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Antioxidant activity of 1,3-dicaffeoylquinic acid isolated from *Inula viscosa*

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ABSTRACT

Inula viscosa is a perennial herbaceous plant used topically in folk medicine as an anti-scabies, anti-inflammatory, and wound-healing agent. We examined the antioxidant activity of the methanolic extract of *I. viscosa*. We isolated and identified several polyphenolic antioxidants from *I. viscosa* leaves and focused on 1,3-dicaffeoylquinic acid (1,3-diCQA). Antioxidant activity was measured using ABTS and DPPH assays, which measure antioxidant activity. The concentrations of 1,3-diCQA required for the inhibition of oxidation were lower than those required by other known antioxidants. 1,3-diCQA inhibited oxidative damage caused by various factors, including $FeSO_4$ and AAPH (2,2′-azobis(2-amidinopropane) dehydrochloride). Antioxidant activity can also be detected by the ability of a compound to scavenge reactive oxygen species (ROS). 1,3-diCQA was found to scavenge hydroxyl radical and superoxide radicals, as measured by electron spin resonance (ESR). These data demonstrate that 1,3-diCQA exhibits antioxidant properties, probably through the involvement of a direct scavenging effect on several free radicals.

1. Introduction

Reactive oxygen species (ROS) are essential for life, as they are involved in cell signaling and are used by phagocytes for their bactericidal action. However, excessive production of ROS, for example, in the physiological processes of oxidative stress, was suggested to be strongly associated with the aging process and certain degenerative diseases, including various cancers, cognitive dysfunction, and coronary heart disease (Wang et al., 2003). Recent investigations have shown that there is a profound link between dietary habits and the incidence of cancer and heart disease in humans (Conklin, 2000; Kitani et al., 2001). Vegetables furnish an abundance of nutrients, especially vitamins and minerals, to the human diet. They also contain nonnutritive constituents, such as fiber and phenolic compounds (Velioglu, Mazza, Gao, & Oomah, 1998; Wettasinghe, Bolling, Plhak, Xiao, & Parkin, 2002). In the past, many of the nonnutritive constituents were ignored because they were considered biologically inert. However, recent studies have demonstrated that nonnutritive constituents may play a role in preventing the development of some diseases (Khanduja et al., 2006; Li, But, & Ooi, 2005). Many of the beneficial health effects of nonnutritive constituents of vegetables have been known to originate from, or be closely associated with, their antioxidant properties (Russo et al., 2005; Wu, Ho, Shieh, & Lu, 2005). Most commercial antioxidants are synthetic and water-insoluble. Because of pathological effects associated with some synthetic antioxidants, more attention is now being paid to antioxidants derived from natural sources (Mishima et al., 2005; Wu et al., 2005).

Inula viscosa is a perennial herbaceous plant that profusely colonizes sub-nitrophile and sub-saline soils in abandoned and plowed fields in the Mediterranean region. It exhibits simple alternate leaves, covered with glands secreting a sticky substance, and bright yellow flowers that bloom between August and November. This species is used topically in folk medicine as an anti-scabies and anti-inflammatory agent, and to promote wound-healing (Ali-Shtayeh & Abu Ghdeib, 1999; Lauro & Rolih, 1990).

Various compounds have been described that exhibit both antioxidant and anticancer activity (Wattenberg, 1983, 1996). In our laboratory, we have succeeded in isolating and characterizing a water-soluble antioxidant from spinach (Bergman, Varshavsky, Gottlieb, & Grossman, 2001), and we described its antioxidant and broad free-radical scavenging properties (Bergman, Perelman, Dubinsky, & Grossman, 2003; Lomnitski, Bergman, Nyska, Ben Shaul, & Grossman, 2003). Recently, we also isolated cucurbitacin B glucoside and cucurbitacin E glucoside from *Citrullus colocynthis*, and explored the mechanism by which their combination (1:1) reduces the proliferation of human breast cancer cells (Tannin-Spitz, Grossman, Dovrat, Gottlieb, & Bergman, 2007). Moreover, we demonstrated that these cucurbitacin glucosides exhibited antioxidant properties, probably through the involvement of a direct scavenging effect on several free radicals (Tannin-Spitz, Bergman, & Grossman, 2007).

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In this study, we describe the isolation of a polyphenolic antioxidant from the leaves of *I. viscosa*, comprised of seven derivatives from the caffeoylquinic acid (CQA) and dicaffeoylquinic acid (diCQA) family. The derivatives are **(1)** 3-CQA; **(2)** 1-CQA; **(3)** 4-CQA; **(4)** 5-CQA; **(5)** 1,3-diCQA; **(6)** 3,4-diCQA; and **(7)** 1,5-diCQA. This study focuses on the antioxidant activity of 1,3-diCQA, the first compound that was isolated from *I. viscosa*. Members of the caffeoylquinic acid and dicaffeoylquinic acid families exhibit a wide range of biological activities in plants and animals. For example, treatment of human promyelocytic leukemia HL-60 cells with 4,5-diCQA, 3,5-diCQA, and 3,4-diCQA suppresses cell growth in a dose-dependent manner (Mishima et al., 2005), and 3,5-diCQA and 3,4-diCQA possess potent antiviral activity against respiratory syncytial virus (RSV) (Li et al., 2005). In addition, diCQA derivatives including 3,5-diCQA and 4,5-diCQA exhibit potent antioxidant, tyrosinase-inhibitory and antiproliferation activities (Iwai, Kishimoto, Kakino, Mochida, & Fujita, 2004).

In the present study, we examined the antioxidant and free-radical scavenger activity of 1,3-dicaffeoylquinic acid (1,3-diCQA). We report here the isolation of this compound from *I. viscosa* leaves and the ability of 1,3-dicaffeoylquinic acid to scavenge free radicals and reduce oxidative stress in cultured cell lines.

