Journal of Functional Foods 94 (2022) 105133

Contents lists available at ScienceDirect

Journal of Functional Foods

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

High production of secondary metabolites and biological activities of *Cydonia oblonga* Mill. pulp fruit callus

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ARTICLE INFO

Keywords:
Callus culture
Quince pentacyclic triterpenes, Enzyme inhibition
Genoprotection
Antioxidant activity

ABSTRACT

This study has developed an innovative method for the production of secondary metabolites starting from *Cydonia oblonga* Mill (quince) pulp callus culture. The qualitative and quantitative content of phenolic and triterpenic acids of quince callus extract were elucidated by GC–MS, GC, and HPLC-DAD-ESI-MSn. The callus extract was rich of 5-O-caffeoylquinic acid (5-CQA), 5-p-coumaroylquinic acid (5-p-CoQA) and maslinic and corosolic acid. Quince callus extract's radical scavenging and antioxidant activity were evaluated by 2,2-diphenyl-1-picrylhydrazyl, 2,2,-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), and Oxygen Radical Absorbance Capacity methods. The genoprotection was evaluated by gel electrophoresis analysis and quantitative Real-Time PCR. In addition to the good antioxidant activity the quince callus extract is a strong inhibitor of α-glucosidase (IC_{50} of 0.25 ± 0.02 mg dw/mL) and lipase (IC_{50} of 1.99 ± 0.005 mg dw/mL), but mild inhibitor of α-amylase. Therefore, this work would be significant for the future development of a nutraceutical approach to the management of hyperglycemia and dyslipidemia.

1. Introduction

Plants represent a valuable source of chemical compounds that are routinely used as the main font in the development of food additives, functional foods, and new drugs (Azmir et al., 2013). These bioactive compounds exert positive effects on humans due to their health-promoting properties (i.e. hypoglycemic, hypocholesterolemic, anti-aging, etc.). Plants synthesize standard bioactive compounds (alkaloids, terpenes, glycosides, polyketide flavonoids, essential oils, quinones, coumarins, resins, etc.) as secondary metabolites (SMs), through various metabolic pathways. SMs play an important role in protecting the plant from biotic or abiotic stress (Dixon, 2001; Azmir et al., 2013) and in the adaptation to the environment. Moreover, based on their chemical nature, SMs exert several activities including antibacterial, antiviral, anti-tumoral, antioxidant, anticonvulsant, analgesic, anti-inflammatory, and antidepressant effects, with great relevance in medicine, and in the field of food and agriculture (Seca & Pinto, 2019). Unfortunately, the amount of SMs produced by plants is quite limited and depends on seasonality or balsamic time. Consequently, their extraction from plant matrices foresees technological difficulties involving solvents, steam, or supercritical fluid uses (Starmans & Nijhuis, 1996). Over the past two decades, plant cell culture-based technologies have been improved for the upscale production of SMs useful for applicability in the pharmaceutical, and food industries (Eibl et al., 2018; Potenza, Minutelli, Stocchi, & Fraternale, 2020).

Our previous research have led to the development of a new method for the production of consistent quantities of SMs through the *in vitro* culture of callus from explants of ripe apple pulp of Mela Rosa Marchigiana (Verardo, Gorassini, Ricci & Fraternale, 2017) and fruits of *Acca sellowiana* Burret (Verardo, Gorassini, & Fraternale, 2019). In the

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https://doi.org/10.1016/j.jff.2022.105133
Received 18 January 2022; Received in revised form 5 May 2022; Accepted 23 May 2022
Available online 4 June 2022

present study, we focused our interest on the quince (*Cydonia oblonga* Mill), a fruit tree belonging to the Rosaceae family and the *Cydonia* genus. It is one of the oldest fruit trees already cultivated in 2000BCE, a plant originally from India and Turkestan but now even cultivated throughout the Mediterranean area. The fruit is an ovoid and yellow apple-like pomus. Its taste is sour and astringent and the scent is aromatic. The fruit phytochemicals are also used with a health-promotion perspective with a wide range of functional and biological activities such as antioxidant, antibacterial, antifungal, anti-inflammatory, antidepressant, antidiarrheal, hypolipidemic, hypoglycemic hepato- and cardiovascular protection (Ashraf, Muhammad, Hussain, & Bukhari, 2016; Carvalho et al., 2010). Moreover, quince is used in the food industry as a source of pectin that protects against colon damage in irritable bowel syndrome, in peptic ulcers (Hamauzu, Irie, Kondo, & Fujita, 2008; Minaiyan, Ghannadi, Etemad, & Mahzouni, 2012) and is used for preparing jams and jellies (Sut et al., 2019).

The present study aimed to fine-tune the method for obtaining a callus culture starting from the ripe quince pulp, to investigate the chemical composition and some biological activities of the phytocomplexes present in the quince callus extract.

2. Materials and methods

2.1. Chemical and reagents

Extraction and derivatization solvents were of analytical grade and were obtained from Sigma-Aldrich (Milan, Italy). Cholesterol (GC-FID Internal Standard; IS), β-sitosterol, ursolic and oleanolic acids, used as standards, Sylon BFT [Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS)], employed as a silylating reagent, 6-benzylaminopurine (BA), 1-naphthaleneacetic acid (NAA), and agar were also obtained from Sigma-Aldrich. Methanol (MeOH) and formic acid (HCOOH) for HPLC-MS, (+)-catechin, (−)-epicatechin, 5-caffeoylquinic acid (chlorogenic acid), and 3-hydroxycinnamic acid (HPLC-DAD Internal Standard; IS) were purchased from Sigma-Aldrich. Quercetin-3-*O*-galactoside and procyanidin B2 were obtained from Extrasynthese (Lyon, France). Milli-Q grade water was produced by the Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK). Solid-phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy). Reagents for antioxidant and enzyme activity experiments were purchased from Sigma Aldrich; reagents for DNA extraction were purchased from Qiagen (Milan, Italy), primers were synthesized by Sigma Genosys Inc. (Milan, Italy). Real-time reactions were performed using Diatheva (Fano, Italy) kit.

