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Procyanidins in fruit from Sour cherry (*Prunus cerasus*) differ strongly in chainlength from those in Laurel cherry (*Prunus lauracerasus*) and Cornelian cherry (*Cornus mas*)

Esra Capanoglu[a], Dilek Boyacioglu[a], Ric C.H. de Vos[b,c], Robert D. Hall[b,c] and Jules Beekwilder[b,*]
[a]*Faculty of Chemical and Metallurgical Engineering, Food Engineering Department, Istanbul Technical University, Maslak, Istanbul, Turkey*
[b]*Plant Research International, Wageningen UR, BU Bioscience, Wageningen, The Netherlands*
[c]*Centre for BioSystems Genomics, Wageningen, The Netherlands*

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Abstract. Sour cherry (*Prunus cerasus*), Laurel cherry (*Prunus lauracerasus*), and Cornelian cherry (*Cornus mas*) fruits are widely used in Turkey, both as food and as traditional medicines. The phytochemical composition and antioxidant capacities of these three cherry types were compared. Fruit flesh was evaluated for procyanidin concentration, subunit composition and degree of polymerization, for anthocyanin composition and for total antioxidant capacity, total phenolic content and total flavonoid content. High concentrations (up to 1 g per 100 g dry weight) of long-chain procyanidins were found in Laurel cherry, whereas concentrations of procyanidins in Cornelian cherry were 25 times lower. Surprisingly, Sour cherry (0.3 g/100 g DW) had a dramatically different procyanidin profile which was dominated by short polymers, with an average chain length of 4 monomer units. This is of particular interest since short-chain procyanidins have recently been suggested to play a role in the prevention of coronary heart disease.

Keywords: Sour cherry, Laurel cherry, Cornelian cherry, procyanidins, anthocyanins

1. Introduction

Traditionally, a large diversity of fruit is grown in Turkey for both food and non-food applications. Among these fruits, stone fruits and especially cherries are widely consumed. Many different cherry types can be consumed fresh, but they may also be dried, pickled and processed in a variety of ways and consumed e.g. as *pekmez* (syrup obtained by condensing juices of the fruit must), *pestil* (a dried form of marmalade), jam, marmalades, fruit juice products and tea infusions [15, 34, 40]. Besides their food uses and their various tastes and flavors, these cherry types have also been used for traditional medicine applications and are known for their positive health effects [8, 30, 38]. In the work

*Corresponding author: Jules Beekwilder, Plant Research International, Wageningen UR, BU Bioscience 6700 AA, Wageningen, The Netherlands. Tel.: +31 317 477164; Fax: +31 317 418094; E-mail: jules.beekwilder@wur.nl.

reported here, attention is focused on the Sour cherry, Laurel cherry, and Cornelian cherry as these are all widely consumed in Turkey both for their attractive taste as well as for their publically-known health-related benefits [17, 67, 72].

Sour cherry (*Prunus cerasus* L.), known as *vişne* in Turkey, is widely consumed as fresh fruit as well as in many processed products such as jams, liqueur, wine and confectionery items. Its most important application is in the form of juice. Sour cherry has the highest production and consumption rates of all fruits in Turkey [22]. Its consumption has been shown to be associated with a reduced risk of cardiovascular disease [8]. Sour cherry extracts have been shown to display dicyclooxygenase inhibition [58], antineurodegenerative activity [38], anti-inflammatory potential [56], and inhibition of human colon cancer cell proliferation [33].

Laurel cherry fruit (*Prunus lauracerasus* L., L. officinalis), which is also known as *taflan* or *karayemiş*, is grown in the eastern Blacksea region of Turkey. Laurel cherries are well known for their high mineral and antioxidant contents [12, 39]. Both the fruit and the stones are favored as traditional medicines in Turkey and have been used for many years for the treatment of a wide variety of ailments including stomach ulcers, digestive system complaints, bronchitis, eczema, haemorrhoids, and as a diuretic agent [40].

Fruits of the Cornelian cherry (*Cornus mas* L.) are still harvested from the wild, and have a notably sour taste. The fruits have been applied as food preservatives, but also in traditional Turkish medicine for treatment of gastrointestinal disorders and diarrhea [15]. Cornelian cherry fruits have also been reported to have anti-allergic, anti-microbial, anti-malarial and anti-diabetic activities [19, 31, 65, 66, 70].

