

In vitro* 12(S)-HETE and leukotriene metabolism inhibitory activity of sesquiterpenes of *Warburgia ugandensis

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Abstract

Twelve drimane and coloratane sesquiterpenes, isolated from the stem bark of Ethiopian *Warburgia ugandensis*, were evaluated for inhibition of 12(*S*)-HETE using human platelets and of leukotriene B₄ formation using activated human neutrophil granulocytes. Among the compounds examined for inhibition of 12-LOX, ugandensidial and muzigadial displayed potent inhibitory activity with IC₅₀ values of 3.3 and 20.2 μM, respectively, in comparison to the positive control baicalein with an IC₅₀ value of 20.0 μM. In the 5-LOX assay muzigadial and ugandensidial also exhibited a dose dependent and potent inhibitory effect on LTB₄ biosynthesis with an IC₅₀ value of 10.2 and 12.7 μM, respectively, compared to the well known 5-LOX inhibitor zileuton with an IC₅₀ value of 10.4 μM. In contrast, none of the other sesquiterpenoids showed significant inhibition of either 12(*S*)-HETE or LTB₄ metabolism.

Keywords: *Warburgia ugandensis*, Canellaceae, 12-LOX, 5-LOX, 12(*S*)-HETE, LTB₄

Abbreviations:

12-LOX 12-lipoxygenase

12(*S*)-HETE 12-hydroxyeicosatetraenoic acid

Metabolism of arachidonic acid through the lipoxygenase and cyclooxygenase pathways produces a host of pro-inflammatory substrates, known as eicosanoids. Among the LOX pathways, 12-lipoxygenase (12-LOX) introduces oxygen at carbon 12 of arachidonic acid to generate a 12-hydroperoxy derivative which in turn is reduced to 12-hydroxyeicosatetraenoic acid (12(*S*)-HETE). Platelet-type 12(*S*)-LOX is one of the 12-LOX isoenzymes found in humans. We have employed this assay due to the fact that 12-lipoxygenase is expressed in a wide variety of tumor cell lines and the 12-LOX metabolite, 12(*S*)-HETE, is implicated as a

critical signalling molecule in tumor metastasis [1], atherosclerotic processes [1-2] and a mediator of hyperproliferation of the skin [1, 3].

Etc.

In this paper, we report the inhibitory effect of twelve sesquiterpenes isolated recently [12] (for structure see Fig. 1) from the stem bark of Ethiopian *W. ugandensis*, on the formation of arachidonic acid metabolites, 12(*S*)-HETE and LTB₄.

In the 12-LOX assay the compounds were tested at concentrations of 20, 10, 5, and 1 µg/mL and compounds **2** and **12**, possessing the dialdehyde functional group, displayed dose dependent potent inhibition of 12(*S*)-HETE (Fig. 2) whereas compounds lacking this group exhibited weak or no inhibition. Comparing the activity of 6 α , 9 α -dihydroxy-4(13),7-coloratadiene-11,12-dial (**1**), having one more hydroxyl group, with that of muzigadial (**2**), it seems that the absence of a free hydroxyl group at position 6 in **2** significantly enhanced the inhibitory effect. The same tendency in activity seems to be true for **11** and **12**, in the latter case the free hydroxyl group at position 6 is blocked by acetylation and inhibitory activity is dramatically enhanced. Of the drimane/coloratane sesquiterpene lactones tested against 12(*S*)-HETE, cinnamolide (**7**) and cinnamolide-3 β -acetate (**8**), lacking the hydroxyl group at positions 6 and 9, caused a slight inhibition at 20 µg/mL. All other compounds tested showed no inhibitory activity (see Table 1).

Etc.

In summary, although there was no correlation between 12(*S*)-HETE and LTB₄ biosynthesis inhibition, **2** and **12** were found to be equipotent inhibitors of both metabolites. The results of

our study, therefore, clearly demonstrate the significant inhibition of 12(*S*)-HETE and LTB₄ formation by the sesquiterpene dialdehydes **2** and **12**.

Materials and Methods

Stem bark of *W. ugandensis* Sprague (Canellaceae) was collected in Harena Forest, approximately 13 km from Dello Menna, Ethiopia in April 2001 and identified by Mr. Melaku Wendafrash, the National Herbarium, Biology Department, Science Faculty, Addis Ababa University. A voucher specimen (collection No 977) was deposited at the National Herbarium for further reference.

The sesquiterpenes subjected to this study, 6 α ,9 α -dihydroxy-4(13),7-coloratadiene-11,12-dial (**1**), muzigadial (**2**), 4(13),7-coloratadiene-12,11-olide (**3**), muzigadiolide (**4**), 11 α -hydroxymuzigadiolide (**5**), 7 β -hydroxy-4(13),7-coloratadiene-11,12-olide (**6**), cinnamolide (**7**), cinnamolide-3 β -acetate (**8**), 7 α -hydroxy-8-drimen-11,12-olide (**9**), ugandensolide (**10**), mukaadial (**11**) and ugandensidial (**12**) (Fig. 1), were isolated from the stem barks of *W. ugandensis* as previously described [12]. Copies of the original spectra are obtainable from the author of correspondence. The purity of the compounds ranged from 93.2 % (**11**) -98.7 % (**1**) as determined by analytical HPLC with PDA detection.

The 12(S)-LOX inhibitory assay was performed *in vitro* using human platelets as reported previously [17]. The bioassay for inhibition of LTB₄ biosynthesis was performed using activated neutrophil granulocytes as described before [18].

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Supporting Information

Detailed protocols for the *in vitro* 12(S)-HETE assay and for the *in vitro* LTB₄ assay are available as Supporting Information.

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Etc.

