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Lupenone Isolated from *Diospyros melanoxylon* Bark Non-competitively Inhibits α-amylase Activity

Mohan Krishna Durgam[1], Praveen Kumar Vemuri[2], Vijaya Lakshmi Bodiga[3], Sreedhar Bodiga[1,*]
[1]Laboratory of Biochemistry, Department of Basic Sciences, Forest College and Research Institute Hyderabad, Mulugu, Telangana, India
[2]Department of Biotechnology, Koneru Lakshmaiah University, Green Fields, Vaddeswaram, Guntur, Andhra Pradesh, India
[3]Department of Biochemistry and Molecular Biology, Institute of Genetics & Hospital for Genetic Diseases, Osmania University, Begumpet, Hyderabad, Telangana, India.

Corresponding author*
sbodiga@gmail.com

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Abstract

Diabetes mellitus is a chronic disease that poses a serious global health problem, due to its associated effects on obesity and aging. Therapeutic strategies for targeting diabetes include the downregulation and/or inhibition of enzymes such as α-amylase and α-glucosidase, hydrolyzing the dietary carbohydrates in intestine. There is increasing interest for α-amylase inhibitors from natural sources. Our objective was to undertake the phytochemical screening of bark extracts of *Diospyros melanoxylon* for potential α-amylase inhibitory activity and further identification of the active principle and the underlying mechanisms of inhibition. Enzyme-assay guided fractionation of the *Diospyros melanoxylon* bark extract led to the isolation of a triterpene, Lupenone as a potential inhibitor of α-amylase, with a non-competitive inhibition and inhibitor constant = 30 μM. Lupenone-mediated inhibition of α-amylase responsible for the breakdown of dietary sugar may be effective in preventing postprandial hyperglycemia in the diabetic subjects.

Keywords: Diospyros melanoxylon; Ebenaceae; Triterpenoid ketone; pancreatic amylase; non-competitive inhibition.

INTRODUCTION

Diabetes is a chronic disease of multiple etiologies characterized by chronic hyperglycemia with alterations in carbohydrate, fat and protein metabolism. This disorder affects various organs, including eyes, kidneys, nerves, heart and blood vessels. The prevalence of diabetes in India is exploding and 2017 estimates by the International Diabetes Foundation indicate that 72.9 million people are affected by diabetes in India. Type 2 diabetes population in India has a high burden (76.6%) of poor glycemic control(Borgharkar & Das, 2019). Inhibition of intestinal absorption and digestion of carbohydrates targeting the carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase is an attractive strategy (Matsui, Ogunwande, Abesundara, & Matsumoto, 2006; Tundis, Loizzo, & Menichini, 2010). Research efforts directed towards screening and developing natural compounds with potential anti-diabetic properties is on the rise.

α-amylase recognizes a consecutive glucose chain as a substrate using its subsite (Brayer et al., 2000). Acarbose, a typical α-amylase inhibitor, has a strong affinity for the enzyme due to pseudo-tetrasaccharide structure (C. Li et al., 2005). Most of the small molecule inhibitors from natural sources against α-amylase include polyphenols with low enzyme specificity (Kim, Kwon, & Son, 2000; H. Li, Tanaka, Zhang, Yang, & Kouno, 2007; Lo Piparo et al., 2008; McDougall et al., 2005). A triterpene glycoside is reported as a specific inhibitor (Tarling et al., 2008), and a simple low-polar ketone, chalcone was also found to exhibit α-amylase inhibitory activity (Najafian et al., 2011). Natural triterpenoids have wide spectrum of biological activities (Siddique & Saleem, 2011). Most triterpenes consist of 30 carbon atoms and can be regarded as a compound of 6 isoprene structural units and distributed in plants with both monocotyledons and dicotyledons. Two main classes containing either tetracyclic or pentacyclic triterpenes exist. Lupene is the most basic structure of lupane-type pentacyclic triterpienoid. It contains A, B, C and D 6-carbon rings and a pentacyclic E ring with an isopropryl group at the 19th position. Lupenone is a typical polar lupine type triterpenoid, and it has a ketone group at position 3 in the nuclear ring (MENG, HUANG, & LIU, 2008). Lupenone is a secondary metabolite in many plants (Lee & Lee, 1999; Na, Kim, Osada, & Ahn, 2009) and possess anti-inflammatory, anti-diabetic, and anti-tumor activity(Wang, Liu, Wang, & Zhong, 2012; Xu et al., 2014).

