TITLE RUNNING HEAD: Tabacchiera peach chemical composition and antioxidant activity

ORIGINAL PAPER

Prunus persica var. *platycarpa* (Tabacchiera Peach): bioactive compounds and antioxidant activity of pulp, peel and seed ethanolic extracts

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Abstract A comparative analysis of ethanol extracts from peel, pulp and seed of *Prunus persica* var. *platycarpa* (Tabacchiera peach) was done. The total phenol, flavonoid and carotenoid content as well as the antioxidant properties by using different *in vitro* assays (DPPH, ABTS, FRAP, Fe-chelating, β-carotene bleaching test) were evaluated. Pulp extract was subjected to liquid chromatography-electrospray-tandem mass spectrometry (HPLC-ESI-MS/MS). Gallic acid, protocatechuic acid, protocatechualdehyde, chlorogenic acid, *p*-coumaric acid, and ferulic acid were identified as main constituents. Pulp extract was characterized by the highest total phytonutrients content and exhibited the highest antioxidant activity in all *in vitro* assays (IC_{50} values of 2.2 μg/mL after 60 minutes of incubation by using β-carotene bleaching test and 2.9 μg/mL by using Fe-chelating assay). Overall, the obtained results suggest that *P. persica var. platycarpa* displays a good antioxidant activity and its consumption could be promoted.

Keywords *Prunus persica* L. Stokes ex Batsch var. *platycarpa* • Phenols • HPLC-ESI-MS/MS• Dietary antioxidants

Abbreviations

ABTS 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

DPPH 2,2-Diphenyl-1-picrylhydrazyl

FRAP Ferric Reducing Ability Power

ROS Reactive oxygen species

TEAC Trolox Equivalent Antioxidant Capacity

Introduction

Several epidemiological studies have confirmed the relationships between diet and diseases [1]. The health-promoting properties of fruits are due to the presence of some secondary metabolites such as phenols that have aroused substantial attention due to their protective potential against diseases [2]. Oxidative stress is considered to play a very important role in the pathogenesis of several health problems [3]. A diet rich in antioxidants can reduce the risk of several diseases. Adequate level of antioxidants provided with diet induces immunological processes and increases defensive abilities of cell in proper way [4]. Several tests were developed to analyse the food antioxidant properties. These tests differ in the generation of radicals, target compounds, and in the mode of assessment the end points. Antioxidants may act *in vivo* through different mechanisms, so the measure of antioxidant activity required a multifactorial approach based on the use of different analytical techniques. Therefore, choosing an adequate assay is critical and in practice several test procedures are carried out for evaluating antioxidant activities with the samples of interest [5, 6]. The growing interest in the substitution of synthetic antioxidants by natural ones has promoted research on vegetable sources for new antioxidants. Oxidation reactions are not an exclusive concern for the food industry, and antioxidants are widely needed to prevent deterioration of other oxidisable products, such as cosmetics and pharmaceuticals products.

P. persica (Rosaceae) is the second most widely cultivated fruit tree [7]. Peach fruit is known for its nutritional value and therapeutic properties as consequence of its bioactive constituents (phenols and carotenoids) [7-13]. Thus, in order to evaluate the potential of peach fruits, significance efforts have been directed on the estimation of

bioactivity and functionality present in different peach varieties. Several research papers reported the analysis of bioactive constituents of *P. persica* using different solvents in the extraction procedure. Among them methanol was the most widely used [7-14] since it offers a high recovery of antioxidant compounds. Differently, the ethanol proves to be least effective solvent for extracting vegetable phenolic compounds. However, due the safety and acceptability for human use of the ethanol, it is very important to encourage the evaluation of the potentiality of ethanol extract as valuable sources of bioactive compounds. The scientific knowledge generated could be used by pharmaceutical and food industries to identify alternative value-added ingredients for the development of new functional intermediates or products with health-benefits.

