

International Journal of Food Properties

ISSN: 1094-2912 (Print) 1532-2386 (Online) Journal homepage: https://www.tandfonline.com/loi/ljfp20

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To cite this article: Nimet Baltas, Semra Pakyildiz, Zehra Can, Barbaros Dincer & Sevgi Kolayli (2017) Biochemical properties of partially purified polyphenol oxidase and phenolic compounds of *Prunus spinosa* L. subsp. *dasyphylla* as measured by HPLC-UV, International Journal of Food Properties, 20:sup2, 1377-1391, DOI: 10.1080/10942912.2017.1343349

To link to this article: https://doi.org/10.1080/10942912.2017.1343349

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Accepted author version posted online: 23 Jun 2017.
Published online: 17 Dec 2017.

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INTERNATIONAL JOURNAL OF FOOD PROPERTIES
2017, VOL. 20, NO. S2, S1377–S1391
https://doi.org/10.1080/10942912.2017.1343349

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Biochemical properties of partially purified polyphenol oxidase and phenolic compounds of *Prunus spinosa* L. subsp. *dasyphylla* as measured by HPLC-UV

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ABSTRACT

Polyphenol oxidase (PPO) enzyme was extracted from *Prunus spinosa* L. subsp. dasyphylla plum, partially purified by acetone precipitation, and its biochemical properties were investigated. Different substrates (*p*-coumaric acid, L-tyrosine, *p*-hydroxyphenyl propionic acid (PHPPA), catechol, 4-methylcatechol (4-MTC), hydrocaffeic acid, gallic acid, quercetin, catechin, and epicatechin) were analysed to determine their affinities with *Prunus spinosa* PPO (PsPPO). The substrate specificity was in the following order: 4-MTC > catechol > hydrocaffeic acid > catechin > epicatechin. 4-MTC was the most suitable substrate (K_m = 0.97 mM and V_{max} = 4753 U/mg protein). The optimal pH values were 7.0 for 4-MTC and catechol, 5.0 for catechin and epicatechin, and 4.0–6.0 for hydrocaffeic acid. Optimal temperatures were 40°C for 4-MTC, 30°C for catechol, and 60°C for catechin, epicatechin, and hydrocaffeic acid. In the inhibition tests, the most potent inhibitor was found to be sodium metabisulphite (IC_{50} = 0.01 mM), followed by ascorbic acid, thiourea, benzoic acid, L-cysteine, and sodium azide. Approximately 80 and 75% of the diphenolase activity was conserved at pH 5.0 and 7.0, respectively, at 4°C after 7 d incubation. Moreover, native polyacrylamide gel electrophoresis (Native-PAGE) of the enriched extract revealed the presence of at least six bands with PPO activity, suggesting the existence of different PPO isoforms. However, the oxidation of diphenol related to browning significantly, so the data obtained in this research provided a basis for the prevention of enzymatic browning of *Prunus spinosa* during processing. High-performance liquid chromatography (HPLC) revealed the plum extract contained protocatechuic acid, *p*-OH benzoic acid, vanillic acid, syringic acid, epicatechin, *p*-coumaric acid, and luteolin.

ARTICLE HISTORY
Received 27 January 2017-01
Accepted 14 June 2017

KEYWORDS
Characterisation; Polyphenol oxidase; *Prunus spinosa* L. subsp. *Dasyphylla*; Plum; Phenolic compound

Introduction

Polyphenol oxidase (PPO) is a common copper-containing enzyme with a dinuclear copper centre that catalyses two distinct reactions involving molecular oxygen, namely the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity; E.C. 1.14.18.1) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity; E.C. 1.10.3.2).[1,2] The resulting *o*-quinones easily polymerise or react with a variety of cellular constituents, such as amino acids and proteins, to form complex brown, red, or black pigments. These reactions also produce undesirable browning in products during food processing, or in post-harvest of plant products, resulting in a decrease in their sensory properties and nutritional and economic value.[1,2,3] Many researchers have characterized or purified

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PPO from various fruits and vegetables, such as Stanley plum,[4] medlar fruits,[5] mulberry fruit,[6] persimmon (*Diospyros kaki* L.),[3,7] mamey,[8] mango,[9] wolf's apple,[10] Yomra apple (*Malus communis* L.),[11] Ataulfo mango,[12] chestnut kernel,[13] Hemşin apple,[14] *Pyrus elaeagnifolia*,[15] Turkish Alyanak apricot (*Prunus armenica* L.),[16] cape gooseberry,[17]and *Diospyros lotus*.[18]

Prunus spinosa subsp. *dasyphylla*, commonly known as "guvem" in Turkish, is a wild plum species of the Rosaceae family that is grown in various regions in Turkey (particularly in central, north, west, and south Anatolia) and bears a red-black-bluish, soft and edible fruit on ripening. Ethnobotanical studies have reported that the fruit of *P. spinosa* subsp. *dasyphylla* has been used as an astringent, diuretic, purgative, and decoction in the treatment of cardiac diseases in the European part of Turkey.[19,20]

Plant polyphenols are a large and diverse group of secondary metabolites commonly found in our diet, particularly in fruits, cereals, vegetables, and beverages. Various classes of polyphenols exist based on their structural characteristics. One of the main classes are the phenolic compounds, such as phenolic acids, flavonoids, procyanidins, and anthocyanins. *P. spinosa* L. extracts are reported to contain a high antioxidant capacity and high phenolic content; however, only a limited number of the phenolic compounds have been identified.[19] *P. spinosa* subsp. *dasyphylla* is widely consumed as fruit, jam, syrup, or marmalade, produced from the whole fruit after deseeding. Enzymatic browning catalyzed by PPO also occurs in *P. spinosa* L. Forestalling of enzymatic browning requires a detailed study of the PPO biochemical properties, which can help reveal suitable methods to control PPO activity and the adverse browning associated with its activity. The aims of this research were to (1) biochemically characterise the PPO of *Prunus spinosa* by determining several selected substrates' specificity, (2) determine their enzyme kinetic parameters, (3) determine the effects of pH and temperature on the enzyme activity in order to find the optimal ranges of work, (4) determine the inhibitory effect of some common PPO inhibitors and metal ions on the enzyme activity, (5) determine the thermal and pH stability of the enzyme, and (6) determine the phenolic compounds in the *Prunus spinosa* plum.

