Miconidin Acetate and Primin as Potent 5-Lipoxygenase Inhibitors from Brazilian *Eugenia hiemalis* (Myrtaceae)

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Abstract

This paper describes the isolation and identification of primin and miconidin acetate as metabolites from the flower bud extract of *Eugenia hiemalis* as well as the anti-inflammatory activity of miconidin acetate by inhibition of 5-lipoxygenase. Miconidin acetate inhibited leukotriene B_4 formation catalyzed by the human recombinant enzyme ($IC_{50} = 0.3 \pm 0.17$ µM) more than primin ($IC_{50} = 1.4 \pm 0.6$ µM) and zileuton ($IC_{50} = 1.1 \pm 0.7$ µM). Miconidin acetate (20 µM) inhibited LTB_4 formation to an extent of 59 ± 12% *in vitro* using a cell-based assay, comparable to the positive control zileuton (69 ± 12% inhibition at a concentration of 10 µM). The binding modes of miconidin acetate were further evaluated *in silico* by molecular docking to the human 5-lipoxygenase crystal structure. The hydroxyl group was predicted to form a hydrogen bond with the terminal Ile676, while the pentyl moiety occupied the hydrophobic substrate channel. The obtained results show that flower buds of *E. hiemalis* are an interesting source of anti-inflammatory compounds, mainly of miconidin acetate.

Key words
Eugenia hiemalis · Myrtaceae · miconidin acetate · primin · anti-inflammatory · 5-lipoxygenase

Supporting information available online at http://www.thieme-connect.de/products

Eugenia is the largest genus in the Myrtaceae family from tropical America [1], growing from Mexico and the Caribbean to Argentina [2]. The plants of this genus are evergreen trees or shrubs with usually edible fruits and are known to be rich in volatile oils [3,4]. Several species are used in folk medicine as anti-inflammatory [5], diuretic, digestive [6], and antimicrobial remedies [7].

Eugenia hiemalis Cambess., commonly known as "guamirim", is a tree that grows in Brazil, Argentina, Uruguay, and Paraguay [8]. There are a few reports on its chemical composition and biological activity. Nevertheless, no report of its traditional use was found for this species. A benzoquinone (primin) and a monoacetyl derivative of the correspondent hydroquinone were isolated from the extract of leaves collected in Southern Brazil [9]. Both compounds showed antiapoptotic activity towards KG1a cells [10]. Moreover, three galloyl arbutins (hyemalosides A–C) along with nine phenolic compounds were isolated from the leaves' extract from plants growing in Paraguay. Some of these compounds inhibited HIV-1 RNase H *in vitro* [11]. The essential oil from the leaves contained sesquiterpene hydrocarbons as the major components [12,13].

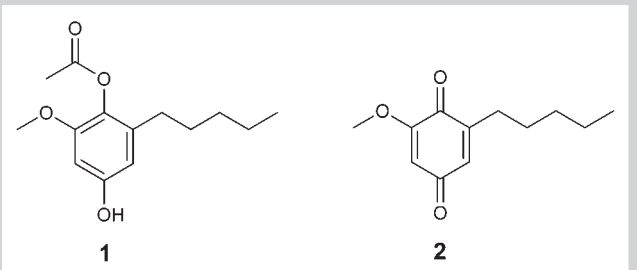

Fig. 1 Chemical structures of MA (**1**) and primin (**2**).

Natural products have long been used in folk medicine for the treatment of inflammatory conditions such as fevers, pain, and arthritis. These bioactive plant-derived substances represent a wide structural diversity. Some examples include curcumin, resveratrol, baicalein, boswellic acid, betulinic acid, ursolic acid, and oleanolic acid, which act on several inflammatory targets like cyclooxygenases (COX-1 and COX-2), 5-lipoxygenase (5-LOX), cytokines, chemokines, or interleukins [14].

Previous work evaluating primin detected a strong selective inhibition of COX-2 over COX-1 [15]. Furthermore, primin was similarly as potent as the 5-LOX inhibitor and reference compound zileuton, which is clinically used for asthma treatment. Docking analysis showed that the benzoquinone structure was essential for this activity [16]. This paper describes the isolation of miconidin acetate (MA) (**1**) and primin (**2**) (**Fig. 1**) by chromatographic fractionation of the flower bud extract from *E. hiemalis*, their anti-inflammatory activities via 5-LOX inhibition, and a docking analysis of MA binding to the target. Both compounds were identified and confirmed by spectral data and comparison to the literature. MA was the major metabolite and constitutes a hydroquinone derivative related to primin.

