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Constituents in Evening Primrose Oil with Radical Scavenging, Cyclooxygenase, and Neutrophil Elastase Inhibitory Activities

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Cold-pressed, non-raffinated evening primrose oil was found to contain lipophilic radical scavengers. A highly enriched fraction of these compounds could be obtained from the oil by extraction with aqueous ethanol and subsequent liquid−liquid partitioning with petroleum. LC-DAD-MS analysis revealed that the fraction contained three aromatic compounds with identical UV and ESI-MS spectra. The compounds were isolated by RP-HPLC and their structures established by chemical and spectroscopic means as 3-*O*-*trans*-caffeoyl derivatives of betulinic, morolic, and oleanolic acid. The morolic acid derivative was a new compound. The three esters exhibited pronounced radical scavenging activity against the stable 2,2-diphenyl-1-picrylhydrazyl radical and were potent inhibitors of neutrophil elastase and cyclooxygenase-1 and -2 in vitro. Commercial samples of evening primrose oils contained only traces of these lipophilic antioxidants.

KEYWORDS: Evening primrose oil; *Oenothera biennis*; Onagraceae; caffeoyl ester; triterpenoid acids; radical scavenger; DPPH radical; neutrophil elastase; cyclooxygenase; in vitro

INTRODUCTION

Oil obtained from the seeds of evening primrose (*Oenothera biennis* L., Onagraceae) has attracted much interest due to its high content of polyunsaturated fatty acids, in particular, γ-linolenic acid (18:3n-6) (*1*). Evening primrose oil (EPO) is widely used as a dietary supplement from which beneficial effects have been reported in rheumatic and arthritic conditions, atopic dermatitis, psoriasis, premenstrual and menopausal syndrome, and diabetic neuropathy (*2*). Hovewer, the present clinical evidence for most uses needs to be substantiated by further rigorous trials (*3*, *4*). The beneficial effects of evening primrose oil are thought to be due to its γ-linolenic acid content, since ω-6 fatty acids are precursors for eicosanoids of the 1-series and exert an inhibitory effect on leucotriene synthesis (*3*).

Parallel to the predominantly clinical investigations on the seed oil, the phytochemistry of the roots and the aerial parts has been studied. Triterpenoids, gallic acid derivatives, and functionalized long-chain alcohols and acids were identified in the roots (*5*). Numerous flavonol glycosides, gallic acid derivatives, phenylpropanoids, and anthocyanins have been reported in polar plant extracts (*6*). The aerial parts are rich in tannins. Indeed, high content of phenolics and hydrolyzable tannins is considered a chemotaxonomic feature of the family Onagraceae (*7*). Recent interest has focused on the biological properties and phytochemistry of the seed cake obtained as a byproduct of oil extraction, as the seed meal has been found to be a rich source of phenolic antioxidants (*8*−*12*). The main phenolics have been recently identified as (+)-catechin, (−)-epicatechin, and gallic acid (*12*).

In contrast, little effort has been expended to characterize the non-triglyceridic constituents of EPO. The non-saponifiable portion, about 1.5−2% of the oil, reportedly consists of sterols, 4-methyl sterols, triterpene alcohols, hydrocarbons, alcohols, and tocopherols (*1*). As part of ongoing investigations on bioactive secondary plant metabolites in medicinal and food plants (*13*), we recently carried out some preliminary analytical screening of EPO. Interestingly, the analytical profiles of various oils showed distinct differences in the qualitative and quantitative composition of the non-triglyceride (NTG) portion. These differences appeared to be linked to the extraction process. It should be mentioned that evening primrose oils are usually obtained either by extraction with hydrocarbons (*7*), by supercritical fluid extraction (SFE) (*7*, *14*, *15*), or by mechanical pressing. Cold-pressed oils, which had a yellow-greenish appearance, showed the highest NTG content. Preliminary thin-layer chromatographic (TLC) analysis of the NTG fraction using various staining reagents revealed the likely presence of lipophilic phenolic and terpenic compounds. We were particularly intrigued by the strong radical scavenging activity which

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was detected upon staining of the TLC plate with an ethanolic solution of DPPH radical. Various edible oils had been screened for their radical scavenger abilities (*16*, *17*), but we could not find any information with respect to antioxidants in EPO. This prompted us to investigate the NTG fraction, and we report here on the purification, identification, and preliminary biological characterization of phenolic esters in EPO.

