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Inhibition of Soybean and Potato Lipoxygenases by Bhilawanols from Bhilawan (*Semecarpus anacardium*) Nut Shell Liquid and Some Synthetic Salicylic Acid Analogues

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Bhilawanol diene (3) isolated from bhilawan nut shell liquid was found to be a potent inhibitor of both soybean and potato lipoxygenases with IC_{50} values of 0.85 μM and 1.1 μM, respectively. However, the monoene (2) and saturated (1) bhilawanols exhibited relatively lower inhibitory activity. In addition, inhibition studies with synthetic analogues of salicylic acid (4–8) suggested that the unsaturated lipophilic side chain may be an absolute requirement for inhibitory activity.

Keywords: Bhilawanols; *Semecarpus anacardium*; Soybean lipoxygenase; Potato lipoxygenase; Inhibition; Urushiol

INTRODUCTION

Lipoxygenases or linoleic acid dioxygenases (EC 1.13.11.12) are the enzymes that catalyze the dioxygenation (hydroperoxidation) of 1Z, 4Z-diene systems using molecular oxygen.[1] The cis form of the diene is generally found to be the substrate requirement for the enzyme to act,[2] though some anomalies are known.[3,4] Fatty acids such as linoleic acid, and arachidonic acid are well known substrates for this enzyme.[5] Even though lipoxygenases are known to exist in different isozyme forms, each having its own specificity,[6] inhibition studies are concentrated on the lipoxygenase-1 from soybean (soy. Lox. 1) and potato lipoxygenase (pot. Lox.). Lipoxygenases from soybean and potato have some degree of similarity, but the potato lipoxygenase is found to have more resemblance to the human lipoxygenase.

Recently, research has been focussed on finding a potent inhibitor for lipoxygenase for medicinal use.[1,7] Notably, some naturally occurring compounds possessing certain structural requirements to act as enzyme substrates. The first naturally occurring lipophilic phenol derivative serving as a substrate was anacardic acid diene (**11**, 39% as active as linoleic acid) from Cashewnut shell liquid (CNSL).[8] In contrast, saturated (**9**, IC_{50} 85 μM), monoene (**10**, IC_{50} 50 μM) and triene (**12**, IC_{50} >250 μM) derivatives of anacardic acid showed inhibitory activity.[9] Furthermore, a few naturally occurring lipophilic phenolic acids and their derivatives were evaluated for their inhibitory action against lipoxygenases.[10] In a previous study, catechol has been shown to be an inhibitor of soybean lipoxygenase with an IC_{50} of 62 μM.[11] Interestingly, introduction of an undecyl chain to catechol enhances its inhibitory activity. Compounds with minor structural differences to lipophilic catechols have shown (cardols from CNSL, **13–16,** IC_{50} of saturated and monoene >250 μM, diene 125 μM, triene 100 μM) reasonably good inhibition of lipoxygenases.[9]

In the light of these observations we checked the inhibitory activity of bhilawanols, isolated from the nut shell of *Semecarpus anacardium* Linn. Bhilawanol is a mixture of 3-pentadec(en)yl catechols unsaturated in the lipophilic side chain. The chief

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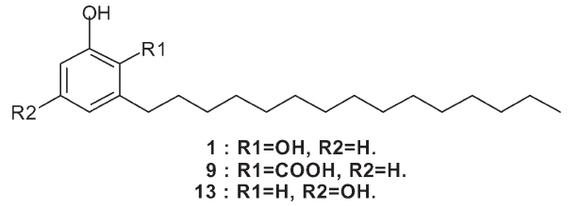
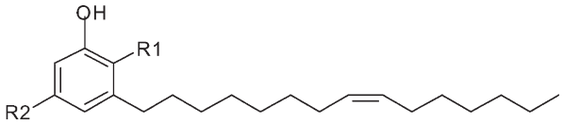
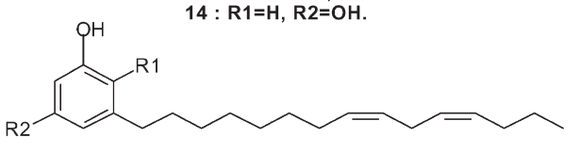
components of bhilawanol are the 8Z, 11Z-diene and 8Z-monoene along with minor amounts of saturated bhilawanol (**1**–**3**).[12]



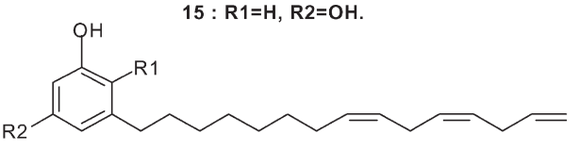
1 : R1=OH, R2=H.
9 : R1=COOH, R2=H.
13 : R1=H, R2=OH.



