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In Vitro Anti-inflammatory Activity of Larch (*Larix decidua* L.) Sawdust

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The influence of larch (*Larix decidua* L.) sawdust extracts on arachidonate metabolism has been evaluated in vitro. Extracts of different polarities were tested for their ability to inhibit prostaglandin formation by COX-1 and COX-2 and LTB_4 formation by 5-LOX. The lipophilic *n*-heptane extract showed the highest activity (IC_{50} values: COX-1, 5 μg/mL; COX-2, 0.1 μg/mL; LTB_4 assay, 11.1 μg/mL). A series of abietane, pimarane, and labdane type diterpenes isolated from this extract turned out to be potent inhibitors of LTB_4 product formation, whereas their COX inhibitory activity was less pronounced. From the abietane type diterpenes, compounds possessing a 7,13-abietadiene skeleton were better inhibitors of LTB_4 formation than those with an 8,11,13-abietatriene skeleton. Compounds bearing an OH group in position 4 were more active than those substituted with a COOH group, and methylation of the COOH group led to an almost complete loss of LTB_4 formation inhibitory activity.

KEYWORDS: ***Larix decidua*** **L.; larch sawdust; cyclooxygenase; lipoxygenase; anti-inflammatory; diterpene; abietane; labdane; pimarane**

INTRODUCTION

European larch (*Larix decidua* L., Pinaceae) is an up to 35 m high, deciduous tree that is mainly native to higher regions of the Alps, the Sudetes, and the Carpathian mountains (*1*). Larch wood is robust, waterproof, and durable, but also flexible in thin strips. The heartwood is particularly weather-proof and therefore mainly used as construction timber. Due to its high resin content and difficult machinability, its use for furniture production is limited. Larch sawdust is a byproduct of larch wood production. At present, larch sawdust is mainly used for producing pellet fuels.

Larch wood is known to contain phenolic compounds such as lignans and flavonols (mainly dihydroquercetin and dihydrokaempferol) (*1*). These compounds have been shown to possess antioxidant and anti-inflammatory effects (*2, 3*). Larch arabinogalactan, a water-soluble polysaccharide, which is predominantly extracted from the wood of *Larix occidentalis*, is approved by the U.S. FDA as a source of dietary fiber and also has potential therapeutic effects as an immunostimulant and cancer therapy adjunct (*4*). By drilling holes into the trunk of *L. decidua* trees, larch turpentine, also known as Venice turpentine, can be obtained. This product is composed of about 15% essential oil, 50−65% resin acids, and about 15% nonsaponifiable resin. The main constituent of the neutral resin fraction is the labdane type diterpene larixyl acetate (*2*), together with lower amounts of larixol (*1*) and 13-epimanool. Larixol and larixyl acetate are characteristic for larch resin as they have only been detected in *L. decidua* and *L. gmelinii* and as they have not been observed in any other genus. Furthermore, abietane and pimarane type diterpenes such as abietic acid, dehydroabietic acid, pimaric acid, and isopimaric acid are present (*1, 5, 6*). Larch turpentine has been traditionally used in external preparations for the treatment of rheumatic and neuralgic disorders and catarrhs of the respiratory tract (*1*).

Cyclooxygenases (COX) and 5-lipoxygenase (5-LOX) are key enzymes in arachidonate metabolism. The isoenzymes COX-1 and COX-2 catalyze the first two steps of the cyclooxygenase pathway of the arachidonic acid cascade, which leads to the production of prostaglandins. As COX-1 is constitutively expressed and COX-1 derived prostanoids play a protective role for the gastrointestinal mucosa, COX-1 is considered to possess housekeeping functions. COX-2 expression, however, is mainly induced by external stimuli. Therefore, inhibition of COX-1-derived prostanoid production has been considered to cause side effects of NSAIDs such as gastrointestinal bleeding and ulcers, whereas inhibition of COX-2 derived prostanoids has been considered to be responsible for their anti-inflammatory, analgesic, and antipyretic effects. Thus, selective COX-2 inhibitors have been preferred over nonselective ones to treat inflammation (*7*). However, several selective COX-2 inhibitors have been found to cause side effects as well, such as cardiovascular problems (*8*), and it was found that also COX-2 is expressed constitutively in some tissues (*9*). In the first step of the

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leukotriene pathway of arachidonate metabolism, 5-LOX catalyzes arachidonic acid oxygenation. Leukotrienes such as LTB_4 are potent mediators of inflammatory and allergic reactions. Therefore, 5-LOX inhibitors are considered to possess therapeutic potential in a range of allergic and inflammatory diseases (*7*).