Materials and methods

Material and chemicals

P. spinosa L. subsp. *dasyphylla* plum was collected directly from Gümüşhane province, Turkey, at an altitude of 1300 m, in 2015 November and was stored at −30°C until used. All chemicals were of reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of crude enzyme extract and acetone precipitation

The crude enzyme extract was prepared as reported in the literature.[5,15,18,21] *P. spinosa* plums (30 g) were placed in a Dewar flask under liquid nitrogen for 15 min to disrupt the cell membranes, and then homogenized in a blender in 100 mL of acetate buffer (pH 7.0, 50 mM) containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $MgCl_2$, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 6% w/v Triton X-114 for 20 min. After that, the homogenate was filtered using three layers of cheesecloth and the filtrate was centrifuged at 15,000 rpm, 4°C for 30 min. An equal volume of cold acetone (−30°C) was added to the supernatant and the mixture was incubated overnight at 4°C for protein precipitation. After centrifugation at 10,000 rpm, 4°C for 20 min to remove the acetone, the precipitate was redissolved in an appropriate volume of 50 mM phosphate buffer (pH 7.0). This solution was used as the crude enzyme extract and characterised for its continued PPO activity during storage at 4°C for 6 weeks.

PPO activity assay

Phenolic compounds are the primary substrates of PPO. PPO activity was determined using *p*-coumaric acid, L-tyrosine, and *p*-hydroxyphenyl propionic acid (PHPPA) as the monophenolic substrates; catechol, 4-MTC, and hydrocaffeic acid as the diphenolic substrates; gallic acid as a triphenolic substrate; and quercetin, catechin, and epicatechin as pentaphenolic substrates. The enzyme activity was assayed using a 1 mL sample cuvette containing 100 µL substrate (stock 100 mM), an equal volume of 10 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) and 20 µL dimethylformamide (DMF), and diluted to 980 µL with 50 mM phosphate buffer (pH 7.0). Subsequently, 20 µL of crude enzyme extract was added. The increase in absorbance was measured at 494 nm for 4-MTC and at 500 nm for the remaining substrates, in a UV/visible spectrophotometer (1601UV, Shimadzu, Australia).[22] The reference cuvette included all the reactants, except for the enzyme. One unit of PPO activity was defined as the amount of enzyme causing a 0.1 increase in absorbance per minute, in 1 mL of the reaction mixture.[10]

Protein determination

The soluble protein content was determined according to the Lowry method using bovine serum albumin as the standard.[23]

Native polyacrylamide gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli,[24] by using 10% separating gel. After running the gel, it was incubated in 24 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in 0.1 M phosphate buffer (pH 7.0) at 40°C for 1 h. Then, an appropriate amount of 1 mM ascorbic acid solution was added to the gel to detect the isoenzyme bands.

Substrate specificity and enzyme kinetics

The stock solutions (100 mM) of catechol, 4-MTC, catechin, epicatechin, hydrocaffeic acid, *p*-coumaric acid, L-tyrosine, PHPPA, gallic acid, and quercetin were used to monitor *P. spinosa* PPO (PsPPO) activity. The rate of the reaction was based on the increase in absorbance at the absorption maxima of the corresponding quinone products for each substrate.

The kinetics of *P. spinosa* diphenolase activity for 4-MTC (0.05–0.80 mM), catechol (0.10–1.50 mM), catechin (1.25–15.00 mM), epicatechin (0.31–10.00 mM), and hydrocaffeic acid (0.15–5.00 mM) substrates were analysed as 1/specific activity (1/V) versus 1/substrate concentration (1/[S]). The Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were obtained from the Lineweaver–Burk plots.[25]

pH optimum and stability

The activity of PsPPO was determined as a function of pH, using 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin as substrates (100 mM stock solutions). HAc-NaAc (pH 4.0 and 5.0), phosphate (pH 6.0 and 7.0), Tris–HCl (pH 8.0 and 9.0), and glycine-NaOH (pH 10.0) buffers were used.

For determining the PsPPO pH stability, 0.010 mL of crude enzyme solution was added to 0.990 mL buffer solution (pH 4–7, which covered the optimum pH values for 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin substrates) and stored at 4°C for 24 d. At the end of each storage period, the diphenolase activity was assayed according to the percentage of residual PPO activity at the optimum pH by mixing 0.1 mL of 100 mM 4-MTC as substrate, 0.1 mL of 10 mM MBTH, and 0.02 mL dimethylformamide with the incubated crude enzyme solution.[5,21,26]

Thermal activity and stability

The optimum temperature for diphenolase activity was measured at 10°C increments from 10 to 80° C by using 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin as substrates. To determine the thermal stability of PsPPO, Eppendorf tubes containing the enzyme solution in 50 mM phosphate buffer (pH 7.0) were incubated in a thermoblock at 30, 40m and 60°C for 60 min, rapidly cooled in an ice bath for 5 min, and then brought to 25°C. The enzyme activity was subsequently assayed under the standard assay conditions in the presence of 4-MTC as the substrate. The percentage of residual PsPPO activity was calculated by comparison with the unincubated enzyme.[15,18]

Effect of inhibitors and metal ions

The inhibitory effects of ascorbic acid (12.50–200 μM), sodium azide (6.25–200 mM), benzoic acid (1.56–50 mM), sodium metabisulphite (3.13–50 μM), thiourea (0.78–25 mM), and cysteine (6.25–200 mM) on diphenolase activity were screened in the presence of 4-MTC as the substrate. The concentration of inhibitor causing 50% inhibition of PPO activity (IC_{50}) was determined from a plot of the percentage of the remaining activity against the inhibitor concentration.

