

# Bioactive compounds of sweet and sour cherry stems obtained by subcritical water extraction

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## Abstract

**BACKGROUND:** Sweet cherries (*Prunus avium* L.) and sour cherries (*Prunus cerasus* L.) contain substantial amounts of anthocyanins and polyphenolics, and their stems have been used in traditional medicine for centuries. However, comparative data on the composition of sweet and sour cherry stems using the same analytical methodologies are limited. Moreover, there is no data in terms of biological activity of subcritical water extracts of cherry stems.

**RESULTS AND DISCUSSION:** Results obtained by using several assays that focused on different mechanisms showed that subcritical water extracts of sour and sweet cherry stems were powerful antiradicals and antioxidants. Anti-proliferative properties measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test showed prominent cytotoxicity against different tumor cell lines. Inhibitory concentrations obtained were in the range 8.38–11.40  $\mu\text{g mL}^{-1}$ . GC-MS (gas chromatography mass spectrometry) analysis showed the presence of alcohols, fatty acids, organic acids and other organic compounds. However, the chemical compositions of the two samples observed were similar, indicating similar chemical composition of sour and sweet cherry stems.

**CONCLUSION:** Subcritical water extracts of cherry stem demonstrated excellent biological activity and the potential to be used for pharmaceuticals or supplements due to confirmed high antioxidant, antiradical and antitumor activity.

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**Keywords:** cherry stems; subcritical water; bioactivity; GC MS

## INTRODUCTION

Cherry fruits are cultivated worldwide and mostly two cultivars are used for human consumption and processing. Both sweet (*Prunus avium*) and sour (*Prunus cerasus*) cherries enjoy great popularity worldwide due to rich refreshing flavor and recognized health benefits.<sup>1–3</sup> Sweet and sour cherries differ in chemical composition, properties and use. Sweet cherries are mainly consumed as fresh fruit, whereas sour cherries are mostly used in processed forms such as frozen or canned products, and juices. In comparison with sour cherries ( $\sim 8 \text{ g } 100 \text{ g}^{-1}$ ) sweet cherries have a higher content of simple sugars ( $\sim 13 \text{ g } 100 \text{ g}^{-1}$ ), however, sour cherries are characterized by higher content of total phenolics, vitamin A, beta-carotene<sup>4</sup> and anthocyanins,<sup>5</sup> accounting for their usual darker colour. In sweet cherries the main anthocyanin is cyanidin-3-O-rutinoside, whereas in sour cherries cyanidin-3-glucosylrutinoside is dominant.<sup>5</sup>

In cherry processing industries great quantities of by-products are generated, including cherry pits, pomace and juice. Valorisation of cherry by-products is not common despite their great value. Cherry pits are rich in oils, minerals and volatiles, however they also contain toxic amygdaline, similarly to kernels of other fruits, such as apricot, apple and peach.<sup>6</sup> One of the proposed valorisation routes of cherry pits, similarly to apricot kernels, is the extraction of oils and volatiles. Cherry pits are also industrially packed into pellets and used as a fuel, and smaller quantities are used for hot therapeutic pillows and crafting.

Valorisation routes of other cherry by-products are mostly focused on the extraction of polyphenols from cherry pomace. Tumbas-Šaponjac<sup>7</sup> encapsulated extracted bioactive compounds from cherry pomace with whey and soy proteins, incorporating them further into food products. There are no reported industrial uses of cherry stems or detailed chemical and biological characterisation, despite their use in traditional medicine. On harvesting, cherry stems can be separated for further use and valorisation, being of great industrial potential.

Cherry stem tea has been used in traditional medicine as a diuretic and for the treatment of urinary tract infections. The infusion has been used also for the prevention of cardiovascular

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diseases and for lowering blood pressure and cholesterol. In traditional medicine the stems of sweet cherries are mostly used, probably owing to its wider availability. Bastos *et al.*<sup>8</sup> studied the chemical characterisation of extracts, infusions and decoctions of sweet cherry stems, describing their high biological potential. Those extracts were obtained by conventional extraction with a methanol/water mixture. Analogous considerations related to sour cherry stems and extracts obtained by modern extraction techniques are, according to our knowledge, unavailable in the scientific literature. Serra *et al.*<sup>2,3</sup> used supercritical carbon-dioxide for the extraction, but focused their work on antiproliferative effect against colon cancer of fruit extracts. In our work we compared biological activity and gas chromatography mass spectrometry (GC-MS) chemical profiles of sweet and sour cherry stems. This research is also important because chemical and bioactivity characterisation of fruit wastes is particularly attractive, taking into consideration global trends on waste valorisation. In this respect, discovery of new and cheap sources of bioactive and added-value compounds is important in emerging waste management strategies and development of circular business models.

