

Article

A Thin-Layer Chromatography Bioautographic Assay for the Rapid Detection of Arachidonate 5-Lipoxygenase Inhibitors

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Abstract

Arachidonate 5-lipoxygenase (ALOX5) is a key enzyme implicated in several inflammatory disorders, including asthma and allergic rhinitis. Despite its therapeutic importance, only one compound is currently approved as an ALOX5 inhibitor in the United States, highlighting the urgent need for new drug candidates. Progress in this area is often hindered by conventional bioassays, which can be labor-intensive, costly, and unsuitable for complex mixtures. To overcome these challenges, we developed a simple thin-layer chromatography (TLC) bioautographic assay for the rapid detection of ALOX5 inhibitors in natural extracts, a rich source of pharmacologically active compounds. The method exploits the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with 3-(dimethylamino)benzoic acid (DMAB) during the ALOX5-catalyzed conversion of arachidonic acid, producing a colored indamine dye. Experimental parameters influencing chromogenic reaction were investigated and optimized to minimize reagent consumption while ensuring accuracy and sensitivity of the method. The assay was then applied to a panel of natural products and to crude mushroom extracts, enabling the rapid identification of several active compounds within complex extracts, including the dual COX2/ALOX5 inhibitor 3 α -acetylpolyporenic acid A. Easy to implement, cost-efficient, and well suited for screening and bioguided fractionation, this TLC bioassay provides a powerful tool to accelerate the discovery of novel anti-inflammatory compounds.

Keywords: thin-layer chromatography; arachidonate 5-lipoxygenase; bioautography; enzyme inhibition; anti-inflammatory drugs; dual COX/LOX inhibitor



Academic Editor: Marcello Locatelli

Received: 26 February 2026

Revised: 17 April 2026

Accepted: 25 April 2026

Published: 6 May 2026

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1. Introduction

Inflammation is a multifaceted process mediated by several enzymatic pathways, among which cyclooxygenases (COX) and lipoxygenases (LOX) play central roles. In particular, arachidonate 5-lipoxygenase (ALOX5) contributes critically to inflammatory, allergic, and other immune-related disorders through the conversion of arachidonic acid into leukotrienes [1]. While numerous COX inhibitors are clinically available, therapeutic options targeting the ALOX5 pathway remain limited and are primarily indicated for the prophylaxis and long-term management of asthma. These include zileuton, an orally active ALOX5 inhibitor, and leukotriene receptor antagonists such as montelukast, although the latter has been linked to potential neuropsychiatric adverse effects [2]. Zileuton itself may

cause headaches, nausea, diarrhea, or sinusitis. Another ALOX5 inhibitor often used as a positive control in bioassays, the plant-derived lignan nordihydroguaiaretic acid (NDGA), has not been approved as a drug due to toxicity concerns and insufficient clinical evidence. Recently, ALOX5 has also been identified as a promising target against cancer and age-related brain disorders [3,4]. The broad clinical relevance of ALOX5, coupled with the limited availability of safe and effective inhibitors, underscores the urgent need to identify and develop novel drugs with improved safety profiles.

Current assays for ALOX5 inhibition primarily rely on quantification of metabolic products using HPLC-MS or ELISA, or employ chromogenic or fluorogenic agents that yield a measurable change in color or fluorescence during enzymatic reactions [5–8]. However, these methods are frequently tedious, expensive, and poorly suited for the evaluation of complex natural extracts, as they fail to provide insights into the activity of individual constituents. Nevertheless, numerous natural products possess significant ALOX5-inhibitory potential [9,10]. In a previous work, we developed a rapid, cost-effective and sensitive TLC assay for detecting COX-2 inhibitors in complex extracts, based on a chromogenic reaction [11]. Building on this approach, we present here an enzyme-based TLC assay designed to detect ALOX5 inhibitors. Several chromogenic reagents have been reported for monitoring lipoxygenase activity in microplate assays, including xylenol orange, used in the ferrous-oxidized xylenol orange (FOX) assay, which forms a pink-purple complex under acidic conditions [12]; a colorless precursor that yields methylene blue in the presence of hemoglobin [13]; and the combination of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with 3-(dimethylamino)benzoic acid (DMAB), which produces a colored indamine dye [14,15]. Based on preliminary testing, we adopted the MBTH-DMAB reagent to develop a TLC assay to detect ALOX5 inhibitors rapidly.