In the search for new anti-inflammatory drugs, the simultaneous blockage of both the COX and 5-LOX pathways by dual inhibitors has been suggested as a possible alternative approach to selective COX-2 inhibition. Drugs possessing these characteristics are believed to be more effective, with lower gastric toxicity due to a stronger anti-inflammatory effect, on the one hand, and because, on the other hand, the enhanced conversion of arachidonic acid to leukotrienes, which is observed for drugs inhibiting only the COX pathway, is avoided (*10*).

As a part of the European research project SAFEWASTES (www.safewastes.info) that aims to discover bioactive constituents in organic byproduct from the food, agricultural, and pharmaceutical industries, the anti-inflammatory potential of larch sawdust was evaluated by investigating larch sawdust extracts and pure compounds isolated thereof for their COX-1, COX-2, and LTB_4 formation inhibitory activities in vitro.

MATERIALS AND METHODS

General Experimental Procedures. Analytical and semipreparative HPLC separations were performed on an Agilent 1100 series HPLC system. For preparative HPLC a Dynamax solvent delivery system and absorbance detector were used. NMR spectra were recorded with a Varian Unity Inova (600 MHz) spectrometer at 25 °C using the parameters described by Seebacher et al. (*11*). $CDCl_3$ was used as solvent and TMS as internal standard.

Plant Material and Extraction. Larch sawdust was kindly provided by Jannach Lärchenholz GmbH, Thalheim, Austria. A voucher specimen of the material (LD02RN090905) is deposited in the herbarium of the Department of Pharmacognosy in Graz.

For in vitro testing, three different extracts were prepared from powdered larch sawdust: one with *n*-heptane, one with 70% ethanol, and one with water. The powdered material was mixed with a 10-fold amount of solvent and stirred at room temperature for 2 h. After filtration, the extracts were concentrated at 40 °C under reduced pressure. The remaining *n*-heptane was removed under a stream of N_2, and the remaining water was removed by lyophilization. The following extract yields (% w/w of starting material) were obtained: water extract, 3.6%; 70% ethanol extract, 1.5%; and *n*-heptane extract, 0.5%.

In Vitro Assay for COX-1, COX-2, and LTB_4 Formation Inhibitory Activity. COX-1 and COX-2 inhibition assays were performed in a 96-well plate format with purified prostaglandin H synthase (PGHS)-1 from ram seminal vesicles for COX-1 and purified PGHS-2 from sheep placental cotyledons for COX-2 (both Cayman Chemical Co., Ann Arbor, MI) as previously described (*12*). The concentration of PGE_2, the main arachidonic acid metabolite in this reaction, was determined by a competitive PGE_2 EIA kit (Assay Designs Inc., Ann Arbor, MI). Indomethacin (ICN, Aurora, OH; IC_{50} COX-1 = 0.9 μM) and NS-398 (Cayman Chemical Co., IC_{50} COX-2 = 2.6 μM) were used as positive controls.

The bioassay for inhibition of 5-LOX-mediated LTB_4 formation was carried out in a 96-well plate format with stimulated human polymorphonuclear leukocytes as described earlier (*13*) with slight modifications (*14*). Zileuton (Sequoia, Oxford, U.K.; IC_{50} = 5.0 μM) was used as positive control.

Test samples were dissolved in absolute ethanol. Extracts were tested at a final concentration of 20 μg/mL in the assay mixture and pure compounds, at 50 μM. Samples were tested in at least three independent experiments run in duplicate. Results are given as means ± SD.

