Constituents of *Hypericum laricifolium* and Their Cyclooxygenase (COX) Enzyme Activities[1)]

Hesham Rushdey EL-SEEDI,* Therese RINGBOM, Kurt TORSSELL, and Lars BOHLIN

Division of Pharmacognosy, Department of Medicinal Chemistry, Biomedical Centre, Uppsala University; Box 574, SE-751 23 Uppsala, Sweden. Received July 22, 2003; accepted September 5, 2003

Investigation of the aerial parts of the medicinal plant *Hypericum laricifolium* led to the isolation of two new natural products, hentriacontanyl caffeate (1a), nonacosanyl caffeate (1b). In addition, stigmasterol, β-sitosterol, 3-*epi*-betulinic acid (2), caffeic acid (3), ferulic acid, docosanol, *p*-hydroxybenzoic acid, 3,4-dimethoxy benzoic acid, quercetin (4), quercetin-3-*O*-galactoside (5), quercetin-3-*O*-rutinoside (6), quercetin-3-*O*-rhamnoside (7), quercetin-3-*O*-glucuronide (8) and shikimic acid were also isolated. The structures were determined by 1D- and 2D-NMR, mass spectrometry, and chemical transformations. The anti-inflammatory effects of the isolated compounds were discussed briefly.

Key words *Hypericum laricifolium*; Guttiferae; caffeate ester; flavonol; cyclooxygenase-1 (COX-1); cyclooxygenase-2 (COX-2)

The genus *Hypericum* (Guttiferae) encompasses approximately 400 species, of which ten morphologically and chemically distinct species grow in Central Europe.[2)] Several species have been used in folk medicine. There is a growing interest in constituents of the genus because a number of species have been found to possess various biological properties.[3)] Preparations of *H. perforatum* L. are now commercialized in Europe for the management of various depressive disorders.[4)] Antifungal γ-pyrone and xanthones,[5)] dianthrones (hypericin),[6)] spiroterpenoids,[7)] antifungal and antimalarial phloroglucinol,[3)] and antibacterial phloroglucinols[8)] have been reported in the literature for this genus. The investigated plant, *H. laricifolium* H.B.K., with the common name "Romerillo", has been used in Ecuadorian traditional medicine as a diuretic and for provoking menstruation.[9)] This herb was selected for this study primarily because it has not previously been investigated, chemically or pharmacologically. In addition, hyperforin and extracts of the related species, *H. perforatum*, has shown effects on inhibition of COX-1 and 5-lipooxygenase,[10)] which prompted an investigation of COX-1 and -2 enzyme activity for *H. laricifolium* as a part of our continuing search for identification of natural products as inhibitors of prostaglandin biosynthesis.[11)] This paper reports methods and results of isolating and characterizing 14 compounds from the EtOAc extract of *H. laricifolium*, and of investigating COX-1 and -2 enzymes activity for the extract and isolated compounds.

Results and Discussion

All the known compounds were identified by their spectral properties and, where appropriate, by their melting points and/or optical rotations. In several cases (all unnumbered compounds), identification was confirmed by direct comparison with authentic samples.

From the less polar fractions of the ethyl acetate extract of *H. laricifolium*, a caffeic acid ester of long-chained aliphatic alcohols (**1**) was isolated as a semisolid. It has been found to be a mixture by analysis of electron impact mass spectrum (EI-MS), which showed a major peak at *m/z* 614, $C_{40}H_{70}O_4$, corresponding to hentriacontanyl caffeate and a minor peak at *m/z* 586, $C_{38}H_{66}O_4$ corresponding to nonacosanyl caffeate with approximately ratio 13 : 3, which was estimated according to the lit.[12)] The MS spectra also showed the characteristic caffeoyl moiety at 163. The ^{1}H-NMR spectrum showed the characteristic AB *trans* spectrum at δ 6.22 and 7.53, J=16.5 Hz, for the two olefinic protons and triplets at δ 0.86 ($-CH_2\underline{CH_3}$) and δ 4.18 ($-O\underline{CH_2}CH_2$). The IR spectrum showed peaks at 3410 (OH), 1712 (C=O), 1630 (CH=CH) and 1588 cm^{-1} (aromatic). Alkaline hydrolysis of (**1**) gave caffeic acid and free alcohols which proved to be hentricontanol and nonacosanol from GC-MS analysis which supported the structures as 3,4-dihdroxy-*trans*-cinnamic acid hentriacontanylester (**1a**) and 3,4-dihdroxy-*trans*-cinnamic acid nonacosanylester (**1b**), respectively. Such long-chained esters are uncommon in nature, although few have been isolated as mixtures[13)] and individuals[14)] from some plants. It is possible that such compounds have a role in the stress management of halophytic plants.[15)]

The isolated compounds were investigated for anti-inflammatory activity on cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) catalysed prostaglandin biosynthesis. Of

