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ORIGINAL ARTICLE

Polyphenol oxidase activity and antioxidant properties of Yomra apple (*Malus communis* L.) from Turkey

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Abstract

In this study, firstly, antioxidant and polyphenol oxidase (PPO) properties of Yomra apple were investigated. Seventeen phenolic constituents were measured by reverse phase-high-performance liquid chromatography (RP-HPLC). Total phenolic compounds (TPCs), ferric reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging activities were performed to measure antioxidant capacity. Some kinetic parameters (K_m, V_{max}), and inhibition behaviors against five different substrates were measured in the crude extract. Catechin and chlorogenic acid were found as the major components in the methanolic extract, while ferulic acid, caffeic acid, *p*-hydroxybenzoic acid, quercetin and *p*-coumaric acid were small quantities. K_m values ranged from 0.70 to 10.10 mM in the substrates, and also 3-(4-hydroxyphenyl) propanoic acid (HPPA) and L-DOPA showed the highest affinity. The inhibition constant of K_i were ranged from 0.05 to 14.90 mM against sodium metabisulphite, ascorbic acid, sodium azide and benzoic acid, while ascorbic acid and sodium metabisulphite were the best inhibitors.

Keywords

Antioxidant, *Malus communis* L., phenolics, polyphenoloxidase, Yomra apple

History

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Introduction

Apples contain many compounds, such as sugars, alcohols, organic acids, vitamins, amino acids and phenolic compounds that are beneficial to humans. The amounts and varieties of these compounds determine apples' nutritional value, taste and flavor[1]. Phenolic compounds are ubiquitous in plants and are the largest secondary metabolite derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways[2]. Composition and amount of phenolic substances depend on genetic and numerous environmental factors[3]. These play an important role in growth and reproduction, as well as providing protection against pathogens and predators, in addition to contributing towards the color and sensory characteristics of fruits and vegetables. When plant foods are consumed, these phytochemicals contribute to the intake of natural antioxidants in the human diet[4]. A direct relationship has also been determined between phenolic content and antioxidant capacity in many natural agents. Epidemiological studies have shown that consumption of natural products reduces cardiovascular diseases and cancers[5,6]. Apple is one of the most popular fruit juices in the world. In addition to its vitamin and mineral content, apple juice is characterized by a high level of phenolic compounds with antioxidant capacity. The main phenolic compounds of apples are flavonols, flavanols, dihydrochalcones and derivates of hydroxycinnamic acid, present as free or mono, di- and oligomers glycosides[7,8].

Enzymatic browning in fruits and vegetables is an undesirable reaction, the prevention of which has always been a challenge to food scientists[9]. Polyphenol oxidase (PPO) is a widely distributed copper containing enzyme in plants. It is known to be responsible for the enzymatic browning reaction occurring during the handling, storage and processing of fruits and vegetables. Its activity in the presence of molecular oxygen occurs with two different reactions. The first activity, monophenolase or cresolase (E.C.1.14.18.1), catalyzes the hydroxylation of monophenols to *o*-diphenols. The second activity of PPO, diphenolase or catecholase (E.C.1.10.3.2), consists of the oxidation of the *o*-diphenols to the corresponding *o*-quinones, which are highly reactive molecules and polymerize to brown, red or black pigments depending on natural components present in a given plant material[10,11]. In plant tissues, the browning pigments lead to organoleptic and nutritional modifications, thus compromising the food product[12,13]. One of the important problems faced by the fruit juice industry is the presence of seeds and browning occurring during processing. Fruit seeds and PPO give juice an unpleasant appearance and taste. Seedless fruits and control of the PPO activity are therefore very important for the fruit juice industry. Moreover, investigation of PPO enzyme in natural extracts of fruits such as apple, which is again important for the fruit industry, will be highly effective and beneficial in the prevention of browning.