In this paper, we present the results of a study on α-amylase inhibition and identification of the active

principle from the extract of leaves of *D. Melanoxylon*. *Disopyros melanoxylon Roxb (Family: Ebenaceae)*, the Coromandel ebony or East Indian ebony (Tendu in Hindi), is a middle sized deciduous tree or shrub native to India and Srilanka. This plant is considered to be a minor forest produce (MFP) in India, as its leaves are used for making bidi, a traditional cigarette.

The leaves are arranged opposite, mostly subopposite or alternate, coriaceous, up to 35 cm long with most of them between 6 and 15 cm; tomentose on both sides when young but when full grown glabrous above, tomentose or pubescent beneath, parrot green; petiolate, with petiole up to 1 cm long; exstipulate, simple and entire; secondary nerve 6-10 pairs, often irregular and branching; tertiary nerves reticulate and raised on upper side; shape variable in the same plant but predominantly of one type out of the four basic forms, namely ellipsoidally lanceolate, ovate, elliptic and orbicular (sometimes cuneate). The bark is pelican in colour, exfoliating in rectangular scales. The wood is also utilized for making boxes, combs, ploughs and beams. The fruits are eaten and sold commercially. The bark is burnt by tribals to "cure" small-pox. The seeds are prescribed as cure for mental disorders, palpitation of heart and nervous breakdown (J. Rathore, 1972). Recent studies have reported anti-adipogenic, anti-diabetic and hypolipidemic pharmacological activities of *D.melanoxylon* leaf extracts (K. Rathore et al., 2014). However, the phytochemicals responsible for the observed effects have not been elucidated.

MATERIALS AND METHODS

Materials

Porcine Pancreatic α-Amylase (PPA) was obtained from Sigma Aldrich, USA. Ethyl acetate, methanol, benzene, n-hexane were obtained from Merck Chemicals Ltd. Acarbose was from Bayer Pharmaceuticals Pvt Ltd. Soluble starch (extra pure) was obtained from HiMedia Laboratories. All the chemicals and reagents procured were of AR grade.

Extraction and isolation of inhibitor from the bark of *D. melanoxylon*

The barks of *D. melanoxylon* were collected from Bhadrachalam district of Telangana. The plant was authenticated by Prof. M. Mamatha from the Forest College and Research Institute, Mulugu. A digital specimen voucher was deposited in the herbarium of FCRI. The bark was shade dried, cut into small pieces, and powdered in a pulverizer. The powdered bark (1.5 kg) of *D. melanoxylon* was extracted with ethanol (3 x 18 L) at room temperature for 24 h. The extract was filtered and concentrated *in vacuo*, suitably diluted with water, then partitioned with dichloromethane (3 x 1.5 L), ethyl acetate (3 x 1.5 L) and n-butanol (3 x 1.5 L), successively. Column chromatography of the dichloromethane-soluble layer (20 g) over silica gel using *n*-hexane-acetone mixture with increasing polarity yielded 15 fractions. Fraction 2 (2.0 g) that exhibited PPA-inhibition was further applied to a flash column chromatography with RP-18 using methanol/water (30:70 to 80:20) yielded four fractions. PPA-inhibitor was isolated form fraction 4 by silica gel column chromatography eluting with dichloromethane-acetone gradient (1:0, 50:1, 20:1, 10:1, 5:1, acetone). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker ADVANCE III 500 MHz NMR spectrometer using $CDCl_3$ as a solvent. Mass spectra were obtained using a Waters Q-TOF micro mass spectrometer. Spectral analysis indicated that the isolated compound is lupenone.

