorimetric method ($\text{NaNO}_2\text{-Al}(\text{NO}_3)_3\text{-NaOH}$ system) with minor modification [12]. Preparation of a standard curve: 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mL of rutin standard solution (0.2 mg/mL) were added into separate volumetric flasks (10 mL). Sodium nitrite (0.3 mL, 5 %) and aluminum nitrate (0.3 mL, 10 %) were added, respectively. After fully mixing and standing for 6 min, sodium hydroxide (4 mL, 4 %) was added. Each flask was finally diluted with ethanol (60 %) to a defined volume (10 mL). The absorbance of each flask was examined at 510 nm with a 1mL blank solution for comparison. The regression equation of the rutin standard curve was obtained as $y = 0.087x - 0.0005$ ($R^2 = 0.9991$), where y is the absorbance, x is the flavonoid content (mg/mL). Using the same method of standard curve preparation, 1mL of prepared sample was used to measure the flavonoids concentration of the crude extract (mg/mL). The extract was concentrated by evaporation of the ethanol and was dried to constant weight. The flavonoids content was calculated as follows:

$$\text{Total flavonoids content (\%)} = (C \times V/M) \times 100\%$$

where C is the total flavonoids concentration of the extract solution (mg/mL), V is the volume of the extract solution (mL), and M is the mass of total flavonoids after drying (mg).

2.4 Purification of broccolini leaves flavonoids

The purification process is summarized in Figure 1. Specifically, the crude extract was vacuum-evaporated to dryness, and the residue obtained was dissolved in 70 % ethanol. Petroleum ether was added to remove impurities like chlorophyll. The petroleum ether layer was concentrated by removing the ethanol. Ethyl acetate (1 L) was added to the concentrated solution for extraction (three times). The combined ethyl acetate layers were evaporated to dryness under vacuum to obtain

Fraction A. Subsequently, *n*-butanol (1 L) was added to the residual solution after ethyl acetate extraction for the extractions (three times). The combined *n*-butanol layers were vacuum-evaporated to dryness to give Fraction B. Then, a column chromatography method based on a polyamide resin (pretreated with 95 % ethanol to remove impurities) was used for further separation of the obtained Fractions A and B. Briefly, the ethanol pretreated polyamide resin was loaded into a glass column (2.0 × 30 cm). Samples were dissolved in ethanol with ultrasonic assistance and passed through the column at a rate of 2 BV (bed volume)/h. After standing for 60 min to allow full adsorption of the flavonoids by the polyamide resin, sample solutions were firstly eluted with distilled water, secondly with 50% ethanol and finally with 70 % ethanol to generate two major layers. Fractions were collected every 10 min. All collected fractions were then freeze-dried. The sorption and desorption capacities were determined by the following equations [3]:

$$Q_e = (C_0 - C_e)V_i/W$$

$$E = (C_0 - C_e)/C_0 \times 100 \%$$

$$D = C_d V_d / ((C_0 - C_e)V_i)$$

where Q_e is the adsorption quantity at equilibrium (mg/g); E is the adsorption ratio (%), defined as the percent mass of total adsorbate adsorbed at equilibrium; D is the desorption ratio (%); C_0 and C_e are the initial and equilibrium concentrations of solutes in the solutions, respectively (mg/mL); V_i is the volume of the initial feed solution (mL); W is the weight of the dry adsorbent (g); C_d is the concentration of the solutes in the effluent (mg/mL); and V_d is the volume of the effluent (mL).

Figure 1. Flowchart for separation and purification of flavonoids from broccolini leaves.

2.5 HPLC and LC-ESI-MS analyses of broccolini leaves flavonoids

The crude flavonoids extract (0.1 mg/mL, dissolved in methanol) was separated using a Waters 1525 system (Waters) with a Waters 2487 tunable absorbance detector and a Waters 717 plus Auto sampler. The mobile phase was composed of

solvents A (methanol) and B (water with 0.3% phosphoric acid). Two elution procedures were tested. For gradient elution: 95 % B at the start; changed to 62.5 % B in 10 min; 57.5 % B in 35 min; 45 % B in 60 min; 5 % B in 80 min; 95 % B in 95 min. For equivalent elution: the binary solvent system was fixed at A/B = 60/40 (v/v). The injection volume was 5 μ L. The flow rate was 0.8 mL/min. The UV absorption of flavonoids was monitored at 360 nm.