Owing to specificity and superiority of subcritical water, cherry stem extracts were obtained by subcritical water extraction (SWE). Literature reports point out higher biological activity of plant extracts obtained by subcritical water when compared with other extraction techniques. For example, Cvetanović *et al.*<sup>9</sup> compared subcritical with ultrasound and microwave extraction of chamomile, concluding that the subcritical extracts exhibited much higher antiradical and antioxidant capacity. Similarly, Pongnaravane *et al.*<sup>10</sup> extracted anthraquinones from *Morinda citrifolia* and found higher antioxidant activity of subcritical water extracts in comparison with ethanolic extracts obtained in stirred vessel and sonicated extracts. Significant improvement was also observed when comparing polyphenolic yields from coriander seeds obtained with subcritical and microwave-assisted extraction.<sup>11,12</sup> Moreover, SWE has shown to be a good alternative for the extraction of essential oils. Saim *et al.*<sup>13</sup> showed that essential oil of coriander obtained by subcritical water was comparable with that obtained by hydrodistillation. Finally, subcritical water is safe and cheap, thus allowing extracts compatible with food, pharmaceutical and cosmetic applications.

Besides comparing chemical profiles, sweet and sour cherry stem extracts were compared with respect to their radical-scavenging, antioxidant and anti-proliferative effects. Radical-scavenging and antioxidant capacity of the observed extracts was evaluated using five different assays enabling better insight into their activities. Anti-proliferative properties of cherry stem extracts were tested against three histologically different cell lines, more specifically, human rhabdomyosarcoma cells (RD), cell line derived from human cervix carcinoma (Hep2c) and cell line derived from murine fibroblast (L2OB).

## MATERIAL AND METHODS

### Chemicals and reagents

Trichloroacetic acid (TCA), linoleic acid 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH), thiobarbituric acid (TBA), 2-deoxyribose, hydrogen peroxide solution, ascorbic acid, phosphate buffer, derivatization agent BSTFA (N,O-bis[trimethylsilyl]trifluoroacetamide), ammonium sulphate and organic solvents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ferric chloride was obtained from Zorka (Šabac, Serbia). Cirsimarin, resazurin, amaricin, nystatin, sabourand dextrose, Tween 80,

Tween-20 and *cis*-diamminedichloroplatinum (*cis*-DDP) were purchased from Tedia Company (USA). Etylenediaminetetraacetic acid (EDTA) was purchased from Centrohem (Stara Pazova, Serbia). Potassium dihydrogen phosphate was obtained from Kemika (Zagreb, Croatia). All other chemicals and reagents were of analytical reagent grade.

### Samples

Sweet (*Prunus avium* L.) and sour (*Prunus cerasus* L.) cherries were collected in the northern region of Serbia in June 2015. Separated stems were stacked in a crate with perforated bottom and air dried at ambient temperature and in the dark to a moisture content of 12%. Dry stems were packed in canvas bags and stored in the dark until use.

### Extraction

Stems of sweet and sour cherry were extracted at 150°C for 30 min at a pressure of 20 bar in a batch-type custom-made subcritical extractor/reactor as described previously.<sup>14</sup> Total capacity of the extraction vessel, made of stainless steel, was 1.7 L. Pressurisation was done with 99.9999% nitrogen (Messer) through a built-in valve. The batch type extractor was equipped with a vibrational platform that allowed convective mass transfer during extraction and variable vibration frequencies. In all extractions sample-to-solvent ratio was maintained at 1:90. The frequency of the platform housing the extraction vessel was 3 Hz, assuring effective agitation and mass transfer. After extraction, cooling of the extraction vessel was done by placing it in a flow-through water bath at the temperature of 20±2°C. After cooling, depressurization was done by valve opening. Obtained extracts were separated by filtration through Whatman qualitative filter paper, grade 1, and stored in a refrigerator at 4 °C for further analysis.

### Antioxidant activity

#### DPPH test

The spectrophotometric method was used to determine antiradical activity against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.<sup>15</sup> The extracts were mixed with methanol (96%) and 90 µmol L<sup>-1</sup> of DPPH to give final concentrations of 0.01, 0.02, 0.05, 0.1 and 0.2 mg mL<sup>-1</sup>. After 60 min at room temperature, the purple color of the test mixtures turned yellow, due to pairing of the odd electron of the DPPH radical with hydrogen to form the reduced DPPH-H form. The resulting discoloration, which is stoichiometric, is proportional to the number of captured electrons. The odd electron in the DPPH radical shows maximum absorption at 515 nm. The activity was expressed as inhibitory concentration at 50% (IC<sub>50</sub>), which is the concentration of the test solution to achieve 50% of the radical scavenging capacity.

#### Determination of the hydroxyl radical scavenging activity

The ability to inhibit non-site-specific hydroxyl radical-mediated peroxidation was carried out according to the method described in the literature.<sup>16</sup> Extracts were mixed with 500 µL of 5.6 mmol L<sup>-1</sup> 2-deoxy-D-ribose, 100 µL of premixed 100 µmol L<sup>-1</sup> FeCl<sub>3</sub> and 104 mmol L<sup>-1</sup> EDTA (1:1 v/v) solution, 100 µL of 1.0 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, and 100 µL of 1.0 mmol L<sup>-1</sup> aqueous ascorbic acid. The reaction mixture was incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% trichloroacetic acid (TCA) and 1 mL of 1.0% thiobarbituric acid (TBA) were added to each tube. The samples were mixed in a vortex mixer and heated in a water bath at 50 °C for 30 min. The degree

of 2-deoxyribose oxidation was calculated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance measured for the control (Ac) and of the sample (As), where the controls contained all the reaction reagents except the extract or a positive control substance. The values were presented as means of triplicate analysis.

