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Erythroivorensin: A novel anti-inflammatory diterpene from the root-bark of *Erythrophleum ivorense* (A Chev.)

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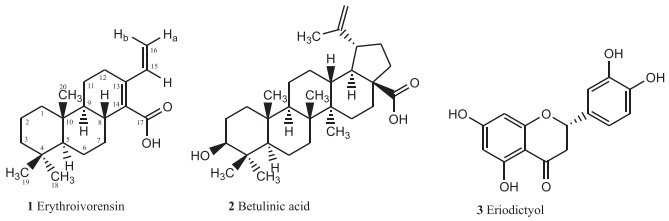
ABSTRACT

The stem- and root-bark of *Erythrophleum ivorense* (A Chev., family, Fabaceae) are routinely employed in the West African traditional medicine to treat inflammation and a variety of other disease conditions. Although the chemistry and pharmacology of cassaine-type diterpene alkaloids isolated from the stem-bark of the plant are fairly established, the root-bark has not yet been investigated. In the present study, the crude aqueous-alcohol extract of the root-bark was demonstrated to display a time- and dose (30–300 mg/kg p.o.)-dependent anti-inflammatory effect in chicks. Comprehensive chromatographic analysis coupled with spectroscopic and X-ray study further allowed the assignment of one of the major anti-inflammatory constituents as a novel cassaine-type diterpene, erythroivorensin. The other major constituents were known anti-inflammatory compounds: a triterpene, betulinic acid and a flavonoid, eriodictyol. The dose (10–100 mg/kg p.o.)-dependent anti-inflammatory effects of the three compounds were either comparable or more significant than the positive control, diclofenac.

1. Introduction

Erythrophleum ivorense (A Chev., family, Fabaceae) is a large evergreen tree widely distributed in tropical regions of Western Africa ranging from Gambia to the Central African Republic and Gabon. With the potential to grow up to 40 m high and source of hard-heavy wood, the plant is among the most exploited timber trees in West Africa. The plant, known in native countries by its local names including "Epo-obo" among Yoruba people of South Western Nigeria and 'potrodum' among the Akans in Ghana, is also widely traded for medicinal uses [1]. The stem-bark and roots of *E. ivorense* are particularly employed to treat convulsive disorders, emesis, pain, edema, smallpox, and laxative and as anti-helminthic [2]. The ethanol extract of the stem-bark has been shown to display anti-convulsant and sedative activities in mice model while the methanol extract has been proven to possess anti-microbial and cytotoxic effects [3,4].

The stem-bark of *E. ivorense* is widely known to contain diterpene-alkaloids, cassaine, cassaidine, cassamidine coumidine, erythropillamine and erythrophleguine [5]. Other cassaine analogues including 19-hydroxycassaine, norcassaide, norcassamide and norerythrophlamide have also been isolated from the stem-bark [6,7]. Although the genus is generally regarded to predominantly contain cassaine, cassaidine and erythrophleguine, the chemistry and pharmacology of the root-bark are yet to be investigated. In the present study, an investigation into the anti-inflammatory activity of the root-bark along with phytochemical analysis has resulted in the identification of a novel diterpene, named as erythroivorensin (**1**), with a significant pharmacological activity. The identification of the compound along with two known anti-inflammatory compounds, betulinic acid (**2**) and eriodictyol (**3**) is discussed.



1 Erythroivorensin **2** Betulinic acid **3** Eriodictyol

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2. Materials and methods

2.1. Chemicals

All organic solvents used for the experiments were of analytical grade and obtained from BDH Laboratory Supplies (Merck Ltd., Lutterworth, UK). The standard reference drug, diclofenac, was purchased from Troge (Hamburg, Germany) while all other chemicals were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK).