2. Materials and methods

2.1. Materials

Linoleic acid, Tween 20, and DCFH were obtained from Sigma Chemical Co. (St. Louis, MO). TEMP (2,2,6,6-tetramethyl-4-piperidone hydrochloride), xanthine, xanthine oxidase, AAPH (2,2′-azobis(2-amidinopropane) dehydrochloride), ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), and DPPH (1,1-diphenyl-2-picrylhydrazyl) were also obtained from Sigma Chemical Co. (St. Louis, MO, USA). DEPMPO (5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) was purchased from Calbiochem (San Diego CA, USA). Trolox was obtained from Acros Organics (Morris Plains, NJ, USA).

2.2. Plant material and extraction

Fresh leaves of *I. viscosa* (collected from an open field at Bar-Ilan University) were mixed 1:4 (w/v) with distilled water and homogenized in a blender for about 5 min. The homogenate was filtered and centrifuged at 20,000*g* for 10 min. The supernatant was boiled for 40 min, frozen in liquid nitrogen, and dried in a lyophilizer (0.07 mbar, −48 °C). Then, the powder was extracted in acetontrile and the precipitate was dried in a lyophilizer and extracted in methanol. The resulting methanol extract was analyzed by thin layer chromatography (silica gel 60 F_{254} plates, Merck Eurolab SA, Strasbourg, France) using the solvent system: butanol/acetic acid/water (6:4:1). The methanol elution fractions were separated on HPLC.

2.3. HPLC analysis and separation

HPLC analysis was performed on a LaChrom system with a LaChrom L-7450 diode array detector. Data for each run were documented using the HSM software package supplied with the LaChrom detector. For analytical separations, a LiChrocart 125-4 column packed with Lichrospher 100, RP8, 10 μm, was used. Solutions: (A) 0.1% trifluoroacetic acid in water; and (B) acetonitrile. The running conditions were as follows: 0–5 min, 100% A; 5–14 min, 0–7% B (linear gradient); 14–22 min, 15% B; 22–26 min, 28% B; 26–30 min, 50% B; flow rate: 1 ml/min; detection at 250 nm. The fractions were collected into clean tubes, acetonitrile was evaporated in a Speed Vac Plus SC110A (Savant), and the fractions were freeze-dried. All the peaks were identified by NMR.

2.4. Identification of molecular structure by NMR

NMR spectra of the 1,3-dicaffeoylquinic acid were obtained on a Bruker DMX-600 spectrometer, at 600.1 (^{1}H) and 150.9 (^{13}C) MHz, respectively, at room temperature. The solvent used was a mixture of 10% CD_3OD and 90% $CDCl_3$, containing 0.1% TMS as an internal reference. NMR analysis was facilitated by the use of 2D experiments including COSY ($^1H \times {}^1H$ correlation), HMQC (one-bond $^{13}C \times {}^1H$ correlation), and HMBC (long-range $^{13}C \times {}^1H$ correlation).

2.5. DPPH assay

Free-radical scavenging activity of antioxidants was estimated by the method described earlier (Hirota, Abe, & Murata, 1997), using DPPH, a stable free radical. Various concentrations of antioxidant prepared in ethanol solution (100 μl) were added to a mixture of 0.1 M acetate buffer (pH 5.5, 100 μl) and 0.5 mM DPPH in ethanol (50 μl) in a 96-wall plate. The absorbances were measured at 517 nm. Trolox was used as a standard. The activity was expressed as the concentration of sample necessary to give a 50% reduction in the sample absorbance (IC_{50}).

2.6. $ABTS^{\cdot+}$ assay

The total antioxidant capacity of the components was measured by the (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) $ABTS^{\cdot+}$ decolorization assay involving preformed $ABTS^{\cdot+}$radical cations, as described previously by Kerem, Bilkis, Flaishman, and Sivan (2006). Activity was calculated relative to trolox, as described previously (Kerem et al., 2006). The activity was expressed as the concentration of sample necessary to give a 50% reduction in the sample absorbance (IC_{50}).

2.7. Determination of antioxidant activity by inhibition of linoleic acid oxidation

Antioxidant activity was determined by measuring the inhibition of linoleic acid oxidation initiated by AAPH. Linoleic acid was prepared in Tween 20, as described (Grossman & Zakut, 1979). In brief, a stock solution of 3×10^{-2} M linoleic acid was prepared by addition of 3 nmoles of linoleic acid to 50 ml of distilled water containing 1 ml of Tween 20. Next, 3–5 ml of 1 N NaOH was added in order to clarify the resulting emulsion, and the volume was adjusted to 100 ml with distilled water. This stock solution was diluted with 100 mM phosphate buffer, pH 7.0, to 7.5×10^{-3} M. The solution was stored at 4 °C for up to 2 weeks. For the oxidation assay, 80 μl of the solution was incubated with 25 mM AAPH in the presence or absence of 1,3-diCQA at 39 °C for 2 h. From this solution, 40 μl was taken for MDA assay.

Oxidation level was determined by measuring the MDA (malonedialdehyde) and 4-HNE (4-hydroxynonenal) linoleic acid chain cleavage products. The determination was based on the reaction of the chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-hydroxyalkenal at 45 °C. The chromophore absorbance measured at 586 nm (Esterbauer, 1993).

Ninety-six well plates were used for the assay. A 40-μl aliquot of the tested sample was added to each well, followed by 130 μl of R-1 (7.725 mM *N*-methyl-2-phenylindole diluted in methanol 3:1) and 30 μl of R-2 (15.4 M methanesulfonic acid). Three replicates were prepared for both controls and samples. The microplate was incubated for 1 h at 45 °C, and then read at 586 nm using a spectrofluorometer (Tecan).