PsPPO activity was measured in the presence (1, 5 and 10 mM final concentrations) of various ionic compounds (Na^+, K^+, Ba^{2+}, Cd^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Al^{3+}) under standard assay conditions, with 4-MTC as the substrate. The percentage of the remaining activity was expressed by comparison against a standard assay mixture with no metal ion added using the following equation: inhibition (%) = ((A0−Ai)/A0) × 100, where A0 is the initial PsPPO activity (no metal ion) and Ai is PsPPO activity with the metal ion.[15,21]

Preparation of the *P. spinosa* L. extract

P. spinosa L. extraction was performed by placing 2 g of the sample in a Falcon tube with 25 mL methanol (99%), which was then stirred using a shaker (Heidolph Promax 2020, Schwabach, Germany) at room temperature for 24 h, and then sonicated for 3 h with a sonicator (Elma® Transsonic Digital, Germany). The suspensions were filtered through filter paper (Whatman) and concentrated in a rotary evaporator (IKA-Werke, Staufen-Germany) under reduced pressure at 40° C. The residue was then resuspended in a minimal volume of methanol and stored at 4°C until use.

High-performance Liquid Chromatography Analysis of the Phenolic Compounds

Approximately 2.5 g of the sample was placed into a falcon tube (100 mL), to which 50 mL 99% methanol was then added. The mixture was continuously stirred with a shaker (HeidolphPromax 2020, Schwabach, Germany) at room temperature for 24 h, and then sonicated for 4 h with an ultrasonicator (ultrasonic Elma Scmidbauer GmbH) Germany. Particles were removed using Whatman filter paper and concentrated in a rotary evaporator (IKA-Werke, Staufen, Germany) at 40°C. The residue was redissolved in methanol to a known final concentration and kept at 4°C until use for phenolic compound analysis.

Samples were prepared for high-performance liquid chromatography (HPLC) analysis by dissolving the residue in 10 mL acidified distilled water (pH 2). The sample was then extracted three times with 15 mL of diethyl ether:ethyl acetate mixtures (1:1 v/v). The organic solvents were subsequently removed using a rotary evaporator. The final residue was redissolved in methanol and completed to a final volume of 2 mL with methanol, before HPLC analysis.

The methanolic extract was evaporated until dryness with a rotary evaporator at 40°C. The residue was dissolved in 15 mL acidified distilled water (pH 2). Liquid–liquid extraction was carried out with 5 × 3 mL diethyl ether and 5 × 3 mL ethyl acetate, consecutively. Fourteen phenolic

standards were analysed using an HPLC system (Elite LaChrom Hitachi, Japan) equipped with a reverse-phase C18 column (150 × 4.6 mm, 5 µm; Fortis) maintained at 30°C. Acetonitrile, water, and acetic acid were used for the mobile phase by applying the programmed gradient. The mobile phase consisted of (A) 2% acetic acid in water and (B) acetonitrile:water (70:30). The sample injection volume was 25 µL and the flow rate was 0.75 mL/min. The programmed solvent used began with a linear gradient held at 95% A for 3 min, decreasing to 80% A at 10 min, 60% A at 20 min, 20% A at 30 min, and finally 95% A at 50 min.[27]

HPLC analysis of the phenolic compounds method validation

The method described in this study was validated according to the method described by Ribani.[28] The validation characteristics evaluated were selectivity, accuracy, precision, linearity, robustness, and limits of detection and quantification. The validation characteristics evaluated were selectivity, accuracy, precision, linearity, robustness, and limits of detection and quantification. Standard solutions of the phenolics were prepared for calibration of the device. The standard solutions were filtered at 0.45 µm and collected in vials. These standard solutions were read in the UV on an HPLC device, and calibration graphs were generated according to their arrival times. Linearity was determined by calculation of the regression plots using the least squares method and was expressed as the determination coefficient (R^2). Concentrations of all compounds in the bee product samples were calculated based on peak area ratios.

Calibration generated at the linear measurement range was calculated from the equation data from the graph and using the formulas given below for limit of detection (LOD) and limit of quantification (LOQ). LOD was calculated using the following formula:

$$3,3 \times \left(\frac{SD}{m}\right)$$

where SD is the standard deviation of the response and m is the slope of the calibration curve.[28] LOQ was established using the following formula:

$$10 \times \left(\frac{SD}{m}\right)$$

LOD and LOQ were experimentally verified by injection of phenolic compounds at the LOD and LOQ concentrations as follows:

$$\mathrm{LOD} = 3,3 \times \left(\frac{SD}{m}\right)$$

$$\text{Limit of quantitative measurement (LOQ)} = 10 \times \left(\frac{SD}{m}\right)$$

where SD is the standard deviation at the lowest level of the calibration curve, and *m is* slope of the calibration curve. Precision was estimated by evaluating intra-batch precision (repeatability) and inter-batch precision (preparation process, repeatability). The precision of the method (within-day variations of replicate determinations) was checked by injecting reference compounds six times at the LOD and LOQ levels. The area values were recorded, and the dimethylformamide (DMF) was calculated (Table 1).

Results and discussion

The results obtained in the present study revealed that PsPPO displaying diphenolase activity with broad substrate specificity, and good pH and thermal stabilities, was successfully biochemically characterized.

Table 1. RP-HPLC-UV validation parameters of the phenolic compounds in *P. spinosa* L.

No.	Compound	R^2	% RSD (Retention time)	% RSD(Area)	LOD[a]	LOQ[a]
1	Gallic acid	0.999	0.210	1.941	0.022	0.067
2	Protocatechuic acid	0.999	0.871	1.920	0.042	0.128
3	*p*-OH benzoic acid	0.998	0.351	3.055	0.036	0.109
4	Catechin	0.997	0.492	4.279	0.040	0.121
5	Vanillic acid	1.000	0.828	2.066	0.025	0.075
6	Caffeic acid	0.998	0.179	4.039	0.062	0.187
7	Syringic acid	1.000	0.550	0.848	0.009	0.027
8	Epicatechin	0.999	0.429	3.819	0.030	0.090
9	*p*-Coumaric acid	0.999	0.204	1.562	0.010	0.030
10	Ferulic acid	0.999	0.222	1.301	0.011	0.033
11	Rutin	1.000	0.234	3.139	0.041	0.123
12	Daidzein	0.998	0.174	1.545	0.018	0.054
13	*t*-Cinnamic acid	1.000	0.262	1.071	0.014	0.042
14	Luteolin	0.994	0.229	5.833	0.043	0.130

[a]: Values are expressed as mg/L.