For IC_{50} determination, active samples were tested in at least three concentrations in at least three independent experiments, each time in duplicate. Calculation of IC_{50} values was performed by semilogarithmic presentation of dose versus activity and logarithmic regression analysis.

Isolation of Compounds 1−6 (Figure 1) from the *n*-Heptane Extract. Thirteen grams of *n*-heptane extract was subjected to VLC on silica gel (130 g, 0.04−0.063 mm, Merck, Darmstadt, Germany) with a stepwise gradient from 0 to 100% ethyl acetate in *n*-hexane, yielding 13 fractions. Fraction 6 (*n*-hexane/ethyl acetate 90:10, 950 mg) was further separated on a Sephadex LH20 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; 2.8 × 60 cm, mobile phase ethyl acetate), yielding fractions 6a−f. Thirty milligrams of fraction 6d (297 mg, elution volume = 67−112 mL) was purified by semipreparative HPLC using a Hypercarb column (10 × 150 mm, 7 μm; Thermo Electron Corp.) as stationary phase and MeCN/THF 95:5 (4 mL/min) as mobile phase. This purification step yielded compounds **3** (isopimaric acid, 2 mg) and **4** (palustric acid, 1.5 mg). Fraction 7 (*n*-hexane/ethyl acetate 85:15, 2.3 g) was separated on a Sephadex LH20 column (2.8 × 60 cm, mobile phase ethyl acetate), yielding fractions 7a−f. Seventy-five milligrams of fraction 7c (370 mg, elution volume = 320−340 mL) was separated by preparative HPLC using a LiChrosorb RP 18 (7 μm) LiChroCART 250-25 column (Merck), mobile phase MeCN/H_2O 65:35−90:10 in 30 min; 30−31 min, 90:10−100:0; 31−45 min, 100:0; 10 min re-equilibration; flow rate, 20 mL/min. The separation yielded compounds **2** (larixyl acetate, 30 mg) and **5** (dehydroabietic acid, 3 mg). Fraction 7d (230 mg, elution volume = 340−370 mL) was further purified on silica gel (1.8 × 26.5 cm, 0.04−0.063 mm, Merck) using a stepwise gradient of petroleum ether/acetone 95:5−80:20, yielding fractions $7d_1$− $7d_{10}$. Semipreparative HPLC of fraction $7d_6$ (petroleum ether/acetone 90:10, 60 mg) using a LiChrosorb RP 18 (7 μm) LiChroCART 250-10 column (mobile phase MeCN/H_2O 65:35−90:10 in 30 min; 30−31 min, 90:10−100:0; 31−45 min, 100:0; 10 min re-equilibration; flow rate, 2 mL/min) resulted in the isolation of compound **6** (dehydroabietinol, 1.5 mg). Purification of 25 mg of fraction 9 (*n*-hexane/ethyl acetate 70:30; 900 mg) using the same HPLC method led to the isolation of compound **1** (larixol, 2 mg). Structures were assigned by comparing their spectroscopic data with data from the literature (*15*−*18*).

Fatty Acid Analysis. Free fatty acids present in the active fractions were converted to their methyl esters as described earlier (*19*), and fatty acid methyl esters were identified by GC-MS analysis on a HP 6890 GC-MS system (Agilent Technologies, Waldbronn, Germany) equipped with a J&W Scientific DB 225 column (30 m, i.d. 0.25 mm, film = 0.25 μm; Agilent Technologies). Helium (0.8 mL/min) was used as a carrier gas, injector and detector temperatures were 220 °C, and the following temperature program was used: 0−0.5 min, 40 °C; 40−195 °C (25 °C/min); 195−202 °C (0.8 °C/min).

Preparation of Compounds 7−9. *Abietic Acid (7).* Compound **7** was purified by recrystallization from its dipentyl ammonium salt according to the method given in ref *20*.

