Journal of Applied Botany and Food Quality 89, 150 - 155 (2016), DOI:10.5073/JABFQ.2016.089.018

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Phytochemical compositions, antioxidant and antimicrobial activities analysis of extracts from *Vaccinium bracteatum* Thunb. leaves

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(Received October 27, 2015)

Summary

Vaccinium bracteatum Thunb. is an edible plant, which has been used for many food products and is also a resource of traditional Chinese medicine. In this study, the antioxidant and antimicrobial activities of ethanol extracts from its leaves were investigated. To study the characteristic compositions, twelve compounds of extracts accumulated by the D-101 macroporous adsorption resin (VBE) were identified by HPLC-DAD and HPLC-ESI/MS techniques, including chlorogenic acid and its isomers, and eight flavonoid compounds. The contents of total flavonoids, orientin and isoorientin in the accumulated part were 601.4, 44.7, and 96.1 mg/g, respectively, which were far more than that in the raw materials. Furthermore, the antioxidant activities were estimated by DPPH, ABTS, and FRAP assays, which showed that the high content accompanied with strong antioxidant activities. Besides, compared to the same type of bamboo leaves (AOB), the accumulated part possesses better activities. At the last, the antimicrobial activities of VBE were assessed by a serial two-fold dilution assay, the results showed that it had good antimicrobial activities. Taken together, extracts from *Vaccinium bracteaturn* Thunb. leaves have better antioxidant activities, which can be used as a natural antioxidant.

Introduction

Antioxidants, as food additive, are used to keep the foods quality during their shelf life. At the present, the most commonly used antioxidants are chemical preservatives, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertbutylhydroquinone (TBHQ). However, some studies showed that the chemical preservatives exhibited certain toxicity, which can affect the respiratory enzymes activity, and cause teratogenesis and carcinogenesis (WETTASINGHE and SHAHIDI, 1999; LUO and FANG, 2008). Nowadays, the demand for food additives is increasingly high, and the utilization of natural antioxidants becomes a necessary research direction. A lot of research had found that the extracts of most plants possessed antibacterial and antioxidant effects (SUN et al., 2011; RYSZARD et al., 2004; ICHIKAWA et al., 2001).

Vaccinium bracteatum Thunb. is mainly distributed in the regions south of the Yangtze River, which belongs to the genus *Vaccinium* (family *Ericaceae*). The leaves were rich in proteins, vitamins and minerals, which contained abundant vitamin C (Vc), calcium, iron, selenium and manganese. With methionine (Met) and histidine (His) being the highest in them, seventeen amino acids were found (CHEN et al., 2008). Furthermore, it showed multiple advantages for Wufanshu being taken as a rootstock species of blueberry, the contents of potassium, sodium, iron, phosphorus, zinc, and calcium element in fruit of Wufanshu were significantly higher than those in none-grafted blueberry (XU et al., 2014). In China, a series of *Vaccinium bracteatum* food was also made of its extracts such as *Vaccinium bracteatum* beverage. Besides, making Wufan with rice was people's custom, it had been developed into a local tourism food (HAO et al., 2010).

Modern medical research found that it had many pharmacological activities, such as improving blood microcirculation and anti-inflammatory (LANDA et al., 2014). In recent years, some studies showed that the main constituents of *Vaccinium bracteatum* Thunb. leaves were flavonoids, polyphenols, and diterpenes. Additionally, it was reported that the flavonoids in extracts from plants had antioxidant activity (BERNONVILLE et al., 2011; CHUA et al., 2011; LI et al., 2009), and the mechanism of antioxidant activity was related to the structure of the flavonoids and the substituents of the heterocyclic rings (KONG et al., 2003).

In our previous studies (LI et al., 2008; ZHANG et al., 2009), a systematical investigation of the chemical components of *Vaccinium bracteatum* Thunb. leaves had been carried out. Twenty-five compounds were isolated from the leaves, and twenty-four of them were identified. Here, the antioxidant and antibacterial activities of extracts from leaves were performed. The results showed that the extracts from *V. bracteratum* leaves could be used as a natural antioxidant in food industry.