Yomra apple is a cultivar of the *Malus communis* type belonging to the family Rosaceae. It is cultivated around the Yomra district of Trabzon, in the Eastern Black Sea region of Turkey, from which it takes its name. The fruit ripens in fall,

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especially in November, and can be kept for a long time after being harvested. It maintains its flavor while the seeds disappear during the ripening period. Yomra apples are red, claret red, orange or yellow against a green background, depending on insolation. The apple has many desirable characteristics, such as being firm, juicy and sourish. Yomra apples are often used as gifts since they are believed to be having healthy benefits in the sick and elderly. The Yomra apple has a thin skin and is generally consumed without being peeled. It does not require the use of agricultural pesticides since it is immune to infestation[14]. There are only limited studies in the literature concerning the Yomra apple, and this is the first detailed investigation of its phenolic composition, antioxidant capacity and PPO enzyme activity.

Material and methods

Chemicals

Seventeen different standards (purity > 99.0%) (gallic acid, protocatechuic acid, *p*-hydroxy benzoic acid, vanillic acid, caffeic acid, chlorogenic acid, syringic acid, epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, *o*-coumaric acid, trans-cinnamic acid, abscisic acid, catechin, rutin, quercetin) and propylparaben, used as internal standard (IS) for high-performance liquid chromatography (HPLC) analysis, and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were supplied by Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany). Methanol, acetic acid, acetonitrile diethyl ether and ethyl acetate used as solvents were obtained from Sigma-Aldrich (St. Louis, MO) and Merck (Darmstadt, Germany). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) and Folin–Ciocalteu's phenol reagent were obtained from Fluka Chemie GmbH (Buchs, Switzerland).

Catechol, 4-methylcatechol (4-MCT), L-3,4-dihydroxyphenylalanine (L-DOPA), L-tyrosine and 3-(4-hydroxyphenyl) propanoic acid (HPPA), dimethylformamide (DMF), 3-methyl-2-benzothiazolinone hydrazone (MBTH), Triton X114 and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO) and the other reagents were of analytical grade and used as obtained.

Plant materials

Ten Yomra apples (~1 kg) were obtained from a local greengrocer after being harvested during the first week of November in Yomra, a small town near Trabzon (Turkey). The fruits were stored at +4 °C until homogenization, and were then stored in a deep freezer (−20 °C) as aliquots.

Extract preparation for antioxidant tests and HPLC analysis

Samples of apple were peeled (2–3 mm thickness), cored and cut into small pieces. The peels and the flesh were pooled separately. Approximately 100 g of samples was blended and extracted with 100 mL bidistilled water and shaken (Heidolph Promax 2020, Schwabach, Germany) for 24 h at room temperature. The crude mixture was then filtered through cotton wool to remove solid particles, and the filtrates were lyophilized (Christ-Alpha 1-4 LD plus, Osterode am Harz, Germany). The lyophilized filtrates were dissolved in 10 mL distilled water, adjusted to pH 2 with 0.1 M HCI and sonicated (Elma® Transsonic Digital, Singen, Germany) for 3 h. Acidic hydrolysis was performed to break down the glycosidic bonds between phenolic substances and carbohydrates. Liquid–liquid extractions with diethyl ether and ethyl acetate were then performed three times consecutively. Organic phases were picked up and evaporated until dry under reduced pressure in a rotary evaporator at 40 °C. The residues were weighed and dissolved in methanol until used for HPLC analysis and antioxidant tests.

Crude enzyme extraction

One hundred grams of Yomra apple fruits were placed in a Dewar flask under liquid nitrogen for 10 min in order to decompose the cell membrane before being cracked with a blender. Cold fruits were homogenized in the blender in 100 mL of 50 mM cold acetate buffer (pH 5.5) containing, 6% (w/v) Triton X-114, 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM PMSF for 5 min at 4 °C. PMSF was used as the protease inhibitor. The homogenate was filtered and kept at 4 °C for 60 min before being centrifuged at 20 000 × *g* for 30 min at 4 °C. The supernatant was used as crude enzyme extracts and maintained PPO activity for 1 month at 4 °C.

