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5 **Poncirus trifoliata (L.) Raf.: chemical composition, antioxidant properties and hypoglycaemic**

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28 **_Poncirus trifoliata_ (L.) Raf.: chemical composition, antioxidant properties and hypoglycaemic**

29 **activity _via_ the inhibition of α-amylase and α- glucosidase enzymes**

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39

40 **Abstract**

41 The aim of this study was to investigate the phytochemicals content of *Poncirus trifoliata* (L.) Raf.

42 (Rutaceae) and to assess its hypoglycaemic and antioxidant effects. Juice and seeds methanol extract

43 were analysed by high performance liquid chromatography-diode array detection (HPLC-DAD).

44 Fourteen selected chemical markers were quantified. Among these phytochemicals, hesperidin,

45 naringin and chlorogenic acid were the most abundant compounds of the juice. Peels components

46 were extracted by hydrosistillation and the essential oil was analysed by gas chromatography-flame

47 ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). The main

48 components were the monoterpene hydrocarbons limonene, myrcene, *p*-cimene and α-pinene.

49 Samples were tested for their efficiency to inhibit carbohydrates-hydrolysing enzymes, α-amylase

50 and α-glucosidase. Juice was the most active with IC_{50} values of 138.14 and 81.27 μg/ml against μ-

51 amylase and α-glucosidase, respectively. Interestingly, neoeriocitrin inhibited α-amylase with an IC_{50}

52 value of 4.69 μM. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Ability Power (FRAP),

and β-carotene bleaching tests were used to investigate the antioxidant activity. IC_{50} values in the range 30.38-39.25 μg/ml for juice and peel oil, respectively, were found in DPPH test. Seed extract showed the highest protection of lipid peroxidation with an IC_{50} value of 46.13 μg/ml after 60 minutes of incubation. Among tested flavonoids, neoeriocitrin was the most active with IC_{50} values of 2.85 and 3.18 μM in DPPH and β-carotene bleaching test, respecetively.
P. trifoliata fruits due to their healthy properties could be an interesting natural source of antioxidant and hypoglycaemic agents.

Introduction

Diabetes mellitus is a metabolic disease, whose prevalence is rapidly increasing. In fact, diabetes is expected to affect about 439 million people by 2030.[1]

Type 2 diabetes is a progressive condition in which the body becomes resistant to the effects of insulin and/or progressively loses the capacity to produce enough insulin from pancreatic β-cells.[2] Pancreatic □-cells become dysfunctional because of the persistent high glucose or lipid levels, release of inflammatory mediators and/or oxidative stress by producing increased amount of reactive oxygen species (ROS).[3] In fact, failure of insulin-stimulated glucose uptake by fat and muscle produces high glucose levels in blood. Increased glucose flux reduces antioxidant defences and enhances oxidant production by several pathways. Oxidative stress plays a key role in the development of diabetes complications.

Many studies evidenced as the consumption of fruits is associated with the protection and/or prevention from several diseases and emphasised the potential use of natural compounds for the treatment of type 2 diabetes and for the management of its complications.[4-6]

The Rutaceae family, that consists of several genera including *Citrus* and *Poncirus*, is rich in flavonoids, limonoids, vitamin C, that are documented to possess health promoting properties.[7,8]

Poncirus trifoliata (L.) Raf., a deciduous or semi-deciduous shrub native of China and Korea, also known as "trifoliate orange", is closely related to the genus *Citrus*. The bitter fruits are used fresh in

marmalade and dried and powdered as a condiment.[9] *P. trifoliata* have been widely used in traditional medicine for the treatment of gastro-intestinal disorders including digestive ulcers and gastritis, dysentery, and inflammation.[10,11] Several works have been demonstrated the *P. trifoliata* anti-inflammatory, antibacterial, anti-anaphylactic effects and antitumor activity.[12-14] The main classes of phytochemicals identified in *P. trifoliata* fruits, roots, bark and seeds are phenolic acids (mainly hydroxycinnamic acids), flavonoids (mainly glycosides such as hesperidin and narirutin), coumarins, alkaloids, triterpenoids and sterols.[15-21] *P. trifoliata* seeds represent also a source of 7□-oxygenated limonoids.[22]

A survey of literature revealed few studies on the characterization of volatiles. Limonene, myrcene and □-phellandrene have been identified as most abundant constituents of the peel essential oil.[23] In a recent work, the volatile compounds of flavedo, pulp and seeds from two cultivars, namely *P. trifoliata* var. *trifoliata* and var. *monstrosa*, grown in Italy were analysed.[24] Peel essential oils were mainly characterized by monoterpene hydrocarbons, with limonene and myrcene as the most representative components.