#### *Determination of lipid peroxidation*

Determination of the inhibitory activity against lipid peroxidation was carried out according to the thiocyanate method.<sup>17</sup> Extracts were diluted in water to a final concentration of 10 mg mL<sup>-1</sup>. This solution was further used for the preparation of serial dilutions, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mmol L<sup>-1</sup>, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween-20 in 50 mL of 40 mmol L<sup>-1</sup> phosphate buffer. The mixture was incubated at 37 °C for 72 h. After that, 0.1 mL of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL of FeCl<sub>2</sub> (20 mmol L<sup>-1</sup>), and 0.1 mL of ammonium thiocyanate (30%). The mixture was stirred for 3 min and absorbance was measured at 500 nm. All tests were performed in triplicate, and results were expressed as IC<sub>50</sub> values.

#### *Ferric reduction activity power assay*

Ferric reduction activity power (FRAP) determinations were obtained by adding 20 µL of sample to 180 µL of FRAP reagent (Fe<sup>3+</sup> - 2,4,6-Tri(2-pyridyl)-s-triazine) and the absorbance was measured at 593 nm.<sup>18</sup> A calibration curve was prepared with ascorbic acid (AA) and results were expressed as ascorbic acid equivalents (AAE).

#### *ORAC assay*

Briefly, the ORAC assay was carried out by mixing 25 µL of sample, standards or phosphate buffer saline (PBS) (for blanks) and 150 µL of fluorescein solution (1.5 × 10<sup>-8</sup> g L<sup>-1</sup>), and incubated for 30 min at 37 °C.<sup>19</sup> Reactions were initiated by the addition of 25 µL of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution (40 g L<sup>-1</sup>), and fluorescence was measured for 100 min at 37 °C, with a sensitivity setting of 60, a 485 nm excitation filter and a 528 nm emission filter. A calibration curve was prepared with Trolox and results were expressed as Trolox equivalents (TE).

#### **Anti-proliferative activity**

The influence of the extracts on the growth of malignant transformed cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. The following cell lines were used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma - HeLa derivative) and L2OB (cell line derived from murine fibroblast). Cells were seeded (2 × 10<sup>5</sup> cells mL<sup>-1</sup>; 100 µL per well) in 96-well cell culture plates in nutrient medium (minimum essential medium (MEM) Eagle supplemented with 5% of Hep2c, RD and L2OB) and grown at 37 °C in humidified atmosphere for 24 h. Then, corresponding extract (stock solution: 5 mg of extract dissolved in 1 mL of absolute ethanol) and control (absolute ethanol) diluted with nutrient medium to desired concentrations were added (100 µL per well) and cells were incubated at 37 °C in humidified atmosphere for 48 h. Pure nutrient medium (100 µL) represented positive control for each cell line. After the incubation period, supernatants were discarded. Then

MTT (dissolved in D-MEM (Dulbecco's modification of Eagle's medium) at a concentration of 500 µg mL<sup>-1</sup>) was added to each well (100 µL per well). Immediately after, all wells were incubated at 37 °C in humidified atmosphere for 4 h. Reactions were ceased by adding 100 µL of sodium dodecyl sulfate-SDS (10% in 10 mmol L<sup>-1</sup> HCl). After overnight incubation at 37 °C, absorbance was measured at 580 nm using a spectrophotometer (Ascent 6-384 [Suomi], MTX Lab Systems Inc., Vienna, VA 22182, USA). The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against A580. Corresponding cells (grown in flasks), after cell count by haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (*ut supra*). The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader at 580 nm. Absorbance (A) at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The blank absorbance was always subtracted from the absorbance of the corresponding sample with target cells. IC<sub>50</sub> concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements were expressed as the percentage of positive control growth taking the cis-diamminedichloroplatinum (cis-DDP) determined in positive control wells as the 100% growth.<sup>20-22</sup> All experiments were done in triplicate.

#### **GC-MS analysis**

Subcritical water extracts (2 mL) were evaporated at 80 °C under the flow of nitrogen. Dry residue was dissolved in 200 µL of acetonitrile and 200 µL of derivatization agent BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) were added. Vials were shaken for 30 s and placed in the autosampler.

Prepared extracts were analysed by GC-MS (Agilent 7890 with mass spectrometer HP 5973C, Agilent Technologies, Palo Alto, CA, USA). System control and data acquisition were achieved by ChemStation Software. The derivatives (1 µL) were injected and separated on a 30 m × 0.25 mm i.d. 0.25 mm film thickness DB5 (methyl 5% phenyl polysiloxane) gas chromatographic column. The injection port operated in splitless mode with an injection temperature of 290 °C. Helium (99.9999%) was used as a carrier gas at the flow rate of 1.0 mL min<sup>-1</sup>. The MS operated in electron impact (EI) mode (70 eV).

