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Phytochemical and biological characterization of dry outer scales extract from Tropea red onion (*Allium cepa* L. var. Tropea)–A promising inhibitor of pancreatic lipase

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ABSTRACT

Background: Allium cepa L. var. Tropea is typically cultivated in Calabria (Italy) and it is certified as "*Cipolla Rossa di Tropea Calabria-PGI*" (Tropea red onion). The use of clinically available anti-obesity drugs such as Orlistat is being gradually dismissed due to their side-effects and this has encouraged the search for alternative inhibitors of intestinal lipases such as phytochemicals showing less side-effects. In this study we aimed to evaluate for the first time the anti-obesity potential of the hydroalcoholic extract from the dry outer scales of Tropea red onion by the assesment of its capacity to inhibit pancreatic lipase. Its possible mechanism of action was also studied with planar lipid membranes (PLMs) surrogate of intestinal membranes.

Methods: Specialized metabolites in the extract were determined by GC–MS, HPLC-DAD, HPLC-UV-DAD and HPLC-HRMS analyses. Inhibition of pancreatic lipase was studied *in vitro* against crude lipase Type II from porcine pancreas. PLMs used in the electrophysiology measurements were made up of DOPS:DOPE:POPC.

Results: The extract contained quercetin-4′-*O*-glucoside, quercetin and quercetin-3,4′-*O*-diglucoside as the most abundant phenolics. Among apolar constituents, γ-sitosterol, linoleic and stearic acids were dominant. The lipase inhibitory effect of the extract had an IC_{50} value equal to 0.77±0.03 mg/mL (positive control, IC_{50} = 0.018 mg/mL). The electrophysiological study demonstrated that the extract is able to incorporate into PLMs and to form transient channel-like events

Conclusions: Taken altogether, the results allow us to suggest that the hydroalcoholic extract from the dry outer scales of Tropea red onion could prevent lipid ester hydrolysis and it has a protective effect against phospholipase as found for interfacially active compounds.

Abbreviations

Ag, silver;
AgCl, silver Chloride;
$AlCl_3$, aluminium chloride;
ANOVA, analysis of variance;
C, capacitance;
CA, chlorogenic acid;
Cf, final capacitance;
CH_3CN, acetonitrile;
CH_3CO_2H, acetic acid;
Ci, initial capacitance;
Cn, capacitances of known values;
DMSO, dimethyl sulfoxide;
DOPE, dioleoyl phosphatidyl ethanolamine;
DOPS, dioleoyl phosphatidyl serine;
DW, dry weight;
EtOAc, ethyl acetate;
EtOH, ethanol;
F, frequency;
FWHM, full width at half maximum;
GC–MS, gas chromatography-mass spectrometry;

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H_2O, water;
HCO_2H, formic acid;
HPLC-DAD, high performance liquid chromatography-diode array detection;
HPLC-HRMS, HPLC-high resolution mass spectrometry;
IC_{50}, half maximal inhibitory concentration;
KCl, potassium chloride;
LoD, limit of detection;
LoQ, limit of quantitation;
MeOH, methanol;
Min, minutes;
MS/MS, tandem mass spectrometry;
Na_2CO_3, sodium carbonate;
NP-PEG, natural products-polyethylene glycol;
NPC, nitrophenyl caprilate;
PGI, protected geographic indication;
PLM, planar lipid membrane;
POPC, palmitoyl oleoyl phosphatidyl choline;
RSD, relative standard deviation;
SD, standard deviation;
SE, standard error;
SEM, standard error of the mean;
TRIS-HCl, tris(hydroxymethyl)aminomethane hydrochloride;
UV, ultraviolet;
V_{lh}, output voltage values;
Λc, central conductance;
τ, lifetime.

Introduction

Allium cepa L. var. Tropea (Amaryllidaceae), commonly known as Tropea red onion, is a two-year herbaceous plant, but annual in cultivation, whose growth is restricted to the Calabria region (southern Italy) (Pignatti, 1982). The environment in the defined area contributes to the high quality and uniqueness of the vegetable, which is widely appreciated all over the world. Information about Tropea red onion dates back to the Middle Ages and during the Renaissance it was considered a staple food also used as trade goods. The bulbs are characterized by a red external tunic and white internal scale leaves (Fig. 1), and they are appreciated for their organoleptic characteristics (tenderness, crispness, sweet taste) which have been approved by the European Union with the Protected Geographical Indication (PGI) as "*Cipolla Rossa di Tropea Calabria-PGI*" (Council Regulation (EC), 2007). In addition, they are also appreciated for the low level of sulphates, which makes them less sour and sharp in taste and more easily digestible, even if consumed raw in high amount. Due to these typical and valuable traits, Tropea red onion represents an important horticultural product for the local and national economy.

Onions are a rich source of valuable phytochemicals with health beneficial effects and they are used both as a food and for medical purposes (Upadhyay, 2016). Previous studies showed that Tropea red onion contains high amounts of flavonoids (anthocyanins and flavonols), fructo-oligosaccharides and sulfur compounds such as thiosulfinates which make it a valuable food of the Mediterranean diet. A high content of saponins was also detected in a methanolic (MeOH) extract of its bulbs. Among these, the four furostanol saponins named tropeosides A1/A2 and B1/B2 were described to have antispasmodic activity (Corea et al., 2005). Gennaro and co-workers (2002) identified and quantified flavonoids and anthocyanins of commercial Tropea red onions separately in the edible part, in the outer fleshy scale leaves and in the dry outer skin, which is usually removed in home processing. Authors reported that Tropea red onion contains a higher amount of quercetin 4′-glucoside, compared to other cultivars of onions. Anthocyanins were found mainly concentrated in the external tunic and consisted of a remarkable amount of delphinidin and petunidin derivatives (Gennaro et al., 2002). Based on this study, the total intake of anthocyanins in the diet should be quite poor due to the fact that about 70% of the onion outer skin is discarded during peeling. Nevertheless, the outer tunic can be used as a natural sustainable source of high-value functional ingredients (Benítez et al., 2011).