2.2. Plant material and callus cultures

The ripe pulp of quince was used as starting material to establish callus culture. The fruits were randomly collected from a tree present in the Botanical Garden of the University of Urbino Carlo Bo. The fruits were surface sterilized, as indicated in Verardo et al. (2017) by washing with ethanol and by flaming in a laminar flow cabinet. After sterilization, the quince fruits were divided lengthwise into two parts under sterile conditions. Disc shapes samples (diameter, 5 mm; height, 3 mm) of ripe quince pulp were collected with a Cork borer at a depth of 1 cm from the fruit surface. The samples were cultured on Murashige and Skoog (MS) media (Murashige & Skoog, 1962) and Gamborg B5 media (GB5) (Dixon, 1985), both containing 30 g/L sucrose with the addition of two growth regulators: BA (0.44, 0.89 and 1.77 μM,) and NAA (2.70, 5.50 and 11.00 μM) or NAA alone. Culture media were adjusted to pH 5.8 before adding 0.8% Agar, autoclaved (120 °C, 104 KPa) for 20 min, and 30 mL were dispensed into 90 mm Petri dishes. For each treatment, three dishes containing five samples of quince ripe pulp were set up and each experiment was performed in triplicate. Cultures were incubated at 25 ± 2 °C in the dark and subcultured after 28 days. After two months biomass productions were estimated (Verardo, Gorassini, Ricci & Fraternale, 2017) based on fresh (fw) and dry (dw) weight measurements in each cultural condition in agreement with Simoes et al. (2009). Callus cultures were maintained under the same conditions described above with subcultures at 4-week intervals. The callus produced was frozen at −20 °C and then freeze-dried before use. Moreover, the pulp of two fresh fruits was cut into pieces, immediately frozen, freeze-dried, and stored in vacuum jars and was used to determine metabolites naturally present in quince pulp.

2.3. Preparation of quince callus extract

Freeze-dried callus of quince (780 mg) was ground in a mortar, overnight stirred, and extracted in 45 mL of 70% (v/v) ethanol/double-distilled water. After centrifugation at 13,000 rpm for 45 min at 4 °C, the supernatant was filtered using a 0.22 μm filter (Millipore, Molsheim, France). The filtrate was concentrated and lyophilized using a Speed Vac Concentrator (Thermo Fisher Scientific, MA, USA). The lyophilized sample was suspended in 6 mL of double distilled water and centrifuged at 13,000 rpm for 45 min at 4 °C. Aliquots of supernatant (1 mL) were lyophilized, weighted, and stored at −80 °C. Except for GC and HPLC analyses, immediately before the use, each lyophilized sample was dissolved in water at a concentration of 30 mg dry weight (dw)/mL.

2.4. Chemical characterization of quince callus and pulp extracts

Each lyophilized sample (30 mg) and 500 μL of the internal standard (IS) solution (cholesterol, 1.244 mg/mL in ethyl acetate (EtOAc)) was suspended in water (5 mL) and extracted with EtOAc (25 mL × 2). The collected organic phases were washed with H_2O (4 mL) and brine (4 mL) then dried (Na_2SO_4 anhydrous), filtered, and evaporated to dryness in vacuo and the residue was dissolved in EtOAc (4 mL) and stored at 4 °C. An aliquot (2 mL) of this solution was evaporated under argon and the residue was dissolved in pyridine (0.5 mL) and Sylon BFT (0.5 mL). Silylation of the sample was performed at 80 °C for 18 h. After this time, the reaction mixture was analyzed by GC–MS and GC-FID.

GC–MS analyses were carried out using a Trace GC Ultra gas chromatograph coupled to an ion-trap mass spectrometer (ITMS) detector Polaris Q (Thermo Fisher Scientific, MA, USA) and equipped with a split-splitless injector. The column was a 30 m × 0.25 mm i.d., 0.1 μm film thickness, fused silica SLB-5 ms (Supelco, Sigma-Aldrich, Milan, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 10 min then the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 20 min. Samples were injected in the split (1:10) mode. The injector, transfer line, and ion source were set at 280, 280, and 200 °C, respectively. Helium was used as carrier gas at a flow of 1 mL/min. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from m/z 50 to 1000 and a scan rate of 0.8 scan/sec. Identification of metabolites was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library. The data acquisition was under the control of Xcalibur software (Thermo Fisher Scientific, MA, USA).

A Fisons GC 8000 series gas chromatograph, equipped with a flame ionization detector (FID) and a split/splitless injector (Fisons Instruments, Milan, Italy) was used for the quantitation of triterpenic acids. The separation was carried out with a fused silica capillary column DB-5MS UI 30 m × 0.250 mm × 0.25 μm film thickness (Agilent, J&W, Milan, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 25 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 13 min. Samples were injected in the split (1:10) mode. The injector and detector were set at 280 °C. Hydrogen was used as carrier gas at a flow of 1.8 mL/min. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Milan, Italy).

Quantification of triterpenic acids in the samples was performed

using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements. A calibration curve was built for each corresponding standard compound in the extracts. For this purpose, working solutions of each available standard (β-sitosterol, ursolic acid, and oleanolic acid) were prepared at a concentration ranging from 5 to 120 μg/mL with a constant concentration of the IS (60.0 μg/mL). The calibration curves obtained had a high level of linearity with a correlation coefficient (R^2) higher than 0.999 for all analytes. When standards were unavailable, the quantification of the target analyte was carried out using the calibration curve of the available standard of a similar chemical structure.

2.5. Sample preparation for HPLC-DAD-ESI-MS^n analyses of polyphenolic compounds

Each lyophilized sample (30 mg) and 30 μL of the IS solution (3-hydroxycinnamic acid, 100 μg/mL in MeOH) was dissolved in H_2O with 0.4% formic acid (2 mL) and loaded on a C18 SPE column previously conditioned with 10 mL of MeOH with 0.4% formic acid and 10 mL of H_2O with 0.4% formic acid. After loading, the column was washed with 10 mL of H_2O with 0.4% formic acid, and the phenolic fraction was eluted with 5 mL of MeOH with 0.4% of formic acid. Solvents were removed, and the residue was diluted in 1 mL of H_2O/MeOH 9:1 (v/v) with 0.4% of formic acid and transferred to an autosampler vial for the HPLC-DAD-ESI-MS^n analysis.

Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Fisher Scientific, MA, USA) equipped with a thermostated autosampler and a column oven. The chromatographic separation was performed with a column InfinityLab Poroshell 120 EC-C18 (4.6 × 150 mm, 2.7 μm; (Agilent Technology, Milan, Italy), thermostated at 30 °C. Elution was carried out at a flow rate of 0.6 mL/min, using as mobile phase a mixture of 0.2% formic acid in methanol (A) and 0.2% formic acid in water (B) with the following gradient: 0–6 min 10% A, 20 min 35% A, 46 min 40% A, 48 min 100% A, 60 min 100% A, 62 min 10% A, 70 min 10% A. The injection volume was 20 μL. The HPLC system was coupled with a diode array detector (DAD) and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by splitting the mobile phase 1:1. The acquisition was carried out in full scan (*m/z* 50–1500) and in full scan MS^2 (*m/z* 50–600) selecting the precursor ion $[M-H]^-$.