2. Materials and Methods

2.1. Chemicals and Reagents

Recombinant human 5-lipoxygenase (ALOX5), arachidonic acid (AA), 3-methyl-2-benzothiazolinone (MBTH), 3-(dimethylamino)benzoic acid (DMAB), Tris-HCl, calcium chloride (CaCl_2), adenosine triphosphate (ATP) disodium salt, bovine hemoglobin, and hematin porcine were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, acetonitrile, and methyl tert-butyl ether were obtained from Fisher Scientific (Hampton, NH, USA), tetrahydrofuran from VWR (Radnor, PA, USA), ethyl acetate from Carlo Erba (Val-de-Reuil, France), and cyclohexane from Sigma-Aldrich. Zileuton, nordihydroguaiaretic acid (NDGA), piperine, uvaol, caffeine, salicylic acid, and paracetamol were also purchased from Sigma-Aldrich. Betulin was from Carl Roth (Karlsruhe, Germany). The other standard natural compounds were purchased either from Sigma-Aldrich or Extrasynthese (Genay, France).

2.2. Mushroom Material

Mushroom samples were collected from natural habitats in and around Strasbourg (Bas-Rhin, France) between 2018 and 2024. The species *Xylaria polymorpha*, *Fomes fomentarius*, *Fomitopsis pinicola*, and *Fomitopsis betulina* were identified using a combination of macroscopic examination and DNA-based analyses. An additional specimen belonging to the genus *Hebeloma* was included in the study; however, its species could not be conclusively determined using macroscopic, microscopic, or molecular sequencing approaches. The fruiting bodies were freeze-dried using a Labconco (Kansas City, MO, USA) Freezone 4.5 L freeze dryer, ground into a fine powder, and extracted with ethyl acetate via ultrasonication with a ratio of 25 mL of solvent per gram of dry matter. The resulting extracts were concentrated under reduced pressure to remove the solvent and stored at 4 °C.

A voucher specimen of each species is deposited at the Laboratory CAMBAP, University of Strasbourg.

2.3. Purification and Identification of 3 α -Acetylpolyporenic Acid A

A finely powdered sample of *Fomitopsis betulina* (37.5 g) was successively extracted by sonication for 30 min with n-heptane and ethyl acetate at room temperature (ratio of 10 mL per gram). A portion (8.2 g) of the dry ethyl acetate extract was subjected to solid-phase extraction (SPE) using a 60 mL Chromabond cartridge (Macherey-Nagel, Düren, Germany) loaded with silica gel (15 g) with mixtures of acetonitrile (ACN) and water of decreasing polarity: 100%, 80%, 60%, and 40% of ACN. The fraction obtained with 80% ACN was purified by preparative HPLC using a C18 Kinetex Axia preparative column (5 μ m, 150 $\times$ 21.2 mm, Phenomenex, Torrance, CA, USA) and a Gilson (Middleton, WI, USA) 322 pump delivering the mobile phases A (H₂O + 0.1% formic acid) and B (ACN + 0.1% formic acid) at a flow rate of 30 mL/min. A sample at a concentration of 20 mg/mL was loaded via a valve equipped with a 2.5 mL loop, and eluted with a gradient from 15% to 85% of solvent B in 12 min, then kept at 85% B to 21 min. The fractions were collected using a Gilson FC 204 fraction collector, and combined on the basis of their TLC and analytical HPLC profiles. The fraction eluted at approximately 13.1 min yielded a product identified as 3 α -acetylpolyporenic acid A, by comparing its spectral data with those in the literature [16].

3 α -acetyl-polyporenic acid A: colorless powder; ¹H NMR (500 MHz, CDCl₃, δ (ppm), mult., J (Hz)): δ 4.95 (m, 2H), 4.73 (t, J = 2.8 Hz, 1H), 4.02 (d, J = 7.9 Hz, 2H), 3.43 (m, 2H), 3.19 (m, 1H), 2.62 (m, 2H), 2.20 (m, 1H), 2.05 (m, 7H), 1.88 (m, 1H), 1.74 (dd, J = 15.0, 3.3 Hz, 2H), 1.53–1.45 (m, 4H), 1.39 (s, 1H), 1.31 (d, J = 7.1 Hz, 4H), 1.28–1.23 (m, 1H), 1.20–1.13 (m, 1H), 1.10 (s, 2H), 1.02 (s, 2H), 1.00 (s, 3H), 0.98 (s, 3H), 0.93 (s, 3H), 0.89 (s, 3H), 0.61 (s, 3H); ¹³C NMR (125 MHz, CDCl₃): δ 179.5, 170.1, 148.2, 134.8, 132.8, 111.5, 80.3, 73.5, 49.5, 49.5, 45.3, 45.2, 42.9, 36.8, 36.6, 35.9, 34.2, 32.3, 32.0, 31.4, 30.4, 27.7, 27.6, 25.9, 24.7, 23.0, 21.6, 21.6, 18.8, 17.9, 17.8, 16.2, 16.1. LC-MS (ESI), *m/z* (rel. int.): [M-H][−] 527 (100).