A. cepa synthesizes many bioactive specialized metabolites and numerous pharmacological properties, including antimicrobial, antioxidant, anti-inflammatory, analgesic, anti-hypertensive, hypolipidemic, anti-diabetic and immunoprotective effects have been reported for this horticultural product (Teshika et al., 2018). Only few studies have been however conducted to highlight the biological properties of Tropea red onion. Tedesco and co-workers (2015) investigated the capacity of the phenolic extract from the bulbs of two southern Italian cultivars (Tropea red onion and copper onion Ramata di Montoro) to defend human erythrocytes against oxidative stress. The main flavonols identified in the two varieties were quercetin and its glucosides, isorhamnetin glucosides, kaempferol-3-*O*-glucoside, and some cyanidin glucosides. In addition, the Tropea variety was found to have a higher content of flavonols (29.35 ± 0.03 mg/mL) compared to the Montoro type (4.79 ± 0.02 mg/mL) (Tedesco et al., 2015).

The aim of our study was to identify a potential anti-obesity agent through the evaluation of the pancreatic lipase inhibitory activity of the hydroalcoholic extract from the dry outer scales of Tropea red onion and highlight its possible mechanism of action by using Planar Lipid Membranes (PLMs) mimicking intestinal lipid composition. The phytochemical composition of the hydroalcoholic extract was determined by Gas Chromatography–Mass Spectrometry, GC–MS (apolar constituents), High Performance Liquid Chromatography-Diode Array Detection (HPLC-DAD), HPLC-HRMS (High Resolution Mass Spectrometry) analyses and HPLC-UV (Ultraviolet)-DAD detection (phenolics).

Many extracts from plants have been reported to have inhibitory

Fig. 1. *Allium cepa* L. var. Tropea.

activity on pancreatic lipase (Birari and Bhutani, 2007; Marrelli et al., 2016a). This enzyme serves to process dietary lipids into simpler forms (monoglycerides and fatty acids) that can be more easily absorbed and transported throughout the body. The major dietary fats (90%) are in fact represented by mixed triglycerides which cannot be directly metabolized by the human body and must be hydrolyzed for absorption by lipases. Lipases involved in human digestion include tongue, gastric and pancreatic lipases which are directly responsible for the absorption of fat in the intestine. Inhibition of pancreatic lipase leading to a reduced absorption of complex dietary lipids may be, therefore, employed to treat obesity. An increasing number of studies have proved that phenolic specialized metabolites from vegetables, fruits, and cereals are able to inhibit some digestive enzymes *in vitro* (de la Garza et al., 2011), and they represent the major class of pancreatic lipase inhibitors (Lunagariya et al., 2014).

To the best of our knowledge, the detailed chemical composition of the dry outer scales of Tropea red onion-IGP, as well as the evaluation of its anti-obesity potential and its physiological action on model intestinal membranes are reported for the first time in this study.

Materials and methods

Chemicals

Ethanol (EtOH), MeOH and *n*-hexane were obtained from VWR International S.r.L. (Milan, Italy). aluminum chloride ($AlCl_3$), chlorogenic acid (CA), Orlistat, 4-nitrophenyl octanoate, quercetin, crude lipase Type II from porcine pancreas (EC 3.1.1.3), Folin-Ciocalteu reagent, Dioleoylphosphatidylserine (DOPS), dioleoyl-phosphatidylethanolamine (DOPE) and all other chemicals used in PLM studies were bought from Sigma-Aldrich S.p.A (Milan, Italy). Avanti Polar Lipids, Inc (Alabaster, AL, USA) provided palmitoyl-oleoyl-phosphatidylcholine (POPC). All solvents and reagents were of analytical grade.

Plant materials, extraction and fractionation

A. cepa L. var. Tropea was collected in Calabria, Italy, during September 2018 (leg. F. Conforti, det. F. Conforti, DFSSN, University of Calabria, Italy). A voucher specimen (CLU 25,978) is available from the Botanic Garden, University of Calabria. The outer dry scales of the bulbs (140 g) were macerated (48 h x 3 times) with 70 % aqueous EtOH (3 L) at room temperature. The resultant extract was filtered and dried under reduced pressure to determine its weight and yield of extraction (9.6 g and 6.9%, respectively). It was then dissolved in EtOH 70% and partitioned with *n*-hexane. The *n*-hexane fraction was analyzed by GC–MS.

GC–MS analysis

Chemical constituents of the hexanic extract were identified by GC–MS. Analyses were run with a Hewlett-Packard 6890 GC connected to a mass selective detector (Hewlett Packard 5973) and equipped with an SE-30 (100% dimethylpolysiloxane) capillary column (30 m x 0.25 mm, 0.25 μm film thickness). Analyses were performed using a programmed temperature from 60 to 280 °C (rate 16°C/min). Column inlet was set at 250 °C. MS operating parameters were: Ion source, 70 eV; ion source temperature, 230 °C; electron current, 34.6 μA; vacuum 10^{-5} torr. Mass spectra were acquired over a 40–800 amu range at 1 scan/sec. Helium was used as the carrier gas (linear velocity 0.00167 cm/sec). Identification of constituents was achieved by comparison of their GC retention times with those of available standards and on the comparison of their mass spectra with those from Wiley Mass Spectral Database of the GC–MS system (Marrelli et al., 2015).

Total phenolic and flavonoid content estimation

The total amount of phenolics in the hydroalcoholic extract was calculated using the Folin-Ciocalteau method (Chun and Kim, 2004; Marrelli et al., 2016b). The extract was mixed with 7.5% w/v Na_2CO_3 and Folin-Ciocalteau reagent and, after two hours, the absorbance was recorded at 765 nm (Perkin-Elmer Lambda 40 UV/VIS (Visible) spectrophotometer). CA was used as the equivalent chemical and total amount was expressed as CA equivalents (mg) *per* g of Dry Weight (DW).

The flavonoid content was determined by the quantification of a flavonoid-aluminum complex as reported in Marrelli et al., 2020. The sample (2 mg/mL in EtOH 80%) was mixed with 2% $AlCl_3$ in EtOH and, 15 min later, the absorbance was measured at 430 nm. Total amount was expressed as quercetin equivalents (mg) *per* g of DW. Both quantifications were conducted in triplicate.

