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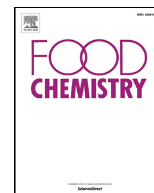

# Extraction, isolation of heat-resistance phenolic compounds, antioxidant properties, characterization and purification of 5-hydroxymaltol from Turkish apple pulps

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ABSTRACT

Apple pulps (AP) were obtained as a side product in fruit juice factories and contains valuable phenolic compounds. The dried AP was subjected to extraction with water, ethyl acetate (APEA) and *n*-butanol (APBU), respectively. 5-Hydroxymaltol (5-HM) was isolated and confirmed by NMR techniques. The HPLC-TOF/MS analysis revealed the presence of 16 components including major components of morine, gentisic, 4-hydroxybenzoic, vanillic and fumaric acid. The antioxidant activities were evaluated with total antioxidant activity, reducing power, inhibition of lipid peroxidation, metal chelating, free radical and $H_2O_2$ scavenging activities. 5-HM, APEA and APBU exhibited the *in vitro* antioxidant activities in a concentration-dependent and moderate manner. The $IC_{50}$ values for free radical scavenging activity of 5-HM (8.22 μg mL$^{-1}$), $H_2O_2$ scavenging activity for APEA (8.12 μg mL$^{-1}$) and inhibition of lipid peroxidation for APEA (0.93 μg mL$^{-1}$). The 5-HM and APEA have antioxidant capacities and also feasible to apply variety *in vivo* tests.

## 1. Introductıon

Apple fruits are a very important food resource and contain minerals, vitamins, amino acids, sugars, organic acids and phenolic compounds which are useful to humans. The varieties of these compounds determine nutritional value, taste, and flavor of apples so it is a very important source of food for human. The apple fruits are consumed as a mineral salt, pasta, dry-fruit, marmalades, fruit juice, sliced in cans, jam and syrup. Therefore, it may be helpful in reduction of risks of some diseases such as some cancers, heart problems, asthma and diabetes due to its content of different chemical constituents (Brown, 2018). Phenolic compounds in plants are products of the secondary metabolites found and play an important role in protection plants against antimicrobial effects since they synthesize phenolics in normal and stress conditions (Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010; Gülçin, Mshvildadze, Gepdiremen, & Elias, 2006). These compounds polymerize or are conjugated with monosaccharides or organic acids and then stored in vacuoles or cell walls. The phenolic compounds contained in the organs grow gradually to full extent during growth and are distributed in different tissues and fractions (Croteau, Kutchan, & Lewis, 2000). Phenolic compounds are the most important secondary compounds of the derivatives of pentose phosphate, shikimate and phenylpropanoid pathways in plants and are the most abundant compounds among phytochemicals (Pirlak, Ünüvar, & Ersoy, 2017). Many studies indicate the apple to be a fruit that has antioxidant activity and are related to health benefits, such as reducing the risk of antiallergic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic, cardioprotective, vasodilator effects cardiovascular disease, lung cancer, asthma and diabetes due to significant quantities of phenolic compounds (Alberti, et al., 2017; Balasundram, Sundram, & Samman, 2006; Zardo, Silva, Guyot, & Nogueira, 2013).

Turkey in apple production (2.550 thousand tons per year) ranks third in the world and has an important contribution to the Turkish trade (Depci, Kul, & Önal, 2012). Although approximately 90% of the annual apple produced is consumed as fruit, the remaining 10% is used for apple juice production. As a result, approximately 12% by weight of solid residue (AP: apple pulp) is obtained from the apples used in fruit juice production. In the food industry, the solid residue of apple fruit, a residual by-product, is usually a waste material (Ozbay & Yargic, 2018). AP is a non-consumable material that usually spills uncontrollably because it does not damage the environment (Suárez-Garcıa, Martınez-Alonso, & Tascon, 2001). Based on the above background and since waste AP possessed no earlier reports related to its antioxidant activity and heat-resistance phenolic compounds, the objective of this research

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was to investigate the effect of ethyl acetate (EA) and *n*-butanol (BU) extracts on *in vitro* antioxidant capacity, and contribute to the isolated 5-hydroxymaltol (5-HM). The analysis of the phenolics in the APEA and APBU extracts, purification and characterization of 5-HM were carried out by Sephadex LH-20 column, silica gel column chromatography and characterized using HPLC-TOF/MS, 1D and 2D NMR and studied the antioxidant properties. The antioxidant activities of 5-HM, APEA, and APBU were determined by the assays such as total antioxidant activity, reducing power, inhibition of lipid peroxidation, metal chelating, free radical and $H_2O_2$ scavenging activity.

## 2. Materials and methods

### 2.1. Chemicals and solvents

Silica gel, formic acid, acetonitrile, trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), EDTA, $K_3Fe(CN)_6$, sodium phosphate, $K_2HPO_4$, $KH_2PO_4$, ascorbic acid, DPPH˙, linoleic acid, α-tocopherol, ammonium molybdate, $FeCl_2$, $FeCl_3$ ve $Na_2S_2O_3$ were obtained from Sigma Chemical Co.

The organic solvents (ethyl acetate, methanol, hexane, dichloromethane, chloroform, *n*-butanol used in analyses were of HPLC grade and purchased from E. Merck. All the other chemicals were supplied from other commercial sources.