MATERIALS AND METHODS

General Procedures. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DMX-400 instrument operating at 400.13 and 100.63 MHz, respectively. All samples were measured in $CDCl_3$.^{13}C NMR and ^{1}H NMR assignments were made with the aid of DEPT subspectra and HSQC and ^{1}H−^{1}H COSY experiments, and by comparison with literature data. Thin-layer chromatography (TLC) was carried out on silica gel GF254-coated Al sheets (Merck, Darmstadt, Germany). A Desaga AS 30 TLC sample applicator (Desaga, Wiesloch, Germany) was used for TLC. LC-DAD-MS analysis was performed with a Agilent 1100 HPLC consisting of a solvent degasser, a binary high-pressure mixing pump, an autosampler, a column oven, and a diode array detector, connected via a T-splitter (4:1) to an Applied Biosystems API 165 mass spectrometer with a turbo ionspray interface. Isolation of compounds **1**−**3** was carried out with a Hewlett-Packard 1050 HPLC instrument. COX and DPPH assays were carried out on a Perkin-Elmer HTS 7000 microplate reader. Optical rotation measurements were carried out with a Polartronic-E polarimeter (Schmidt/Haensch, Berlin, Germany). UV−vis spectra were recorded with a Beckman DU 640 spectrophotometer.

Evening Primrose Oils. Cold-pressed, non-raffinated oil (lot no. 55) was obtained from Kroppenstedter Oil Mill (Kroppenstedt, Germany). Ropufa "10" n-6 oil (lot no. E007004) was purchased from Roche Lipid Technologies (Heanor, UK). CO_2-extracted oil, refined, deacidified, and deodorized (lot no. R0568-6A), was provided by Paninkret (Pinneberg, Germany).

Reference Compounds and Biochemicals. Caffeic acid was purchased from Merck (Darmstadt, Germany). Gallic acid and (+)-catechin were from our own laboratories (*18*). Oleanolic acid was purchased from Extrasynthese (Genay, France) and betulinic acid from Bio-Service (Halle, Germany). Morolic acid was provided by Dr. Y. Rios, Universitad Autonoma del Estado de Morelos. Purified cyclooxygenase-1 (COX-1) from sheep and human recombinant COX-2 were purchased from Cayman Chemicals (Ann Arbor, MI). ELISA kits for thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) were from R&D Systems (Minneapolis, MN). The assays were performed according to package instructions. Unless stated otherwise, all other reagents were from Sigma (Deisenhofen, Germany).

Extraction of Radical Scavengers. Initial purification of the NTG fraction was carried out according to the procedure of Brenes et al. (*19*), with some modifications. Briefly, 15 mL of cold-pressed oil was extracted with 4 × 15 mL of methanol/water (80:20). The methanol was removed under reduced pressure. The oily residue was redissolved in acetonitrile and washed with 3 × 20 mL of hexane. The acetonitrile layer was taken to dryness. For the cold-pressed oils, typical yields of the NTG fraction were 60−70 mg; commercial EPO samples yielded 10−20 mg.

In the modified procedure, the NTG fraction was extracted with 4 × 15 mL of ethanol/water (80:20). The combined extracts were washed with 3 × 20 mL of hexane. The ethanol was removed in vacuo to afford a pale solid residue. Typical yields of the NTG fraction from the cold-pressed oils were 60 mg. For the isolation of compounds, 340 g of cold pressed oil was extracted likewise to afford 1.85 g of solid residue.

TLC Analysis. The residue was redissolved in methanol (7 mg/mL). Solutions (5 mg/mL) of gallic acid and catechin were used for reference purposes. Aliquots of 5 μL of these solutions were applied onto the silica gel TLC plate. Chloroform/methanol (90:10) (system 1) and chloroform/ethyl formiate/formic acid (30:60:10) (system 2) were used as mobile phases. UV detection was carried out at 254 and 366 nm, and by staining of TLC plates with Godin's reagent (*20*), natural products/poly(ethylene glycol) reagent (NP/PEG) (*21*), or 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical (1 mg/mL in ethanol) (*22*).

LC-DAD-MS Analysis. HPLC separation was carried out on a Hypersil ODS column (5 μm; 4.6 × 250 mm i.d.) with a water/acetonitrile (both containing 2% acetic acid) gradient from 100:0 to 0:100 in 30 min, then maintaining 0:100 for 15 min. The flow rate was 1.0 mL/min, the column temperature was set at 25 °C, and the injection volume was 20 μL. Chromatograms were recorded at 330 nm, while DAD spectra were measured from 200 to 500 nm. MS spectra were recorded in the negative ion mode using the following settings: scan range, *m*/*z* 150−1000; source temperature, 350 °C; ion spray voltage, −5400 V.