The gas chromatographic temperature programme was as follows: initial temperature 70 °C, held 1 min, increased to 290 °C at a rate of 10 °C min<sup>-1</sup> with a total run of 30 min and solvent delay 5 min. The EI source, the quadrupole mass analyser, and the interface temperature were maintained at 230, 150 and 280 °C, respectively. The MS was equipped with the mass spectra library NIST MS search 2.0. It operated in total ion current mode, scanning from m/z 50 to 400 for identification purposes.

#### **Statistical analysis**

All analyses were run in triplicate and were expressed as mean values ± 2 standard deviations (SD). Mean values were considered significantly different at *P* < 0.05 confidence level after the performance of the one-way ANOVA statistical analysis followed by Tukey test.

**Table 1.** Antiradical activity of subcritical water extracts of sweet and sour cherry stems

Sample	IC <sub>50</sub> values (µg mL <sup>-1</sup> )	
	DPPH test	OH radicals
Sweet cherry stems	23.7 ± 0.3 *	471.4 ± 4
Sour cherry stems	25.7 ± 2.5	553.5 ± 40

\*Mean values of triplicates ±2 SD.

## RESULTS AND DISCUSSION

### Antioxidant activity

In the present study, antiradical activity of studied extracts was determined by measuring their ability to neutralize DPPH and OH radicals. Results obtained are presented in Table 1.

The investigated extracts showed dose-dependent radical scavenging activities (data not shown). Based on these data, IC<sub>50</sub> values (the concentration of extracts necessary to decrease the initial concentration of DPPH or hydroxyl radicals by 50%) were defined. As can be seen, inhibitory concentrations for both samples were very close for both radicals. The differences between antiradical activities of sweet and sour cherries were statistically insignificant at  $P < 0.05$  ( $P = 0.2291$ ). Close IC<sub>50</sub> values may indicate similar chemical composition of examined extracts and it can be assumed that concentrations of compounds participating in DPPH-scavenging mechanism were similar. Antiradical activity of sweet cherry petioles by using DPPH assay was also described in the study of Prvulović *et al.*<sup>23</sup> The authors concluded that antiradical activity of petioles extracts was correlated with polyphenolics and flavonoids contents, but could not have been related to tannins content. Acetone extracts obtained by sonication neutralized from 29.88 to 86.94% of DPPH radicals. Bastos *et al.*<sup>8</sup> studied DPPH-radical scavenging properties of different preparations obtained from cherry fruit and stem. In this study hydromethanolic stems extracts showed to be more potent in neutralising DPPH radicals in comparison with its infusions and decoctions. Moreover, stems extracts with IC<sub>50</sub> values of 0.36 mg mL<sup>-1</sup> expressed better anti-radical properties than fruit extracts (IC<sub>50</sub> = 0.99 mg mL<sup>-1</sup>). In our study extracts obtained by subcritical water showed better activity in comparison with the study of Bastos *et al.*<sup>8</sup> In the work of Yu *et al.*<sup>24</sup> subcritical water extracts of XiLan olive (*Elaeocarpus serratus* L.) pomace inhibited DPPH radicals for 4% in concentration of 94.03 mg mL<sup>-1</sup> indicating much lower activity towards these radicals in comparison with the studied cherry stem extracts. Lower activity was also seen for subcritical water extracts of onion peel<sup>25</sup> and fig pulp.<sup>26</sup> Onion peel extracts obtained at 110°C inhibited 64.72% of DPPH radicals at a concentration of 0.2 mg mL<sup>-1</sup>, whereas extracts obtained at 165°C exhibited even lower activity, inhibiting only 11.45% of radicals at the same concentration. Subcritical water extracts of fig pulp inhibited ~65% of DPPH radicals in much higher concentration (1 mg mL<sup>-1</sup>) than both cherry samples.

In the case of neutralization of OH radical a significant difference was noticed ( $P = 0.0248$ ). Extracts prepared from sweet cherry stems at a concentration of 0.4714 mg mL<sup>-1</sup> were able to neutralize 50% of hydroxyl radicals while the same effects with sour cherry stems extracts were achieved at a concentration of 0.5535 mg mL<sup>-1</sup>. Results obtained indicated quite good antiradical activity of the extracts. Hydroxyl radical scavenging ability of cherry stem extracts was slightly lower in comparison with subcritical water extracts of *Inonotus Obliquus*,<sup>27</sup> a parasitic fungus used

in traditional medicine for anticancer preparation. IC<sub>50</sub> values for cherry stem extracts were approximately two-fold higher in comparison with fungus extracts (0.13 mg mL<sup>-1</sup>). There is no data in the literature about hydroxyl radicals scavenging activity of cherry stems, thus this study provides first evidence. However, the ability of cherry leaves to neutralize OH radicals has been investigated by Kutlu *et al.*<sup>28</sup> Methanolic extracts of cherry leaves (0.2 mg mL<sup>-1</sup>) neutralized 43.99% of hydroxyl radicals. In comparison with these data cherry stems extracts studied in this work indicated slightly lower OH-scavenging activity.