The quantitative analysis was carried out using an Ultimate 3000 RS Diode Array detector (Thermo Fisher Scientific, MA, USA) controlled by Chromeleon software (version 6.80). Spectral data from all peaks were accumulated in the range of 200–400 nm and chromatograms were recorded at 280 nm for (+)-catechin, (-)-epicatechin, procyanidin B2, and 3-hydroxycinnamic acid (IS), 328 nm for 3- and 5-caffeoylquinic acid, 4-*p*- and 5-*p*-coumaroylquinic acid, 258 nm for quercetin-3-O-glucoside, respectively.

Calibration curves ($R^2 > 0.999$) were prepared by diluting a stock solution of each standard [(+)-catechin, (-)-epicatechin, procyanidin B2, 5-caffeoylquinic acid, quercetin-3-O-glucoside] in H_2O/MeOH 9:1 (v/v) with 0.2% of formic acid in the range 12–3000 ng/ with a constant concentration of IS (500 ng/mL). When standards were unavailable, the quantification of the analyte was carried out using the calibration curve of available standards presenting similar chemical structures.

2.6. Total phenolic content (TPC)

The dosage of the polyphenols was conducted as reported by Zhang et al. (2006). The method involves the construction of a standard calibration curve that uses gallic acid at different concentrations (0.5–0.11 mg/mL). The quince callus extract (30 mg dw/mL) was then diluted at 1:10, 1:20, and 1:40 v/v in double-distilled water.

The procedure was performed on a 96-well flat-bottom microtiter plate. To 100 μL of Folin-Ciocalteu reagent, 1:10 v/v 10 μL of gallic acid were added at various concentrations or the callus extract at various dilutions. The plate was incubated for 5 min in the dark at room temperature (RT). After this time, 140 μL of Na_2CO_3 at 7.5% w/v were added to each well. The plate was further incubated at RT in the dark for 90–120 min. The absorbance at 630 nm was measured using a Microplate Reader (Bio-Rad, CA, USA). Total polyphenols are expressed as milligrams of gallic acid equivalent per gram of dry weight of callus extract (mg GAEq/100 mg dw).

2.7. Determination of vitamin C content

Folin-Ciocalteu reagent in a range of acid pH (from 1.0 to 7.0) was used to estimate the vitamin C content (Jagota & Dani, 1982) in the callus extract. 200 μL of a 30 mg dw/mL quince callus extract were added to 800 μL of 10% v/v trichloroacetic acid, stirred, and left on ice for 5 min. Subsequently, the solution was centrifuged for 5 min at 14,000 rpm and 500 μL of supernatant were added to 500 μL H_2O, and 100 μL of Folin-Ciocalteu reagent, was stirred and left on ice in the dark for 10 min. Distilled water was used as a blank. The standard calibration curve was obtained using a solution of vitamin C in water (5–45 μg/mL) using a UV Beckman spectrophotometer (CA, USA) at 760 nm.

2.8. Antioxidant activity of quince callus extract

2.8.1. DPPH scavenging activity

The antioxidant activity was evaluated using the stable free radical DPPH• (2,2-diphenyl-1-picrylhydrazyl) as reported in Saltarelli et al. (2019) with slight modifications. Briefly, 850 μL of a freshly prepared 100 μM DPPH• ethanol solution was added to 150 μL of quince callus extract (30 mg dw/mL) diluted 1:2 v/v in double-distilled water. After 30 min in the dark at RT, the absorbance decrease at 517 nm was measured; distilled water was used as a blank.

The DPPH radical scavenging ability (DSA) of the extract was determined as a decrease in DPPH absorbance at 517 nm using a UV Beckman Spectrophotometer (CA, USA) with the following equation:

DPPH scavenging activity (% DSA) = [(A_{517} nm of blank - A_{517} nm of sample)/ A_{517} nm of blank] × 100.

2.8.2. ABTS scavenging activity and Trolox equivalent antioxidant capacity (TEAC)

Antioxidant activity against ABTS• [2,2,-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] radical was performed as described by Loizzo et al. (2019) with some modifications. Briefly, the reaction mixture was prepared by mixing 7 mM ABTS solution and 2.45 mM potassium persulphate followed by 12–16 h incubation in the dark at RT to produce ABTS• radical. Before use, the solution was diluted with ethanol to obtain an absorbance of 0.80 ± 0.05 at 734 nm. Aliquots of quince callus extract at concentrations ranging from 0.01 to 0.40 mg dw/mL were added up to 1 mL of ABTS ethanolic solution and incubated in the dark at RT for 6 min. The absorbance was measured at 734 nm using a UV Beckman spectrophotometer (CA, USA). Distilled water was used as a blank. The ABTS radical scavenging activity was calculated following the equation:

ABTS scavenging activity (%) = [(A_{734} nm of blank – A_{734} nm of sample)/ A_{734}4 nm of blank] × 100.

The standard TEAC assay was performed as described by van den Berg et al. (1999) with slight modifications. The Trolox standard calibration curve was obtained using a solution of Trolox in ethanol (2.5–20 μM). The TEAC of the antioxidant was calculated by relating this decrease in absorbance to that of a Trolox solution on a molar basis.

2.8.3. ORAC assay

The antioxidant capacity of callus extract was determined by ORAC (Oxygen Radical Absorbance Capacity) method as previously reported (De Bellis et al., 2019) using a Fluostar Optima Plate reader fluorimeter (BMG Labtech, Offenburg, Germany). The following mix was used: 200 μL of 0.096 μM fluorescein in 0.075 M Na-phosphate buffer (pH 7.0), 20

μL of sample or Trolox or 0.075 M Na-phosphate buffer (pH 7.0) as blank. The reaction was started with 40 μL of 0.33 M of AAPH [2,2′-azobis-(2-amidinopropane)hydrochloride]. Fluorescence was measured at 485 nm ex. and 520 nm em. until complete extinction. A calibration curve was set up using the Trolox (from 50 to 500 μM) as a positive standard in 0.075 M Na-phosphate buffer (pH 7.0). ORAC values were expressed as mmol Trolox Equivalents (TEq)/100 mg dw and a twenty-time diluted callus extract (30 mg dw/mL), was prepared.