2.4. Preparation of Solutions

ALOX5 was prepared in 100 mM Tris-HCl buffer (pH 7.5) to a final concentration of 2 U/mL, supplemented with 5 μ M CaCl₂. Stock solutions of arachidonic acid (AA), DMAB, and MBTH were prepared in ethanol. Immediately before use, the chromogenic mixture was prepared by combining these stock solutions in Tris-HCl buffer to yield final concentrations of 20 μ M AA, 0.1 mM MBTH, and 0.1 mM DMAB. To evaluate their influence on the chromogenic reaction, hematin or hemoglobin was added to the reaction mixture at a final concentration of 100 μ M, while ATP (1 μ M) was added directly to the ALOX5 enzyme solution. Standard compounds were dissolved in ethanol and mushroom extracts were resuspended in ethyl acetate at appropriate concentrations for TLC analysis.

2.5. Thin-Layer Chromatography

Experiments were performed on aluminum TLC silica gel plates 60 F₂₅₄ Merck (Darmstadt, Germany) pre-washed with methanol. Appropriate volumes of standard compound solutions were applied as dot spots on the TLC plate without any elution. Ethyl acetate crude extracts of mushrooms (50 μ g) were deposited onto TLC plates as bands of 8 mm in length, with a Camag (Muttens, Switzerland) Linomat 5 equipped with a 100 μ L Hamilton (Reno, NV, USA) syringe under a stream of nitrogen. The plate was then developed over 8 cm with methyl tert-butyl ether/tetrahydrofuran/cyclohexane (5:1:4 v/v/v).

2.6. Arachidonate 5-Lipoxygenase Inhibition Assay

After thorough drying to remove any residual solvent that can prevent chromogenic reaction, the TLC plates (10 × 20 cm) were uniformly sprayed with 2 mL of ALOX5 solution using a Camag Derivatizer. The plates were then placed in a Petri dish lined with moist filter paper and incubated at 37 °C in a Binder KB 23 incubator (Tuttlingen, Germany). Following a 5 min incubation, the chromogenic reaction was initiated by spraying 2 mL of arachidonic acid/MBTH/DMAB solution with the Derivatizer. After an additional 5 min, images of the TLC plates were captured under visible light using a Camag Reprostar 3.

2.7. Data Processing

To obtain semi-quantitative values during method development and assess the influence of experimental parameters on the contrast between inhibition zones and the colored background, plate images were analyzed using ImageJ 1.53k (National Institutes of Health, Bethesda, MD, USA). Photographs were first converted to 32-bit grayscale to standardize intensity measurements. This enabled the evaluation of background homogeneity and variations in intensity.

2.8. Optimization of Reagent Concentrations

To evaluate the effects of enzyme, substrate, and chromogenic reagent concentrations on the contrast obtained with ALOX5 inhibitors (zileuton, NDGA, and piperine), a two-level factorial design was implemented, resulting in eight experimental conditions, each performed in triplicate. The tested factors and levels were ALOX5 concentration (0.5 and 2 U/mL), arachidonic acid concentration (0.02 and 1 mM), and both DMAB and MBTH concentrations (0.1 and 1 mM), with the MBTH:DMAB ratio fixed at 1:1. Contrast values were analyzed using descriptive statistics, with a significance threshold of $\alpha = 0.05$, in MATLAB R2023b (MathWorks, Natick, MA, USA). Analysis of variance (ANOVA) was applied to assess the overall effect of experimental conditions, followed by Tukey's HSD post hoc test to identify pairwise differences between conditions.

2.9. Sensitivity and Linearity

The linearity and limit of detection (LOD) were assessed by applying decreasing amounts of inhibitors (ranging from 0.01 to 100 µg) using a Linomat 5 (Camag). Bioassays were conducted in triplicate without chromatographic elution. Regression equations were derived for each inhibitor, and the visual LOD was determined by independent observers.

3. Results and Discussion

3.1. Development and Validation of the TLC-Based Enzyme Assay

3.1.1. Specificity and Selectivity

In order to assess the suitability of the method for TLC bioautography, preliminary tests were performed in the absence of inhibitors to evaluate color development on silica gel plates and identify any potential interactions. The initial concentrations of enzyme and reagents were adapted from literature-reported conditions used in microplate assays, as follows: ALOX5 2 U/mL, AA 20 µM, MBTH 1 mM, and DMAB 10 mM. During the enzymatic reaction, MBTH was oxidized to an intermediate that condenses with DMAB to form an indamine dye (Figure 1).

