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ORIGINAL ARTICLE

Apple pomace: antiradical activity and antiproliferative action in HeLa and HT-29 human tumor cell lines

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Summary

Purpose: *Apple pomace is an easily accessible source of bioactive compounds which can be used for various purposes in the food, pharmaceutical and cosmetic industry. Six types of apple pomace extracts were tested to study their health benefits, free radical scavenging and antiproliferative activities.*

Methods: *The radical scavenging activity was determined by electron spin resonance (ESR) spectroscopy. Antiproliferative action was measured using MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] colorimetric assay in cervix epithelioid carcinoma (HeLa) and colon adenocarcinoma (HT-29) human cancer cell lines.*

Results: *All extracts suppressed the formation of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and hydroxyl-free radical in a dose-dependent manner. In the presence of 12.5 mg/ml Pinova, Reinders and Nectar pomace extract, the ESR $DPPH^{\bullet}$ signals vanished. The $^{\bullet}OH$ was completely scavenged in the presence of 45 mg/ml or higher concentration of the investigated extracts. Pinova and Braeburn pomace extracts showed the strongest antiproliferative activity against the investigated human cancer cell lines. Also, HeLa cells were found more sensitive than HT-29 cells to all extracts.*

Conclusion: *Although the relationship between radical scavenging activities and phenolic contents or flavonol glycosides ($R^2 \geq 0.80$) was high, there were no significant correlations between the total phenolic contents or individual phenolic compounds and the antiproliferative activity.*

Key words: antioxidant activity, antiproliferative action, apple pomace, cancer cell lines, polyphenolics

Introduction

Nowadays, there is increasing scientific focusing on by-products of fruit and vegetable food processing in terms of their antioxidant contents and their contribution to health-promoting effects [1-3]. The antioxidant compounds from waste products of food industry could be used for increasing the stability of foods by preventing lipid peroxidation and also for protecting oxidative damage in living systems by scavenging oxygen free radicals [4]. Application of "non-chemical" ingredients in health products is indispensable for the pharmaceutical and cosmetic industry [5]. Products with natural supplements could be named functional food, nutraceuticals or cosmeceuticals.

A number of investigations pointed out that consumption of fruits and vegetables imparts health benefits, e.g. reduced risk of coronary heart disease and stroke, as well as certain types of cancer [6-8]. One of the most frequently cultivated fruit are apples, which mostly (~70%) are used for direct consumption; they also (~30%) are processed mainly into juice. In numerous diets, apples are a very significant part and represent an important source of sugars, minerals, organic acids, dietary fibre, and bioavailable compounds such as vitamin C and phenolic compounds (flavonoids and phenolic acids). Many of the phenolic compounds have shown strong antioxidant properties as oxygen scavengers, peroxide decomposers, metal chelating agents, and free radical inhibitors [9-11]. Beside antioxidant activity, phenolic compounds have a wide range of action, which includes antitumoral, antiviral, antibacterial, cardiopro-

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tective, and antimutagenic activities [12-14]. Conventional apple juice production (straight pressing of apple pulp or pressing after pulp enzyming) resulted in a juice poor in phenolics and with only 3-10% of the antioxidant activity of the fruit they were produced from [15].

Apple pomace, the primary by-product in the apple juice processing, is used successfully for pectin production and recent investigations regard it as a rich source of polyphenols, minerals and dietary fibre [16-18]. In view of the fact that most of the phenolic compounds remained in the apple pomace, this research deals with the apple pomace as a potential source of bioactive phenolics, which can be used for various purposes in the food, pharmaceutical and cosmetic industry. Therefore, the antioxidant and antiproliferative activities of 6 different types of apple pomaces (obtained from apple varieties - Pinova, Reinders, Jonagold, Iduna, Braeburn and a sample obtained from Nectar factory) were examined. ESR spectroscopy, a very sensitive analytical method, was employed to determine the antioxidative activity of apple pomace extracts against stable DPPH and reactive hydroxyl radicals. The effects of apple pomace extracts on the proliferation of HeLa and HT-29 human cancer cell lines were investigated. The possible correlation between the content of phenolics and antioxidant activity/antiproliferative activity was investigated.