Native PAGE

Under native PAGE, six isoenzyme bands were detected by in-gel enzymatic activity staining with L-DOPA (Fig. 1). Various numbers of PPO isoenzyme bands obtained from diverse sources are reported in the literature. For instance, two isoenzymes have been detected in wild pear (*P. elaeagnifolia*),[15] and an additional third and fourth PPO isoenzyme have been

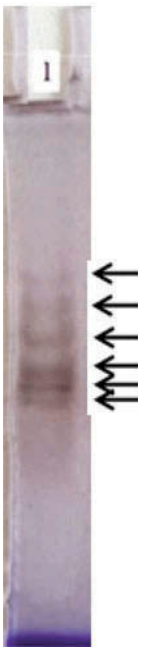

Figure 1. Native PAGE electrophoresis gel revealed by incubating with 100 mM of L-3,4-dihydroxyphenylalanine (L-DOPA) as a substrate of polyphenol oxidase. 1: Crude enzyme after acetone precipitation. Each isoenzyme band was marked.

reported in Turkish Alyanak apricot (*P. armenica* L.)[16] and persimmon (*D. kaki* L.),[7] respectively.

Substrate specificity and enzyme kinetics

The substrate specificity of PsPPO at pH 7.0, room temperature, was investigated spectrophotometrically, using 10 commercial-grade substrates, namely catechol, 4-MTC, catechin, epicatechin, hydrocaffeic acid, *p*-coumaric acid, L-tyrosine, PHPPA, gallic acid, and quercetin. The calculated PPO activity using 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin substrates at 100 mM decreased in the following order: 3795 > 2240 > 1840 > 1133 > 908 U/mg protein (Table 2). Therefore, it can be summarized that PsPPOs have substrate binding sites with high affinity for hydrocaffeic acid and catechol, while 4-MTC is the best substrate for PsPPOs. The PsPPO showed no or little activity towards *p*-coumaric acid, PHPPA, and L-tyrosine. These results suggested that PPOs in *P. spinosa* plums lack monophenolase (cresolase) activity. Catechol and 4-MTC are common substrates for determining the activity of PPO isolated from different food and plant sources.[7,10,13–16,18,29]

The Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) values for the diphenolase activity of PsPPO were determined using five substrates (4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin) at seven different concentrations and under optimum analysis conditions. The Lineweaver–Burk double reciprocal plots of 1/V and 1/[S] concentration (Table 3, Fig. 2A–2E) confirmed that the maximum catalytic efficiency and substrate affinity were achieved in the presence of 4-MTC, followed by catechol, hydrocaffeic acid, catechin, and then epicatechin (Table 3). Using 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin as substrates, the K_m and V_{max} values were calculated as 0.97, 2.35, 2.49, 4.26, and 6.04 mM; 4753, 3964, 7628, 1686, and 2093 U/mg protein, respectively, under optimum reaction conditions (Table 3, Fig. 2A–2E). The obtained K_m values for 4-MTC and epicatechin were 0.97 and 6.04, respectively (an increase of 6.2-fold). The isoenzymes exhibited different kinetic properties. The K_m value can change depending on the structure of the enzyme, the number of enzyme isoforms, pH, temperature, and ionic strength.[13,14,16,29]

The respective K_m and V_{max} values obtained using 4-MTC as the substrate were previously reported as 3.57 mM and 4781 U/mg protein for *P. elaeagnifolia*,[15] 3.88 mM and 38.51 U_{PPO}/

Table 2. Substrate specificity and activity (U/mg protein) of *P. spinosa* polyphenol oxidase.

Substrate	Type of phenolic compound	Activity (U/mg protein)	Wavelength (nm)
p-Coumaric acid	Monohydroxy	3	500
PHPPA	Monohydroxy	5	500
L-Tyrosine	Monohydroxy	33	500
Gallic acid	Trihydroxy	38	500
Quercetin	Pentahydroxy	155	500
Epicatechin	Pentahydroxy	908	500
Catechin	Pentahydroxy	1133	500
Hydrocaffeic acid	Dihydroxy	1840	500
Catechol	Dihydroxy	2240	500
4-Methylcatechol	Dihydroxy	3795	494

Table 3. Some biochemical parameters of *P. spinosa* polyphenol oxidase towards various substrates.

Substrate	pH	Temperature (°C)	K_m (mM)	V_{max} (U/mg protein)
4-Methylcatechol	7.0	40	0.97	4753
Catechol	7.0	30	2.35	3964
Hydrocaffeic acid	4.0-6.0	60	2.49	7628
Catechin	5.0	60	4.26	1686
Epicatechin	5.0	60	6.04	2093