Methyl Abietate (8). Compound **8** was prepared by esterification of compound **7** with $(CH_3)_3SiCHN_2$ according to the method given in ref *21*.

Abietinol (9). Five hundred milligrams (1.58 mmol) **8** was dissolved in 20 mL of dry Et_2O and added dropwise to a suspension of 96 mg (2.53 mmol) $LiAlH_4$ in 10 mL of dry Et_2O. After refluxing for 60 min, excessive hydride was destroyed by the addition of a few drops of water. The reaction mixture was diluted with 10 mL of Et_2O, washed with 2 N H_2SO_4 and brine, dried over Na_2SO_4, and evaporated. Column chromatography of the residue with CH/EtOAc (3:2) on silica gave **9** as a colorless crystallizing oil, 387 mg (85%). The recorded data are in accordance with those reported in ref *22*.

RESULTS AND DISCUSSION

Aqueous, 70% ethanolic, and *n*-heptane extracts prepared from larch sawdust were investigated for their inhibitory activity on prostaglandin formation by COX-1 and COX-2 and on LTB_4 formation by 5-LOX. As shown in **Figure 2**, the lipophilic *n*-heptane extract possessed pronounced inhibitory activity in all three assays at the screening concentration of 20 μg/mL. IC_{50} values for the *n*-heptane extract were found to be 5 μg/mL for COX-1, 0.1 μg/mL for COX-2, and 11.1 μg/mL for the LTB_4 assay. The 70% ethanolic and water extracts were less active.

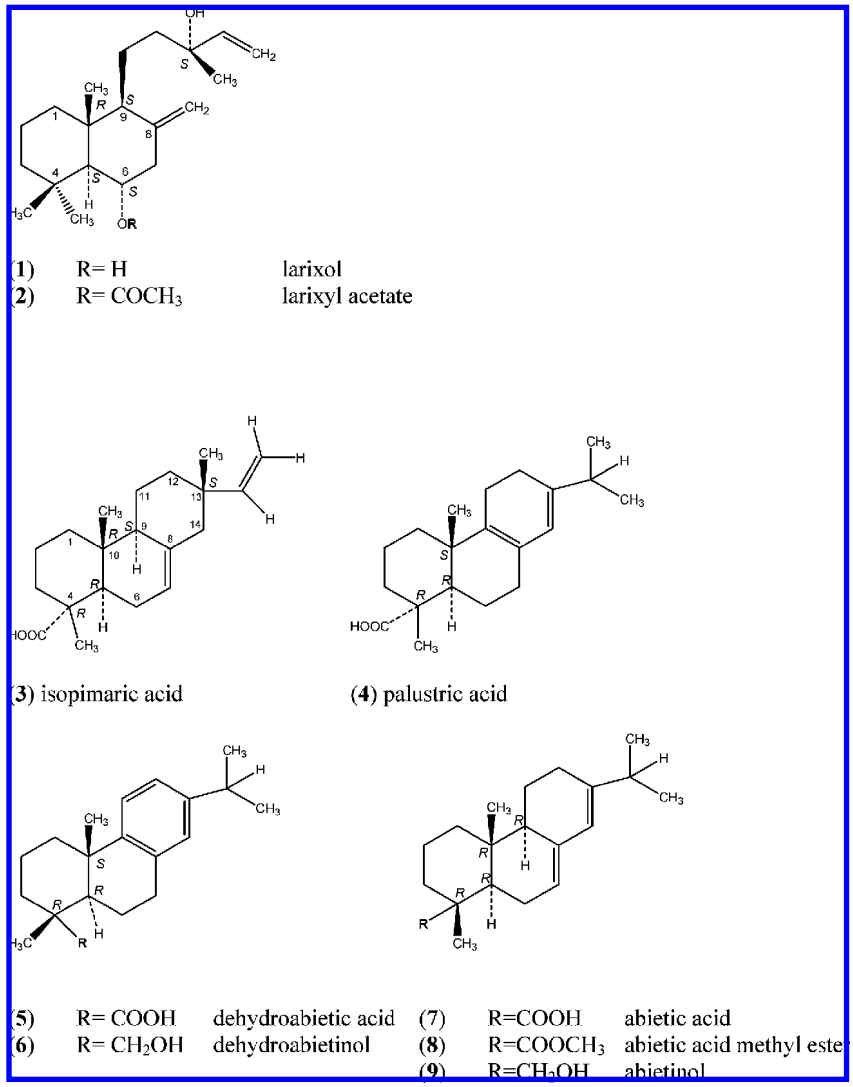

Figure 1. Compounds **1**−**9**.

