Griffonianone D, an Isoflavone with Anti-inflammatory Activity from the Root Bark of *Millettia griffoniana*[1]

Emmanuel Yankep,[†] Dieudonné Njamen,[‡] Maurice Tagatsing Fotsing,[†] Zacharias Tanee Fomum,[†] Jean-Claude Mbanya,[§] Rosa M. Giner,[⊥] M. Carmen Recio,[⊥] Salvador Máñez,[⊥] and José Luis Ríos*,[⊥]

Department of Organic Chemistry, Faculty of Science, University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon, Laboratory of Animal Physiology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde 1, P.O. Box 812 Yaounde, Cameroon, Department of Internal Medicine, Faculty of Medicine and Biomedical Sciences, University of Yaounde 1, Cameroon, and Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

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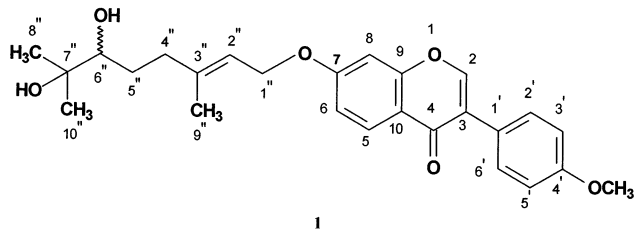
A new isoflavone, griffonianone D (**1**), and the previously known compounds durmillone and odorantin were isolated from a chloroform extract of the root bark of *Millettia griffoniana*. The structure of **1** was established as (7*E*)-(6″,7″-dihydroxy-3″,7″-dimethyloct-2″-enyl)oxy-4′-methoxyisoflavone on the basis of its spectral data. The chloroform extract of the root bark of *M. griffoniana* and compound **1** showed anti-inflammatory effects in different experimental models of inflammation.

The genus *Millettia* (subfamily Papilionoideae of the Leguminosae) is widespread in Africa,[2] with many of its species exhibiting various biological and pharmacological properties.[3] A key phytochemical characteristic is their ability to produce isoflavonoids.[4] Several species of this genus are used in traditional medicine, and, for example, potions made from the root bark of *Millettia griffoniana* Baill. are employed by some village communities of Cameroon as an oral treatment for boils. In previous publications[5−7] we have reported the isolation and structural elucidation of a chalcone, a rotenoid, a phenylcoumarin, and several isoflavones from the hexane extract of the root bark of *M. griffoniana*. The present study focuses on the anti-edema effect of the chloroform extract of this plant as well as the anti-inflammatory activity of an isolated metabolite, griffonianone D (**1**).



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The root bark of *M. griffoniana* was extracted initially successively with *n*-hexane and chloroform, and the chloroform extract then underwent column chromatographic separation followed by recrystallization to afford three isoflavones, **1**, durmillone (6-methoxy-3′,4′-methylenedioxy-2″,2″-dimethylpyrano[7,8;5″,6″]isoflavone),[8] and odorantin (5,6,7-trimethoxy-3′,4′-methylenedioxyisoflavone).[9] The two known compounds were identified by comparison of their physical and spectral data with literature values.[8,9]

Compound **1** gave a negative $FeCl_3$ reaction, indicating the absence of any free phenolic hydroxyl groups. The HREIMS of **1** gave a molecular ion at *m*/*z* 438.2049, corresponding to a molecular formula of $C_{26}H_{30}O_6$. The IR spectrum disclosed characteristic absorption maxima for a conjugated carbonyl group (1634 cm^{-1}) and in the OH region (3421 cm^{-1}). The NMR spectra of compound **1** displayed a characteristic one-proton singlet for H-2 of an isoflavone at δ 7.92, as well as C-2 and C-4 signals at δ 152.0 and 175.8. The ^{1}H NMR spectrum of compound **1** also clearly indicated that ring B was oxygenated at the C-4′ position, from the AA′BB′ spin coupled system (J = 8.7 Hz) shown by H-2′, H-3′, H-5′, and H-6′. This was confirmed by the signal sequences of C-2′, C-3′, C-4′, C-5′, and C-6′ observed in the ^{13}C NMR spectrum. The ^{1}H NMR spectrum of compound **1** further revealed the presence of a methoxyl group and a (*E*)-(6″,7″-dihydroxy-3″,7″-dimethyloct-2″-enyl)oxyl substituent.[10,11] On the basis of the above evidence, compound **1** was assigned as (7*E*)-(6″,7″-dihydroxy-3″,7″-dimethyloct-2″-enyl)oxy-4′-methoxyisoflavone, which is described for the first time and for which we suggest the trivial name griffonianone D.