2.9. Genoprotective effect of quince callus extract

The quince callus extract genoprotective effect was investigated on the DNA of HaCaT human keratinocytes UVC (280–100 nm) damaged for 1–15 min. An Ultraviolet Crosslinker UVC oven (Amersham Life Science, UK) was used as a source of UVC. Quince callus extract concentrations employed were 0.14, 0.28, and 0.56 mg dw/mL. The samples were analyzed on 0.8% agarose gel. Quantitative analysis was carried out with the Delta cT method after Real-Time PCR Syber Green in a volume of 20 μL using 1 μL of each DNA in triplicate, 0.3 μM of each ND1 primer (ND1: F- 5′- ACGCCATAAAACTCTTCACCAAAG −3′; ND1 R:- 5′ – TAGTAGAAGAGCGATGGTGAGAGCTA – 3′) (Accession MZ457933.1), Power up Syber Green Master Mix (Thermo Fisher Scientific, MA, USA) and Quant Studio1 thermal cycler (Thermo Fisher Scientific, MA, USA). The qRT-PCR thermal conditions were carried out as follows: initial denaturation at 95 °C for 10 min, then 40 cycles at 95 °C for 15 sec and 60 °C for 1 min.

2.10. Enzyme inhibition activity

2.10.1. α-Glucosidase inhibition

The α-glucosidase inhibition by quince callus extract was performed according to the literature data with minor modifications (Lordan et al., 2013). The activity of α-glucosidase (EC 3.2.1.20) was determined by measuring the release of pNP (*p*-nitrophenol) from the substrate pNPG (*p*-nitrophenyl-α-glucopyranoside) at 405 nm. The acarbose, a pharmacological inhibitor, was included as positive control while distilled water was a negative control. A volume of 50 μL containing 125–1000 μg dw of quince callus extract or acarbose (32 and 64 μg) and 100 μL of α-glucosidase (0.2 U/mL) in 100 mM Na-phosphate buffer pH 6.9 was mixed in a 96 well microplate and incubated for 10 min at 37 °C. Then, 50 μL di pNDG 5 mM was added to each well. Due to the natural color of the quince callus extract, a set of blanks was set up without the presence of the enzyme and the absorbance was subtracted from the sample absorbance. The absorbance was read at 405 nm using a Microplate Reader (Bio-Rad, CA, USA). Each incubation was conducted in quadruplicate and the activity of α-glucosidase was calculated as follows:

α-glucosidase activity (%) = [(A_{405} nm sample- A_{405} nm blank)/(A_{405} nm control- A_{405} nm blank)] × 100.

2.10.2. α-Amylase inhibition

The α-amylase inhibition by callus extract of quince was performed according to Lordan et al. (2013).

Equal volumes (100 μL) of 1% (w/v) starch solution, α-amylase (EC 3.2.1.1) from porcine pancreas 0.5 mg/mL (in 100 mM Na-Phosphate buffer pH 6.9), and quince callus extract (125–500 μg dw) or acarbose (32 μg) were added to each tube and incubated for 10 min at 25 °C. The α-amylase reaction was blocked by the addition of 200 μL of dinitrosalicylate reagent (1% w/v 3,5-dinitro salicylic acid, 0.05% w/v sodium sulfite, 1% w/v sodium hydroxide and 0.25% v/v phenol). The mixture was boiled for 5 min and cooled to room temperature. 100 μL was removed from each tube and transferred to a 96-well plate, 100 μL of water was added to each well, and absorbance was measured at 540 nm using a Microplate Reader (Bio-Rad, CA, USA). Due to the natural color of quince callus extract, a set of blanks was set up without the presence of the enzyme and the absorbance was subtracted from the sample absorbance. Each incubation was conducted in quadruplicate and the activity of α-amylase was calculated as follows:

α-amylase activity (%) = [(A_{540} nm sample- A_{540} nm blank) /(A_{540} nm control- A_{540} nm blank)]x100.

2.10.3. Lipase inhibition

The porcine pancreas lipase (EC 3.1.1.3) inhibition by quince callus extract was performed according to Jaradat et al. (2017) with minor modifications. 100 μL of lipase 1 mg/mL in TRIS HCl 0.5 M + $CaCl_2$ 0.01 M, pH 7.5 was added to 50 μL of quince callus extract (125–500 μg dw) or orlistat (5 μg) as positive control; distilled water was a negative control. The mixture was made up to 0.9 mL by adding TRIS HCl buffer and incubated at 37 °C for 10 min; then 100 μL of 4-nitrophenyl butyrate (pNPB) 10 mM was added to each tube. The amount of 2,4-dinitrophenol released in the reaction was measured at 410 nm using a UV–visible spectrophotometer UV (Beckman CA, USA). Due to the natural color of quince callus extract, a set of blanks was set up without the presence of the enzyme. The change rate K, which is the change of absorbance over 10 min was measured. Each data point was conducted in triplicate and the inhibition rate of lipase was calculated follows:

Lipase activity (%) = [(A_{410} nm sample- A_{410} nm blank) / (A_{410} nm control- A_{410} nm blank)] × 100.

2.11. Statistical analyses

The statistical analyses were performed using GraphPad Software (GraphPad Prism version 6 for Windows). Values were expressed either as the mean ± Standard Error of the Mean (S.E.M.) or Standard Deviation (S.D.). The treated and control sample variables were compared using one-way ANOVA. Dunnett's multiple comparisons test was employed for calculating the significant difference. The differences between samples were considered significant if P values were < 0.05. To analyze the differences between the callus biomasses produced, the statistical software MSTAT-C with the Tukey test at a 5% level of significance was used.

3. Results and discussion

3.1. Callus induction from quince fruit pulp

The key to establishing callus culture is the choice of the optimum medium components and plant growth regulator concentrations. We observed that both culture media used (MS and B5), without growth regulators, did not induce callus production. The same results were also obtained using the lower dose of NAA (2.70 μM) and the explants assumed a necrotic appearance after 2-weeks of culture (Table 1). Quince fruit pulp explants formed friable callus on the upper face and along the cutting surface in both culture media used (NAA 5.50 and 11.00 μM), but after the first subculture of 20 days, the callus darkened, stopped growing, and died. To maintain and subculture the callus culture, we added cytokinin BA to the culture medium, already containing NAA, at three different concentrations (0.44 μM, 0.89 μM, and 1.77 μM). Thus, the optimal conditions for growing, maintaining, and inducing the highest biomass production of quince callus were found to be B5 medium plus 1.77 μM BA and 5.40 μM NAA (Table 1). Similar results are reported in our studies on Apple "Mela Rosa Marchigiana" and Apple "Golden delicious" (Verardo, Gorassini, Ricci & Fraternale, 2017) and on the fruit of *A. sellowiana* (Verardo, Gorassini, & Fraternale, 2019), where the cytokinin BA was necessary for the maintenance of callus culture. This culture condition was maintained over time and subcultures were done every four weeks to obtain the plant material for the extraction and analysis of the secondary metabolites. To the best of our knowledge, this is the first time that callus cultures have been obtained starting from the ripe pulp of quince.