Regarding their phytochemical composition, cherries are known for their high levels of phenolic antioxidants and in particular, anthocyanins and procyanidins. Both categories of compounds have been particularly associated with health effects, apart from their general antioxidant activity. Cherries are ranked among the richest sources of procyanidins [9]. Procyanidins represent oligomers or polymers of flavan-3-ol units, while anthocyanins are essentially monomeric molecules which carry glycosidic moieties. The degree of procyanidin polymerization, which may vary from two subunits up to several hundred, appears to be relevant for their absorption in the intestinal tract [20, 26, 37], and is therefore relevant for their general efficacy and their contribution e.g. to cardiovascular health.

Procyanidins have been linked to some of the health-related effects associated with cherry fruits [17, 67, 72]. *In vivo* and *in vitro* studies have shown that procyanidins increase nitric oxide synthesis, inhibit platelet activation, stimulate the production of anti-inflammatory cytokines, and inhibit the production of certain proinflammatory cytokines [45, 57, 71]. They limit free radical formation by inhibiting enzymes or chelating metals involved in their generation and lower the rate of low-density lipoprotein (LDL) oxidation [60, 68]. These activities have been associated with reduced risks for coronary heart disease. There is a remarkable correspondence of procyanidin activities to those of Sour cherry juice, as reported from a study on diabetic women, which were given a Sour Cherry juice concentrate [8]. In this study, reduced blood pressure, body weight and LDL cholesterol were reported.

In the present study we have compared the content of bio-active molecules of these three cherry types. The qualitative and quantitative composition of anthocyanins and procyanidins were compared, as well as their overall antioxidant activities, phenolic content and flavonoid content.

2. Materials and methods

2.1. Fruit material

Replicate samples of Sour cherry and Cornelian cherry were obtained separately from a bazaar in Istanbul. Laurel cherry replicate samples were provided from a local market in Trabzon, Turkey. The stones of the fruit were removed and the de-stoned fruits were snap-frozen in liquid nitrogen after which they were transported in still frozen state to the Netherlands in dry ice. Samples were subsequently ground to a fine powder in liquid nitrogen using a pre-cooled electric grinder. All powder samples were then individually freeze-dried to compensate for the differences in water content, and stored at –80ºC until analysis. The water content was generally found to be around 80% for all three cherry types.

2.2. Spectrophotometric assays

Extracts for spectrophotometric assays for total antioxidant activities, total phenolics, and total flavonoids were prepared as described previously [13].

Total antioxidant activities were evaluated by the ABTS (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) method [47], the FRAP (Ferric Reducing Antioxidant Power) method [10], and the CUPRAC (Copper Reducing Antioxidant Capacity) method [2, 3]. In all assays, Trolox was used as a reference compound and results were expressed in terms of mM Trolox Equivalent Antioxidant Capacity (TEAC) per 100 g DW.

The total phenolic content was estimated using the Folin-Ciocalteu reagent [62], using 100 μl of extract, 900 μl pure water and 5 ml reagent. For the preparation of a standard curve, 0.10–0.50 mg/ml gallic acid was used and data were expressed in mg gallic acid equivalents (GAE) per 100 g dry weight (DW).

Total flavonoid content was determined according to Dewanto et al. [21]. Absorbance of the samples was measured at 510 nm against a reagent blank. Catechin at concentrations of 0.01–0.25 mg/ml was used to create a calibration curve and data were expressed in mg catechin equivalents ((+) CE) per 100 g DW.

2.3. Anthocyanin analysis

For the analysis of anthocyanin content, 25 (± 0.05) mg freeze-dried sample was extracted with 2.0 ml 75% methanol in ultrapure water following the procedure described before [11]. Sample extracts were analyzed by HPLC-PDA as described before [39]. Briefly, compounds were separated using a Luna C18 column (Phenomenex, USA) in a Waters HPLC system (W600), employing a gradient of 0.1% formic acid and acetonitrile. Eluting compounds were detected by PDA (Waters 996) detector at 512 nm for anthocyanins.