Analysis of phenolics

HPLC-DAD

Phenolics in the extract were analyzed and quantified by reverse-phase HPLC-DAD on a Gemini C18 column as already described in Marrelli et al. (2018). Reported results were the average of three replicates under the following conditions: solvent A, H_2O-HCO_2H, 0.1%, pH 2.7; solvent B, CH_3CN-HCO_2H, 0.1%; elution gradient, 10–60% B in 60 min; flow rate, 1 mL/min. Aliquots of 20 μL (60 mg/mL) at the flow rate of 1 mL/min were injected. HPLC-DAD chromatograms were acquired at 210, 270, 310, 350 and 510 nm. Identification of each eluted component was based on comparison of spectroscopic and chromatographic data with those available from literature (Bonaccorsi et al., 2005; Marrelli et al., 2018; 2020) and on co-elution with reference phenolics analyzed in the same experimental conditions.

Quantitative analysis was carried out by the external standard method according to Marrelli et al. (2020) by injection of isoquercitrin. The 7 level (0.0078–0.5 mg/mL MeOH) calibration curve in the linear plot showed a correlation coefficient r^2=0.9997 (y=5E+07x+166,437), indicating a positive correlation between isoquercitrin concentrations and peak areas within the assayed range of concentrations.

Precision of the adopted analytical method was calculated by the intra-day % RSDs of isoquercitrin retention times (0.81% to 1.04%) and peak areas (3.83% to 1.86%). Calculated % RSD for inter-day peak areas was 1.73%, and 0.39% for retention times. Limit of detection, LoD, was 0.03 μg/mL; limit of quantification, LoQ, was 0.12 μg/mL. All analyses for quantitative determination of phenolics and validation of the adopted HPLC method were run in three replicates.

HPLC-HRMS

Analyses were performed with an HPLC system Accela coupled to a Q Exactive single-stage MS system equipped with HESI II-electrospray ion source (Thermo Fisher Scientific, Waltham, MA, USA). The HESI ion source was used both in the negative and positive mode. Settings were as follows: capillary temperature, 300 °C; capillary voltage, 4 kV (positive mode) and 4.5 kV (negative mode); sheath gas flow rate, 15 and 30 au; auxiliary gas flow rate, 15 and 10 au. Mass spectra were acquired over the scan range of 50.2–1003.1 m/z, with a resolution power of 10,000 FWHM. The Xcalibur software (v 2.1.0; Thermo Fisher Scientific, Waltham, MA, USA) was utilized for acquisition and processing of data (Marrelli et al., 2018). For separation of the components in the extract, the same column and elution program as for HPLC analyses (see above) were used. Constituents of the extract were identified on comparison with mass spectra of available reference phenolics analyzed in the same conditions and/or mass spectra from library files (Stobiecki, 2000; Cuyckens and Claeys, 2004; Ferreres et al., 2007).

Measurement of pancreatic lipase activity

The anti-obesity potential of the hydroalcoholic extract from Tropea red onion outer scales was assessed *in vitro* by the pancreatic lipase inhibitory assay according to the following procedure: a 1 mg/mL solution (25 μL) of type II crude porcine pancreatic lipase in distilled H_2O

was added to 5 mM 4-nitrophenyl caprilate (NPC, 25 μL), Tris–HCl buffer (pH= 8.5, 1 mL) and *A. cepa* raw extract (25 μL, concentrations in the range of 5 to 0.06 mg/mL). After 25 min of incubation of the mixture at 37 °C, the absorbance was monitored at 412 nm using a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer. Orlistat (20 μg/mL) was used as positive control. All experiments were performed in triplicate (Marrelli et al., 2016c).

Planar membrane experiments (PLMs)

Channel-like events were estimated using PLMs made up of DOPS: DOPE:POPC (27:27:18, w:w:w) in 1% *n*-decane. A Teflon chamber with two aqueous compartments (4000 μL; unbuffered water solutions) joined by a small circular hole (300 μm) was employed; bilayers were formed across the hole and the two compartments held symmetrical (1 M KCl solutions, pH=7, temperature 23±1 °C). The assayed concentrations (0.005, 0.01, 0.02 and 0.04 mg/mL) of the extract were obtained by adding known volumes (4, 8, 16 and 32 μL, respectively) of the stock solution (5 mg/mL) to the *cis* side of the membrane under stirring for 2 min.

The electrophysiology set-up for single-channel measurements allowed to monitor the membrane current with an oscilloscope and to record the membrane capacitance and current for further analysis (Micelli et al., 2008; Meleleo, 2021). Two Ag/AgCl electrodes in series with a voltage generator and a current amplifier (OPA129; Micelli et al., 2002; Meleleo, 2021) were used to link the two compartments to the amplifier head stage. The time resolution of single-channel instrumentation was in the range of 1–10 ms and was dependent on the magnitude of the conductance of the conductive unit. The *cis* side added with the *A. cepa* extract, defined the polarity of the voltage. A negative (-) potential value was applied to the *trans* side, opposite to the *cis* side. Single-channel recordings are considered to be a sensitive tool for the study of membrane-active substances (Tien and Ottova-Leitmannova, 2000; 2001).

The biophysical parameters used in this study to characterize the extract incorporation and channel-like formation were the following:

- Conductance levels of channel-like events: values were obtained for each experiment from the analysis of the conductance amplitude generated histograms, fitted with a Gaussian distribution function. Results were expressed as central conductance ± standard error (Λ_c±SE) and were processed by the ANOVA-Tukey test and Student t test.
- PLM capacitance, calculated using a calibration curve as reported in our previous studies (Meleleo, 2021; Micelli et al., 2002). In brief, a standard curve was constructed by simulating the C of PLMs with a discrete set of known values, C_n, and registering the related output voltages, V_{lh}. Data were fitted with the Eq. (1):

$$V_{lh} = \frac{A \times C_n}{(B + C_n)} \quad (1)$$

where A and B are free parameters derived by the fitting method and used to transform V_{lh} into C data. Data were expressed as mean capacitance ± standard error (C±SE).

- Channel-like event lifetime, expressed by the exponential distribution of the duration of channel-like events according to the Eq. (2):

$$N = A_1 e^{\left(\frac{-t}{\tau_1}\right)} + A_2 e^{\left(\frac{-t}{\tau_2}\right)} \quad (2)$$

where N defines the number of channels which stay open for a time equal to or greater than a certain time t, A_1 and A_2 are the amplitudes at zero time, and τ_1 and τ_2 are correlated to the fast and slow components of the time constant, respectively. The single-exponential distribution was expressed by the formula (A_2= 0). To choose the right experimental model, a suitable statistical analysis was carried out (F-test, Graph Pad Prism™ version 3.0).