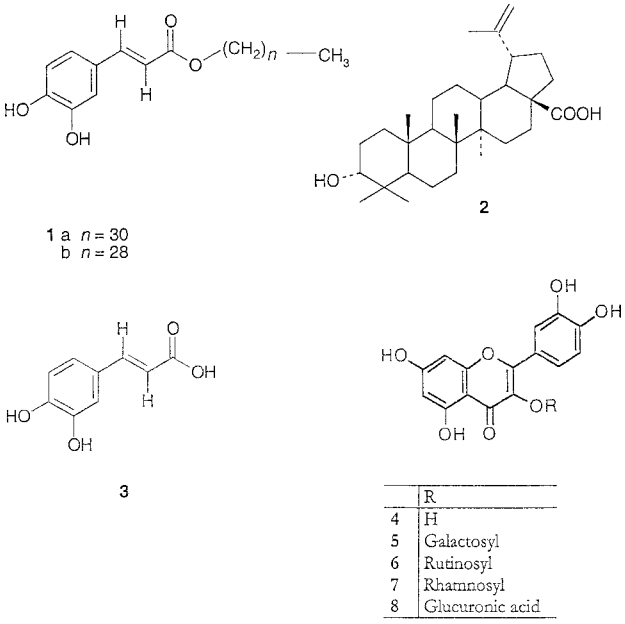

Fig. 1. Structure of Compounds **1**—**8**

* To whom correspondence should be addressed. e-mail: Hesham.El-Seedi@fkog.uu.se

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the tested compounds, quercetin (**4**) inhibited COX-1 by 44±2% at 200 μM concentration, and ester caffeates (**1**) inhibited the same enzyme at 1000 μM by 52±2%. Indomethacin (1.7 μM) was used as a positive control yielding 43±3% inhibition of COX-1. Due to the solubility problems of both compounds no IC_{50} value was obtainable. On the other hand, however, caffeic acid (**3**) inhibited COX-2 by 32±16% at 100 μM concentration, it showed no dose-dependent inhibition as we explained in our previous paper.[16] The other isolated compounds and the extract showed less than 30% inhibition of COX-1 and COX-2, and were considered inactive.

Experimental

General Procedures 1D- and 2D-NMR spectra were recorded on a 400 MHz Varian VXR-400 NMR instrument. The EI-MS; and the EI-MS (with glycerol as matrix) spectra, with a JEOL JMS SX/SX102A instrument.

MPLC was performed using SEPARO AB MPLC equipment (Baeckstrom Separo AB, Lidingo, Sweden). For this, SEPARO variable-length glass columns with inner diameter of 1.5 or 2.5 cm were used, packed with silica gel 60, 40—63 mm (Merck). An FMI Lab pump, model QD (Fluid Metering Inc., Oyster Bay, NY, U.S.A.) was used at a flow rate of 20—30 ml/min. Fractions of 9 ml were collected with a Gilson 201 fraction collector. The columns were eluted with continuous gradients running from hexane, over CH_2Cl_2 to MeOH and H_2O afforded by a SEPARO constant-volume-mixing chamber combined with an open reservoir. The mixing chamber initially contained 50-ml non-polar solvent, and the reservoir contained the first of 15—20 premixed binary (less polar/more polar solvent) gradient mixtures, of 20—40 ml each, which were successively fed to the reservoir during the separation.

Plant Material *Hypericum laricifolium* H.B.K. consisting of leaves, stems, and flowers was collected by Dr. Felipe Ghia in July, 1992 at Hacienda El-Tablón, Sitio Palugillo via Quito-Papallacta, 3100 m altitude, Provincia de Pichincha, Ecuador. Voucher specimens, F.G. 845, are deposited in the Herbario Economico, Escuela Politecnica Nacional (EPN), Quito, Ecuador.

Extraction and Isolation *H. laricifolium* was dried in the dark at 40 °C in a ventilated hood. The plant (1.78 kg), after being ground, was extracted (at room temp.) three times with light petroleum (40—60°) (6 l) and then three times with ethanol (5 l) for 48 h each time with occasional stirring. The extracts were evaporated *in vacuo* to give 15.1 g and 135 g of semisolid material respectively. The ethanol extract was partitioned between ethyl acetate and water to give 41.3 g of an ethyl acetate soluble fraction. An insoluble residue (2.8 g) was discarded. The water phase was freeze dried to give 90.7 g that according to the ^{1}H-NMR consisted mainly of carbohydrates. That phase was not further investigated chemically.