The aim of this study is to evaluate the compositional profile of *Prunus persica* var. *platycarpa* var. "Tabacchiera" or "Saturnina" ethanolic extracts and to teste the antioxidant activities using different *in vitro* methods. The phenol composition of peach pulp was elucidated by HPLC- ESI-MS/MS.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Co. Ltd (Milan, Italy) and VWR International (Milan, Italy) and, unless specified otherwise, were analytical grade or higher.

Plant Material and Extraction Procedure

Prunus persica var. *platycarpa* fruits were purchased at commercial maturity as assessed by peel fruit colour and pulp firmness maturity in the local market in Catania

(Sicily, Italy) in June 2012. *P. persica* var. *platycarpa* "Tabacchiera" or "Saturnina" is a deciduous tree cultivated in Etna volcanic area (Sicily, Italy). Fruits were examined for integrity and absence of dust and insect contamination, were cleaned by using distilled water and were peeled. Pulp was separated by seeds. Peel (60.80 g), pulp (483.31 g), and seed (33.05 g) were exhaustively extracted at 25 °C by ethanol (absolute, ≥ 99.5%) (400, 1500 and 250 mL, respectively) (3 × 72 h).

Determination of Total Phytochemicals Content

The total phenol and carotenoid content was performed as previously described [15]. The total flavonoid content follows the procedure described by Loizzo et al. [16].

High Performance Liquid Chromatography (HPLC) – Tandem Mass Spectrometry (MS) Condition and Analysis of Phenolic Compounds in Pulp

The determination of the phenolic profile of pulp peach was performed by means of liquid chromatography-electrospray-tandem mass spectrometry, as already described [17]. Twenty-six selected compounds belonging to different phenolic classes (gallic acid, (+)-catechin hydrate, *p*-coumaric acid, *p*-salicylic acid, caffeic acid, chlorogenic acid, (−)-epicatechin, (−)-epigallocatechin ethyl gallate, gallate, ferulic acid, fisetin, gentisic acid, homogentisic acid, polydatin, protocatechuic acid, protocatechualdehyde, quercetin dehydrate quercitrin hydrate, resveratrol, syringic acid, syringaldehyde, taxifolin, umbelliferon, sinapic acid, kaempferol, and vanillic acid) were quantified.

ABTS and DPPH Radical Scavenging Activity Assay

Radical scavenging activity was evalòuated by ABTS and DPPH assays [5, 18].

β-Carotene Bleaching Test

Antioxidant activity was determined as previously described [19]. Briefly, β-carotene solution was added to linoleic acid and 100% Tween 20. The absorbance of the samples, standard and control was measured at 470 nm against a blank at t= 0 and successively at 30 and 60 minutes.

FRAP (Ferric Reducing Ability Power) Assay

The FRAP test is based on the redox reaction that involves TPTZ (2,4,6-tripyridyl-*s*-triazine)-Fe^{3+} complex [20].

Fe^{2+}Chelating Activity Assay

The chelating activity was measured according to the previously reported method [21]. Briefly, extract was mixed with water, 2 mM $FeCl_2$ and 5 mM ferrozine. After 10 min at room temperature, the absorbance was measured at 562 nm.

Pulp Peach Nutritional Analysis

The total nitrogen content, moisture content, ash content, fat content, crude fiber content, total carbohydrates, minerals, and energy values were evaluated [22, 23].

Statistical Analysis

The inhibitory concentration 50% (IC_{50}) was calculated by non-linear with the use of Prism Graphpad Prism version 4.0 for Windows, GraphPad Software, San Diego, CA, USA. Differences were evaluated by ANOVA test followed by multicomparison

Dunnett's test ($p< 0.01$).The concentration-response curve was obtained by plotting the percentage of inhibition *versus* the concentrations.