An additional three different antioxidant assays (inhibition of lipid peroxidation, FRAP and ORAC test) were applied to assess activity of the examined extracts. When observing lipid peroxidation the results obtained showed that all tested extracts demonstrated the activity (Table 2). The difference between the extracts was at the limit to be estimated as statistically significant ( $P = 0.0499$ ), and the overall conclusion is that the extracts of sweet and sour cherry stems had very similar potential to inhibit lipid peroxidation. Inhibitory concentrations of subcritical water extracts of sweet and sour cherry stems were 3.27 mg mL<sup>-1</sup> and 4.01 mg mL<sup>-1</sup>, respectively. In comparison with literature data, results obtained demonstrated lower activity in comparison with the study conducted by Bastos *et al.*<sup>8</sup> For hydromethanolic extracts, infusions and decoctions of sweet cherry stems, the authors determined IC<sub>50</sub> values of 0.07, 0.24 and 0.13 mg mL<sup>-1</sup>, respectively, indicating much better activity in lipid peroxidation inhibition measured by TBARS assay. This can be explained by possible decomposition of components playing a key role in the prevention of lipid peroxidation, taking into consideration the high reactivity of subcritical water. On the other hand, geographical and climate variations may have been responsible for different chemical composition.

Antioxidant activity of sweet and sour cherry extracts assayed by FRAP and ORAC tests demonstrated almost the same activity (Table 2). The differences between the activities of sweet and sour cherry stem extracts in both FRAP ( $P = 0.3476$ ) and ORAC ( $P = 0.7738$ ) assays were statistically insignificant ( $P < 0.05$ ).

According to the research of Bastos *et al.*<sup>8</sup> cherry stem extracts, but also infusions and decoctions, were more potent in respect of antioxidant activity than extracts prepared from the fruit. Antioxidant and antiradical activities of cherry fruit are well studied,<sup>2,29–31</sup> however, there is a lack of data for the activity of its stems. According to previous research it could be stated that antioxidant activity of sour cherry fruit was comparable with the activity of some other berries such as strawberry, but higher than that of apple or kiwi fruit.<sup>32,33</sup> Serra *et al.*<sup>31</sup> investigated antioxidant ability of nine different cherry cultivars by four *in vitro* assays. Results obtained by ORAC, HORAC and LDL assays showed correlation between total phenolic content and antioxidant activity. The activities were also in agreement with total anthocyanin content.

When comparing the studied extracts with other herbal subcritical water extracts it should be noted that ferric reducing antioxidant power (FRAP) of cherry stem extracts studied in this work was comparable with that measured in subcritical water extracts of mandarin peel<sup>34</sup> and barley straw.<sup>35</sup> The highest FRAP activity of mandarin peel extracts was seen in samples obtained at 180°C (0.13 mmol FeSO<sub>4</sub>·7H<sub>2</sub>O/g dry extract) whereas barley straw extracts were most active when obtained at 160°C (0.60 mmol FeSO<sub>4</sub>·7H<sub>2</sub>O g<sup>-1</sup> dry extract). Cherry stem extracts, expressed in units per gram of dried extract, exhibited the activity of ~0.53 mmol FeSO<sub>4</sub>·7H<sub>2</sub>O g<sup>-1</sup> dry extract.

Cherry stem extracts analysed in this work had comparable oxygen radical absorbance capacity (ORAC) with subcritical water

**Table 2.** Antioxidant capacity of subcritical water extracts of sweet and sour cherry stems

Sample	Lipid peroxidation (IC <sub>50</sub> value, mg mL <sup>-1</sup> )	FRAP (μg AAE mL <sup>-1</sup> )	ORAC (μg TE mL <sup>-1</sup> )
Sweet cherry stems	3.27 ± 0.63 *	433.8 ± 10.8	11 ± 1.1
Sour cherry stems	4.01 ± 0.46	448.1 ± 33.7	10.8 ± 0.9

\*Mean values of triplicates ±2 SD.

**Table 3.** Anti-proliferative activity of subcritical water extracts of sweet and sour cherry stems extracts

Sample	IC <sub>50</sub> (μg mL <sup>-1</sup> )		
	Hep2c cells	RD cells	L2OBcells
Sour cherry stems	8.38 ± 0.44 *	11.40 ± 0.66	9.69 ± 0.39
Sweet cherry stems	10.65 ± 0.29	10.55 ± 0.83	8.87 ± 0.43
Cis-diamminedichloro-platinum	0.94 ± 0.55	1.40 ± 0.97	0.72 ± 0.64

\*Mean values of triplicates ±2 SD.

extracts of dried red grape skin, but slightly lower than its extracts obtained by sulfured water.<sup>36</sup> The authors explained higher oxygen radical absorbance capacity of sulfured water extracts by higher anthocyanins content in those extracts.