Determination of phenolic compounds using HPLC-UV

High-performance liquid chromatography analyses were performed on a Shimadzu LC-UV (Shimadzu, Kyoto, Japan) system. A reverse phase Agilent Zorbax Eclipse XDB-C_{18} column (Agilent Technologies, Waldbronn, Germany) (4.6 mm × 150 mm, 5 μm) and a gradient program with two solvent systems were used[15,16], (A: 80% acetonitrile in methanol, B: 2% acetic acid in distilled water). The elution gradient was established from high polarity and low pH to low polarity and high pH. Gradient; 0–2 min, 95% B; 2–8 min, 95–90% B; 8–11 min, 90–85% B; 11–13 min, 85–75% B; 13–17 min, 75–70% B; 17–30 min, 70–65% B; 30–33 min, 65–0% B; 33–38 min, 0–0% B; 38–40 min 95% B; 40–48 min, 95% B. The injection volume was 50 μL, and the column temperature was regulated at 30 °C in a column oven. A flow rate of 1 mL/min was used, and detection was performed at 280 and 315 nm. In this system, the phenolic compounds were analyzed simultaneously at 280 and 315 nm. The internal standard (IS) technique was applied to the analysis to increase repeatability. Propylparaben was a suitable IS for this system[17]. Limit of detection (LOD) and limit of quantification (LOQ) were calculated to identify and quantify the standards of phenolic compounds (Table 1).

Determination of total phenolic content

Total phenolic content (TPC) was analyzed using Folin–Ciocalteu assay[18], with gallic acid as the standard. For this, 680 μL distilled water, 20 μL aquatic extracts and 400 μL of 0.2 N Folin–Ciocalteu were mixed and then vortexed. After 2 min, 400 μL Na_2CO_3 (% 7.5) was added and the mixture was incubated for 2 h at room temperature. Absorbance was then measured at 760 nm. The concentration of TPCs was calculated as mg of gallic acid equivalents (GAE) per 100 g of fresh weight (FW), using a standard curve for gallic acid in the concentration range between 0.02 and 0.5 mg/mL ($r^2 = 0.997$)

Ferric reducing/antioxidant power assay

Working ferric reducing antioxidant power (FRAP) reagent was obtained as required by mixing 25 mL acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) solution dissolved in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ solution[19]. Next, 100 μL of the sample was mixed with 3 mL of freshly prepared FRAP reagent and incubated for 4 min at 37 °C. Absorbance was read at 595 nm against reagent blank containing distilled water. $FeSO_4 \cdot 7H_2O$ was used a positive control to construct a reference curve (15.625–500 μM, $r^2 = 0.999$), FRAP values were expressed as μM $FeSO_4 \cdot 7H_2O$ equivalent of 100 FW g.

Scavenging of free radical (DPPH) assay

The scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals was assayed using the technique described by Hatano et al.[20].

Table 1. The standard chromatogram values of 17 individual phenolic substances and an internal standard.