Results and Discussion

Extraction Yield and Phytonutrients Content

Tabacchiera peach peel, pulp and seed are exhaustively extracted with ethanol (yield of 12.09, 7.83 and 3.69%, respectively). The values of total phenols, flavonoids and carotenoids content for peach pulp, peel and seed ethanol extracts were reported in Table 1. The total phenols content of Tabacchiera peach varied significantly among the tree fruit part tested. The pulp exhibited the highest phenols content (921.8 mg chlorogenic acid equivalent/100 g FW), followed by the peel (448.6 FW) and the seed (111.3 FW). This trend was observed with total flavonoid content. Correlation analysis revealed that phenols are correlated with flavonoids ($r= 0.89$, $p< 0.01$), implying that this group of phytochemicals an important group of phenols in peach fruit [7, 13].

Previously, Legua et al. [14] reported the methanol extract of the pulp of *P. persica* var. *platycarpa* cultivar "Sweet-cap" and "ASF-06-83" as a rich source of phenols (35.97 and 41.37 mg gallic acid equivalent/100 g FW, respectively). Our results demonstrated that pulp extract from Tabacchiera is richer in phenols and flavonoids than peel extract. These results are in contrast with previous reports in a wide range of peach cultivars including the flat peach varieties [11-14, 24].

The observed differences might be related to the kind of solvent used for the extraction. It is well documented that the quali-quantitative profile of phytonutrient fraction is markedly affected by the polarity of extracting solvent and the solubility of the compounds in the solvent used for the extraction process [25]. Generally, extraction

with aqueous methanol results in a higher recovery of total extractable compounds, whereas ethanol gave the lowest recovery of antioxidant compounds [26]. Nevertheless, due the largely use of ethanol as solvent in human, it is very important to investigate the healthy potentiality of ethanolic extract. Unlike to phenol and flavonoid contents, the peel had significantly higher concentration of carotenoids than the pulp (about 6-fold). This content was similar to those reported for other white-fleshed peaches and nectarines, in which the major constituent was *β*-carotene followed by *β*-criptosanthin and lutein [12, 13, 24, 27]. Carotenoids are effective against free radicals, singlet oxygen suppressors and work as chain-breaking antioxidants, protecting cells against attack from radicals [28].

HPLC-ESI-MS/MS Phenolic profile of Pulp Extract

The phenolic profile of pulp extract, that showed the most interesting antioxidant activity, was outlined by means HPLC-ESI-MS/MS analysis. The used methodology was able to provide a comprensive evaluation of twenty-six selected phenols belonging to different phenolic classes, such benzoic and cinnamic acids, flavanols and flavones. Among them, the Tabacchiera pulp extract contained benzoic acids (*i.e.* gallic and protocatechuic acids), cinnamic acids (*i.e. p*-coumaric and ferulic acids), chlorogenic acid and protocatechualdehyde (Figure 1, Table 3). The present study confirmed that chlorogenic acid is the main phenolic compound in peach pulp. In fact, according to previously reported [9] in round peaches and nectarines, our results showed that chlorogenic acid represent the most abundant compound in Tabacchiera pulp extract, followed by gallic acid, protocatechuic acid, protocatechualdehyde, *p*-coumaric acid and ferulic acid. Singularly, the Tabacchiera pulp extract resulted devoid of catechin,

189 epicatechin, epigallocatechin and quercitin whom were previously reported in aqueous
190 methanol extract of pulp of round peaches and nectarines [29]. This difference could be
191 related to the peach variety as well as to the kind of the solvent used for the phenolic
192 extraction from the pulp.