### Anti-proliferative activity

The cell growth inhibitory activity of subcritical water extracts of cherry stems was evaluated *in vitro* by MTT assay. Cis-diamminedichloroplatinum was applied as a standard compound and results obtained are presented in Table 3. Treatment of three cell lines with both extracts and a standard resulted in considerable dose-dependent inhibition of the cell growth. However, in the case of RD ( $P = 0.3846$ ) and L2OB ( $P = 0.1645$ ) cell lines significant difference between the extracts was not noticed. On the other hand, in the case of Hep2c cells the difference could be characterised as statistically significant ( $P = 0.0072$ ). In the case of sour cherry stems calculated IC<sub>50</sub> values for of Hep2c, RD and L2OB cells were 8.38, 11.40 and 9.69 μg mL<sup>-1</sup>, while in the case of sweet cherry stems these values were 10.65, 10.55 and 8.87 μg mL<sup>-1</sup>, respectively. Results presented demonstrated the high cytotoxic potential of the studied extracts. This claim is supported by the fact that criterion for cytotoxic activity for plant extracts is IC<sub>50</sub> < 30 μg mL<sup>-1</sup>.<sup>37</sup>

In the study reported by Bastos *et al.*<sup>8</sup> for methanolic extracts of sweet cherry stems calculated IC<sub>50</sub> values for colon carcinoma cells (HTC-15) were above 400 μg mL<sup>-1</sup>, while in the case of fruit methanolic extracts IC<sub>50</sub> value was 73.51 μg mL<sup>-1</sup>. Anti-proliferative activity of infusions and decoctions of sweet cherry stems showed relatively high IC<sub>50</sub> values (> 400 μg mL<sup>-1</sup>). In our study much better cytotoxic activity against all three tested cell lines was observed, since IC<sub>50</sub> values were around 10 μg mL<sup>-1</sup>. Such results indicate that extraction with subcritical water provides enhanced recovery of compounds with anti-proliferative activity.

Other studies reported superiority of SWE over other extraction techniques in terms of anti-proliferative activity of extracts.<sup>9,38</sup> Cvetanović *et al.*<sup>9</sup> reported better antitumor properties of chamomile extracts obtained by SWE in comparison to microwave, ultrasound and Soxhlet extraction. In this study, activity against the same cell lines as in this work was evaluated. Subcritical water extracts of

chamomile were inferior to cherry stem extracts indicating that both sour and sweet cherry stems are excellent sources of biologically active molecules. Anti-proliferative activities of subcritical water extracts of sherry stems and ginger were comparable,<sup>14</sup> whereas the extracts of ginseng leaves and stems obtained by the same extraction technique were less active.<sup>38</sup> In the study of Lee *et al.*<sup>38</sup> cytotoxic potential of extracts increased with the extraction temperature. The lowest determined IC<sub>50</sub> value was around 200 μg mL<sup>-1</sup> representing much higher inhibitory concentration than in the case of cherry stems.

This study reports for the first time the activity of cherry stem extracts against human cervix carcinoma, human rhabdomyosarcoma and murine fibroblast cells. The activity of other plant extracts against these cells has been evaluated. Mašković *et al.*<sup>39</sup> reported that ethanolic extracts of *Onosma aucheriana* inhibited the growth of all three cell lines but IC<sub>50</sub> values obtained (IC<sub>50(Hep2C)</sub> = 40.34 μg mL<sup>-1</sup>, IC<sub>50(RD)</sub> = 50.57 μg mL<sup>-1</sup>, IC<sub>50(L2OB)</sub> = 25.54 μg mL<sup>-1</sup>) were much higher than that reported in this study. Such high anti-proliferative activity of subcritical cherry stem extracts could be related to its characteristic phytochemical composition and possible neof ormation of cytotoxic products during the treatment, as well as with high extraction yields of phenolic and other anticancer compounds.

Results obtained demonstrate that both sweet and sour cherry stems extracts exhibited anticancer properties *in vitro* and demonstrate that the efficiency of the SWE technique in the recovery of cheap bioactive compounds.

### GC MS analysis

Chemical screening of subcritical water extracts of cherry stems was carried out by gas chromatography coupled to mass spectrometry. Derivatisation allowed identification of organic compounds belonging to different chemical classes, including alcohols, organic acids, sugars, fatty acids, heterocyclic compounds and others (Table 4). The presence of compounds of different polarities in aqueous extracts confirmed the versatility of subcritical water as an extraction medium and its ability to extract simultaneously different chemical classes.

Detected polyols in extracts probably resulted from hydrothermal conversion of cellulose.<sup>31</sup> Among the alcohols formed, sorbitol was the most abundant, followed by glycerol. The presence of these alcohols in extracts obtained even at moderate conditions supports the fact that pure hot pressurised water can be used for the production of added-value chemicals from plant biomass. Cellulose and other plant polysaccharides can be converted to sugars by simple treatment even without catalysts, as seen in this work.

Production of sorbitol from biomass is interesting since this alcohol sugar is widely used in food, pharmaceutical and cosmetic industry as a dietary sweetener, humectants and thickener.