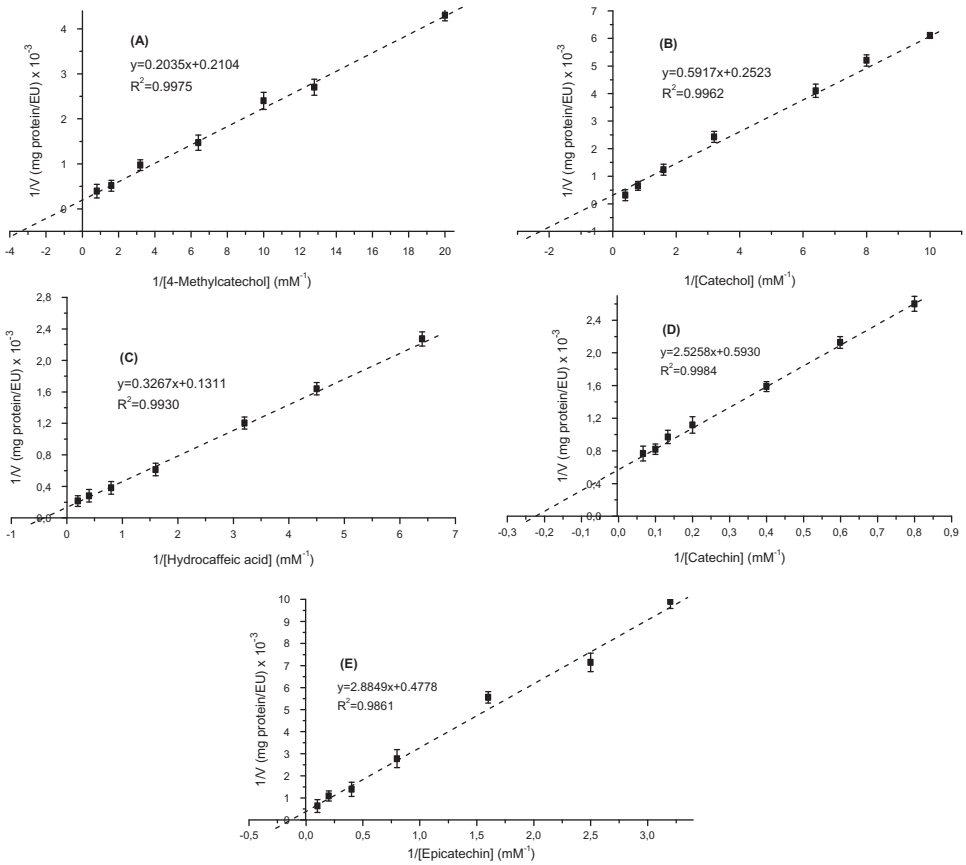

Figure 2. The Michaelis–Menten equation of diphenolase activity of *P. spinosa* for various substrates. **(A)** 4-methylcatechol (0.05–0.80 mM), **(B)** catechol (0.10–1.50 mM), **(C)** hydrocaffeic acid (0.15–5.00 mM), **(D)** catechin (1.25–15.00 mM), and **(E)** epicatechin (0.31–10.00 mM) substrates were analysed as 1/specific activity (1/V) versus 1/substrate concentration (1/[S]). The Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were obtained from the Lineweaver–Burk plots.

mL/min for Cape gooseberry,[17] 3.8 mM and 1250 U/mg protein for *D. lotus*,[18] 0.15 mM and 3.01 U/mL for Wolf's apple,[10] 3.69 mM and 0.24 IU/mL for Ataulfo mango,[12] and 94 mM and 1.3 mmol/L for chestnut kernel.[13] The corresponding values using catechol as the substrate were expressed as 6.47 mM and 3.42 U/mL for Wolf's apple,[10] 5.7 mM and 88 μM/min for medlar fruits,[5] 3.40 mM and 333.3 EU/mL/min for Hemşin apple,[14] 14.62 mM and 0.28 IU/mL for Ataulfo mango,[12] and 12.4 mM and 55.2 μM/min for persimmon.[3] These previous reports reveal the variability in the apparent K_m and V_{max} values of PPOs from various sources.

Optimum pH and stability

The optimum pH for the diphenolase activity of crude PsPPO towards 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin was pH 7.0, 7.0, 4.0–6.0, 5.0, and 5.0, respectively (Fig. 3A–3E, Table 3). Similar values are reported in the literature, such as pH 7.0 for *P. elaeagnifolia*,[15] chestnut kernel,[13]and Yomra apple (*M. communis* L.),[11] pH 6.5 for medlar fruits,[5] pH 5.5 for Hemşin apple,[14] and between pH 4.5–8.0 for mulberry fruit.[6] It was reported that the differences in optimum pH for PPO activity depended on several factors, such as the plant source, extraction method, temperature, purity of the enzyme, the number of enzyme isoforms, buffer system used, and

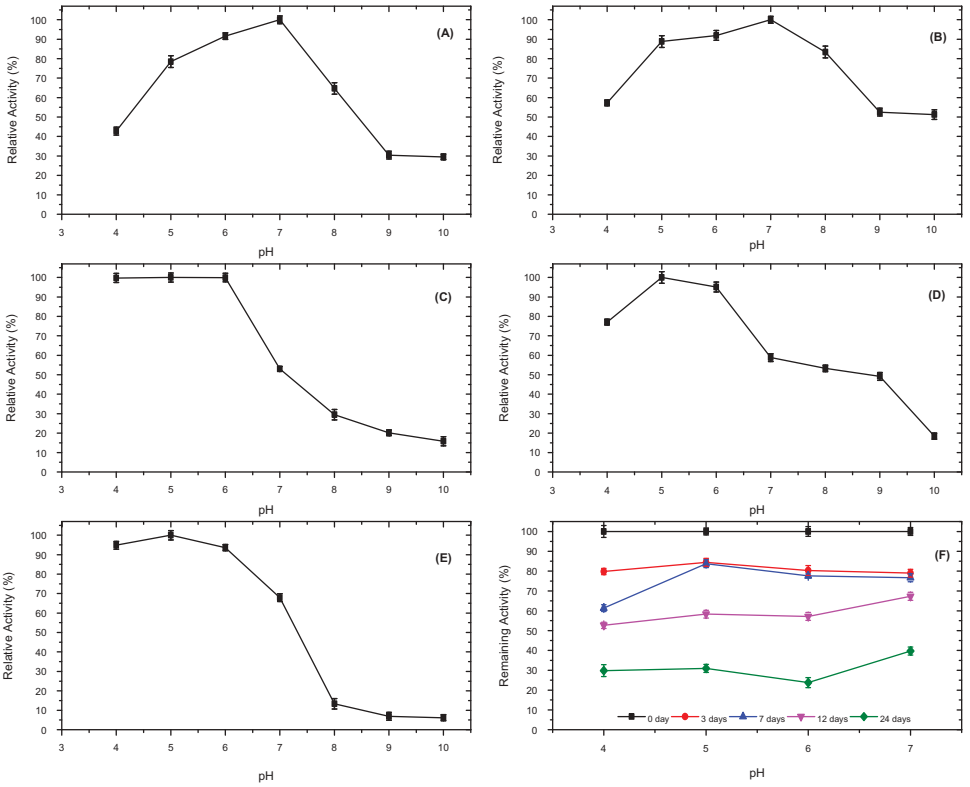

Figure 3. pH-activity profile for *P. spinosa* polyphenol oxidase in the presence of various substrates; **(A)** 4-methylcatechol, **(B)** catechol, **(C)** hydrocaffeic acid, **(D)** catechin, and **(E)** epicatechin, and **(F)** pH stability of *P. spinosa* polyphenol oxidase at 4°C, at pH 4.0, 5.0, 6.0, and 7.0, incubated in the presence of 4-methylcatechol as a substrate for 24 days.

type of phenolic substrate.[30] According to Kolcuoğlu,[31] the pH can alter the ionisation of the substrate; hence, it is the determining factor in the expression of enzymatic activity.