α-amylase inhibitory activity

α-amylase inhibition assay was carried out according to the method of Sudha et al. (2011) based on the starch-iodine test with little modification (Sudha, Zinjarde, Bhargava, & Kumar, 2011). 75 µl of different fractions (100-500 mg/ml) were added to 150 µl 0.02 M sodium phosphate buffer (pH 6.9 containing 6 mM sodium chloride) and 75 µl α-amylase solution, and incubated at 37°C for 15 min. Subsequently, 100 µl from each sample reaction solution was taken and added to 750 µl soluble starch (1%, w/v). 500 µl phosphate buffer solution was added and incubated at 37 °C for 45 min. 25 µl of the above mixture was taken and added to 2.5 ml of iodine reagent (5 mM I_2 and 5 mM KI) and mixed thoroughly. The color change was observed and the absorbance was taken at 565 nm. Control sample was without any inhibitor representing 100% enzymatic activity. To eliminate the absorbance produced by plant fractions, appropriate fraction controls without the enzyme were also included. The standard drug acarbose (α-amylase inhibitor) was used as a positive control. It is observed the dark-blue color which indicates the presence of starch, a brownish color indicates partially degraded starch and a yellow color indicates the absence of starch in the reaction mixture. In the presence of inhibitors from the fraction the starch added to the enzyme assay mixture is not degraded and gives a dark blue color complex whereas no color complex is developed in the absence of the inhibitor, indicating that starch is completely hydrolyzed by α-amylase. The percentage inhibition of α-amylase was calculated using the following formula:

$$\%\ inhibition = \frac{(Absorbance\ of\ control - Absorbance\ of\ test)}{Absorbance\ of\ Control} * 100$$

Statistical analysis: Data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using two-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Bioassay-guided fractionation yielded a potential inhibitor against porcine pancreatic α-amylase, which was obtained as a white amorphous powder, with a melting point of 168-170°C.

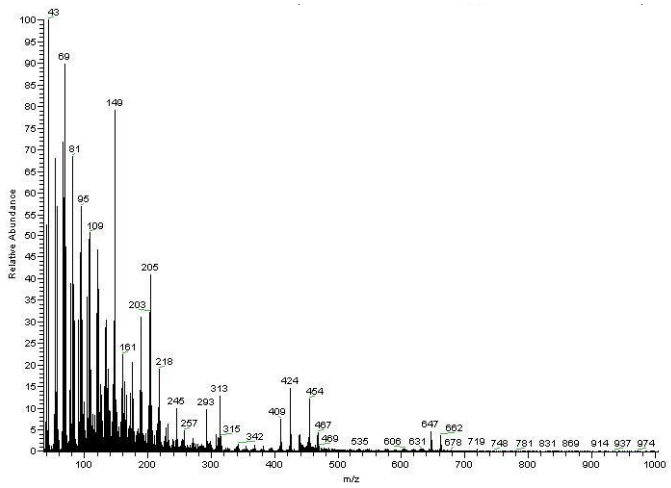

Figure 1. EI-MS spectrum of the isolated inhibitor.

The mass spectrum of the isolated inhibitor as shown in Figure 1exhibited a molecular ion peak $[M]^+$ at *m/z* 424, corresponding to a molecular formula C30H48O. Fragments at *m/z* 203 and 218, characteristic of pentacyclic triterpenes were also present in the mass spectrum of compound. The mass spectrometry data also suggested the presence of a carbonyl group (fragment at *m/z* 205) and the accurate mass of this fragment indicated that it was attached to rings A or B of the pentacyclic ring. The fragment at 409 corresponding to $[M\text{-}CH3]^+$ was also observed.

The ^{1}H NMR data of the isolated inhibitor represented in Figure 2 showed signals for six tertiary methyl groups, which were observed as singlets at 0.79 ppm, 0.97 ppm, 1.04 ppm, 0.84 ppm, 0.74 ppm and 0.92 ppm. The same spectrum also showed resonances for olefinic methylene protons at 4.74 ppm and 4.60 ppm and a vinyl methyl singlet at 1.68 ppm which was shown to be coupled to one of two vinylic protons (H-29a, 4.74 ppm), thus indicating the presence of an isopropenyl group as well as a lupane skeleton.