No	RT_{Mean}*	Compound	*m*†	*c*‡	r^2	%RSD (RT)	%RSD (AREA)	LOD¶,§	LOQ¶
1	2.97	Gallic acid	0.23	0.16	0.9998	0.320	2.377	0.042	0.126
2	5.77	Protocatechuic acid	0.11	0.076	0.9998	0.183	3.842	0.048	0.144
3	10.35	*p*-Hydroxy benzoic acid	0.15	0.10	0.9998	0.119	4.966	0.038	0.114
4	12.06	Catechin	0.05	0.03	0.9998	0.115	2.124	0.028	0.085
5	12.97	Chlorogenic acid	133.32	−74.34	0.9998	0.107	8.732	0.039	0.119
6	14.13	Vanillic acid	0.13	0.09	0.9998	0.094	2.692	0.029	0.088
7	14.62	Caffeic acid	239.10	−49.54	0.9997	0.094	1.928	0.037	0.112
8	16.08	Syringic acid	0.24	0.16	0.9998	0.085	2.474	0.046	0.138
9	17.02	Epicatechin	0.05	0.03	0.9998	0.073	5.222	0.036	0.108
10	18.61	*p*-Coumaric acid	350.6	−95.40	0.9995	0.054	0.517	0.104	0.312
11	19.42	Rutin	44.76	−10.64	0.9990	0.077	3.709	0.032	0.097
12	19.83	Ferulic acid	287.69	72.03	0.9999	0.745	0.897	0.054	0.162
13	21.63	Benzoic acid	0.03	0.02	0.9995	0.075	1.854	0.033	0.101
14	22.29	*o*-Coumaric acid	0.48	0.34	0.9998	0.076	1.687	0.036	0.109
15	26.45	cis.trans-Abscisic acid	0.38	0.26	0.9998	0.089	2.138	0.024	0.072
16	28.41	Quercetin	51.96	−6.73	0.9999	0.131	3.956	0.027	0.083
17	29.53	trans-Cinnamic acid	0.73	0.49	0.9998	0.114	4.554	0.036	0.109
18	36.91	Propylparaben (IS)				0.026	3.193	0.293	0.880

*Time is expressed as minute.
†Slope.
‡Intercept.
¶Values are expressed as mg/L.
§The number of significant digits of values of LOD and LOQ is given three-digit after comma.

The method is based on the fact that the radical is purple in color, and that the purple color of DPPH decays in the presence of an antioxidant agent. The change in absorbance can then be spectrophotometrically monitored at 517 nm. Briefly, 1.5 mL of the aquatic solution was mixed with 1.5 mL of 0.1 mM DPPH (dissolved in methanol), and the mixture was vortexed and incubated for 50 min in the dark at room temperature until stable absorbance values were obtained. After the incubation period, the absorbance was recorded at 517 nm against a blank and control. The control solution contained DPPH solution with no sample. The results were expressed as SC_{50} (mg/mL), which was calculated from the curves by plotting absorbance values, the SC_{50} values representing the concentration of the extract (mg/mL) required to inhibit 50% of the radicals.

Crude PPO activity

Polyphenol oxidase activity was determined by the method described previously[21]. Activity was determined using different substrates by measuring the increase in absorbance at 494 nm for 4-MCT and 500 nm for the other substrates[21]. For enzyme activity, 1 mL of the extract was placed in a cuvette containing various concentrations of substrates (stock 100 mM), an equal volume of substrate solution, 3-methyl-2-benzothiazolinone hydrazone (MBTH, stock 10 mM), 20 µL DMF, the solution was diluted to 950 µL with buffer and 50 µL enzyme extract was lastly added. The blank sample contained the same concentration of solution, except for 50 µL enzyme extract in 1 mL[21]. One unit of PPO activity was defined as the amount of enzyme causing an increase in absorbance of 0.001 per minute in 1 mL reaction mixture[22].

Optimum pH and temperature

In order to determine optimum pH values for each of the substrates, crude PPO activity was measured at different pHs (4.5–9.5) using 50 mM buffer systems, namely acetate (pH 4.5–5.5), MOPS (pH 6.5–7.5), phosphate (pH 7.5–9.5) and Tris–HCl (pH 8.5–9.5).

Similar to optimum pH, crude PPO activity was determined at various temperatures controlled using a circulation water bath (Heidolph D-91126, Schwabach, Germany). The crude enzyme in a reaction mixture was incubated for 5 min at various temperatures in the range of 5–80 °C at the optimum substrate pH, prior to the addition of the enzyme solution. The relative activity of PPO at a specific temperature was determined spectrophotometrically by addition of enzyme extract into the mixture as quickly as possible.