The present study was designed to investigate *P. trifoliata* juice, seeds extract and peel essential oil for their chemical profile, antioxidant and hypoglycaemic properties. For this purpose, HPLC-DAD, GC-FID and GC-MS analyses were performed. The antioxidant activity was investigated trough three different *in vitro* assays, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Ability Power (FRAP), and □-carotene bleaching tests. The hypoglycaemic properties were evaluated *via* the inhibition of carbohydrate-hydrolysing enzymes, □-amylase and □-glucosidase. The inhibition of these enzymes, with consequently decrease post-prandial hyperglycaemia, is of interest because represents one of the therapeutic approaches for the management of diabetes type 2.

Materials and methods

Chemicals and reagents

104 Caffeic acid and clorogenic acid were purchased from Sigma-Aldrich Chem. Co. (Milwaukee, WI,
105 USA). Hesperetin, narirutin, naringin, hesperidin, neohesperidin, quercetin, rhamnetin, isorhamnetin,
106 rutin, neoeriocitrin, didymin and poncirin were supplied by Extrasynthese (Genay, France).
107 Acetonitrile, formic acid and water were obtained from Carlo Erba Reagents (Milan, Italy). Ethanol,
108 NaOH, chloroform, sodium acetate buffer, and phosphate buffer were obtained from VWR
109 International s.r.l. (Milan, Italy). -Carotene, tripyridyltriazine (TPTZ), butylated hydroxytoluene
110 (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, Tween 20, ascorbic acid,
111 α-amylase from porcine pancreas (EC 3.2.1.1), and α-glucosidase from *Saccharomyces cerevisiae*
112 (EC 3.2.1.20 were purchased from Sigma-Aldrich S.p.a. (Milan, Italy). Acarbose from *Actinoplanes*
113 sp. was purchased from Serva (Heidelberg, Germany).
114
115 **Plant materials and extraction procedure**
116 The fruits of *Poncirus trifoliata* were collected during October 2014 in the Botanic Garden of
117 University of Calabria and were identified by Dr. N.G. Passalacqua, Natural History Museum of
118 Calabria and Botanic Garden, University of Calabria (Italy). Fruits were examined for integrity and
119 the absence of dust and insect contamination.
120 Eighty-seven fruits (3.16 kg) were squeezed and the juice (300 ml) was centrifuged and filtered until
121 analysis. To obtain the *P. trifoliata* seeds, foreign materials, including the peel, etc., were removed.
122 Seeds (657 g) were exhaustively extracted by methanol at room temperature (5 x 700 ml).
123 Immediately after collection, the peels (1.37 kg) were subjected to hydro-distillation in a Clevenger
124 type apparatus for 3 h. The oil was collected in sealed dark brown glass vials and stored at -20 °C
125 until analysis.
126
127 **High performance liquid chromatography-diode array detection (HPLC-DAD) analyses**

128 *P. trifoliata* juice and seeds extract were analysed by a Knauer (Asi Advanced Scientific Instruments,
129 Berlin) system equipped with two pumps Smartiline Pump 1000, a Rheodyne injection valve (20 µl),
130 a photodiode array detector UV/VIS equipped with a semi micro-cell.
131 Processing data were carried out using Clarity Software (Chromatography Station for windows).
132 Compounds were separated on a Knauer RP C18 (250 mm x 4.6 mm, 5 µm). The mobile phase was
133 a gradient prepared from formic acid in water (pH= 3, solvent A) and formic acid in acetonitrile (pH=
134 3, solvent B): 0.01-20.00 min 5% B isocratic; 20.01-50.00 min, 5-40% B; 50.01-55.00 min, 40-95%
135 B; 55.01-60.00 min 95 % B isocratic.[25] The column temperature was 30 °C and the flow rate was 1.0
136 ml/min. Samples were filtered through a 0.45 µm millipore filter (GMF Whatman) before injection.
137 The injection volume was 20 µl. Peaks were monitored at 280 and 350 nm. Fourteen selected
138 compounds belonging to different phenolic classes (caffeic acid, chlorogenic acid, didymin,
139 hesperedin, hesperetin, naringenin, naringin, narirutin, neohesperedin, neoeriocitrin, poncirin,
140 quercitin, rhamnetin, and rutin) were quantified. A standard mixture were prepared by adding
141 accurately weighed amount of each compounds (100 mg) to a 100 ml volumetric flask and brought
142 to the mark with methanol (90:10). A calibration straight for each standard was obtained by analysing
143 the standard solution diluted at different concentrations. All solutions were filtered through a 0.45
144 m millipore filter (GMF Whatman) and inject to HPLC system for retention times determination.
145 The identification and quantification were carried out from the retention times in comparison with
146 authentic standards. Analyses were performed in triplicate.
147