Figure Legends

Table 1: Inhibitory activities of sesquiterpenes **1-12** from *Warburgia ugandensis* on 12(*S*)-HETE and LTB₄ formation *in vitro*.

Figure 1: Chemical structures of compounds **1-12**.

Table 1

Compound	12(S)-HETE assay		LTB ₄ assay	
	Inhibition at 20 µg/mL ± S.D.	IC ₅₀ (µM) ± S.D.	Inhibition at 40 µM ± S.D.	IC ₅₀ (µM) ± S.D.
1	43.1 ± 4.6	n.d.	57.5 ± 9.8	n.d.
2	86.4 ± 0.8	20.2 ± 0.6	99.0 ± 2.1	10.2 ± 1.2
3	-17.6 ± 7.7	n.d.	28.0 ± 3.0	n.d.
4	-15.1 ± 3.6	n.d.	37.2 ± 2.9	n.d.
5	- 7.48 ± 0.20	n.d.	35.5 ± 6.3	n.d.
6	-17.6 ± 3.6	n.d.	28.5 ± 4.2	n.d.
7	11.1 ± 0.4	n.d.	31.0 ± 2.6	n.d.
8	5.9 ± 10.0	n.d.	35.0 ± 1.4	n.d.
9	-8.6 ± 2.0	n.d.	53.0 ± 13.2	n.d.
10	- 22.0 ± 1.8	n.d.	27.5 ± 4.7	n.d.
11	30.54 ± 1.86	n.d.	50.1 ± 5.7	n.d.
12	91.5 ± 0.1	3.3 ± 0.1	99.2 ± 3.1	12.7 ± 1.3
Baicalein	74.3 ± 0.7	20.0 ± 0.	n.d.	n.d.
Zileuton	n.d.	n.d.	87.8 ± 11.3 ^a	10.4 ± 0.5

n.d. not determined; ^a at 20 µM

Supporting Information

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***In vitro* 12(S)-HETE assay**

For the 12(S)-lipoxygenase assay peripheral venous blood from healthy volunteers was drawn into trisodium citrate-containing (9 : 1, v/v) blood collection tubes (BD Vacutainer Systems). Platelet-rich plasma (PRP) was collected by centrifugation at 200 g for 10 min at 20 °C. PRP was further centrifuged at 1200 g for 15 min at 20 °C and the platelet pellet was washed twice with PBS-buffer (Fluka) containing 1 mM EDTA (Fluka). Thereafter, the platelets were resuspended in PBS to a final concentration of 0.9×10^8 platelets/mL. 970 µL of platelet suspension were preincubated at 37 °C for 7 min in the presence of 2 mM reduced glutathione (Sigma) with test solutions or controls. Test samples were dissolved in absolute EtOH (final EtOH concentration of 1 % in the assay mixture). The suspensions were incubated with 33 µM arachidonic acid (Sigma) for another 7 min at 37 °C. The reaction was stopped by 2 M HCl and by cooling with ice. 12(S)-HETE was quantified by 12(S)-HETE-EIA using a Correlate-EIA™-12(S)-HETE-kit (96-well; Assay Designs). Before quantification, the samples were centrifuged at 2000 g for 15 min at 4 °C. 12(S)-HETE-concentrations were calculated in relation to a 12(S)-HETE-standard (Sigma). The results are means of at least two and in most cases three single experiments. Limits of quantification of 12(S)-HETE were 146 pg/mL for EIA. Positive control measurements were performed with baicalein (Sigma-Aldrich; purity > 98% by HPLC).

***In vitro* LTB₄ assay**

Principle: Activated neutrophil granulocytes with 5-LOX activity are incubated with a defined concentration of test sample and arachidonic acid. After stopping the enzymatic reaction by addition of formic acid and centrifugation to remove cellular fragments, the

produced LTB₄ is quantified in the supernatant by means of an LTB₄ EIA Kit (Cayman Chemical). Zileuton (Cayman Europe; purity > 98% by HPLC) was used as positive control.

Isolation of human neutrophilic granulocytes: 30 mL of venous human blood from healthy voluntary donors were collected with a Vacutainer™ (BD) system containing a 0.219 M pre-analytical citric acid solution. The blood was immediately transferred to a falcon tube containing 20 mL of sedimentation solution (1 % NaCl, 6 % dextran T-500) and left to separate for 60 min at 4 °C. While most of the dense erythrocytes sink into the dextran layer, the lighter blood fractions remain in the upper layer which is then removed and centrifuged at 1600 rpm at 4 °C for 10 min to concentrate the leukocytes, the plasma supernatant is discarded, the pellet is resuspended in 10 mL of a wash buffer (7.4 % CaCl₂ dihydrate p. a.; 0.1 % anhydrous *D*-glucose; 0.2 % MgCl₂·6 H₂O; 0.04 % KCl; 1.75 % Tris p. a.; with the pH adjusted to 7.6 with 1 N HCl). After centrifugation at 1400 rpm at 4 °C for 10 min and removal of the supernatant the resulting pellet is resuspended in 10 mL of hypotonic lysis buffer (0.17 % NH₄Cl; 0.2 % Tris; pH 7.2) and gently shaken for 5 min at room temperature to destroy remaining erythrocytes. The suspension is submitted to another centrifugation at 1400 rpm at 4 °C for 5 min. The pellet is resuspended in 10 mL of wash buffer and then centrifuged at 1400 rpm at 4 °C for 15 min. The resulting pellet which now mainly contains neutrophilic granulocytes is resuspended in 2 mL of Tris buffer (1.75 % Tris p. a., 0.9 % NaCl, pH 7.4), tested for vitality, quantified and then diluted to a cell concentration of 5000 cells/μL with Tris buffer.

Etc.