LC analysis was performed using an Agilent 1100 series LC (Agilent, USA) equipped with a diode array detector. Elution with solvent A (methanol) and solvent B (water with 0.3 % acetate acid) in a step gradient manner at a flow rate of 0.8 mL/min was carried out as follows: 95 % B at the start; changed to 62.5 % B in 10 min; 57.5 % B in 35 min; 45 % B in 60 min; 5 % B in 80 min; 95 % B in 95 min. The column was at room temperature. The sample injection volume was 5 μ L. The detection was at 365 nm. For identification, MS analysis was performed on a triple quadrupole instrument (Thermo Electron Corporation, USA), equipped with an ESI positive and negative ion source. Samples were dissolved in methanol and infused in the ESI source for detection with ESI+3.0 kV and ESI-2.8 kV. The total ion scanning range was 100-3000 m/z. Nitrogen was used both as a drying gas and as a nebulising gas at a flow rate of 350 L/h. The desolvation temperature and ionization temperature were 300 °C and 100 °C, respectively.

2.6 Cell culture and MTT assay

A human colon cancer cell line SW480 was purchased from the Guangdong province peoples hospital (Guangzhou, China). Complete DMEM medium was obtained from GIBCO (Grand Island, NY, USA). For routine maintenance, SW480 cells were cultured in suspension in DMEM medium with 10% fetal bovine serum (GIBCO) in a water-jacketed 5 % CO₂ incubator at 37 °C (Forma Scientific, Marietta, OH).

Cell growth inhibition was evaluated using a standard colorimetric MTT assay. Briefly, cells in logarithmic growth phase were plated in 96-well microtitre plates at a density of $3 \times 10^3 \sim 10^4$ cells/100 μ L/well and allowed to incubate for 24 h at 37 °C for attachment. Subsequently, the broccolini leaves flavonoids samples were diluted with the complete medium to the desired initial concentration (15, 30, 60, 80, 120, 160 μ g/mL) and then added to the cells in the logarithmic growth phase. A blank was used as a negative control and a quercetin standard was used as positive control. After incubation for another 24 h, the MTT solution (Bornem, Belgium) (20 μ L of 5 mg/mL) was added to each well, and incubated for an additional 4 h at 37 °C. The MTT-containing medium was removed and the formazan crystals, formed within the cells, were solubilized by the addition of DMSO (Bornem, Belgium) (150 μ L/well) and agitation. The absorbance of the samples and control cultures were read at 490 nm with a spectrophotometer (Model 550 Microplate Reader). The half inhibition rate IC₅₀ was calculated. Each experiment was in triplicate.

3. Results and Discussion

3.1 Extraction and identification of broccolini leaves flavonoids

In the present work, the dried leaf powder samples were firstly extracted by ethanol under ultrasonic-assistance to obtain crude extract of flavonoids. In Figure 2, the chromatogram of crude extract from broccolini leaves are presented.

Figure 2. Total ion current (TIC) of the EtOH extract from broccolini leaves.

ESI-MS was frequently used for the detection and identification of compounds in the crude extract from the plants. The identification of each analyte was carried out by comparison of peak retention times, molecular masses, MS/MS fragmentation and the characteristic product ions with those of the available reference standards. The MS and MS/MS spectra in ESI positive and negative mode were particularly good for the detection of both molecular weight and the characteristic fragmentation of the compounds. By comparing retention times with those reported in the literature, no flavonoids compounds are found beyond a retention time of 80 min, so the author ignored peaks 9-12 in Figure 2. The positive ion MS/MS spectra of chromatographic peaks 1-8 for the crude extract from broccolini leaves are shown in Figure 3.

Figure 3. The ESI-MS fragments of peaks in Figure 1. (A) Peak 1, (B) Peak 3, (C) Peak 5, (D) Peak 7.

Chromatographic peaks 1 and 2 with a retention time of 26.4 min showed an $[M+H]^+$ ion at 610.9 m/z and an $[M-H]^-$ ion at 608.9 m/z. MS/MS studies (Figure 3 A) revealed main fragments at 303.1, 304.1 and 465.0 m/z. The fragment 465 m/z could be attributed to the loss of glycoside 6-deoxy-L-mannose from the parent ion, m/z 303.1 could indicated the loss of β -D-glucoside from 465 m/z, 303.1 m/z could be quercetin and become $[M+2H]^+$ 304.1 m/z after transference of hydrogen. The compound was identified as rutin.

Chromatographic peaks 3 and 4 with a retention time of 46.8 min presented a parent ion of 301.0 m/z in negative ion mode and 303.1 m/z in positive ion mode (Figure 3 B). The fragments observed were: 273, 257, 179, 151 and 122 m/z. 273 m/z indicated the loss of CO, 257 m/z was attributed to the loss of CO₂, 179 m/z could be produced by the breakage of two C-2 bonds and transference of two hydrogens, as well as 151 m/z resulting from the breakage of two C-3 bonds. These results allowed the identification of quercetin.