Materials and methods

Materials and chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from the Wako Pure Chemical Company (Osaka, Japan). 2,4,6-Tris(2-pyridyl)-1,3,5-triazine (TPTZ), and 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from the Tokyo Chemic Industry Company (Tokyo, Japan). Ethanol, methanol (HPLC-grade), acetonitrile (HPLC-grade), iron vitriol, ferric trichloride, sodium acetate, and potassium persulfate were obtained from the Sinpharm Chemical Reagent Company (Shanghai, China). All solvents and chemicals were of analytical grade, unless otherwise specified. HPLC grade water was obtained from Milli-Q System (Millipore, Billerica, MA, USA).

Extraction and accumulation of total flavonoids from *Vaccinium bracteatum* Thunb. leaves

Vaccinium bracteatum Thunb. leaves were harvested in Zhejiang Province, China. The leaves were dried in the shade for a week, and then were ground to powder using a grinder. A portion (100 g) of powder was extracted with 70% ethanol solution. One part of ethanol extracts was dried in a rotary evaporator, which was the raw materials part. Another part of ethanol extracts was added into the column contained D-101 macroporous resin. The column was successively washed by water and 70% ethanol. The eluted solution from the column was collected and dried, which was the macroporous adsorption resin part (VBE). All samples were packed in the bag and stored at room temperature until later analysis.

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HPLC analysis

The extract (25 mg) was dissolved in 10 mL of 70% (v/v) ethanol and filtered through a 0.45 μm cellulose acetate filter before HPLC analysis. The samples were analyzed by Shimadzu LC-10AT HPLC system (Shimadzu Co., JAPAN), equipped with CBM-20A Triton System Controller, SPD-M20A detector, SIL-20A auto sampler and CTO-10A column oven. The analytical column was a C18 column of Agilent TC-C18 (250 mm × 4.6 mm, 5 μm i.d., Agilent Technologies, USA) with a flow rate of 1 mL min^{-1} at 40 °C. The mobile phase consisted of 1% acetic acid in water and 100% methanol. The applied gradient program was: 0-10 min (5% to 15% methanol), 10-40 min (15% to 20% methanol), 40-60 min (20% to 30% methanol), 60-100 min (30% to 50% methanol), 100-120 min (50% to 95% methanol), and 120-130 min (95% methanol). 20 μL of the standard solutions and samples were used and all of the samples were analyzed in triplicate.

HPLC-ESI/MS analysis

The chromatographic separation condition was described above. An Agilent 1100 analytical HPLC system was used, with a G1312 Binpump, G1314A variable-wavelength detector (VWD), model 7725 injector fitted with a 20 μL sample loop, and an Agilent chemstation data system. LC/ESI-MS analyses were performed by using the Agilent HPLC system described above combined with a Bruker Esquire 3000 plus ion trap mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany), which was equipped with an electrospray ionization. Instrument control and data acquisition were performed by using Esquire 5.0 software. The ion source temperature was 270 °C, and the needle voltage was always set at 4.0 kV. Nitrogen was used as the drying and nebulizer gases at a flow rate of 10 L min^{-1} and a back-pressure of 30 psi.

Total flavonoids content

The total flavonoids content of the extracts was determined by the method described in the Chinese Pharmacopoeia (2010 edition). 100 mg of extract was dissolved in 100 mL of 70% (v/v) ethanol solvent. 1 ml solution was taken, being mixed with 1 mL of 5% $NaNO_2$. 6 min later, 1 mL of 10% $AlCl_3$ was added, and then 10 ml of NaOH (1M) was added after the same time. Immediately, the mixture was constant volume to 25 mL and was kept in dark place to react for 15 min. With 70% ethanol as a blank control, the absorbance (A) was measured at the wavelength of 510 nm. Rutin was used as standard compound and the concentration ranged from 0.01 to 0.80 mg ml^{-1}. The total flavonoids content was expressed as mg of rutin, which was equivalent to per g of extracts.

