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Original research article

# Analysis of phenolics in the peel and pulp of wild apples (*Malus sylvestris* (L.) Mill.)

Nevena R. Mihailović[a,*], Vladimir B. Mihailović[a], Samo Kreft[b], Andrija R. Ćirić[a], Ljubinka G. Joksović[a], Predrag T. Đurđević[a]

[a] Faculty of Science, Department of Chemistry, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia
[b] Faculty of Pharmacy, Department of Pharmaceutical Biology, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

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ABSTRACT

A simple and efficient ultrasound extraction method for phenolic compounds in the pulp and peel of *Malus sylvestris* (wild apples) was developed using multivariate methodology. Optimal extraction conditions were obtained using this methodology for peel and pulp samples (2.0 g), including solvent volume 100 mL and 79 mL, methanol concentration 100% (v/v) and 20% (v/v), extraction time 33 min and 40 min, extraction temperature 65 °C and 80 °C, respectively. The peel and pulp extracts were analysed by HPLC-PDA using a $C_{18}$ Phenomenex Kinetex column. Among the phenolic compounds present in our samples, we quantified (mg/g fresh weight) chlorogenic acid, 0.791; epicatechin, 1.18; and phloridzin, 0.106 in the pulp extract, whereas the peel extract contained chlorogenic acid, 0.568; epicatechin, 1.36; phloridzin, 0.207; catechin, 0.187; hyperoside, 0.261; and quercitrin, 0.256 mg/g. The antioxidant activity of the extracts was measured by spectrophotometric methods Peel extract proved to be a better antioxidant than pulp extract. Additionally, the stability of the analysed phenolic compounds was tested by *in vitro* digestion procedures. Simulated *in vitro* digestion showed that the concentrations of all analysed phenolic compounds (except chlorogenic acid) decreased during the intestinal phase of digestion.

## 1. Introduction

Apples are one of the most widely produced and consumed fruits in the world. Fresh apples and their processed products, such as dried apple, applesauce, juice and cider, are usually available in the market for the entire year and represent a significant part of human nutrition in many parts of the world (Šavikin et al., 2014). In addition to its nutritive value, the consumption of apples has been associated with a decreased risk of chronic diseases, including cardiovascular diseases, asthma, various cancers and type II diabetes (Gossé et al., 2005; Boyer and Liu, 2004; Knekt et al., 2002; Le Marchand et al., 2000; Hyun and Jang, 2016). Several studies have characterized the bioactive compounds and pharmacological functions of cultivated apples (*Malus domestica* Borkh.) (Xu et al., 2016; Panzella et al., 2013; Lata et al., 2009, Mikulič Petkovšek et al., 2009). However, data on wild European apples (*Malus sylvestris* (L.) Mill.) are scarce because their populations are very rare, and some European countries (i.e., Belgium and the Czech Republic) have classified *M. sylvestris* as an "endangered species" (Schnitzler et al., 2014).

The European wild apple, which is a member of the Rosaceae family, is widely spread in European forests as dispersed single trees. The genus *Malus* is native to the temperate zones of the northern hemisphere, Europe, Asia, and North America, and it comprises approximately 30–35 species of small deciduous trees or shrubs of the Rosaceae family. *M. sylvestris* species grow throughout Serbia in the woods and thickets at boundaries from lower to hilly regions, and they can reach up to 10 m in height. Their sour apples are consumed as fresh fruit or used for traditionally produced apple vinegar (Josifović, 1974). Because of its many health benefits, apple cider vinegar is a product of increasing interest (Solieri and Giudici, 2009; Morgan and Mosawy, 2016). Additionally, in Serbian traditional medicine, vinegar from wild apple fruits is used to strengthen the immune system to ward against cold, digestive, and hypertensive ailments (Zlatković et al., 2014). In Europe, wild apples have been used to treat infant intestinal disorders (diarrhoea, dyspepsia and dysentery), and for adults, they have been used against constipation (Duke and Wain, 1981). The examination of wild apples has been neglected despite their use in traditional medicine (Šavikin et al., 2014). Although wild apples are not used as much as cultivated apples because of their tart taste and because they are economically less significant, there is a possibility that they are rich in

* Corresponding author.
*E-mail address:* i.nevena@kg.ac.rs (N.R. Mihailović).

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bioactive compounds and antioxidants. Some studies have shown that older varieties of apples possess a higher level of phenolic compounds and antioxidant activity than most commonly cultivated apples (Golden Delicious and Stark Delicious) (Iacopini et al., 2010). Similar research that included several ancient apple varieties and wild apples grown in Croatia showed that the wild apple contained much higher flavonol and phenolic acid content in their flesh compared to other apples (Jakobek and Barron, 2016). Therefore, testing the wild apples is important to consider if they could be a valuable source of bioactive phenolic compounds.