2.2. General experimental procedures

^{1}H NMR, ^{13}C NMR and ^{2}D NMR (COSY, NOESY, HMQC and HMBC) spectra were obtained on a JEOL 500 MHz instrument. Homonuclear ^{1}H connectivities were determined by using the COSY experiment. One bond ^{1}H–^{13}C connectivities were determined with HMQC while two- and three-bond ^{1}H–^{13}C connectivities were determined by HMBC experiments. Chemical shifts were reported in δ (ppm) using the solvent ($CDCl_3$ or methanol-D_4) standard and coupling constants (J) were measured in Hz. Optical activity was recorded using an ADP410 polarimeter (Bellingham and Stanley, Kent, UK) while IR and UV spectra were obtained using Perkin Elmer spectrum two FTIR (Coventry, UK) and Hitachi U-2900 spectrophotometers respectively. The high resolution mass spectroscopy instrument, Thermo Fisher LTQ Orbitrap XL (Thermo Fisher Scientific, UK), with an electrospray ionization probe was used for accurate mass measurement over the full mass range of m/z 50–2000. Nano-electrospray analyses were performed in positive ionization mode by using NanoMate to deliver samples diluted into MeOH + 10% NH_4OAc. The temperature was set at 200 °C, sheath gas flow of 2 units and capillary (ionizing) voltage at 1.4 kV. The accurate mass measurements obtained from this system were far better than 3 ppm.

2.3. Plant material

The root-bark of *E. ivorense* (Leguminosae) was harvested from Adukrom, a village in Nzema East Metropolis of Ghana, in October 2014 and was identified by curators of the University of Cape Coast Herbarium (Ghana). A voucher specimen (BHM/Eryth/017R/2014) has also been identified and deposited at the Herbarium of the Department of Herbal Medicine, Faculty of Pharmacy, Kwame Nkrumah University of Science and Technology, Ghana.

2.4. Isolation of the anti-inflammatory compounds

Powdered air-dried root bark of *E. ivorense* (1.2 kg) was cold macerated with 70% ethanol for 72 h. The resulting extract was then filtered and concentrated under reduced pressure (40 °C) to give the crude extract in a yield of 8.7% ($^{w}/_{w}$). A portion of this extract (100 g) was successively partitioned with petroleum ether (5 L), ethyl acetate (5 L), and methanol (5 L), to afford fractions in the yield of 15, 36.3 and 41.2 g respectively. The ethyl acetate fraction (25 g) was subjected to silica gel (70–230 mesh) column chromatography and elution was done with a gradient of petroleum ether–EtOAc to yield five major fractions: I (pet-ether/EtOAc 9:1; 0.72 g), II (pet-ether/EtOAc 4:1; 0.26 g), III (pet-ether/EtOAc 7:3; 4.2 g), IV (pet-ether/EtOAc 3:2, 5.9 g) and V (pet-ether/EtOAc 1:4; 10.8 g). Fractions I and II were combined on the basis of their TLC profile and subjected to repeated smaller column chromatography (on silica gel) as above to give compound **1** (yield 400 mg). Further recrystallization of this compound from acetone yielded colorless needle crystals. An oily yellow mass of fraction III was dried in a dessicator after which it was washed several times with petroleum ether to afford a yellow amorphous powder (compound **2**, 200 mg). Fraction IV was column chromatographed over silica gel as above, eluting with pet-ether and EtOAc mixtures of 7:3, 1:1 and 2:3. The fractions eluted with pet-ether/EtOAc 7:3 were combined, concentrated under reduced pressure and purified by repeated silica gel column chromatography to afford compound **3** (yield, 300 mg).

2.5. X-ray analysis

A single crystal was mounted on a nylon loop and X-ray diffraction data were recorded on an Agilent Super Nova Dual Diffractometer (Agilent Technologies Inc., Santa Clara, CA) with Cu-Kα radiation ($\lambda = 1.5418$ Å) at 150 K. Unit cell determination, data reduction and absorption corrections were carried out using CrysAlisPro [8]. The structure was solved by direct methods and refined by full matrix least squares on the basis of F [9] using SHELX 2013 [8] within the OELX2 GUI [10]. The asymmetric unit contains two molecules of $C_{20}H_{30}O_2$. Non-hydrogen atoms were refined anisotropically and hydrogen atoms were included using a riding model. The absolute stereochemistry was confirmed by successful refinement of the Flack parameter (−0.12(4)). Crystal data are presented in Table 1.