148 **Gas chromatography (GC) and gas chromatography-mass spectrometry (GC–MS) analyses**

149 *P. trifoliata* essential oil was analysed by gas chromatography (GC) using Shimadzu GC17A gas
150 chromatograph (Shimadzu, Milan, Italy) fitted with a HP-5 MS capillary column (30 m x 0.25 mm
151 i.d.; 0.25 m film thickness) (Agilent, Milan, Italy) and controlled by Borwin Software. Flame
152 ionization detection (FID) was performed at 280 °C. Nitrogen was the carrier gas (1 ml/min). Column
153 temperature was initially kept at 50 °C for 5 min, then gradually increased to 280 °C at 13 °C/min,

154 held for 10 min at 280 °C. Diluted *P. trifoliata* essential oil (1/100 v/v, in *n*-hexane) was injected (1.0
155 l).
156 Gas chromatography-mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard
157 6890 gas chromatograph fitted with a fused silica HP-5 capillary column (30 m length, 0.25 mm i.d.,
158 0.25 m film thickness). Ionization energy voltage 70 eV was used. The carrier gas was helium and
159 the gas chromatographic conditions were as given above. Constituents were tentatively identified by
160 gas chromatography by comparison of their retention indices either with those of the literature or with
161 those of authentic compounds available in our laboratories. The retention indices were determined in
162 relation to a homologous series of *n*-alkanes (C_9-C_{31}) under the same operating conditions. Further
163 identification was made by comparison of their mass spectra on both columns with either those stored
164 in Wiley 275 and NIST 98 libraries or with mass spectra from the literature and our home made
165 library.[26-28]
166 Component relative concentrations were calculated based on GC-FID peak areas without using
167 correction factors.
168

169 **Physico-chemical parameters of *P. trifoliata* juice**

170 The pH was measured in sample juices by pH meter (Basic Model 20, Crison) and the total titratable
171 acidity (TA) was assessed by titration with NaOH (0.1 N) to pH 8.1 and expressed as citric acid %.
172 The ascorbic acid content was evaluated using iodometric titration with a iodine 0.01 N solution.
173 Results are expressed in mg/100 ml of juice. The total soluble solids were estimated by a digital
174 refractometer PR-201α (Atago, Tokyo, Japan) and expressed as °Brix (sucrose percentage) at 20 °C.
175 Colour of fresh juice was measured at 25 °C using a Konica Minolta CM-700/600d spectrophotometer
176 (Konica Minolta Sensing, Japan). Data were expressed as L* (lightness/darkness in a range 0-100),
177 a* (greenness/redness in a range between -60 and + 60) and b* (blueness/yellowness in a range
178 between – 60 and + 60).
179

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The measurement of the radical scavenging activity was performed by DPPH assay according to the procedure previously described.[29] This assay is based on the measurement of the scavenging ability of antioxidants towards DPPH radicals. The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants to the corresponding hydrazine. When DPPH reacts with an antioxidant, which can donate hydrogen, it is reduced. The change in colour from violet to yellow was read at 517 nm (Perkin Elmer Lambda 40 UV/VIS spectrophotometer). A decrease in the absorbance of the DPPH solution indicates an increase of DPPH radical scavenging activity. The positive control was ascorbic acid.