Orientin and isoorientin content

The content of orientin and isoorientin in extracts was measured with HPLC method. The mobile phase consisted of 0.1 % phosphoric acid in water (A) and 2 % Tetrahydrofuran in methanol (B). The gradient profile was established as follows: the ratio of A and B was 77:23 (v/v) within 60 min. The flow rate was 1.0 mL min^{-1}, and the column temperature was 40 °C. All of the samples were analyzed in triplicate.

Assays for antioxidant activities

DPPH assay

The DPPH assay of the extracts was determined following the method used by Shimada, Fujikawa, Yahara, and Nakamura (Kazuko et al., 1992). DPPH was dissolved in ethanol and the reaction solution concentration was 0.1 mM. 1 mL of the DPPH reaction solution was added to 5 mL of extract solution with being shaken vigorously. The mixture reacted for 30 min in the dark at room temperature, and then was measured at a wavelength of 517 nm using a UV spectrophotometer. Ascorbic acid and the antioxidant composition of bamboo leaves (AOB) were used as control groups. All of the samples were analyzed in triplicate. The DPPH radical scavenging activity was calculated by the following equation:

Scavenging activity % = [1 - A_{sample} / A_{blank}] × 100%

According to different concentrations of the clearance curve, the scavenging activities were expressed as half maximal inhibitory concentration (IC_{50}).

ABTS assay

The ABTS assay was based on the method of RE et al. (Re et al., 1999) with some modifications. ABTS stock solution was dissolved in deionized water to a 7 mM concentration. The ABTS reagent solution was generated by 5 mL of ABTS stock solution with 88 μL of 140 mM potassium persulfate. This solution was stored at room temperature in the dark for 12-16 h. Before it was used, the ABTS reagent solution was diluted with PBS (pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. For the ABTS assay, 0.3 mL sample of different concentrations was added to 6 mL of diluted ABTS solution to react in the dark at 30 °C for 6 min, and then the absorbance was measured at a wavelength of 517 nm. Ascorbic acid and AOB were used as control groups. All of the samples were analyzed in triplicate. The ABTS radical scavenging activity was calculated by the following equation:

Scavenging activity % = [1 - A_{sample} / A_{blank}] × 100%

According to different concentrations of the clearance curve, the scavenging activities were expressed as IC_{50}.

FRAP assay

The FRAP assay was determined by the method of Benzie and Strain (Benzie and Strain, 1996) with minor modifications. The FRAP reagent included the following solution: 300 mM acetate buffer; 10 mM TPTZ in 40 mM hydrochloric acid; and 20 mM $FeCl_3 \cdot 6H_2O$. The FRAP reagent was prepared by mixing 25 mL acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, and then warmed at water bath to 37 °C before using. 0.3 mL of sample solutions was added into 6 mL of FRAP solution, and reacted for 10 min. Absorbance was measured at a wavelength of 593 nm. Ascorbic acid and AOB were used as control groups. All of the samples were analyzed in triplicate.

Antimicrobial activities

The antimicrobial activity of the extracts was determined by the serial 2-fold dilution method (Charles et al., 1979; Shadomy and Espinel, 1980). Three bacterial strains were used in this study: *Staphylococcus aureus* (ATCC6538), *Escherichia coli* (8099), and *Candida albicans* (10231). All strains were obtained from the Zhejiang Academy of Medical Sciences, China. The bacterial strains were incubated on micrococcus, nutrient, and Yeast Maltose (YM) media then cultured at incubator at 35 °C for 24 h. VBE was dissolved in PBS at the highest concentration (100 μg/mL), then diluted to serial two-fold dilutions. Tetracycline was used as standard antibiotics. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that inhibited microorganism growth, which was based on the degree of turbidity visually.