Enzyme kinetics and substrate specificity

Crude PPO activity of Yomra apple was assayed using catechol, 4-MTC and L-DOPA as diphenolic substrates, and L-tyrosine and HPPA as monophenolic substrates[21] in buffers at optimum pH and temperature values for each substrate. The kinetic data for each substrate were plotted as reciprocals of activities versus substrate concentrations following the method described by Lineweaver–Burk[23]. Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were measured from the linear regression curve.

Effect of inhibitors

Sodium azide (1–20 mM), benzoic acid (1–10 mM), ascorbic acid (0.1–1 mM) and sodium metabisulphite (0.1–0.5 mM) were evaluated for their effectiveness as inhibitors of crude PPO activity using 4-MCT as the substrate. One milliliter of reaction mixture contained 0.1 mL of 4-MCT at various concentrations (1.0–32 mM) in 50 mM phosphate buffer (pH 6.5), 0.05 mL of crude enzyme extract and 0.1 mL of inhibitor solutions at fixed concentrations. I_{50} values were calculated from the plots of inhibitor concentration versus percentage inhibition of catechol oxidation. The Lineweaver–Burk curves obtained were used for the determination of K_i and the inhibitor type. To determine K_i values, three different inhibitor concentrations (1.0–10 mM) were also tested.

Results and discussion

This is the first study to investigate the phenolic compounds, antioxidant properties and PPO enzyme activity of Yomra apple. TPCs responsible for the apple's antioxidant and other biological properties were measured as gallic acid equivalent using Folin's technique. TPC and antioxidant values measured in the two separate parts of the apple are shown in Table 2. TPC level in the peel was ~2.5 times higher than that in the pulp. Other studies

Table 2. Antioxidant activity measured by TPC, FRAP and DPPH assays in the peel and flesh of Yomra apple.

Tissue	Total phenolics* (mg GAE/ 100 g FW)	FRAP* (μmol $FeSO_4 \cdot 7H_2O$/ 100 g FW)	DPPH SC_{50}* (mg/mL)
Peels	182.5 ± 37.25†	123.60 ± 45.62†	0.93 ± 0.01†
Flesh	70.2 ± 1.30‡	51.50 ± 3.03‡	2.59 ± 0.17‡
Total	252.70 ± 38.55	175.20 ± 97.12	3.83 ± 0.18

*Values are means of triplicate determinations.
†,‡Values with differences symbols in a column are significantly different to each other (Mann–Whitney test $p < 0.05$).

have also reported greater levels of phenolic content in apple peel compared to the pulp[4,8,24,25]. Vieira et al.[25] studied 11 varieties of apple from Brazil and reported TPC in peel (expressed as mg GAE/100 g fresh matter) ranging from 128.3 to 712.6. Vega-Gálvez et al.[26] reported that TPC ranged from 27.04 to 44.84 mg GA/100 g FM in sliced apple (var. Granny Smith) dried with different velocities of air. Compared with these values reported in the literature, Yomra apple has a high phenolic content. Amount of TPC is a reflection of biological activity, and plants with a high phenolic content always have antioxidant, antibacterial, anti-tumor, anti-viral and anti-inflammatory capacities[27–30]. Yomra apple also therefore possesses a high biological activity potential.

Only the antioxidant properties of Yomra apple were investigated as a test of biological activity in this study. Various different techniques are used to measure natural products and antioxidant capacities. However, TPC, FRAP and DPPH are the most widely employed tests, and are also sensitive, reliable and easy to perform. The FRAP test measures the ability of components in a polar solution environment to convert the Fe-III–TPTZ complex into the Fe-II–TPTZ complex, and the emerging colored complex gives absorbance at 595 nm[19]. High absorbance shows high antioxidant activity. FRAP value results are also given in Table 2. Parallel to TPC, antioxidant or FRAP, activity in the peel was ~2.5 times greater than that in the pulp. Many studies have shown that antioxidant capacity rises in line with phenolic material content[6,31,32]. The antioxidant properties of phenolic agents may result from the greater H-atom donating ability of phenolic acids and flavonoids to several radicals, thus terminating the chain radical reaction[33]. As with TPC, since there are no previous antioxidant studies involving Yomra apple, we had no means of comparing the FRAP values. However, Yomra apple has a high antioxidant capacity compared with other different plants or natural products reported in the literature[24–26].