In the search for protein targets mediating a possible anti-inflammatory effect of MA, a similarity ensemble approach (SEA) was pursued using the publicly available SEA search tool [17,18]. The structure of MA was uploaded as smiles code. MA was then compared to structurally similar molecule ensembles from the ChEMBL16 binding database. If the similarity was high, MA was predicted to act on the same biological targets at the same concentration as the respective molecules from the ChEMBL. We considered all predictions with E-values ≤ −4, as suggested by Lounkine et al. [19]. Among the 54 predicted targets for MA, the 8th-ranked target, rat 5-lipoxygenase (5-LOX), was linked to inflammation. Overall, 5-LOX from different species was proposed five times as a target by the SEA tool.

MA inhibited leukotriene B_4 (LTB_4) formation in the human recombinant enzyme ($IC_{50} = 0.3 \pm 0.17$ µM) more potently than primin ($IC_{50} = 1.4 \pm 0.6$ µM) and zileuton ($IC_{50} = 1.1 \pm 0.7$ µM). Primin previously inhibited LTB_4 biosynthesis in a cell-based assay with an $IC_{50} = 4.0$ µM (IC_{50} of zileuton was 4.1 µM) [16]. Therefore, MA was also evaluated for 5-LOX inhibition *in vitro* using a cell-based

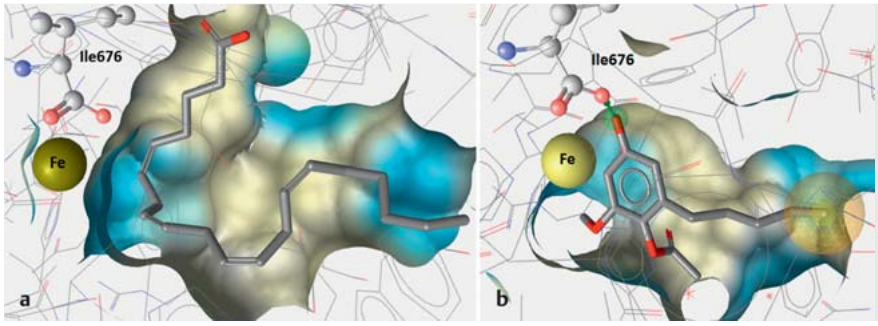

Fig. 2 The substrate arachidonic acid docked into the active site of 5-LOX (**a**). Predicted binding pose and protein-ligand interactions of MA and its target 5-LOX (**b**). Chemical interactions are color-coded: yellow – hydrophobic contact; green – hydrogen bond donor. The surface of the binding site is displayed according to aggregated hydrophilicity (blue)/hydrophobicity (yellow).

assay and detecting LTB_4 with an ELISA kit [20]. MA (20 µM) inhibited LTB_4 formation to an extent of 59 ± 12%, while the positive control zileuton (10 µM) inhibited 5-LOX by 69 ± 12%. The inhibitory activity in the cell-free assay demonstrated direct interferences of MA and primin with 5-LOX's catalytic activity. In the case of cell-based assays, tested compounds can actually interact with several targets within the 5-LOX activation and reaction cascade [21]. In our case, the activity of MA in the cell-based assay demonstrated mainly its ability to enter the cells.

To further elucidate the mode of inhibition, MA was docked into an X-ray crystal structure of human 5-LOX (PDB entry 3o8 y) [22] using GOLD 5.2 (CCDC, GB). As shown in **Fig. 2**, MA was predicted to occupy part of the active site going by the iron ion. It formed a hydrogen bond with the iron-coordinating residue Ile676 and hydrophobic contacts in the tunnel leading to the catalytic center.

Miconidin has been considered the biosynthetical precursor of primin [23,24], and the isolation of MA as a major compound in the floral buds suggests that this metabolite is a storage form for primin, which could be involved in protection against microorganisms and herbivore attacks during the flowering stage. MA was identified chromatographically as a minor compound from *Primula obconica* Hance (Primulaceae) leaves [25]. Nevertheless, its isolation as a major natural product was just reported for *E. hiemalis*. Furthermore, no quinones or related compounds were reported so far for the *Eugenia* genus, except for *E. hiemalis* leaves [9] and flower buds.