When the crude enzyme eluate was incubated at pH 4.0, 5.0, 6.0, and 7.0 at 4°C for up to 24 d (Fig. 3F), 80, 84, 80, and 79% of the original diphenolase activity was, respectively, maintained at the end of 3 d. After 24 d, the enzyme preserved its activity by 40 and 31% at pH 5.0 and 7.0, respectively (Fig. 3F). It was earlier reported that PPO from *P. elaeagnifolia* subsp. Pallas was extremely stable, retaining at least 95% of its original activity over pH in the range of 5.0–8.0 after incubation at 4°C for 72 h.[15] In contrast, persimmon,[3] *D. kaki* L.,[7] *D. lotus*,[18]and cherry laurel[21] lost PPO activity, while *P. elaeagnifolia*,[15] Yomra apple (*M. communis* L.),[11] and Hemşin apple[14] conserved their respective diphenolase activity at their optimum pH for 24 h incubation.

Optimum temperature and stability

The temperature dependence for the diphenolase activity of crude PsPPO (Fig. 4A–4E) indicates the optimum temperature towards 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin substrates occurred at 40, 30, 60, 60, and 60°C, respectively (Fig. 4A–4E, Table 3). It appears that in the presence of hydrocaffeic acid, catechin, and epicatechin, the diphenolase activity shows low sensitivity to temperatures above its optimum. Comparing other published results for PPO activity, the optimum temperature towards catechol substrate was 55°C for medlar fruits[5] and persimmon (*D. kaki* L.),[7] 40°C for Cape gooseberry[17] and Yomra apple,[11] and 30–40°C for Hemşin apple.[14] In contrast to Navarro et al.,[7] Özen et al.[3] reported an optimum of 20°C for persimmon (*D. kaki* L.).

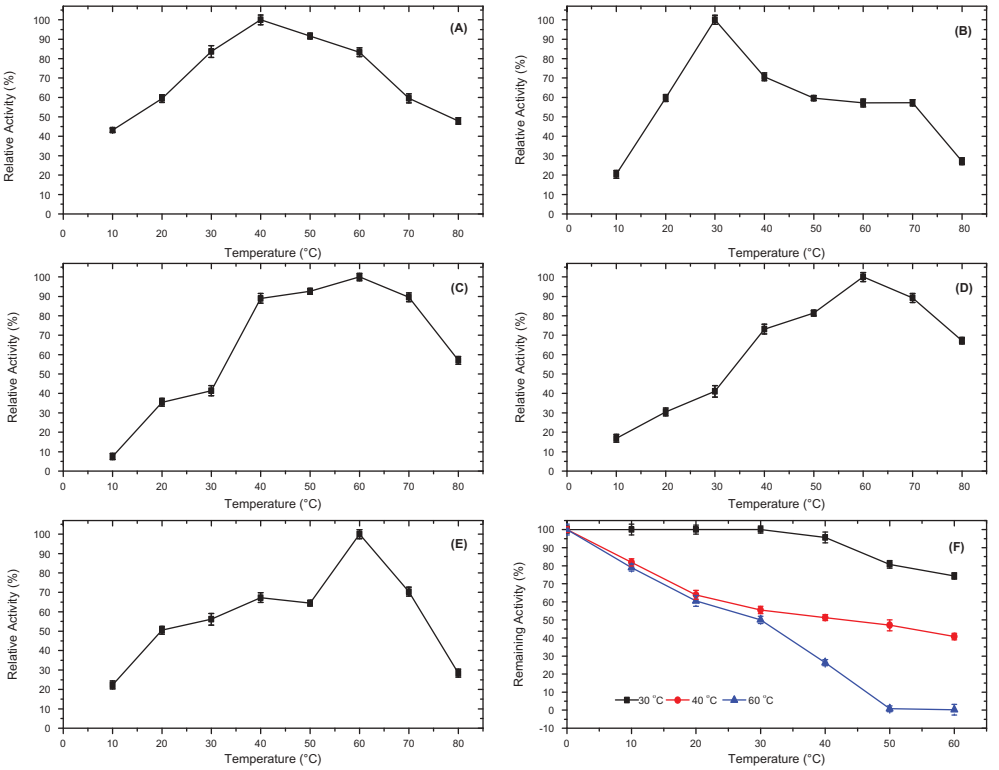

Figure 4. Optimum temperature of *P. spinosa* polyphenol oxidase in the presence of various substrates; **(A)** 4-methylcatechol, **(B)** catechol, **(C)** hydrocaffeic acid, **(D)** catechin, and **(E)** epicatechin, and **(F)** thermal stability of *P. spinosa* polyphenol oxidase at 30, 40, and 60°C, at pH 7.0, for 60 min.

In the presence of 4-MTC substrate, the optimal temperature for diphenolase activity reported by other researchers was between 25 and 40°C.[3,5,11,15,18]

The heat stability profiles for the diphenolase activity of PsPPO (Fig. 4F) showed that 100, 82, and 79% of the original activity were maintained at 30, 40, and 60°C, at 10 min, respectively. At 1 h incubation, the enzyme conserved its diphenolase activity by 74, 41, and 0.24% at 30, 40, and 60°C, respectively (Fig. 4F). In comparison, the diphenolase activity of PPO from persimmon was stable at 20–45°C for 30 min,[3] while Stanley plum PPO was stable at 70°C for 30 min.[4] The thermal stability of the PPO may be related to the ripeness of the plant from which it is sourced, while different molecular forms from the same source may also exhibit variations in their thermostability.[32]

Effect of various inhibitors and metal ions on PPO activity

Polyphenoloxidase is the target for the development of several food anti-browning agents. The ability of ascorbic acid (12.50–200 μM), sodium azide (6.25–200 mM), benzoic acid (1.56–50 mM), sodium metabisulphite (3.13–50 μM), thiourea (0.78–25 mM), and L-cysteine (6.25–200 mM) to inhibit 4-MTC oxidation by PsPPO demonstrated that the variation observed in the IC_{50} values were associated with differences in the mechanism of inhibition concerning the compound used. Sodium metabisulphite had the greatest inhibitory effect on diphenolase activity, with an IC_{50} value of 0.01 ± 0.00 mM (Table 4, Fig. 5A–5F). Sodium metabisulphite can act as a reducing agent for *o*-benzoquinones, while thiourea can react with quinones, interfering with PPO activity. Ascorbic acid acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial

Table 4. The IC_{50} values of several inhibitors on the diphenolase activity of *P. spinosa* polyphenol oxidase using 4-MTC as substrate.