The ethyl acetate extract (17 g) was chromatographed on silica gel (42 g) using SEPARO MPLC equipment with gradient elution by hexane–CH_2Cl_2, then CH_2Cl_2–EtOAc, and finally EtOAc–MeOH. The first fractions contained saturated hydrocarbons, glycerides, polyprenols and ethyl esters of fatty acids (oleic, palmitic and stearic) as identified from NMR spectra and GC-MS analysis. Increasing the polarity of the eluent gave compounds (**1**—**8**) (see Fig. 1). Compound (**1**) was eluted with CH_2Cl_2 and purified by repeated MPLC using petroleum ether–CH_2Cl_2 as eluent followed by prep. TLC on silica using $CHCl_3$–MeOH (9 : 1) as eluent, 22 mg; compound (**2**) was eluted with CH_2Cl_2–EtOAc and purified by prep. TLC using $CHCl_3$–MeOH (9.5 : 0.5) as eluent, 25 mg; compound (**3**) was eluted with CH_2Cl_2–EtOAc and purified by prep. TLC using $CHCl_3$–MeOH (9 : 1) as eluent, 65 mg; compound (**4**) was eluted with EtOAc, and purified by cc with $CHCl_3$–MeOH (8 : 2) as eluent, 1.82 g; compound (**5**) was eluted with EtOAc–MeOH and purified by chromatography on Sephadex LH-20 using $CHCl_3$–MeOH (2 : 8) as eluent, 35 mg; compound (**6**) was eluted with MeOH, purified by chromatography on Sephadex LH-20 using $CHCl_3$–MeOH (1 : 9) as eluent, 150 mg; compound (**7**) was eluted with MeOH and purified by chromatography on Sephadex LH-20 using *n*-PrOH as eluent, 38 mg; compound (**8**) was eluted with MeOH and purified by chromatography on Sephadex LH-20 using MeOH as eluent, 59 mg.

Cyclooxygenase-1 and -2 Catalysed Prostaglandin Biosynthesis Assays Inhibition on COX-1 and COX-2 catalysed prostaglandin biosynthesis *in vitro* was performed according to Noreen *et al.*[17] COX-1 (prostaglandin endoperoxide H synthase-1) was prepared from bovine seminal vesicles, and COX-2 (prostaglandin endoperoxide H synthase-2) was obtained from sheep placental cotyledons (Cayman Chemical Company, Ann Arbour MI, U.S.A.). The crude extract and the substances were tested in 4% DMSO at 20—1000 μM concentration using indomethacin and NS-398 as positive controls for COX-1 and COX-2, respectively. The inhibitory effect was measured after 10 min preincubation of enzyme and test compound at 4 °C. The percent inhibition of COX catalysed prostaglandin biosynthesis was calculated as the decrease in radioactivity (disintegrations per minute), relative to the solvent vehicle, of the samples containing test substance.

Hydrolysis of **1**: 30 mg was dissolved in 7 ml 2 M KOH in MeOH (20 ml) and stirred for 3 h at 60 °C under N_2. The reaction mixture was diluted with H_2O (15 ml) and extracted with Et_2O (20 ml) then adjusted to pH 2 by conc. HCl and re-extracted with Et_2O (4×20 ml). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated to give 17 mg residue. This substance was separated on prep. TLC using CH_2Cl_2 as eluent to give 5 mg of caffeic acid which was identical with an authentic sample and 9 mg of the free alcohols which proved to be hentricontanol and nonacosanol from GC-MS analysis.

Compound (**1**): UV λ_{max}^{MeOH} nm: 330, 299, 244 and 220. IR ν_{max}^{KBr} cm^{-1}: 3410, 2924, 2865, 1712, 1630, 1588, 1518, 1468, and 1275. ^{1}H-NMR ($CDCl_3$–CD_3OD, 2 : 1) δ: 0.86 (3H, t, J=7 Hz, CH_3-31′), 1.21 [(*ca.* 49H, s, $CH_2)_{n\text{-}2}$], 1.70 (2H, m, CH_2-2′), 4.18 (2H, t, CH_2-1′, J=7.5 Hz), 5.51—6.01 (2H, br peak, exchangeable with D_2O, 2×OH), 6.22 (1H, d, J=16.5 Hz, H-8), 6.80 (1H, d, J=8.3 Hz, H-5), 6.93 (1H, dd, J=8.8, 1.5 Hz, H-6), 7.15 (1H, J=1.5 Hz, H-2), 7.53 (1H, d, J=16.5 Hz, H-7). ^{13}C-NMR ($CDCl_3$–CD_3OD, 2 : 1) δ: 168.10, 147.31, 145.21, 144.73, 126.43, 121.67, 115.02, 114.30, 113.78, 64.49, 31.62, 29.42, 29.33, 29.27, 29.09, 29.04, 28.45, 25.70, 22.39, 13.73, with several ^{13}C absorptions overlapping. EI-MS: *m/z* (%) 614 $[M]^+$ of **1a** (22.1) and 586 $[M]^+$ of **1b** (5.1), 491 (12.6), 343 (100), 257 (15.6), 163 (9). HR-EI-MS: *m/z* 614.5278 $[M]^+$ of **1a** (Calcd for $C_{40}H_{70}O_4$: 614.5274 and HR-EI-MS: *m/z* 586.4971 $[M]^+$ of **1b** (Calcd for $C_{38}H_{66}O_4$: 586.4961).

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