2.4. Procyanidin analysis

For analysis of procyanidins, terminal units and extension units should be distinguished. Under acidic conditions, procyanidins depolymerize, which results in the release of terminal subunits as flavan-3-ol monomers, while extension subunits are released as electrophilic flavan-3-ol intermediates. The electrophilic intermediates can be trapped by a nucleophilic reagent (in this case phloroglucinol), to generate analyzable adducts. Thus, the concentration of terminal subunits and extension units can be determined, and the degree of polymerization can be determined. In this study, procyanidins were evaluated using the phloroglucinol hydrolysis method with slight modifications [35]. To 50 (± 0.05) mg freeze-dried material, 0.8 ml of solution A (10 g/l Vitamin C + 50 g/l phloroglucinol), and 0.4 ml of solution B (0.3 M HCl diluted in methanol) were added. The phloroglucinol-treated samples were then incubated at 50°C for 30 min for hydrolysis, shaking every 10 min. Lastly, 1.2 ml of 200 mM Na Acetate was added and the samples were filtered and analyzed by HPLC. For HPLC analysis, the same set-up was used as for the anthocyanins, using a Waters 2475 Fluorescence Detector with excitation at 275 nm and emission at 310 nm for the detection of (+) catechin and (–) epicatechin. To determine (+) catechin and (–) epicatechin monomers in the original samples, another 50 (± 0.05) mg of freeze-dried material was weighed, and 2.4 mL 75% methanol was added for extraction. Free flavan-3-ol monomers ((+) catechin and (–) epicatechin) were detected in the unhydrolyzed sample, while the end-positioned flavan-3-ols plus the originally free flavan-3-ols were observed as flavan-3-ols in the phloroglucinol-treated hydrolyzed sample. The non-end positioned flavan-3-ols of procyanidins were observed as phloroglucinol conjugates in the hydrolyzed sample. The Degree of Polymerization (DP) was calculated by the following equation: DP = (end flavan-3-ols plus non-end flavan-3-ols)/(end flavan-3-ols).

2.5. Statistical analysis

All analyses were performed with three technical replicates. For Cornelian cherry and Laurel cherry, two samples were individually purchased (biological replicates) and for the Sour cherry three samples (biological replicates) were available. $N = 6$ (2 biological replicates × 3 technical replicates) samples for Cornelian cherry and Laurel cherry samples, $N = 9$ (3 biological replicates × 3 technical replicates) for Sour cherry samples were independently analyzed and the data from all analyses were taken for statistical analyses. The calculated standard deviation (SD) of

the biological replicates was not different from the SD of the technical replicates. Data were subjected to statistical analysis using SPSS software (version 11.5, SPSS Inc.) for the Analysis of Variance (ANOVA). Duncan's New Multiple Range Test was used to analyze differences between treatments.

3. Results

3.1. Spectrophotometric methods

The total antioxidant activities of the cherry samples were determined by three different methods (Table 1). All three methods indicated that the Cornelian cherries had three times higher total antioxidant capacity than Sour cherries, while Laurel cherries had an intermediate antioxidant capacity.

The total phenolic content of the different cherries followed the same trend as observed for the antioxidant capacity, but the differences were less pronounced (Table 2). The Cornelian cherry samples showed a total phenolic content which was roughly 50% higher than both the Laurel cherry and Sour cherry samples.

In contrast, the total flavonoid content values showed a different trend than the other spectrophotometric methods (Table 2). Here, the Laurel cherry showed an almost two-fold higher value, relative to both the Cornelian cherry and the Sour cherry.

3.2. Anthocyanins

The absorbance spectra of anthocyanins detected in the cherries analyzed indicated that the compounds mainly consisted of glycosylated forms of cyanidin and, in case of Laurel cherry, also of pelargonidin derivatives (Fig. 1). The dominant compounds were cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside, as was verified using authentic standards. The total quantity of anthocyanins did not differ greatly between the different cherry types (Table 3).

Table 1
Antioxidant capacities of cherry samples[a]

mmole TEAC/100 g DW	ABTS	CUPRAC	FRAP
Sour cherry	17.8 ± 1.7 c	29.7 ± 1.2 c	7.6 ± 0.3 c
Laurel cherry	28.4 ± 1.4 b	46.9 ± 1.9 b	13.3 ± 0.9 b
Cornelian cherry	50.8 ± 2.0 a	76.3 ± 3.6 a	22.3 ± 0.9 a

[a]The values having different letters in columns are significantly different according to Duncan's New Multiple Range Test, $N = 6$ for Laurel cherry and Cornelian cherry samples, and $N = 9$ for Sour cherry samples ($P < 0.05$).

