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Lipoxygenase inhibition by anadanthoflavone, a new flavonoid from the aerial parts of *Anadenanthera colubrina.*

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Abstract

Chemical investigation of aerial parts of *Anadenanthera colubrina* led to the isolation of a new flavonoid named anadanthoflavone (**1**), along with 11 known compounds: alnusenol, lupenone, lupeol, betulinic acid, α–amyrin, β–amyrin, β–sitosterol, stigmasterol, apigenin, 4–hydroxy benzoic acid and cinamic acid. The isolated compounds were evaluated for their inhibitory activity on human platelet 12-lipoxygenase (12-hLO), human reticulocyte 15-lipoxygenase (15-hLO) and soybean lipoxygenase-1 (15-sLO). Compound **1** was found to be active against 12-hLO and 15-hLO with IC_{50} values of 13 ± 3 μM and 17 ± 3 μM, respectively. Apigenin selectively inhibited the activity of 15-hLO (IC_{50} 4.0 ± 1 μM), while lupenone, lupeol and α–amyrin were found active against 15-sLO (IC_{50} 22 ± 3 μM, 35 ± 9 μM and 15 ± 3 μM, respectively).

Anadenanthera colubrina (Fabaceae) is a South American rain forest tree known as "Yopo", "Cohoba", "Vilca" and "Angico". Its bark is used as a tanning agent and its gum exudate, characterized as a high-arabinose polysaccharide, is employed as an adhesive and as a remedy for respiratory problems [1-3]. The seeds of *Anadenathera* species have been employed via snuff, enema or smoked preparations for medicinal and ceremonial purposes by some cultures of Argentina and Southern Peru. Bufotenin was found to be the main component in seeds of *A. peregrina* [4].

As a part of our research program to discover bioactive agents from dryland plants from Latin America [5], a CH_2Cl_2-MeOH extract of the aerial parts of *A. colubrina* was selected for chemical study on the basis of its inhibitory activity on human platelet 12-lipoxygenase (12-hLO), human reticulocyte 15-lipoxygenase (15-hLO) and soybean lipoxygenase-1 (15-sLO). Lipoxygenases (LOs) are widely distributed in plants and animals [6]. They catalyze the oxidation of fatty acids through thee generally accepted mechanism of hydrogen atom abstraction at C-3 of the 1,4 diene by Fe (III) with subsequent trapping of the pentadienyl radical by oxygen, forming the hydroperoxide product [7]. Inhibition of lipoxygenases is a significant area of research due to its implications in cancer, atherosclerosis and a variety of inflammatory diseases [8], [9], [10]. Various natural products have been discovered that inhibit LO, such as nordihydroguaiaretic acid (NDGA) [11], boswellic acid [12] and puupehenone [13], to name a few. Flavonoids, such as baicalein and apigenin, have also been shown to inhibit lipoxygenase, yet vary in activity and selectivity [14], [15]. In this report, we describe the isolation and characterization of a new flavonoid, anadanthoflavone, together with other

known compounds from the aerial parts of *A. colubrina*, as well as their effect on human and soybean LOs.

Bioassay guided isolation of the active extract of *A. colubrina* led to the isolation of a new flavonoid named anadanthoflavone (**1**), along with 11 known compounds characterized as alnusenol (**2**), lupenone (**3**), lupeol (**4**), betulinic acid (**5**), α–amyrin (**6**), β–amyrin (**7**), β–sitosterol (**8**), stigmasterol (**9**), apigenin (**10**), 4–hydroxy benzoic acid (**11**) and cinamic acid (**12**) through analysis of their NMR spectra.

The molecular formula of compound **1** was determined as $C_{19}H_{14}O_7$ using HR-FABMS. Its ^{13}C NMR spectrum exhibited 19 signals, 15 of which corresponded to the flavone nucleus, one methoxyl group and three additional carbons due to two methines (δ 119.8 and 135.6) and a conjugated carbonyl group (δ 169.1). On the other hand, evident in the ^{1}H NMR spectrum of **1** were two signals at δ 8.86 (1H, *J* = 16.1 Hz, H-1'') and δ 7.66 (1H, *J* = 16.1 Hz, H-2'') attributable to olefin protons. In addition, the presence of two magnetically equivalent methines at δ 7.86 and δ 7.16 indicated 4' substitution in the ring B of the flavone skeleton. The UV, IR and NMR data suggested that **1** was a flavonoid having a conjugated carboxylic group in the A- ring of the flavone nucleus [16], [17]. The proposed structure was further confirmed by the analysis of its HMBC spectrum (Fig. **1**). Structurally, compound **1** appears to be related to torosaflavone D [16] and demethyltorosaflove D [17] reported from *Cassia torosa* and *C. nomame*, respectively. However, **1** lacks an oxygenated functional group at C-3' position as in torosaflavone D and its demethyl derivative.