Although this dye should exhibit an intense blue coloration in acidic aqueous solution, incubation on TLC plates resulted in a pinkish-beige coloration. This shift can be attributed to the markedly different environment on silica, where interactions with surface silanol groups and increased exposure to air could alter chromophore stability. While coloration was observed across all plates, the intensity was markedly higher on the plate

treated with the complete reaction mixture. These findings confirm the feasibility of the bioautographic approach.

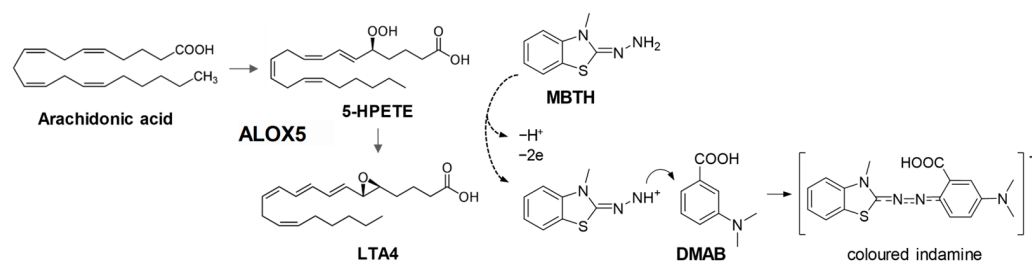


Figure 1. Chromogenic reaction of MBTH with DMAB during ALOX5 conversion of arachidonic acid into leukotriene A4 (LTA4), via 5-hydroperoxyeicosatetraenoic acid (5-HPETE).

Selectivity was subsequently evaluated using zileuton, NDGA, piperine, caffeine, and salicylic acid. Inhibition of ALOX5 by piperine has been previously reported with an IC_{50} value of 54 μM [17], whereas only a very weak effect was documented for salicylic acid (IC_{50} value of 168 μM) [18]; the lack of reported data for caffeine may suggest an absence of inhibitory activity. As expected, zileuton, NDGA, and piperine yielded distinct clear spots against the colored background, whereas caffeine and salicylic acid showed no effect. Zileuton and piperine produced uniform clear spots, while NDGA generated a halo-shaped spot with a darker core (Figure 2).



Figure 2. Preliminary ALOX5 TLC assay with 10 μg of (a) zileuton, (b) NDGA, and (c) piperine, used as positive controls, and (d) caffeine and (e) salicylic acid, used as negative controls.

3.1.2. Optimization of Experimental Parameters

• Heme cofactors

Our assay is based on the work of Anthon and Barrett, used for plant linoleate lipoxigenase [15], which itself builds upon the peroxidase assay described by Ngo and Lenhoff [14]. Anthon and Barrett added a heme cofactor (hemoglobin) in their chromogenic reaction, acting as a peroxidase-type catalyst, thus accelerating the oxidation of MBTH by lipid peroxide. We decided to run experiments, adding hemoglobin or hematin (100 μM) to the reaction mixture without any heme factor. Experiments using hemoglobin and hematin were not conclusive. Nebulization of hemoglobin in the derivatizer resulted in excessive foaming, which prevented uniform application onto the TLC plate. Although background staining was slightly increased in the presence of heme cofactors, the intrinsic coloration of both hemoglobin and hematin posed a significant limitation: it obscured the inhibition zones, thereby substantially diminishing their contrast and complicating result interpretation. Subsequent experiments were therefore carried out without any heme compounds.

• Incubation conditions

Regarding incubation, conditions were set at 37 $^{\circ}C$ in a moist atmosphere, with a pH of 7.5 (100 mM Tris-HCl buffer) for an optimized enzyme activity. During optimization steps, coloration and contrast were evaluated after 5, 10, 20, 30, 40, and 60 min of incubation, once the reaction mixture was applied. Five minutes of incubation were found to be sufficient to clearly detect an inhibition zone, with the highest contrast obtained between 5 and 10 min, and the coloration was relatively stable during 40 min (it has to be noted that the inhibition zone of NDGA turned darker than the background after 25 min).

- ATP and calcium

In vivo, the activity of ALOX5 is critically dependent on calcium ions and ATP: Ca^{2+} facilitates the enzyme translocation to the nuclear membrane while ATP stabilizes its active conformation [19]. Although these cofactors are not strictly required for assays using purified enzyme, some studies include ATP and Ca^{2+} in their in vitro assays. We investigated the influence of these two factors in our TLC bioassay and found that coloration—and thus contrast—was slightly enhanced in the presence of Ca^{2+} (5 μM) in the enzyme solution, whereas ATP had no observable effect.