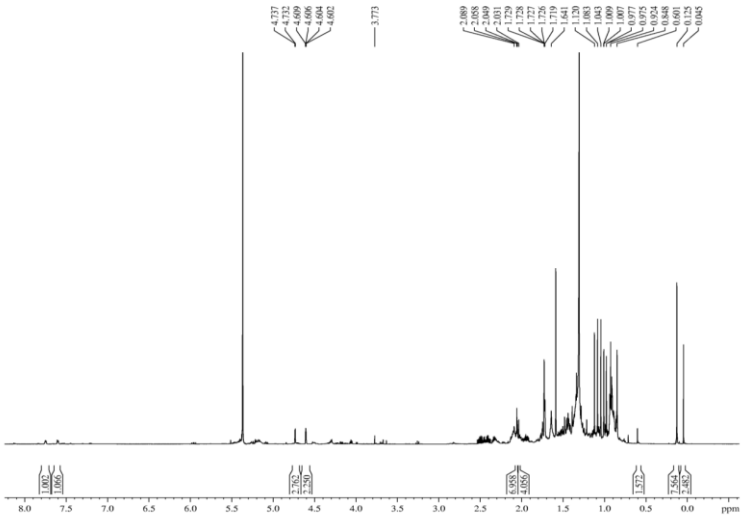

Figure 2. ^{1}H NMR spectrum of the isolated inhibitor.

The ^{13}C NMR data of the isolated inhibitor shown in Figure 3 were in agreement with the mass spectral data, as it revealed the presence of 30 carbon atoms. ^{13}C NMR data of compound was also in agreement with the existence of an isopropenyl group, evidenced by the characteristic vinylic carbon atom resonances at 151.6 ppm and 109.6 ppm, corresponding to carbon atoms 20 and 29 respectively. The existence of an isopropenyl

group was also supported by the olefinic methylene protons seen as singlets at 4.74 ppm and 4.60 ppm in the ^{1}H NMR spectrum of compound. The carbon resonance at 218.0 ppm, in the 13C NMR spectrum was assigned to a carbonyl carbon and a resonance at 1.55 ppm, in the ^{1}H NMR spectrum, to two alpha protons of the carbonyl group.

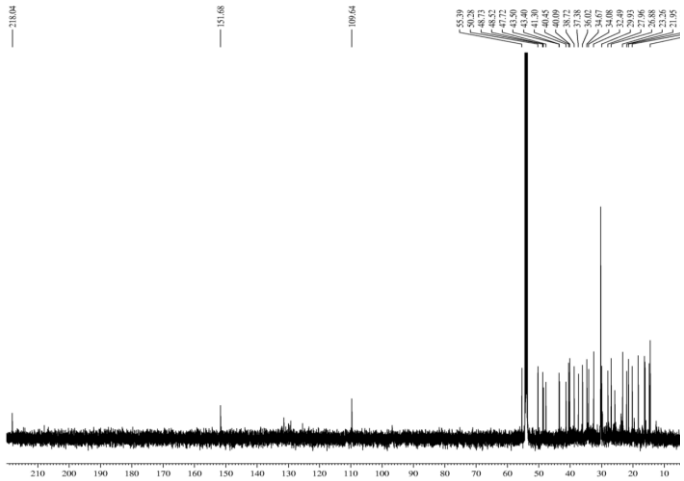

Figure 3. ^{13}C NMR spectrum of the isolated inhibitor.

The chemical structure of the inhibitor ascertained from the chemical shifts of NMR data and mass spectra is presented in Figure 4.

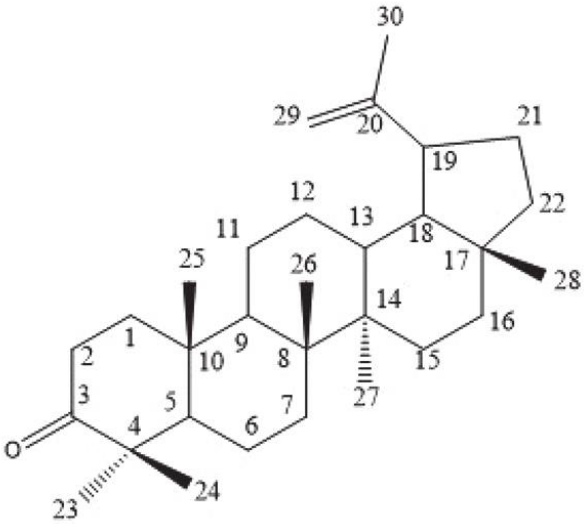

Figure 4. Structure of lupenone.