Table 1
Callus biomass production from *in vitro* culture of quince fruit pulp at different conditions after 2 months.

Media and growth regulators (μM)	Fresh weight[a] (g)	Dry weight[a] (g)
MS (Murashige and Skoog)		
0.0 NAA	No callus	No callus
2.70 NAA	No callus	No callus
5.50 NAA	3.26 ± 0.09a	0.111 ± 0.002a
11.00 NAA	2.98 ± 0.08a	0.113 ± 0.004a
0.00 BA	No callus	No callus
0.44 BA + 2.70 NAA	3.88 ± 0.21a	0.141 ± 0.006a
0.44 BA + 5.40 NAA	3.21 ± 0.08a	0.112 ± 0.010a
0.44 BA + 11.00 NAA	3.35 ± 0.09a	0.123 ± 0.006a
0.89 BA + 2.70 NAA	3.61 ± 0.11a	0.122 ± 0.010a
0.89 BA + 5.40 NAA	3.74 ± 0.08a	0.131 ± 0.013a
0.89 BA + 11.00 NAA	3.45 ± 0.12a	0.121 ± 0.008a
1.77 BA + 2.70 NAA	3.31 ± 0.09a	0.112 ± 0.005a
1.77 BA + 5.40 NAA	5.36 ± 0.10a	0.184 ± 0.010a
1.77 BA + 11.00 NAA	5.19 ± 0.31a	0.161 ± 0.010a
GB5 (Gamborg's B5)		
0.0 NAA	No callus	No callus
2.70 NAA	No callus	No callus
5.50 NAA	4.13 ± 0.16b	0.142 ± 0.008b
11.00 NAA	4.56 ± 0.12b	0.153 ± 0.011b
0.00 BA	No callus	No callus
0.44 BA + 2.70 NAA	4.25 ± 0.08b	0.141 ± 0.012b
0.44 BA + 5.40 NAA	3.86 ± 0.11b	0.135 ± 0.012b
0.44 BA + 11.00 NAA	3.61 ± 0.11b	0.121 ± 0.009b
0.89 BA + 2.70 NAA	3.16 ± 0.03b	0.111 ± 0.012b
0.89 BA + 5.40 NAA	3.84 ± 0.04b	0.132 ± 0.012b
0.89 BA + 11.00 NAA	3.35 ± 0.11b	0.115 ± 0.011b
1.77 BA + 2.70 NAA	4.22 ± 0.06b	0.141 ± 0.004b
1.77 BA + 5.40 NAA	**13.29 ± 0.06a**	**0.441 ± 0.006a**
1.77 BA + 11.00 NAA	5.52 ± 0.09b	0.174 ± 0.006b

[a] Data represent mean ± standard deviation; $n = 9$ repetitions. In each column, the values with the same letters are not significantly different by Tukey test at 5% of significance.

3.2. Content of secondary metabolites in the quince extract obtained from pulp and pulp callus culture.

The quince pulp showed a highly variable content of phenolic compounds, from 11.7 to 3436.56 mg/100 g dw (Silva et al., 2002; Wojdyło, Oszmiański, & Bielicki, 2013; Zhang et al., 2021). Our data showed a total phenolic content in quince pulp of 10.98 μg/100 mg dw (Table 2). Surprisingly, the total content of phenolic compounds in the quince pulp callus extract was 91.58 μg/100 mg dw. In callus extract, 5-CQA (72.18 μg/100 mg) is the major compound followed by 5-*p*-coumaroylquinic acid (5-*p*-CoQA) 12.98 μg/100 mg, and 3-O-caffeoylquinic acid (3-CQA) 1.82 μg/100 mg. In the pulp extract, 5-CQA (4.02 μg/100 mg) is the major constituent followed by 3-CQA (3.29 μg/100 mg), and procyanidin B2 (3.11 μg/100 mg) (Fig. 1). The production of 5-*p*-CoQA is higher in the quince pulp callus compared to the pulp (12.98 μg/100 mg *vs* 0.05 μg/100 mg), while 3-CQA and procyanidin B2 are more expressed in the pulp than in the callus: 3.29 μg/100 mg *vs* 1.82 μg/100 mg and 3.11 μg/100 mg *vs* 0.94 μg/100 mg, respectively (Table 2). To the best of our knowledge, only *Thevetia peruviana* pulp callus produces dihydroquercetin, a flavonoid with anti-cancer properties (Mendoza, Arias, Cuaspud, & Arias, 2020).

Table 2
Specific content of phenolic compounds in the extract obtained from 100.0 mg of lyophilized pulp and callus culture of quince, identified and quantified by HPLC-DAD-ESI-MS^{n}.

	Callus culture	Pulp
Compound	**μg**[a]	**μg**[a]
5-CQA	**72.18 ± 0.47**	**4.02 ± 0.03**
5-*p*-CoQA	**12.98 ± 0.34**	0.05 ± 0.01
(-)-Epicatechin	**2.02 ± 0.05**	0.43 ± 0.03
3-CQA	1.82 ± 0.04	**3.29 ± 0.03**
Procyanidin B2	0.94 ± 0.02	**3.11 ± 0.07**
4-*p*-CoQA	0.65 ± 0.03	0.02 ± 0.01
(+)-Catechin	0.60 ± 0.02	n.d.
Quercetin-3-O-glucoside	0.18 ± 0.01	0.05 ± 0.01
Procyanidin B type dimmer	0.11 ± 0.01	n.d.
Procyanidin B type dimmer	0.10 ± 0.01	n.d.
Total	**91.58 ± 0.91**	**10.98 ± 0.56**

[a] Data are expressed as the mean value ± standard deviation; n = 3 repetitions. n.d.: not determined.