Isolation of Compounds 1−3. The NTG fraction (1.85 g) was separated by column chromatography on silica gel (40−63 μm; 3 × 30 cm i.d.) with chloroform/methanol (90:10) as the mobile phase. Ten fractions were collected on the basis of the TLC pattern. Fractions 9 (130 mg) and 10 (85 mg) contained the radical scavenging compounds. For the isolation of pure substances, a portion of fraction 9 was submitted to HPLC. The separation was carried out on a Hypersil ODS column (5 μm; 4.6 × 250 mm i.d.) equipped with a precolumn. Acetonitrile/water (98:2) was used as the eluent. The flow rate was 0.75 mL/min, with detection at 360 nm. Repeated injections (20 μL) of a purified fraction (60 mg/mL in acetonitrile) were carried out, and peaks 1−3 were collected individually to afford compounds **1** (17 mg), **2** (20 mg), and **3** (5 mg), respectively. The purity of the compounds was checked by HPLC and TLC.

Base Hydrolysis of Compounds 1−3. Compounds **1**−**3** (0.5 mg) were dissolved in methanol (0.5 mL). After addition of aqueous NaOH (0.5 N, 2.5 mL), the mixture was kept overnight at room temperature. The solution was brought to pH 4 by addition of dilute HCl and extracted with ethyl acetate. The organic layer was rinsed with water, concentrated, and analyzed by co-chromatography with authentic reference samples of betulinic, oleanolic, and morolic acids. TLC on silica gel was carried out with toluene/ethyl acetate/methanol (80:20:2); detection was with Godin reagent.

Acidic Hydrolysis of Compounds 1−3. Compounds **1**−**3** (0.5 mg) were dissolved in methanol (0.5 mL). Ascorbic acid (10 mg) and aqueous trifluoroacetic acid (1 mL, 1 N) were added. The mixture was flushed with nitrogen and heated in a sealed vial for 1 h at 110 °C. After cooling to room temperature, the solution was extracted with ethyl acetate. The organic layer was concentrated and analyzed by TLC on silica gel in comparison with an authentic sample of caffeic acid. The eluent was chloroform/methanol/acetic acid (88:10:2); UV detection was at 254 and 366 nm after spraying with an ethanolic solution of NP/PEG reagent.

Physicochemical Data of Isolated Compounds. *3β-trans-(3,4-Dihydroxycinnamoyloxy)-20(29)-lupen-28-oic Acid (**1**).* Colorless glass, $[\alpha]^{20}_D$ −60° (MeOH, *c* 0.2). UV: λ_{max} (MeOH, nm) (log ϵ): 243 (3.84), 297sh (3.89), 328 (3.99). ESI-MS (negtive ion mode): *m*/*z* 617 $[M - H]^-$, 1236 $[2M - H]^-$. ^{1}H and ^{13}C NMR: signals in agreement with literature data (*23*).

*3β-trans-(3,4-Dihydroxycinnamoyloxy)olean-18-en-28-oic Acid (**2**).* Colorless glass, $[\alpha]^{20}_D$ 0° (MeOH, *c* 0.2). UV: λ_{max} (MeOH, nm) (log ϵ): 243 (3.85), 297sh (3.90), 328 (4.00). ESI-MS (negative ion mode): *m*/*z* 617 $[M - H]^-$, 1236 $[2M - H]^-$. HR-ESI-MS (positive ion mode): *m*/*z* 641.38206 $[M + Na]^+$ (calcd for $C_{39}H_{54}NaO_6$ 641.38181). ^{1}H NMR: 1.04 (H-1a),[a] 1.77 (H-1b),[a] 1.68 (H_2-2),[a] 4.55 (1H, dd, J = 6.2, 11.1 Hz, H-3), 0.87 (H-5),[a] 1.38 (H-6a),[a] 1.52 (H-6b),[a] 1.35 (H-7a),[a] 1.42 (H-7b),[a] 1.35 (H-9),[a] 1.58 (H_2-11),[a] 1.23 (H-12a),[a] 1.58 (H-12b),[a] 2.25 (1H, br s, H-13), 1.21 (H-15a),[a] 1.65 (H-15b),[a] 2.10 (H-16a),[a] 1.40 (H-16b),[a] 5.10 (br s, H-19),[a] 1.37 (H-21a),[a] 1.30 (H-21b),[a] 1.90 (H-22a),[a] 1.60 (H-22b),[a] 0.87 (s, H_3-23),[a] 0.93 (s, H_3-24),[a] 1.02 (s, H_3-25),[a] 0.93 (s, H_3-26),[a] 0.81 (s, H_3-27),[a] 0.95 (s, H_3-29),[a] 0.92 (s, H_3-30),[a] 7.00 (1H, d, J = 1.9 Hz, H-2′), 6.75 (1H, d, J = 8.2 Hz, H-5′), 6.91 (1H, dd, J = 8.2, 1.9 Hz, H-6′), 7.51 (1H, d, J = 15.9 Hz, H-7′), 6.21 (1H, d, J = 15.9 Hz, H-8′). [[a]The multiplicity pattern or integration was unclear due to signal overlapping.] ^{13}C NMR: δ 38.4 (C-1), 23.5 (C-2), 80.9 (C-3), 37.7 (C-4), 55.7 (C-5), 17.8 (C-6), 34.3 (C-7), 40.5 (C-8), 51.1 (C-9), 37.0 (C-10), 20.8 (C-11), 25.7 (C-12), 41.1 (C-13), 42.3 (C-14), 29.2 (C-15), 33.2 (C-16), 48.2 (C-17), 137.5 (C-18), 132.1 (C-19), 31.6