- Reagent concentrations

Our optimization approach focused on three key parameters: enzyme concentration, substrate concentration, and chromogenic reagent concentration, as well as their respective ratios. According to Ngo and Lenhoff [14], a DMAB:MBTH ratio of 50:1 yields the most intense staining in a 96-well plate format, though this was established for fluorescence detection. In our bioassay, which is read under visible light, we determined that a DMAB:MBTH ratio of 1:1 produced more intense staining. Then, we employed a two-level factorial design, generating eight experimental conditions, each performed in triplicate. The tested factors and levels were as follows: ALOX5 concentration (0.5 and 2 U/mL), arachidonic acid concentration (0.02 and 1 mM), and DMAB and MBTH concentrations (0.1 and 1 mM), with the MBTH:DMAB ratio fixed at 1:1. Statistical analysis revealed that, while one-way ANOVA did not indicate a significant overall difference ($p = 0.083$), Tukey's multiple comparisons test identified certain conditions as more favorable. These conditions represented the best compromise among contrast, detection limit, and linearity, while minimizing the diffusion halo observed for NDGA. Results were validated through contrast quantification using ImageJ 1.53k Software.

- Final protocol

The optimized protocol involves pre-incubation of the developed TLC plate at 37 °C for 5 min in a moist atmosphere with 100 mM Tris-HCl buffer (pH 7.5) containing 2 U/mL of 5-lipoxygenase and 5 μM CaCl_2 . The reaction is initiated by spraying a reaction mixture containing 20 μM arachidonic acid, 0.1 mM MBTH, and 0.1 mM DMAB, followed by an additional 5 min incubation.

3.1.3. Limit of Detection and Linear Regression

The visual limit of detection (LOD) was determined for five compounds: zileuton, NDGA, piperine, uvaol, and betulin (Table 1). Zileuton exhibited a detectable inhibition zone at quantities as low as 1 μg , whereas the LOD of both nordihydroguaiaretic acid (NDGA) and piperine was determined at 0.01 μg . The limit of detection for the triterpene derivatives betulin and uvaol was 0.05 μg and 0.5 μg , respectively. These findings demonstrate that ALOX5 inhibitors can be visually detected below the microgram level according to their inhibitory potency.

Regression analyses were performed for each inhibitor, yielding good coefficients of determination ($R^2 = 0.98$ for zileuton, piperine and betulin). The signal–response relationships were shown to be compound-dependent; linear relationships were observed for zileuton, betulin, and uvaol, whereas NDGA and piperine exhibited logarithmic regression profiles (Table 1). The nonlinear responses may be attributed to diffusion effects or, in the case of piperine, to interference from its yellow coloration during luminance measurements. Even among compounds exhibiting linear equations, substantial differences in slope are observed. Consequently, it is important to note that comparison of inhibitory potency of different compounds based on the intensity of inhibition spots is inappropriate.

Table 1. Limit of detection (LOD), regression equation, and correlation coefficient of five ALOX5 inhibitors.

Compound	LOD (μg)	LOD (nmol)	Regression Equation	R ²
zileuton	1.00	4.23	$y = 2.4175x + 232.75$	0.98
NDGA	0.01	3.31	$y = -15.64 \ln(x) + 140.72$	0.97
piperine	0.01	1.65	$y = -10.01 \ln(x) + 176.94$	0.98
uvaol	0.50	1.13	$y = 0.3882x + 241.03$	0.91
betulin	0.05	0.226	$y = 0.0194x + 241.53$	0.98

3.2. Application to Natural Products

3.2.1. Application to Pure Compounds

A total of 40 natural products were evaluated for their ability to inhibit ALOX5 using the developed bioassay. The compounds were selected to represent a broad range of metabolite classes, including 13 phenolic compounds, 19 terpene and sterol derivatives, 4 alkaloids, 2 vitamins (ascorbic acid and α -tocopherol), caffeine, and sinigrin. Zileuton and NDGA were used as positive controls, while paracetamol, known to have no effect on ALOX5 activity, served as the negative control. Each compound (10 μg) was spotted onto the TLC plate without elution. After incubation, 21 of the 40 compounds induced inhibition zones (Table 2), more or less clearly defined (Figure A1); these slight diffusion effects—which did not affect screening—could be due to the physicochemical properties of compounds, affecting interactions with both the silica phase and the enzyme/reagent solutions.

Table 2. Evaluation of ALOX5 inhibitory activity of selected natural products using the developed TLC bioassay (for a tested quantity of 10 μg ; see Figure A1).

