Warangalone, the Isoflavonoid Anti-inflammatory Principle of *Erythrina addisoniae* Stem Bark

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The prenylisoflavone warangalone has been isolated from the bark of *Erythrina addisoniae*. This compound, previously recognized as a powerful inhibitor of protein kinase A, showed marked effectiveness as an anti-inflammatory on the phospholipase A_2-induced paw edema and on the 12-*O*-tetradecanoylphorbol 13-acetate-induced ear edema in mice, after systemic and local administration, respectively.

The genus *Erythrina* (Leguminosae) comprises 107 species distributed in tropical and subtropical regions of the world.[1,2] Extracts of the leaves, stem bark, and roots have a significant history of use in indigenous medicinal practice for the treatment of diseases such as skin tumors due to insect bites and pathological inflammations.[2,3] Previous studies[4−9] have shown that the stem bark of many *Erythrina* species principally contains flavonoids, which are known to display interesting biological activities. In our ongoing research on the medicinal plants of Cameroon, we have studied the stem bark of *Erythrina addisoniae* Hutchinson & Dalziel, which is widely used in traditional medicine to treat various diseases, including dysentery, asthma, venereal diseases, boils, and leprosy. In this note we present the experimental data that have led to the identification of the isoflavonoid warangalone as the main anti-inflammatory principle of the EtOAc extract of *E. addisoniae* stem bark.

The anti-inflammatory activity of the *Erythrina* extract was assayed on several models of inflammation, including both phospholipase A_2 (PLA_2)- and carrageenan-induced mouse paw edema, 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema, and a model of chronic dermatitis caused by repeated administration of TPA. After systemic administration on the paw models, the anti-inflammatory effect of the extract was moderate, but when applied to the mouse ear surface, the extract greatly reduced the swelling. The activity of warangalone, the main isoflavonoid from this extract, followed the same patterns. Although its activity developed more slowly at the same dose, warangalone was as effective as the standard drug cyproheptadine on the PLA_2-induced paw edema at 60 min. However, when carrageenan was used to induce the inflammation, the effect of orally administered warangalone was low throughout the experiment, especially after 60 min, when it produced only one-third of the effect of the standard drug indomethacin.

On the TPA-induced ear edema, the effectiveness of warangalone reached its highest level, reducing swelling by 78% when applied at a dose of 0.25 mg/ear. Histological examinations indicated that, in comparison with the TPA-control group, warangalone markedly reduced the neutrophil afflux into the dermis, suppressed the edema, and induced a moderate papillary fibrosis. In the epidermis, its effects were radical since no signals of hyperkeratosis, acanthosis, or papillomatosis appeared. The ID_{50} for warangalone in this test was 0.54 μmol/ear (0.25−0.62, $P < 0.01$), well within the range of that of indomethacin, which was 0.29 μmol/ear (0.013−0.52, $P < 0.01$). However, in a model of chronic dermatitis caused by repeated administration of TPA, the results were not as positive since only a modest reduction in myeloperoxidase (MPO) activity was observed. Although there was no ear weight reduction, the epidermal cell layer was mildly reduced (6.66 ± 0.14 for control group, 5.12 ± 0.10 for test group) and the morphological features associated with skin inflammations were generally softened. When tested against elastase release by polymorphonuclear leukocytes (PMNL), warangalone exhibited no activity, whereas very slight inhibitions (<20%) of LTB_4 and 12-hydroxyheptadecatrienoic acid (12-HHTrE) production were noted. Finally, the compound produced no effect in the free diphenylpicrylhydrazyl (DPPH) radical scavenging test. Due to the fact that warangalone was one of the main constituents of the EtOAc extract of *E. addisoniae* and particularly because of the similar effects that both the pure compound and the extract had on the inflammation models in vivo, it appears that this principle essentially accounts for the plant's anti-inflammatory effect. Assuming that the PLA_2-induced mouse paw edema is mediated by the effect of vasoactive amines,[10] the effectiveness of warangalone in this test would arise from an antagonism of histamine or serotonin, in possible combination with some kind of membrane-stabilizing properties. It should be noted, however, that the latter hypothesis is rendered somewhat doubtful by the absence of any effect of the

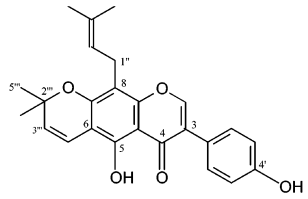

Figure 1. Structure of warangalone.

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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
control	6.50 ± 0.84		6.30 ± 0.21	
E. addisoniae extract	3.50 ± 2.45[c]	46	3.83 ± 0.47[d]	39
warangalone	5.16 ± 1.01[e]	21	2.00 ± 0.63[d]	68
cyproheptadine	2.16 ± 0.60[d]	66	2.32 ± 0.61[d]	63

[a] *Erythrina addisoniae* EtOAc extract (50 mg/kg), warangalone (5 mg/kg), and cyproheptadine (5 mg/kg) were intraperitoneally administered 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 mL × 10^{-2} (mean ± SEM). [b] Inhibition percentage with respect to the control group (PLA_2) value. [c] $P < 0.05$. [d] $P < 0.01$. [e] Not significant after Dunnett's *t*-test.