- Channel-like event frequency, derived by counting the number of successful events in 60 s. Results were expressed as frequency (F) ±SD.

Statistical analyses

Pancreatic lipase inhibitory assays were run in triplicate. Results are presented as SEM. Homogeneity of variances and normality of data were estimated using Levene's test and D'Agostino–Pearson's K2 test and t, respectively. A nonlinear regression was used to transform raw data (GraphPad Prism™ version 3.0, San Diego, CA, USA). One-way ANOVA was applied to compare samples and controls at different concentrations (Dunnett's multiple comparison test, SigmaStat Software, SanRafael, CA, USA).

Values of biophysical parameters were obtained from the analysis of experimental data resulting from at least three experiments (more than 150 single channel-like events). GraphPad Prism 3 software was used for Gaussian data transformation, Student t test, ANOVA and the fitting procedures.

A value of $P < 0.05$ was considered significant for all the statistical tests.

Results and discussion

Chemical composition of the extract

Recovery yield from the extraction of the dry outer scales from *A. cepa* var. Tropea bulbs was 6.9%: starting from 140 g of plant material we have obtained 9.6 g of extract. Table 1 reports the chemical composition of the hexanic fraction as obtained from the GC–MS analysis: γ-sitosterol was the major component (29.4%), followed by the three fatty acids palmitic, linoleic and stearic acid (17.6%, 9.8% and 9.5%, respectively). The hydroalcoholic extract contained a phenolic and flavonoid amount of 40.00 ± 1.39 mg CA/g DW and 2.40 ± 0.17 mg quercetin/g DW, respectively.

Specialized metabolites were identified with a combination of analytical data from chromatographic, spectrometric and spectroscopic methods and co-elution with available authentic compounds. HPLC-

Table 1
Apolar compounds identified in the *n*-hexane fraction of *A. cepa* L. var. Tropea extract.

Compound	Rt[a]	RAP[b]
Myristic acid	17.068	Tr[c]
Neophytadiene	17.645	Tr
Palmitic acid	18.639	17.6
Heptadecanoic acid	19.365	Tr
Linoleic acid	19.874	9.8
Oleic acid	19.908	Tr
Eicosanoic acid	21.383	1.5
Stearic acid	22.663	9.5
Cholesta-3,5-diene	25.738	Tr
Cholesterol	29.161	Tr
γ-Sitosterol	33.442	29.4
Stigmasta-3,5-dien-7-one	35.774	Tr

[a] Retention time (as min).
[b] Relative peak area percentage (peak area relative to total peak area in TIC %).
[c] Tr–Traces percentages < 0.1%.

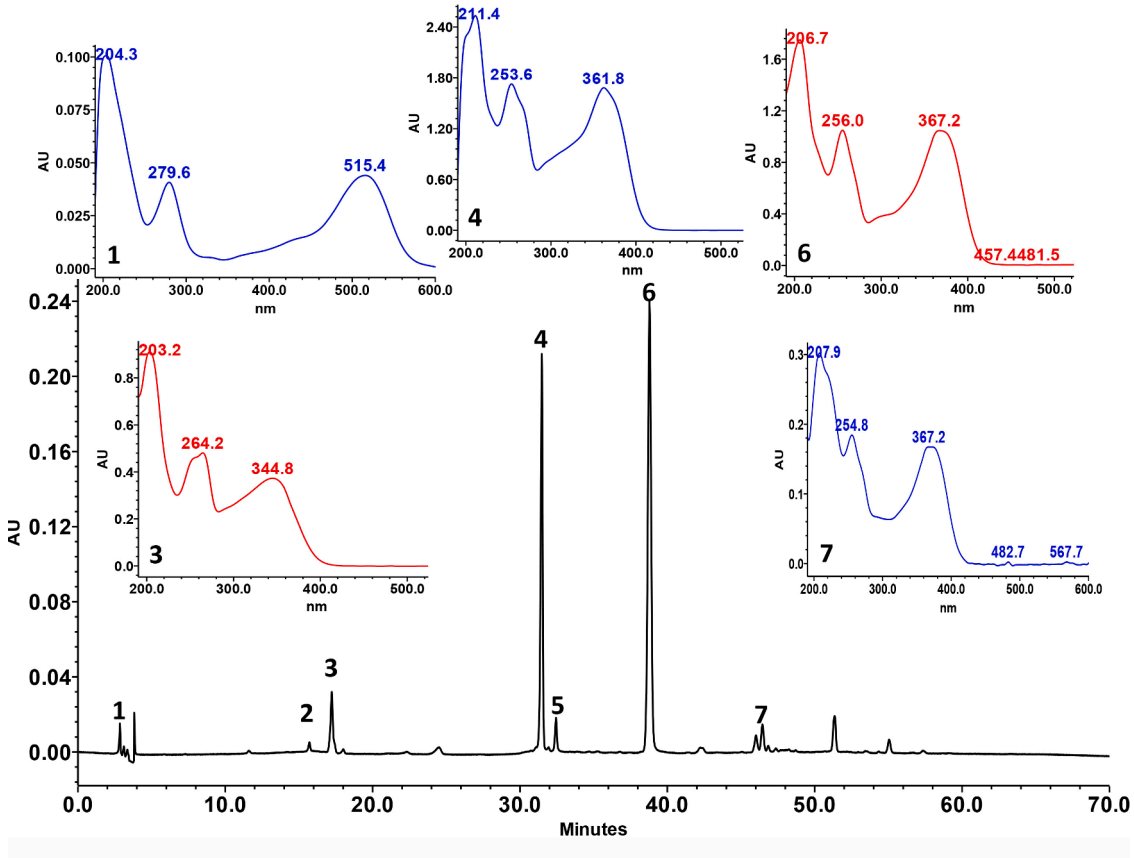

Fig. 2. HPLC-DAD chromatogram and UV-DAD spectra of major components in the hydroalcoholic extract from the outer scales of Tropea red onion.