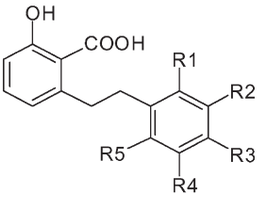
2 : R1=OH, R2=H.
10 : R1=COOH, R2=H.
14 : R1=H, R2=OH.



3 : R1=OH, R2=H.
11 : R1=COOH, R2=H.
15 : R1=H, R2=OH.



12 : R1=COOH, R2=H.
16 : R1=H, R2=OH.



4 : R1, R4, R5 = OH, R2, R3 = H.
5 : R1, R4 = OH, R2, R3, R5 = H.
6 : R1, R3 = OH, R2, R4, R5 = H.
7 : R2, R3 = OH, R1, R4, R5 = H.
8 : R3 = OH, R1, R2, R4, R5 = H.

Though these three compounds(**1**–**3**) in *Semecarpus anacardium* are identical to the three isomers of Urushiol from *Rhus Vernicifera*,[13] they have retained their identity in the literature. Isolation and the presence of other positional isomers of bhilawanol diene lead bhilawanols to possess different physical and chemical properties to those of urushiols. Also, both these class of compounds are utilized for entirely different purposes in the country of their origin. Even when bhilawanols are considered very toxic, treated bhilawan nuts and the milk extract of the nuts find application in Indian medicinal system for curing more than 50 dreaded diseases.[14,15] However, to our knowledge, there are no reports of urushiol having been tested for lipoxygenase inhibition.

MATERIALS AND METHODS

Semecarpus anacardium nuts were purchased from the local market and compared with an authentic sample from the Arya Vaidya Sala, Kottakkal, Kerala, India. All the solvents were purchased from Merck, India and were distilled before use. The buffer components such as boric acid, tetrasodium borate, sodium dihydrogen phosphate, disodium hydrogen phosphate were purchased from Qualigen, India.

Enzyme Purification

The procedure followed for the purification of soybean lipoxygenase was reported earlier.[17] The enzyme preparation containing 0.3 mg/ml protein with specific activity (towards linoleic acid) of 60 μmol/min/mg protein was used in the study. Potato lipoxygenase was purified using the method essentially similar to that used for soybean lipoxygenase-1. The enzyme had a protein concentration of 15 mg/ml and a specific activity (towards linoleic acid) of 36 μmol/min/mg protein. Enzyme assay reactions were monitored at 234 nm after starting the reaction with the addition of 0.1 mM linoleic acid.

Assay Method

All the reactions were carried out at 25°C in 0.2 M borate buffer (pH 9.0) containing 0.1 mM linoleic acid dispersed in Tween 20 for soybean lipoxygenase and in 0.2 M phosphate buffer (pH 6.8) containing 0.1 mM linoleic acid dispersed in Tween 20 for potato lipoxygenase. For inhibition studies, 5 μl of an ethanol solution of the test compound was added to 5 μl of suitably diluted (1:200) enzyme (and 980 μl suitable buffer solution) and incubated for 2 min on ice and then brought to room temperature. As a control experiment, 5 μl of ethanol was added to 5 μl of the enzyme solution with buffer solution and incubated for 2 min on ice and then brought to room temperature. Total volume of the assay mixture was 1 ml. The enzyme reaction was started by the addition of substrate (10μl) and production of diene hydroperoxide was monitored on a Shimatzu 2100 double beam spectrophotometer. The concentration (μM) of the test compound required for 50% decrease in velocity in the reaction are given as IC_{50} values. All values reported are from averaged triplicated experiments.

Isolation and Characterization of Bhilawanol Enes

Bhilawanol (mixture of enes) was purified from bhilawan nut shell liquid by column chromatography. The shell, after removing the kernel (70 g), was extracted with dichloromethane and the extract was evaporated *in vacuo* to give a dark liquid (33.2 g). A portion of this (10 g) was subjected to column chromatography on silica gel 60 (100–200 mesh size, 100 g) using ethyl acetate–hexane (1: 4) as mobile phase and bhilawanol ene mixture was obtained (5.73 g) as one of the major fractions.

