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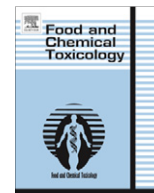

Antioxidant properties and cytotoxic effects on human cancer cell lines of aqueous fermented and lipophilic quince (*Cydonia oblonga* Mill.) preparations

Severina Pacifico [a,*], Marialuisa Gallicchio [a], Antonio Fiorentino [a], Anna Fischer [b], Ulrich Meyer [b], Florian Conrad Stintzing [b]

[a] *Laboratorio di Fitochimica, Dipartimento di Scienze della Vita, Seconda Università degli Studi di Napoli, Caserta, Italy*
[b] *WALA Heilmittel GmbH, BadBoll/Eckwälden, Germany*

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ABSTRACT

In the course of a screening program on quince phytochemicals, two complex preparations were in the focus of the present study, i.e., a lipophilic quince wax extract (QWE) and an aqueous fermented one (QAFE). While the phytochemical composition has been described earlier, the intention of the current investigation was to complement these data with an extensive antioxidant screening of these preparations including their radical scavenging and reductive power as well as their antilipoperoxidative properties. The Quince Aqueous Fermented Extract (QAFE) effectively scavenged the radical target species exhibiting ID_{50} values equal to 68.8 μg/mL towards DPPH· and 73.7 μg/mL towards the anion superoxide radical. Quince wax extract (QWE) was more effective at preventing the formation of thiobarbituric reactive species than QAFE exhibiting an ID_{50} value equal to 48.9 μg/mL. Moreover the cytotoxic effects towards human HepG2, A549, and HeLa cell lines were evaluated. The two preparations exerted a different effect on the proliferation of the three tested cell lines. Noteworthy, QAFE was almost always more active than QWE but, sometimes, its effects seemed to be strongly dependent on exposure time. Data obtained demonstrate clearly that both hydrophilic and lipophilic quince preparations are non-toxic and exert health-promoting properties.

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1. Introduction

Nowadays, it is commonly recognized that there is a positive relationship between a diet rich in plant foods and a reduced incidence of degenerative diseases such as cancer and cardiovascular events (Gibney et al., 2009). The decrease in the incidence of diseases is usually attributable to the antioxidant properties of the respective plant foods that may retard or even prevent some of the processes involved in cancer development. Besides classical antioxidants, plant foods contain further bioactives not classified as traditional essential nutrients, but are able to act beneficially on health, such as flavonoids and other phenolics, carotenoids, sterols, glucosinolates.

The growing research on dietary antioxidants and the protective role of phytocomplexes and pure plant metabolites, have favored the development of the functional food market. Food can be considered functional if, beyond the normal nutritional effects, it exerts positive effects on one or more specific body functions. Furthermore, there is a parallel development to identify bioactive phytocomplexes and/or compounds from classical food sources for non-food applications.

Health beneficial properties of quince fruit (*Cydonia oblonga* Mill.) are known from ancient times. Quince is the only species in the genus *Cydonia*, which falls into Pomoideae subfamily of the Rosaceae along with apple and pear. The yellow fruit may be pear- or apple-shaped and is characterized by a bright yellow leathery skin, a firm, acidulous, highly astringent fruit flesh full of stone cells making the raw fruit impalatable. Its strong flowery smell makes it a popular fruit to make jam, jelly and quince pudding. As traditional medicine quince fruits were used to treat cough (Kültür, 2007), cystitis (Sezik et al., 2001), constipation, and as a skin emollient (Pieroni et al., 2004). Preparations from quince leaves and seeds complete the list of using the fruit for non-food applications (Khoubnasabjafari and Jouyban, 2011). Recent *in vivo* studies have revealed that quince fruits and leaves have cell-protecting properties owing to the abundance of antioxidants they contain, for example, phenolic acids and flavonoids (Oliveira et al., 2007; Fattouch et al., 2007; Hamauzu et al., 2006).

Quince leaf has also been attested to possess anti-hemolytic (Costa et al., 2009; Magalhães et al., 2009), anti-diabetic and anti-lipoperoxidant (Aslan et al., 2010), as well as lipid-lowering properties (Osman et al., 2010). Aqueous preparations from whole

* Corresponding author. Tel.: +39 0823 274572; fax: +39 0823 274571
E-mail address: severina.pacifico@unina2.it (S. Pacifico).