DAD chromatogram and HPLC-UV-DAD spectra of major eluted components are depicted in Fig. 2. As shown, the extract was relatively poor of components and made up by two major peaks (4 and 6, Fig. 2). Overall analytical data from HPLC-UV-DAD and MS/MS fragmentation of the seven identified phenolics are reported in Table 2. Based on their major UV absorption bands (240–280 nm, Band I-benzoyl A-ring and 330–380 nm, Band II-shikimate derived B-ring), the presence of flavonols or flavones could be established (Mabry et al., 1970). In addition, the absorption peaks with maxima around 250–260 nm and 350–370 nm indicated the presence of quercetin derivatives (peaks 3–4, 6–7; Table 2, Fig. 1). This was also supported by the fragmentation pattern of most of the components showing mass spectra characterized by the [Aglycone + H]$^+$ ion at *m/z* 303 (Table 2). Based on its UV absorption, pseudomolecular ion at 479 m/z and [Aglycone+H]$^+$/ [M+H-162, Gluc]$^+$ ion at *m/z* 317, peak 5 was instead identified as a glycoside of isorhamnetin, namely isorhamnetin-4′-*O*-glucoside or alliumoside (Table 2, Fig. 1) (Bonaccorsi et al., 2005; Lee and Mithcell, 2011; Marrelli et al., 2018; 2020). Characteristic absorption of peak 1 (Table 2, Fig. 1) at 515 nm suggested the presence of anthocyanins. Moreover, the fragment ion at 287 m/z indicated the presence of a cyanidin glucosylated aglycone [M (449 m/z)+H-162]$^+$ (Tedesco et al., 2015; Kim and Lee, 2020). Identification of the components in the extract was also corroborated by co-elution with available authentic reference phenolics.

The quantitative analysis revealed that the predominant phenolic constituents in the extract were quercetin-4′-*O*-glucoside (spiraeoside) and quercetin which amounted to 43 ± 2 and 84 ± 4 μg/mL, respectively. In addition, quercetin-3,4′-*O*-diglucoside represented another specialized metabolite present in good amount (13 ± 0.4 μg/mL). The other identified compounds (Table 2) accounted for lower amounts (in the range of 1 to 6 μg/mL).

To the best of our knowledge, this is the first detailed phytochemical investigation of the dry outer scales of the bulbs of *A. cepa* L. var Tropea-IGP. Gennaro et al. (2002) also studied Tropea red onion from Calabria, analyzing different parts of the bulb. In their work, main attention was however payed to the characterization of anthocyanins. Among flavonoids, only quercetin-4′-glucoside was detected in the dry skin. Other investigations (Fossen et al., 1998; Fossen and Anderson, 2003) using the same horticultural product, have been mainly carried out on the dry

Table 2
Analytical data of phenolics identified in the EtOH skin extract of *A.cepa* var. Tropea.

Peak*	Compound	UV-DAD (λ_{max}, nm)	[M+H]$^+$, m/z	MS/MS, m/z, (I%)
1	Cyanidin-3-O-glucoside	204.3, 279.6, 515.4	449	287 (37) [M-162+H]$^+$
2	Qercetin-7,4′-diglucoside	200.8, 253.6, 264.2 *sh*, 359.8	627	465 (45) [M-162+H]$^+$; 303(4) [Aglycone + H]$^+$
3	Quercetin-3,4′-O-diglucoside	203.2, 253.0 *sh*, 264.2, 344.8	627	465 (100) [M-162+H]$^+$; 303(22) [Aglycone + H]$^+$
4	Quercetin-4′-O-glucoside (Spiraeoside)	211.4, 253.6, 270.0 *sh*, 305.0 *sh*, 361.8	465	303 (100) [Aglycone + H]$^+$
5	Isorhamnetin-4′-O-glucoside (AlliumosideA)	203.2, 252.4, 304.4 *sh*, 362.9	479	317 (100) [Aglycone + H]$^+$
6	Quercetin	206.7, 256.0, 300.0 *sh*, 367.2	303	-
7	Quercetin 3-O-glucoside (Isoquercitrin)	207.9, 254.8, 268.0 *sh*, 300.0 *sh*, 367.2	465	303 (100) [Aglycone + H]$^+$

* For peak numbering refer to Fig. 2

outer scales from the bulbs of other varieties of red onion, namely the "Red Baron" or an unspecified "southern italian red onion" (Bonaccorsi et al., 2005). Nevertheless, in agreement with previous reports (Gennaro et al., 2002; Bonaccorsi et al., 2005; Fossen et al., 1998; Kwak et al., 2017; Olsson et al., 2010), quercetin glycosylated derivatives represent the characteristic specialized metabolites identified also in our extract. In addition, as already reported for an extract of the entire bulb from different varieties of onion including the red onion *var* Benny, isorhamnetin-4′-glucoside (alliumoside) was also - identified in our extract (Park and Lee, 1996). As shown in Table 2, cyanidin-3-*O*-glucoside, likely responsible for the red color of the outer scales of Tropea red onion, was another compound contained in our extract. This was the only anthocyanins detected in our study, whereas a previous investigation (Tedesco et al., 2015) carried out on the extract from the whole bulbs of the plant showed the presence of other cyanidin derivatives, namely of some malonyl acylated cyanidins.

Quantitative data from the analysis of other varieties of *A. cepa* or different plant material, slightly differ from ours. Namely, the main flavonols found in the extract from the entire bulbs of Tropea red onion and Montoro onion were quercetin-4′-*O*-glucoside and quercetin-3,4′-*O*-diglucoside (Tedesco et al., 2015), amounting to 85% of the total flavonol content. Similarly, these compounds were reported as the main constituents of the outer layers of a number of common onions varieties (Lee and Mithchell, 2011). Quercetin-7,4′-*O*-diglucoside (17.3 mg/g DW) and quercetin-4′-*O*-glucoside (41.4 mg/g DW) were instead identified as the dominant constituents in the extract from the scaly leafs of an unspecified red onion type studied by Kwak et al. (2017). This extract, differently from our extract, contained a very low amount of quercetin (0.6 mg/g DW).

Anti-obesity activity

The potential anti-obesity activity of the extract of Tropea red onion outer scales was determined using the *in vitro* porcine pancreatic lipase inhibitory assay, based on the measurement of the hydrolysis of NPC and of the release of the yellow chromogen *p*-nitrophenol (Marrelli et al., 2020).