Statistical Analysis

All samples were measured in triplicate. SPSS statistical software was used for the statistical evaluation of results, which were presented as the mean ± standard deviation (SD). Statistical analysis was done by Independent-Samples t-test.

Results and discussion

HPLC analysis and identification compounds by HPLC–ESI/MS analysis

In order to get the information of the VBE compounds, the analysis of HPLC and HPLC–ESI/MS was needed. The HPLC chromatogram of the VBE was showed in Fig. 1 (A), the separation of the compounds was excellent under the optimized conditions. As to the LC-MS information, the TIC negative and the TIC positive were shown in Fig. 1 (B), Fig. 1 (C), the TIC positive intensity was obviously less than the TIC negative, it was related to noise level and the compound structure, especially flavonoid compositions (PETSALOA et al., 2006). Compared to previous research, the compounds 1-12 in Fig. 1 (A) were identified as shown in Tab. 1 which include eight flavonoids: (4) orientin; (5) isoorientin; (6) hyperoside; (7) quercetin-3-O-β-D-glucuronide; (8) quercetin-3-O-α-L-arabinopyranoside; (10) quercetin-3-*O*-*α*-*L*-rhamnoside; (11) quercetin-3-*O*-*β*-*D*-glucuronide methyl ester; (12) isolariciresinol-9-*O*-*β*-*D*-xyloside. Compared with the HPLC chromatogram of chlorogenic acid and the VBE, the result showed the existence of chlorogenic acid in the *Vaccinium bracteatum* Thunb. leaves in Fig. 1 (D).

