Journal of Enzyme Inhibition and Medicinal Chemistry, June 2005; 20(3): 297–302

Taylor & Francis
Taylor & Francis Group

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

Purification and characterization of polyphenol oxidase from nettle (*Urtica dioica* L.) and inhibitory effects of some chemicals on enzyme activity

İLHAMİ GÜLÇİN[1], Ö. İRFAN KÜFREVİOĞLU[1], & MÜNİR OKTAY[2]

[1]*Atatürk University, Faculty of Science and Arts, Department of Chemistry, 25240, Erzurum-Turkey, and* [2]*Atatürk University, Kazim Karabekir Education Faculty, Department of Chemistry Education, 25240, Erzurum, Turkey*

(Received 23 June 2003; in final form 21 August 2003)

Abstract
Polyphenol oxidase (PPO) of nettle (*Urtica dioica* L.) was extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis, and CM-Sephadex ion-exchange chromatography and was used for its characterization. The PPO showed activity to catechol, 4-methylcatechol, L-3,4-dihydroxyphenylalanine (L-DOPA), L-tyrosine, *p*-cresol, pyrogallol, catechin and trans-cinnamic acid. For each of these eight substrates, optimum conditions such as pH and temperature were determined and L-tyrosine was found to be one of the most suitable substrates. Optimum pH and temperature were found at pH 4.5 and 30°C respectively and K_m and V_{max} values were 7.90×10^{-4} M, and 11290 EU/mL for with L-tyrosine as substrate. The inhibitory effect of several inhibitors, L-cysteine chloride, sodium azide, sodium cyanide, benzoic acid, salicylic acid, L-ascorbic acid, glutathione, thiourea, sodium diethyl dithiocarbamate, β-mercaptoethanol and sodium metabisulfite were tested. The most effective was found to be sodium diethyl dithiocarbamate which acted as a competitive inhibitor with a K_i value of 1.79×10^{-9} M. In addition one isoenzyme of PPO was detected by native polacrylamide slab gel electrophoresis.

Keywords: *Polyphenol oxidase, enzyme, nettle,* Urtica dioica L

Introduction

Polyphenol oxidase (PPO; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase, E.C. 1.14.18.1) is a copper protein widely distributed on the phylogenic scale and responsible for the enzymatic browning reaction occurring during the handling, storage and processing of the damaged tissue of fresh fruits and vegetables, as well as some animal products. PPO catalyzes two types of oxidative reactions: hydroxylation of monophenols to *o*-diphenols, which are oxidized to the corresponding *o*-quinones, and subsequently polymerized to brown, red or black pigments, depending on natural components present in a given plant material [1,2]. In plant tissues, the browning pigments lead to organoleptic and nutritional modifications, thus depreciating the quality of the food product [3–5].

PPO characteristics have been widely studied in various plants such as grapes [6,7], yam tubers [8], banana [9], plums [10], potato [11], tea [12], papaya [13], chickpea,[14] alliums [15], dog-rose [16], cabbage [17], apple [18], and peaches [19]. However, there is no information about isolation, purification, and characterization of nettle PPO.

Nettle (*Urtica dioica* L.) has been consumed as a fresh vegetable in Turkey. Also, nettle has been commonly used as a natural pigment instead of synthetic pigments in canned foods. Therefore, characterization of PPO from nettle is very important. The browning reaction during storage or processing is a serious problem and in the most cases is considered detrimental to the quality of the products [20].

It is known in traditional therapy that nettle (*Urtica dioica* L.) has a hypotensive[21] and anti-inflammatory[22,23] effects. Some other actions of this plant

Correspondence: Ö. İ. Küfrev, Atatürk University, Faculty of Science and Arts, Department of Chemistry, 25240, Erzurum-Turkey, Tel: 90 442 2314438. Fax: 90 442 2360948. E-mail: okufrevi@atauni.edu.tr

ISSN 1475-6366 print/ISSN 1475-6374 online ©2005 Taylor & Francis Group Ltd
DOI: 10.1080/1475636032000141890

RIGHTSLINK

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

have also been reported such as stimulation of proliferation of human lymphocytes [24]. The effects of the nettle are also evoked in the therapy of prostatic hyperplasia [25–27] and this plant has been used in the traditional therapy of hypertension [28].

