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Novel Biological Properties of *Oenothera paradoxa* Defatted Seed Extracts: Effects on Metallopeptidase Activity

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In this study, for the first time, we used the *in vitro* metallopeptidase model for the identification of a potential novel activity of defatted evening primrose seed extracts. Prepared extracts of different polarity (aqueous, 60% ethanolic, isopropanolic, and 30% isopropanolic) at concentrations of 1.5−100 μg/mL exhibited a significant and dose dependent inhibition of three tested enzymes. The 50% inhibition of enzymes activity showed that aminopeptidase N (APN) was the enzyme affected to the greatest extent with IC_{50} at the level of 2.8 μg/mL and 2.9 μg/mL for aqueous and 30% isopropanolic extracts, respectively. The activity of neutral endopeptidase (NEP) was quite strongly inhibited by the extracts as well. The HPLC-DAD analysis and bioguided fractionation led to the identification of four active compounds: (−)-epicatechin gallate, proanthocyanidin B3, oenothein B, and penta-*O*-galloyl-β-D-glucose (PGG). Oenothein B has been shown previously to inhibit metallopeptidases. The three other compounds are known to inhibit angiotensin-converting enzyme (ACE), but they have not been previouly reported to inhibit the NEP and APN activity. PGG and procyanidins with different degrees of polymerization, as the dominating compounds in *O. paradoxa* seeds, seemed to play a role in the crude extract activity.

KEYWORDS: ***Oenothera paradoxa*; evening primrose; polyphenols; procyanidins; pentagalloylglucose; metallopeptidases; angiotensin-converting enzyme; neutral endopeptidase; aminopeptidase N; biological activity**

INTRODUCTION

Oenothera sp. (*Oenotheraceae*) are native to Central and South America, where they have been widely used for various medicinal purposes. Today, these species are also cultivated in Europe for the production of seeds. Oil from seeds of *Oenothera biennis* L., as well as *Oenothera paradoxa* Hudziok, is the main source of γ-linolenic acid (GLA), a precursor of prostaglandin E_1 (PGE_1) exhibiting anti-inflammatory, immunoregulatory, and vasodilatory properties. Evening primrose oil (EPO) is used for the treatment of atopic eczema, premenstrual syndrome, multiple sclerosis, hypercholesterolemia, rheumatoid arthritis, and diabetic nephropathy (*1*−*4*).

In traditional medicine, the whole plant is reputed for its sedative, analgesic, astringent, and wound healing properties (*5*). In Eastern Europe, the roots have been used for upper respiratory tract diseases.

In the past few years, there has been a growing interest in the evening primrose because of its polyphenolics content. It is well known that *Oenothera* herb contains flavonoids, phenolic acids, and hydrolyzable tannins (ellagitannins and gallotannins). Two macrocyclic ellagitanins, oenotheins A and oenotheins B, were first isolated from *O. erythrosepala* and *O. biennis* and are also present in many species of the *Oenotheraceae* family (*6*−*8*). A number of flavonoids have been found in *Oenothera* sp., including myricetin, quercetin, and kaempferol glycosides, as well as phenolic acids (mainly gallic acid) (*9*−*11*). A phenolic fraction purified from defatted seeds of *Oenothera biennis* promoted selective apoptosis of human and mouse bone marrow-derived cell lines. Analysis of the fraction has revealed that it contains gallic acid, which showed selective cytotoxicity against a variety of tumor cells with a higher activity than against normal cells (*12*). Other studies have demonstrated that the defatted seed extract of *O. biennis* could induce apoptosis in Ehrlich ascites tumor cells, by increasing the activity of superoxide dismutase and intracellular peroxides levels, which resulted in cytochrome *c* release. However, no activation of caspase-3 was observed (*13, 14*).

A number of studies have been conducted to demonstrate the antioxidant and iron (II) chelation activity of Oenothera (*O. biennis* and *O. paradoxa*) defatted seed extracts (*15*−*21*). Fractionation of the extracts showed the presence of hydrolyz-

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Table 1. Total Polyphenolics and Proanthocyanidins Content in Obtained Extracts and Their Inhibitory Activity against Metallopeptidases at a Concentration of 25 μg/mL with IC_{50} values (μg/mL)[a]

extract	total polyphenols [mg/g]	total proanthocyanidins [mg/g]	ACE inhibition at 25 μg/mL (IC_{50} μg/mL)	NEP inhibition at 25 μg/mL (IC_{50} μg/mL)	APN inhibition at 25 μg/mL (IC_{50} μg/mL)
aqueous	459.9±0.1	443.3±2.7	36.9 ± 1.3 (**55**)	84.0 ± 2.8 (**4.7**)	91.2 ± 1.8 (**2.8**)
60% ethanolic	653.3±0.8	643.4±0.2	33.3 ± 2.3 (**37**)	84.6 ± 2.5 (**4.9**)	92.4 ± 0.7 (**3.3**)
isopropanolic	272.1±0.6	402.6±2.5	n.in (**130**)	70.5 ± 5.4 (**15**)	91.4 ± 5.0 (**5.6**)
30% isopropanolic	581.3±1.6	518.9±1.6	44.7 ± 2.7 (**26**)	90.4 ± 2.5 (**6.1**)	94.9 ± 1.1 (**2.9**)
lisinopril			IC_{50} 0.0045 μg/mL	(−)	(−)
phosphoramidon			(−)	IC_{50} 0.0053 μg/mL	(−)
bestatin			(−)	(−)	IC_{50} 1.8 μg/ml

[a] Data present the mean ± SEM of at least three independent experiments, each performed in duplicated samples ($P < 0.05$). IC_{50} values were obtained from dose−effect curves by linear regression. n.in., no inhibition; (−), not determined.

able and condensed tannins, but only gallic acid, (+)-catechin, (−)-epicatechin, and a tetrameric procyanidin gallate were isolated as pure compounds from *O. biennis* seeds (*18, 19, 21*).

Angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP), and aminopeptidase N are zinc metallopeptidases located on the outer membrane of various cell types (ectoenzymes). They play an important role in the metabolism of a number of regulatory peptides of the human nervous, cardiovascular, inflammatory, and immune systems. ACE (EC 3.4.15.1) is a dipeptidyl carboxypeptidase that converts angiotensin I into angiotensin II (AngII) and degrades kinins. ACE plays a central role in the renin−angiotensin−aldosterone system (RAAS). The neutral endopeptidase (NEP; EC 3.4.24.11) catalyzes the degradation of a variety of renal and CNS-active peptides, including substance P, bradykinin, enkephalins, atrial natriuretic peptides, endothelin, and AngII. NEP inhibitors prevent the degradation of natriuretic peptides and enhance diuresis and natriuresis. Dual ACE/NEP inhibitors have been developed as effective and broad-spectrum antihypertensive drugs, and NEP inhibitors are also hoped to be useful in the future to treat pain and diarrhea (*22*−*25*). In contrast to NEP and ACE, a relatively specifically acting enzyme, aminopeptidase N (APN; EC 3.4.11.2), is an α-aminoacylpeptide-hydrolase with a low substrate specificity. APN plays an important role in the invasion and metastasis of cancer cells and in inflammation (*26, 27*).

Companies manufacturing evening primrose oil may produce 50 tons of defatted seeds each year, on average, and therefore, there is a growing need for utilization of this material. With this in mind, we chose the *in vitro* metallopeptidases model for the purpose of identification of a potential novel activity of defatted seed extracts and for defining the active compounds present in them.

MATERIALS AND METHODS

General Procedures. Proton ^{1}H and carbon ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at room temperature or at 30 °C for compound **3** on a Varian 500 INOVA-500 instrument (Varian Inc., Palo Alto, CA) at 500.61 MHz and 125.88 MHz, respectively, using tetramethylsilane (TMS) as internal standards. Techniques such as DEPT and HSQC were also utilized. ESI-MS was carried out using a Mariner biospectrometry workstation (PersSeptive Biosystem Inc., Germany). HPLC-DAD analysis was performed at room temperature on LC-10AT equipment consisting of two high-pressure mixing pumps and a diode array detector (SPD-M10A) (Shimadzu Corp., Kyoto, Japan). Enzymatic assays were carried out on 160A UV−vis spectrophotometer and a RF-5000 spectrofluometer (Shimadzu Corp., Kyoto, Japan).

Chemicals. Leucine-*p*-nitroanilide, Hip-His-Leu, Suc-Ala-Ala-Phe-7-amino-4 methylcoumarin (SAAP-AMC), phosphoramidon, bestatin hydrochloride, and leucine aminopeptidase (type IV−S from porcine kidney microsomes) were purchased from Sigma (Sigma-Aldrich Chemie Inc., Steinheim, Germany). *O*-Phthalaldehyde was obtained from Merck KgaA (Daremstadt, Germany). Lisinopril was a gift from the Drug Institute (Warsaw, Poland). Source of NEP and ACE was a boar sperm preparation made available by the Mazovian Center of Animal Breeding and Reproduction (Łowicz, Poland).

Gallic and ellagic acids were purchased from ChromaDex (Santa Ana, CA), (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate, and quercetin from Carl Roth (Karlsruhe, Germany), and caffeic acid from Fluka (Fluka Sigma-Aldrich Chemie Inc., Buchs, Switzerland). Oenothein B and quercetin-3-*O*-glucuronide were isolated as described previously (*28*). All substances used in the biological assays were of >95% purity.

The Folin−Ciocalteu reagent was purchased from POCh (Gliwice, Poland). All solvents were of analytical or HPLC grade.

Plant Material. Defatted seeds of *Oenothera paradoxa* Hudziok and the 30% isopropanolic extract consisting of approximately 58% of polyphenolics were obtained from Agropharm S.A. (Tuszyn, Poland).

Preparation of Crude Extracts and Fractionation. Accurately weighed 20 g of powdered defatted seeds of *Oenothera paradoxa* were extracted with (a) water (2 × 100 mL), (b) 60%ethanol (v/v, 2 × 100 mL), (c) isopropanol (2 × 100 mL), and (d) 30%isopropanol (v/v, 2 × 100 mL) for 60 min in an ultrasonic water bath at room temperature. The isopropanolic extract was dried under vacuum at 40 °C giving a residue of 2.36 g. The aqueous extract and the aqueous residues after organic solvents evaporation were lyophilized, giving residues of 1.93 g for a, 2.58 g for b, and 2.05 g for d.

Accurately weighted 20 g of 30% isopropanolic extract from Agropharm was dissolved in water (200 mL) and extracted with ethyl acetate (3 × 200 mL). The collected ethyl acetate fractions were dried under vacuum at 45 °C (giving a residue of 3.2 g). The aqueous residue was lyophilized (giving a residue of 16.2 g).

A 2.5 g portion of the ethyl acetate extract was dissolved in 10 mL of methanol, adsorbed on 5 g of polyamide, applied to a column (5 cm in diameter and 8 cm in height) packed with polyamide 6 (particle size 0.05−0.16 mm, Carl Roth) and eluted with methanol (50 mL each), and with acetone/water (7:3; 50 mL each). One hundred fractions were collected and pooled into 1A−6A main fractions based on their polyphenolic TLC profile. All fractions were lyophilized.

