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In vitro assays of the antibacterial and antioxidant activity of aqueous leaf extracts from different *Prunus salicina* Lindl. cultivars

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ABSTRACT

The growing interest in the substitution of synthetic food antioxidants and antimicrobial additives by natural ones has fostered research on vegetable sources and on the screening of raw materials, for identifying new antioxidants and antimicrobial natural agents. The aim of the present study was to assess total phenolic contents and determine polyphenolic composition, related antioxidative and antimicrobial properties of plum leaves extracts from six cultivars. It was observed that the content of total phenolic compounds was cultivar dependent. High antioxidant capacity has been observed and related to the relative amounts of polyphenolic compounds with good antioxidant properties. The antimicrobial activity was confirmed against *Listeria innocua* and *Escherichia coli*, and it has found to be related with the high phenolic contents. Our results suggest that the use of plum leaf extracts is a feasible alternative as antibacterial and antioxidant agents to prevent the deterioration of stored foods by bacteria and oxidation.

1. Introduction

Extraction is one of the most widely used unit operations in the food industry. It is mainly used for obtaining certain desired components initially retained in a food matrix (Pinelo et al., 2005). Molecules obtained by extraction may be used as food additives or for exerting peculiar beneficial effects on human health (Delgado-Adámez et al., 2012; Lee and Lee, 2010).

In the past several years, a large number of scientific reports have described the properties of phenolic compounds from numerous natural products. These secondary plant metabolites, naturally present in fruit and vegetables, are part of our everyday diet. Phenolics display a wide variety of structures, ranging from simple moieties containing a single hydroxylated aromatic ring to highly complex polymeric substances. These compounds arise biogenetically from two main primary synthetic pathways: the shikimate pathway and the acetate pathway. Based on their carbon skeleton polyphenols can be classified into non-flavonoid compounds (stilbenes) and flavonoid compounds. Abiotic environmental factors (temperature, moisture, soil and climatic conditions, elevation, etc.) as well as biotic effects (human disturbance, herbivores, etc.) were proven to influence both essential oil and polyphenol production (Pluhár et al., 2007). The synthesis of flavonoid and non-flavonoid plant polyphenols such as stilbenes is increased in plant tissues following wounding or infection by pathogenic organisms (Montealegre et al., 2006).

Antioxidants are needed for preventing degenerative reactions produced by reactive oxygen and nitrogen species *in vivo* and lipid peroxidation in foods (Cevallos-Casals and Cisneros-Zevallos, 2003). The most widely used antioxidants, to prevent the oxidation of lipids in foods and in addition to increase product shelf life, are butylated hydroxyanisole (BHA), butylated hydroxytolune (BHT) and propyl gallate and 2-tert-butylhydroquinone (TBHQ) (Moure et al., 2001). However, there has been growing concern over the safety of some of the commercial antioxidants because several studies documented they might be carcinogenic (Whysner et al., 1994) or toxic (Moure et al., 2001), although other authors reported anti-carcinogenic effects (Williams et al., 1999). For this reason, a great effort has been made to characterize phenols occurring in different plant tissues.

Phenolics as efficient free radical scavengers they can potentially interact with biological systems and play a role in anticarcinogenic, antiatherogenic, anti-inflammatory, antimicrobial and antioxidant activities (Delgado-Adámez et al., 2012; Moure et al., 2001). Since the prevention of chronic diseases is a more effective strategy than their treatment, reducing the risk of diseases such as cardiovascular disease and cancer is a subject of great interest for doctors, scientists in general, consumers and the food industry (Liu, 2003). For this reason, many functional foods are nowadays

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BHA, butylated hydroxyanisole; BHT, butylated hydroxytolune; CECT, Spanish type culture collection; TAA, total antioxidant activity; TBHQ, 2-tert-butylhydroquinone; TPC, total phenolic contents.

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aimed at boosting intakes of antioxidants in order to reduce the risk of chronic disease linked to oxidative stress. Phenolics have also been found to be natural antimicrobial compounds, which are important for increasing the shelf life of food and inhibiting the growth of pathogenic microorganisms (Davidson and Branen, 1981; Davidson, 2001).

Plum (*Prunus salicina* Lindl.) is a deciduous tree native to China of the Rosaceae family. It is now also grown in fruit orchards of Korea, Japan, United States, Australia and Europe. The fruit of the plum tree (*P. salicina* Lindl.) has been used as a traditional medicinal food in humans to enhance immunity against infectious agents and to treat cancers (Lee et al., 2009). The plum fruits are a highly nutritious food, which are rich in phenolic composed. Antioxidant and antimicrobial effects of isolated polyphenols obtained from plums have been previously reported (Cevallos-Casals et al., 2006). Positive correlation between total phenolic content of plant extracts and related antioxidant capacity has been reported (Cevallos-Casals et al., 2006).

Knowledge of polyphenolic content of plum leaf is relevant for their future use as they are a cheap and valuable raw material for the production of biologically interesting polyphenolic compounds (or products based on them). The aim of this study was to determine polyphenolic composition, related antioxidative and antimicrobial properties of plum leaves extracts from six cultivars grown in Extremadura (Spain), with the same agricultural, geographical and climatic conditions, and know the potential of individual varieties with regard to the concentration of each of these biologically-active compounds.

2. Materials and methods

2.1. Plant material and preparation of the water extracts

P. salicina leaves were collected during summer, September 2010, in Badajoz, Southwest Extremadura (Spain). A total of six plums cultivars were studied: 'Ambra' (Am), 'Angeleno' (An), 'Red Beauty' (RB), 'Fortune' (F), 'Larry Ann' (LA), 'Black Diamond' (BD). After collecting, leaves were dried at 40 °C temperature. Right after that, leaves were ground in a knife mill and powdered samples were sieved to select particles between 0.5 and 3.0 mm, and they were stored at −80 °C in vacuum conditions until further used. Voucher specimens were preserved in our laboratory for further reference. Dried leaves were powdered and bioactive compounds were extracted with water (70–80 °C) for 3 h in Soxhlet. These extracts were filtered and kept in a dark place at −80 °C until analysis.