Lipase inhibitory activity is reported in Fig. 3. Results were consistent with a concentration-dependent inhibitory activity, with an IC_{50} value of 0.77 ± 0.03 mg/mL. At the highest concentration (5 mg/mL), inhibition of the enzyme was 90.19 ± 3.08%, while inhibitory percentages of 83.30 ± 2.79 and 70.95 ± 1.05% were detected at 2.5 and 1 mg/mL, respectively. Observed activity could be reasonably referred to the presence of flavonoid compounds such as quercetin and its glycosides for which previous studies showed inhibition effect on pancreatic lipase (Guo et al., 2016; Alizadeh and Ebrahimzadeh, 2021).

Several studies on the potential anti-obesity activity of common onion have been published until now and different mechanisms of action, such as pancreatic lipase and adipogenesis inhibition, have been suggested. Furthermore, different studies have documented the efficacy of onion extracts in the treatment of obesity-related co-morbidities, such as hyperlipidaemia and diabetes (Marrelli et al., 2019).

Kim (2007) described the inhibitory activity of a MeOH extract of common onion skin on pancreatic lipase and its effect on body weight gain and related parameters. He found that this extract inhibited the enzyme activity in a dose-dependent manner, having an activity about 1.300 times lower than Orlistat (53.70/0.041 μg/mL), used as positive control. In our study the extract obtained from Tropea red onion skin showed instead an activity 40 fold less than Orlistat (0.77/0.02 mg/mL).

Moreover, Yu et al. (2021) investigated the anti-obesity potential of the peel extract of a yellow type of onion both *in vitro*, on 3T3-L1 adipocytes, and *in vivo*, in an obesity mouse model. The extract was able to lower the lipids amount in 3T3-L1 cells and to inhibit their accumulation. Furthermore, treated mice showed lower serum concentrations of triglycerides, total cholesterol, and low density lipoprotein cholesterol compared to mice fed with a high-fat diet.

As far as we know, this is the first study on the anti-obesity potential of *A. cepa* var. Tropea-IGP.

Insertion of A. cepa extract into PLMs and channel-like activity

Many studies have shown that biomembranes are the main target of membrane-active compounds (Gallucci et al., 2003; Haney et al., 2010; Matsuzaki et al., 1998). The membrane lipid models are always preferred to study physiologically interactions of drugs because the cell membrane is a complex structural and biological system.

To analyze the ability of the extract from Tropea red onion dry outer scales to incorporate and induce ion conductance, PLMs made up of DOPS:DOPE:POPC (see Material and Methods) were used. In addition, incorporation and pore formation, as induced by the extract concentration, were investigated. Two types of experiments were performed: - control experiments to evaluate the PLMs stability and to exclude non-specific effects on the PLMs with the lipid mixture alone; - experiments in which the *A. cepa* var. Tropea extract was added.

Results from the control experiments showed that C (0.30 μF/cm^2) and Λ (25 pS) remain constant over long periods of time (18 h) at the voltages of ±80 and ±100 mV or when the membrane bilayer was damaged and reconstituted by the operator.

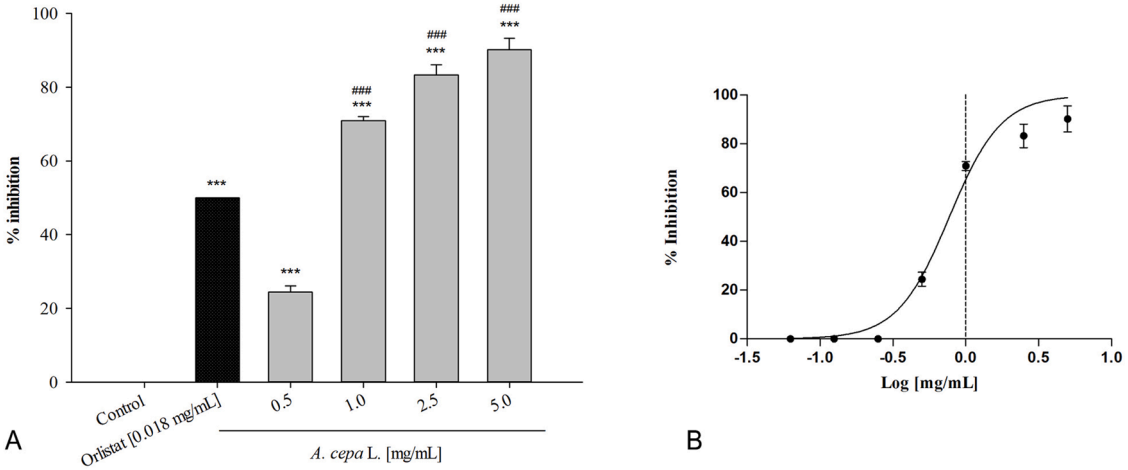

Fig. 3. Concentration-dependent pancreatic lipase inhibition induced by *A. cepa* L. var. Tropea extract. (A) Data were expressed as SEM (n = 3). Orlistat (0.018 mg/mL) was used as positive control. Significant difference *versus* control: *** $p < 0.001$; significant difference *versus* positive control: $^{###}$ $p < 0.001$ (Dunnett's multiple comparison test). (B) Non-linear regression analysis.

In the second type of experiments, before the addition of known volumes of the extract in the medium facing the PLMs, the basic Λ (25 pS) and C (0.30 μF/cm^2) remained constant at the voltage of ±100 mV for 12–15 min and no channel-like activity was detected. On the contrary, the addition of the *A. cepa* extract (at the addition voltage of 100 mV) produced in all the experiments channel-like activity which occurred as discrete non-random events of conductive or non-conductive states consistent with channel-type openings and closures. These events appeared at different lag times depending on the concentration of the extract. In experiments at 0.02 and 0.04 mg/mL extract concentrations, after the first channel-like event, it was possible to observe alternating periods of channel-like activity, during which the number of events could be rigorously monitored, followed by intervals of quiescence and paroxystic channel-like activity that often lead to destabilization of the membrane until rupture.