A 7.5 g portion of the aqueous residue was dissolved in 40 mL of methanol−water (7:3) and applied to a column (4 cm in diameter and 40 cm in height) packed with Sephadex LH-20 (particle size 0.025−0.100 mm, Pharmacia, Uppsala, Sweden) and eluted with methanol−water (7:3; 50 mL each). Forty fractions were collected and pooled into 1B−5B main fractions based on their polyphenolic TLC profile. All fractions were lyophilized. Extracts and fractions were stored at −18 °C.

Total Phenolic Content. The total phenolic content was determined with the Folin−Ciocalteu reagent using catechin as the standard. Accurately weighed 0.05 g of each extract was dissolved in 25 mL of methanol−water (7:3), then 0.2 mL of each extract was mixed with 0.5 mL of the reagent and 10 mL of 10% Na_2CO_3 solution, and then filled up with water to 50 mL. The mixture were incubated in the dark for 30 min. The absorbance was measured spectrophotometrically at 700 nm.

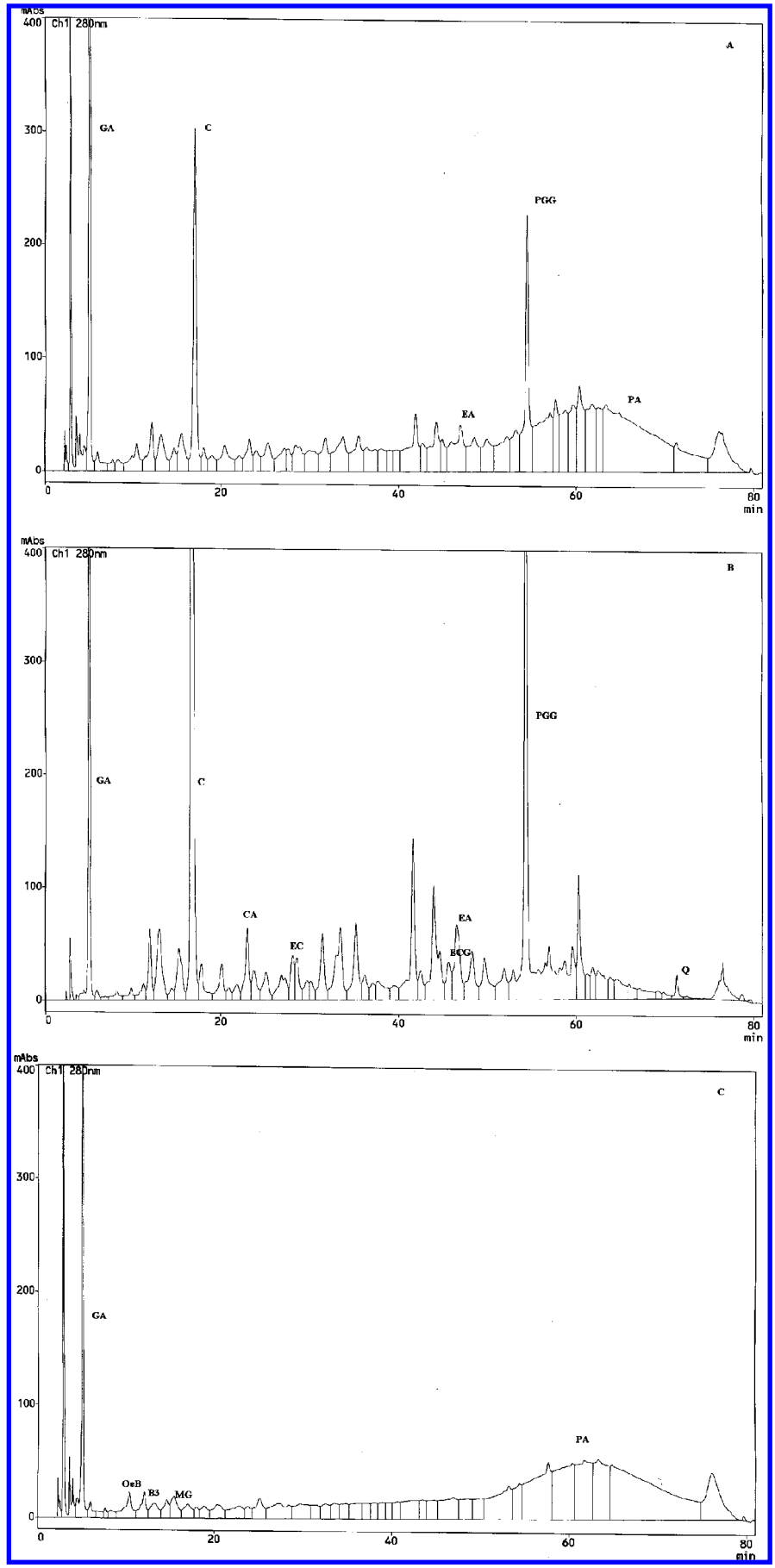

Figure 1. HPLC chromatograms of (**A**) 30% isopropanolic extract, (**B**) ethyl acetate extract, and (**C**) aqueous residue. Gallic acid (GA), oenothein B (OeB), procyanidin B3 (B3), methyl gallate (MG), (+)-catechin (C), caffeic acid (CA), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), ellagic acid (EA), pentagalloyl glucose (PGG), quercetin (Q), and procyanidins (PA).