Ferric Reducing Ability Power (FRAP) assay

The reduction of TPTZ (2,4,6-tripyridyl-s-triazine)-Fe^{3+} to the TPTZ–Fe^{2+} in the presence of antioxidants is measured in the Ferric Reducing Ability Power (FRAP) assay.[30] Concisely, 0.2 ml of samples solution (concentration of 1 μg/ml) was mixed with 1.8 ml of FRAP reagent freshly prepared and the absorption of the reaction mixture was measured at 595 nm. FRAP value represents the ratio between the slope of the linear plot for reducing Fe^{3+}–TPTZ reagent by *P. trifoliata* samples compared to the slope of the plot for $FeSO_4$.

β-Carotene bleaching test

The β-carotene bleaching assay is based on the oxidation of linoleic acid. Linoleic acid hydroperoxides react with β-carotene resulting in the rapid disappearance of colour. The presence of an antioxidant can obstruct the extent of β-carotene by acting on linoleate free radicals and other free radicals formed in the system. So, the absorbance rapidly decreased in samples without antioxidants whereas in the presence of an antioxidant, they maintained their absorbance and colour for a longer period. Propyl gallate was used as positive control. In this assay, 1 ml of β-carotene (0.2 mg/ml) was added to 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20.[29] After evaporation of solvent and

dilution with water, 5 ml of the emulsion were transferred into different tubes containing 0.2 ml of *P. trifoliata* samples at different concentrations. The absorbance was measured at 470 nm.

□-Amylase inhibition assay

The □-amylase inhibition assay was performed as previously described.[29] The enzyme solution was prepared by adding 0.0253 g of enzyme in 100 ml of cold distilled water. The starch solution was prepared by stirring 0.125 g of potato starch in 25 mL of sodium phosphate buffer 20 mM and sodium chloride 6.7 mM (65 °C for 15 minutes). The colorimetric reagent was prepared mixing a sodium potassium tartrate solution and 96 mM 3,5-dinitrosalicylic acid solution. *P. trifoliata* juice, seeds extract, peel essential oil and control were added to the starch solution and left to react with □-amylase solution at 25 °C for 5 min. The enzyme inhibition was calculated spectrophotometrically at 540 nm by using the following equation: % Inhibition= 100 – [([maltose] test/[maltose] control)] × 100. Acarbose was used as positive control.

α-Glucosidase inhibition assay

In α-glucosidase inhibition assay a maltose solution was prepared by dissolving 12 g of maltose in 300 ml of 50 mM sodium acetate buffer.[29] The enzyme solution was prepared by adding 1 mg of enzyme (10 units/mg) in 10 ml of ice-cold distilled water. DIAN solution was prepared by dissolving 1 tablet in 25 ml of distilled water. PGO system-colour reagent solution was obtained by dissolving 1 capsule in 100 ml of ice-cold distilled water. Samples and control were added to maltose solution and left equilibrate for 5 minutes at 37 °C. The addition of α-glucosidase solution started the reaction. After 30 minutes of incubation at 37 °C, the reaction was stopped by adding a solution of perchloric acid. The supernatant of tube of step one was mixed with DIAN and PGO and was left to incubate at 37 °C for 30 minutes. Acarbose was used as positive control. The α-glucosidase inhibition was calculated by using spectrophotometric data at 500 nm and by the equation: % Inhibition= 100 – [([glucose] test/[glucose] control)] × 100.

232

233 **Analysis of data**

234 The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use
235 of GraphPad Prism version 6 for Windows (Graph Pad Software, San Diego, CA, USA). The
236 concentration-response curve was obtained by plotting the percentage inhibition *vs* concentration.
237 Differences concerning parameters were analysed by the one-way ANOVA test and multicomparison
238 Dunnet's test.

239

240 **Results and discussion**

241

242 **Chemical profile of *P. trifoliata***

243 *P. trifoliata* juice and seeds were investigated for their polar constituents by HPLC-DAD. Peels were
244 subjected to hydrodistillation and analysed by GC-FID and GC-MS. Flavonoids constitute one of the
245 most important groups of naturally occurring phenols that are beneficial to plants as protective agents
246 and to human health.[31-34] The juice of *P. trifoliata* was characterized by having a large amount of
247 flavonoids, in particular flavanones.[35] Herein, four-teen compounds were choose as markers and
248 quantified in *P. trifoliata* juice and seeds extract. Data are reported in Table 1. Nine flavanones,
249 namely narirutin, naringin, hesperidin, neohesperidin, neoeriocitrin, didymin, poncirin, hesperetin
250 and naringenin, were identified and quantified in juice.