Table 2. Effect of *Erythrina addisoniae* Extract on Carrageenan-Induced Mouse Paw Edema[a]

	1 h		3 h		5 h	
	$\Delta V \pm$ SEM	% I[b]	$\Delta V \pm$ SEM	% I	$\Delta V \pm$ SEM	% I
control	7.8 ± 0.3		12.6 ± 0.5		12.3 ± 1.2	
E. addisoniae extract	6.7 ± 0.6[c]	14	9.5 ± 1.3[c]	21	9.7 ± 1.0[c]	21
indomethacin	6.7 ± 0.4[c]	14	4.8 ± 0.6[c]	60	8.5 ± 0.8[c]	31

[a] *Erythrina addisoniae* EtOAc extract (100 mg/kg) and indomethacin (10 mg/kg) were orally administered 1 h before subcutaneous plantar injection of carregeenan, and the edema was measured 1, 3, and 5 h later. Numerical values express increase in paw volume in mL × 10^{-2} (mean ± SEM). [b] Inhibition percentage with respect to the control group (carrageenan) value. [c] $P <$ 0.01 after Dunnett's *t*-test.

Table 3. Effect of Test Products on TPA-Induced Ear Edema[a]

	ear swelling[b]	% I[c]	MPO activity[d]	% I
control	14.25 ± 0.92		155 ± 17	
E. addisoniae extract	1.80 ± 0.49[e]	87	n.d.[f]	
warangalone	3.07 ± 0.45[e]	78	91 ± 5[e]	41
indomethacin	2.57 ± 0.70[e]	82	99 ± 6[e]	36

[a] *Erythrina addisoniae* EtOAc extract, warangalone, and indomethacin were administered at 1, 0.25, and 0.5 mg/ear, respectively, on mouse ear surface together with TPA. [b] Increase in ear weight in mg (mean ± SEM). [c] Inhibition percentage with respect to the control group (TPA) value. [d] Myeloperoxidase (MPO) activity in mOD units (mean ± SEM). [e] $P < 0.01$ after Dunnett's *t*-test. [f] Not determined.

compound in the assay on elastase release by PMNLs. As for the weak effect on the carrageenan-induced paw edema, even at the rather high dose of 100 mg/kg, this result is coherent with warangalone's lack of activity on platelet cyclooxygenase and DPPH scavenging since prostaglandins and oxygen free radicals are among the best characterized mediators in this model.[11] By far, the highest potency was shown in the single-dose TPA-induced ear edema, which is a model of protein kinase C (PKC)-mediated acute inflammation. On the basis of the low estimated potency of warangalone in inhibiting PKC in vitro,[12] a direct inhibition of the enzyme might be discarded in principle, but it must be taken into account that this inhibition has previously been measured only against rat brain PKC, which is too limited an approach to the complex role of PKC in inflammatory conditions. Although TPA imitates diacylglycerol, the powerful endogenous PKC activator, with certain specificity, its downstream effects are pleiotropic; thus, many different mechanisms may be implicated in the inhibition of the edema. Moreover, warangalone has been reported to be a potent inhibitor of the catalytic subunit of PKA ($IC_{50} = 3.5\ \mu M$), which, in the light of recent research of the role of cAMP in PGE_2-governed endothelial adhesion by integrin $a V\beta 3$,[13] would explain the compound's anti-inflammatory activity. Taking the results of the histological study into account, we hypothesize that the control of inflammatory response in the single-dose TPA-induced ear edema is partially independent of neutrophil recruitment, since the MPO level, which serves as an indicator of neutrophil infiltration, is moderately reduced in comparison with the total decrease in ear weight.

Experimental Section

Isolation and Identification. Stem bark of *E. addisoniae* was collected in Etoug-Ebe (Yaounde) in April 1996. The plant was authenticated and deposited at the Cameroon National Herbarium, Yaounde (voucher No. 41617/HNC). The dried plant material (7 kg) was ground and successively extracted with EtOAc and MeOH. A pharmacological screening on a model of ovoalbumin hind paw edema indicated a promising anti-inflammatory activity of the EtOAc extract, which was therefore fractionated by column chromatography over silica gel using *n*-hexane, EtOAc, and MeOH in various proportions. From the fractions eluted with hexane−EtOAc (95:5), 150 mg of yellowish crystals was obtained. The structure of this compound was elucidated by spectral analyses principally involving MS (on a Fisons VG Auto Spec) and NMR experiments including ^1H and ^{13}C 1D spectra and HMBC and HMQC 2D experiments. NMR data were acquired in CD_3OD on a Varian Unity 400 instrument. Comparison of ^1H NMR data with those existing in the literature indicated that the compound is the isoflavonoid warangalone (8(3,3-dimethylallyl)-4′-hydroxy-2‴,2‴dimethylpyran[6,7-*b*]isoflavone, $C_{25}H_{24}O_5$) first described by Pelter and Stainton.[14] NMR spectral signals for warangalone were assigned as follows: ^1H NMR ($CDCl_3$, 400 MHz) δ 7.89 (1H, s, H-2), 7.35 (2H, d, $J = 8.6$ Hz, H-2′, H-6′), 6.84 (2H, d, $J = 8.6$ Hz, H-3′, H-5′), 6.74 (1H, d, $J =$ 10.0 Hz, H-4″), 5.63 (1H, d, $J = 10.0$ Hz, H-3″), 5.17 (1H, dt, $J = 7.2$ Hz, H-2‴), 3.40 (2H, d, $J = 7.6$ Hz, H-1‴), 1.81 (6H, s, H-5‴), 1.68 (3H, s, H-4‴), 1.47 (6H, s, H-5″,H-6″); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 181.39 (C-4), 156.97 (C-4′), 155.91 (C-7), 154.85 (C-5), 154.76 (C-9), 152.67 (C-2), 131.70 (C-3‴), 130.32 (C-2′, C-6′), 128.05(C-3″), 123.27 (C-1′), 121.95 (C-2‴), 121.07 (C-3), 115.84 (C-4″), 115.65 (C-3′, C-5′), 107.50 (C-8), 105.90 (C-6), 105.47 (C-10), 77.83 (C-2″), 28.21(C-5″, C-6″), 25.75 (C-4‴), 21.27 (C-1‴), 17.87 (C-5‴).

