Food Chemistry 230 (2017) 24–29

Contents lists available at ScienceDirect

Food Chemistry

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

Antioxidant and antibiofilm activities of secondary metabolites from *Ziziphus jujuba* leaves used for infusion preparation

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ARTICLE INFO

Article history:
Received 10 October 2016
Received in revised form 14 February 2017
Accepted 28 February 2017
Available online 3 March 2017

Chemical compounds studied in this article:
Alphitolic acid (PubChem CID: 12305768)
Quercitrin (PubChem CID: 5280459)
Catechin (PubChem CID: 9064)
Gallocatechin (PubChem CID: 65084)

Keywords:
Ziziphus jujuba
Jujube leaf infusion
Antibiofilm activity
Antioxidant activity
Alphitolic acid
Anti-caries agents

ABSTRACT

Ziziphus jujuba Mill., jujube, leaf infusions are popular bedtime beverages as they improve sleep by soothing the nerves. With the aim of providing further insights into the polyphenol content of jujube leaf infusion and their antioxidant activities, a 2,2-diphenyl-1-picrylhydrazyl (DPPH)-guided purification of the aqueous extract of *Z. jujuba* Mill. leaves was conducted. Three major antioxidant polyphenols (i.e. quercitrin, catechin and gallocatechin) of the leaves were identified. In addition, a significant antibiofilm bioactivity against *Streptococcus mutans*, a causative agent of human dental caries, was detected for the aqueous leaf extract and its bio-guided separation resulted in the identification of the lupane triterpenoid alphitolic acid as the main antibiofilm metabolite. The results herein presented further promote the consumption of jujube leaf infusion as a healthy antioxidant bedtime beverage, and associate it to an unreported anti-caries activity.

1. Introduction

Ziziphus jujuba Mill (Rhamnaceae), known as jujube or Chinese red date, is a small deciduous tree naturally occurring in Southern Asia, now rife also in Madagascar and South-Eastern Europe. Many studies have been published on both jujube fruit and seed preparations, stimulated by their long use in Chinese and Korean traditional medicine as antifungal, antibacterial, antiulcer, anti-inflammatory and antioxidant remedies (Mahajan & Chopda, 2009). Conversely, little has been reported on the plant leaves that for centuries have been harvested and dried to prepare bedtime infusions (Yook, 1972). A recent investigation has demonstrated that jujube leaf infusion is in fact able to improve sleep, since it contains purportedly sedative flavonoids, while stimulant agents, such as caffeine and theophylline, are absent (Zhang et al., 2014). Thus, the many ascertained beneficial effects for both the heart and the nervous system (Ho, Lee, & Huang, 1992) have prompted the use of jujube leaf infusion as a healthy bedtime beverage not only across Asia but also in the Western hemisphere.

To the best of our knowledge, a complete qualitative and quantitative analysis of jujube leaves has yet to be conducted. Only a list of quercetin and kaempferol diglycosides, in addition to other primary metabolites, such as tryptophan and tyrosine, has been recently reported, as a result of High Performance Liquid Chromatography (HPLC)-electrospray ionization-time of flight mass spectrometry experiments (Zhang et al., 2014).

With the aim of providing further insights into the polyphenol content of jujube leaf infusion and their biochemical activities, we conducted a 2,2-diphenyl-1-picrylhydrazyl (DPPH)-guided purification of the aqueous extract of *Ziziphus jujuba* Mill. leaves. This resulted in the isolation and identification of three major antioxidant polyphenols (i.e. quercitrin, catechin and gallocatechin) in the analyzed leaves. Furthermore, a parallel analysis on the aqueous leaf extract revealed a significant antibiofilm bioactivity against *Streptococcus mutans*, a facultative anaerobic Gram-

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http://dx.doi.org/10.1016/j.foodchem.2017.02.141
0308-8146/

positive member of the oral microflora and one of the primary causative agents of human dental caries (Kleinberg, 2002; Loesch, 1986). A bio-guided separation of the aqueous leaf extract resulted in the identification of the lupane triterpenoid alphitolic acid as the main antibiofilm metabolite contained in jujube leaves.

2. Materials and methods

2.1. Plant material, extraction and separation

Ziziphus jujuba Mill fresh leaves were collected from the cultivated fields of Azienda Agricola Si.Gi. (C.da Acquevive, 25, 62100, Macerata, Italy) and identified by the farm owners. A voucher specimen (ZJ 2015-08) has been deposited at the Herbarium of the School of Pharmacy, University of Camerino, Italy. The leaves were dried at room temperature, pulverized using a blender and stored until biological and phytochemical analysis. Powdered plant material (50.0 g) was extracted at room temperature either in 500 mL methanol or in 500 mL distilled water by overnight maceration. Methanol was removed under reduced pressure at 40 °C using a rotary evaporator, giving a sticky dark-green crude extract of approximately 20% w/w yield. The water extract was freeze-dried giving a light-green crude extract of approximately 24% w/w yield. The crude extracts were diluted with 10% DMSO in distilled water or distilled water respectively to make appropriate doses for further use. Crude water extract was partitioned against ethyl acetate (EtOAc) and then butanol using a separatory funnel at room temperature. The solvents were removed by lyophilization in the case of water and using a rotary evaporator at 40 °C under vacuum in the case of EtOAc and *n*-butanol. The EtOAc extract (2.89 g), selected for further investigation, was separated through a 40-g Combiflash silica column eluted with a *n*-hexane/EtOAc gradient whose ratio changed linearly from *n*-hexane to EtOAc in 60 min, and then the column was further eluted with a EtOAc/methanol gradient whose ratio changed linearly form EtOAc to EtOAc/methanol 1:1 in 30 min. Thirteen fractions were collected according to their increasing polarity and named A-M.