Table 2. Metallopeptidase Inhibition by Pure Polyphenols Identified and Isolated at a Concentration of 100 μg/mL with IC_{50} values (μM)[a]

compounds	ACE inhibition at 100 μg/mL (IC_{50} μM)	NEP inhibition at 100 μg/mL (IC_{50} μM)	APN inhibition at 100 μg/mL (IC_{50} μM)
(+)-catechin	27.2 ± 4.1 (**>350**)	12.0 ± 1.0 (**>350**)	n.in
(−)-epicatechin	47.0 ± 2.2 (**>350**)	13.0 ± 2.1 (**>350**)	82.3 ± 0.9 (**173**)
(−)-epicatechin gallate	60.8 ± 4.0 (**165**)	26.4 ± 2.0 (**>350**)	85.2 ± 0.4 (**12**)
gallic acid	37.4 ± 4.8 (**>500**)	41.1 ± 6.5 (**480**)	n.in
ellagic acid	57.0 ± 2.0 (**400**)	26.0 ± 2.0 (**>500**)	20.2 ± 3.7 (**>500**)
caffeic acid	79.0 ± 2.0 (**220**)	28.0 ± 6.0 (**>500**)	n.in
oenothein B	27.0 ± 6.0 (**250**)	70.0 ± 5.0 (**20**)	39.0 ± 1.0 (**165**)
quercetin	51.0 ± 4.2 (**330**)	38.7 ± 1.2 (**>350**)	32.4 ± 3.9 (**>350**)
quercetin glucuronide	54.0 ± 3.0 (**200**)	50.0 ± 4.1 (**250**)	n.in
pentagalloyl glucose	76.7 ± 0.8 (**35**)	81.7 ± 2.0 (**12.5**)	98.8 ± 1.0 (**6.5**)
methyl gallate	52.5 ± 2.8 (**500**)	80.0 ± 0,5 (**65**)	62.2 ± 2.5 (**380**)
procyanidin B3	64.1 ± 2.5 (**135**)	88.0 ± 0.6 (**18**)	73.4 ± 1.4 (**58**)
lisinopril	IC_{50} **1nM**	(−)	(−)
phosphoramidon	(−)	IC_{50} **9nM**	(−)
bestatin	(−)	(−)	IC_{50} **5.3 μM**

[a] Data present the mean ± SEM of at least three independent experiments, each performed in duplicated samples ($P < 0.05$). IC_{50} values were obtained from dose−effect curves by linear regression. n.in., no inhibition; (−), not determined.

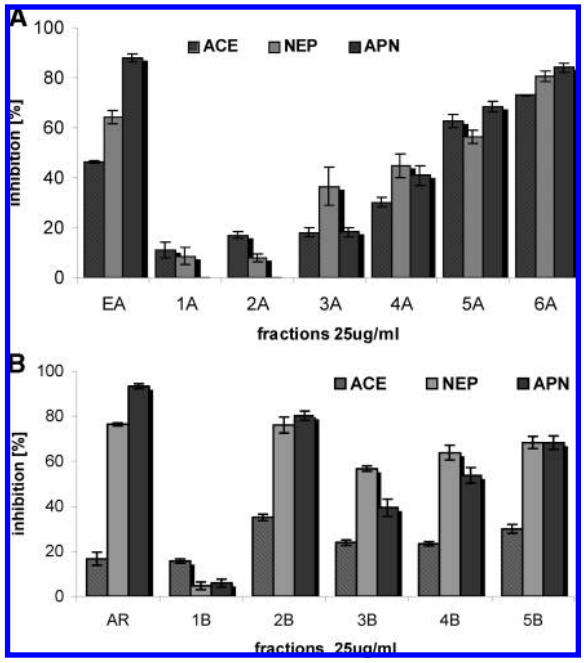

Figure 2. Metallopeptidase inhibition by fractions A and B at 25 μg/mL. EA, ethyl acetate extract; AR, aqueous residue. Data present the mean ± SEM of at least three independent experiments, each performed in duplicated samples ($P < 0.05$).

Total Proanthocyanidin Content. The total proanthocyanidin content was determined according to the fourth European Pharmacopoeia as cyanidin chloride.

Accurately weighted 0.2 g of each extract was dissolved in 30 mL of methanol−water (7:3) and heated under reflux for 80 min with 15 mL of 25% HCl. After cooling, each extract was filtered and filled up with methanol−water (7:3) to 250 mL. Then 50 mL of each solution was evaporated to about 3 mL and transferred to a separating funnel with 15 mL of water. The solutions were than extracted with butanol (3 × 15 mL, each). The organic layers were transferred to volumetric flasks and filled up with butanol to 100 mL. The absorbance was measured spectrophotometrically at 545 nm.

Thin-Layer Chromatography (TLC) Analysis. TLC was performed on silica gel 60 F_{254} (0.25 mm layer thickness) and polyamide 11 F_{254} precoated plates (Merck KgaA) and developed in a horizontal sandwich chamber (Chromdes, Lublin, Poland).

Polyphenolics. TLC was performed on a polyamide plate with 60% CH_3COOH as solvent systems or on a silica gel plate with $CHCl_3$/EtOAc/HCOOH (5:4:1). The spots were detected under $UV_{254,\ 366}$ and by spraying with 5% methanolic $FeCl_3$ solution (blue spots) or 1% vanillin solution in H_2SO_4 (pink spots).

Flavonol Glycosides. TLC was performed on a silica gel plate with EtOAc-HCOOH-H_2O (9:1:1 v/v) as the solvent system. The spots were detected under $UV_{254,\ 366}$ before and after spraying with methanolic 1%$AlCl_3$ and alkalizing with ammonia solution.

High-Performance Liquid Chromatography (HPLC) Analysis. The phenolics of different extracts and fractions were analyzed by HPLC with diode array detection. Separation was performed on a Luna C-18, 25 × 4.6 mm, 5 μm column (Phenomenex, Torrance, CA). The eluent was (A) 2.5% CH_3COOH and (B) CH_3CN + 2.5% CH_3COOH (80:20). A gradient solvent system was used: 7−20% B (45′); 20−40% B (70′); 40−100% B (75′); 100% B (80′). The flow rate was 1 mL/min, and the injection volume was 20 μL. UV spectra were recorded in the range of 200−400 nm, and chromatograms were acquired at 280 and 350 nm.