Compound	ALOX5 Inhibition ^a	Compound	ALOX5 Inhibition ^a
NDGA	+	lobeline	±
betulin	+	caffeine	—
digitoxin	+	anethol	—
ginsenoside Rd	+	eucalyptol	—
α -hederin	+	menthol	—
lupeol	+	α -tocopherol	—
madecassic acid	+	ascorbic acid	—
piperine	+	epigallocatechin	—
quinine	+	kampferol	—
senecionin	+	naringin	—
β -sitosterol	+	protocatechuic acid	—
stigmasterol	+	quercetin	—
tomatine	+	quercetol	—
uvaol	+	resveratrol	—
borneol	±	rutin	—
carvacrol	±	salicylic acid	—
caryophyllene	±	scopoletin	—
farnesene	±	taxifolin	—
humulene	±	tyrosol	—
santonin	±	sinigrin	—

^a (+) indicates a clearly visible inhibition zone, (±) indicates a diffuse inhibition zone, and (—) indicates no inhibition zone observed on the TLC plate after bioautography.

Steroid and triterpenoid derivatives produced clearly demarcated inhibition spots, with triterpenoids exhibiting particularly sharp and well-defined outlines. The activities observed for uvaol, madecassic acid, and α -hederin, are in line with previous reports: inhi-

bition of human ALOX5 by oleanane- and ursane-type triterpenoids has been described, predominantly for boswellic acid derivatives, and for uvaol, with an IC_{50} of 42 μM [20,21]. In our bioassay, lupeol, betulin, and ginsenoside Rd also produced detectable inhibition zones. Although in vitro inhibition of ALOX5 by lupane-type triterpenes has not been demonstrated, inhibition of other lipoxygenase isoforms suggests that the lupane scaffold can interact with lipoxygenase active sites, as exemplified by the activity of lupeol against soybean 15-LOX (IC_{50} 35 μM) [22]. Furthermore, the inhibitory activity of ginsenoside Rd was recently predicted using machine learning models [23]. This computational approach also unveiled the inhibitory potential of gitoxin, a cardenolide closely related to digitoxin, found to be active in our TLC bioassay [23]. Stigmasterol, β -sitosterol, and the steroidal glycoalkaloid tomatine also generated an inhibition zone on the plate, although no inhibition of ALOX5 activity by phytosterols has been reported to date. Alkaloids were also active, with piperine demonstrating the most pronounced inhibitory effect. While the activity of piperine and its derivative against ALOX5 is well known [17,24,25], information about the LOX inhibitory effect of quinine, senecionine, or lobeline is scarce.

Regarding mono- and sesquiterpenes, they yielded absent or poorly defined inhibition zones, which might be attributable to their volatile nature and evaporation during incubations. Carvacrol exhibited a large diffuse inhibition zone, in accordance with the literature [26]. No inhibition was observed for ascorbic acid and α -tocopherol; given that vitamins C and E are recognized as potent antioxidants, these findings further substantiate that antioxidant activity does not yield false-positive results in this assay, despite the oxidative nature of the enzymatic reaction.

Although most of our results are consistent with the literature, this was not the case for phenolic compounds, for which no inhibitory effect was observed. Because the yellow coloration of flavonoid derivatives could potentially interfere with the bioassay, luminance data were analyzed. These data showed a slight shift following the enzymatic reaction, indicating the formation of the colored reaction product and thereby confirming the absence of inhibition of ALOX5 activity. This finding contrasts with several reports describing ALOX5 inhibitory activity for polyphenols [27,28]. The discrepancy may be explained by the low amounts applied to the TLC plate, which were well below the reported IC_{50} values. For example, 10 μg of kaempferol corresponds to approximately 35 nmol, whereas its IC_{50} has been reported to be 20 μM [29]. These results demonstrate that the proposed bioautographic assay is applicable to a broad range of compounds. However, certain limitations may arise when analyzing highly pigmented substances, such as cyanidins or quinone derivatives, as their intrinsic coloration may interfere with detection. In such cases, the use of complementary or alternative methods should be considered.

3.2.2. Application to Mushroom Extracts

The TLC bioautographic assay was subsequently applied to complex crude natural extracts from mushrooms: 50 μg of ethyl acetate extracts from *Hebeloma* sp., *Xylaria polymorpha*, *Fomes fomentarius*, *Fomitopsis pinicola*, and *Fomitopsis betulina* were applied onto a silica TLC plate and eluted with an appropriate solvent system. After thorough drying to remove residual solvent, the bioassay was initiated. Following incubation, the plate developed a uniform pinkish-beige background, against which distinct inhibition zones were observed in all extracts except that of *F. fomentarius* (Figure 3). A single inhibition zone was detected for *X. polymorpha* (Rf 0.86), while the *Hebeloma* extract exhibited two zones (Rf 0.04 and 0.87). Extracts from *Fomitopsis* species displayed the highest diversity of ALOX5 inhibitors, with *F. pinicola* producing seven distinct inhibition zones most likely corresponding to at least seven different active compounds within the crude extract.