2.6. Erythroivorensin ***(1)***

Colorless needle (Ac_2O); mp 187–189 °C; $[\alpha]^{21}{}_{D} = -3.3°$ (*c* 0.15, MeOH); UV (MeOH) λ_{max} nm (ε): 231 (999); IR (Universal Attenuated Total Reflectance) ν_{max}; 2919, 2863, 1685, 1293, 1263, 904 cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$): Table 1. ESI-MS m/z $[M+H]^+$ 303.2321 (calc $C_{20}H_{31}O_2$ plus H 303.2319).

2.7. Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid, ***2***) and eriodictyol ((2S)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-2,3-dihydro-4H-chromen-4-one, ***3***)

The spectroscopic data were identical with those published previously [11,12].

Table 1
Crystal data and structure refinement for erythroivorensin (**1**).[a]

Empirical formula	$C_{20}H_{30}O_2$
Formula weight	604.88
Temperature/K	150 (1)
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
a/Å	11.99271 (7)
b/Å	14.66394 (7)
c/Å	19.91449 (10)
α/°	90
β/°	90
γ/°	90
Volume/Å^3	3502.17 (3)
Z	8
ρ_{calc} g/cm^3	1.147
μ/mm^{-1}	0.554
F(000)	1328.0
Crystal size/mm^3	0.28 × 0.1 × 0.08
Radiation	CuKα (λ = 1.54184)
2Θ range for data collection/°	7.486 to 147.294
Index ranges	−14 ≤ h ≤ 14, −18 ≤ k ≤ 18, −24 ≤ l ≤ 24
Reflections collected	59,230
Independent reflections	7031 [$R_{int} = 0.0305$, $R_{sigma} = 0.0141$]
Data/restraints/parameters	7031/0/405
Goodness-of-fit on F^2	1.021
Final R indexes [I ≥ 2σ (I)]	$R_1 = 0.0309$, $wR_2 = 0.0843$
Final R indexes [all data]	$R_1 = 0.0317$, $wR_2 = 0.0853$
Largest diff. peak/hole/e Å^{-3}	0.23/−0.15
Flack parameter	−0.12 (4)

[a] The details of the structural analysis are described in the Supporting information along with the cif file, which has been deposited at the Cambridge Crystallographic Data Centre (CCDC No. 1051612) and can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif.

2.8. Experimental animals

One day old post-hatch chicken (*Gallus gallus*; strain shaver 579) obtained from Akati Farms, Kumasi, Ghana were housed in stainless steel cages ($34 \times 57 \times 40$ cm^3) at a population density of 10–13 per cage. Feed (Chick Mash, GAFCO, Tema, Ghana) and water were available ad libitum through 1-quart gravity-fed feeders and water trough. Room temperature was maintained at 29 °C, and overhead incandescent illumination was maintained on a 12 hour light–dark cycle. Daily maintenance of the cages was conducted during the first quarter of the light cycle. Chicks were used for anti-inflammatory assay at 7 days of age.

2.9. Determination of anti-inflammatory activity

The carrageenan foot edema model in chick was used to evaluate the anti-inflammatory properties of test samples [13]. Seven day-old chicks (weighing between 40 and 60 g) were put into groups of five animals. After carrageenan (2% $^{w}/_{v}$) was injected intraplantar into right footpads, the initial foot volumes (time zero) were taken by water displacement plethysmography using an electronic Von Frey plethysmometer (Model 2888, IITC Life Science Inc., Ca 91367, Canada) as described by Feridoni et al. [14]. The foot volumes were then measured every hour for a total period of 5 or 6 h. For the anti-inflammatory activity measurements, crude extracts (30, 100, and 300 mg/kg) and **1** or diclofenac (10, 30, 100 mg/kg) were administered orally 1 h prior to carrageenan injection. Animals receiving 2 ml/kg normal saline served as control. The foot volumes were individually normalized as percentage of change from their values at time zero and then averaged for each treatment group. The total inflammation during the entire observation period for each treatment was also calculated in arbitrary unit as the area under the curve (AUC) and compared with the untreated control group [13]. All experimental protocols were in compliance with the National Institute of Health guidelines for the care and use of laboratory animals and were approved by the Department of Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST Ethics Committee.