(C-20), 33.2 (C-21), 33.3 (C-22), 27.1 (C-23), 15.9 (C-24), 15.2 (C-25), 15.8 (C-26), 14.1 (C-27), 179.1 (C-28), 29.4 (C-29), 28.1 (C-30), 126.3 (C-1′), 113.7 (C-2′), 145.4 (C-3′), 148.1 (C-4′), 115.1 (C-5′), 121.5 (C-6′), 145.2 (C-7′), 114.2 (C-8′), 167.8 (C-9′).

*3β-trans-(3,4-Dihydroxycinnamoyloxy)olean-12-en-28-oic Acid (**3**).* Colorless glass, $[\alpha]^{20}_D$ +50° (MeOH, *c* 0.2). UV: λ_{max} (MeOH, nm) (log ϵ): 243 (3.95), 297sh (4.00), 328 (4.10). ESI-MS (negative ion mode): *m/z* 617 $[M - H]^-$, 1236 $[2M - H]^-$. ^1H and ^{13}C NMR: signals in agreement with literature data (*24*).

Evaluation of Radical Scavenging Activity. The radical scavenging potential was assessed in a microtiter-based assay according to Gamez et al. (*25*) with some modifications. Briefly, test compounds and 300 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical in ethanolic solution were shaken for 10 min at 20 °C, and absorbances were measured at 517 nm. The 0% inhibition was determined with cinnamic acid (1.70 mM) as negative control; a dilution range of ascorbic acid (0.02−1.42 mM) was used as positve controls, with the highest concentration providing 100% inhibition.

Elastase Assay. Inhibition of neutrophil elastase was determined with human leucocyte elastase according to Löser et al. (*26*). Briefly, 250 μL of substrate solution (700 μM MeO-Suc-Ala-Ala-Pro-Val-pNA in Tris-HCl-buffer, pH 7.5) was mixed with 100 μL of test solution (test substances solubilized in Tris-HCl-buffer, pH 7.5) and vortexed. After the addition of 250 μL of enzyme solution (approximately 0.5 mU), the samples were incubated for 1 h at 37 °C. The reaction was stopped by addition of 500 μL of soybean trypsin inhibitor solution (2 mg/mL Tris-HCl-buffer, pH 7.5) and placed in an ice bath. After vortexing, the absorbance was read at 405 nm. The assays were performed three times with duplicate samples. Inhibition rates were calculated in percent to blank controls, and IC_{50} values were calculated from the dose−inhibition curves by linear regression.

Cyclooxygenase Assays. The procedure of Mitchell et al. (*27*) was used, with some modifications. Briefly, 40 μL of a COX-2 enzyme solution (100 U/mL) was diluted with 140 μL of Tris buffer, and 80 μL of test compound solution in Tris buffer (final DMSO concentration 1.25%) was added and incubated for 10 min at 37 °C. A 140 μL portion of a substrate solution consisting of an ethanolic stock solution of arachidonic acid and a cofactor solution containing epinephrine, glutathione, and hematin in Tris buffer was added. The final volume of the reaction mixture was 400 μL; the concentrations in the assay were 6.6 μM for arachidonic acid, 5 mM for epinephrin and glutathione, 1 μM for hematin, 0.25% for DMSO, and 0.16% for ethanol. After incubation for 10 min (COX-1) and for 2 min (COX-2) at 37 °C, the reaction was stopped by addition of 20 μL of 1 N HCl and immediately centrifuged (13000 rpm for 3 min). The supernatant was removed and neutralized with 1 N NaOH prior to determination of the 6-keto-$PGF_{1\alpha}$ concentration (COX-1) or the TXB_2 concentration (COX-2), respectively, by ELISA. The assays were performed in triplicate, and inhibition rates were calculated in percent to blank controls.