In the experiments at the 0.005 and 0.01 mg/mL concentrations, channel-like activity occurred after about 110 and 90 min lag time at a voltage of 100 mV. The applied voltage was lowered to 80 mV after the first channel-like event and the channel amplitude was measured. The channel-like activity was recorded at the voltages of 80 and ±100 mV, each applied for 90 min beginning from 80 mV. No channel-like activity was detected in the voltages range of -80÷60 mV. By increasing the extract concentrations to 0.02 and 0.04 mg/mL, the lag time reduced to 60 and 30 min, respectively, and channel-like events appeared at the voltage of 100 mV. The channel-like activity in the voltage range of ±60 ÷ ±100 mV and ±20 ÷ ±100 mV at the 0.02 and 0.04 mg/mL concentrations, respectively, was recorded following the same experimental procedure as described above for the lowest concentrations of the extract. Fig. 4. describes a typical example of channel-like event recordings.

Values of the biophysical parameters (Λ_c±SE; F±SD; τ and C±SE) at the various applied voltages for the different extract concentrations are reported in the Tables 3 and 4. It should be noted that recorded frequencies were symmetrical for negative and positive applied voltages, indicating that the turnover of channel-like events was not dependent on the applied voltages. Another parameter used to characterize the extract channel-like event was its lifetime (Table 3). The results indicate that the channel-like event has the slow channel lifetime component at all applied voltages for the different assayed concentrations, whereas at the applied voltages of 20, -40 and -100 mV and at the concentration of 0.04 mg/mL, the channel-like event shows both slow and fast lifetime components. Capacitance is considered the best parameter to express the stability and structural properties of the double lipid layer and for this reason it is monitored for the timespan of each experiment. In all experiments, before the addition of the *A. cepa* extract, the basic capacitance of the PLMs was in the range of 0.28–0.31 μF/cm^2, remaining constant. After the addition of the extract, regardless of the concentrations used, it was observed that the capacitance remained constant during the lag time; slowly decreased, reaching lower values than those of basic capacitance when the first channel-like events appeared and these values remain almost constant to the end of the experiment. Table 4 reports the C±SE values calculated just before the addition of the extract (Ci) and at the end of the experiment (Cf). The capacitance variation observed after its insertion into the PLM bilayer could be interpreted as due to the effect of the Tropea red onion extract on the packaging of the fatty acids.

Results from the electrophysiological experiments indicated that the extract from the dry outer scales of *A. cepa* var. Tropea incorporates into PLMs made up of DOPS:DOPE:POPC as a surrogate for the intestinal membrane lipids (Schwarz et al., 1985) and causes the formation of transient channel-like events with a lag time dependent on its concentration. The lag time defines the time at which a conductance variation first occurs after the extract addition and it depends on achieving an appropriate extract/lipid ratio on the PLMs surface. Once this threshold

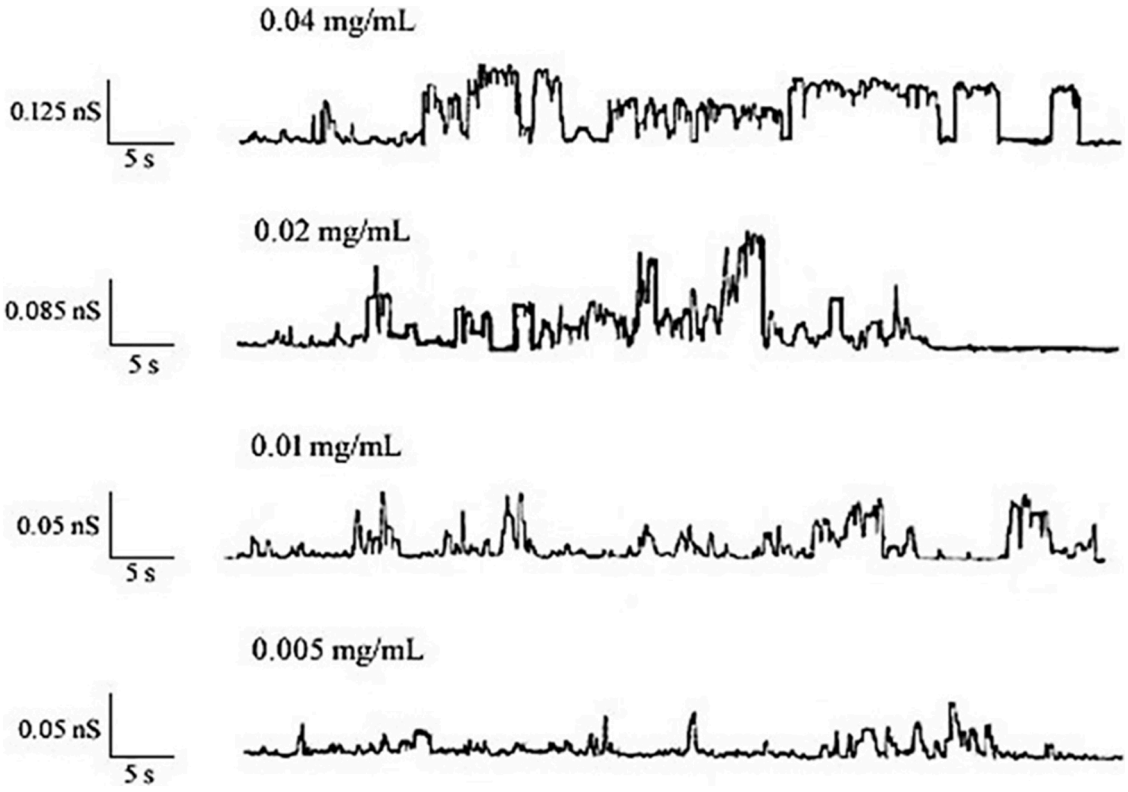

Fig. 4. *A. cepa* extract channel-like activity in DOPS:DOPE:POPC PLMs. Example of chart recordings of channel activity of *A. cepa* extract in membranes made up of DOPS:DOPE:POPC at different *A. cepa* extract concentrations ([AE]). Applied voltage was set to: 40 mV, [AE] 0.04 mg/mL; 60 mV, [AE] 0.02 mg/mL; 100 mV, [AE] 0.01 and 0.005 mg/mL. Experiments were performed in the presence of *A. cepa* extract added to the *cis* side, while the aqueous phase contained 1 M KCl (pH 7) and T = 23±1 °C.