The ability of the aquatic extract of Yomra apple to scavenge free radicals was measured as DPPH radicals. Values for SC_{50} are given in Table 2. Since low SC_{50} values indicate a high antioxidant capacity, the scavenging property of the pulp was calculated as ~3 times greater than that of the pulp.

Plants possess the ability to synthesize countless phenolic compounds. The presence of ~4000–6000 phenolic compounds has been described to date[34]. However, it is impossible to elucidate all phenolic compounds in studies of the phenolic composition of natural products. It is, however, possible to establish levels of TPC using the Folin Ciocalteu assay. Since it is impossible to describe every phenolic compound in studies performed, only the major phenolic acids and some flavonoids that may be present in plants are clarified using chromatographic analysis. For that reason, we measured only 17 phenolic substances using reversed phase-HPLC (RP-HPLC) in this study. Detection in the range of 315–280 nm is the most generally used wavelength for separation of mixtures of phenolic acids. These were identified by comparison of retention times (peak normalization) with those of authentic standards. The calibration parameters of the phenolic compound standards with their respective standard deviation (RSD), correlation coefficient (r^2), limit of detection (LOD) and limit of quantification (LOQ) values are given in Table 1. Linear results were obtained for all the phenolic compounds ($r^2 > 0.9998$). The phenolic profiles and quantities are given as mg/100 g FW in Table 3. Of the 17 phenolic standards, 7 substances were detected in the sample extract of Yomra apple: *p*-OH benzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, catechin and quercetin. Although both parts of the Yomra apple contain the same compounds, the peel is richer in quantitative terms.

Catechin (19.20 mg/100 g FW) was the most abundant phenolic in hydrolyzed extracts, followed by chlorogenic acid (12.15 mg/100 g FW) and ferulic acid (0.73 mg/100 g FW) among the compounds detected. These findings are in accordance with those reported for other apple species[8,35]. Chlorogenic acid is derived from hydroxycinnamic, and catechin is substituent of flavonoid derivative. Chlorogenic acid and epicatechin have been reported as the predominant polyphenolic compounds in apple cores (cultivar Idared) and to represent 77% of total polyphenols[36]. It is interesting to note that gallic acid, rutin, epicathecin, vanillic acid, syringic acid, ferulic acid, benzoic acid and trans-cinnamic acid were not detected in the Yomra apple. Chlorogenic acid and catechin have been reported as the major phenolic compounds in Honeycrisp apple[1], that value being similar to that in Yomra apple. Epicatechin, gallic acid, rutin, ferulic acid, syringic acid have also been detected in the Honeycrisp appple. However, gallic, protocatechuic, chlorogenic, caffeic, ferulic and *p*-coumaric acids, epicatechin, catechin, quercetin and rutin have been identified in different apple varieties[37].