Methods

Samples and chemicals

Apple varieties (Pinova, Reinders, Jonagold, Iduna, Braeburn) harvested in Serbia in the 2009 season, were collected from the Department for fruit growing and viticulture, Faculty of Agriculture, University of Novi Sad.

DPPH, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), Folin-Ciocalteu reagent, chlorogenic acid, caffeic acid, (+)-catechin, (-)- epicatechin, rutin, phloridzin and quercetin were purchased from Sigma Chemical Co. (USA). These chemicals were of analytical reagent grade. Other used chemicals and solvents were of the highest analytical grade and obtained from "Zorka" Sabac (Serbia).

Pomace and extract preparation

Pomace of selected apple varieties was obtained during apple juice production in our laboratory. Also, one sample of apple pomace from juice processing was supplied by the Nectar factory, Bačka Palanka. Extractions of apple pomaces were carried out at room temperature in ultrasonic bath, Heidolph DIAX 900, as previously described [19].

DPPH radical scavenging activity (SA)

A water solution of 200 μl of each pomace extract (or water itself as control) was added to 400 μl DPPH (0.4 mM) dissolved in methanol. The range of the investigated extract concentrations was 2.5-30 mg/ml. After mixing for 2 min, the solution was transferred to a quartz flat cell ER-160FT and ESR spectra were recorded on an ESR Bruker 300E spectrometer (Rheinstetten, Germany). The following instrument settings were used: field modulation 100 kHz, modulation amplitude 0.256 G, receiver gain 2×10^4, time constant 40.96 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 7.96 mW, temperature 23° C.

The SA_{DPPH} value of the extract was defined as:

$$SA_{DPPH}\,(\%) = 100(h_0 - h_x)/h_0$$

where h_o and h_x are the height of the second peak in the ESR spectrum of DPPH radicals of the control and the probe, respectively.

Hydroxyl radical scavenging activity (SA)

Hydroxyl radicals, generated by the Fenton reaction, reacted rapidly with DMPO spin trap and formed nitroxide adducts (stable free radicals form) which were detectable by ESR spectrometer. The Fenton reaction was conducted by mixing 0.2 ml 10 mM H_2O_2, 0.2 ml 10 mM $FeCl_2 \times 4H_2O$ and 0.2 ml 80 mM DMPO (control). The influence of pomace extracts on the formation and stabilization of hydroxyl radicals was reconsidered by adding the investigated extract to the Fenton reaction system in a concentration range 10-55 mg/ml. ESR spectra were recorded after 5 min using ESR Bruker 300E spectrometer (Rheinstetten, Germany) set at the following conditions: field modulation 100 kHz, modulation amplitude 0.512 G, receiver gain 5×10^5, time constant 81.92 ms, conversion time 163.84 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23° C.

The SA_{OH} value of the extract was defined as:

$$SA_{OH}\,(\%) = 100(h_0 - h_x)/h_0$$

where h_o and h_x are the height of the second peak in the ESR spectrum of hydroxyl radicals of the control and the probe, respectively.

Cell antiproliferation assay

The antiproliferative activity of apple pomace extracts was assessed by measuring the inhibition of HeLa

and HT-29 human cancer cell proliferation. Cell proliferation was measured using MTT colorimetric assay [20]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL, UK), supplemented with 10% heat inactivated fetal calf serum (FCS, NIVNS, Serbia) and antibiotics: 100 IU/ml of penicillin and 100 μg/ml of streptomycin (ICN Galenika, Serbia) in Kartel (Kartel, Switzerland) 25 ml flasks at 37° C in 5% CO_2 atmosphere and high humidity. Cells were maintained in the logarithmic phase of growth and subcultured twice a week using 0.5% trypsin (SIGMA, USA). The cell density (number of cells per unit volume) and the percentage of viable cells were defined as previously described [21]. Tumor cells were dispensed into each well of 96-well Corning microtiter plates (Corning, USA) at seeding density of 3×10^3 cells per well, in a volume of 180 ml, and preincubated in complete medium at 37° C for 24 h. Extracts were added (20 μl/well) to achieve the required final concentrations (10-50 mg/ml). An equal amount of solvent was added in the control wells. Microplates were incubated at 37° C for 48 h. After incubation, 20 μl of MTT (Sigma, USA), dissolved in DMEM medium solution (5 mg/ml) and filtered through 0.45 μm filter (Sartorius, UK), were added to each well. After a further 3 h incubation, the MTT reaction medium was removed and the purple formazan was dissolved by adding 100 μl of 0.04 mol/l HCl-isopropanol. Cell proliferation was measured by the ability of viable cells to reduce MTT to formazan, whose absorbance can be analyzed photometrically. Absorbance (*A*) was measured in a microplate reader (Multiscan Ascent, Labsystems) at 540/620 nm.

