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Isolation and *in silico* Studies of New Diterpene from *Phyllanthus amarus* Linn.

TANVEER ALAM[1], BAHAR AHMED[1], LUBNA NAJAM[2] and SHAH ALAM KHAN[1,3,*]

[1]Antihepatotoxic Research Lab, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi-110 062, India
[2]Department of Chemistry, D.A.V. College, Muzaffarnagar-251 001, India
[3]Department of Pharmacy, Oman Medical College, Muscat, Sultanate of Oman

*Corresponding author: E-mail: shahalamkhan@yahoo.com

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We report herein the isolation of a new diterpenoid methyl *labda*-5(6),11(12),14(15)-triene-17-oate (**1**) from the whole plant of *Phyllanthus amarus* Linn for the first time. The structure of the isolated compound was established on the basis of IR, ^{1}H NMR, ^{13}C NMR and mass spectral studies. The *in silico* studies were carried out to predict the biological activity and to calculate bioactivity score, molecular and pharmacokinetic properties of isolated diterpene using Molinspiration and PASS cheminformatics software. The diterpene showed Pa values of 0.502 and 0.411 for antifungal and antihypertensive activity by PASS software. The bioactivity score obtained by Molinspiration indicated that the diterpene is most likely to act either as a nuclear receptor ligand or by enzyme inhibition. The compound was predicted to be lipophilic (mi log P = 5.3) and thus showed one violation of Lipinski's rule of five.

Keywords: *Phyllanthus amarus*, Diterpenoid, Euphorbiaceae, Labdane.

INTRODUCTION

Phyllanthus amarus Linn., is a small erect, annual glabrous herb that belongs to the family Euphorbiaceae. It is widely grown in tropical and subtropical regions of the world and has a long history of use as a folkloric medicine in Asia and America [1]. It is commonly known as 'Bhuinanvalah' in Hindi and is used in Indian System of Medicine for the treatment of edema, stomach ache, intermittent fever, ophthalmology, diseases of urino-genital system, scabies, ulcer and wounds, *etc*. It is also used as a traditional medicine to cure liver diseases [2,3]. A number of previously isolated phytochemicals from the leaf, stem and root of the plant includes lignans, glycosides, flavonoids, ellagitannins and phenylpropanoids along with common lipids, sterols and flavonols [4]. It has been reported that mainly lignans (phyllanthin, hypophyllanthin, niranthin and nirtetralin) are responsible for the liver protective activity of this plant [5]. The various parts of the plant have been a subject for numerous scientific studies owing to its proven hepatoprotective and antiviral activity against the hepatitis B virus. Therefore, the present study aimed to isolate and characterize the active principle from the whole plant which could be responsible for exhibiting hepatoprotective action.

The present paper deals with the isolation, structure elucidation of the new diterpenoid **1** from the whole plant along with the prediction of its biological activity and molecular properties.

EXPERIMENTAL

The melting point was recorded using a Perfit melting point apparatus and is uncorrected. An IR spectrum was recorded in KBr pellet on Win IR FTS 135 instrument. Column chromatography was performed on silica gel (60-120 mesh) and thin layer chromatography on silica gel coated TLC plates. ^{1}H NMR spectra was recorded in DMSO on Bruker DRX 300 NMR spectrophotometer using TMS as an internal reference. Chemical shift values are expressed as δ ppm and coupling constant (*J* values) are expressed in Hz. ^{13}C NMR spectra was recorded on DRX-300 with TMS as an internal standard in 5 mm spinning tubes at 27 °C. A FAB mass spectrum was scanned at 70 eV on a Jeol D-300 instrument.

The whole plant of *Phyllanthus amarus* was collected from Trivendrum, Kerala and identified by the Taxonomist of Department of Botany, Jamia Hamdard University, New Delhi, where a voucher specimen of the plant is deposited in the herbarium.

Extraction and isolation: The air dried plant material (8 kg) was crushed to a coarse powder and extracted exhaustively by cold percolation method with ethanol (95 %). The alcoholic extract was concentrated under reduced pressure to yield a viscous mass (500 g). It was then dissolved in hot water and partitioned with petroleum ether (60-80 °C), acetone and methanol. The concentrated methanol soluble part was adsorbed on silica gel (60-120 mesh) to prepare slurry. The