In the present work the isolation and characterization of PPO from nettle was studied in terms of substrate specificity, thermal activation and stability, pH-optimum and stability, and potency of general PPO inhibitors, so as to be able to determine the behavior of the nettle enzyme. Specifically, we have investigated the inhibitory effects of thiourea, L-ascorbic acid, sodium metabisulfite, glutathione, sodium azide, β-mercaptoethanol, dithioerythritol, sodiumdiethyl dithiocarbamate and potassium cyanide.

Materials and methods

Plant materials and chemicals

Nettle was harvested fresh from a local garden at Erzurum, Turkey in May and June and stored deep-frozen at −85°C until use. Sodium metabisulfite, β-mercaptoethanol, L-ascorbic acid, L-cysteine chloride, thiourea, benzoic acid, sodium diethyl dithiocarbamate, catechol, 4-methylcatechol, L-Dopa, L-tyrosine, *p*-cresol, catechin, and trans-cinnamic acid were obtained from Sigma Chemical Co. (St. Louis, USA). Sodium azide, salicylic acid, sodium cyanide, and pyrogallol were purchased from Merck, Germany. All other chemicals use were of analytical grade.

Protein determination

Protein concentration was determined according to the dye-binding method of Bradford [29] using bovine serum albumin as standard.

Polyphenol oxidase activity assay with different substrates

PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). For enzyme activity, the sample cuvette contained 0.1 mL of the enzyme solution and 2.9 mL of substrate solution at various concentrations. The blank sample contained the same solution except 0.1 mL enzyme. The reaction was carried out at various temperatures and pH values with the substrates mentioned as described later. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min [9,18].

Purification of the enzyme

Nettle (50 g) was placed in a dewar flask under liquid nitrogen for 10 min in order to decompose cell membranes. The cold nettle was homogenized by using a blender in 100 ml of 100 mM cold phosphate buffer (pH 4.5), containing 2 mM EDTA, 0.5% PVP and 0.01 M ascorbic acid for 2 min at 4°C. The homogenate was filtered and kept at 4°C before being centrifuged at 20.000 × g for 30 min at 4°C. The supernatant was used as a crude enzyme extract. The crude extract was further fractionated with ammonium sulfate by being first brought to 20% saturation, centrifuged, the supernatant collected and then brought to 70% saturation by an additional amount of $(NH_4)_2SO_4$. The protein precipitate was dialyzed against 10 mM phosphate buffer, pH 6.0 and chromatographed on a CM-Sephadex column (2.6 × 42 cm) pre-equilibrated with the same buffer. After loading the enzyme, the column was thoroughly washed with the same buffer until the UV absorbance of the eluate returned to the base line. The bound protein was eluted with a linear gradient of 0–1.0 M NaC1 in the same buffer at a flow rate of 0.5 ml min^{-1}. Active fractions were collected and concentrated by ammonium sulfate precipitation. The precipitated protein was re-dissolved in a small volume of 100 mM phosphate buffer (pH 6.0) and dialyzed against the same buffer. Finally, the 10 ml purified enzyme obtained from the nettle had specific activity of 425.7 EU/ml proteins with 17.8 fold purification and in 12.4% yield (Table I).

Characterization of nettle PPO

pH-optimum and Stability. PPO activity was determined in the pH range 3.0–5.5 in 0.2 M

Table I. Purification steps for polphenol oxidase from nettle (*Urtica dioica*).