2.10. Statistical analysis

All data were presented as mean and standard error of mean (SEM) values. Where appropriate, the significance of difference between two means was analyzed by using unpaired *t*-test.

3. Results and discussion

The stem- and root-barks of *E. ivorense* are routinely employed in traditional medicine practices to treat a variety of conditions including inflammatory pain and edema. Although cassaine and related diterpene alkaloids isolated from the stem-bark of the plant are known to display numerous pharmacological activities [5], little is known about the pharmacology of the root-bark. The carrageenan-induced edema model in rodents is one of the most accepted in vivo methods of anti-inflammatory activity evaluations [15,16]. We have also previously shown that injection of carrageenan into footpads of the chicks results in induction of inflammation in a time-dependent manner [13]. This model of inflammation has been shown to be attributed to the rapid release of acute phase mediators such as histamine and bradykinins followed by cyclooxygenase products including prostaglandins [17,18]. Consistent with our own previous reports [13,16], the maximum level of edema formation under this experimental condition could be obtained in less than 3 hour post-carrageenan injection and the increased edema was persistently evident during the whole observation period (Fig. 1). In order to further validate our experimental protocol, we first employed a standard positive control, diclofenac, which showed a time-dependent anti-inflammatory effect at all time points (Fig. 1). The AUC calculation further revealed that the three tested doses (10, 30 and 100 mg/kg) of diclofenac suppressed the Carrageenan-induced edema during the entire observation period by 36.8 ± 4.7, 47.3 ± 3.2 and 58.6 ± 4.6 respectively. The closeness of these data with previous reports [13] once again validates the chick's edema model of inflammation as an economically viable alternative to rodent-based assays. As shown in Fig. 1, oral administration of the crude extract of root-bark of *E. ivorense* similarly resulted in suppression of the carrageenan-induced inflammation in a dose- and time-dependent manner.

Fractionation of the crude extract of root-bark of *E. ivorense* followed by repetitive column chromatography over silica gel resulted in the isolation of one of the major compounds as a white powder which was subsequently crystallized to give colorless needles. Accurate mass analysis using high resolution ESI-MS instrument showed the $[M+H]^+$, $C_{20}H_{30}O_2$ plus H, ion at m/z 303.2321 (expected/theoretical 303.2319). The ^{13}C and Dept-135 NMR spectra showed signals indicating the presence of a 20-carbon diterpene skeleton. Of these, one was carbonyl; 4 olefinic of which two quaternary, one methine and one methylene; and in the saturated region, 2 quaternary, 3 methine, 7 methylene and 3 methyl groups (Table 2). Further assignment of all NMR signals and the structure as **1** came from comprehensive 2-D NMR studies (COSY, HMQC, HMBC and NOESY). Given the molecular formula allows 6 double bond equivalents and two double bonds and a carbonyl group (strong IR signal at 1685 cm^{-1} for α,β-unsaturated carbonyl) were evident from the NMR data, the structural assignment was based on a three-ring diterpene skeleton. The classical ABX system in the side chain (C15 methine and C16 methylene olefinic system) was established from the coupling pattern in the ^{1}H NMR spectrum (10.9 Hz as *cis*- and 17.2 Hz as *trans*-coupling) and COSY studies. The key ^2J and ^3J HMBC connectivities shown in Table 2 further allowed the assignment of the extended conjugation of this system with the second double bond (C13–C14) and the carboxylic acid at C-17. The C-4 gem-dimethyl (C18, C19) and C-20-methyl groups were also good reference points to establish the HMBC-based assignment of the structure as **1**. Finally, the assignment of ^{1}H NMR signals of the C-16 protons were based on NOESY studies where only H-16B showed an interaction with one of the H-12 protons at δ 2.46. Although the NOESY studies were helpful in determining the stereochemistry of the compound,