Compounds **1**–**12** were evaluated against 15-sLO, 15-hLO and 12-hLO. The most active LO inhibitors were found to be compounds **1**, **3**, **4**, **6** and **10**. Compound **1** inhibited both

12-hLO (IC_{50} 13 ± 3 μM) and 15-hLO (IC_{50} 17 ± 3 μM), while **10** inhibited selectively the activity of 15-hLO (IC_{50} 4 ± 1 μM), with little activity towards 12-hLO (IC_{50} 100 ± 40 μM). Since both flavonoids are similar in structure, the results suggest that the change in specificity may be attributed to the appended vinyl-ester to ring A of **1**, as this is the only structural difference between **1** and **10** and suggests that the active site of 15-hLO is larger than 12-hLO. Nevertheless, this analysis is complicated by the inhibition data of baicalein obtained in our lab. Baicalein is a potent inhibitor to both 12-hLO (IC_{50} 0.6 ± 0.1 μM) and 15-hLO (IC_{50} 1.6 ± 0.2 μM) and yet its structure is more similar to **10**, which does not inhibit 12-hLO. These results suggest a critical role for the placement of the hydroxyl groups on the flavonoid scaffold as well as size for inhibition of 12-hLO. Apigenin has previously been shown to inhibit rabbit reticulocyte LO weakly (IC_{50} 180 μM), suggesting a difference in its active site from that of the human reticulocyte LO [14].

With regards to soybean LO inhibition, compounds **3**, **4** and **6** selectively inhibited the activity of 15-sLO. **6** was the most potent compound (IC_{50} 15.0 ± 3 μM), whereas **7** (structurally related to **6**), was not effective at all. This is quite an intriguing result since there is no apparent solubility difference between the two and yet switching the bulk of the methyl from C-19 to C-20 effectively eliminates inhibition. Finally, Compounds **2**, **5**, **7**–**9** and **11**–**12** were inactive against 12-hLO, 15-hLO and 15-sLO at the concentration of 200 μM.

Materials and methods

General experimental procedures: NMR spectra were recorded on a Bruker DRX 500 or DRX 600 spectrometer at 298 K either in $CHCl_3$-*d* or pyridine-d_5. Solvent signals δ_H 7.2 and δ_C 77.0 ($CDCl_3$) and δ_H 7.55, and δ_C 135.5 (pryridine-d_5) were used to reference the spectra. HR-FABMS spectra were recorded on a JEOL HX110 spectrometer with a resolution of 10,000 (mixed matrix: glycerol, thioglycerol and m-NBA). IR and UV spectra were obtained on a Thermo Nicolet Avatar 360 FT-IR as a film on a diamond cell and Beckman DU-600 spectrophotometers, respectively. Melting points (uncorrected) were measured in a Fisher–Johns apparatus. TLCs were sprayed with 0.5 % anisaldehyde in methanol and heated until colored spots appeared.

Plant material: Aerial parts of *Anadenanthera colubrina* (Vell.) Brenan var cebil (Griseb.) Alschul were collected and identified by Renée H. Fortunato in November 1995, in La Gruta, Santiago de Estero, Argentina. A specimen has been deposited in the herbarium at Instituto Nacional de Tecnologia Agropecuaria (INTA), Castelar, Buenos Aires, Argentina (coll. no. RF5144). Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between The University of Arizona and INTA.

Extraction and isolation: The air-dried aerial parts (400 g) of *A. colubrina* were extracted by maceration using of a mixture of CH_2Cl_2-MeOH (1:1) (10L X 3) at room temperature. The resulting organic extract (54 g) was then fractionated by column chromatography on silica gel (400 g, Merck 63-200 μm, 0.2 mm) and eluted with a stepped gradient of hexane, ethyl acetate and methanol starting with 100% hexane followed by mixtures of two solvents (hexane:ethyl acetate and ethyl acetate:methanol) in the proportions 95:5,

9:1, 8:2, 7:3, 6:5, 5:5 (~3L each) and final wash with methanol. Fractions (100 ml) were collected and pooled on the basis of their TLC profiles to yield 34 primary fractions (F001-F034). Fractions F010-F018, F022 were found active in the 15-sLO inhibition assay while F026 was active against 12-hLO and 15-hLO. Direct HPLC purification of F026 (65 mg) using a Reliasil C_{18} column (10 x 250 mm, Column Engineering), eluted with a gradient of 10 % methanol in 0.15 % HCOOH to 100 % methanol in 22 minutes (5.2 ml/min) and detection at 280 nm, yielded compounds **1** (1.7 mg, R_t=19.6 min), **10** (2.0 mg, R_t=17.1 min) **11** (3.1 mg, R_t=9.2 min) and **12** (3.7 mg, R_t=15.2 min). Compounds **2** – **4** and **6** – **8** were isolated from fractions F010 to F018 (1.0 g) by the use of RPHPLC (Lichosphere C_{18}, 10 x 250 mm, Column Engineering, elution with methanol, 5.2 ml/min, detection at 200 nm). The isolated compounds showed retention times of 10.4 min (**3**, 8.0 mg), 11.2 min (**2**, 10.0 mg), 13.3 min (**8**, 12.0 mg), 15.4 min (**9**, 10 .0 mg), 16.6 min (**4**, 3.0 mg), 21.1 min (**7**, 5.0 mg) and 23.4 min (**6**, 6.0 mg), respectively. Finally, compound **5** (2.5 mg, 7.2 min) was isolated from fraction F022 (45 mg) by the use of HPLC (Echonosphere, Altech, eluting gradient of hexane-isopropanol-methanol (99:0.5:0.5 → 90:5:5) in 10 min.