Chemicals. Butylated hydroxytoluene, calcium ionophore A23187 (calcimycin), carrageenan, cyproheptadine hydrochloride, dexamethasone, dimethyl sulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), glycogen, 12-hydroxyheptadecatrienoic acid (12-HHTrE), hexadecyltrimethylammonium bromide (HTAB), indomethacin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), *N*,*N*-dimethylformamide, phosphate buffer saline, phospholipase A_2 (PLA_2) from *Naja mossambica* venom, prostaglandin B_2 (PGB_2), 12-*O*-tetradecanoylphorbol 13-acetate (TPA), tetramethylbenzidine, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO); acetone and methanol of analytical grade from Baker (Deventer, Holland); and ethanol 96% and sodium acetate of analytical grade from Panreac (Barcelona, Spain).

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).

DPPH Scavenging Activity.[15] DPPH in MeOH (1.5 mL, 20 mg/L) was added to 0.75 mL of a solution of test compounds in MeOH (100 μM). Increase in absorbance at 517 nm was determined after 5 min. Butylated hydroxytoluene was used as a reference compound.

Cytotoxicity Assay.[16] Rat polymorphonuclear leukocytes (PMNLs) were exposed to the test compound (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 (LTB_4) Production from Rat PMNLs.[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 with 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 LTB_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 Cyclo-oxygenase 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 A23187 (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 MeOH−H_2O (74:26) containing 0.007% trifluoroacetic acid. The results obtained were expressed as percentage of 12-HHTrE production.

Phospholipase A_2 (PLA_2)-Induced Paw Edema in Mouse.[10] 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 product cyproheptadine and the test compounds were dissolved in Tween 80−ethanol−saline (1:1:10). The 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.

12-*O*-Tetradecanoylphorbol 13-Acetate (TPA)-Induced Mouse Ear Edema.[19] Edema was induced by topical application of 2.5 μg per ear of TPA dissolved in acetone. Warangalone (0.25 mg/ear) and the standard drug indomethacin (0.5 mg/ear) were applied simultaneously with TPA. The 50% inhibitory dose (ID_{50}) was determined by applying warangalone at four different doses ranging from 500 to 50 μg/ear. The ear swelling was measured before TPA application and 4 h after, and the edema was expressed as the increase in thickness.

Myeloperoxidase (MPO) Assay.[20] Each ear sample was placed in an eppendorf tube with 0.75 mL of 80 mM sodium phosphate buffer (pH = 5.4) containing 0.5% HTAB. Enzyme activity was determined through the hydrogen peroxide breakdown-induced oxidation of tetramethylbenzidine, using a Labsystems Multiskan MCC/340 plate reader set at 620 nm. Details of the method have been described earlier.[21]

Mouse Ear Inflammation Induced by Multiple Applications of TPA.[22] Skin inflammation was induced by topical application on alternate days (5 applications) of 2 μg of TPA (20 μL) in each ear. Warangalone (0.2 mg/ear) and dexamethasone (0.05 mg/ear) were applied topically twice daily for 4 days. On the last day the compounds were applied only in the morning. The mice were killed by cervical dislocation, and two ear punches from each animal were taken (n = 5 animals). Details of the method have been described earlier.[21]

Histological Analysis.[21] Ear samples were fixed in 4% neutral buffered formalin. Each sample was cut longitudinally into equal halves. Half of each was embedded in paraffin, cut into 3−4 μm sections and stained with haematoxylin-eosin. Epithelium thickness was evaluated using an objective ×100 and expressed as the mean ± SD of the number of epidermal layers from the basal to the granulous stratum, both included. The use of an arbitrary four-level range allowed the semi-quantitative estimation of infiltrating cells.

Carrageenan-Induced Paw Edema in Mouse.[23] Edema was induced on the right hind paw by subplantar injection of carrageenan (3% w/v in saline, 25 μL). The EtOAc extract of the stem bark of *E. addisoniae*, 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.

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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