The hexane and chloroform extracts from *M. griffoniana* root bark (100 mg/kg, per os) were tested in carrageenan-induced paw edema and 12-*O*-tetradecanylphorbol 13-acetate (TPA)-induced mouse ear edema (1 mg/ear, topical application) models. The hexane extract showed no significant activity in both edema models (data not shown). In the first experiment, the chloroform extract showed a weak but significant effect at 3 h (the extract inhibited edema by 27%, $P < 0.05$, at a dose of 100 mg/kg, whereas the reference drug indomethacin inhibited edema by 60%, $P < 0.01$, at a dose of 10 mg/kg). In the second experiment, a dose of 1 mg/ear inhibited edema by 86% at 4 h ($P < 0.01$), an effect similar to that obtained for the reference drug indomethacin at a dose of 0.5 mg/ear (82%, $P < 0.01$). The hexane extract was not included in the following tests because it showed no significant activity in the first two general tests. In contrast, the chloroform extract gave good results when assayed in the phospholipase A_2 (PLA_2)-induced paw edema model, inhibiting edema by 36% and 68% at 30 and 60 min, respectively (Table 1).

We were able to isolate three pure compounds (the new derivative, griffonianone D (**1**), odorantin, durmillone) from the active crude chloroform extract, and they were tested in the in vivo tests. Only compound **1** was found to be

* To whom correspondence should be addressed. Tel and fax: +34 963 544 973. E-mail: riosjl@uv.es.
[†] Department of Organic Chemistry, University of Yaounde 1.
[‡] Laboratory of Animal Physiology, University of Yaounde 1.
[§] Faculty of Medicine and Biomedical Sciences, University of Yaounde 1.
[⊥] Universitat de València.

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Table 1. Effect of Test Products on PLA_2-Induced Mouse Paw Edema[a]

	30 min		60 min	
	$\Delta V \pm$ SEM	% I[b]	$\Delta V \pm$ SEM	% I[b]
control	55 ± 7		57 ± 10	
$CHCl_3$ extract	35 ± 9[c]	36	18 ± 8[c]	68
1	32 ± 9[c]	42	40 ± 7[d]	30
cyproheptadine	22 ± 6[c]	82	23 ± 6[c]	82

[a] *Millettia griffoniana* $CHCl_3$ extract (50 mg/kg), **1** (5 mg/kg), and cyproheptadine (5 mg/kg) were administered intraperitoneally 30 min before subcutaneous plantar injection of *Naja mossambica* PLA_2, and the edema was measured 30 and 60 min later. Numerical values express increase in paw volume in μL (mean ± SEM); $n = 6$ animals. [b] Inhibition percentage with respect to the control group (PLA_2) value. [c] $P < 0.01$. [d] Not significant after Dunnett's *t*-test.

Table 2. Effect of **1** on TPA-Induced Acute Mouse Ear Edema[a]

	$\Delta T \pm$ SEM	% I[b]
control	183 ± 21	
1	42 ± 6[c]	77
indomethacin	34 ± 11[c]	81

[a] Griffonianone D (**1**, 0.25 mg/ear) and indomethacin (0.5 mg/ear) were administered topically together with TPA, and the edema was measured 4 h later. Ear thickness expressed as the mean of the difference between thickness in μm before and after challenge ± SEM. $n = 6$ animals. [b] Inhibition percentage with respect to the control group (TPA) value. [c] $P < 0.01$ after Dunnett's *t*-test.

effective when assayed against TPA-induced mouse ear edema, inhibiting swelling by 77% at a dose of 0.25 mg/ear (Table 2). Odorantin and durmillone both showed activity that was not significant (data not shown). In the PLA_2-induced paw edema test, compound **1** inhibited edema by 42% at 30 min at a dose of 5 mg/kg, but showed no effect at 60 min (Table 2).

In addition, in vitro studies were performed to determine the possible effect of griffonianone D (**1**) on arachidonic acid metabolism. The results indicated that this compound did not modify either the production of prostaglandins via cyclooxygenase or that of leukotriene via lipoxygenase (data not shown).

The present results add to the many studies concerning the potential pharmacological effects of isoflavones. Diverse mechanisms for these compounds have been described, including their roles as weak phytoestrogens, inhibitors of tyrosine kinase-dependent signal transduction processes, and antioxidants.[12]

The results of the in vitro experiments, together with the results obtained in the in vivo tests, suggest that compound **1** may act by modifying the proinflammatory vasoactive amine release from mast cells,[13] together with having potential antioxidant effects. The present work has shown that the mechanism of this compound is not related to arachidonate metabolism.