The antiproliferative activity (*APA)* of the extract was defined as:

$$APA\ (\%) = 100(A_c - A_t)/A_c$$

where A_t and A_c are the absorbance of the test sample and the control, respectively. All measurements were performed in quadriplicate.

Statistical analysis

All measurements were carried out at least in triplicate. The analysis of tendency was performed using logarithmic function with Microsoft Excel using data from the dose-response curve.

Results

The radical scavenging activities, as a function of total phenolic contents, obtained by the Folin-Ciocalteu method and HPLC method, are shown in Figure 1. The radical scavenging activities of 6 types of apple pomace extracts were characterized according to their activity to-

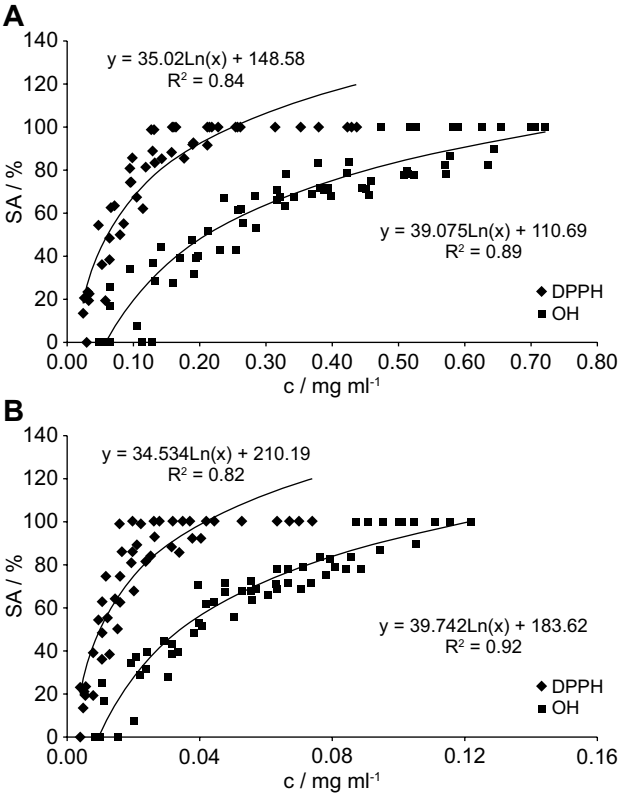

Figure 1. Relationship between radical scavenging activity (SA) values (SA_{DPPH} and SA_{OH}) and total phenolic content obtained by (**A**) Folin-Ciocalteu and (**B**) HPLC methods.

wards the stable DPPH and reactive hydroxyl radicals. The scavenging activity (SA_{DPPH}) of different concentrations of the pomace extracts is presented in Figure 2. All extracts showed strong radical scavenging activity on $DPPH^{\bullet}$ in a dose-dependent manner at the investigated concentrations. In the presence of 12.5 mg/ml Pinova, Reinders and Nectar pomace extracts, the ESR signals vanished indicating that these apple varieties possess effective antioxidant activity, while the other apple varieties exerted the same activity at higher concentrations (≥ 20 mg/ml). The typical 1:2:3:2:1 five lines of ESR sig-