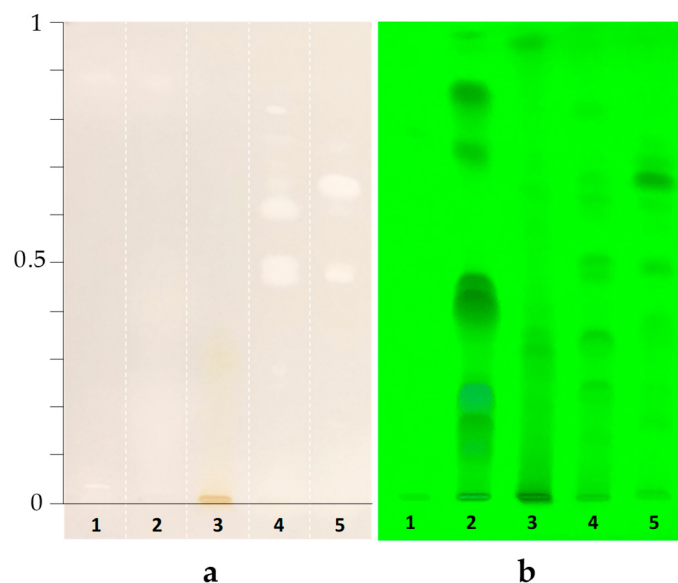


Figure 3. TLC of mushroom extracts (50 μ g); (a) after ALOX5 bioassay under visible light; (b): prior to bioassay at 254 nm; (1) *Hebeloma* sp., (2) *Xylaria polymorpha*, (3) *Fomes fomentarius*, (4) *Fomitopsis pinicola*, (5) *Fomitopsis betulina*.

Interestingly, we previously demonstrated the presence of COX-2 inhibitors in *Fomitopsis pinicola* using a TLC-based enzymatic assay developed by our group [11]. Comparison of the COX-2 and ALOX5 bioautograms revealed that several compounds act as dual COX/LOX inhibitors (Figure 4), a feature considered promising for the development of safer drug candidates [30–32]. This rapid insight was enabled by the chromatographic separation inherent to bioautography, which allows for a direct comparison of multiple biological activities without prior purification, thereby substantially accelerating the identification of compounds with complementary properties. One of these compounds was purified and identified as 3 α -acetylpolyporenic acid A, a lanostane-type triterpenoid previously isolated from *Fomitopsis betulina*, known to inhibit COX-1 and 3 α -hydroxysteroid dehydrogenase [16].

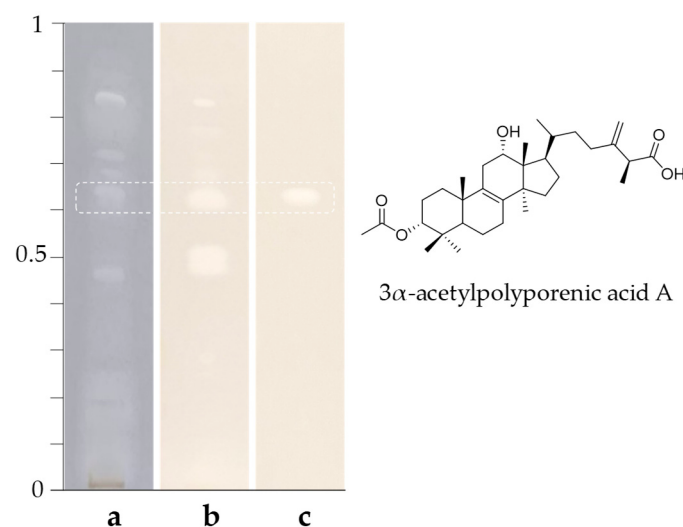


Figure 4. (a) COX-2 bioautogram of *F. pinicola* EtOAc extract (50 μ g) [11]; (b) ALOX5 bioautogram of *F. pinicola* EtOAc extract (50 μ g); (c) ALOX5 bioautogram of 3 α -acetylpolyporenic acid A (1 μ g). The dotted outline highlights the inhibition spots induced by 3 α -acetylpolyporenic acid A.

The other dual COX-2/ALOX5 inhibitors detected in *Fomitopsis* species have also been preliminarily characterized as triterpenoid derivatives, and more in-depth studies of their anti-inflammatory activity are currently underway. These results underline the anti-inflammatory potential of macromycete metabolites [33].