Several works reported the production of phenolic compounds in callus cultures obtained from other plant organs (Khan, Abbasi, Khan, & Shinwari, 2016; Nazir et al., 2021). It is noteworthy that the quince pulp callus produced in our laboratory showed the presence of pentacyclic triterpene acids which have never been determined in the pulp or peel of quince. GC–MS analysis allowed the identification of the following compounds: maslinic acid (13.92 ± 0.12 μg/100 mg), corosolic acid (8.74 ± 0.06 μg/100 mg), annurcoic acid (6.81 ± 0.06 μg/100 mg) and tormentic acid (3.70 ± 0.08 μg/100 mg). The results are expressed as the mean value ± standard deviation of triplicate replicates. However, these compounds have already been determined in callus culture starting from apple pulp (Verardo, Gorassini, Ricci & Fraternale, 2017) and from the pulp of exotic fruit such as *A. sellowiana* (Verardo, Gorassini, & Fraternale, 2019).

3.3. Total phenolic and vitamin C contents of quince callus extract

Total phenolic and vitamin C contents of quince callus extract were also determined by using spectrophotometric methods and Folin-Ciocalteu reagent in different pH conditions, as previously reported in section 2.7. Total phenolic content was 1.08 ± 0.008 mg GAEq/100 mg dw of quince pulp callus extract (values are expressed in Gallic Acid Equivalents) while vitamin C was 27.0 ± 0.006 μg AAEq/100 mg dw (values are expressed in Ascorbic Acid Equivalents). All the values are the mean of quadruplicate replicates ± standard deviation.

3.4. Antioxidant capacity assays (DPPH, ABTS, ORAC) of quince pulp callus extract

Antioxidant activity of the callus extract was examined against different free stable radicals such as DPPH (Moon & Shibamoto, 2009), ABTS (MacDonald-Wicks, Wood, & Garg, 2006), and peroxyl radicals (ORAC) (Prior, Cao, Prior, & Cao, 2000). The callus extract from quince shows a DPPH• scavenging activity of 73.8% at 30 min at a concentration of 1.25 mg dw/mL, and the free radical 50% inhibition value (EC_{50}) was 0.30 mg dw/mL.

The ABTS scavenging activity was 16.1% ± 0.79 at 30 μg dw/mL (EC_{50} = 19.4 μg dw/mL). TEAC assay resulted in 16.0 ± 1.90 mM Trolox equivalents/100 mg dw.

The ORAC assay on quince callus extract was 14.0 ± 0.20 μM Trolox equivalents/100 mg dw.

The antioxidant capacity of quince callus extract obtained from fruit pulp is probably due to the contents of polyphenols, vitamin C, and triterpenes (Moradi, Koushesh Saba, Mozafari, & Abdollahi, 2016). All reported values are the mean of at least three replicates ± standard deviation.

3.5. Genoprotective activity of quince callus extract

The genome derived from HaCaT cells was used to evaluate the effect of quince extract on UVC irradiated DNA. Exposure to 1–15 min UVC leads to partial or total DNA degradation. HaCaT genomic DNA appears in the form of smear in UVC exposed samples, less evident in the samples without quince extract treatment (280 μg dw/L) (Fig. 2). In an attempt to quantify the loss of DNA due to UVC irradiation, we performed a quantitative PCR. A 100 bp region from the NADH dehydrogenase subunit 1 (ND1) of the previously treated samples was amplified.

The treatment of UVC for 15 min which corresponds to 30,000 μJ/

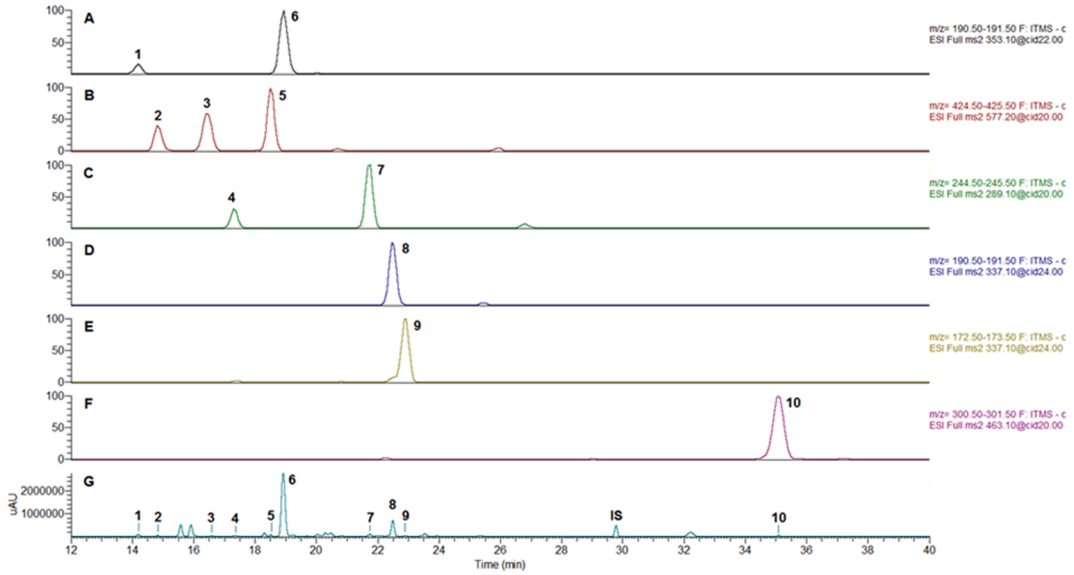

Fig. 1. HPLC-ESI-MSn extracted ion chromatograms (EIC) (A-F) and HPLC-DAD chromatogram at 280 nm (G) of phenolic compounds identified in the extract obtained from lyophilized callus culture of quince. On the right of the figure are reported, for each analyte, the precursor ion [M−H]- and the most intense fragment ion selected for EIC. 1 (3-CQA), 2–3 (procyanidin B type dimers), 4 [(+)-catechin], 5 (procyanidin B2), 6 (5-CQA), 7 [(-)-epicatechin], 8 (5-*p*-CoQA), 9 (4-*p*-CoQA), 10 (quercetin-3-O-glucoside).