Crude PPO enzyme was characterized with no purification, since this study was only a preliminary one. The crude enzyme extraction was prepared in cold aquatic mixture containing Triton X114, EDTA, $MgCI_2$ and PMSF to characterize properties of the PPO from Yomra apple. Crude enzyme activity was assayed at different pHs (4.0–9.0) using assorted buffers (acetate, pH 4.5–5.5; MOPS, pH 6.5–7.5 and Tris–HCl, pH 8.5–9.5) in the presence of the five different substrates. The optimum pH of PPO enzyme against the five substrates of the crude PPO was determined between 5.0 and 7.0 (Table 4). It seems that although tyrosine and catechol were oxidized by PPO in acidic solution (pH 5.0), HPPA, 4-MCT and L-DOPA were oxidized at neutral pH (7.0). The optimum pH for PPO enzyme varies in many fruits, but the maximum PPO activity in most plants is between 5.0 and 7.0[38]. However, the optimal pH value of PPO has been reported as 8.5 for catechol and 6.5 for 4-MCT and L-DOPA as substrates in medlar (*Mespilus germanica* L.) fruits[39]. Similar to our study, optimum pH for PPO enzyme has been reported to range from 5.5 to 7.5 for persimmon[42] and Jonagored apple[40] against 4-methyl catechol, catechol and DOPA. Similar pH behavior has also been reported for other fruits of the family Rosaceae[38,41,42]. Similarly, pH optimum for PPO enzyme was reported as 7.0 for Amasya apple[43]. The pH optimum for a PPO enzyme is clearly highly dependent on the enzyme sources and the nature of the substrate[41].

The optimum temperature of the crude enzyme was measured over an initial time period and determined at different temperatures ranging from 0 °C to 80 °C. The results indicated that the crude PPO activity against the substrates ranged from 20 °C to 40 °C (Table 4). The optimum temperature for monophenolase activity of Yomra apple PPO was 30 °C for HPPA and 20 °C for L-tyrosine. The optimum temperature was 40 °C for catechol, 4-MCT and L-DOPA as diphenolic substrates for diphenolase activity. Optimum temperatures for PPO of peach[44], grape[45] and plum[46] have been reported as 20 °C, 25 °C and 37 °C, respectively.

It appears that crude PPO is sensitive to increases in temperature. Yomra apple PPO was almost inactivated >30 °C in the presence of monophenol substrates, while diphenolic substrates were more activated >30 °C. The optimal temperature values for PPO from persimmon fruits using 4-MCT, catechol and L-DOPA were 40 °C, 20 °C and 10 °C, respectively[42], 25 °C from medlar with catechol as substrate[39] and 60 °C from leaves of *Cleome gynandra* L.[47].

Polyphenol oxidase activities for Yomra apple were assayed using catechol, 4-MCT and L-DOPA as diphenolic substrates and L-tyrosine, HPPA as monophenolic substrates in this study. Either monophenolic or diphenolic substrates were oxidized by the crude enzyme from the Yomra apple. Lineweaver–Burk plots for the kinetic analysis of the reaction rates, at a series of concentrations for each substrate, are presented in individual V_{max} and K_m values (Table 4). All the kinetic investigations were carried out at optimum pH and temperature. The V_{max}/K_m ratio was taken as the criterion in order to evaluate the substrate specificity[48].

Although the catalytic efficiency (V_{max}) was lowest, substrate binding (lower K_m value) was highest for L-DOPA in diphenolic substrates. In contrast, 4-MCT was oxidized by Yomra apple PPO at a much higher rate (V_{max} 24.80 μM/min), with the highest K_m (10.10 mM) value. The values of catalytic efficiency, V_{max}/K_m, indicated that catechol and 4-MCT were the most suitable phenolic substrates for Yomra apple PPO (Table 4). This result agrees with previous reports in which diphenolic susbtrates were most suitable for PPOs[39,41,42].

Table 3. Phenolic constituents of Yomra apple of determined by RP-HPLC-UV.

Phenolic compounds	Peels (mg/100 g FW)	Flesh (mg/100 g FW)
Gallic acid	–	–
Protocatechuic acid	–	–
p-Hydroxybenzoic acid	–	0.12
Catechin	19.20	4.00
Chlorogenic acid	12.15	7.86
Vanillic acid	–	–
Caffeic acid	0.23	0.12
Syringic acid	–	–
Epicatechin	–	–
p-Coumaric acid	–	0.02
Rutin	–	–
Ferulic acid	0.73	0.07
Benzoic acid	–	–
o-Coumaric acid	–	–
Abscisic acid	–	–
Quercetin	0.04	0.08
t-Cinnamic acid	–	–
Proplyparaben	IS*	IS*

–, not detected; *, Internal Standard.