4. Conclusions

The developed ALOX5 TLC bioautographic assay demonstrates high sensitivity, enabling the detection of biologically active compounds even at low concentrations in crude extracts. Although commercial kits offer high sensitivity and allow for quantitative evaluation of inhibition relative to a control, they are not well suited for complex mixtures. In contrast, the present bioautographic assay offers several key advantages: it provides early insight into both the number and physicochemical nature of active constituents prior to fractionation, and it minimizes the influence of synergistic or antagonistic interactions that may obscure individual activities and lead to false-negative outcomes [34]. The assay is simple to implement, cost-effective, and well suited for preliminary high-throughput screening as well as for bioguided fractionation monitoring.

Moreover, the oxidative coupling reaction between MBTH and DMAB is not specific to ALOX5. It has previously been employed to investigate other lipoxygenases, including human ALOX15 and plant lipoxygenases [15,35], suggesting that the present assay could be extended to a broader range of lipoxygenase isoforms. In such applications, the choice of substrate should be adapted accordingly (e.g., arachidonic acid for human lipoxygenases and linoleic acid for plant lipoxygenases).

Finally, its compatibility with complementary bioautographic assays enables the parallel detection of compounds acting through multiple mechanisms. This feature facilitates and accelerates the discovery of novel anti-inflammatory agents, as demonstrated in this study by the rapid identification in a crude natural extract of a potential dual COX/LOX inhibitor.

Author Contributions: Conceptualization, A.U.; methodology, A.U.; validation, A.U. and Y.P.; formal analysis, Y.P., M.M. and A.U.; investigation, Y.P., M.M. and A.U.; data curation, A.U., Y.S., L.B., C.C. and V.B.; writing—review and editing, A.U., Y.S., L.B., C.C., V.B. and Y.P.; supervision, A.U.; project administration, A.U., L.B., C.C. and V.B.; funding acquisition, V.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: This work was supported by PiLeJe. We thank Antoine Mamet, training student, for his contribution to this project, Elodie Lepeltier for preliminary research and assays, and Claude Blondeau (PiLeJe) for proofreading.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AA	Arachidonic acid
ACN	Acetonitrile
ALOX5	Arachidonate 5-lipoxygenase
ATP	Adenosine triphosphate
COX	Cyclooxygenase
DMAB	3-(dimethylamino)benzoic acid

ELISA	enzyme-linked immunosorbent assay
EtOAc	Ethyl acetate
FOX	Ferrous-oxidized xylenol orange assay
5-HPETE	5-hydroperoxyeicosatetraenoic acid
HPLC	High-performance liquid chromatography
LTA4	Leukotriene A4
MBTH	3-methyl-2-benzothiazolinone hydrazone
NDGA	Nordihydroguaiaretic acid
SPE	Solid-phase extraction
TLC	Thin-layer chromatography

Appendix A

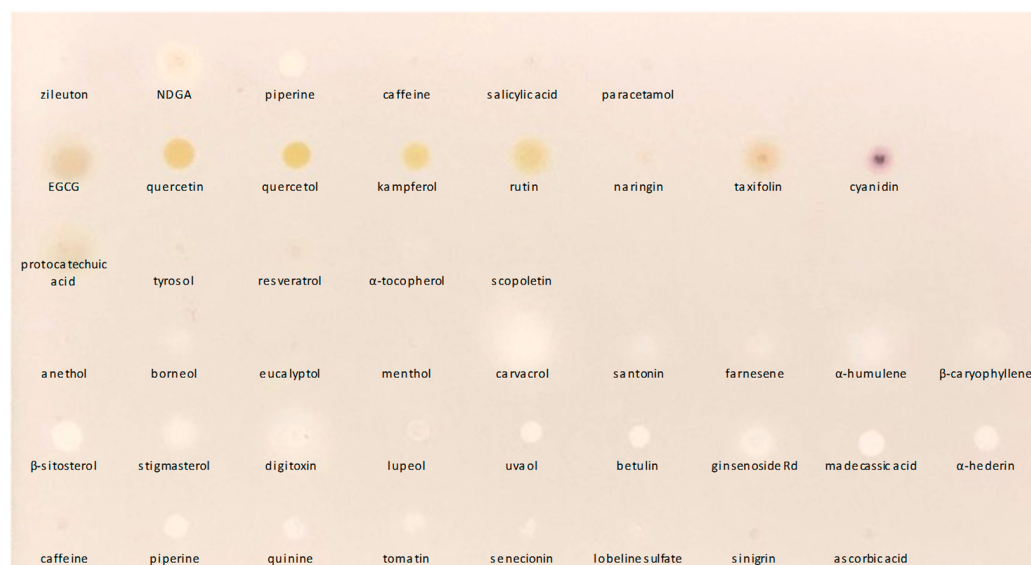


Figure A1. ALOX5 TLC bioassay of standard natural products (spotted at 10 µg).

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