Samples were dissolved in MeOH + 2.5% CH_3COOH (1:1) to the concentration of 10, 5, or 2.5 mg mL^{-1}. The R_t and UV_{max} of the analyzed standards and isolated compounds are presented in **Table 3**.

Thiolysis. Thiolysis of procyanidins was performed as described by Guyot et al. (*29*). Samples were dissolved in methanol (50 μL) and mixed with 50 μL of 3.3% hydrochloric acid in methanol, and 100 μL of 5% benzyl mercaptan in methanol. The solution was incubated in 40 °C for 30 min and then cooled in an ice bath. Products were analyzed by HPLC in the above mentioned conditions.

Isolation of Active Compounds. The ethyl acetate fraction 5A (225 mg) was applied to a column (2.5 cm in diameter and 35 cm in height) packed with Toyopearl HW-40, fine grade (Tosoh, Tokyo, Japan), and eluted with acetone/water (7:3) (10 mL each). Fractions 7−9 (5A) were separated again in the same column conditions, eluted with acetone/water (4:6) (10 mL each) to obtain compound **1** (53 mg).

The water residue fraction 2B (70 mg) was applied to a column (2.5 cm diameter and 20 cm height) packed with Toyopearl HW-40, fine grade (Tosoh), and eluted with methanol−water (7:3) (10 mL each) to obtain compound **2** (10 mg).

The water residue fraction 4B (150 mg) was applied to a column (2.5 cm diameter and 20 cm height) packed with Toyopearl HW-40, fine grade (Tosoh), and eluted with methanol−water (7:3) (10 mL each) to obtain compound **3** (31 mg).

1,2,3,4,6-O-Penta-O-galloyl-β-D-glucose (**1**). Off-white powder; UV, λ_{max} 281 nm; ESI-MS (positive ion mode) *m/z* 963.1 [M + Na]+; ^{1}H NMR (MD3OD) glucose moiety: δ 6.46 (1H, d, $J = 8$ Hz, H-1), 6.20 (1H, t, H-3), 5.85 (1H, m, H-4), 4.78 (1H, m, H-5), 4.51 (1H, m, H-6). Galloyl moieties: δ 7.16, 7.18, 7.23, 7.25, 7.32 (each 2H, s). ^{13}C NMR (MD3OD) glucose moiety: 93.85 (C-1), 74.45 (C-5), 74.13 (C-3), 72.21 (C-4), 69.82 (C-2), 63.15 (C-6). Galloyl moieties: 167.99, 167.35,

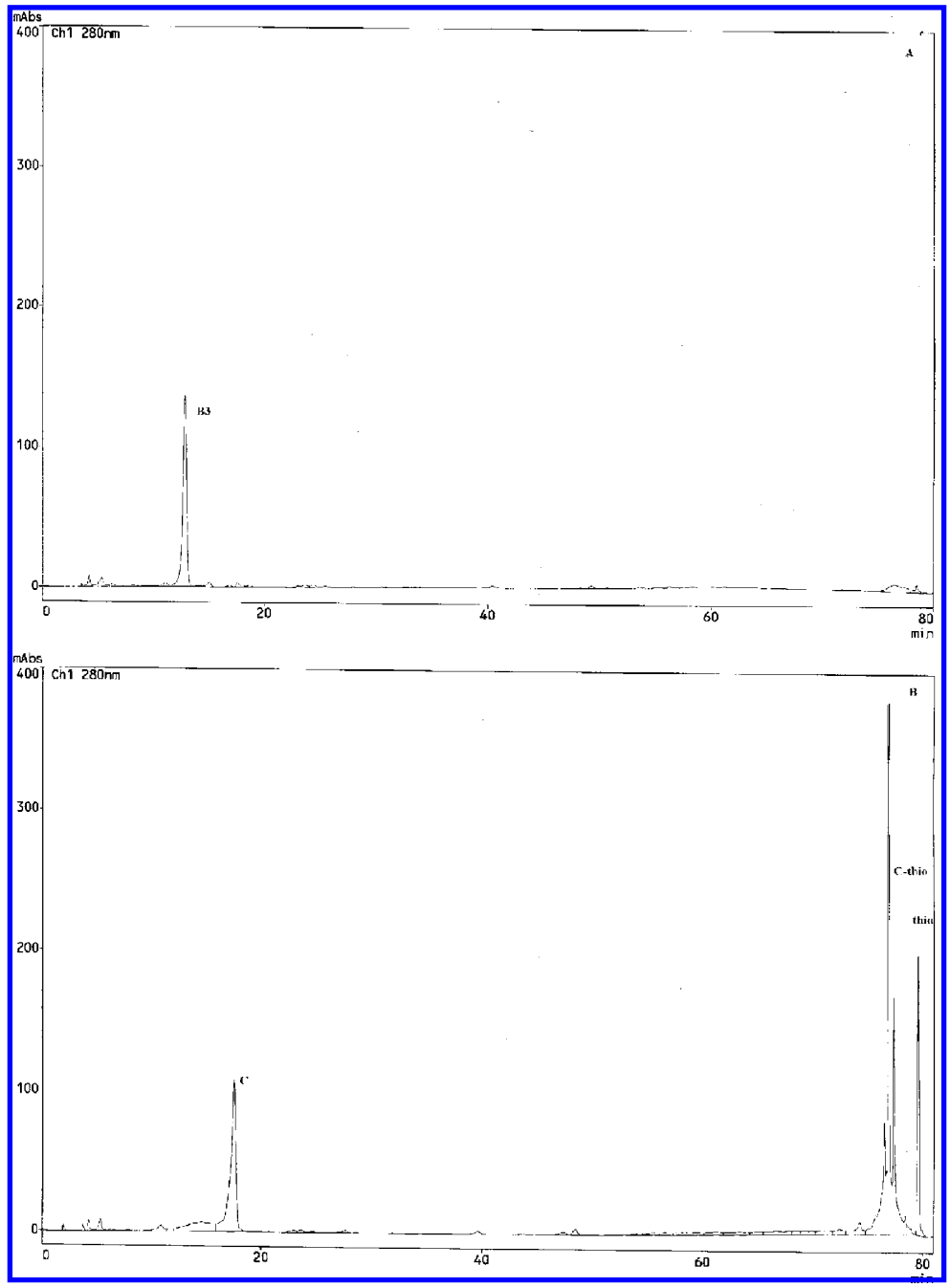

Figure 3. HPLC chromatograms of (**A**) procjanidin B3 (B3) and (**B**) products of thiolytic degradation of procjanidin B3: (+)-catechin (C), catechin benzylthioether (C-thio), and benzylmercaptan (thio).

