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Influences of ripening stages and extracting solvents on the polyphenolic compounds, antimicrobial and antioxidant activities of blueberry leaf extracts

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ABSTRACT

This study investigated polyphenols, antimicrobial and antioxidant activities of blueberry leaf extracts harvested at three fruit ripening stages (immature, semi-mature, and commercial-mature) and extracted by three solvents (95% ethanol, 70% acetone, and 100% methanol). Quercetin, kaempferol, gallic, protocatechui, caffeic, vanillic, syringic, p-coumaric, and ferulic acids were identified in the extracts with varied amount depending on ripeness stage and extraction solvent. Protocatechui and ferulic acids were not detected at semi-mature samples, and none protocatechui, syringic and p-coumaric acids in ethanol-extracts from commercial-mature samples. All extracts exhibited antimicrobial activity against *Escherichia coli* and fungi, but only semi-mature and commercial-mature samples were against *Listeria monocytogenes* and *Staphylococcus aereus*. Methanol- and acetone-extracts had higher polyphenol content, DPPH and reducing power than those of ethanol-extracts, whereas opposite trend was observed in ORAC values. These results indicated the potentials of using blueberry leaf extracts as natural antimicrobial and antioxidant substance for food applications.

1. Introduction

Blueberry (*genus Vaccinium*) fruits are well known for their high amount of health-promoting substances, such as anthocyanins, phenolics and flavonols (Howard, Clark, & Brownmiller, 2003; Naczk, Grant, Zadernowski, & Barre, 2006). Blueberry leaves, the primary plant parts, have generally been considered as a waste material. However, several recent studies have reported that plant leaf extracts have functional activities such as antimicrobial, anti-hypertensive, anti-inflammatory and neuroactive properties (Gurjar, Ali, Akhtar, & Singh, 2012; Gutiérrez-Larraínzar et al., 2012; Khaing, 2011; Piljac-Žegarac, Belščak, & Piljac, 2009; Shan, Cai, Brooks, & Corke, 2009; Skupień, Oszmiański, Kostrzewa-Nowak, & Tarasiuk, 2006). The plant leaf extracts are considered attributing to the exceptionally high antioxidant activity that strongly correlates with the phenolic compounds in the leaves (Cowan, 1999; Gurjar et al., 2012).

Plant maturity status reflects physiological, biochemical, and structural processes of plant tissue. The antioxidant and antimicrobial activities as well as the physicochemical properties of plants are often related to the ripening stage of plants (Gurjar et al., 2012; Moulehi, Bourgou, Ourghemmi, & Tounsi, 2012; Ribera, Reyes-Diaz, Alberdi, Zuñiga, & Mora, 2010). Howard et al. (2003) reported that genetics plays a more significant role than growing season in oxygen radical absorbing capacity (ORAC) and phenolic content of blueberries. Ribera et al. (2010) showed that the total antioxidant activity is high in unripen green and fully ripen highbush blueberry fruits and vary among the cultivars, and the levels of phenolic acids (mainly chlorogenic acid) and flavonols (mainly rutin) are high at immature fruits. Increased fruit maturity at harvest could increase ORAC, anthocyanin, and total phenolic content in four different cultivars of blueberries (Prior et al., 1998). Although intensive research has investigated the effect of maturity status on the antioxidant and antimicrobial activities of blueberry fruits, little

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information is available about the impact of ripening stage on the phenolic composition, antimicrobial and antioxidant activities of blueberry leaves. Therefore, it is important to investigate the impact of harvesting maturity of the leaves on their bioactive compounds, and in turn the antioxidant and antimicrobial activities.

In addition, accurate quantitation of the bioactive substances in plant tissues depends largely on the type of extraction conditions used (Gurjar et al., 2012), same as the yield and bioactivities of the extracts (Fernández-Agulló et al., 2013; Kaneria & Chanda, 2012; Kchaou, Abbès, Blecker, Attia, & Besbes, 2013). The characteristics of the extraction solvents noticeably affected the total phenolic content (±25% variation) and antioxidant activity (up to 30% variation) of the plants (Michiels, Kevers, Pincemail, Defraigne, & Dommes, 2012). Different solvents, such as water, ethanol, methanol, and acetone have been used to extract the phenolic compounds in the blueberry leaves. Li, Feng, Huang, and An (2013) found that the ethanolic extracts of rabbiteye blueberry leaves contain higher total phenolic content and total flavonoid content, and show higher antioxidant activity than those of blueberry fruit. Piljac-Žegarac et al. (2009) reported that the water extracts of blueberry leaf infusions have significant reducing capacity and radical scavenging potential. Hicks et al. (2012) quantified the chlorogenic acid (64.0 mg chlorogenic acid/g powdered leaf) and hyperoside (8.58 mg hyperoside/g powdered leaf) from crude extract material of blueberry leaveswith NMR spectroscopy. Naczk et al. (2006) cstated that the total phenols contents of ethanolic (95%) crude extracts was slightly higher than those of acetone (70%) extracts from the both blueberry leaves and fruits. Howard et al. (2003) found that the changes in ORAC and phenolic contents of the blueberry extracts with ethanol/acetone/water/acetic acid (40:40:20:0.1) were related to genotype and growing season. However, no of these studies systematically investigated the impacts of different extraction solvents on the phenolic composition, antioxidant and antimicrobial potential of the extracts from blueberry leaves. Therefore, it is necessary to compare the extraction efficacy and the antioxidant and antimicrobial activities of the extracts using different solvents for producing more effective and economically feasible natural antimicrobial and antioxidant substances.