2.2. Determination of total polyphenols contents

The concentration of total phenolic compounds in the extracts was determined by the Folin–Ciocalteu colorimetric method (Singleton and Rossi, 1965). All the analysis were conducted in triplicate and calculated from a calibration curve using gallic acid as standard. The results were expressed in milligrams of gallic acid per liter of plum leaf extracts (mg L^{-1}).

2.3. Total antioxidant activity (ABTS method)

Total antioxidant activity (TAA) was determined according to Cano et al. (1998) on freshly prepared plum leaf extracts. The measurement was carried out in a UV-2401 PC Shimadzu spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland, USA). The results were expressed as Trolox equivalents in grams Trolox L^{-1} of extract.

2.4. HPLC conditions for phenolic compounds separation and quantification

The analysis of the individual phenolic compounds was achieved following the method proposed by González-Gómez et al. (2009) with some minor modifications. Chromatographic separation was accomplished with a Phenomenex C18 HPLC column (150 mm × 4.6 mm, 5 μm) heated to 35 °C. The mobile phase used for the separation was composed of aqueous TFA 0.1% (A) and acetonitrile (B) in gradient mode set as follows: initial conditions 10% B; from 0 to 3 min 10% B; from 3 to 15 min 15% B; from 15 to 20 min the composition was kept constantly at 15% B; from 20 to 25 min 18% B and from 25 to 40 min 30% of B. A period of 5 min was necessary for column equilibration. The flow was fixed at 0.500 mL min^{-} for all the experiments. The injected volume was 5 μL. For all chromatographic studies, an Agilent 1100 series liquid chromatographic instrument, equipped with a diode array detector (DAD) was used for analyte detection and quantification. Phenolic acids were detected and quantified at 320 nm, flavanol (epicatechin) at 280 nm and flavonols at 360 nm. Chromatographic data processing was done using Agilent ChemSation software and the quantification was done by external standard method. Phenolic acids results were expressed in milligrams of chlorogenic acid per liter of plum leaf extracts, flavanol in milligrams of epicatechin per liter of plum leaf extracts and flavonols in quercetin-3-*O*-rutinoside per liter of plum leaf extracts.

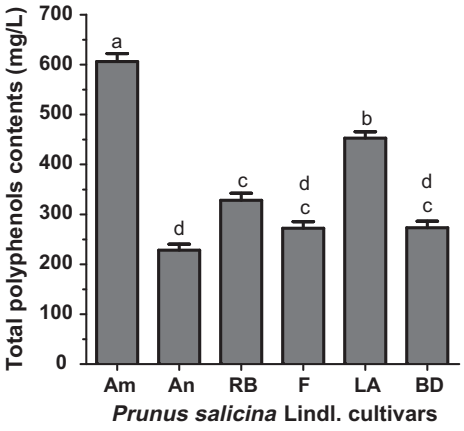

Fig. 1. Total polyphenols contents (TPC) of plum leaf extracts from six *Prunus salicina* Lindl. cultivars. Am: 'Ambra', An: 'Angeleno', RB: 'Red Beauty', F: 'Fortune', LA: 'Larry Ann', BD: 'Black Diamond'. Values expressed are mean ± SD of five experiments. Different letters mean significant differences for $p < 0.05$. TPC were expressed in milligrams per liter of plum leaf extracts.

2.5. Antimicrobial activity

2.5.1. Microbial strains

The bacteria strain used in the experiment were obtained from the Spanish type culture collection (CECT) of Valencia University. The antimicrobial activities of the extract were individually tested against a panel of bacteria strains including: one Gram-positive bacteria such as 910 *Listeria innocua* and one Gram-negative bacteria such as 45 *Escherichia coli*.

2.5.2. Determination antimicrobial activity

The antimicrobial activity values were studied for the leaf extracts following the procedure previously established by Delgado-Adámez et al. (2012). The target microorganisms were cultured in Mueller–Hinton broth (MHB) at 37 °C for 24 h. To measure the CFU, the suspensions were diluted with 0.5 McFarland standard turbidity and diluted again (1/1000 ratio) by Mueller–Hinton broth. To 24-well microliters plates, 1.8 ml of Mueller–Hinton broth containing diluted bacteria (about 10^5 CFU mL^{-1}) and 200 μL of aliquot from the extracts were added. A positive control (containing inoculums but no extracts) and negative control (containing extracts but no inoculums) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 37 °C for 24 h under aerophilic conditions.

2.6. Statistical analysis

Data were analyzed by Statistical Package SPSS 17.0 version for Windows (SPSS Inc., Chicago, IL, USA). Tukey's test for pairwise comparison was used to determine significant differences at a level of 5%. Pearson correlation was carried out to understand any relation between all analyzed parameters. Mean values with standard deviations are reported.

3. Results and discussion

The contents of total polyphenols in plum leave extracts are given in Fig. 1. The total polyphenols contents showed great variation in different leaf extracts, and the range was from 606.01 ± 16.14 to 228.29 ± 12.19 mg L^{-1}. 'Ambra' had the highest total polyphenols content, followed by 'Larry Ann', 'Red Beauty', 'Black Diamond' and 'Fortune', whereas 'Angeleno' had the lowest total polyphenols content.

The antioxidant capacity of leaf extracts was determined by the ABTS method and the results are presented in Fig. 2. The free radical scavenging activity of leaf extracts ranged from 5.08 ± 0.44 to