Purification step	Volume (mL)	Activity (EU/mL)	Total activity	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg of protein)	Yield (%)	Purification fold
Crude extract	40	30	1200	1.25	50.0	24.0	100	–
$(NH_4)_2SO_4$ precipitation	8	104	832	2.06	16.48	50.4	69.33	2.1
Dialysis	9.2	19.0	174.8	1.92	17.62	9.92	41.3	0.41
CM-Sephadex ion-exchange chromatography	10	14.9	149	0.035	0.35	42.57	12.4	17.8

RIGHTSLINK

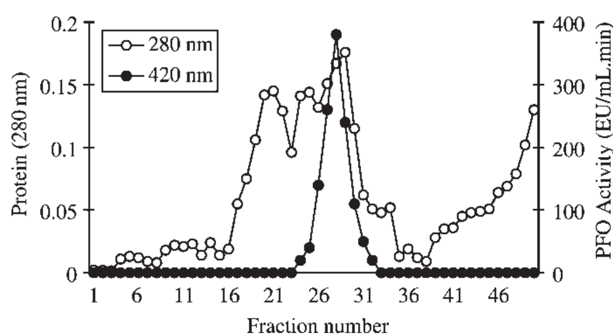

Figure 1. Elution pattern of nettle PPO on CM-Sephadex C-50.

phosphate/0.1 M citrate buffer, 5.5–7.0 in 0.2 M phosphate buffer and 7.5–9.0 in 0.2 M Tris/HCl buffer. PPO activity was assayed using catechol, 4-methylcatechol, L-3,4-dihydroxyphenylalanine (L-DOPA), L-tyrosine, *p*-cresol, pyrogallol, catechin, and trans-cinnamic acid as substrates. To determine the pH stability of nettle PPO, 0.5 ml of crude enzyme solution was incubated in 1 ml buffer solution pH 3.0–9.5 for 96 h at 4°C. Aliquots were then taken at intervals and enzyme activity was assayed with catechol as the substrate. Briefly, all of the solutions except for the enzyme solution were heated in a water bath for 5 min. Enzyme was then added to this mixture and the activity measurement commenced. Finally, increased absorbance was recorded during a 3 min period. The optimum-pH values obtained from this assay were used in all the other experiments.

The effect of temperature. PPO activity was determined at various temperatures controlled by a circulatory water bath. The PPO activity at a definite temperature was determined spectrophotometrically by addition of enzyme to the mixture as rapidly as possible. For determining optimum temperature ranges of the enzyme for each of the above mentioned eight substrates, activity was measured at different temperatures in the range from 0–80°C.

Enzyme kinetics and substrate specificity. PPO activity was assayed using catechol, 4-methylcatechol, L-Dopa, L-tyrosine, *p*-cresol, pyrogallol, catechin, and trans-cinnamic acid in buffers at optimum-pH values and 25°C for each substrate. The rate of the reaction was measured in terms of the increase in absorbance (Table I). To determine the Michaelis–Menten constant (K_m) and maximum velocity (V_{max}), PPO activities were measured with variable substrate concentrations in the standard reaction mixture. The K_m and V_{max} of PPO for each substrate was calculated from a plot of 1/V and 1/[S] by the method of Lineweaver and Burk [30].

Effect of inhibitors. The inhibitors L-cysteine chloride, sodium azide, benzoic acid, salicylic acid, L-ascorbic acid, glutathione, thiourea, sodium diethyl dithiocarbamate, β-mercaptoethanol and sodium metabisulfite at three different concentrations were evaluated for their effects on nettle PPO activity using catechol as substrate. The reaction mixture (3 ml) contained 0.1 ml of catechol as a substrate, 2.7 mL phosphate buffer (pH 4.5, 0.2 M), 0.1 mL enzyme solution, and 0.1 mL of inhibitor solution at a fixed concentration. A graph of PPO activity was constructed for five inhibitor concentrations to find the I_{50} value (50% inhibition effect). Then, using five different concentrations of the substrates, PPO activities were measured at three constant inhibitor concentrations with the inhibitors indicated above. 1/V and 1/[S] values obtained from these activity measurements were used for drawing Lineweaver–Burk graphs. K_i values were determined from the graphs and the types of inhibition from plot.