RESULTS AND DISCUSSION

Purification of the Non-Triglyceride (NTG) Portion. Initial small-scale purification of the NTG fraction was carried out according to a procedure previously used for the extraction of the phenolic fraction in olive oil (*19*). A crude NTG fraction was obtained by extracting cold-pressed and commercial EPO samples with methanol/water (80:20). Upon evaporation of the solvent, the remaining triglycerides were removed by partitioning between acetonitrile and hexane. While this protocol was acceptable for analytical purposes, handling of large amounts of the toxic acetonitrile was cumbersome, considering a preparative purification of NTG. Experiments aimed at replacing acetonitrile and methanol showed that comparable results could be obtained with a simplified procedure in which ethanol/water (80:20) was used. The need for an intermediate evaporation and redissolution step was eliminated at the same time.

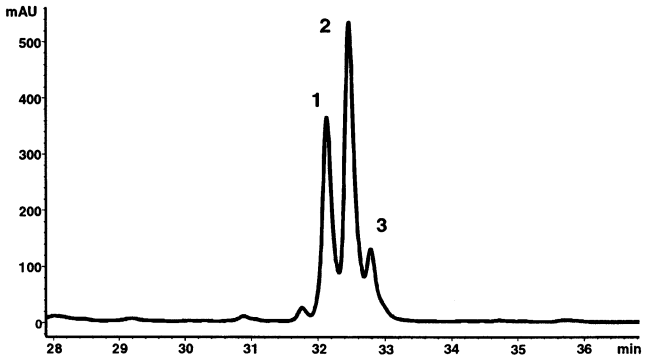

Figure 1. Gradient HPLC profile of the non-triglyceride (NTG) fraction of a cold-pressed, non-raffinated oil. The time window from 28 to 37 min is shown, with peaks 1–3 corresponding to compounds **1**–**3**. Detection was at 330 nm.

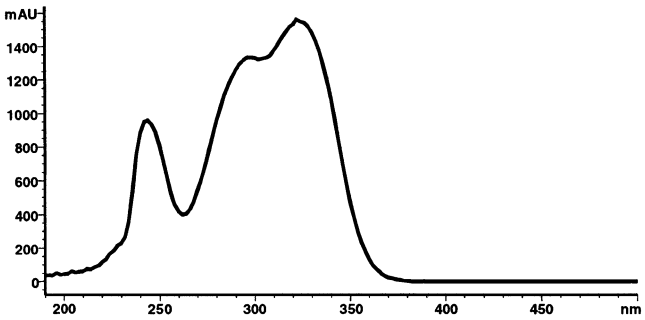

Figure 2. HPLC-DAD spectrum of peak 2, corresponding to compound **2**.

Chromatographic and On-Line Spectroscopic Characterization. TLC analysis of the NTG fraction using solvent system 1 (see Material and Methods), and staining with Godin reagent, revealed purple spots at $R_f = 0.45$, 0.59, and 0.80. A zone with pronounced DPPH radical scavenging activity (*22*) appeared at $R_f = 0.45$ and corresponded to a blue fluorescent spot under 366 nm UV irradiation. Spraying with NST/PEG reagent led to a distinct change in fluorescence color, indicative of a phenolic structure (*21*). With TLC system 2, several purple spots appeared upon staining between $R_f = 0.60$ and 0.90. The DPPH radical scavenging activity was in a zone at $R_f = 0.74$. HPLC analysis proved to be difficult. Due to poor separation of the main UV-absorbing peaks, several RP-HPLC sorbents had to be tested for suitability. A satisfactory separation was finally achieved on Hypersil ODS. **Figure 1** shows the relevant time window from the HPLC fingerprint of the NTG fraction of a cold-pressed oil recorded at 330 nm. A very clean chromatogram was observed at this wavelength, displaying three peaks, numbered 1−3, which eluted only with 100% acetonitrile. DAD spectra of these peaks were virtually identical and displayed absorption maxima around 240, 295, and 325 nm (**Figure 2**). ESI-LC-MS spectra for peaks 1−3 recorded in the negative ion mode also were similar to each other, showing intense $[M - H]^-$ ions at *m/z* 617 and $[2M - H]^-$ ions at *m/z* 1236 (**Figure 3**). The ionization behavior in the acidic eluent was in support of the phenolic nature of the analytes (*28*). Given the identical mass and UV spectra, compounds **1**−**3** were isomers. Comparative LC-MS analysis of cold-pressed, non-raffinated oil and commercial EPO samples revealed that the concentrations of **1**−**3** in the latter were significantly lower.