This mixture of enomers was subjected to preparative reverse phase high performance liquid chromatography (prep HPLC) on a Waters (Waters associates, Milford, MA) HPLC instrument with a UV detector monitored at 280 nm, connected to a Waters Bondapak C18 column (40 × 100 mm, particle size 15–20 μM and pore size 300 Å). A ternary mixture of acetonitrile, water and acetic acid in the ratio 66:33:1 (v/v/v) at a flow rate of 30 ml/min was used as mobile phase. Individual peaks were separately collected, and the solvent evaporated under reduced pressure to isolate the compounds. Analytical HPLC of the individual compounds were performed using a Novapak C18 column (4 × 150 mm) with a mobile phase of 80% aqueous acetonitrile (v/v) containing 1% acetic acid at a flow of 1.8 ml/min. Since the saturated bhilwanol occurs in low concentration in nature, a portion of bhilawanol ene mixture (50 mg) was subjected to catalytic hydrogenation using 10% Pd-C catalyst to isolate the saturated bhilawanol (M.p. 57°C, lit. 57–58°C) with Mass spectral (m/z) splitting pattern 320 (M^+), 207, 179, 123 (100%), 55 in quantitative yields. Analytical chromatography for the individual components obtained gave retentions (R_f) of 4.23 (diene), 6.90 (monoene) and 13.59 min. (saturated), and all the components were >95% pure.

Synthesis of Compounds 4–8

Compounds **4–8** were synthesised using a simple general procedure of treating the ethyl 6-chloromethyl-2-methoxybenzoate with triethylphosphite and treating the resulting phosphonate with the corresponding methoxy benzaldehydes using Horner–Wittig reaction conditions followed by catalytic hydrogenation and demethylation. The overall yields of compounds **4–8** were approximately 50%. The synthetic protocols for these compounds have been discussed elsewhere.[16]

Preparation of Stock Solution

Bhilawanol enes obtained by chromatography were dissolved in alcohol to give 10 mM stock solutions. Since 5 μl of the solution was used in the assay, the highest concentration was 50μM. Upon suitable dilution, final concentrations from 0.15625–50 μM of the test solutions were obtained. In the case of synthetic compounds, higher concentrations of the compounds were used as indicated.

RESULTS AND DISCUSSION

Among the compounds from *Semecarpus anacardium* species, bhilawanol diene (**3**) was the most potent, having an IC_{50} value of 0.85 μM (Figure 1) for the soybean lipoxygenase and 1.1 μM for the potato lipoxygenase (Figure 1). In the case of soybean lipoxygenase, the diene inhibited the enzyme to about 90% at a concentration of 20 μM whereas at a concentration of 10 μM a similar inhibition of potato lipoxygenase was observed.

Monoene, **2** was less potent than the diene with IC_{50} values of 10 μM and 14.5 μM for soybean lipoxygenase and potato lipoxygenase respectively (Figure 2). The saturated bhilawanol, **1** was least

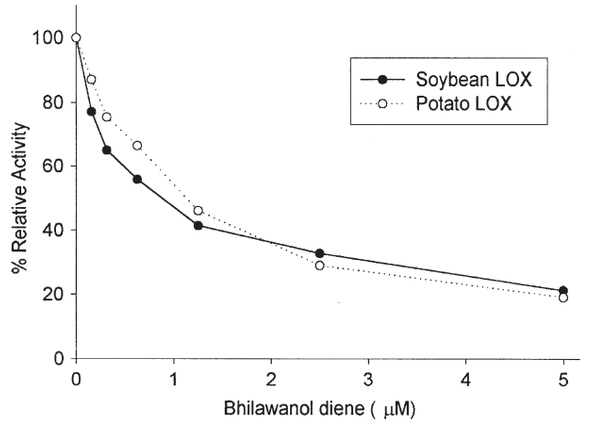


FIGURE 1 Inhibition of soybean and potato lipoxygenases by bhilawanol diene.

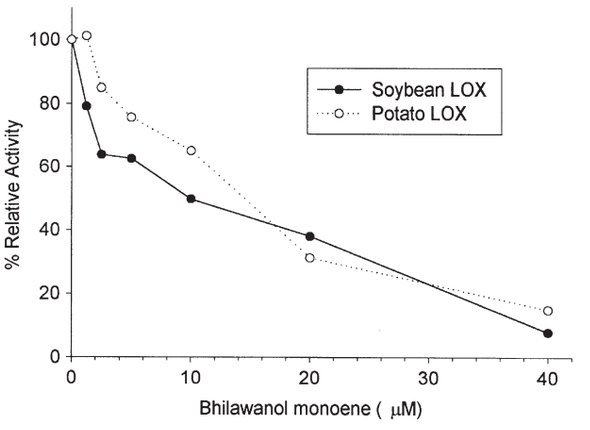


FIGURE 2 Inhibition of soybean and potato lipoxygenases by bhilawanol monoene.