Native polyacrylamide gel electrophoresis (PAGE). Polyacrylamide slab gels electrophoresis was performed according to Laemmli [31] under native conditions (i.e. without sodium dodecyl sulfate) for separating nettle PPO isoenzymes under natural condition. The experiment was conducted in a cold room at 4°C with the electrode buffer Tris/glycine (pH 8.3) using 3% stacking gel and 10% separating gels. The enzyme samples were loaded on to each space of the

Figure 2. Relative mobility of nettle PPO on native polyacrylamide gel electrophoresis

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

RIGHTSLINK

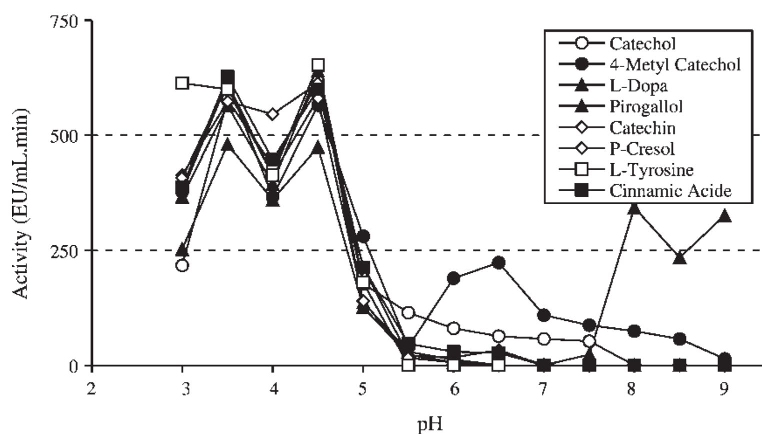

Figure 3. The effect of pH on nettle PPO activity.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

stacking gel at 0.1 mL. Initially, an electric current on 80 V was applied until the bromophenol dye extended into the separating gel and then increased to 150 V for 5–6 h until the tracking dye migrated to 1–2 cm from the bottom. After running, gels were incubated in 15 mM L-DOPA in 0.1 M phosphate buffer (pH 4.5) at 37°C for 1 h and then in 1 mM ascorbic acid solution until appearance of the isoenzyme bands. Gels were stored in 30% ethanol and then photographed.

Results and discussion

Extraction and purification of PPO

Extraction of PPO was carried out in 0.1 M phosphate buffer pH 4.5, containing 1% PVP and 10 mM ascorbic acid and then the enzyme precipitated with $(NH_4)_2SO_4$. Several precipitations with solid $(NH_4)_2SO_4$ between 0–10%, 10–20%, 20–30%, 30–40%, 40–50%, 50–60%, 60–70%, 70–80%, 80–90%, 90–100% were conducted to find the optimum saturation point. As a result, PPO activity of the precipitate from 20–70% $(NH_4)_2SO_4$ was found the highest and this saturation point was used in all the extraction processes. PVP was used during extraction to bind the phenols which may inactive PPO. Ascorbic acid was also, used to reduce quinones to phenolic substrates during extraction. Following ammonium sulfate precipitation, the enzyme extract was dialyzed and used for purification of PPO. Results on the purification of the PPO are shown in Table I. The degree of purification of PPO was 17.8-fold after CM-Sephadex ion-exchange chromatography (Figure 1), showing that nettle PPO has only one isoenzyme (Figure 2).