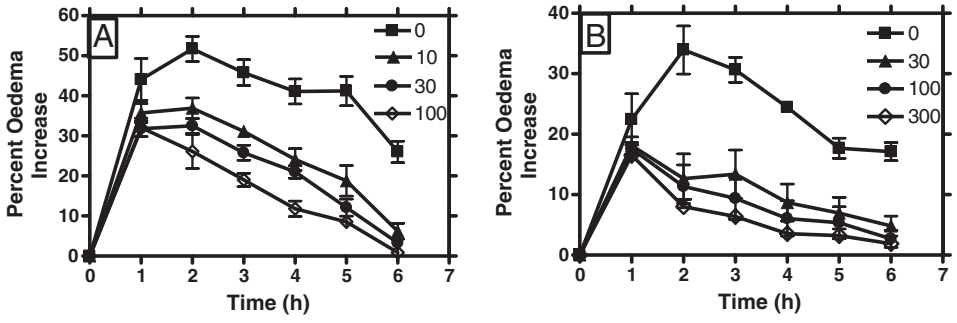

Fig. 1. Time-course edema development following carrageenan injection into chick paws and dose (mg/kg)-dependent anti-inflammatory effect of the standard positive control, diclofenac (A) and the crude root-bark extract of *E. ivorense* (B). All data from 2 to 6 h of the treated groups are significantly different ($p < 0.05$) from the negative control group at each time point.

Table 2
NMR spectroscopic data (500 MHz, $CDCl_3$) for erythroivorensin (**1**).

Position	δ_C, type	δ_H	HMBC (major ^2J and ^3J correlations)
1	38.7, CH_2	1.75 dd (12.6) 0.91 m	C2, C5
2	31.9, CH_2	2.01 dd (12.2, 2.2)	
		1.12 m	
3	42.2, CH_2	1.43 m	
		1.38 m	
4	33.2, C	–	
5	55.4, CH	0.92 m	C4, C9, C20, C18, C19
6	21.8, CH_2	1.68 dd (13.3, 3.0)	C8
		1.39 m	
7	18.9, CH_2	1.61 dt (12.9, 3.4)	
		1.43 m	
8	37.4, CH	2.48 m	C14
9	52.9, CH	0.97 m	C7, C20
10	36.8, C	–	
11	20.6, CH_2	1.86 dd (12.6, 5.9)	C8, C9, C12, C13
		1.15 m	
12	26.1, CH_2	2.46 m 2.18 m	C13, C13, C14 C13, C13, C14
13	136.5, C	–	
14	134.5, C	–	
15	134.9, CH	6.84 dd (17.2, 10.9)	C12, C13, C16
16	115.6, CH_2	16a 5.16 d (10,9)	C13, C15
		16b 5.35 d (17.2)	C13, C15
17	174.3, C	–	
18	33.4, CH_3	0.85 s	C3, C4, C5, C19
19	21.6, CH_3	0.83 s	C3, C4, C5, C18
20	14.2, CH_3	0.87 s	C1, C5, C9, C10

unambiguous assignment of the structure as the novel compound named erythroivorensin **(1)** came from X-ray analysis study (Fig. 2). Details of the crystal data are presented in Table 1. The identification of the other two major constituents as betulinic acid (**2**) and eriodictyol (**3**) was based on comparison of spectroscopic data (UV, IR, NMR and MS) with those published before [11,12] and authentic samples.

The time course study clearly shows that all the three major compounds isolated from *E. ivorense* displayed anti-inflammatory activity in a dose dependent manner (Fig. 3).

The overall anti-inflammatory activity of the isolated compounds (**1**–**3**) during the entire observation period was also assessed through the AUC analysis with due comparison with the positive control, diclofenac. All doses (3–100 mg/kg) of **1**–**3** and diclofenac displayed significant ($p < 0.0001$) edema reduction when compared with the untreated control group (Fig. 4.). Interestingly, all doses of **1**–**3** administered through the same (oral) route displayed either comparable or better anti-inflammatory activity as diclofenac (Fig. 4). While the presence of other minor constituents with a similar pharmacological effect cannot be ruled out, the novel diterpene, erythroivorensin (**1**), along with betulinic acid (**2**) and eriodictyol (**3**) as major constituents of the root-bark of *E. ivorense* are likely to play major role for the reported medicinal uses of the plant.