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quince fruits have been reported to be effective to treat allergic disorders (Degen et al., 2000; Gründemann et al., 2011; Huber et al., 2012). Finally, a lipophilic quince extract was demonstrated to exhibit skin moisturizing properties (Lorenz et al., 2008a).

The phytochemical composition of quince has been extensively investigated earlier: Silva et al. (2002) scrutinized the levels and ratios of organic acids present in quince fruit (pulp and peel) and quince jam samples. Carotenoid metabolites responsible for the specific rich flavor of quince, including C-13 norisoprenoids, C-10, C-12, and C-15 compounds (Lutz and Winterhalter, 1992; Fiorentino et al., 2006; Fiorentino et al., 2007) have been isolated. Furthermore, quince contains considerable amounts of hydroxycinnamic derivatives mainly characterized by 3-caffeoylquinic and 5-caffeoylquinic acids as well as polymeric procyanidins (Hamauzu et al., 2005; Fiorentino et al., 2008). Methanolic extracts from quince fruit (pulp, peel, and seed) and jam were fractionated into a phenolic fraction and an organic acid one. Each extract was analyzed by HPLC/diode array and HPLC/UV and screened for its antioxidant capability. The partially purified extracts were more active than their parental one. The antioxidant activity seemed to be strongly correlated with the caffeoylquinic acids total content and with the respective fruit component analyzed. In fact, peel extract was responsible for the highest activity detected (Silva et al., 2004; Silva et al., 2005). The different quince fruit parts contain significant amounts of phenolic compounds (Magalhães et al., 2009). In particular, the pulp extract is characterized by 3-*O*- and 5-*O*-caffeoylquinic acids, 3,5-*O*-dicaffeoylquinic acid, and rutin. Caffeoylquinic acids were the major phenolic compounds (99%) and the most abundant was 5-*O*-caffeoylquinic acid (57%). In previous studies, 4-*O*-caffeoylquinic acid was also found in quince pulps (Silva et al., 2004; Silva et al., 2005), but in small amounts.

Besides caffeoyl quinic acids, several kaempferol and quercetin glycosides were detected in peel extracts.

Previous investigations on an aqueous and an aqueous fermented extract (QAFE) revealed that phenolic compounds were dominating (Gründemann et al., 2011; Huber et al., 2012).

Alesiani et al. (2010) reported the isolation and spectroscopic characterization of phenolic and terpenic secondary metabolites from quince peels emphasizing the antiproliferative activity of their triterpene constituents and the antioxidant effectiveness due to the phenolic portion. The complete lipophilic fraction of the fruit including peels has also been thoroughly explored (Lorenz et al., 2008b).

Since previous reports have mainly focused either on the phytochemical composition or the bioactivity, the present paper intended to complement data on two recently chemically characterized extracts from quince by assessment of the antioxidant and cytotoxic evaluation of the lipophilic quince wax extract QWE (Lorenz et al., 2008b) and the hydrophilic aqueous fermented extract QAFE (Huber et al., 2012) from the whole fruit.

The extracts were tested for their radical scavenging power by measuring their capacity to scavenge DPPH radical, anion superoxide radical ($O_2^{\cdot -}$), and peroxyl radicals. The Mo(VI) reducing capabilities were also determined. The antioxidant efficacy of the extracts was tested by the TBARS method, using vegetal fat as oxidable substrate. Finally, the cytotoxic effects towards HepG2, A549, and HeLa human cell lines were evaluated.

2. Materials and methods

2.1. Chemicals and reagents

All of the solvents and reagents used for assessing antioxidant screening were purchased from Sigma–Aldrich Chemie (Buchs, Switzerland). The cell culture medium and reagents for cytotoxicity testing were purchased from Invitrogen (Paisley, UK), MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was from Sigma–Aldrich Chemie.

The reference compound betulinic acid was from Phytolab (Vestenbergsgreuth, Germany), oleanolic acid was from Roth (Karlsruhe, Germany) and betulin was from Sigma Aldrich (Steinheim, Germany), all of them with a purity of at least 98%. Ursolic acid (purity >98% assessed by HPLC and NMR) was previously isolated from *Cydonia vulgaris* peels (Alesiani et al. 2010).

2.2. Preparation of quince extracts

Quince wax. Quince wax, obtained via supercritical fluid CO_2 extraction of quince pomace (blend from pear-shape-fruited and apple-shape-fruited quince, *C. oblonga* var. *pyriformis* and *C. oblonga* var. *maliformis*, respectively, origin, Spain and Italy), was provided by Flavex Naturextrakte (Rehlingen, Germany).