Table 3
Characteristic parameters of *A. cepa* var. Tropea extract channel-like event in DOPS:DOPE:POPC PLM. The mean conductance fitted by Gaussian distribution ($\Lambda c \pm SE$), the frequency (F ± SD), the lifetime (τ) of *A. cepa* extract [AE] channel-like events at different applied voltages. The minimum and maximum number of channel-like events considered (N) out of a total number of channel-like events considered (Nt) was: *A. cepa* extract 4 mg/mL, 195<N<1013, Nt=4655; *A. cepa* extract 2 mg/mL, 112<N<784, Nt=2803; *A. cepa* extract 1 mg/mL, 194<N<554, Nt=1042; *A. cepa* extract 0.005 mg/mL, 140<N<955, Nt=1702.

	[AE]=0.04 mg/mL				[AE]=0.02 mg/mL				[AE]=0.01 mg/mL				[AE]=0.005 mg/mL			
Vs	$\Lambda_c \pm SE$	F±SD	τ_1	τ_2	$\Lambda_c \pm SE$	F±SD	τ_1	τ_2	$\Lambda_c \pm SE$	F±SD	τ_1	τ_2	$\Lambda_c \pm SE$	F±SD	τ_1	τ_2
mV	nS		s	s	nS		s	s	nS		s	s	nS		s	s
100	0.020±0.0005	6.37±0.29	2.31		0.016±0.003	3.23±0.14	1.40		0.017±0.003	3.80±0.16	1.07		0.023±0.002	8.65±0.0.27	0.93	
80	0.028±0.002	8.03±0.25	2.20		0.030±0.002	4.89±0.17	2.03		0.029±0.002	5.60±0.0.40	1.20		0.029±0.001	11.35±0.46	1.01	
60	0.030±0.002	4.62±0.38	2.54		0.044±0.004	6.72±0.27	1.19									
40	0.060±0.004	5.76±0.22	2.07													
20	0.088±0.002	6.85±0.65	0.75	5.26												
-20	0.090±0.01	4.69±0.33	2.07													
-40	0.065±0.002	5.23±0.30	1.08	3.90												
-60	0.026±0.003	8.85±0.58	2.50		0.022±0.003	15.13±0.85	0.83									
-80	0.028±0.001	6.41±0.33	2.00		0.017±0.006	4.87±0.24	2.94									
-100	0.023±0.001	7.64±0.34	0.70	4.97	0.012±0.003	2.01±0.0.19			0.013±0.002	3.55±0.0.21	0.78		0.024±0.0007	5.33±0.45	1.56	

Table 4
Capacitance variation in DOPS:DOPE:POPC PLM. Mean values of the membrane capacitance (C±SE) calculated just before the addition of *A. cepa* extract (Ci) and at the end of the experiment (Cf). The mean value was obtained from at least four experiments.

[AE] mg/mL	Ci±SE μF/cm^2	Cf±SE μF/cm^2
0.005	0.28±0.01	0.21±0.01
0.01	0.30±0.002	0.16±0.003
0.02	0.31±0.02	0.18±0.01
0.04	0.28±0.02	0.20±0.02

has been reached, the extract is able to form transient conductive units permeabilizing the membrane. Collapse of the conductive units inducing rapid flip-flopping of the membrane lipids also contributes to the paroxystic activity which was observed. This mode of interaction with lipids was also reported for other natural compounds (Meleleo, 2021). Correlation observed between time lags and concentrations of the *A. cepa* extract indicates that it is necessary for the extract to reach an appropriate concentration on the membrane surface before it can incorporate and form conductive units. The formation of channel-like events and the appearance of paroxystic activity support the affinity of the *A. cepa* var. Tropea extract for the lipid core of PLMs and indirectly, provide information on its location within the lipid double layer. The results achieved by the electrophysiological study provides the basis for a possible mechanism of action by which the *A. cepa* extract may display its anti-obesity effect.

Conclusions

In conclusion our results demonstrate that the hydroalcoholic extract prepared from the outer scales of a certified PGI variety of onion, Tropea red onion, has a significant inhibitory activity *in vitro* against pancreatic lipase in a dose-dependent manner.

Obesity is the most prevalent nutritional disease and a major public health problem that, according to WHO, has reached epidemic proportions worldwide. Moreover it is a factor of risk for several chronic diseases including diabetes, cancer and cardiovascular diseases (Rani et al., 2016). One of the strategies to prevent or to treat it, relies on the inhibition of gastrointestinal lipases. Thus, the *in vitro* inhibitorial activity of our extract from Tropea red onion poses the basis for finding anti-obesity agents alternative to synthetic drugs.

The beneficial effects of polyphenols in treating or preventing obesity is increasingly reported (Buchholz and Melzig, 2015). We can therefore assume that the high content of phenolic compounds in our extract favors its inhibitory activity. In particular, the presence of quercetin and its glycosides as the major components. Based on its polyhydroxylated nature, this phytochemical has in fact a very high bioactivity (Materska, 2008; Batiha et al., 2020; Adorisio et al., 2021).

Although several papers describe the pancreatic lipase inhibition of phenolic compounds, their inhibitory mechanism of action remains unknown (Sergent et al., 2012). Electrophysiological data obtained in our study using PLMs with a composition mimicking that of intestinal membranes, proved that the extract of *A. cepa* var Tropea is able to promote the ionic flux across the bilayer forming channel-like pathways and affecting the biophysical properties of the membranes such as thickness, fluidity and packaging of lipids. These evidences indicate that the extract from the dry outer scales of Tropea red onion prevents lipid ester hydrolysis and has a protective effect against phospholipase as found for interfacially active molecules. The results obtained by the measurement of pancreatic lipase activity seem to confirm this hypothesis.

Ethical statement

Hereby, I declare that all the data presented in the manuscript were

produced by the Authors partnership and used with the permission of all the Authors.

Author contributions

All data were generated in-house, and no paper mill was used. All Authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

CRediT authorship contribution statement

Mariangela Marrelli: Writing – original draft, Investigation. **Concetta Russo:** Writing – original draft, Investigation. **Giancarlo Statti:** Writing – original draft, Investigation. **Maria Pia Argentieri:** Writing – original draft, Investigation. **Daniela Meleleo:** Writing – original draft, Formal analysis. **Rosanna Mallamaci:** Writing – original draft, Formal analysis. **Pinarosa Avato:** Investigation, Writing – original draft, Writing – review & editing. **Filomena Conforti:** Investigation, Writing – original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

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