193 Antioxidant Activity of *P. persica* var. *platycarpa* Extracts

194 The antioxidant capacity of fruits varies in relation to antioxidant moieties present in the
195 different species, although variations can occur among cultivars within a single species
196 [9, 12, 24]. A concentration-effect relationship was found for all tested samples (Table
197 3). The radical scavenging activity of peach extracts was evaluated using DPPH and
198 ABTS systems. Pulp extract exhibited the highest DPPH scavenging ability with an IC_{50}
199 value of 12.0 μg/mL followed by peel (IC_{50} 45.3 μg/mL). This trend was observed also
200 against ABTS radical with value of 6.8 and 8.4, respectively. The Ferric Reducing
201 Ability Power of Tabacchiera peach was screened by using FRAP assay. Both pulp and
202 peel extracts resulted the most active (values of 30.2 and 78.9 μM Fe(II)/g,
203 respectively). The reducing ability of both extract was strongly correlated with the
204 phenolic levels ($p<0.01$, $r= 0.99$). A strong correlation between high level of phenol and
205 FRAP value was previously reported also by Hong *et al*. [30]. Phenols exhibited redox
206 properties acting as reducing agents, hydrogen donators and singlet oxygen quenchers.
207 The redox potential of phenolic phytochemicals plays a crucial role in determining the
208 antioxidant properties. Pulp extract chelates ferrous ions with an IC_{50} value of 2.9
209 μg/mL (value 2.2-time higher than the positive control BHT). Carotenoid content was
210 the only value that correlated significantly ($p< 0.01$) with antioxidant activity ($r= 0.98$,
211 0.99, 0.98, 0.98, 0.98, and 0.99 for DPPH, ABTS, β-carotene bleaching test after 30 and
212 60 minutes of incubation, FRAP and Fe-Chelating activity assay, respectively).

Tabacchiera peach is a rich source of phytochemicals with known antioxidant activity. Phenols and carotenoids are able to inhibit oxidation process by acting as free radical scavengers, metal chelators and also trough their effects on cell signaling pathways and on gene expression. The antioxidant activity results from a complex interaction between phytochemicals, which produce synergistic responses.

Pulp Nutritional Analysis

Tabacchiera peach pulp nutritional analysis is reported in Table 4. Moisture content and ash content of pulp were 84.76 and 0.43%, respectively. The fat content was determined by using petroleum ether founding a percentage of 0.21% that revealed that Tabacchiera is a low fat fruit that can be eaten to prevent weight gain and obesity. Protein content was found to be 0.68%. Gravimetric method was used for the determination of dietary fiber founding a value of 1.78 %. Carbohydrates present in the sample were calculated to be 12.14%. The energy value was calculated to be 53.17 Kcal *per* 100 g of pulp. The mineral content showed the presence of high level of sodium and potassium, a moderate iron content which can be utilized to metabolize proteins and helps red blood cells in the production of haemoglobin.

Conclusion

Prunus persica var. *platycarpa* ethanolic extract was analysed for its potential as a functional food. Our results highlight that pulp is a rich source of bioactive compounds particularly phenols. Due the safety and acceptability for human use of this solvent it is very important verify the potentiality of ethanolic extract as valuable sources of bioactive compounds. Moreover, the commercial and domestic uses of large quantity of

fruits, especially for the purposes of juice, and/or processed sauces and slice production, result in the generation of large quantity of seeds and peel as agro-wastes. The results could be useful to provide a solid base for the development and utilization of peach ethanolic extracts, and also for the agro-wastes containing peel and seed as ingredient in formulations of functional or nutraceutical products useful in diseases prevention such as neurodegenerative diseases, cardiovascular diseases, cancer etc. Further *in vivo* studies are warranted to confirm these findings and support the use of this matrix.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Table 1 Total phenols, flavonoids and carotenoids content in *Prunus persica var. platycarpa* fruits.

Phytochemicals	Pulp	Peel	Seed
Phenols [a]	921.8 ± 2.5	448.6 ± 3.5	111.3 ± 1.5
Flavonoids [b]	726.5 ± 8.2	231.9 ± 2.0	76.8 ± 1.1
Carotenoids [c]	61.9 ± 1.8	344.7 ± 1.6	109.3 ± 1.7

Values represent means (*n*= 3) ± S.D. [a]mg chlorogenic acid equivalents/100 g FW; [b]mg quercetin equivalents/100 g FW; [c]mg β-carotene equivalents/100 g FW.