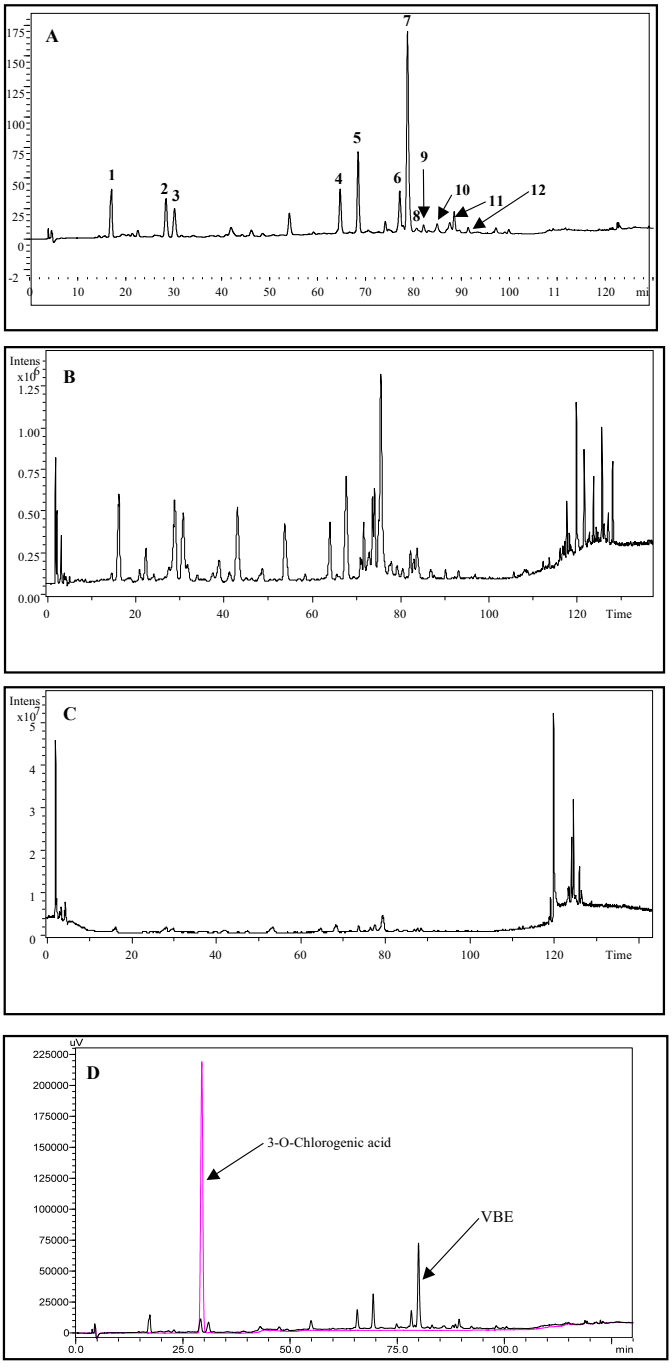

Fig. 1: (A) HPLC chromatography of the VBE at the 254 nm; for peak identification see Tab. 1.
(B) The TIC negative;
(C) The TIC positive;
(D) Chromatography contrast between chlorogenic acid reference and the VBE HPLC.

Tab. 1: Identification of 12 compounds.

Peak	Retention time	Λmax (nm)	m/z	Identification
1	16.4	240, 324, 443	354	5-O-Chlorogenic acid
2	29.0	240, 324, 443	354	3-O-Chlorogenic acid
3	30.9	240, 324, 443	354	4-O-Chlorogenic acid
4	64.0	252, 266, 348	448	Orientin
5	67.6	255, 269, 348	448	Isoorientin
6	74.9	253, 267, 352	464	Hyperoside
7	75.5	255, 354	478	Quercetin-3-O-β-D-glucuronide
8	77.9	247, 443	434	Quercetin-3-O-α-L-arabinopyranoside
9	79.0	220, 247, 342	536	10-O-*trans*-*p*-coumaroyl-sandoside
10	82.9	246, 286, 321	448	Quercetin-3-*O*-*α*-*L*-rhamnoside
11	86.7	266, 344, 324	492	Quercetin-3-*O*-*β*-*D*-glucuronide methyl ester
12	90.0	248, 443	494	Isolariciresinol-9-*O*-*β*-*D*-xyloside

The total flavonoids, orientin and isoorientin content

The contents of total flavonoids, orientin, and isoorientin were compared between the raw materials and the VBE. The results are shown in Tab. 2. Orientin and isoorientin have a typical flavonoid structure, so we choose the two standards as index compositions for measurements. The total flavonoids content was expressed as rutin equivalents in mg/g of extracts. Results indicated that the content of the total flavonoids in the extracts was 601.4 ± 8.50 mg/g, which was nearly ten times than that in the raw materials. Furthermore, the contents of orientin and isoorientin in the extracts were 44.7 ± 0.60 mg/g and 96.1 ± 0.92 mg/g, respectively, which were far more than that in the raw materials. The macroporous resins D-101 had good effect in enrichment of flavonoids, which was also confirmed in many studies (FANG et al., 2008; QI et al., 2007). As active ingredients, it would enhance the antioxidant activity and other activities by increasing flavonoids content.

Tab. 2: The content between the raw materials and the extracts. (Mean ± SD, n = 3)

Sample	Total flavonoid (mg/g)	Orientin (mg/g)	Isoorientin (mg/g)
Raw materials	56.3 ± 0.27	5.31 ± 0.05	9.42 ± 0.04
VBE	601.4 ± 8.50	44.7 ± 0.60	96.1 ± 0.92

The antioxidant activities

Antioxidant activities were evaluated by three methods, and the results were displayed in Fig. 2 and Tab. 3. The results of DPPH assay found that all samples had scavenging action. The IC_{50} value was 42.2 ± 1.2 μg/ml for VBE, while 449.5 ± 10.6 μg/ml for the raw materials. Additionally, the results of ABTS assay showed that the IC_{50} value of the raw materials, ascorbic acid, VBE, and AOB were 381.8 ± 12.4, 48.7 ± 1.0, 71.1 ± 1.1, and 104.3 ± 2.2 μg/ml, respectively. Furthermore, using 0.3 mmol/L $FeSO_4$ as the standard, the result of FRAP assay showed that the concentrations up to the same absorbance of the raw materials, ascorbic acid, VBE and AOB were 327.9 ± 7.5, 28.2 ± 0.5, 65.0 ± 1.8, and 101.7 ± 1.1 μg/ml, respectively.