Inhibitor	IC_{50} (mM)
Sodium metabisulphite	0.01 ± 0.00
Ascorbic acid	0.04 ± 0.00
Sodium azide	50.02 ± 1.01
Benzoic acid	5.92 ± 0.28
Thiourea	3.95 ± 0.32
L-cysteine	23.25 ± 1.45

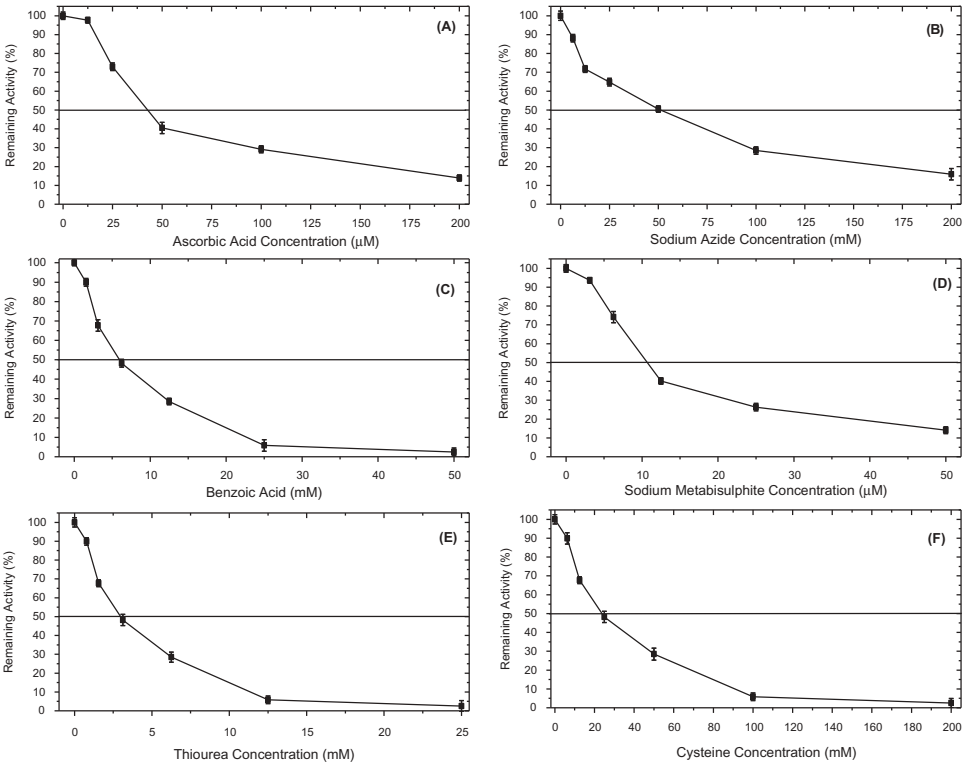

Figure 5. Inhibition effect of common polyphenol oxidase inhibitors on the diphenolase activity of *P. spinosa*. **(A)** Ascorbic acid (12.50–200 µM), **(B)** sodium azide (6.25–200 mM), **(C)** benzoic acid (1.56–50 mM), **(D)** sodium metabisulphite (3.13–50 µM), **(E)** thiourea (0.78–25 mM), and **(F)** L-cysteine (6.25–200 mM).

o-quinone formed by the enzyme to the original diphenol before it undergoes a secondary reaction, which leads to browning.[10] The IC_{50} values of the other inhibitors studied are given in Table 4. Can et al.[11] reported IC_{50} values of 0.07, 0.05, 30.04, and 9.80 mM for sodium metabisulphite, ascorbic acid, sodium azide, and benzoic acid, respectively. Many studies have highlighted ascorbic acid, sodium metabisulphite, sodium diethyldithiocarbamate, L-cysteine, tropolone, and sodium ascorbate as the most effective PPO inhibitors.[3,5,7,10–16,18,25,27,33,34]

PPO activity was inhibited by K^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Mn^{2+}, Cd^{2+}, and Hg^{2+}, while it was stimulated by Co^{2+}, Ni^{2+}, Zn^{2+}, and Al^{3+}, at 1 mM final concentration (Table 5). The diphenolase activity was inhibited by 10 mM final concentration of all the metal ions studied (Table 5). Previous studies showed that various metal ions either inhibited or stimulated PPO activity.[3,5,10] According to the researchers, metal ions may have different coordination numbers and geometries in their coordination compounds, and potential as Lewis acids; thus, they may behave differently towards

Table 5. Effect of various metal ions on *P. spinosa* polyphenol oxidase activity in the presence of 4-MTC as substrate.