Although the major source of the pentacyclic lupane-type triterpenoid betulinic acid (**2**) is the bark of the common birch tree

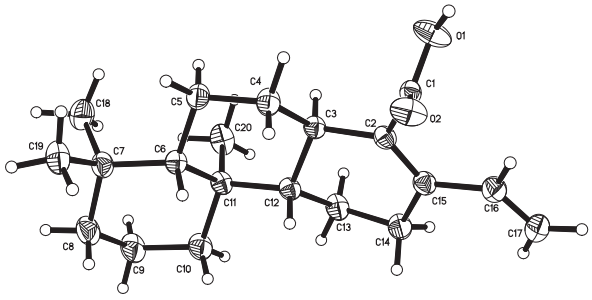

Fig. 2. ORTEP plot of erythroivorensin (**1**) showing the atom numbering scheme of one of the two molecules in the asymmetric unit. Thermal ellipsoids are drawn at the 50% probability level (hydrogen atoms are of arbitrary radius).

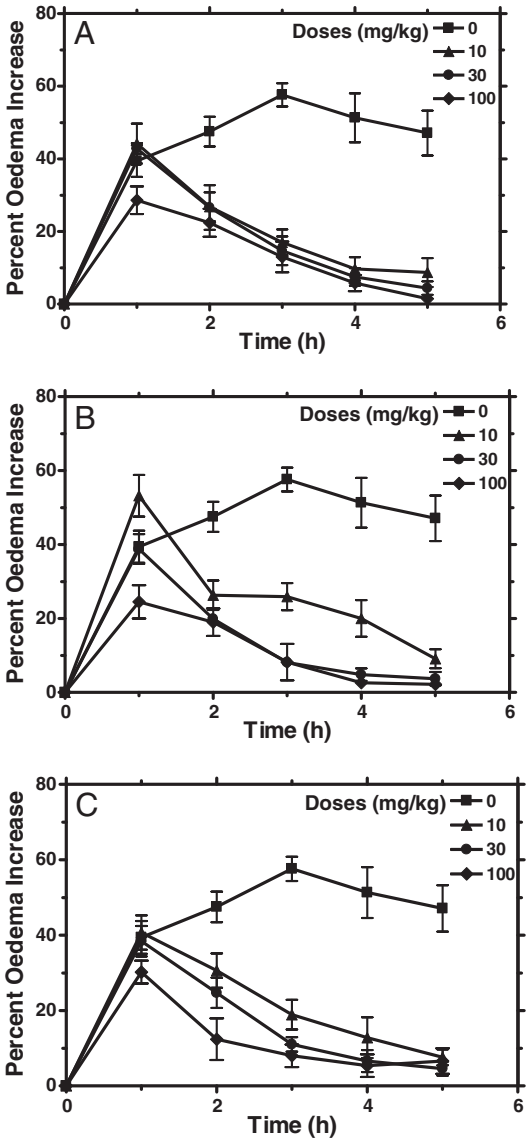

Fig. 3. Time-course edema development following carrageenan injection into chick paws and dose (mg/kg)-dependent anti-inflammatory effect of compounds isolated from the root-bark extract of *E. ivorense*. A — erythroivorensin; B — betulinic acid; C — eriodictyol. At time 2–5 h, data for all doses of the isolated compounds were significantly different ($p < 0.001$) from untreated control group.