Table 4. Optimization and kinetic characterization of Yomra apple PPO-catalyzed oxidation reactions againts monophenolic and diphenolic substrates.

Substrate	pH* optimum	Temperature optimum* (°C)	V_{max}* (μM/min)	K_m* (mM)	V_{max}/K_m (min^{-1})
Monophenols					
L-Tyrosine	5.0	20	7.60	9.30	$0.82.10^{-3}$
HPPA	7.0	30	1.02	0.70	$1.46.10^{-3}$
Diphenols					
Catechol	5.0	40	21.50	7.90	$2.72.10^{-3}$
4-MCT	7.0	40	24.80	10.10	$2.46.10^{-3}$
L-DOPA	7.0	40	1.60	2.50	$0.64.10^{-3}$

*Values are means of triplicate determinations.

Table 5. Sensitivity of Yomra apple PPO catalyzed oxidation of 4-MCT to some common PPO inhibitors.

Inhibitor	K_i (mM)	I_{50} (mM)
Sodium metabisulphite	0.05 ± 0.01*	0.07 ± 0.02*
Ascorbic acid	0.05 ± 0.01*	0.05 ± 0.01*
Sodium azid	14.90 ± 1.10†	30.04 ± 2.55†
Benzoic acid	7.80 ± 0.85‡	9.80 ± 1.76†

*,†,‡Values with different symbols in a column are significantly different to each other (Mann–Whitney test. $p < 0.05$).

The effects of well-known inhibitors, sodium azide (1.0–50 mM), benzoic acid (1.0–10 mM), ascorbic acid (0.05–1.0 mM) and sodium metabisulphite (0.05–0.5 mM), were examined to determine their potential to inhibit 4-MCT oxidation by the crude PPO of Yomra apple (Table 5). The I_{50} values and inhibition constants (K_i) were compared with those of the control (100% activity). The decreased K_i value indicates a better binding (lower K_i) effect of the enzyme. A high positive correlation (r^2: 0.9982) was determined between the inhibition constant (K_i) and I_{50} values of the studied inhibitors for crude PPO. Although the inhibitors used in this study exhibited complete inhibition on the crude PPO of Yomra apple, ascorbic acid and metabisulphite were the most effective inhibitors. The inhibition constants for the reducing agents of ascorbic acid and metabisulphite are several times lower than those for benzoic acid and azide. The general mechanisms of PPO inhibition have been reviewed previously[49,50]. The inhibition values indicate that ascorbic acid and thiol compounds are potent in inhibiting the crude PPO of Yomra apple, and the results are in agreement with previous reports regarding the inhibition of PPO enzyme in many fruits[39,42,51,52].

We conclude that crude extracts prepared from the Yomra apple have a PPO activity very similar to those of other apples and fruits. The enzyme exhibited oxidase activity towards diphenolic and monophenolic substrates. Yomra apple PPO has the greatest substrate specificity to catechol for diphenolase activity and to HPPA for monophenolase activity among the substrates employed. In accordance with the results obtained from other studies on plant PPOs, the optimal pH and temperature for the enzyme activity in the presence of these substrates were ~5.0–7.0 °C and 30–40 °C, respectively. Moreover, Yomra apple PPO activity was very sensitive to some of the general PPO inhibitors, and especially to ascorbic acid and metabisulphite.

To summarize, evaluated in terms of antioxidant activity, the Yomra apple is a fruit with a high phenolic content and associated antioxidant capacity. This means that it is more beneficial to human health for the apple to be consumed without removing the peel. These excellent characteristic of the Yomra apple mean that the species is suitable for organic agriculture, and cultures should be prepared and production increased.

Declaration of interest

The authors declare that they have no conflict of interests. One of author (H.S.) was supported by TUBITAK BIDEB for his Ph.D. study.

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