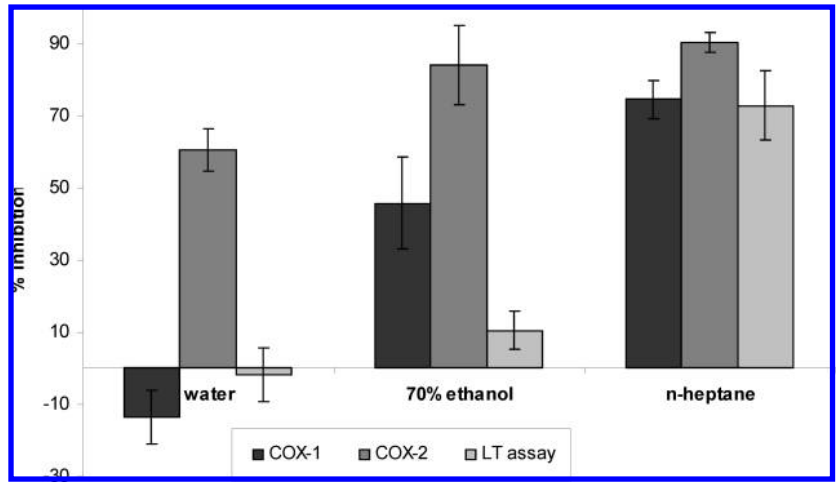

Figure 2. COX-1, COX-2, and LTB_4 formation inhibitory activities of larch sawdust water, 70% ethanol, and *n*-heptane extracts (screening concentration = 20 μg/mL in the assay mixture).

The IC_{50} of the 70% ethanolic extract against COX-2 was found to be 0.8 μg/mL; for the other assays IC_{50} values were not determined due to the low activity at the screening concentration.

Due to its potent dual inhibitory effect against COX isoenzymes with a certain COX-2 selectivity, on the one hand, and against LTB_4 product formation, on the other hand, the *n*-heptane extract was considered to be most interesting for further phytochemical and pharmacological evaluation to identify the active principles.

Fractionation of the *n*-heptane extract resulted in the isolation of a series of diterpenes (compounds **1**−**6**, **Figure 1**). The bioactivity of the compounds was evaluated in the COX-1, COX-2, and LTB_4 formation assays (**Figure 3**; **Table 1**). In contrast to the findings for the crude *n*-heptane extract, for which

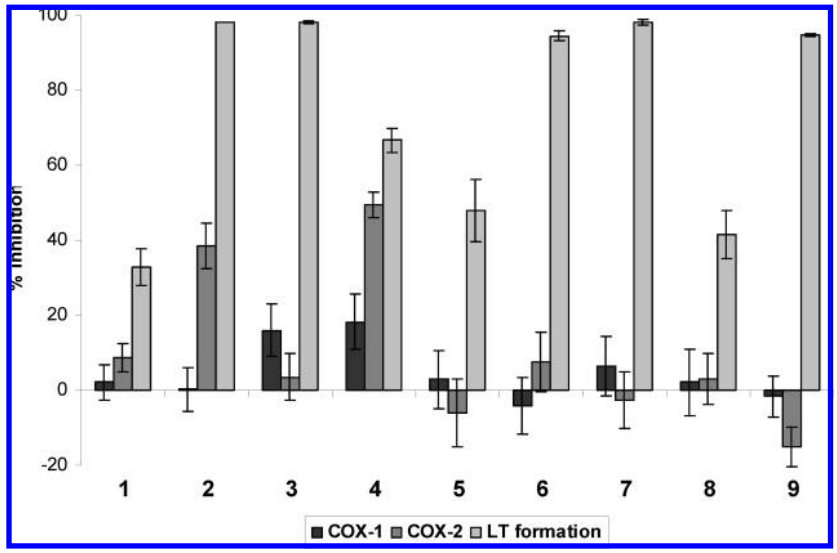

Figure 3. COX-1, COX-2, and LTB_4 formation inhibitory activities of compounds **1**−**9** at a screening concentration of 50 μM in the assay mixture.