α-amylase catalyzes the hydrolysis of internal α-(1→4) glucosidic bonds. The porcine pancreatic amylase, a 469- amino acid containing protein exhibits $(\beta/\alpha)_8$ barrel along with a C-terminal β-stranded domain with an α-crystalline topology (Buisson, Duee, Haser, & Payan, 1987; M. Qian, Haser, & Payan, 1993). Lupenone showed a dose-dependent decrease in amylase activity with 50% inhibition at 30 μM, 85% inhibition at 50 μM and 89% inhibition at 100 μM.

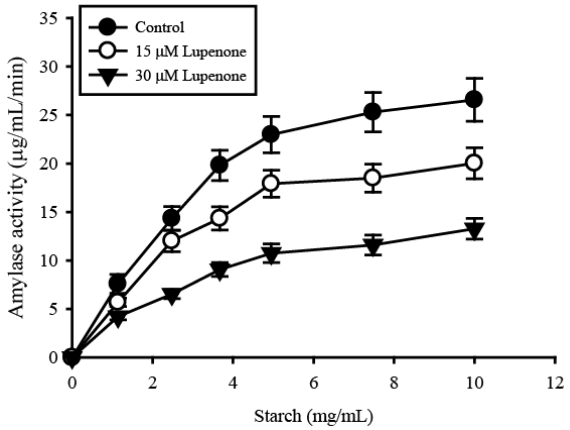

Figure 5. Effect of different concentrations of lupenone on amylase activity.

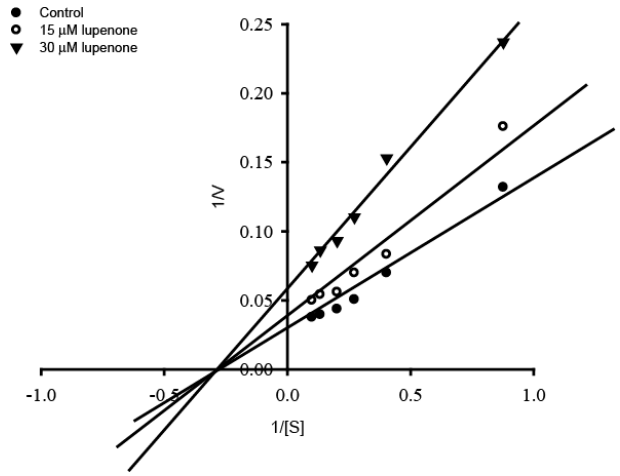

Figure 6. Non-competitive inhibition of amylase by lupenone as demonstrated by Lineweaver-Burk plot.

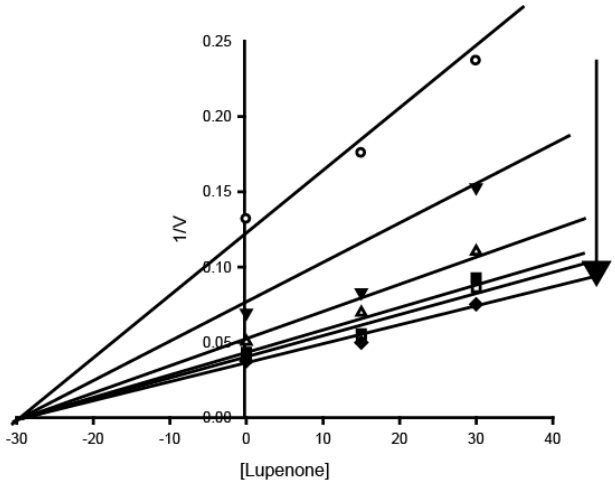

Figure 7. Dixon plot for calculation of inhibitor constant.