Antioxidant capacities varied among these three antioxidant assays, all the samples showed certain antioxidant ability. Compared to the extracts of the raw materials, the antioxidant activity of VBE was better ($P < 0.05$), which also showed that the high content accompanied with strong antioxidant activities. As chemical antioxidant, ascorbic acid had the highest antioxidant ability, however, the activity was significantly higher in VBE compared to AOB ($P < 0.05$). It is reported that all of the three bamboo extracts from different parts had antioxidant activities, which had approved the development of natural antioxidants (Gong et al., 2015). Besides, its main active ingredients were flavonoids, azalides, and phenolic acid, and four major flavonoids were orientin, isoorientin, vitexin and isovitexin. The VBE and AOB had the same compound ingredients, but the VBE flavonoids content was much higher and the antioxidant activity of VBE was better than that of AOB. It was indicated that the *Vaccinium bracteatum* Thunb. leaves had large potential to be used as natural antioxidant.

The antimicrobial activities of VBE

The results of antimicrobial activities were presented in Tab. 4, which showed that the serial 2-fold dilution method was feasible. Some researches had found that chlorogenic acid had strong antimicrobial activity (Zhao et al., 2010; Chakraborty and Mitra, 2008), there were also many antimicrobial reports on flavonoids (Mellou et al., 2005; Sousa et al., 2009; Sohn et al., 2004). As representative compositions of the flavonoids, there were many studies on the antimicrobial activities of orientin and isoorientin (Zu et al., 2010; Becker et al., 2005). Based on these researches reported previously, we can infer that the VBE has good antimicrobial activity. The results obtained showed that the VBE has the inhibition to the three kinds of bacteria, and the minimum inhibitory concentration was 12.5 mg/ml with high level of antimicrobial activity.

Conclusions

In this research, twelve compounds were identified by using HPLC-DAD and HPLC–ESI/MS data analysis, which contained chlorogenic acid and its isomers, and eight flavonoids. Furthermore, we quantitatively analyzed the total flavonoids, orientin and isoorientin content of the extracts from *Vaccinium bracteatum* Thunb. leaves. These substances provided the basis of chemical compositions to explain the antioxidant activities. The antioxidant activities of the extracts were estimated by DPPH, ABTS, and FRAP assays. The results suggested that the antioxidant activity of the VBE could be significantly stronger than the AOB. Finally, the antibacterial activity of the VBE was determined by a serial twofold dilution method. Based on these results, the *Vaccinium bracteatum* Thunb. leaves could be a potential natural antioxidant. For further research, more works are needed to be carried out; the VBE will be added to the meat or the fried food as a readily accessible source of new natural food antioxidants.