Table 2 Phenolic compounds present in ethanolic extract obtained from fresh pulp peach samples

Compound	Mean (mg/kg extract)
Gallic acid	1.654 ± 0.9
Protocatechuic acid	0.190 ± 0.06
Protocatechualdehyde	0.020 ± 0.04
Chlorogenic acid	15.029 ± 1.3
p-Coumaric acid	0.153 ± 0.09
Ferulic acid	0.226 ± 0.08

Values represent means (*n*= 3) ± S.D.

Table 3 Radical scavenging and antioxidant capacities of *Prunus persica var. platycarpa* extracts

Sample	DPPH (IC_{50}µg/mL)	ABTS (TEAC value)	β-Carotene bleaching test (IC_{50}µg/mL)		FRAP (µM Fe(II)/g)	Fe-Chelating activity (IC_{50}µg/mL)
			30 min	60 min		
Pulp	12.0 ± 1.9**	6.8 ± 0.6**	2.7 ± 0.4**	2.2 ± 0.2**	30.2 ± 1.5**	2.9 ± 0.5**
Peel	45.3 ± 2.7**	8.4 ± 1.0**	9.2 ± 0.8**	5.2 ± 0.8**	78.9 ± 2.9**	8.7 ± 0.9**
Seed	85.3 ± 3.4**	11.7 ± 1.4**	15.7 ± 1.0**	12.3 ± 1.0**	130.5 ± 3.9**	18.8 ± 1.7**
Propyl gallate[a]	-	-	1.0 ± 0.01	1.0 ± 0.01		
Ascorbic acid[a]	2.0 ± 0.01	0.96 ± 0.03	-	-		
BHT[a]					63.2 ± 4.5	1.3 ± 0.05

Data are given as media ± S.D. (n = 3); DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation ($ABTS^+$), β-Carotene bleaching test, FRAP Ferric ion reducing antioxidant power, ; [a] Propyl gallate, ascorbic acid and BHTwere used as positive control. Differences within and between groups were evaluated by one-way analysis of variance test *** $P< 0.0001$ followed by a multicomparison Dunnett's test: ** $P< 0.01$ compared with the positive controls.

Table 4 Nutritional analysis and minerals in pulp from Tabacchiera peach

Nutritional constituents	Content (%)
Moisture	84.76
Ash	0.43
Fat	0.21
Protein	0.68
Fiber	1.78
Carbohydrates	12.14
Energy	53 Kcal/100g
Minerals	**Content (mg/100 g)**
Sodium	18
Potassium	37
Zinc	0.64
Copper	0.14
Iron	1.40

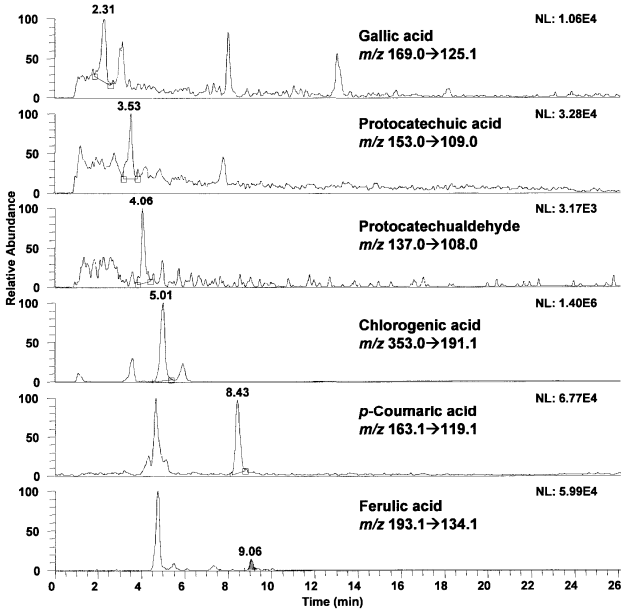

Figure 1. LC-ESI-MS/MS chromatogram of polyphenols found in *Prunus persica var. platycarpa* pulp