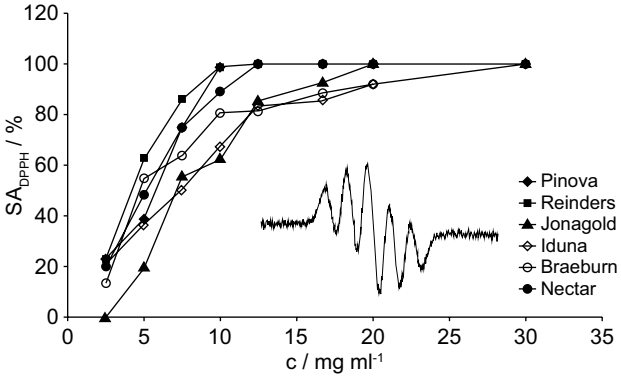

Figure 2. Scavenging activity of different concentrations of pomace extracts on DPPH radicals. ESR spectrum of the DPPH radical is shown in the insert.

nal of DPPH radical with hyperfine splitting constant a_N=9.03 G is inserted in Figure 2.

High reactive hydroxyl radicals are major reactive oxygen species inducing biological damage and lipid peroxidation. Hydroxyl radicals generated by the Fenton reaction (Fe^{2+}/H_2O_2) were trapped by DMPO, forming DMPO-OH spin adducts which were detectable by ESR. The hydroxyl radical scavenging activity of the apple pomace extracts increased with the increase of concentration, reaching complete scavenging at 45 mg/ml and higher concentrations (Figure 3). The insert shows the typical ESR spectrum of DMPO-OH adduct which is characterized by its 1:2:2:1 quartet of lines and hyperfine splitting constant a_N and a_H=14.9 G.

The apple pomace extracts influenced cell growth depending on apple variety, cell line and dose (Figure 4).

The antiproliferative activities of apple pomace extracts were expressed as the median effective dose (IC_{50}), with a lower IC_{50} value indicating a higher antiproliferative activity (Table 1).

All apple pomace extracts exhibited strong cytotoxic effect on HeLa cell line within the examined concentration range (Figure 4A), with IC_{50} values below 10 mg/ml for all extracts except Iduna pomace extract (IC_{50}=11.05 mg/ml). The curves showed that the growth of HeLa cells was inhibited to a highest degree in all concentrations of Pinova, Reinders and Braeburn pomace extracts (between 85 and 97%). However, Jonagold and Iduna pomace extracts reached a similar plateau-type antiproliferative effects (~90%) at the concentrations ≥20 mg/ml. Figure 4B shows that HT-29 cells were sensitive to cytotoxic effect of all investigated apple pomace extracts. Strong plateau-type antiproliferative effect on colon adenocarcinoma cells with IC_{50} values below 10 mg/ml (Table 1) was observed for Pinova, Iduna and Braeburn pomace extracts. Reinders, Jonagold and Nectar pomace extracts exhibited dose-dependent antiproliferative effect on this cell line with IC_{50} values 34.05, 38.28 and 20.45 mg/ml, respectively (Table 1).

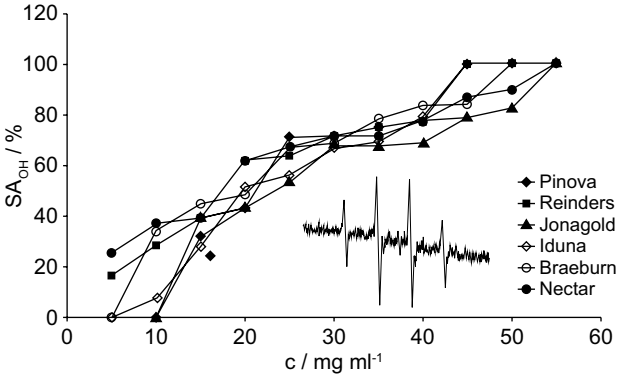

Figure 3. Scavenging of different concentrations of pomace extracts on hydroxyl radicals generated in the Fenton reaction. The insert shows the ESR spectrum of DMPO-OH adduct.