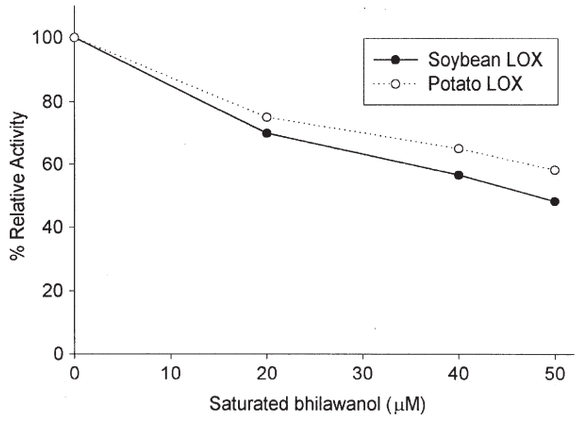


FIGURE 3 Inhibition of soybean and potato lipoxygenases by saturated bhilawanol.

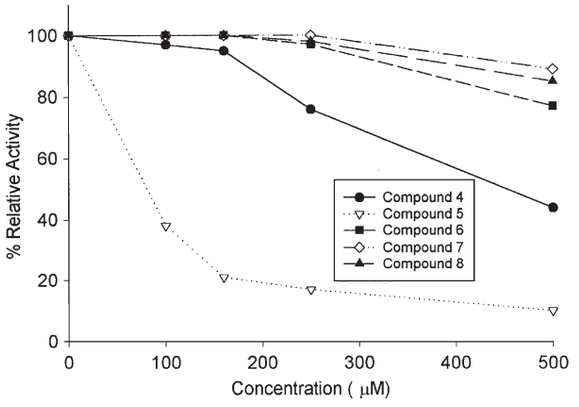


FIGURE 4 Inhibition of potato lipoxygenase by synthetic salicylic acid analogues.

effective as an inhibitor from the species with IC_{50} of 48.5 μM for the soybean lipoxygenase while the IC_{50} for potato lipoxygenase could not be determined due to turbidity in the enzyme assay mixture at concentrations higher than 50 μM (Figure 3).

However, from Figure 3 it is evident that the IC_{50} of saturated bhilawanol for the potato enzyme is close to 50 μM. This trend is similar to the one shown by cardols from CNSL wherein the inhibitory potency increases with increase in unsaturation in the side chain of the molecule.[9]

It has been well known that lipoxygenases contain iron, and the metal ion is essential for enzyme activity. Also, the iron in the Fe (II) oxidation level has to be oxidised to the Fe (III) form in order to activate the enzyme (pink) from its inactive (yellow) form.[6] Previously, inhibition of lipoxygenase has been attributed to metal chelation by the inhibitors.[9] Any molecule either inhibiting the oxidation of the metal or chelating it irreversibly could be considered as an enzyme inhibitor. If this is so, bhilawanols should act as good inhibitors since they can function both as a chelator of metals and as an antioxidant and thus should be more potent than most of the naturally occurring long chain phenols studied so far. Results obtained here strongly suggested that our assumption regarding bhilawanols being good inhibitors was true. However, further experiments involving some synthetic analogues of salicylic acid proved to be useful in ascertaining the structural units responsible for enzyme inhibition. Salicylic acid analogues with different phenolic substituents were synthesised[16] and were checked for their activity against potato lipoxygenase. For this reason, we chose five compounds (compounds **4**–**8**) having salicylic acid (the functional unit in anacardic acid) along with the two-carbon side chain linked to a phenol, catechol, resorcinol, quinol or a trihydroxy benzene unit. Of these five synthetic model compounds, compound **5** showed the best inhibition activity against potato lipoxygenase. Even when analogue **5** acted as the most effective of the synthetic inhibitors tested, the inhibitor concentration was much higher than that observed with the bhilawanols. Since none of the synthetic model compounds showed any significant inhibition in the concentration range of 50 μM, a concentration as high as 500 μM was used. Only compound **5** showed 80% inhibition at that concentration, while compound **4** exhibited about 60% inhibition (Figure 4).

This suggests that the lipophilic chain plays a major role in lipoxygenase enzyme inhibition as evidenced in the literature. In this regard, a study on lipoxygenase inhibition by bhilawanols is of some biological significance. The powerful inhibitory activities of these lipophilic phenols are one of the many biological and medicinal properties exhibited by bhilawanols at such low concentrations.

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