The most important phenolic compounds in apples are quercetins (in glycosylated forms), catechin and epicatechin, which are responsible for astringency and bitterness. An esterified form of caffeic and quinic acid (chlorogenic acid) and *p*-coumaric acid are generally present in apples (Asha et al., 2013). Phloretin and its glucoside form, phloridzin, are two dihydrochalcones present in particularly high concentrations in an apple peel (Marks et al., 2007; Vrhovsek et al., 2004; Tsao et al., 2003; Sanoner et al., 1999). Each of these phenolic compounds may have specific health benefits. The phenolic compound composition in apples varies between different apple cultivars and between different apple parts (Francini and Sebastiani, 2013). Data about the phenolic composition and content of the peel and pulp of *M. sylvestris* and their antioxidant potential are still limited. In this context, data about the extraction and identification of biologically valuable compounds from wild apple fruits may be needed to obtain better knowledge about their potential use as nutraceuticals and functional foods. Currently, the methods used for extraction of phenolic compounds from natural products include conventional (e.g., Soxhlet extraction and maceration) and non-conventional (e.g., ultrasound-assisted extraction, microwave-assisted extraction and supercritical fluid extraction) extraction methods (Chandrapala et al., 2013; Chemat et al., 2011). In this study, an ultrasound-assisted extraction method, which is a non-conventional extraction method and is a time-saving approach that requires less organic solvent, was developed for the extraction of phenolic compounds from the fruit of *M. sylvestris*. Mechanical effects of ultrasound can promote eddy and internal diffusion, which increases the mass transfer and penetration of the solvent into the sample matrix (Ji et al., 2006). Cavitation can break cell walls and accelerate the release of the contents (Zhou et al., 2017). Extraction parameters (e.g., the concentration of the solvent, the solvent/material ratio, temperature, extraction time) can significantly affect the extraction efficiency and could also interact with each other (Zhou et al., 2017). Therefore, it is important to optimize these parameters to obtain high extraction efficiency.

The aim of this work was to find optimal extraction conditions for total phenolic content in the pulp and peel of wild apples and, due to their biological importance, the quantification of their main phenolics. To elucidate the health benefits of wild apples, an evaluation of their antioxidant potential and simulation of their gastrointestinal digestion was also conducted.

## 2. Materials and methods

### 2.1. Chemicals and standards

All chemicals and reagents that were used were analytical grade. Quercitrin, rutin, caffeic acid, gallic acid, phloridzin, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)), PMS (phenazine methosulphate), NBT (nitro blue tetrazolium), and TCA (trichloroacetic acid) were purchased from Sigma-Aldrich (Deisenhofen, Germany). The (+)-catechin was purchased from Serva (Heidelberg, Germany), the hyperoside was purchased from Carl Roth (Karlsruhe, Germany) and the epicatechin was purchased from Thermo Fisher Scientific (Geel, Belgium). TBA (2-thiobarbituric acid) was purchased from ABCR (Karlsruhe, Germany). In the HPLC analyses, we used HPLC-grade acetonitrile, water and trifluoroacetic acid (Merck, Darmstadt, Germany). Pepsin from porcine gastric mucosa, pancreatin from a porcine pancreas and porcine bile extract were also purchased from Sigma-Aldrich (Deisenhofen, Germany). Standard solutions of chlorogenic acid, catechin, epicatechin, quercitrin, hyperoside and phloridzin were used for quantifying the compounds identified in extracts using the HPLC method. The concentration of all stock solutions was 100 μg/mL and additional dilutions were made for calibration curves. The solvent and diluent was absolute methanol. Standard solutions of phenolic compounds used for calibration curve construction and characterization of the HPLC method were stored at +4 °C for 3 days.

### 2.2. Plant material

*Malus sylvestris* (wild apple) fruits (approximately 1 kg) were collected in Central Serbia at the village Čestin (43°53′21″N 20°48′56″E) at an altitude of 378 m in October 2014 (see Supplementary material). Fruits were harvested from 5 trees to obtain approximately 20 kg a year and by a random sampling method, 5 kg of apples were separated. After that, a quartering method was used to reduce the amount to 1 kg. Apples were stored in the refrigerator no more than one week before sample preparation. The voucher specimen (no. 118/015) was prepared and deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia, after the identification of species. The apples were washed with tap water, and the peel and pulp were separated mechanically (peel thickness was 0.5–1 mm), chopped into small pieces, frozen in liquid nitrogen, and stored at −22 °C for further use for less than 1 month. There are scarce data in the literature concerning the stability of phenolic compounds in apple samples during the freezing, but according to Türkben et al. (2010), freezing could reduce degradation of phenolic compounds.

### 2.3. Preparation of the apple fruit extracts

At the time of analysis, the samples were homogenized using a laboratory mill (IKA homogenizer, IKA − Werke GmbH & Co. KG, Staufen, Germany). Samples (2.0 g) were extracted using methanol:-water solvent with different concentrations of methanol (20, 60, 100 v/v%) at different temperatures (20, 50, 80 °C) and for different extraction times (20, 30, 40 min) in an ultrasonic bath (Bandelin Sonorex, Bandelin electronic GmbH & Co. KG, Berlin, Germany). The volume of the solvent was 30, 65 or 100 mL for 2.0 g of sample. Thirty extracts were prepared with different combinations of these four factors, as presented in Table S1 (Supplementary material). Liquid extracts were separated using filtration and frozen until further analysis. The concentration of extracted phenolics from the peel and pulp of wild apples was measured according to the procedure described below (Section 2.8.). The optimal concentration of extraction solvent, extraction temperatures and extraction times for the preparation of apple fruit extracts under optimal conditions were predicted according to the experimental model designed using Design Expert 7.0.0. software (Stat-Ease Inc., Minneapolis, MN, US) based on the results obtained in determining the total phenolic compounds, flavonoids and phenolic acids. After obtaining the optimal conditions for total phenolics, flavonoids and phenolic acids, additional extracts were prepared under the optimal conditions for further HPLC analysis and evaluation of antioxidant activities. Homogenized samples of apples (peel and pulp), which were prepared as described in the procedure for extraction optimization, were extracted under obtained optimal conditions with three replications. The obtained extracts were evaporated on a vacuum evaporator and lyophilized (LIO 2000, Kambič, Laboratory Equipment, Semič, Slovenia) for further HPLC analysis and antioxidant activity measurements.