Peaks 5 and 6 detected at 64.6 min are due to a deprotonated molecular ion at 285.1 m/z and a protonated molecular ion at 287.2 m/z (Figure 3 C). The second-generation fragmentation ions include: 257.1, 229.1, 211.1, 185.1, 169.1 and 151.1 m/z. 257 m/z corresponded to the loss of CO. 229 and 211 m/z could be result of the loss of CO and HCOOH, respectively. 185 m/z was attributed to the loss of CO and H₂O, 169 m/z was produced by the loss of O, while 151 m/z was generated due to the loss of H₂O. The compound was identified as kaempferol.

Chromatographic peaks 7 and 8 with a retention time of 67.7 min were identified as apigenin, which exhibited an $[M+H]^+$ ion at 271.3 m/z and an $[M-H]^-$ ion at 269.2 m/z (Figure 3 D). The second-order fragmentations consisted of: 243.2, 227.3, 199.3, 183.2, 152.2, 153.2 and 118 m/z. The fragments 243.2 and 227.3 m/z were produced due to the loss of CO and CO₂ from parent ion 271.3 m/z, respectively. Similarly, 199.3 and 183.2 m/z were attributed to the loss of CO and CO₂ from 227.4 m/z. 152.2 and 118 m/z could be produced by the breakage of C-ring bonds, and the shift of hydrogen leading to $[M+2H]^+$ 153.2 m/z.

In general, the breakage of these compounds primarily occurred at 1,2 or 3,4 C-ring bonds (i.e. clock-wise counted from the top position in the benzene structure), other fragments were formed mainly by rearrangement and the loss of the neutral molecules CO or CO₂, consistent with previous observations [9, 13]. The information about retention time, MS and MS/MS spectra characteristic of each chromatographic peak, along with the flavonoids identified is presented in Table 1, indicating that the crude extract of broccolini leaves flavonoids contains 4 major constituents: rutin, quercetin, kaempferol and apigenin.

Table 1. The LC-ESI-MS/MS data for analysis of flavonoids in broccolini leaves

Peak	tR/min	$[M+H]^+$ m/z	$[M-H]^-$ m/z	MS fragments	Identification
1,2	26.4	610.9	608.9	303.1,304.1,465.0,	Rutin
3,4	46.8	303.1	301.0	273.3,257.2,179.6,151.8,122.2	Quercetin
5,6	64.6	287.2	285.1	257.1,229.1,211.1,185.1,169.1,151.1	Kaempferol
7,8	67.7	271.3	269.2	243.2,227.3,199.3,183.2,152.2,153.2,118	Apigenin

3.2 Separation and purification of broccolini leaves flavonoids

The crude extract of broccolini leaves flavonoids contains many impurities and its flavonoids content was only 10.2%. Subsequently, the crude extract was treated with petroleum ether, and the flavonoids content was increased to 28.4%. Then, ethyl acetate and *n*-butanol were used for further extractions to obtain two extracts, Fraction A and Fraction B, respectively. The flavonoids contents in Fraction A and Fraction B were elevated to 34.3 % and 41.6 %, respectively.

Finally, polyamide resin-based column chromatography was applied for further purification of the extracts. The experiments showed that after adsorption of the flavonoids on the polyamide resin elution firstly with distilled water was very useful to remove impurities, otherwise, the extract was yellow-brown and viscous and contained many polysaccharides and other impurities. In addition, lowering the ethanol concentration had no significant effect on the adsorption of the flavonoids on the polyamide resin and in fact led to obvious tailing and lower desorption efficiency. So, in the present work, 50 % and 70 % ethanol were used for gradient elution. Fraction A was eluted with 50 % ethanol to give a broccolini leaves flavonoids fraction 1 (BLF1). Fraction B was eluted with 50 % and 70 % ethanol to obtain broccolini leaves flavonoids fractions 2 and 3 (BLF2 and BLF3), respectively. The flavonoids contents of BLF1, BLF2 and BLF3 before and after column chromatography are shown in Table 2.

Moreover, the crude extract and adsorption chromatographic fractions were analyzed by HPLC on a Waters liquid chromatographic system. The elution experiments showed that the major components can be well separated both by gradient elution and equivalent elution, but a long time (more than 1 h) was required for gradient elution, therefore, equivalent elution (A/B (v/v)=60/40) was employed for the crude extract and the purified extracts (BLF1, BLF2 and BLF3) (Figure 4). Based on HPLC chromatograms and the comparison with standards and the results of LC-MS/MS above, the major composition of the three adsorption chromatographic fractions could be determined, BLF1 primarily consisted of quercetin (content up to 85.4) , BLF2 was primarily composed of kaempferol (content up to 78.5 %), and BLF3 contains two major constituents kaempferol and apigenin (contents up to 82.6 %). This is partly consistent with previous reports that the predominant flavonoids in broccoli and kale are quercetin and kaempferol [14,15], as broccolini is a cross of broccoli and Chinese kale.