Glycerol has myriad of uses in cosmetic, pharmaceutical and tobacco industries. Regarding extraction processes, subcritical water treatment of a biomass for the purposes of biofuel production requires more rigorous operational parameters and

**Table 4.** Comparison of GC MS profiles of sweet and sour cherry stem extracts

Sweet cherry		Sour cherry	
m/z	Compound	m/z	Compound
<b>Alcohols</b>		<b>Alcohols</b>	
217, 147	Arabitol	217, 147	Arabitol
		297, 147	Ethylene glycol, 3-hydroxy-4-methoxyphenyl
147, 205	Glycerol	147, 205	Glycerol
319, 205	Sorbitol	319, 205	Sorbitol
<b>Fatty acids</b>		<b>Fatty acids</b>	
31, 117	Hexadecanoic acid	313, 117	Hexadecanoic acid
		271, 147	3-Hexenoic acid, 3-methyl-5-keto
		215, 117	Nonanoic acid
		117, 341	Octadecanoic acid
<b>Organic acids</b>		<b>Organic acids</b>	
147, 188	Acetic acid	147, 188	Acetic acid
179, 105, 135	Benzoic acid	179, 105, 135	Benzoic acid
147, 233	Butanoic acid	147, 233	Butanoic acid
147, 245	2-Butenedioic acid	147, 245	2-Butenedioic acid
183, 273	Butenoic acid, 2-isopropyl-3-hydroxy		
125, 169, 95	2-Furancarboxylic acid	125, 169, 95	2-Furancarboxylic acid
147, 217	Gulonic acid	147, 217	Gulonic acid
147, 233	Malic acid	147, 233	Malic acid
117, 147	Propanoic acid	117, 147	Propanoic acid
		147, 72, 219	Propanoic acid, 3-hydroxy
240, 166	Pyrrole-2-carboxylic acid		
<b>Sugars</b>		<b>Sugars</b>	
217, 147	Arabinose	217, 147	Arabinose
217, 147	D-Fructose	217, 147	D-Fructose
		191, 147	D-Galactose
217, 129	D-Glucose	217, 129	D-Glucose
204, 147	Mannose		
103, 147	Xylulose	103, 147	Xylulose
129, 103	D-Ribose	129, 103	D-Ribose
204, 147	D-Xylose	204, 147	D-Xylose
<b>Other organic compounds</b>		<b>Other organic compounds</b>	
103, 217	Dihydroxyacetone dimmer		
87, 177	Dithian, t-tutyl [1,3]		
179, 268	m-Guaiacol (3-methoxyphenol)		
239, 254	Hydroxyquinol (1,3-dihydrophenol)		
		152, 167	Pyridine
		411, 43, 245	Lanost-8-en-3-one

higher temperatures and pressures. Hydrothermal treatment of oils or oil-rich biomass yields primarily n-alkanes (green diesel) as opposed to a mixture of alkyl esters of long chain fatty acids (biodiesel) which are produced by a transesterification approach in biofuel production. The formation of green diesel in sub-/supercritical water treatment of fatty samples is a two-step process. In the first step free fatty acids are liberated by hydrolysis, whereas in a second step, which is catalytically favored, a deoxygenation takes place.

Saturated and unsaturated medium-size and long chain fatty acids have been detected in subcritical water extracts of cherry stems. In sweet cherry stems only hexadecanoic (palmitic) acid was detected. A derivative of hexenoic acid, 3-methyl-5-keto-3-hexenoic acid was seen only in the extracts of sour cherry stems. This specific fatty acid was recognized as one of the auxiliary odor precursors of human apocrine gland secretion, responsible for characteristic smell in humans. Nonanoic (pelargonic) acid is

naturally present in fruits, vegetables and grains, but also in dairy products and meat, at levels ranging from 0.2–400 mg kg<sup>-1</sup>.<sup>40</sup> The compound exhibits herbicidal properties and is of low toxicity to humans.

In water extracts of sour cherry stems another long-chain saturated fatty acid was identified – octadecanoic (stearic) acid. In general, this fatty acid is more abundant in animal fats than in the plant kingdom.

Organic acids are normal constituents of plant and animal tissues. Numerous organic acids have been identified in analysed extracts and may have also been partially formed in the course of hydrothermal conversion of plant constituents. Hydrolytical reactions taking place in subcritical water potentiate formation of organic acids. In human nutrition organic acids are important for acetic-alkali equilibrium that affects all biochemical processes. Their intake affects digestion by stimulating the stomach and pancreas, and increasing the intestine motor function. In food organic

acids act as antiseptics and contribute to flavor. Certain foods disturb an alkali/acid balance, further impairing the absorption of certain nutrients, like magnesium, potassium, calcium, and sodium. Organic acids detected in subcritical water extracts can be considered as desirable products from sensory and functional point of view.

The profiles of organic acids for sweet and sour cherry stem extracts were similar. Pyrrole-2-carboxylic and 2-isopropyl-3-hydroxy butanoic acids were detected in sweet cherry stem extracts but not in sour cherry stem extracts. Pyrrole-2-carboxylic acid is a nitrogen-containing heterocyclic acid, probably formed by Maillard reactions. Its history as a compound of biological origin is rather recent. In mammals it was first identified as a degradation product of sialic acids, then as a derivative of the oxidation of the D-hydroxyproline isomers. In plants it is a secondary metabolite and is formed by the activity of amine oxidase from hydroxy-D- and alhydroxy-D-proline.<sup>41</sup> This metabolite was also detected in boron-deficient orange leaves.<sup>42</sup>

In sweet cherry stems malic acid was the most abundant among other organic acids, as opposed to benzoic and butanoic acids that were most represented in sour cherry stems. Malic acid is beneficial for conditions such as fibromyalgia and chronic fatigue syndrome.<sup>43</sup> In cosmetic products the acid balances pH and exhibits exfoliating properties beneficial in the treatment of different skin irregularities, such as hyperpigmentation, acne and scars. The acid was detected in many fruits and vegetables like apple, grapes and rhubarb, contributing to their flavor. Malic acid is also approved food additive E296 used as a stronger sour alternative to citric acid.