Table 2
Total flavonoid and total phenolic content of the cherry samples

	Total flavonoids (mg (+)CE/100 g DW)	Total phenolics (mg GAE/100 g DW)
Sour cherry	420.5 ± 21.1 b	3371.1 ± 134.8 b
Laurel cherry	952.5 ± 47.6 a	3129.2 ± 125.0 b
Cornelian cherry	477.3 ± 22.9 b	4918.8 ± 195.7 a

The values having different letters in columns are significantly different according to Duncan's New Multiple Range Test, $N = 6$ for Laurel cherry and Cornelian cherry samples, and $N = 9$ for Sour cherry samples ($P < 0.05$).

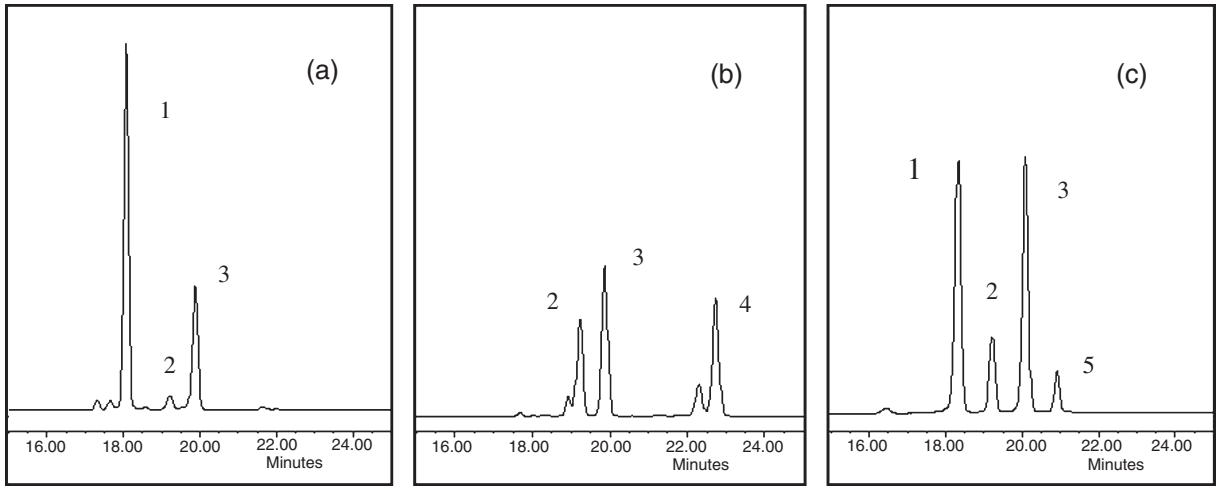

Fig. 1. HPLC profiles, recorded at 512 nm, of aqueous-methanol extracts from (a) Sour cherry, (b) Laurel cherry, (c) Cornelian cherry. Peaks indicated correspond to: (1) cyanidin- glucoside-rutinoside, (2) cyanidin-3-glucoside, (3) cyanidin-3-rutinoside, (4) pelargonidin-3-glucoside, (5) a cyanidin derivative.

Table 3

Content of main anthocyanins in three Turkish cherry types. Levels are in mg/100 g DW (means ± SD, $N = 6$ for Laurel cherry and Cornelian cherry samples, and $N = 9$ for Sour cherry samples), using cyanidin glucoside (for the cyanidin derivatives) or pelargonidin glucoside as standards. nd: not detectable

	Cyanidin-glucoside-rutinoside	Cyanidin-3-glucoside	Cyanidin-3-rutinoside	Pelargonidin-3-glucoside	Cyanidin derivative
Figure 1	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Sour cherry	16.3 ± 2.0	0.7 ± 0.1	6.5 ± 1.4	nd	nd
Laurel cherry	nd	4.6 ± 1.1	6.6 ± 1.2	11.0 ± 2.1	nd
Cornelian cherry	11.8 ± 1.5	4.4 ± 1.1	11.7 ± 1.9	nd	1.9 ± 0.3

3.3. Procyanidins

The qualitative and quantitative procyanidin content of the different cherries varied significantly (Table 4). While in Laurel cherries the total flavan-3-ol content (as a sum of (+) catechin and (–) epicatechin concentrations) was 1% of their dry weight, in Sour cherries it was 0.3% and in Cornelian cherries only 0.04%. While (–) epicatechin units dominated the procyanidin polymers in all samples, the (+) catechin contribution differed strongly. The Laurel cherry procyanidins consisted of 0.2% of (+) catechin, while this was 5% in Sour cherries and 10% in Cornelian cherries. Remarkably, in Sour cherries the mean degree of polymerization was much lower (10–15-fold) than in the other two fruit types. Apparently, short-chain procyanidins are dominant in Sour cherry fruit.