(*Betula* spp., Betulaceae), the compound is widely reported from various plant sources. For example, the bark of many tree species that are utilized for timber production are known to contain up to 2.5% of **2** [19]. Among the various pharmacological activities reported for compound **2** are potent anti-inflammatory activities both in vitro and in vivo. For example, in vivo studies on bacterial lipopolysaccharide-induced lung damage demonstrated the anti-inflammatory and anti-oxidant properties of **2** at oral doses as small as 25 mg/kg [20]. The therapeutic potential of **2** in oxidative damage has also been established as protection from ischemia/reperfusion-induced renal damage was reported [21]. Similarly, the induction of NF-κB activation by carcinogens has been shown to be suppressed by **2** through inhibition of IκBα kinase and p65 phosphorylation as well as abrogation of cyclooxygenase-2 and matrix metalloprotease-9 [22]. The suppressive effect of **2** against pro-inflammatory prostaglandin E_2 production in vitro [23] and neutrophil recruitment and inflammatory mediator expression in lipopolysaccharide-induced lung inflammation in vivo [20,24] were also demonstrated. Interestingly, the expression of the cytokines (e.g. TNF)-mediated adhesion molecules on endothelial cell surface has

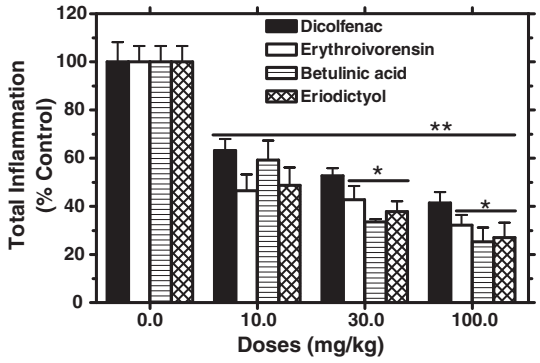

Fig. 4. Total anti-inflammatory activity of erythroivorensin (**1**), betulinic acid (**2**), eriodictyol (**3**) and diclofenac during the 6 h observation period. The dose-dependent effects of test compounds and diclofenac are shown from data obtained from the AUC as described in the Materials and methods section. Data are mean and SEM values (n = 5). Symbols indicate: **, all drug treated groups are significantly different ($p < 0.0001$) from untreated control group and *, significantly different ($p < 0.001$) from diclofenac treatment at the same dose.

been shown to be downregulated by **2** [25]. Hand in hand with these anti-inflammatory properties, the anti-nociceptive effects of **2** have been reported [26]. Other pharmacological effects of betulinic acid (**2**) include anti-bacterial [27,28], anti-HIV [29], anti-HSV-1 [30,31], anthelmintic [32] and anti-cancer effects [33–36]. The other known compound isolated from the root-bark of *E. ivorense* was the common flavonoid eriodictyol (**3**) that is also known to possess anti-inflammatory properties in a variety of test models. For example, it has been demonstrated to suppress nitric oxide (NO) production, expression of pro-inflammatory cytokines, inducible nitric oxide synthase and macrophage inflammatory protein in LPS-stimulated activated monocytes (Raw 264.7 cells) and B cells [37,38]. These activities were also shown to be associated with suppression of NF-κB activation and phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), COX-2 and c-Jun N-terminal kinase (JNK) [37,38]. Eriodictyol (**3**) is also a known antioxidant polyphenolic compound that possesses numerous pharmacological activities in a variety of assay models [39,40]. The biochemical mechanism of action of the novel compound, erythroivorensin (**1**), remains to be elucidated but given its structural similarities with the steroidal skeleton and/or that of the triterpene betulinic acid (**2**), a similar mechanism of action is anticipated. In view of the present finding and abovementioned numerous pharmacological activities of the known compounds **2** and **3**, the traditional medicinal uses of the *E. ivorense* for inflammatory conditions, pain suppression and as anti-helminthic agent appears to be justified.

In conclusion, the present study for the first time has established the scientific basis of the traditional uses of *E. ivorense* roots as an anti-inflammatory agent. Our phytochemical analysis study on the root-bark has also resulted in the identification of a novel compound (**1**) along with known compounds (**2**, **3**) that displayed anti-inflammatory activity comparable with the positive control diclofenac. Future studies must be directed on further pharmacological characterization of the novel compound, **1**.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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