Table 3. R_t and λ_{max} of Analyzed Compounds by HPLC-DAD

compounds	R_t [min]	λ_{max} [nm]
gallic acid (GA)	5.3	273
oenothein B (OeB)	11.1	238; 265
procyanidin B3 (B3)	12.9	236; 279
methyl gallate (MG)	16.9	273
(+)-catechin (C)	17.9	236; 279
caffeic acid (CA)	24.3	242; 295sh; 325
(−)-epicatechin (EC)	28.9	236; 279
(−)-epicatechin gallate (ECG)	48.5	236; 279
ellagic acid (EA)	49.3	254; 366
pentagalloyl glucose (PGG)	55.2	281
quercetin glucuronide (QG)	56.04	256; 356
quercetin (Q)	72.9	254; 374

167.08, 166.98, 166.28 (carbonyl group signals), 146.63, 146.53, 146.50, 146.44, 146.34 (C-3, C-5), 141.00, 140.53, 140.48, 140.27, 140.14 (C-4), 121.00, 120.31, 120.17, 120.13, 119.62 (C-1), 110.63, 110.48, 110.42, 110.40, 110.35 (C-2, C-6).

*Methyl Gallate (***2***).* Light brown powder; UV, λ_{max} 273 nm; ESI-MS (positive ion mode) *m/z* 207.0 [M + Na]+; ^{1}H NMR (MD3OD): δ 6.96, (2H, s, H-2, H-6), 3.75 (3H, s, -OCH_3). ^{13}C NMR (MD3OD): 169.2 (-COOH), 144.8 (C-3, C-5), 138.5 (C-4), 120.7 (C-1), 110.0 (C-2, C-6), 52.7 (-OCH_3).

*(+)-Catechin (4*α *→ 8)-(+)-Catechin (Procyanidin B3) (***3***).* Light brown powder; UV, λ_{max} 236 nm, 279 nm; ESI-MS (positive ion mode) *m/z* 601.1 [M + Na]+; thiolysis has shown the presence of (+)-catechin and (+)-catechin thioether (**Figure 3**); ^{13}C NMR (MD3OD): 157.9−156.9 (C-5u, C-5t, C-7u, C-7t), 146.29, 145.96, 145.89, 145.58 (C-3′u, C-3′t, C-4′u, C-4′t), 132.28 (C-1′u, C-1′t), 120.09 (C-6′u), 119.43 (C-6′t), 116.13, 115.94, 115.39, 115.31 (C-2′u, C-2′t, C-5′u, C-5′t), 110.09 (C-8t), 100.87 (C-10u, C-10t), 96.35 (C-6t), 95.90 (C-6u), 95.56 (C-8u), 82.91 (C-3u), 77.13 (C-2u), 73.19 (C-2t), 68.86 (C-3t), 37.21 (C-4t), 28.57 (C-4u).

Angiotensin-Converting Enzyme Assay. Determination of metallopeptidase activity was performed as described previously (*28*). Briefly, the substrate Hip-L-His-L-Leu solution (20 μL, 4 mM in water) was added to 30 μL of phosphate buffer (83 mM K_2HPO_4 + 326 mM NaCl, pH 8.3), 50 μL of test compounds or extract solution, and 200 μL of ACE from the boar sperm preparation (1:600). The reaction mixture was incubated for 30 min (at 37 °C) after which the reaction was stopped with 0.4 M NaOH (1000 μL), and a methanolic *o*-phthalaldehyde solution (2%, 100 μL) was added for the formation of the fluorescence His-Leu-*o*-phthalaldehyde complex. The mixture was incubated in the dark for 10 min, and the reaction was stopped by an addition of 2 M HCl (300 μL). Fluorescence was measured at λ_{exit} = 365 nm and λ_{emiss} = 500 nm. The inhibition rate was calculated in comparison to the control without inhibitor, taking into account the absorbance of fluorescence light by test extracts/compounds.

Neutral Endopeptidase Assay. A two-step assay was used for the determination of NEP activity (*28*). Lisinopril (50 μL, 8 μM), 50 μL Suc-L-Ala-L-Ala-Phe-7-amino-3-methyl-coumarin (SAAP-AMC 400 μM) and 350 μL of HEPES buffer (50 mM + 154 mM NaCl, pH 7.4) with or without the test extracts or compounds were combined and mixed. The first enzymatic reaction was started by an addition of 150 μL of NEP from the boar sperm preparation (1:2000) and incubated for 60 min (37 °C). The reaction was stopped by adding 50 μL of phophoramidon solution (50 μM).

The second reaction was initiated by the addition of 20 μL of a leucine aminopeptidase (APN) solution (1:235), and the reaction mixture was incubated again for 60 min (56 °C). The reaction was stopped by adding 800 μL of acetone. The fluorescence of the released 7-amino-3-methyl-coumarin (AMC) was measured at λ_{exit} = 367 nm and λ_{emiss} = 440 nm. The inhibition rate was calculated in comparison to the control without inhibitor, taking into account the possibility of an influence on APN or/and fluorescence of test extracts/compounds.