The positive control, acarbose exhibited 50% inhibition at 21.28 ± 1.42 μM. Enzyme kinetics data as shown in Figure 5 shows a dose-dependent decrease in amylase activity with increasing concentrations of lupenone. Further kinetic analysis using Lineweaver-Burk plot shown in Figure 6 indicated that V_{max} decreased, without change in K_m (2.4 mg/mL) with increasing concentrations of lupenone. These data suggest that lupenone is a non-competitive inhibitor of PPA. Further, Dixon plot of 1/V vs. [lupenone] shown in Figure 7 with increasing concentrations of starch as substrate indicated the inhibitor constant to be 30 μM. Considering the characteristics of noncompetitive inhibition, lupenone likely binds at an allosteric site separate from the active site of substrate binding, regardless of the presence of a bound substrate. Lupenone thus may have the same affinity for both the enzyme alone and the enzyme-substrate complex. Binding of lupenone to the enzyme or enzyme-substrate complex inhibits the enzyme, disallowing the production of its end product. Inhibition of the enzyme decreases the maximum rate of the reaction (V_{max}), defined as the rate of the reaction at a substrate concentration that fully saturates all active sites of the specific enzyme. The affinity of the enzyme for its substrate (K_m) remained unchanged as the active site is not competed for by the inhibitor. Inhibition of intestinal enzyme such as α-amylase responsible for the breakdown of dietary sugar by lupenone may be effective in preventing postprandial hyperglycemia in the diabetic subjects.

A large number of *in vitro* and *in vivo* studies have shown that lupenone- containing plants and lupenone have significant anti-diabetic activity. According to the reports, the plants and herbs containing lupenone have good anti-diabetic activity, including Rhizoma Musae (the root of *Musa basjoo* Sied. et Zucc.), banana peel (Musa nana Lour.), Thespesia Populnea bark and leaf, Acanthus ilicifolius L., Adenophora triphylla var. japonica, Pueraria lobata root, etc. (Ahmad et al., 2015; Ahn & Oh, 2013; Callies et al., 2015; Parthasarathy, Ilavarasan, & Karrunakaran, 2009; Seong, Roy, Jung, Jung, & Choi, 2016). The ethyl acetate and petroleum ether fraction of Rhizoma Musae could inhibit the activity of α-glucosidase *in vitro*, and the inhibitory activity of Rhizoma Musae petroleum ether fraction is higher (Zhang, Chang, & Kang, 2010). The Rhizoma Musae fraction with anti-diabetic activity could improve the glucose metabolism and increase the oral glucose load in alloxan-induced diabetic mice (H. Qian, Hao, & Wang, 2012). The chemical constituents of *Euonymus alatus* (Thunb.) Sied. have good inhibitory effects against the protein tyrosine phosphatases 1B (PTP1B) and α-glucosidase enzyme activity, and the lupenone isolated from *Euonymus alatus* (Thunb.) Sied. and *Sorbus commixta* also could inhibit the PTP1B enzyme activity, while lupenone could not inhibit the α-glucosidase enzyme activity (Jeong et al., 2015; Na et al., 2009). The lupenone from *Abrus precatorius* leaves has α-amylase-inhibitory activity (Yonemoto, Shimada, Gunawan-Puteri, Kato, & Kawabata, 2014). In another study, the ethyl acetate and petroleum ether fraction of banana peel (*Musa nana* Lour.) show the anti-hyperglycemic activity. Furthermore, the lupenone isolated from banana peel show promising anti-hyperglycemic activity (Wu, Xu, Hao, Yang, & Wang, 2015). Our study provides mechanistic evidence that lupenone isolated from *D. melanoxylon* exhibits potent inhibitory activity against α-amylase and therefore could be tested further in animal models of diabetes.

CONCLUSION

α-amylase enzyme assay guided fractionation of the *Diospyros melanoxylon* bark extract yielded a triterpene, lupenone that exhibited non -competitive inhibition. Thus, lupenone-based natural inhibitors of α-amylase may aid in limiting the breakdown of dietary sugar and also may be effective in preventing postprandial hyperglycemia in the diabetic subjects.

Conflict of interest: The authors declare that there is no conflict of interest.

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