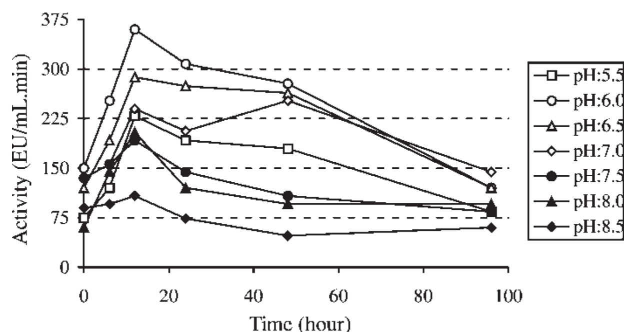

Figure 4. pH stability of nettle PPO.

pH-optimum and stability

The activity in eluates of the CM-Sephadex C-50 ion exchange chromatography was quite low, hence the characterization was not possible. Therefore, the enzyme samples obtained from ammonium sulfate precipitation and dialysis were used for characterization of the PPO. Maximum PPO activities were observed at pH 4.5 for catechol; pH 3.5 for 4-methylcatechol, pH 3.5 for L-3,4-dihydroxyphenylalanine (L-DOPA); pH 4.5 for L-tyrosine, pH 3.5 for p-cresol; pH 3.5 for pyrogallol; pH 4.0 for catechin, and pH 3.5 for trans-cinnamic acid (Figure 3). These results are supported in a general way by Aylward and Haisman (1969) who reported that the optimum pH for maximum activity of PPO varies from about 4.0 to 7.0 depending on the extraction methods, substrates and localization of the enzyme in the cell. Nettle PPO was almost inactive above 6.5 in the presence of these substrates. As can be seen from Figure 4, stability of the enzyme at different pH values (5.5–8.5) was also investigated using catechol. Nettle PPO, at acidic pH, was more stable than at basic pH.

Thermal activity and stability

The effects of temperature between 5–80°C on PPO activity were assayed and optimum temperatures for each substrate are presented in Figure 5. As seen in

RIGHTSLINK

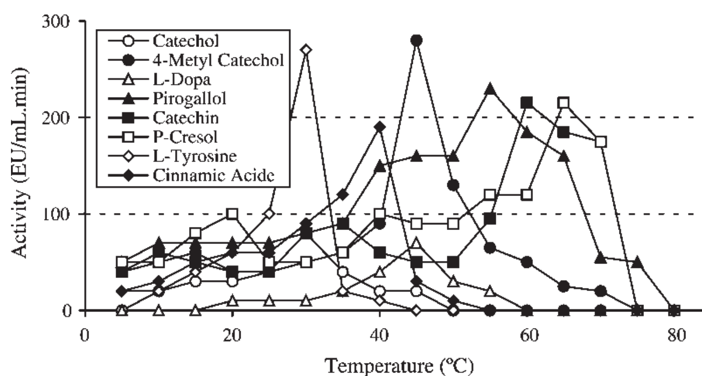

Figure 5. The effect of temperature on nettle PPO activity.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

Table II, the optimum temperatures for all of substrates ranged from 30–65 °C. The enzyme was completely inactivated at 80 °C after 3 minutes.

Substrate specificity

The enzyme oxidized all of substrates significantly including the phenolic substrates used in the study. The substrate with the highest activity was found to be L-tyrosine, and that with the lowest pyrogallol (Table II). For determination of K_m and V_{max} values of the enzyme, PPO activities were measured with the eight substrates at varying concentrations at the optimum pH and 25°C. K_m and V_{max} values of PPO for each substrate were calculated from a Lineweaver and Burk plot [30]. L-Tyrosine had the highest V_{max} value and catechol had the lowest K_m value and was the most efficient phenolic substrate for PPO.

It appears that the nettle PPO has a substrate-binding site with a high affinity for small *o*-diphenols such as catechol, 4-methylcatechol. In contrast to other studies,[32,33] nettle PPO has activity toward monophenols such as L-tyrosine.