4. Discussion

Cherry fruits are well known for being rich in phenolic compounds and in particular, for their high levels of procyanidins [9, 24]. In this study we observed a large qualitative and quantitative variation in the procyanidin content of the three types of cherry. Laurel cherries and Sour cherries were found to have a total flavan-3-ol content of 1089 mg/100 g DW and 328 mg/100 g DW, respectively. This corresponds to about 218 mg/100 g FW and 66 mg/100 g FW, respectively. These levels are in the range of other well-known rich sources of procyanidins, such as grape seeds

Table 4
Flavan-3-ol contents, in mg per 100 g DW, and mean degree of procyanidins polymerization in the cherry samples[a]

	Total (+) catechin	Free (+) catechin	Total (–) epicatechin	Free (–) epicatechin	Mean degree of polymerization[b]
Sour cherry	15.4 ± 0.8 a	0.14 ± 0.01 a	312.5 ± 15.6 b	0.10 ± 0.02 ab	3.9 ± 0.2 c
Cornelian cherry	3.5 ± 0.1 b	0.08 ± 0.01 b	35.5 ± 4.5 c	0.01 ± 0.005 b	62.9 ± 3.1 a
Laurel cherry	2.0 ± 0.1 b	0.10 ± 0.01 ab	1087.7 ± 53.5 a	0.17 ± 0.04 a	45.2 ± 2.1 b

[a]Values having different letters in columns are significantly different according to Duncan's New Multiple Range Test, $N = 6$ for Laurel cherry and Cornelian cherry samples, and $N = 9$ for Sour cherry samples ($P < 0.05$). [b]Total bound (epi)catechin units/end units.

(17–250 mg/100 g) [27] and dark chocolate (31–37 mg/100 g) [46], and also correspond to the content of total (+) catechins found in sweet cherries (*Prunus dulcis*; 117 mg/kg FW) [7].

Antioxidant activity was measured by three different assays. The different standards used to report the antioxidant capacity as well as the wide variations in analytical techniques make comparisons between various studies harder and also raise the question whether apparently conflicting results are associated with non-standardized assay techniques [14]. The differences between test methods might be as a result of the variations in the principle of the antioxidant assay, the radical that is generated, the end-point detection, and the allocated reaction time [1, 4]. Evaluation of antioxidant capacity of fruits, vegetables, and other plant products cannot be performed accurately by any single method due to the complex nature of phytochemicals present [16]. So, it is highly recommended to apply several test procedures for a full evaluation of antioxidant capacity [1, 4, 14]. In case of the cherry fruits, the three methods used gave consistent results, in that the Cornelian cherry has a very high antioxidant capacity, relative to the other cherries (Table 1), and also the highest level of phenolic compounds (Table 2). In contrast, Cornelian cherries had a much lower content of procyanidins (39 mg/100 mg DW) then the other two types, and also a lower level of total flavonoids. Therefore phenolic compounds other than flavonoids are probably more prominent in Cornelian cherry fruit.

Contents of flavonoid, anthocyanins and other antioxidants may be influenced by differences in the variety, ripeness of the fruits and the growing conditions. It was reported that sweet cherry cultivars may show pronounced differences in the concentrations of individual phenolic compounds [24]. Likewise, fruit ripeness and seasonal variations may strongly affect concentrations of anthocyanins in particular [7, 24]. It is unclear if such variations also account for procyanidin polymers. According to Kennedy et al. [36], the relative proportion of procyanidin extension units in grape seeds did not vary with maturity, and fruit ripening did not alter the mean degree of polymerization of extracted procyanidins, at least when analyzed intact by HPLC, but decreases by thiolytic degradation. Further research in cherry fruit samples collected during several years will have to be performed to monitor changes in procyanidins concentration and polymer length.