Experimental Section

General Experimental Procedures. Melting points were determined on a Kofler hot-stage apparatus and were uncorrected. Optical rotations were measured in $CHCl_3$ at ambient temperature using a Perkin-Elmer polarimeter. UV spectra were recorded in MeOH on a Perkin-Elmer model 1600 spectrophotometer. IR spectra were recorded in KBr disks with a 1001 Milton Roy spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were taken in $CDCl_3$ on a Bruker AXR 300 spectrometer operating at 300 MHz for ^{1}H and at 75 MHz for ^{13}C using TMS as internal standard. The EIMS were measured with a Finnigan MAT-112 spectrometer. The HREIMS were measured with a JMS HX-110 spectrometer. For column chromatography or for TLC, silica gel $60F_{254}$ (Merck) was used.

Chemicals. Acetone and methanol of analytical grade (Baker, Deventer, Holland) and ethanol 96° and sodium acetate of analytical grade (Panreac, Barcelona, Spain) were employed. The other chemical and biological reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Material. *Millettia griffoniana* Baill. (Leguminosae) root bark was collected in July 1994 at Onguesse, in the Central Province of Cameroon. A voucher specimen documenting the collection was identified at the National Herbarium, Yaoundé, Cameroon, and is on deposit there (No. 32315/SRF/HNC).

Extraction and Isolation. The air-dried and powdered root bark (20 kg) of *M. griffoniana* was defatted with hexane and then extracted at room temperature with chloroform (24 h, 3 × 20 L), yielding 550 g of a yellowish viscous mass. This $CHCl_3$ extract (50 g) was subjected to vacuum-liquid chromatography (VLC) over silica gel (200 g). Fractions (500 mL each) were collected and combined on the basis of TLC analysis, resulting in two main series (A and B). Series A, fractions 6 and 7 (9.3 g) (petroleum ether−EtOAc, 6:4) gave durmillone (5.2 g) upon filtration and recrystallization from EtOAc. Series B, fractions 13 and 14 (14.4 g) (petroleum ether−EtOAc, 3:7) gave odorantin (1 g) upon filtration and recrystallization from EtOAc. The mother liquor was concentrated to afford a dark oil (10.1 g). This oil was subjected to flash-chromatography over silica gel (250 g) (EtOAc), leading to 2.1 g of a oily solid, which was then defatted through Sephadex LH-20 column chromatography (MeOH−$CHCl_3$, 1:1) to yield an amorphous solid (1.3 g) from which a white fine solid (670 mg) was filtered upon addition of EtOAc. Fractional recrystallization (EtOAc−$CHCl_3$, 1:1) of this solid gave **1** (15 mg).

Griffonianone D (1): light white needdles, mp 128−129 °C; $[\alpha]^{23}_D$ −7.97° (*c* 0.042, $CHCl_3$); UV (MeOH) λ_{max} 248 and 297 nm; IR (KBr) ν_{max} 3421 (OH), 2927 (C═C), 1634 (C═O), 1590 (aromatic), 1445, 1383, 1258, 1179, 1092, 824, 514, and 467 cm^{-1}; ^{1}H NMR δ 7.92 (1H, s, H-2), 8.20 (1H, d, $J = 8.7$ Hz, H-5), 6.99 (1H, dd, $J = 8.7$, 2.2 Hz, H-6), 6.85 (1H, d, $J = 2.2$ Hz, H-8), 7.51 (2H, d, $J = 8.7$ Hz, H-2′, H-6′), 6.97 (2H, d, $J = 8.7$ Hz, H-3′, H-5′), 4.65 (2H, d, $J = 6.4$ Hz, H-1″), 5.55 (1H, t, $J = 6.4$ Hz, H-2″), 2.30 (2H, m, H-4″), 1.57 (2H, m, H-5″), 3.36 (1H, m, H-6), 1.18 (3H, s, H-8″), 1.80 (3H, s, H-9″), 1.22 (3H, s, H-10″), 3.84 (3H, s, O*CH*$_3$); ^{13}C NMR δ 152.0 (CH, C-2), 124.2 (C, C-3), 175.8 (C, C-4), 127.7 (CH, C-5), 115.0 (CH, C-6), 163.2 (C, C-7), 100.9 (CH, C-8), 157.9 (C, C-9), 118.3 (C, C-10), 124.9 (C, C-1′), 130.1 (2CH, C-2′, C-6′), 113.9 (2CH, C-3′, C-5′), 159.6 (C, C-4′), 65.4 (CH_2, C-1″), 118.8 (CH, C-2″), 142.3 (C, C-3″), 36.5 (CH_2, C-4″), 29.5 (CH_2, C-5″), 73.0 (CH, C-6″), 78.0 (C, C-7″), 23.3 (CH_3, C-8″), 16.8 (CH_3, C-9″), 26.5 (CH_3, C-10″), 55.3 (CH_3, OCH_3); EIMS (acetylated derivative) *m/z* 480 $[M]^+$ (19), 465 $[M^+ - CH_3]$ (0.7), 420 $[M^+ - CH_3COOH]$ (1), 268 (100), 153 (10), and 81 (16); HREIMS *m/z* 438.2049 $[M]^+$ (calcd for $C_{26}H_{30}O_6$, 438.2042).