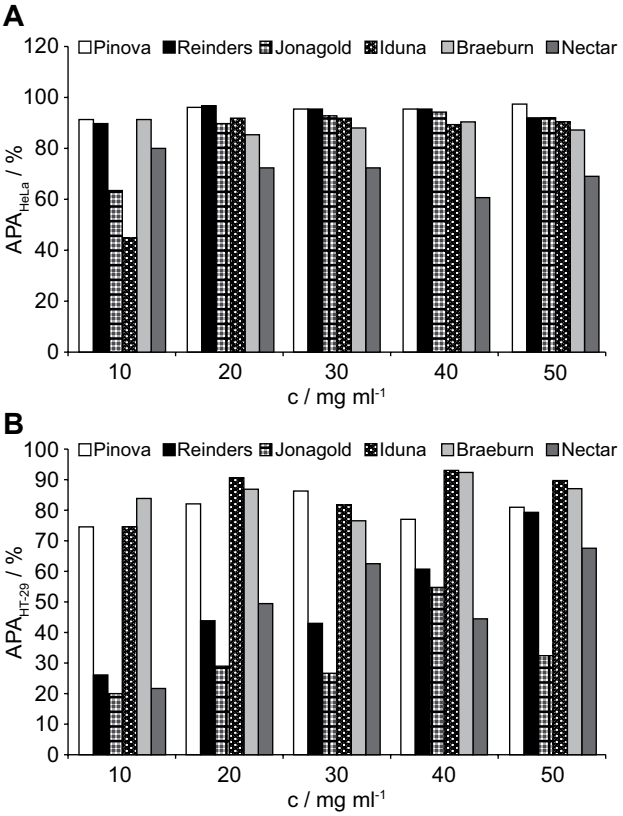

Figure 4. Antiproliferative activities (APA) of apple pomace extracts against (**A**) HeLa and (**B**) HT-29 cells.

The logarithmic correlation coefficients (R2) are listed in Table 2. The correlation coefficients (Table 3),

Table 1. IC_{50} values of different apple pomace extracts in human tumor cell lines

Apple pomace	$IC_{50,HeLa}$ / mg ml^{-1}	$IC_{50,HT-29}$ / mg ml^{-1}
Pinova	<10	<10
Reinders	<10	34.05
Jonagold	<10	38.28
Iduna	11.05	<10
Braeburn	<10	<10
Nectar	<10	20.45

Table 2. Correlation matrix between the chromatographic results and the *SAs* in extracts

Phenolic compounds	*Correlation coefficient, R^2*	
	DPPH	*OH*
Chlorogenic acid	0.59	0.69
Caffeic acid	0.75	0.82
(+)-Catechin	0.55	0.65
(–)-Epicatechin	0.50	0.63
Rutin	0.84	0.91
Quercetin-glycosides	0.80	0.83
Phloridzin	0.35	0.41

Table 3. Correlation matrix between the radical scavenging activities (SA) and inhibition of HeLa and HT-29 cell proliferation (APA)

Correlation coefficients, R^2	SA_{DPPH}	SA_{OH}
APA_{HT-29}	0.0053	0.0153
APA_{Hela}	0.5332	0.2271

calculated from logarithmic regression analysis, indicated that the radical scavenging activities were not interrelated with inhibition of HeLa and HT-39 cells' proliferation.

Discussion

Phenolic compounds are important constituents of apples and contribute to color and flavor of the fresh fruit. Also, the health-protecting properties of apples have been attributed to the presence of these classes of compounds [22-24].

Processing apples into juice has been found to affect their phenolic content. Namely, apple pomace, a by-product in the apple juice processing, contains a variety of bioactive compounds, including polyphenols [1,16,18,24]. In our previous work, the total phenolics, flavonoids and flavan-3-ols in apple pomaces were determined by the Folin-Ciocalteu method, Markam method and vanillin assay, respectively. The HPLC analysis was employed to identify and quantify the major phenolic compounds present in the investigated apple pomaces [19].

It is generally accepted that phenolic compounds behave as antioxidants as a result of the reactivity of the phenolic moiety. But in our study, the obtained results show that all the investigated apple pomace extracts were less effective on hydroxyl radical scavenging than in DPPH test.

DPPH is a synthetic stable free radical which has the ability to become a stable diamagnetic molecule by accepting an electron or hydrogen. The DPPH assay is one of the most popular spectrophotometric methods for determination of the antioxidant activity of food [25,26], plant extracts [27-29] and beverages [30,31]. However, in our study ESR spectroscopy was used to identify DPPH radicals.