Aminopeptidase N Assay. Determination of aminopeptidase activity was performed as described previously (*28*). L-Leucine-*p*-nitroanilide solution (250 μL, 2 mM in HEPES buffer) was added to 200 μL of HEPES buffer (50 mM + 154 mM NaCl, pH 7.4) with or without the test extracts or compounds. The reaction was started by an addition of 50 μL of APN solution (1:5000 in HEPES buffer) and incubated for 60 min (37 °C). Addition of 800 μL of acetone stopped the reaction. The samples were measured spectrophotometrically at 405 nm to determine the *p*-nitroaniline formed.

Statistical Analysis. All results are presented as the mean ± standard error (SEM) of at least three independent experiments (each performed in duplicate). Statistical analysis was performed by using Student's *t*-test, $p < 0.05$. IC_{50} values were obtained from dose−effect curves by linear regression.

RESULTS AND DISCUSSION

A number of studies have previously shown a strong antioxidant activity of *O. biennis* and *O. paradoxa* defatted seed extracts, and the results obtained suggested a high biological potential of evening primrose polyphenols (*15*−*21*). In the present study, for the first time, we have used the *in vitro* metallopeptidases model for identification of a potential novel activity of defatted evening primrose seed extracts and for defining the active compounds contained in them.

All of the tested seeds extracts of different polarity (aqueous, 60% ethanolic, isopropanolic, and 30% isopropanolic) at concentrations of 1.5−100 μg/mL showed a significant and dose-dependent inhibition of metallopeptidases (**Table 1**). The analysis of 50% inhibition of enzyme activity (IC_{50}) showed that aminopeptidase N was the enzyme most affected with IC_{50} at the level of 2.8 μg/mL and 2.9 μg/mL for the aqueous and the 30% isopropanolic extracts, respectively. Notably, the IC_{50} values for those extracts were only slightly lower than those of bestatin, a synthetic inhibitor of this enzyme. Neutral endopeptidase was quite strongly inhibited by the extracts as well; however, the inhibition was lower than that of the selective inhibitor phosphoramidon. There were no significant differences among the aqueous, 60% ethanolic, and 30% isopropanolic extracts in APN and NEP inhibition, probably due to the good extraction of active compounds by all used solvents.

The extracts also exhibited an, albeit weaker, influence on angiotensin-converting enzyme. The most active extract was the 30% isopropanolic one, showing IC_{50} = 26 μg/mL, a result comparable to that obtained for the *Pinus maritima* procyanidin-rich extract (IC_{50} = 26 μg/mL), which was reported to have cardiovascular benefits (*30*). In fact, the analysis of the total polyphenol and total proanthocyanidin content revealed a very high level of the latter in all extracts. In our study, only the isopropanolic extract contained significantly less polyphenols, which also correlated with a lower activity of this extract (**Table 1**). The HPLC analysis of all extracts showed similar qualitative profiles with a wide peak characteristic for polymeric proanthocyanidins. In order to get more detailed information about the active compounds present in those extracts, we performed liquid−liquid extraction to divide the 30% isopropanolic extract in a more lipophilic ethyl acetate extract (EA) and a hydrophilic aqueous residue (AR). The enzyme inhibition analysis showed a noticeably lower activity of EA and AR in comparison with the crude extract (**Table 1**). Only APN appeared to be affected by AR more strongly, with IC_{50} = 2.5 μg/mL. The results suggested that the active compounds were present in both fractions.

The HPLC-DAD analysis of the 30% isopropanolic, EA, and the AR extract showed, by comparison of the retention time (R_t) and UV spectra maximum (λ_{max}) with the standards, the presence of gallic acid, (+)-catechin, caffeic acid, (−)-epicatechin, (−)-epicatechin gallate, ellagic acid, and quercetin (**Figure 1**). Apart from the gallic acid and (+)-catechin, the remaining identified compounds were present in low quantities. The AR extract was rich in polymeric procyanidins (PA), while the EA extract was abundant in a compound later identified as penta-*O*-galloyl-β-D-glucose (PGG). In the enzymatic assay, among the pure compounds tested at the concentration of 100 μg/mL, (−)-epicatechin gallate (ECG) was the only one that exhibited significant activity, but even that was observed against APN only. The obtained IC_{50} = 12 μM for ECG was quite low when compared with IC_{50} = 5.3 μM for bestatin (**Table 2**). At the same time, epicatechin was a much weaker inhibitor, while gallic acid showed no activity. Unfortunately, (−)-epicatechin gallate was only a minor compound in the evening primrose seed extracts and could not be responsible for the crude extract activity observed. To get a more in-depth insight into this issue, we performed the fractionation of both the EA and AR extracts. The ethyl acetate extract was fractionated using CC chromatography on polyamide, with methanol and acetone/water (7:3) as eluents. The collected fractions were pooled into the 1A−6A main fractions based on their polyphenolic TLC profile. The aqueous residue was fractionated on Sephadex LH-20 using methanol−water (7:3) as the eluent, giving the 1B−5B main fractions, collected on the basis of their polyphenolic TLC profile. Following the fractionations, the HPLC-DAD profiles and the biological activity of the described fractions (at 25 μg/mL) were evalueted. As shown in **Figure 2**, fractions 5A, 6A, 2B, 4B, and 5B were the most active ones. The activities of those fractions were similar to, or only slightly lower than, that of the initial extracts, suggesting that there might be more compounds with inhibitory properties present. From fraction 5A, 1,2,3,4,6-*O*-penta-*O*-galloyl-β-D-glucose (PGG) was isolated as the main compound, and it exhibited a significant and dose-dependent inhibition of all free metallopeptidases (**Table 2**).