Fraction E, eluted with *n*-hexane/EtOAc 6:4 (117.9 mg), found positive to the biofilm formation assay was further separated on a 4-g Combiflash silica column eluted with an *n*-hexane/EtOAc gradient whose ratio changed linearly from *n*-hexane to EtOAc in 45 min. The fraction eluted with *n*-hexane/EtOAc 8:2, positive to the biofilm formation assay, was identified as alphitolic acid (**1**, 32.0 mg).

Fractions I and K were found positive to the DPPH assay (see below). Fraction I, eluted with EtOAc from the 40-g Combiflash silica column (160.7 mg), was further purified on a 43-g Combiflash C-18 column eluted with a H_2O/methanol gradient whose ratio linearly changed from 1:1 to methanol in 60 min. The antioxidant fraction eluted with H_2O/methanol 2:8 was finally purified on a HPLC Gemini 10u column with a mixture of H_2O/methanol 3:7, thus affording catechin (**2**, 180.5 mg) and gallocatechin (**3**, 50.4 mg). Likewise, fraction K, eluted with EtOAc/methanol 1:1 from the 40-g Combiflash silica column after 90 min (847.8 mg), was purified on a 43-g Combiflash C-18 column eluted with a H_2O/methanol gradient whose ratio linearly changed from 1:1 to methanol in 60 min. The antioxidant fraction eluted with H_2O/methanol 3:7 after 20 min was finally purified on a HPLC Gemini 10u column using H_2O/methanol 4:6 as eluent, affording quercitrin (**4**, 640.0 mg).

2.2. Analysis of total phenolic content

The amount of total phenolic compounds was determined spectrophotometrically on microplate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) according to the Folin–Ciocalteu method applied to a 96-well microplate assay, using gallic acid as calibration standard (Prior, Wu, & Schaich, 2005). A 50 μL aliquot of the different concentrations of extracts and standard were added to 150 μL of freshly prepared Folin–Ciocalteu reagent (1:4 v/v in distilled water) in a 96-well microtitre plate (Falcon® 96). After 10 min at 37 °C, 50 μL of saturated solution of sodium carbonate were added to each well, and the plate was incubated for 10 min at 37 °C. Absorbance of each solution was measured at 765 nm on Spectrophotometer Tecan Italia and on an Automated Microplate Reader EL 311 s (Bio-Tek Instruments, Winooski, VT, USA). The standard curve was linear between 2 and 0.03 mM gallic acid solution. Total amount of phenolics was calculated as mg gallic acid equivalent (GAE)/g of dried leaves.

2.3. Evaluation of the total antioxidant capacity

The antioxidant activity of leaf extracts was evaluated by means of three different antioxidant assays: 1,1-diphenyl-2-picrylhydrazyl (DPPH·), 2-2′-azino-bis(3-ethylbenzo-thiazoline-6-sulphonate) diammonium salt ($ABTS^{\cdot+}$) and Ferric Reducing Antioxidant Power (FRAP) assays. 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox™) was used as calibration standard. DPPH, ABTS, $K_2S_2O_8$, 2,4,6-tripyridyltriazine (TPTZ), Folin-Ciocalteu reagent (FCR), Trolox™, gallic acid, manganese dioxide and Gly-Pro-p-nitroanilide hydrochloride were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Ferric chloride was from Avantor (Center Valley, PA, USA). Values are expressed as IC_{50}, defined as the concentration of the sample required to cause a 50% decrease in initial DPPH, ABTS or ferric iron concentration, as well as μmoles Trolox™ equivalent (TE)/g of dried leaves.

2.3.1. DPPH radical scavenging assay

DPPH free radical scavenging activity was evaluated on a microplate analytical assay according to literature procedures (Srinivasan, Chandrasekar, Nanjan, & Suresh, 2007). A 50 μL aliquot of the different concentrations of extracts and standard was added to 200 μL of DPPH in methanol solution (100 μM) in a 96-well microtitre plate (Falcon® 96). After incubation at 37 °C for 20 min, the absorbance of each solution was determined at 490 nm using a microplate reader (FLUOstar Optima, BMG Labtech).

2.3.2. ABTS radical cation scavenging assay

ABTS assay was performed following the reported procedure (Re et al., 1999), applied to a 96-well microtitre plate assay. $ABTS^{\cdot+}$ solution (5 mM) was prepared by oxidizing ABTS with MnO_2 in distilled water for half an hour in the dark. Then, the solution was filtered through filter paper. The absorbance of the $ABTS^{\cdot+}$ working solution was read at 734 nm in a spectrophotometer, adjusting solution absorbance to 0.700 by diluting with distilled water. A 50 μL aliquot of the different concentrations of extracts and standard were added to 200 μL of $ABTS^{\cdot+}$ working solution. After incubation at room temperature for 10 min, the absorbance of each solution was determined at 734 nm using a microplate reader (FLUOstar Optima, BMGLabtech).

2.3.3. FRAP assay

FRAP assay was performed according to the published procedure (Firuzi, Lacanna, Petrucci, Marrosu, & Saso, 2005) applied to a 96-well microplate assay, monitoring the reduction of Fe^{3+}-TPTZ to blue-colored Fe^{2+}-TPTZ. Stock solutions of acetate buffer (300 mM) pH 3.6, $FeCl_3{\cdot}6H_2O$ (20 mM) and 10 mM TPTZ in 40 mM in HCl (10 mM) were prepared. The fresh working solution was prepared by mixing ten volumes acetate buffer, one volume TPTZ solution and one volume $FeCl_3{\cdot}6H_2O$ solution and then