### 2.2. Preparation of samples

Apple pulps were supplied from DIMES juice factory (Tokat, Turkey) at 2016 September, and dried room temperature in sunless storage throughout one week. 5 kg dry AP was boiled in reflux system for 2 h with distilled water and subjected to aqueous ethyl acetate and *n*-butanol extraction, respectively (Demirtas, Erenler, Elmastas, & Goktasoglu, 2013; Ipek, Ozen, & Demirtas, 2017). The water extract was separated by filtration and extracted with ethyl acetate. This process was continued until the significant quantities of the aqueous components were transferred to the ethyl acetate. When this process was carried out three times, the extraction with ethyl acetate was completed. The remaining water extract was subjected to extraction in the same manner as *n*-butanol extract (Supplementary Fig. 1). The APEA and APBU extracts were separated from the solvent by rotary evaporation to receive dry crude extracts.

### 2.3. Fractionation process

To determine the target fraction, the AP was subjected to extraction. The fractions obtained from the raw fruit pulp extracts and taken into the separating funnel were fractionated using the changing solvent system from nonpolar to polar. Each fraction was collected separately and the fraction solvents were removed on a rotary evaporator.

### 2.4. Column chromatography

For column chromatographic studies, different sizes and specific columns were used. Column chromatography was performed to separate out the extracted components resulting from the fractionation process. The extract was dissolved in a suitable solvent and then a quantity of silica gel was added. The solvent was removed by rotary evaporation and allowed the mixture of extract to be absorbed on silica gel. After the silica gel and extract solvent were left to stand overnight at room temperature, the solvent was completely removed. The column was filled with silica gel, placed in its walls, and allowed to fill the column with silica gel. The mixture of silica and extract was filled into the colon, and then the glass wool/cotton/sea sand was placed.

### 2.5. HPLC-TOF/MS analysis

Phenolic component analysis of AP agents was quantitatively performed with Agilent brand 1260 Infinity HPLC model and 6210 Time of Flight LC/MS detector and ZORBAX SB-C18 (4.6 × 100 mm, 3.5 μm) column. Ultrapure water and acetonitrile with 1% formic acid were used as the mobile phase. As an application program, the flow rate was 0.6 mL per minute, the injection volume was 10 μL and the column temperature was 35 °C. Programming was performed so that the solvent was passed through the column according to the solvent application program in the range of 0–1 min, 10% water, 1–20 min, 50% acetonitrile, 20–23 min, and 23–30 min.

### 2.6. NMR spectroscopy

The exact structure of 5-hydroxymaltol isolated by chromatographic methods was determined by $^1H$, $^{13}C$, $^{13}C$-DEPT, $^1H$–$^{13}C$-HSQC, $^1H$–$^{13}C$-HMBC and COSY NMR using a 600 MHz NMR instrument.

### 2.7. Biological activity

#### 2.7.1. Total antioxidant activity by phosphomolybdenum method

The activity was evaluated by phosphomolybdenum method (Prieto, Pineda, & Aguilar, 1999). This method was based on the reduction of Mo(VI) to Mo(V) by the sample and the subsequent formation of specific green phosphate-Mo(V) compounds at acidic pH. Briefly, a reagent mixture (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was combined with 0.3 mL of samples and standards in a test tube. All samples were incubated in water bath at 95 °C for 90 min. After cooling to room temperature, the absorbance of the mixture was recorded at 695 nm. The total antioxidant activity was expressed as μmol α-tocopherol equivalent/g.

#### 2.7.2. Reducing capacity

The sample was estimated monitoring the $Fe^{3+}$/ferricyanide complex the reducing power test at 700 nm (Oyaizu, 1986). Of 1 mL sample was mixed with of 0.2 M phosphate buffer (2.5 mL, pH 6.6) and $K_3Fe(CN)_6$ (2.5 mL, 1%). After incubating at 50 °C, TCA (2.5 mL, 10%) was added. The 2.5 mL of the mixture was mixed with $FeCl_3$ (0.5 mL, 0.1%) and 2.5 mL water. The absorbance of the blue-green color was measured at 700 nm and recorded changes to green depending on the reducing capacity. It is indicated that a higher absorbance of the sample at 700 nm has good reducing capacity in the assay.

#### 2.7.3. Free radical (DPPH˙) scavenging activity

The activity was evaluated by bleaching of the purple-colored solution of DPPH˙ according to the technique reported by Blois with a slight modification (1958). In this assay, the bleaching rate of a stable free radical (DPPH˙) is monitored at a characteristic wavelength (517 nm) in the presence of samples. The test sample was mixed with DPPH˙ (0.1 mM, 0.5 mL). The mixtures were incubated at room temperature and set for 10 min. The absorbance was recorded at 517 nm due to proton donating activity ($DPPH_2$) by samples and compared with standards. The result of the reaction was the change of color from purple to yellow with the antioxidant agent and measured a decrease of molar absorptivity of DPPH˙ radical. The percentage radical scavenging activity was calculated by the following formula: Free radical scavenging activity, % = $[(A_{control}-A_{sample})/A_{control}] \times 100$.

#### 2.7.4. Metal chelating activity

The activity was determined to inhibit the formation of ferrozine-$Fe^{2+}$ after adding of a sample with $Fe^{2+}$, tested according to references (Dinis, Madeira, & Almeida, 1994). Ferrozine can form complex with ferrozine-$Fe^{2+}$, effectively. In the presence of a chelating sample, this complex is disrupted with a chelator phenolic compounds. The rate of red color reduction allows evaluating a level of the chelating capacity.

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