In vitro assays point to strong protective properties of procyanidins regarding oxidative damage, microbial infection, prevention of colon cancer, and prevention of cardiovascular disease [6, 42, 50, 55]. However, the degree of polymerization of procyanidins may be highly influential and determine the relevance of these activities to their therapeutic or profilactic properties [25, 29, 59]. Fruits characterized by a high content of procyanidins with relative high degree of polymerization, i.e. long-chain polymers, such as Laurel cherry, may have medicinal applications in the treatment of ulcers and intestinal infections [40]. However, the absorption into the blood system and bioavailability to body tissues both depend strongly on the molecular size of the procyanidin polymers [26]. In the gastro-intestinal tract, procyanidin monomers, dimers, and trimers are absorbed into the blood system to a much larger extent than larger oligomers and polymers [37, 43]. While the Cornelian cherries and Laurel cherries tested in this study show high degrees of polymerization, the Sour cherry procyanidins, which were present at a significant concentration (0.3 g/100 g DW), had an average polymer length slightly lower than 4, indicating relative high levels of these better absorbable short-chain procyanidin species. In plant materials, the lower molecular weight procyanidins are usually present at relatively low concentrations, as compared to the larger-sized oligomers and polymers [23, 53]. For instance, the degree of polymerization in apple skin is 12.5 and 11.3 for the apple pulp [28]. In grape seed it is about 30 [63, 64], in the Laurel cherries about 45 and in the Cornelian cherries about 63 (Table 4). Therefore, this study has identified Sour cherries as being an exceptional source of short-chain procyanidins and this finding deserves further attention regarding health beneficial properties.

The DP value is one of the most important properties in fruits and vegetables since antioxidant activity has been shown to depend on the DP values [29, 32]. The results of our study indicated that cornelian cherry sample which had the highest DP value also showed highest antioxidant activities according to ABTS, CUPRAC, and FRAP methods (Table 1). Antioxidant activities of grape seed procyanidins are positively related to their degree of polymerization: from high to low in the order of polymer, oligomer, and monomer [49, 63]. According to Arteel and Sies [6], long-chain procyanidins are better scavengers than short-chain procyanidins. Similarly, the antioxidant activity of procyanidin oligomers was found to increase significantly with the degree of polymerization in chocolate [18]. However, according to Ndhlala et al. [48], fruits with high degree of polymerization should present lower antioxidant activity.

It has been reported that flavanols and procyanidin oligomers have a number of antioxidative properties and can provide protection against the oxidation of human low-density lipoprotein [41, 52]. They have been reported to exhibit several health beneficial effects by acting as antioxidant, anticarcinogen, cardiopreventive, antimicrobial, anti-viral, and neuro-protective agents [5]. Inhibitory effects of flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis were also shown by Zhu et al. [72]. Even though it was reported that the antioxidant potential of procyanidins is affected in part by oligomer chain length [6, 41, 44], the exact effect and importance of chain length and configuration of procyanidins remain unclear.

The degree of polymerization is also important for the absorption and bioavailability characteristics of procyanidin polymers [26]. It has been reported that procyanidin monomers, dimers, and trimers can be absorbed, but higher oligomers and polymers are poorly absorbed [37]. Recent studies suggest that only the low molecular weight oligomers (polymerization degree up to 3) might be absorbed in the gastrointestinal tract [43]. Considering that the polymeric procyanidins are not depolymerized in the stomach, food material containing procyanidins with a low DP value may be of particular interest [25]. Sour cherry samples in our study, which was found to have a DP value around 3 is therefore interesting for its more bioavailable procyanidin oligomers. And, taking into account the information that the lower molecular weight procyanidins are usually present in plant tissue in relatively low concentrations compared to that of larger oligomers and polymers [23, 53], sour cherry samples deserve particular attention.

Short-chain procyanidins may occur in several coupling configurations (A and B types), each of which may have different bio-activities [5]. Further research should determine which procyanidin type is predominantly present in Sour cherry fruits, and it should be tested if these compounds are, at least partly, responsible for the bio-activities described for Sour cherry fruit extracts [51, 61]. Such studies will allow a better understanding of the health effects observed, such as reported in studies with diabetic women [8], and could lead to an improved awareness of the importance of these fruits for consumer health.

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