Isolation. Initial purification of the radical scavengers in the NTG fraction was achieved by open column chromatography and was monitored by TLC and DPPH spray reagent. Separation of individual compounds from the radical-scavenging fraction

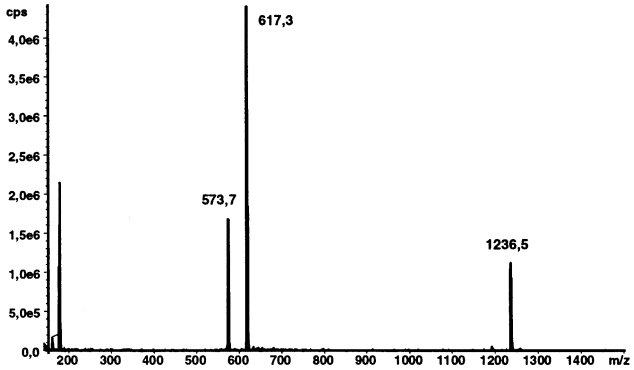

Figure 3. Negative-ion ESI-MS of compound **2** recorded on-line. The signals at *m/z* 617.8 and 1236.5 correspond to the $[M - H]^-$ and $[2M - H]^-$ ions.

was carried out in analogy to analytical separation on a Hypersil ODS column. Compounds **1**−**3** were purified by repeated injections.

Structure Elucidation of Compounds 1−**3.** 1D ^{1}H and ^{13}C NMR spectra of **1**−**3** revealed the presence of a phenylpropanoid ester moiety. The remaining 30 resonances in the ^{13}C NMR spectra of **1**−**3** were indicative of triterpene framework. Functional groups identified on the basis of ^{13}C chemical shifts and DEPT spectra were a double bond, an oxygenated sp^3 carbon, and a carboxyl moiety. A particular feature of the ^{13}C NMR spectrum of **1** was the exocyclic methylene at 109.9 ppm which, in conjunction with quaternary carbon resonance at 150.3 ppm, suggested a 20(29)-lupene derivative. DEPT multiplicities of **1** were in accord with this skeleton, and the ^{13}C NMR chemical shifts showed good agreement with those of betulinic acid (*29*), with the exception of the resonances attributable to C-2, C-3, C-4, and C-25. The characteristic shift differences were indicative of an ester moiety at the C-3 position (*30*). The ^{1}H and ^{13}C NMR data of the phenylpropanoid residue were in agreement with the values reported for *trans*-caffeic acid. Base hydrolysis of **1** afforded betulinic acid, and caffeic acid was confirmed after acidic hydrolysis in the presence of an antioxidant. Hence, **1** was 3β-*trans*-(3,4-dihydroxycinnamoyloxy)-20-(29)-lupen-28-oic acid, a compound which has been reported from *Celastrus stephanotifolius* (*23*).

For compound **3**, the ^{13}C chemical shift of the olefinic resonances (122.2 and 144.8 ppm) and the DEPT multiplicities suggested a triterpenoid moiety of the olean-12-ene type. Indeed, the ^{13}C resonances were in excellent accord with oleanolic acid (*29*), with the exception of the the C-2, C-3, C-4, and C-25 signals, which showed typical shift differences due to esterification at the C-3 oxygen. Again, the NMR data for the phenylpropanoid portion of the molecule were indicative of a caffeoyl moiety. On acid and base hydrolysis of **3**, caffeic and oleanolic acids were confirmed by co-chromatography with authentic samples. 3β-*trans*-(3,4-Dihydroxycinnamoyloxy)olean-12-en-28-oic acid (**3**) (**Figure 4**) has been reported from the bark of *Betula pubescens* (*24*).