Aqueous fermented extract. *Cydonia oblonga* (Mill.) fruits (Rosaceae) were harvested at the WALA company garden (Bad Boll/Eckwaelden, Germany). Aqueous fermented extracts were produced from whole crushed quince fruits (batch no. 210507 B IIIe) according to an official production method (vs. 33b) of the German Homeopathic Pharmacopoeia (GHP, 2010) and provided by WALA Heilmittel GmbH (Bad Boll/Eckwaelden, Germany).

2.3. Assessment of the reducing effectiveness

The development of different methods in terms of substrate, probe, and reaction conditions was helpful to the definition of the samples' antioxidant effectiveness (Pacifico et al., 2011). The investigated quince extracts (QWE, QAFE) were tested at six dosage levels (5.0, 10.0, 50.0, 100.0, 250.0, and 500.0 μg/mL). Tests were carried out for three samples of each examined extracts of three replicate measurements each (in total, 3 × 3 measurements). Recorded activities were compared to a blank treated equally to the samples.

2.3.1. Determination of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity

The determination of DPPH· scavenging capability was estimated as follows: QWE and QAFE extracts were dissolved in 1.0 mL of a DPPH· methanol solution (9.4×10^{-5} M) at room temperature. After 30 min of incubation the absorption at 515 nm was measured by a Shimadzu UV-1700 spectrophotometer in reference to a blank. The results are expressed in terms of the percentage reduction of the initial DPPH radical adsorption by QWE and QAFE test samples.

2.3.2. Determination of superoxide anion radical ($O_2^{\cdot -}$) scavenging capacity

The inhibition of nitroblue tetrazolium (NBT) reduction by photochemically generated $O_2^{\cdot -}$ was used to determine the superoxide anion radical scavenging activity of the extracts (Fiorentino et al., 2006; Fiorentino et al., 2007). QWE and QAFE extracts were dissolved at room temperature in 3.0 mL of a reaction mixture containing sodium phosphate buffer (50.0 mM, pH 7.8), methionine (13.0 mM), riboflavin (2.0 μM), EDTA (100.0 μM) and NBT (75.0 μM). The $O_2^{\cdot -}$ production was followed by monitoring the increase in absorbance at 560 nm after 10 min illumination with a fluorescent lamp.

2.3.3. Determination of Mo(VI) reducing power

To carry out the quantitative determination of antioxidant capacity, extracts from *C. oblonga* were dissolved in 1.0 mL of a reagent solution containing sulfuric acid (0.60 M), sodium phosphate (28.0 mM), and ammonium molybdate (4.0 mM). The samples were then incubated at 95 °C for 90 min. At the end of the incubation period, the samples were cooled to room temperature, and their absorption was measured at 650 nm by a Shimadzu UV-1700 spectrophotometer against the blank. The increase in absorption in reference to the blank estimates the reducing power.

2.3.4. Determination of oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed essentially as described by Prior et al. (2003). QWE and QAFE extracts (20.0 μL, 5.0, 10.0, 50.0, 100.0, 250.0, and 500.0 μg/mL, final concentrations) and fluorescein (120.0 μL, 70 nM, final concentration) solutions were preincubated for 15 min at 37 °C in 75.0 mM phosphate buffer (pH 7.4). Then 2,2′-Azobis-(2-amidinopropane)-dihydrochloride (AAPH) solution (60.0 μL, 12.0 mM, final concentration) was rapidly added. In parallel with the samples, a blank (FL + AAPH) and solutions of the standard antioxidant Trolox® (1–8 μM, final concentrations) were properly prepared in PBS. The fluorescence (λ_{ex} = 485 nm, λ_{em} = 525 nm) was recorded every minute for 80 min using a Tecan SpectraFluor fluorescence and absorbance reader. Antioxidant curves (fluorescence vs. time) were first normalized to the curve of the blank by multiplying original data by the factor fluorescence blank, *t* = 0/ fluorescence sample, *t* = 0. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as follows:

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=80} \frac{f_i}{f_0}$$

where f_0 was the initial fluorescence reading at 0 min and f_i was the fluorescence reading at time *i*. Linear regression equations between net AUC ($AUC_{antioxidant} - AUC_{blank}$) and antioxidant concentration were calculated for all the samples.

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