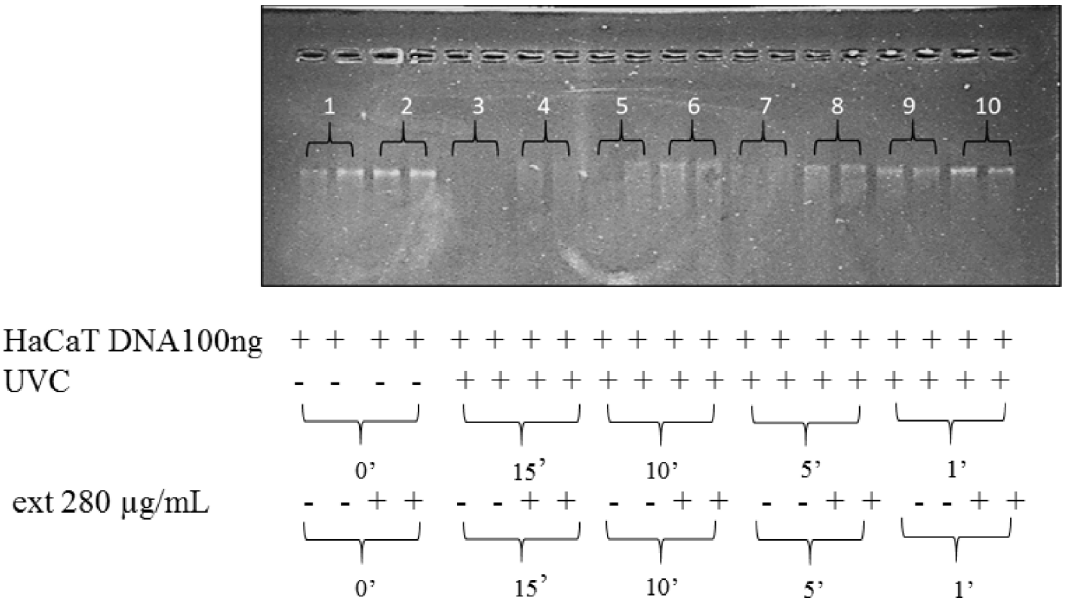

Fig. 2. Protective effect of quince extract on UVC damaged HaCaT cell DNA. Each treatment was performed in duplicate. Sample 1 received no treatment. Sample 2 received quince treatment. Samples 3, 5, and 7 were injured by decreasing the time of UV (15,10. 5, and 1 min). The higher DNA concentration in samples 4, 6, 9, and 10 cotreated with 128 µg/mL of quince extract showed the partial protective effect of the callus in a dose-dependent manner.

cm^2 (Fig. 3) causes a reduction in the amount of DNA of about 16 folds, which is reduced to 10 folds in the presence of the quince callus extract. This result indicates a statistically significant genoprotective effect of quince callus extract. Reducing the UVC exposure time, the DNA damage also decreases, and the genoprotection of quince callus extract did not present statistically significant differences although the protection trend is maintained. Genoprotective effect was also observed using a plasmid DNA oxidatively injured and the ethanolic extract from the above-mentioned callus of Mela Rosa Marchigiana, whose qualitative chemical composition is similar to that of the extract under analysis.

In previous work, the genoprotective effect of the extract from the callus of Mela Rosa Marchigiana on oxidatively injured plasmid DNA was also observed. Moreover, this extract had a chemical composition similar to quince callus extract (Potenza, Minutelli, Stocchi, & Fraternale, 2020).

3.6. *In vitro enzyme inhibitory activity of quince callus extract*

3.6.1. *Anti α-glucosidase anti α-amylase activity*

The most important food carbohydrate (e.g. starch) is hydrolyzed to monosaccharide by α-glucosidase, an enzyme located on the intestinal brush border, and then absorbed into the blood. Usually, these processes take place in the upper portion of the small intestine increasing the glycemic level. Retarding the postprandial glucose level by inhibition of intestinal α-glucosidase complex is an interesting effect of vegetable extract (Ortiz-Andrade et al., 2007). Therefore, anti-α-glucosidase and

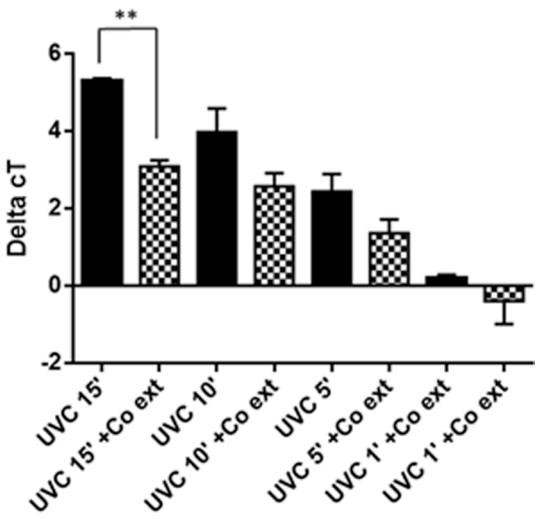

Fig. 3. Genoprotective effect quantitation of quince extract by Real Time PCR. The results are expressed as mean ± SD of three independent experiments. cT of control DNA (no treatment) was subtracted to obtain delta Unpaired Student's *t*-test was performed between the couples of UVC and UVC + quince extract-treated DNA. (**p < 0.01).

anti-α-amylase of quince callus extract were investigated in this study. The extract inhibited α-glucosidase activity *in vitro* in a concentration-dependent manner. As shown in Fig. 4, the quince callus extract at 250 μg/ dw mL already demonstrates a highly significant reduction of α-glucosidase activity (62%). (IC_{50} of 250 ± 2 μg dw/mL) (Fig. 4). Acarbose was used as a positive control (IC_{50} of 27.17 ± 0.28 μg/mL).

α-amylases have also an important role in the hydrolysis of polysaccharides during digestion. The activity of quince callus extract on α-amylase (Fig. 5) shows a moderate, not dose-dependent inhibition, of about 20% (IC_{50} not detectable). Acarbose had an inhibition percentage of 75% at 32 μg/mL with an IC_{50} of 23.0 ± 2.9 μg /mL.

As previously reported by Etxeberria et al. (2012) only moderate α-amylase inhibition is recommended to prevent the bacterial fermentation of undigested polysaccharides in the colon, which results in flatulence and diarrhea. Effectively quince callus extract exhibits a modest inhibitory effect against α-amylase and better inhibitory activity against α-glucosidase, it may establish the basis of notably effective therapy for postprandial hyperglycemia.