Effect of inhibitors

The effects of ten inhibitors, namely L-cysteine chloride, sodium azide, sodium cyanide, benzoic acid, salicylic acid, L-ascorbic acid, glutathione, thiourea, sodium diethyl dithiocarbamate, β-mercaptoethanol and sodium metabisulfite, were examined, to determine their potential for inhibition of 4-methyl-catechol oxidation by nettle PPO (Table III) Inhibition modes of the inhibitors were determined from the Lineweaver-Burk plots: salicylic acid uncompetitive; sodium azide, sodium cyanide, and benzoic acid noncompetitive, and the remainder, competitive. Sodium diethyl dithiocarbamate was the most effective inhibitor. The general mechanisms for PPO inhibition have been reviewed previously [34–38]. The prevention of enzymatic browning by a specific inhibitor may involve a single mechanism or be the result of interplay of two or more inhibitory mechanisms.[15,39]

It can be concluded that crude extracts prepared from nettle have a PPO activity very similar to that of other plants. The enzyme is a catecholase, active toward diphenols, and as the greatest substrate specificity towards catechol and L-tyrosine among the substrates tested. Consistent with previous results, obtained from other plant PPOs, pH and temperature optima for the enzyme, in the presence of L-tyrosine, were 6.5 and 35 °C, respectively. Moreover, the nettle PPO activity was very sensitive to some of the general PPO inhibitors, especially to sodium diethyl dithiocarbamate and L-cysteine chloride.

Table II. Optimum pH, optimum temperature, and K_m and V_{max} values for PPO.

Substrate	Optimum pH	Optimum Temp. (°C)	K_M (M)	V_{max} (EU/ml min)
Catechol	4.5	30	4.14×10^{-4}	8554
4-Methylcatechol	3.5	45	12.55×10^{-4}	6599
Pyrogallol	3.5	55	10.04×10^{-4}	628
L-Dopa	3.5	45	21.4×10^{-3}	744
L-Tyrosine	4.5	30	7.90×10^{-4}	11290
p-Cresol	3.5	65	6.43×10^{-4}	715
(+)-Catechin	4.0	60	9.88×10^{-4}	1098
Trans-cinnamic acid	3.5	40	5.44×10^{-4}	907

RIGHTSLINK

Table III. K_i values and inhibition modes for different inhibitors.

Inhibitors	K_i means ± S.D (M)	Type of inhibition
Sodium azide	$2.50 \times 10^{-4} \pm 0.24 \times 10^{-4}$	Noncompetitive
Sodium metabisulfite	$1.64 \times 10^{-5} \pm 0.83 \times 10^{-5}$	Competitive
Sodium diethyl dithiocarbamate	$1.79 \times 10^{-9} \pm 0.40 \times 10^{-9}$	Competitive
Sodium cyanide	$0.74 \times 10^{-4} \pm 0.20 \times 10^{-4}$	Noncompetitive
L-Ascorbic acid	$1.68 \times 10^{-5} \pm 0.61 \times 10^{-5}$	Competitive
Salicylic acid	$0.67 \times 10^{-3} \pm 0.23 \times 10^{-3}$	Uncompetitive
Benzoic acid	$1.31 \times 10^{-5} \pm 0.31 \times 10^{-5}$	Noncompetitive
β-Mercaptoethanol	$8.40 \times 10^{-5} \pm 3.28 \times 10^{-5}$	Competitive
L-Cysteine chloride	$1.37 \times 10^{-6} \pm 0.57 \times 10^{-6}$	Competitive
Thiourea	$2.13 \times 10^{-4} \pm 1.28 \times 10^{-4}$	Competitive

Acknowledgements

The authors are grateful to the Research Fund of Atatürk University for financial support (Project no: 2001/35).