Animals for Pharmacological Experiments. Female Wistar rats weighing 180−200 g and groups of six Swiss female mice weighing 25−30 g were used. All animals were fed a standard diet ad libitum. Housing conditions and all in vivo experiments were approved by the institutional Ethics Committee of the Faculty of Pharmacy, University of Valencia (Spain), according to the guidelines established by the European Union on Animal Care (CEE Council 86/609).

Carrageenan-Induced Mouse Paw Edema.[14] Edema was induced on the right hind paw by subplantar injection of carrageenan (3% w/v in saline, 25 μL). The $CHCl_3$ extract of the stem bark of *M. griffoniana*, dissolved in Tween 80−ethanol−saline (1:1:10), was administered orally at a dose of 100 mg/kg (0.2 mL), 1 h before carrageenan injection. A group received the reference drug indomethacin (10 mg/kg, po). The right and left volumes were measured on a plethysmometer (Ugo Basile) 1, 3, and 5 h after inflammation induction. The edema was expressed as the difference between right and left paw volume, and edema inhibition was expressed as the percentage of volume reduction referred to the control group.

12-*O*-Tetradecanoylphorbol 13-Acetate (TPA)-Induced Mouse Ear Edema.[15] Edema was induced by topical application of 2.5 μg per ear of TPA dissolved in acetone. The extract (1 mg/ear) and pure compound (0.25 mg/ear) dissolved in acetone were applied topically simultaneously with TPA. The standard drug indomethacin was administered topically (0.5 mg/ear). The ear swelling was measured before TPA application and 4 h after, and the edema was expressed as the increase in thickness.

Phospholipase A_2 (PLA_2)-Induced Mouse Paw Edema.[13] PLA_2 from *Naja mossambica* (2 units in 25 μL of saline) was injected sc into the right hind mouse paw. The left paw received the same volume of vehicle. The test compounds (5 mg/kg) were injected ip 30 min prior to the induction of inflammation with PLA_2. Both the reference compound, cyproheptadine, and the test compounds were dissolved in Tween 80−ethanol−saline (1:1:10). Edema was measured using a plethysmometer (Ugo Basile) 30 and 60 min after challenge and was expressed as the difference between the right and left paw volume.

Cytotoxicity Assay.[16] Rat polymorphonuclear leukocytes (PMNL) were exposed to the test compounds (100 μM) in microplate wells for 30 min, and then 100 μL of MTT (5 mg/mL) was added and incubated at 37 °C. The blue deposits were dissolved in DMSO. Absorbance was measured at 490 nm using a Labsystems Multiskan MCC/340 plate reader.

Inhibition of Leukotriene B_4 Production from Rat Leukocytes.[17] For 5-lipoxygenase product formation from endogenous arachidonic acid (AA), leukocytes were stimulated with calcium ionophore A23187 (1.9 μM) and Ca^{2+} (1.8 mM). Separation of AA-derived products was performed by reversed-phase (RP-18)-HPLC, eluting wih MeOH−H_2O mixtures containing 0.007% trifluoracetic acid, followed by diode array detection. The results obtained from peak areas were normalized to PGB_2 (17 μg/mL) internal standard and expressed as a percentage of leukotriene B_4 production. IC_{50} values were calculated by means of the lineal regression plotted from the inhibition percentages obtained from four different concentrations.

Assay of Cyclooxygenase Activity from Human Platelets.[17,18] Blood platelets were obtained from healthy human donors and were separated by sequential centrifugation. Stimulation was performed with Ca^{2+} (2.5 nM) and calcium ionophore 23187 (1.9 μM). Separation of 12-HHTrE was achieved by HPLC coupled to diode array detection. A RP-18 column was used and eluted with methanol−water (74:26) containing 0.007% trifluoroacetic acid. The results obtained were expressed as a percentage of 12-HHTrE production.

Statistical Analysis. Inhibition percentages were calculated from the differences between the mean value of the control group and those of the drug-treated groups. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons of unpaired data was used for statistical evaluation.

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