Unambiguous assignments of the ^{13}C and ^{1}H spectra of compound **2** were achieved with the aid of homonuclear COSY and HMBC experiments. The resonances of an olefinic quaternary carbon at 137.5 ppm and a methine carbon at 132.1 ppm, and the DEPT multiplicities, were indicative of a olean-18-ene skeleton. The ^{13}C chemical shifts were in good agreement with literature values for morolic acid (*29*), again with the noticeable exceptions of the C-2, C-3, C-4, and C-25 resonances, which again were indicative of an acyl moiety at C-3. Caffeic and morolic acids were confirmed by co-chromatography with

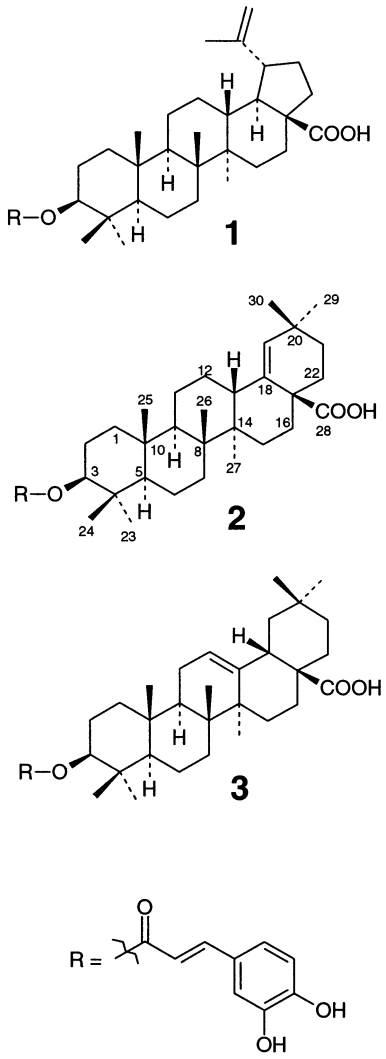

Figure 4. Chemical structures of caffeoyl esters **1**–**3**.

authentic samples. Compound **2** was thus 3β-*trans*-(3,4-dihydroxycinnamoyloxy)olean-18-en-28-oic acid (**Figure 4**). The caffeoyl ester of morolic acid is, to our knowledge, a new natural product.

Biological Activity. Compounds **1**−**3** exhibited strong radical-scavenging activity in a DPPH assay system (see **Figure 5**). The IC_{50} values were 52−64 μM, compared to 94 μM for the positive control ascorbic acid. The NTG fraction and isolates **1** and **2** were potent inhibitors of human leucocytic elastase. The IC_{50} value of the NTG fraction obtained from cold-pressed oil was 0.3 μg/mL, whereas the IC_{50} value for compounds **1** and **2** was 0.2 μg/mL (0.32 μM). Compound **3** was not tested due to scarcity of material. Caffeoyl esters **1**−**3** also strongly inhibited eicosanoid synthesis catalyzed by COX-1 and COX-2 (**Figure 6**). In an assay using purified COX-1 from sheep, the formation of 6-keto-$PGF_{1\alpha}$ was inhibited with IC_{50} values of approximately 0.12 μM, and synthesis of TXB_2 catalyzed by recombinant human COX-2 was inhibited with IC_{50} values in the range of 0.4−2.5 μM. The IC_{50} values for the positive control diclofenac, a clinically used non-steroidal anti-inflammatory drug (NSAID), were 0.05 (COX-1) and 0.013 μM (COX-2).

CONCLUSIONS

In the NTG fraction of cold-pressed evening primrose oil, three isomeric radical scavengers, **1**−**3**, were detected by TLC

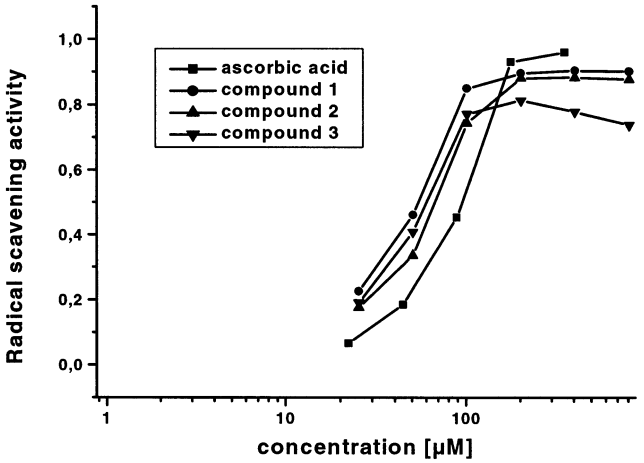

Figure 5. Radical-scavenging activity on the DPPH radical of compounds **1**–**3** and ascorbic acid.