The capacity of apple pomace extracts to remove hydroxyl radicals generated by the Fenton reaction could be due to direct scavenging effect and/or the inhibition of hydroxyl generation. The second mechanism could occur by ion chelation [32].

It is interesting to consider the correlation between radical scavenging activities of the apple pomace extracts and their phenolic constituents, as phenolic compounds contribute directly to antioxidant activity [33]. The high correlation coefficients ($R^2 \geq 0.82$), calculated from logarithmic regression analysis, indicated that there is a significant positive relationship between total phenolic content and DPPH/hydroxyl radical scavenging activity.

In order to determine the relative importance of individual phenolic compounds in radical scavenging activities, correlation analysis was carried out between the measures of *SA* and results of the HPLC analysis, which were presented in our previous work [19]. It was observed that flavonol glycosides (rutin and other quercetin glycosides), which were the dominant compounds in the investigated extracts, were highly correlated with the SA_{DPPH} and SA_{OH} ($R^2 \geq 0.80$). On the other hand, phloridzin seemed to weakly influence the DPPH and hydroxyl radical scavenging activities ($R^2 \leq 0.40$).

The inhibition of the cellular growth was estimated using the MTT assay. In viable cells MTT is reduced to an insoluble purple formazan by mitochondrial succinate dehydrogenase. Cell proliferative activity was measured by comparison of the purple color formation. Dead cells, on the other hand, do not form the purple formazan due to their lack of enzyme. The antiproliferative activity of different apple pomace extracts was assessed by measurement of the growth inhibition of two histologically different human cancer cell lines: HeLa and HT-29. Furthermore, tumor cell proliferation was strongly inhibited *in vitro* by apple pomace extracts. The relationship between radical scavenging activities and phenolic contents for the apple pomace extracts was high, while there were no significant correlations between the total phenolic contents or individual phenolic compounds and the antiproliferative activities ($R2 \leq 0.20$). Therefore, the inhibition of *in vitro* cancer cell proliferation by the investigated extracts cannot be explained only by the content of phenolic compounds. This suggests that other phytochemicals may play a major role in the antiproliferative activity of apple pomace extracts.

Based on the obtained results, it can be concluded that Pinova and Braeburn pomace extracts showed the strongest antiproliferative activity against the investigated human cell lines. Also, HeLa cells were more sensitive than HT-29 cell to all extracts.

Data about the effect of phenolic compounds in cell culture of various carcinoma cell lines are often controversial. It is interesting to point out that some authors reported that polyphenols from fruits and vegetables contributed to anticarcinogenic effect and some of them, such as flavonoids, have also been shown to inhibit cancer cell proliferation *in vitro* [14,34,35]. The flavonol quercetin, especially abundant in apples, is reported to have antiproliferative effects in many cancer cell lines.

Antioxidant or pro-oxidant activities and kinase inhibition have been proposed as molecular mechanisms for these effects. Although none of the tested quercetin concentrations increased reactive oxygen species (ROS) generation in HeLa cells, quercetin stimulation prevented the H_2O_2-induced ROS production both in the presence and in the absence of estrogen receptor α (ERα). However, this flavonoid induced the activation of p38/MAPK, leading to the pro-apoptotic caspase-3 activation and to the poly (ADP-ribose) polymerase cleavage only in the presence of ERα. These findings suggest that quercetin resulted in HeLa cell death through an ERα-dependent mechanism involving caspase and p38 kinase activation [36]. Our results are in agreement with some other investigations in which no correlation could be found between inhibition of growth of cancer cells and total phenolic or flavonoid content in some fruit and berry extracts [37-39].

Conclusions

Apple pomace is often discarded in the apple juice processing, but this study clearly indicated that polyphenols responsible for the antioxidant activity in apples are still present in pomace. Selected apple pomace extracts were evaluated for their antioxidant, DPPH and hydroxyl radicals scavenging activity, and inhibition of HeLa and HT-29 cell lines proliferation. The presented results demonstrate that combination of phytochemicals in apple pomace extracts contributes to their potent antioxidant and antiproliferative activities. In summary, apple pomace could represent a cheap and readily available source of value-added ingredient for functional food and some other health products.

Acknowledgements

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