Table 1. IC_{50} Values of the Active Diterpenes in the COX-2 and LTB_4 Formation Assays

compound	IC_{50} COX-2 assay (μM)	IC_{50} LT assay (μM)
larixol (**1**)		>125
larixyl acetate (**2**)	95.1	10.4
isopimaric acid (**3**)		10.2
palustric acid (**4**)	57.9	17.8
dehydroabietic acid (**5**)		44.6
dehydroabietinol (**6**)		10.9
abietic acid (**7**)		13.5
abietic acid methyl ester (**8**)		>125
abietinol (**9**)		5.9

a very high COX-2 inhibitory activity had been observed, the isolated diterpenes were mainly active in the LTB_4 formation assay. They did not inhibit COX-1, and only compound **2**, a major constituent of the *n*-heptane extract, and compound **4**, which is present only in minor amounts, were found to possess moderate COX-2 inhibitory activity. Their IC_{50} values were determined to be 95.1 and 57.9 μM, respectively. Therefore, compounds other than the isolated diterpenes must be responsible for the COX-1 and high COX-2 inhibitory activity of the crude *n*-heptane extract. One class of compounds that might contribute to this activity is unsaturated fatty acids. Fatty acid analysis of the fractions showing COX inhibitory activity after the first VLC fractionation step revealed that these fractions contained linoleic acid together with minor amounts of α-linolenic acid and oleic acid. These compounds have been shown to inhibit the COX isoenzymes as well as 5-LOX-mediated LTB_4 formation (*12, 19*), linoleic acid possessing a particularly high COX-2 inhibitory activity ($IC_{50} = 0.5$ μM) (*23*). Therefore, it can be concluded that not the isolated diterpenes but the unsaturated fatty acids present in the active fractions, probably together with other, yet unidentified constituents, account for the COX inhibitory activity of larch sawdust *n*-heptane extract.

However, some of the isolated diterpenes were found to be good inhibitors of 5-LOX-mediated LTB_4 formation. Larixol (**1**) and larixyl acetate (**2**) are neutral labdane type diterpenes, which differ only by the acetylation of the OH group in position 6. This acetylation seems to be necessary for LTB_4 formation inhibition as for larixyl acetate (**2**), a good inhibitory activity was observed, whereas larixol (**1**) was inactive in this assay.

Isopimaric acid (**3**), a pimarane type diterpene acid, was found to selectively inhibit LTB_4 formation with an IC_{50} similar to that of compound **2**. Concerning the abietane type diterpenes isolated from the extract, dehydroabietinol (**6**) was one of the most potent LTB_4 formation inhibitors with an IC_{50} similar to that of compounds **2** and **3**. Dehydroabietic acid (**5**), possessing a COOH instead of an OH group in position 4, was much less active. Palustric acid (**4**), lacking the double bond in position 11, showed intermediate LTB_4 formation inhibition and, in addition, moderately inhibited COX-2.

Diterpene acids from conifers are known to possess various bioactivities (for reviews see refs *24* and *25*): Several abietane acids display antimicrobial activity against a variety of microorganisms. Dehydroabietic acid derivatives have been shown to act as antiulcer agents (*26*), and some abietane acids also display cardiovascular effects. Also, some adverse effects of diterpene acids are known: resin acids present in pulp and paper mill effluents are known to be toxic for aquatic organisms such as fish (*27, 28*). The allergenic and antiallergic effects of resin acids are still a controversial subject; however, especially the oxidation products of resin acids seem to possess a dermal allergenic potential (*24, 29*).