251 In agreement with previous studies,[35, 36] hesperidin was the dominant flavanone glycoside (129.33
252 μg/ml), while hesperitin was the principal flavanone aglycones (55.13 μg/ml). Naringin was the
253 second most abundant flavanone in *P. trifoliata* juice (115.79 μg/ml), followed by didimin (78.83
254 μg/ml) and narirutin (75.73 μg/ml). Lower values of naringin were previously found.[37] Neohesperidin
255 and naringenin were the least abundant flavanones in the samples (32.75 and 28.65 μg/ml,
256 respectively).

Three flavones namely rutin, quercitin and rhamnetin have been identified. Rutin was the most abundant with a value of 1.85 µg/ml of juice, followed by quercetin (0.76 µg/ml). Unlike the flavanones their content in the juice is lower. Two phenolic acids, such as caffeic and chlorogenic acid, were quantified. Chlorogenic acid was the most abundant (112.54 µg/ml). Among selected standards, four compounds were detected in seeds methanol extract.

Naringin was the most abundant (156.42 µg/g ex), followed by neohesperedin (80.12 □g/g ex), narirutin (37.62 µg/g ex) and caffeic acid (32.85 µg/g ex).

The quality parameters, including pH, total soluble solids (TSS), total acidity (TA), ascorbic acid content, colour and TSS/TA ratio of *P. trifoliata* juice, are shown in Table 2. These parameters are important for the sensory characteristics of fruit.[38]

The values of total acidity and total soluble solids are in agreement with those found in the literature.[35]

P. trifoliata juice is a rich source of ascorbic acid (352.5 mg/l).

The chemical composition of the essential oil of *P. trifoliata* peel was analysed by GC-FID and GC-MS (Table 3). Forty-eight constituents, representing 98.62% of the total oil composition, were identified. The essential oil was mainly composed of monoterpene hydrocarbons (76.26%). Limonene 41.73%) was the main component, followed by myrcene (15.68%), (*E*)-□-ocimene (5.05%), □-phellandrene (4.11%) and □-pinene (3.95%). Sesquiterpene hydrocarbons are also present in good amount (8.18%) with *trans*-caryophyllene (3.59%) and -farnesene (1.16%) as principal ones.

Oxygenated monoterpenes accounted for 3.06%, where linalool (0.69%) and nerol (0.65%) were the main compounds.

Oxygenated sesquiterpenes constituted a minor quantity (1.48%) with farnesol as the most representative.

Noteworthy was the abundance of esters, such as ethyl octanoate (2.12%) and ethyl hexanoate (2.22%). Esters are significant aroma constituents of many fruits.

Previously, Papa et al.[24] found monoterpene hydrocarbons as the main class of volatile constituents of *P. trifoliata* var. *trifoliata* and var. *monstrosa* peel essential oils. Limonene (41.3-54.1%) and

283 myrcene (18.2-23.2%) are the main representative compounds of this fraction, followed by α-pinene

284 (2.2-4.8%) and (*E*)--ocimene (0.7-4.2%).

285

286 **Antioxidant activity**

287 Several methods have been developed to determine the antioxidant capacity; the most frequently used

288 are *in vitro* methods based on capturing or scavenging free radicals generated in the reaction or in the

289 reduction of metal ions. Antioxidant activity is a complex process, including decomposition of

290 peroxides, free radical scavenging activity, reducing ability, prevention of hydrogen abstraction, and

291 binding of transition metal ion catalysts. Therefore, in this work *P. trifoliata* samples were

292 investigated for their potential antioxidant capacity by using three *in vitro* methods (DPPH, β-

293 carotene bleaching and FRAP) that measure different types of antioxidant function (Table 4). *P.*

294 *trifoliata* juice, seeds extract and peel essential oil exerted DPPH radical scavenging activity in a

295 concentration-dependent manner.

296 IC_{50} values in the range 30.38-39.25 μg/ml for juice and peel essential oil, respectively, were found.

297 Significant differences were in β-carotene bleaching test, in which seed extract showed the highest

298 protection of lipid peroxidation with an IC_{50} value of 46.13 μg/ml after 60 minutes of incubation.