Table 2 The adsorption and desorption results of polyamide resin chromatography on broccolini leaves flavonoids

Items	Total solids (g)	Total flavonoids (g)	Flavonoid content (%)
Fraction A before on-column	10.0	3.43	34.3
Fraction A after on-column	3.08	2.00	65.2
Fraction A elution with (50% ethanol) (BLF1)	2.14	1.83	85.4
Adsorption ratio	58.3%	Desorption ratio	91.5%
Fraction B before on-column	10.0	4.16	41.6
Fraction B after on-column	4.34	2.75	63.4
Fraction B elution with 50% ethanol (BLF2)	2.05	1.61	78.5
Fraction B elution with 70% ethanol (BLF3)	1.15	0.95	82.6
Adsorption ratio	66.1%	Desorption ratio	93.1%

3.3 Anti-cancer effects of broccolini leaves flavonoids on human colon cancer cells

The human colon cancer cell line SW480 was employed to compare anti-cancer effects of the crude extract of

broccolini leaves flavonoids (BLF0) and the purified extracts (BLF1, BLF2 and BLF3). A blank was used as a negative control and the quercetin standard was used as the positive control. The MTT measurement results showed that broccolini leaves flavonoids (15~160 $\mu\text{g/mL}$) can exert a remarkable inhibitory effect on the growth of SW480 cells. Treating the cells with broccolini leaves flavonoids (15~160 $\mu\text{g/mL}$) resulted in a dose-dependent inhibition of cellular proliferation. The IC₅₀ values of BLF0, BLF1, BLF2, BLF3 and positive control quercetin in SW480 cells were estimated to be 88.14, 65.06, 72.62, 79.42 and 54.64 $\mu\text{g/mL}$, respectively. This suggests that the purified extracts exhibit stronger (increased by 10-26 %) anti-cancer ability in SW480 cells than the crude extract. It has been reported that various mechanisms were involved in the anticancer activity of flavonoids, such as cell cycle arrest [16], apoptosis [17], DNA topoisomerase inhibition [18], mitochondrial dysfunction [19], etc. Different flavonoids possess different antitumor activity. For example, Kanakis *et al.* [20] reported that the antioxidant activity of flavonoids changes in the order delphinidin > quercetin > kaempferol > morin > naringin > apigenin.. Zhang *et al.* [21] showed that flavones (luteolin, apigenin, chrysin) and flavonols (quercetin, kaempferol, myricetin) were all able to induce cytotoxicity in the human oesophageal adenocarcinoma cell line (OE33) in a dose- and time-dependent manner, and the cytotoxic potency of these compounds was in the order quercetin > luteolin > chrysin > kaempferol > apigenin > myricetin.. Thus, our present results are consistent with these reports with respect to quercetin, kaempferol, and apigemin.

A

B

C

D

Figure 4. HPLC analysis. (A) Gradient elution for crude extract BLF0, (B) Equivalent elution for crude extract BLF0, (C) Quercetin standard, (D) Purified extract BLF1 obtained by elution of the ethyl acetate layer with 50 % ethanol, (E) Purified extract BLF2 obtained by elution of the *n*-butanol layer with 50 % ethanol, (F) Purified extract BLF3 obtained by elution of the *n*-butanol layer with 70 % ethanol.

4. Conclusion

In this study, for the first time, a purification method of flavonoids from broccolini (not broccoli) leaves based on extraction with solvents (petroleum ether, ethyl acetate and *n*-butanol) and polyamide resin chromatography is reported. With the help of this method, the flavonoids content of the purified products increases from 10.2 % to 41.6 %, and three purified fractions (BLF1, BLF2 and BLF3) were obtained. HPLC and LC-MS/MS analysis indicated that BLF1 primarily consisted of quercetin (content up to 85.4) , BLF2 was primarily composed of kaempferol (content up to 78.5%), and BLF3 contains two major constituents kaempferol and apigenin (contents up to 82.6 %). In addition, bioactivities tests demonstrated that the crude extract BLF0 and the purified extracts (BLF1, BLF2 and BLF3) exhibited anti-cancer activity on human colon cancer SW480 cells, and the IC₅₀ values were estimated to be 88.14, 65.06, 72.62 and 79.42 µg/mL, respectively, i.e. the anti-cancer activities on human colon cancer cells were increased by 10-26 %.

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