However, inhibition of ACE was the weakest among the effects observed, with $IC_{50} = 35$ μM, as compared to the inhibition of NEP ($IC_{50} = 12.5$ μM) and APN ($IC_{50} = 6.5$ μM). The results obtained correlated with the lower activity of the extracts against ACE observed in our study. A nonspecific inhibition of the converting enzyme by PGG has been described before by Lui et al. (*31*) Interestingly, the authors also found that an *in vivo* administration of a related compound 1,2,3,4-*O*-tetra-*O*-galloyl-β-D-glucose, had a strong hypotensive effect in spontaneously hypertensive rats (SHR) after angiotensin I administration (*31*). It is noteworthy that PGG also inhibits neutral endopeptidase, an enzyme that is involve in blood pressure control.

The HPLC chromatogram of fraction 6A showed a wide peak characteristic for oligomeric and polymeric procyanidins. Thiolytic degradation revealed the presence of (+)-catechin and (−)-epicatechin gallate (ECG) and their thioether analogues. The degree of polymerization calculated according to the method by Jerez et al. (*32*) was DPn = 6. Previously, Lu et al. (*21*) in their analysis of the *O. biennis* seed extract found the presence of tetrameric procyanidin containing catechin and ECG as the subunits. Therefore, it seems feasible that procyanidins are the active compounds in fraction 6A analyzed here. Additionally, from fraction 4B, we were able to isolate the dimeric procyanidin B3, which also showed an inhibitory activity, particularly against NEP ($IC_{50} = 18$ μM). The main difference between the two analyzed fractions was that fraction 6A strongly inhibited the angiotensin-converting enzyme activity, while procyanidin B3 is a relatively weak inhibitor of this enzyme (**Table 2**). Although inhibition of ACE by procyanidins is a well established fact (*30, 33*−*35*), there is no information on NEP and APN inhibitory activity for this particular class of compounds, nor is there any information for gallotannins such as PGG. Proanthocyanidins demonstrate hypotensive properties not only by inhibiting ACE activity but also by blocking angiotensin II binding to human AT_1 receptors and by endotelium-dependent nitric oxide-mediated relaxation (*36, 37*).

When analyzing fraction 2B, we isolated methyl gallate (MG), which proved to be considerably active against NEP ($IC_{50} = 65$ μM) but showed only a weak inhibition of the APN activity. Because of the strong activity observed for this fraction, particularly against aminopeptidase, we analyzed this fraction by TLC, HPLC-DAD, and by MS methods and, as a result, found the presence of gallic acid (GA) and quercetin glucuronide (QG). However, upon further analysis, both of those compounds proved to be weak inhibitors, suggesting the presence of a minor compound (or compounds) with higher activity.

In the last fraction analyzed, 5B, we identified ellagitannin oenothein B (OeB) by means of TLC, HPLC-DAD, and MS analysis in comparison with the authentic standard. Thiolytic degradation of this fraction showed the presence of procyanidin(s) containing, (+)-catechin subunits only, with the degree of polymerization DPn = 6. Those two classes of compounds seemed to be responsible for both the NEP and APN inhibition. Using Sephadex LH-20 for fractionation of AR, we were unable to elute procyanidins with a higher degree of polymerization than DPn = 6. It seems probable that those compounds affect the metallopeptidase activity as well; however, they might be active upon topical application rather than oral application.

In summary, we identified four active compounds present in defatted primrose seed extracts, classified as (−)-epicatechin gallate, proanthocyanidin B3, oenothein B, and penta-*O*-galloyl-β-D-glucose. Oenothein B has been previously shown to inhibit metallopeptidases (*28*). The three other compounds have been known to inhibit ACE, but there has been no reports to date on their inhibitory activity against NEP and APN. Notably, we showed here that PGG is a dominating compound in the *O. paradoxa* seed extract. The presence of high amounts of procyanidins with different degrees of polymerization also appeared to play a role in crude extract activity. We should emphasize that almost all of the polyphenols identified in this study have not been repoted for *O. paradoxa* seeds before.

Dual NEP/ACE inhibitors (vasopeptidase inhibitors) increase the levels of natriuretic and vasodilatory peptides (bradykin), and simultaneously inhibit the renin−angiotensin−aldosteron system. This results in a reduced blood pressure, improved local blood flow, sodium−water balance, and target organ protection (*22, 23*). Extracts that contain these particular metallopeptidases inhibitors may potentially have beneficial effects on the cardiovascular system by reduction of blood pressure, by improving endothelium-dependent relaxation and cardiac function (*22, 23*). However, those suppositions should be confirmed in an *in vivo* study after oral administration in order to confirm the pharmacological effects and the bioavailability of active compounds.

In our study, the investigated extracts and PGG showed, at the level comparable to that of the synthetic inhibitor bestatin, the inhibition of aminopeptidase N, the enzyme that plays an important role in the invasion and metastasis of cancer cells. It appears that this inhibition could be one of the mechanisms by which an evening primrose extract might act as an antitumor agent. Interestingly, APN activity has been found in keratinocytes isolated from the skin of patients with various inflammatory skin diseases as well as in epithelial skin tumors. It seems that APN could play a role in the regulation of keratinocyte growth (*38, 39*). Synthetic inhibitors of APN actononin and bestatin in keratinocytes and sebocytes were able to suppress proliferation and restore cytokine balance altered in early acne pathogenesis (*40*). The strong APN inhibition by the evening primrose seeds extract appears to be in agreement with the fact that the *Oenothera* sp. plants are reputed for wound healing properties in traditional medicine (*5*). APN is an ectoenzyme situated in the epidermis, and its activity may be quite easily affected by polyphenols from the extract, when applied topically. This aspect of extract activity is worth further investigation.

Oenothera paradoxa seed extracts are rich in polyphenols and appear to have considerable biological potential. However, more *in vitro* and *in vivo* studies need to be performed to support the finding contained in this article.

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