299 Two time-lower activity was found for peel essential oil and juice (IC_{50} value of 84.89 and 86.77

300 μg/ml, respectively). Mitochondrial dysfunction and ROS are often implicated in diseases involving

301 oxidative stress and elevated iron. Iron is a component of numerous oxidases and oxygenases and an

302 essential element for the utilization of oxygen. Data obtained by FRAP test demonstrated that *P.*

303 *trifoliata* samples are not very active as antioxidant by reduction of iron. Seeds extract and juice

304 showed values of 3.42 and 0.64 μM Fe(II)/g, respectively. Peels essential oil was not active at the

305 tested concentration.

306 Previously, Eom et al.[16] investigated dried powered of *P. trifoliata* extracts and fractions for its

307 antioxidant potential by using several assays, including DPPH, hydroxyl, alkyl radicals, and

308 superoxide. Water extract and dichloromethane fraction showed the most promising scavenging

309 activity against reactive radicals. In addition, *P. trifoliata* water extract reduced the hydrogen
310 peroxide-induced intracellular reactive oxygen species on CCL 13 cell line and improved cell
311 viability against hydrogen peroxide-induced oxidative damage.
312 Natural products such as phenols and flavonoids showed to be efficient antioxidant agents. Taking
313 into account that several synthetic antioxidants have demonstrated to be toxic and/or mutation
314 inducers, many researchers have directed their study in search of natural antioxidants. *P. trifoliata*
315 juice has proven to be a rich source of flavonoids and ascorbic acid.
316 Table 5 reported the antioxidant properties of narirutin, poncirin, didymin, naringin, hesperidin and
317 neoeriocitrin. Tested flavonoids demonstrated a DPPH radical scavenging activity in a concentration-
318 dependent manner. The most active was neoeriocitrin with an IC_{50} value of 2.85 μM, followed by
319 hesperidin and naringin with IC_{50} values of 16.54 and 21.53 μM, respectively. In β-carotene bleaching
320 test interesting results, in comparison to the positive control propyl gallate (IC_{50} value of 4.71 □M),
321 were obtained with neoeriocitrin (IC_{50} value of 3.18 μM) and narirutin (IC_{50} value of 6.72 μM).
322 Flavonoids were also investigated by using FRAP assay, but not activity was evidenced at the test
323 concentration of 1 mg/ml for all samples except seed (0.40 μM Fe(II)/g).
324

325 **Hypoglycaemic properties**

326 The hypoglycaemic potential effects of *P. trifoliata* were analysed by the inhibition of □-amylase
327 and □-glucosidase assays. Data are reported in Table 6. All samples are able to inhibit both enzymes
328 in a concentration-dependent manner. However, the most interesting activity was found against □-
329 glucosidase enzyme. In particular, juice exhibited an IC_{50} value of 81.27 □g/ml, followed by seeds
330 extract (IC_{50} value of 170.54 □g/ml).
331 The same trend was observed against □-amylase with IC_{50} values of 138.14 and 459.58 □g/ml for
332 juice and seed extract, respectively.

Flavonoids have revealed positive effects in the treatment of hyperglycaemia probably inhibiting enzymes, such as glucosidase.[39] The main flavonoids identified in *P. trifoliata* juice were investigated for their α-amylase and α-glucosidase inhibitory activity. Data are reported in Table 7.

All tested compounds demonstrated α-amylase and α-glucosidase inhibitory properties in a concentration-dependent manner. All tested compounds were more active than the positive control acarbose (IC_{50} of 4.69-70.80 µM *vs* IC_{50} of 77.45 µM). The most active against α-amylase is neoeriocitrin with an IC_{50} value of 4.69 µM.

Didymin was the most active against α-glucosidase (IC_{50} of 4.20 µM), followed by naringin (IC_{50} of 10.33 µM) and narirutin (IC_{50} of 14.30 µM). Except for poncirin, all tested flavonoids were more active than acarbose (IC_{50} value of 54.99 µM).

Sixteen flavonoids, divided into six groups, are previously investigated for their ability to inhibit yeast and rat small intestinal α-glucosidases and porcine pancreatic α-amylase.[40]

Anthocyanin, flavonol and isoflavone groups strongly inhibited yeast α-glucosidase with IC_{50} values less than 15 µM.