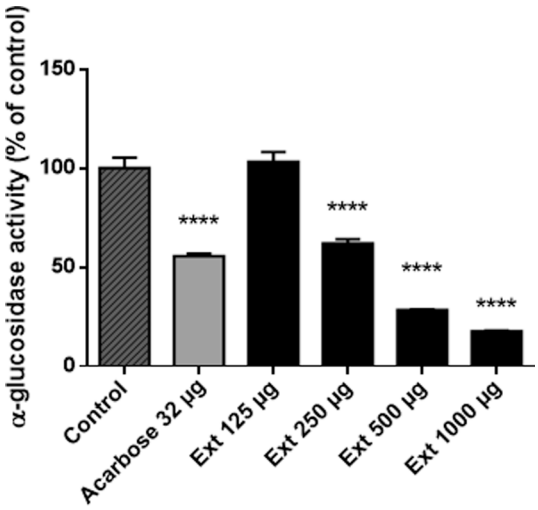

Fig. 4. Inhibitory effect of quince callus extract on α-glucosidase activity. Data represent the mean ± SD of four independent experiments. (**** p < 0,0001; ANOVA followed by Multiple Comparison Test).

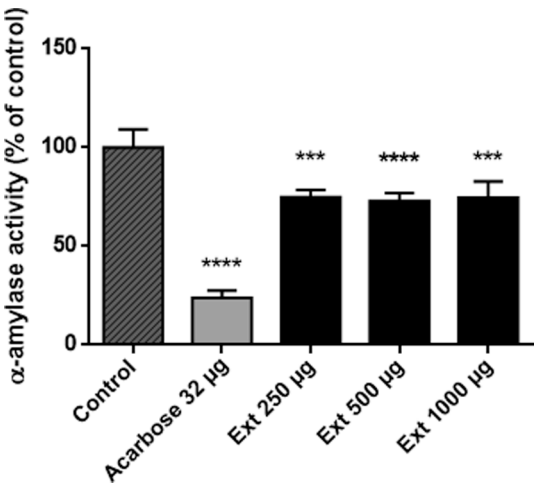

Fig. 5. Inhibitory effect of quince callus extract on α-amylase activity. Data represent the mean ± SD of four independent experiments. (*** p < 0.001; ****p < 0.0001; ANOVA followed by Multiple Comparison Test).

Vegetal origin inhibitors of carbohydrate hydrolases can be useful therapeutic approaches in the management of obesity and diabetes and complications associated with these diseases (Apostolidis & Lee, 2010; Rahman, Ambigaipalan, & Shahidi, 2018). A virtual screening study based on molecular docking evaluated the α-amylase and α-glucosidase inhibitory activity of naturally occurring polyphenols (Rasouli, Hosseini- Ghazvini, Adibi, Khodarahmi, 2017).

Hou et al. (2009) reported that corosolic acid, oleanolic acid, and maslinic acid from *L. speciosa* had a strong inhibition activity on α-glucosidase and a weak one on α-amylase. Literature data on quince chemical profiles are difficult to be comparable based on different extraction matrices and different analytical methods used. Nevertheless, Zhang et al. (2021) recently published encouraging data on the antioxidant properties and enzyme inhibitory activity of different parts of the quince plant. The results obtained in this study evidenced the important role of the co-presence in quince callus extract of the phenolic compounds and the triterpene acids in the inhibition of relevant digestive enzymes.

3.6.2. *Anti lipase activity*

Pancreatic lipase hydrolyzed 50–70% dietary fat (in triglycerides form) into absorbable free fatty acids and monoglycerols. Undigested triglycerides are eliminated by feces. Developing an anti-lipase inhibitor as an anti-obesity drug is valuable. For this purpose, Orlistat, a type of long-term, specific, irreversible inhibitor of lipase was used as a positive control (Heck, Yanovski, & Calis, 2000).

However, this drug produces several gastrointestinal effects (fatty diarrhea, fecal incontinence, etc.) and the development of safer lipase inhibitors from natural sources is of great interest (Derosa & Maffioli, 2012). Fig. 6 shows that quince callus extract moderately inhibited lipase activity *in vitro* in a concentration-dependent manner (IC_{50} of 1.99 ± 0.005 mg dw/mL). Orlistat was used as a positive control.

4. Conclusion

In conclusion, *Cydonia oblonga* (quince) callus represents an attractive sustainable *in vitro* technique under aseptic conditions, which provides an unlimited supply of bioactive high-value secondary metabolites independent of external factors such as soil and the environmental conditions, growth period season, or geographical location. Moreover, our results demonstrated that quince callus extract is characterized by a high production of pentacyclic triterpenes and phenol compounds with antioxidant and genoprotective effects. Quince as well as other pomace fruit can be used as food, feed, or to make bioproducts for the

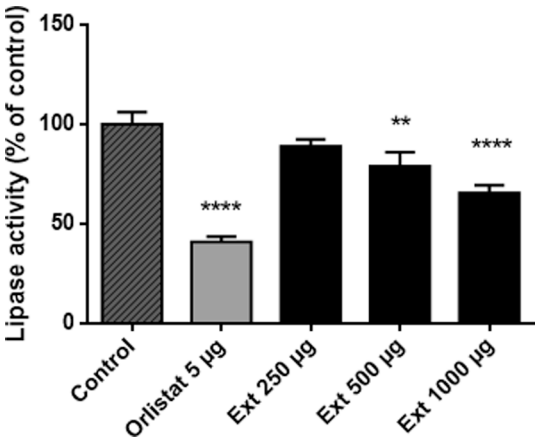

Fig. 6. Inhibitory effect of quince callus extract on lipase activity. Data represent the mean ± SD of three independent experiments. (** $p < 0.01$; ****$p < 0.0001$; ANOVA followed by Multiple Comparison Test).

pharmaceutical industry. The production of a high quantity of secondary metabolites through callus *in vitro* culture could reduce the risk of diverting farmland and crops for SMs production to the detriment of the food supply.

Furthermore, results obtained on digestive enzyme inhibition could offer a complementary approach to the management of hyperglycemia and dyslipidemia by modulating the digestion and the subsequent adsorption of dietary carbohydrates and lipids. The results herein obtained will move our interest toward the use of ripe pulp *Cydonia oblonga* callus extract as a nutraceutical product.

CRediT authorship contribution statement

Roberta De Bellis: Conceptualization, Methodology. **Laura Chiarantini:** Conceptualization, Methodology. **Lucia Potenza:** Conceptualization, Methodology. **Andrea Gorassini:** Methodology. **Giancarlo Verardo:** Methodology. **Rossella De Marco:** Methodology. **Leila Benayada:** Methodology. **Vilberto Stocchi:** Visualization, Supervision. **Maria Cristina Albertini:** Visualization, Supervision. **Daniele Fraternale:** Methodology, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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