References

[1] Mayer AM. Phytochemistry 1987;26:11–20.
[2] Zawistowski J, Biliaderis CG, Eskin AM. Polyphenol oxidase. In: Robinson DS, Eskin AM, editors. Oxidative enzymes in foods. London: Elsevier; 1991. p 217–273.
[3] Friedman M. J Agric Food Chem 1996;44:631–653.
[4] Matheis G, Whitaker JR. J Food Biochem 1984;8:137–162.
[5] Sanchez-Ferrer A, Rodriguez-Lopez JN, Garcia-Carnovas F, Garcia-Carmona F. Biochim Biophys Acta 1995;1247:1–11.
[6] Nakamura K, Amano Y, Kagami M. Am J Enol Vitic 1983;34:122–127.
[7] Yokotsuka K, Makino S, Singleton VL. Am J Enol Vitic 1988;39:293–302.
[8] Anosike EO, Ayaebene AO. Phytochemistry 1981;20: 2625–2628.
[9] Galeazzi MAM, Sgarbieri VCJ. J Food Sci 1981;46: 1404–1406.
[10] Siddiq M, Sinha K, Cash JN. J Food Sci 1992;57:1177–1179.
[11] Batistuti JP, Lourenco EJ. J Food Chem 1985;18:251–263.
[12] Coggon P, Moss GA. Phytochemistry 1973;12:1947–1955.
[13] Cano MP, Lobo MG, de Ancos B, Galeazzi MAM. J Agric Food Chem 1996;44:3075–3079.
[14] Singh U, Jambunathan R. J Food Sci 1981;46:1364–1367.
[15] Arslan O, Temur A, Tozlu I. J Agric Food Chem 1998;46:1239–1241.
[16] Sakiroglu H, Küfrevioglu OI, Kocacaliskan I, Oktay M, Onganer Y. J Agric Food Chem 1996;44:2982–2986.
[17] Fujita S, Tono T. J Sci Food Agric 1988;46:115–123.
[18] Oktay M, Küfrevioğlu ÖI, Kocaçalıskan I, Sakiroğlu H. J Food Sci 1995;60:494–496.
[19] Wong TC, Luh BS, Whitaker JR. Plant Physiol 1971;48:19–23.
[20] Baytop T. Therapy with medicinal plants in turkey (Past and Present). No. 3255. Istanbul: Publications of Istanbul, University, Istonbul 1999. p 231–232.
[21] Garnier G, Bezanger-Beauquesne L, Debraux G. Resources medicinales de la flore Française. Vigots Freres, Paris, pp 1961;962–964.
[22] Obertreis B, Giller K, Teucher T, Behnke B, Schmitz H. Arzneimittel Forsch 1996;46:52–56.
[23] Riehemann K, Behnke B, Schulze-Osthoff K. FEBS Lett. 1999;442:89–94.
[24] Wagner H, Willer F, Kreher B. Planta Med 1989;55: 452–454.
[25] Krzeski T, Kazon M, Borkowski A, Kuczera J. Clin Ther 1993;6:1011–1020.
[26] Hiramo T, Homma M, Oka K. Planta Med 1994;60:30–33.
[27] Lichius JJ, Muth C. Planta Medi 1997;63:307–310.
[28] Ziyyat A, Legssyer A, Mekhfi H, Dassouli A, Serhrouchni M, Benjelloun W. J. Ethnopharmcol 1997;58:45–54.
[29] Bradford MM. Analyt Biochem 1976;72:248–254.
[30] Lineweaver H, Burk D. J Am Chem Soc 1934;56:658–661.
[31] Laemmli UK. Nature 1970;227:680–685.
[32] Mayer AM, Harel E. Phytochemistry 1979;18:193–215.
[33] Wesche-Ebeling P, Montgomery MW. J Food Sci 1990;55:1320–1324.
[34] Duangmal K, Owusu Apenten RK. Food Chem 1999;64:351–359.
[35] Iyengar R, McEvily AJ. Trends Food Sci Tech 1992;3:60–64.
[36] Martinez M, Whitaker JR. Trends Food Sci Tech 1995;6:195–200.
[37] Sapers GM, Hicks KB, Phillips JG, Garzarella L, Pondih DL, Matulaitis RM, McCormack TJ, Sondy SM, Seib PR, El-Ataway YS. J Food Sci 1989;54:997–1002.
[38] Valero E, Varon R, Garcia-Carmona F. J Agric Food Chem 1992;40:904–908.
[39] Roudsari MH, Signoset A, Crovzet J. Food Chem 1981;7:227–235.

Journal of Enzyme Inhibition and Medicinal Chemistry Downloaded from informahealthcare.com by Malmo Hogskola on 12/24/11
For personal use only.

RIGHTSLINK