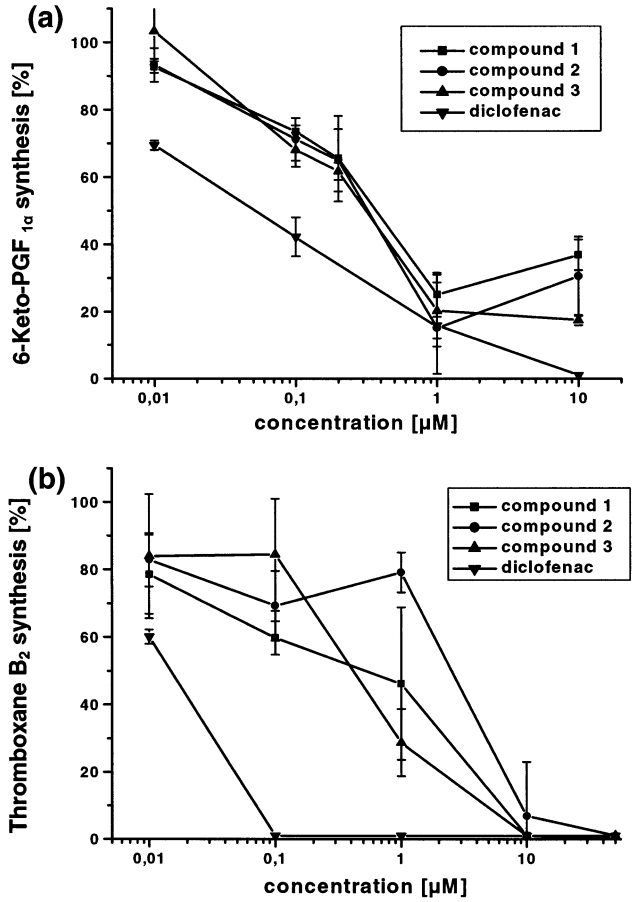

Figure 6. Effect of compounds **1**–**3** and diclofenac on (a) COX-1-catalyzed 6-keto-$PGF_{1\alpha}$ synthesis and (b) COX-2-catalyzed formation of TXB_2.

and LC-DAD-MS. The catechol moieties are responsible for their good scavenging properties (*31*). More important though, compounds **1**−**3** showed high potency in mechanism-based assays directed at clinically relevant enzymes of inflammatory processes. Leucocytic elastase is one of two main proteinases of neutrophils released in connection with inflammation. It cleaves fibrous elastin, an important extracellular matrix protein, collagens, cartilage proteoglycans, and other matrix proteins. Elevated plasma levels of neutrophil elastase is a characteristic feature of an active inflammation (*32*, *33*). The NTG fraction as well as substances **1** and **2** were strong inhibitors of the enzyme. The elastase inhibititory properties are in line with earlier findings by Melzig et al. (*34*, *35*), who reported various caffeic acid derivatives as inhibitors of this enzyme. Compounds **1** and **2** are among the most potent caffeic acid esters found to date, and these findings corroborate the notion that lipophilic esters are particularly active. In comparison, the IC_{50} values reported for caffeic acid and for the selective inhibitor elastatinal are approximately 300- and 3600-fold higher (93 and 1200 μM, respectively) (*26*). Cyclooxygenases (COX) are key enzymes in the synthesis of pro-inflammatory prostaglandins and thromboxanes and the molecular target of the non-steroidal anti-inflammatory drugs (NSAIDs) (*36*, *37*). Esters **1**−**3** inhibited COX-1- and COX-2-catalyzed eicosanoid synthesis in vitro at high nanomolar to low micromolar concentrations. Whether the caffeoyl esters may possibly exert an anti-inflammatory effect via additional mechanisms similar to caffeic acid phenethyl ester (*38*) remains to be studied. It is likely that the triterpenoid portion in **1**−**3** also contributes to their biological properties. Betulinic acid has demonstrated anti-inflammatory and anti-apoptotic properties (*39*), whereas oleanolic acid exhibits anti-inflammatory and anti-edematous activity in various animal models (*40*). The *p*-coumaroyl ester of oleanolic acid, a compound closely related to **3**, had been shown to exert potential cancer chemopreventive activity (*41*).

The discovery of the triterpenoid caffeates in EPO and our preliminary findings on their biological properties add a new facet to this dietary supplement. Considering the current interest in phenolic constituents in dietary oils such as olive oil and in their presumed disease preventing properties (*19*, *42*−*44*), further investigation of the NTG fraction of EPO and the caffeoyl esters **1**−**3** is warranted in order to clarify their possible relevance in the context of EPO usage. As next steps, cell-based functional assays and testing in validated animal models will be required. Also, the factors affecting the yield of the NTG portion during extraction need to be investigated. The low content of **1**−**3** in the commercial EPO samples may be linked to the extraction and raffination processes (*7*, *14*, *15*).

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