Lipoxygenase assay: 12-hLO, 15-hLO and 15-sLO were expressed and purified as described previously [13], [18]. IC_{50} values and corresponding errors were determined in triplicates as previously described [11], [13].

Anadanthoflavone (**1**); yellow powder: mp 290 °C (dec), UV λ_{max} nm (log ε) 202 (4.23), 206 *sh* (4.21), 233 *sh* (3.96), 274 *sh* (3.96), 317 (4.19) and 341 *sh* (4.04) nm. IR 3648-3040, 2931, 1697, 1626, 1602, 1492. HRFABMS *m/z* 355.0805 ($[M+H]^+$), monoisotopic

Calcd. 354.0735, $C_{19}H_{14}O_7$. ^{13}C NMR (125.0 MHz, in pyridine): 182.9 (C-4), 169.1 (C-3”), 164.6 (C-2), 163.7 (C-7), 162.9 (C-4’), 161.2 (C-5), 158.5 (C-9), 135.6 (C-1”), 129.0 (C-2’, C-6’), 121.9 (C-1’), 119.8 (C-2”), 116.9 (C-3’, C-5’), 107.5 (C-6), 104.5 (C-10), 103.9 (C-3) 94.3 (C-8), 51.3 (3’’-O<u>C</u>H_3). ^1H NMR (600 MHz, in pyridine): 8.86 (d, *J* 16.1, H-1”), 7.86 (d, *J* 8.6, H-2’, H-6’, 2H), 7.66 (d, *J* 16.1, H-2”), 7.16 (d, *J* 8.6, H-3’, H-5’, 2H), 6.92 (s, H-3), 6.77 (s, H-8), 3.76 (s, 3’’-OC<u>H</u>$_3$).

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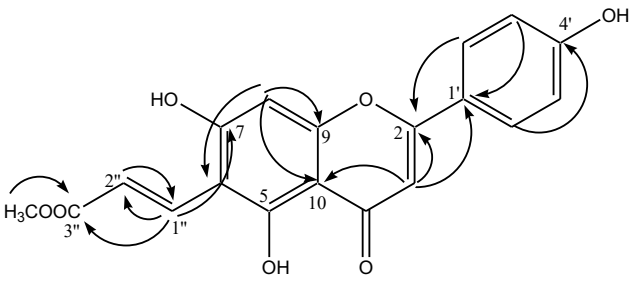

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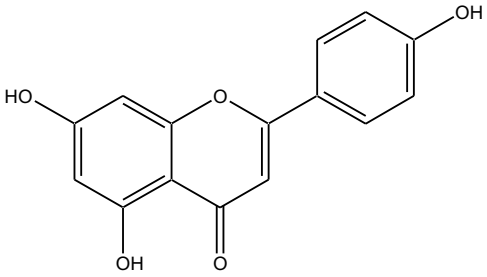

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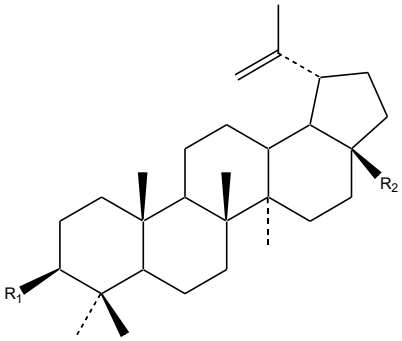
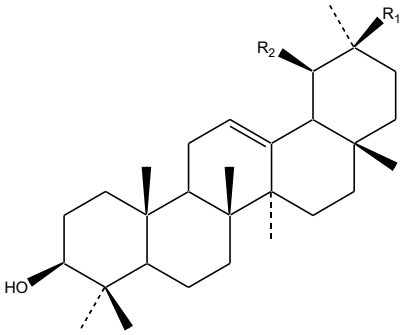


3; R_1=O, R_2 CH_3

4; R_1 OH, R_2 CH_3



6; R_1 H, R_2 CH_3

7; R_1 CH_3, R_2 H

Figure 1. Structure with HMBC correlations of Anadanthoflavone (**1**) and structure of Compounds **3**, **4**, **6** and **7**.