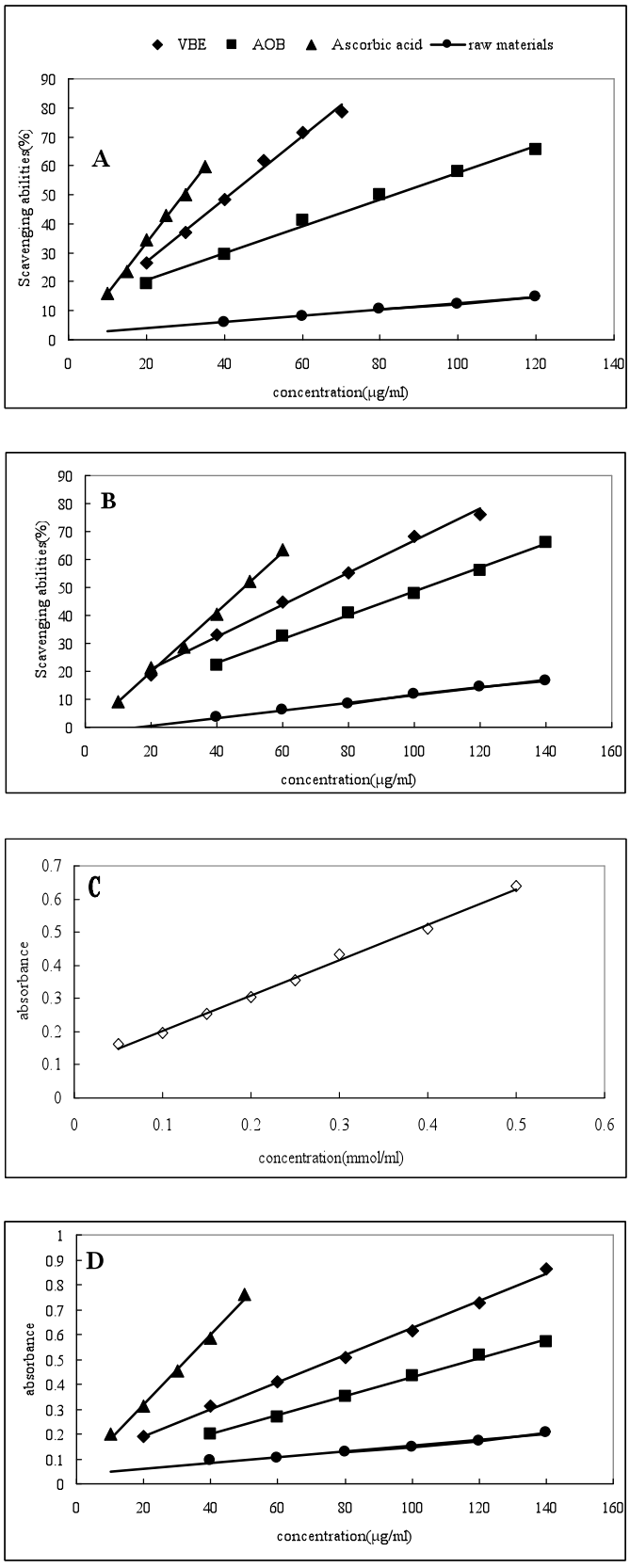

Fig. 2: Antioxidant activities of the raw materials, VBE, AOB and ascorbic acid. (A) DPPH radical scavenging assay;
(B) ABTS radical scavenging assay;
(C) FRAP-$FeSO_4$ standard curve;
(D) Different concentration samples absorbance curve in FRAP assay. The value in the figure is average value, n = 3.

Tab. 3: The antioxidant activity results. (Mean ± SD, n = 3)

Sample	IC_{50} (μg/ml)		FRAP assay (μg/ml) [a]
	DPPH	ABTS	
VBE	42.2 ± 1.2**	71.1 ± 1.1**	65.0 ± 1.8**
Raw materials	449.5 ± 10.6**	381.8 ± 12.4**	327.9 ± 7.5**
AOB	105.2 ± 3.4**	104.3 ± 2.2**	101.7 ± 1.1**
Ascorbic acid	29.7 ± 0.5	48.7 ± 1.0	28.2 ± 0.5

[a]: Result was expressed as the concentration of antioxidants up to the same absorbance that of 0.3 mmol/L $FeSO_4$.
**: Significantly different at the 0.05 level (2-tailed).

Acknowledgements
This work was supported by the Jiangsu Technology Support Program (Grant No. BE2014654), and the Changshu Technology Support Program (Grant No. CS201405).

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Tab. 4: Antimicrobial activities of the VBE

Bacterial strain	The concentration of VBE (mg/ml)							Negative control	Positive control
	50	25	12.5	6.25	3.125	1.5625	0.78125		
Staphylococcus aureus (ATCC6538)	–	–	–	+	+	+	+	+	–
Escherichia coli (8099)	–	–	–	+	+	+	+	+	–
Candida albicans (10231)	–	–	–	+	+	+	+	+	–

+: Instructions bacterial growth was positive;
–: Instructions bacterial growth was negative.

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