2-Furancarboxylic or 2-furanoic acid was detected in both plant extracts. This acid is known for its bactericidal, fungicidal and nematocidal properties<sup>44</sup> and is often added as a food preservative. Furthermore, 2-furoic acid was shown to be effective in lowering both serum cholesterol and serum triglyceride levels in rats with elevated HDL cholesterol levels.<sup>45</sup>

Adicarboxylic acid – 2-butanedionic acid, or succinic acid – was detected in both plant samples. In plant metabolism succinate serves as the electron donor in electron transport chain in the citric acid cycle.<sup>46</sup> Succinic acid is used as a flavoring agent for food and beverages, and is also an important raw material in the polymer industry. Because of its importance succinic acid is largely produced at commercial-scale by fermentation processes. Gulonic acid, detected in both sweet and sour cherry stem extracts, is produced in plants by the oxidation of l-gulose, an aldohexose sugar. Biosynthesis of ascorbic acid in plant proceeds via this intermediate. For short-chain organic acids like acetic and propanoic that were detected in the stems of both cherry types antifungal and antibacterial effects have been recognized.<sup>47</sup> Other beneficial health effects have been ascribed to these acids. Propanoic acid lowers fatty acids content in liver and plasma, reduces food intake, exerts immuno-suppressive actions and improves tissue insulin sensitivity.<sup>48</sup> Butanoic acid was detected in sweet cherry stem extracts but not in sour cherry stem extracts. According to organic acid profiles these extracts, thus, can be considered as functional nutritional supplements beneficial in the prevention and treatment of different health conditions such as obesity and diabetes type 2, high cholesterol, etc.

Different monomeric sugars have been detected in subcritical water extracts, indicating strong hydrolysis of plant polysaccharides under extraction conditions. Arabinose was a dominant monosaccharide in both extracts, with sour cherry stem extract also containing high quantities of D-ribose. Functionality of two identified

sugars, D-ribose and D-fructose, can be ascribed to their effects to increase cellular energy synthesis in heart and skeletal muscle alleviating symptoms of fibromyalgia and chronic fatigue syndrome.<sup>49</sup> Fructose is often recommended for diabetics because it does not trigger the production of insulin by pancreatic  $\beta$  cells. Furthermore, it is about 73% sweeter than sucrose, requiring lower consumption. In addition, fructose has a low glycemic index of 19, compared with 100 for glucose and 68 for sucrose.<sup>50</sup>

Mannose was detected in sweet cherry stem extracts but not in sour cherry samples, whereas D-galactose was seen only in the latter. Mannose is important in human metabolism, especially in glycosylation of certain proteins and it is believed that this sugar aids in urinary tract infections.<sup>51</sup> Xylose, detected in sweet cherry stem extracts probably originated from hemicellulose hydrolysis.<sup>52</sup>

In subcritical water extracts of sweet cherry stems compounds of phenolic structure were detected, namely m-guaiacol and hydroxyquinol. These have probably been released by hydrolysis of plant polyphenolic constituents, such as lignin. Derivatives of guaiacol are used medicinally as an expectorant, antiseptic, and local anesthetic.<sup>53</sup>

Hydroxyquinol commonly occurs in nature as a biodegradation product of catechin. Dihydroxyacetone that was detected in the extracts of sweet cherry stems could have been formed in the course of hydrothermal conversion from glycerol that was abundant in the extracts. On the other hand the compound is involved in carbohydrate metabolism in higher plants and animals through glycolysis and photosynthesis.

Lanostenone was detected only in the extracts of sour cherry stems. This molecule of steroidal structure is formed by oxidation of lanosterol, a tetracyclic triterpenoid. Biosynthesis of sterols in plants proceeds via a mevalonic acid pathway. Cyclization of squalene occurs via the intermediate 2,3-oxidosqualene that forms either lanosterol or cycloartenol via a series of enzymatic cyclizations.<sup>54</sup> Lanostenone can easily be converted by reduction to lanosterol and recently has attracted lot of attention as an anti-cataract agent.<sup>55</sup>

## CONCLUSIONS

The extracts of sweet and sour cherry stems obtained by subcritical water were compared with respect to their chemical profiles and biological activity. Chemical profiles of the tested extracts defined by GC–MS analysis did not differ significantly and revealed the presence of alcohols, fatty acids, organic acids and other organic compounds, confirming the complexity of the composition. Antioxidant, anti-radical and cytotoxic activities of sweet and sour cherry stem extracts were also very similar.

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