	Residual activity (%)		
Metal ion	1 mM	5 mM	10 mM
Control	100	100	100
Na^{+}	103.74	71.63	64.19
K^{+}	84.79	77.85	66.40
Mg^{2+}	90.92	89.37	88.94
Ca^{2+}	94.13	64.50	35.16
Ba^{2+}	76.51	28.59	15.92
Mn^{2+}	94.16	55.92	31.27
Co^{2+}	146.33	91.78	77.96
Ni^{2+}	103.05	89.07	70.81
Zn^{2+}	119.24	95.86	92.65
Cd^{+2}	88.60	49.73	32.62
Hg^{2+}	10.29	2.95	0.67
Al^{3+}	110.82	21.54	8.36

proteins as ligands. These differences may also result in metal binding to different sites, and therefore perturb the enzyme structure in different ways.[35]

Analysis of phenolic compounds by HPLC-UV

The Rosaceae family consists of approximately 175 species distributed worldwide,[36,37] such as *P. armeniaca, P. mume, P. persica, P. salicina, P. domestica, P. genus, P. tomentosa*, and *P. cerasus*. Recent reports confirm that these plants contain high levels of flavonoids. The chromatographic separation of the phenolic compounds in *P. spinosa* are presented in Fig. 6 and their mean quantities (μg/g sample) in Table 6. Five phenolic acids were detected in the methanolic plum extract, namely protocatechuic acid (20.40 ± 6.00 μg/100g sample), *p*-OH benzoic acid (21.81 ± 9.01 μg/100g sample), vanillic acid (155.92 ± 12.60 μg/100g sample), syringic acid (20.70 ± 5.22 μg/100g sample), *p*-coumaric acid (58.60 ± 6.50 μg/100g sample), as well as flavonoids, as epicatechin (59.80 ± 10.10 μg/100g sample), and flavones, as luteolin (93.91 ± 12.40 μg/100g sample). In an earlier study, *P. spinosa* fruits presented 29.78 mg phenolic acids/100 g sample.[38] Kaempferol 3-O-arabinofuranoside (juglanin) and quercetin 3-O-arabinofuranoside (avicularin) were isolated from extracts of *P. spinosa* flowers.[39] *P. spinosa* L. (blackthorn) has also been found to contain substantial quantities of

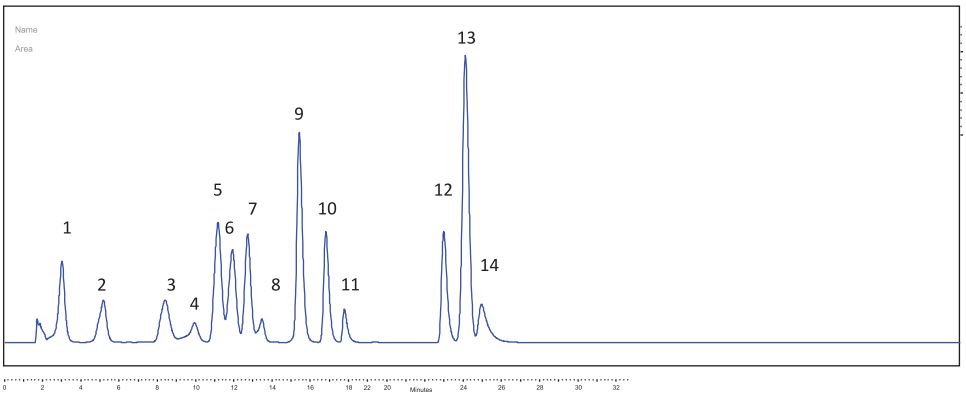

Figure 6. Chromatograms of phenolic acid standards (1. gallic acid; 2. protocatechuic acid; 3. *p*-OH benzoic acid; 4. catechin; 5. vanillic acid; 6. caffeic acid; 7. syringic acid; 8. epicatechin; 9. *p*-coumaric acid; 10. ferulic acid; 11. rutin; 12. daidzein; 13. t-cinnamic acid; 14. luteolin).

Table 6. Phenolic acid content of *Prunus spinosa* L. plum.

	Standard	Plum (μg phenolic/g sample)
1	Gallic acid	N.D.
2	Protocatechuic acid	0.20 ± 0.06
3	*p*-OH benzoic acid	0.22 ± 0.09
4	Catechin	N.D.
5	Vanillic acid	1.56 ± 0.13
6	Caffeic acid	N.D.
7	Syringic acid	0.21 ± 0.05
8	Epicatechin	0.60 ± 0.10
9	*p*-Coumaric acid	0.59 ± 0.07
10	Ferulic acid	N.D.
11	Rutin	N.D.
12	Daidzein	N.D.
13	*t*-Cinnamic acid	N.D.
14	Luteolin	0.94 ± 0.12

N.D.: Not determined.

phenolic antioxidants,[40,41] in particular, flavonol heterosides (quercetin and kaempferol) and phenolic acids (neochlorogenic and caffeic derivatives).

Conclusion

The characterization of biochemical properties of PPO is important because of the browning reactions, which occur in fruits and vegetables. For this reason, PPO enzyme from *Prunus spinosa* L. subsp. dasyphylla plum was extracted and has been characterised for the first time. The optimum pH varies from about 4.0 to 7.0, depending on the substrate. The optimum temperature for maximum enzyme activity was 60°C (for hydrocaffeic acid, catechin, and epicatechin), which is much higher than the values commonly reported for PPOs from other fruits. Based on the enzyme kinetic, which was performed by means of the model of Lineweaver–Burk, the kinetic parameters (V_{max}, K_m) were determined for the substrates. The enzyme seemed to have the highest affinity (lowest K_m) for 4-MTC, catechol, hydrocaffeic acid, catechin, and epicatechin. L-tyrosine was also tested, but was little oxidised by PPO. The partially purified PPO enzyme appears to have biochemical characteristics similar to several plant PPOs in terms of substrate specificity, pH, and temperature of assay and kinetic parameters. Among the various PPO inhibitors tested, sodium metabisulphite was the most effective inhibitor of the enzyme with an IC_{50} 0.01 ± 0.00 mM. Due to its nontoxic properties, ascorbic acid with an IC_{50} 0.04 ± 0.00 mM may be promising alternatives to sulphite in preventing the browning of plant foods. In addition, six activity bands were detected in the "*Prunus spinosa* L. subsp. dasyphylla" extract assayed by native gel electrophoresis, suggesting for the first time the presence of at least six PPO izoenzymes in plum fruits. Besides these results, it was revealed the plum extract contained protocatechuic acid, *p*-OH benzoic acid, vanillic acid, syringic acid, epicatechin, *p*-coumaric acid, and luteolin.

Nomenclature

DHPPA	3-(3,4-dihydroxyphenyl) propionic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
4-MTC	4-methylcatechol
PPO	polyphenol oxidase
PsPPO	*Prunus spinosa* polyphenol oxidase

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