The objectives of this work were 1) to investigate the influences of different ripening stages of fruit and extraction conditions on the phenolic composition and antioxidant activities of blueberry leaf extracts, 2) to evaluate the antimicrobial properties of blueberry leaf extracts from leaves harvested at different fruit maturity stages, and 3) to investigate the relationships among the determined bioactive compounds and their antioxidant and antimicrobial activities.

2. Materials and methods

2.1. Blueberry leaves

Blueberry (*Vaccinium formosum, V3*) leaves were manually picked from Qingpu Modern Agricultural Park (Shanghai, China) in 2012 at three different ripening stages of blueberry fruits based on the fruit color: immature (IM, green), semi-mature (SM, red) and commercial-mature (CM, dark blue) (Zheng, Zhang, & Cheng, 2013). The leaves were immediately freeze-dried (Freezone 2.5 L Triad, Labconco Inc., USA) and then stored at −80 °C until usage.

2.2. Reagents and standards

Gallic, protocatechuic, caffeic, vanillic, syringic, p-coumaric, and ferulic acids, quercetin, kaempferol, 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (trolox), 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH), ciprofloxacin, and triphenyl tetrazolium chloride (TTC) standards were all obtained from Sigma Aldrich (St. Louis, USA). Fluoroscein, 2,2-diphenyl-1-picrylhydrazyl, iron(III) chloride, sodium chloride, sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), trichloroacetic acid, Folin–Ciocalteu's phenol reagent, sodium carbonate anhydrous ethanol, and methanol were all purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Nutrient broth and plate count agar were obtained from Beijing Land Bridge Technology Co., Ltd (Beijing, China). Potato dextrose AGAR medium was from Hangzhou Tianhe Microorganism Reagent Co., Ltd. (Hangzhou, China).

2.3. Polyphenol extraction

Previous study by Naczk et al. (2006) showed that the 70% (v/v) aqueous acetone and 95% (v/v) ethanol are commonly used for extraction of tannins and other classes of phenolic compounds from plant materials. And pure methanol was the best solvent for extracting phenolic compounds in plant tissues, followed by ethanol then diethyl ether and hexane (Moulehi et al., 2012). Therefore, 95% ethanol (v/v), 70% acetone (v/v) and 100% methanol were used for extracting phenolic compounds from the blueberry leaves, respectively in this study. For ethanol extraction, leaf powders were transferred to dark-colored flasks, and mixed with 95% (v/v) aqueous ethanol at a material to solvent ratio of 15:100 (m/v) at 50 °C for 30 min. For acetone and methanol extractions, the leaf samples were mixed with 70% (v/v) aqueous acetone or pure methanol at 25 °C for 30 min with a material to solvent ratio of 1:10 (w/v). After twice extractions with respectively selected solvent, two extracts were collected and pooled into a round-bottom flask, and then evaporated to near dry under vacuum at 40 °C using a rotary evaporator (Models RE-52AA, Yuhua Instruments Co., Henan, China). The residual was re-dissolved in 1% (v/v) the corresponding solvent to 50 mL, and then stored at −80 °C until further analysis.

2.4. Total phenolic content (TPC) assay

Total phenolic content was determined using the Folin-Ciocalteu assay following the method by Moulehi et al. (2012) with some modifications. Briefly, the obtained polyphenol extract described above was diluted with water to 50 mL. A 7.5 mL of deionized water and a 0.5 mL of Folin-Ciocalteau reagent were added into 0.5 mL of diluted sample extract or 0.5 mL gallic acid solutions (0, 50, 100, 150, or 200 mg/kg) in a series of test tubes, respectively. After sitting at room temperature for 10 min, the solutions were mixed with 3 mL of 20% (m/v) Na_2CO_3, placed in a water bath at 40 °C for 20 min, and then immediately cooled to room temperature in an ice bath for 3 min. The absorbance of the samples and standards were measured spectrophotometrically (UV-1800, Kyoto, Japan) at 765 nm. Triplicate extractions were performed for each sample. TPC was calculated as mg of gallic acid equivalents (GAE)/g dry mass (DM) sample.

2.5. Profile of phenolics identified by HPLC

The phenolic profile in the different solvent extracts was analyzed on an Agilent 2489 infinity HPLC using a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 μm, Waters, CA, USA). A complete procedure is illustrated in Fig. 1. The phenolic compounds were separated using a gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v). Separation was achieved through a gradient elution as: solvent B from 10% to 100% within 42 min and then 33 min of post-run for reconditioning. An