The analysis of structure-activity relationships revealed that the unsaturated C ring, a carbonyl group at 4 position, a hydroxyl group at the 3 position or the linkage of the B ring at the 3 position, and the presence of hydroxyl substituents on the B ring enhanced the inhibitory activity. Luteolin, myricetin and quercetin were the most active flavonoids against a-amylase. The following structures enhanced the inhibitory activity: the presence of a 2,3 double bond, a hydroxyl group at 5 position, the linkage of the B ring at the 3 position and the presence of hydroxyl substituents on the B ring. The inhibitory activity against α-amylase is reduced by the presence of a hydroxyl group at 3 position.

Conclusions

The ability of antioxidant molecules to protect against the effects of hyperglycaemia and to improve glucose metabolism and intake must be considered as leads of choice for diabetes management. In addition to their antioxidant activity, many phytochemicals, and in particular flavonoids,

demonstrated to act on biological targets involved in type 2 diabetes mellitus such as □-amylase and □-glycosidase inhibition. In this context, flavonoids behaving as antioxidants were studied as potential drugs by acting as biological targets involved in diabetes development. Of particular interest is the juice of *P. trifoliata* and its flavonoids that demonstrated carbohydrate hydrolysing enzymes inhibition higher than the commercial drug acarbose.

Thus, the present study suggests *P. trifoliata* fruits as promising source for the development of functional ingredients for development of functional foods or supplements for the treatment and/or prevention of several diseases associated with oxidative stress such as type 2 diabetes.

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447 **Table 1**. Identified constituents of juice and seeds extract of *P. trifoliate*

Compound	**Juice (µg/ml)**	**Seeds extract (µg/g ex)**
Flavanone-O-glycosides		
Narirutin	75.73 ± 0.03	37.62 ± 0.53
Naringin	115.79 ± 0.06	156.42 ± 0.35
Neohesperedin	32.75 ± 0.04	80.12 ± 0.25
Hesperidin	129.33 ± 0.12	nd
Neoeriocitrin	12.44 ± 0.14	nd
Didymin	78.83 ± 0.12	nd
Poncirin	49.37 ± 0.07	nd
Flavanone aglycones		
Hesperetin	55.13 ± 0.10	nd
Naringenin	28.65 ± 0.08	nd
Flavone-O-glycosides		
Rutin	1.85 ± 0.06	nd
Flavone aglycones		
Quercetin	0.76 ± 0.07	nd
Rhamnetin	0.37 ± 0.06	nd
Phenolic acids		
Caffeic acid	18.46 ± 0.20	32.85 ± 0.11
Chlorogenic acid	112.54 ± 0.22	nd

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450 **Table 2.** Physico-chemical parameters of *Poncirus trifoliata* juice.

Parameters	
pH	3.17 ± 0.05
Acidity[a]	1.9 ± 0.06
Total soluble solids (°Brix)	14.0 ± 1.0
Ascorbic acid (mg/l)	352.5 ± 0.25
L	9.57 ± 0.04

a*	2.31 ± 0.04
b*	9.96 ± 0.06
Chroma	10.22 ± 0.02

Table 3. The main chemical components of the essential oil of *P. trifoliata*.