Despite its relatively simple structure, MA and primin showed that they are potent 5-LOX inhibitors with similar activities compared to the reference inhibitor zileuton, both in cell-based and human recombinant enzyme-based LTB_4 formation assays. These results, together with the molecular docking study, suggest that the 3D structure of MA is important for its ability to access the catalytic center of 5-LOX.

Material and Methods

General experimental procedures: NMR spectra were obtained by Bruker DRX 400, Bruker AMX 500, and Varian XL 300 spectrometers for the ^1H NMR, ^{13}C NMR, correlation spectroscopy (COSY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC). The mass spectrum was recorded by a spectrometer MS 50 (Kratos). IR spectra were obtained by a Prestige-21 Shimadzu, and UV-Vis spectra by Perkin Elmer Lambda 10.

Plant material: The aerial parts of *E. hiemalis* were collected and identified in April 2014 in Porto Alegre (Rio Grande do Sul, Brazil) by Dr. J. A. Jarenkow (Department of Botany, Federal University of Rio Grande do Sul, Brazil). A voucher specimen (ICN 127 910) was deposited in the herbarium of the Instituto de Ciências Naturais (Federal University of Rio Grande do Sul).

Extraction and isolation: The flower buds (27 g) were extracted with dichloromethane (0.25 L) by maceration for 7 days. The extract was concentrated under reduced pressure affording 0.5 g of CH_2Cl_2 extract that was fractionated in a gravity chromatography column on silica gel (40–63 µm, Sigma-Aldrich; 2.5 × 40 cm) with hexane-ethyl acetate (100/0 to 0/100, flow rate 1 mL/min) to give **1** (138.2 mg) and **2** (43.6 mg).

In vitro 5-lipoxygenase assay: Inhibition of 5-LOX was determined in two assays: a cell-based assay [20] and a cell-free assay using human recombinant enzyme (modified method of Albert et al.) [26]. Human neutrophil granulocytes for the cell-based assay were isolated from buffy coat (50 mL) obtained from healthy donors. Dextran solution was used for sedimentation, and subsequent lysis and washing were performed. Isolated cells were diluted to a final concentration of 4500 cells/µL. The incubation mixture consisted of 225 µL of cell suspension, 10 µL of 2 mM $CaCl_2$, 10 µL of 10 µM eicosatetraenoic acid, 5 µL of tested substances dissolved in DMSO, 10 µL of 21 µM calcium ionophor A23187, and 5 µL of 120 µM arachidonic acid. The reaction was stopped after 10 min incubation at 37 °C and the concentration of LTB_4 was measured using a commercial LTB_4 ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions. Absorbance relative to the LTB_4 concentration was measured at 405 nm using a Tecan Infinite M200 (Tecan Group). The results are expressed as percentage of inhibition of LTB_4 formation against untreated samples (blanks).

Human recombinant 5-LOX (Cayman Chemical) was used for the cell-free assay. 5-LOX (1 unit/reaction) was added to 180 µL of incubation mixture consisting of phosphate buffer saline (pH 7.4), 1 mM of Na_2EDTA, and 200 of µM ATP. After the addition of the test substances (10 µL) dissolved in DMSO (or pure DMSO in case of blank), the mixture was incubated for 10 min at 4 °C. Then 5 µL of 80 mM $CaCl_2$ and 5 µL of 800 µM arachidonic acid were added and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 10 µL of 10% formic acid. All samples were diluted 1 : 15 in assay buffer and the main product of reaction, LTB_4, was quantified using an LTB_4 ELISA kit (Enzo Life Sciences) according to the manufacturer's instructions. At least four concentrations were used for the calculations of the IC_{50} values. Three independent experiments with at least two replicates were used for the calculations of the inhibition curves. IC_{50} values were determined by regression analyses using Microsoft Excel.

Docking analysis: A putative binding mode for MA was calculated employing molecular docking using GOLD 5.2 software (www.ccdc.cam.ac.uk). Before docking, MA was geometrically opti-

mized using Biovia DiscoveryStudio (www.biovia.com). The binding site was defined in a 10-Å radius around the iron ion in the catalytically active center. For all other options, default settings were used.

Supporting information

Spectral and physicochemical data of MA (**1**) and primin (**2**), and also a graphic of the inhibition of LTB_4 formation in the human recombinant enzyme by these compounds, are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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