Furthermore, some evidence exists concerning the anti-inflammatory potential of diterpene acids: A mixture of laevopimaric, abietic, neoabietic, palustric, and isopimaric acid in combination with triglycerides has been suggested for the external treatment of chronic diseases such as rheumatism and gout (*30*). Abietic acid, which is also known to be present in larch resin, has been described as an inhibitor of soybean 5-lipoxygenase (*31*). The compound also has been found to inhibit PGE_2 production in lipopolysaccharide-treated macrophages in vitro and to inhibit rat-paw and mouse-ear edema after oral or topical application in vivo (*32*).

Therefore, abietic acid (*7*) as well as its partial synthetically prepared derivatives abietic acid methyl ester (*8*) and abietinol (*9*) were tested for COX-1, COX-2, and LTB_4 formation inhibitory activities. Compound **9** was the most active of all tested compounds, possessing an IC_{50} of 5.9 μM, which is about the same as that of the positive control used in the assay (5 μM). The IC_{50} of compound **7** was in the range of that of compounds **2**, **3**, and **6**. Compound **8** was virtually inactive ($IC_{50} > 125$ μM).

In conclusion, all tested abietane type diterpenes were selective LTB_4 formation inhibitors, except for compound **4**.

Compounds possessing a 7,13-abietadiene skeleton were better LTB_4 formation inhibitors than those with an 8,11,13-abietatriene skeleton. Compound **4**, possessing an 8,13-abietadiene skeleton, showed intermediate LTB_4 formation and moderate COX-2 inhibitory activity. Compounds bearing an OH group in position 4 were more active than those substituted with a COOH group, and methylation of the COOH group led to an almost complete loss of LTB_4 formation inhibitory activity. The only pimarane type diterpene tested (**3**) was a selective LTB_4 formation inhibitor. Concerning the two labdane diterpenes tested, only larixyl acetate (**2**) was a good LTB_4 formation and a moderate COX-2 inhibitor, whereas larixol (**1**) was inactive in all three assays.

To be able to estimate the relevance of the bioactivities observed in vitro for an anti-inflammatory potential in humans, further studies will be necessary, as the pharmacokinetics of the investigated compounds are virtually unknown. One study has been performed concerning the biotransformation of dehydroabietic acid in rabbits after oral administration (*33*). Dehydroabietic acid also has been detected in the urine of humans after inhalation exposure to rosin-based soldering fluxes (*34*); however, no studies exist investigating the pharmacokinetics of resin acids in humans after oral administration, and the pharmacokinetics of larixol and larixyl acetate have not yet been studied as well. Apart from that, the toxic and allergenic potential of resin acids also has to be considered.

To summarize, the phytochemical and in vitro pharmacological investigation of larch sawdust led to several interesting results: On the one hand, a series of diterpene acids that selectively inhibit 5-LOX-mediated LTB_4 formation was isolated. These compounds represent lead structures for the development of leukotriene formation inhibitors. On the other hand, the *n*-heptane extract and (to a lesser extent) compounds **2** and **4** were found to act as dual inhibitors of COX- and of 5-LOX-mediated LTB_4 formation. Therefore, on the one hand, these compounds might be lead structures for dual COX/LOX inhibitors. On the other hand, the potent dual COX/LOX product formation inhibition observed for the lipophilic extract makes the further evaluation of larch sawdust and its lipophilic constituents as potential anti-inflammatory agents highly interesting. Further studies investigating the pharmacokinetics as well as the toxic and allergenic potential of the active constituents will be necessary.

ABBREVIATIONS USED

COX, cyclooxygenase; EtO_2, diethyl ether; EtOAc, ethyl acetate; LOX, lipoxygenase; LTB_4, leukotriene B_4; MeCN, acetonitrile; NSAID, nonsteroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; PGHS, prostaglandin H synthase; THF, tetrahydrofurane;VLC, vacuum liquid chromatography.

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