Components[a]	*RI*[a]	*Relative amount (%)*	*I.M.*[b]
Monoterpene hydrocarbons			
□-Pinene	938	1.19	1,2,3
Sabinene	973	1.17	1,2,3
□-Pinene	980	3.95	1,2,3
Myrcene	993	15.68	1,2,3
□-Phellandrene	1005	4.11	1,2
□-Terpinene	1012	tr	1,2,3
p-Cimene	1025	1.50	1,2
Limonene	1030	41.73	1,2,3
(*E*)-□-Ocimene	1052	5.05	1,2
□-Terpinene	1057	0.70	1,2,3
Terpinolene	1086	0.54	1,2,3
p-Mentha-1,3,8-triene	1108	0.46	1,2
Oxygenated monoterpenes			
Linalool	1098	0.69	1,2,3
Terpinen-4-ol	1176	tr	1,2,3
□-Terpineol	1189	0.30	1,2,3
Nerol	1236	0.65	1,2,3
Geraniol	1240	0.52	1,2,3
Neryl acetate	1370	0.34	1,2
Geranyl acetate	1388	0.56	1,2
Sesquiterpene hydrocarbons			
□-Cubebene	1352	0.28	1,2
trans-Caryophyllene	1415	3.59	1,2,3
□-Farnesene	1441	1.16	1,2
□-Humulene	1455	0.72	1,2
Germacrene B	1554	0.51	1,2
□-Elemene	1387	0.59	1,2
γ-Cadinene	1515	0.20	1,2,3
δ-Cadinene	1526	0.73	1,2,3
Oxygenated sesquiterpenes			
(*E*)-Nerolidol	1564	0.42	1,2
Spathulenol	1578	0.44	1,2
(*E,Z*)-Farnesol	1742	1.02	1,2
Miscellaneous			
Ethyl hexanoate	1000	2.22	1,2
Hexyl butanoate	1065	0.98	1,2
Nonanal	1102	tr	1,2
Ethyl octanoate	1198	2.12	1,2
Decanal	1205	0.42	1,2
Ethyl decanoate	1396	0.72	1,2
Dodecanal	1417	0.22	1,2
Ethyl laurate	1601	0.46	1,2
Myristic acid	1780	0.26	1,2
Ethyl myristate	1793	0.31	1,2
Octadecane	1800	0.14	1,2,3
Nonadecane	1900	0.12	1,2,3
Eicosane	2000	0.35	1,2,3
Ethyl linoleate	2160	0.23	1,2
Ethyl stearate	2193	0.35	1,2
Pentacosane	2500	0.42	1,2,3
Heptacosane	2700	0.32	1,2,3

Nonacosane	2900	tr	1,2,3
Total identified		98.62	
Monoterpene hydrocarbons		76.26	
Oxygenated monoterpenes		3.06	
Sesquiterpenes hydrocarbons		8.18	
Oxygenated sesquiterpenes		1.48	
Miscellaneous		9.64	

Table 4 Antioxidant activity of *P. trifoliata.*

P. trifoliata	DPPH test IC_{50} (μg/ml)	β-carotene bleaching test IC_{50} (μg/ml)	FRAP test μM Fe(II)/g
Juice	30.38 ± 2.5[***]	86.77 ± 3.7[***]	0.64 ± 0.08[***]
Peels oil	39.25 ± 2.3[***]	84.89 ± 3.7[***]	NA
Seeds extract	33.34 ± 2.1[***]	46.13 ± 2.4[***]	3.42 ± 0.009[***]
Positive control			
Ascorbic acid	5.0 ± 0.8		
Propyl gallate		1.0 ± 0.04	
BHT			63.2 ± 4.3

Table 5 Antioxidant activity (IC_{50} μM) of identified flavonoids.

Flavonoids	DPPH test	β-Carotene bleaching test
Narirutin	45.30 ± 1.4	6.72 ± 0.09
Poncirin	44.23 ± 1.6	47.40%[a]
Didymin	36.16 ± 1.5	35.42%[a]
Naringin	21.53 ± 2.0	13.44 ± 1.1
Hesperedin	16.54 ± 1.3	10.81 ± 1.0
Neoeriocitrin	2.85 ± 0.04	3.18 ± 0.08
Positive control		
Ascorbic acid	28.40 ± 2.1	
Propyl gallate		4.71 ± 0.8

Table 6 *In vitro* α-amylase and α-glucosidase inhibitory activity [IC_{50} (□g/ml)] of *P. trifoliata* samples.

P. trifoliata	□-Amylase	□-Glucosidase
Juice	138.14 ± 3.1[a]	81.27 ± 3.5[a]
Seeds extract	459.58 ± 4.8[a]	170.54 ± 4.4[a]
Peel essential oil	664.54 ± 4.7[a]	300.17 ± 4.4[a]
Acarbose	50.0 ± 0.9	35.5 ± 1.2

Table 7 α-Amylase and α-glucosidase inhibitory activity (IC_{50} μM) of identified *P. trifoliata* constituents.

Flavonoids	□-Amylase	□-Glucosidase
Narirutin	70.80 ± 2.5	14.30 ± 3.5
Poncirin	39.19 ± 1.3	64.58 ± 2.6

Didymin	31.62 ± 2.8	4.20 ± 0.6
Naringin	36.35 ± 1.9	10.33 ± 1.1
Hesperedin	26.04 ± 1.7	15.89 ± 1.8
Neoeriocitrin	4.69 ± 0.9	25.31 ± 1.2
Positive control		
Acarbose	77.45 ± 1.8	54.99 ± 1.3

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