slurry was dried in air, loaded in silica gel column prepared in petroleum ether (60-80 °C). The column was eluted successively with petroleum ether and mixture of petroleum ether, benzene and methanol in increasing order of their polarity. Elution with benzene and methanol (95:5) afforded diterpene **1** (100 mg), m.p.: 65 °C, R_f = 0.523 (benzene:ethylacetate, 8:2); IR (KBr, ν_{max}, cm^{-1}): 2950, 2850 (-CH_3,-CH_2), 1750 (-C=O), 1640 (C=C), 1460, 1380, 1060 (-C-O), 980, 960, 940, 840 and 800 cm^{-1}. EIMS *m/z*: 316 [M^+; $C_{21}H_{34}O_2$], 257 [M^+-$COOCH_3$], 269 [M^+-69], 176 [M^+-$COOCH_3$-C_6H_9], 152 [M^+-$COOCH_3$-ring B, fission *via* 9(10)-6(7)], 137 [$C_{11}H_{20}$-CH_3], 111 [137-C_2H_4], 97 [111-CH_3], 82 [97-CH_3], 71 (100) (Fig. 2). ^{1}H NMR and ^{13}C NMR (Table-1).

TABLE-1
^{1}H NMR AND ^{13}C NMR OF COMPOUND **1**

Position	^{1}H NMR	^{13}C NMR
1a	1.01 *ddd* (3.7, 3.7, 13.1)	39.9
1b	1.89 *m*	-
2a	1.45 *m*	18.6
2b	1.53 *m*	-
3a	1.16 *ddd* (3.7, 3.7, 13.4)	42.0
3b	1.40 *m*	-
4	-	33.3
5	-	50.3
6	5.82 *dd* (1.5, 4.0)	137.0
7a	1.87 *m*	23.7
7b	1.97 *m*	-
8	4.135 *ddd* (7.2,7.0, 5.2)	134.5
9	2.07 *m*	50.3
10	-	36.8
11	5.53 *d* (6.7)	133.1
12	5.88 *d* (6.7)	136.62
13	2.33 *m*	26.2
14	5.79 *dd* (10.5, 17.5)	141.7
15a	4.93 *dd* (2.5, 10.5)	110.0
15b	4.99 *dd* (2.5, 17.5)	-
16	1.75 *s* Me	11.7
17	-	169.5
18	0.85 *s* Me	32.8
19	0.87 *s* Me	22.1
20	0.79 *s* Me	14.5
-COO*Me*	3.665 *s* Me	51.3
-*C*OOMe	-	175.1

***in silico* studies:** The SMILES notation of the diterpene was fed in the online Molinspiration software version 2011.06 (www.molinspiration.com) and molecular properties such as mi log P, topological polar surface area, number of hydrogen bond donors and acceptors, molecular weight, number of atoms, number of rotatable bonds and violations of Lipinski's rule of five, *etc.* were calculated. It was also used to predict bioactivity score for drug targets *viz.* GPCR ligands, kinase inhibitors, ion channel modulators, enzymes and nuclear receptors [6]. Biological activities of natural product were predicted with the help of PASS computer program [7]. It helps in finding and optimizing most probable new leads with required pharmacological activity from the data bases of compounds.

RESULTS AND DISCUSSION

Compound **1** *viz.*, methyl *labda*-5(6),11(12),14(15)-triene-17-oate was obtained as colourless needles and had molecular composition $C_{21}H_{34}O_2$ established on the basis of mass spectrum (M^+, 316). The IR spectrum indicated a double bond (1640 cm^{-1}) and a ketonic group (1750 cm^{-1}). The presence of double bond was further confirmed by ^{13}C NMR and by ^{1}H NMR spectra exhibiting a double doublet at δ 5.82 (*J* = 1.5, 4.0 Hz) at $\Delta^{5(6)}$ position, a doublet at 5.53 (*J* = 6.7, H-11) and at δ 5.88 (*J* = 6.7, H-12) due to double bonds at $\Delta^{11(12)}$ and a third double bond at δ 5.79 (*dd*, *J* = 2.5, 17.5, H-14, *trans*), δ 4.93 (*dd*, *J* = 2.5, 10.5, H-15a) and δ 4.99 (*dd*, *J* = 2.5, 17.5, H-15b, *trans*). ^{1}H NMR also showed four signals of two protons each which were accounted for the cyclic methylene protons at positions 1, 2, 3 and 7, respectively. ^{1}H NMR, ^{13}C NMR and mass spectral data indicated that it contains labdane type nucleus [8-10]. ^{1}H NMR spectrum exhibited four signals of three protons each at δ 1.75, 0.85, 0.87 and 0.79 assignable to four methyl protons at C-16, C-18, C-19 and C-20, respectively. A three proton singlet at 3.665 was assigned to methoxy group of carbonyl moiety assignable at C-8 as evidenced by the elimination of 59 mass units in the mass spectrum. Other peaks in the mass spectrum at 164, 152, 234 and 176 were also structurally indicative (Fig. 1).

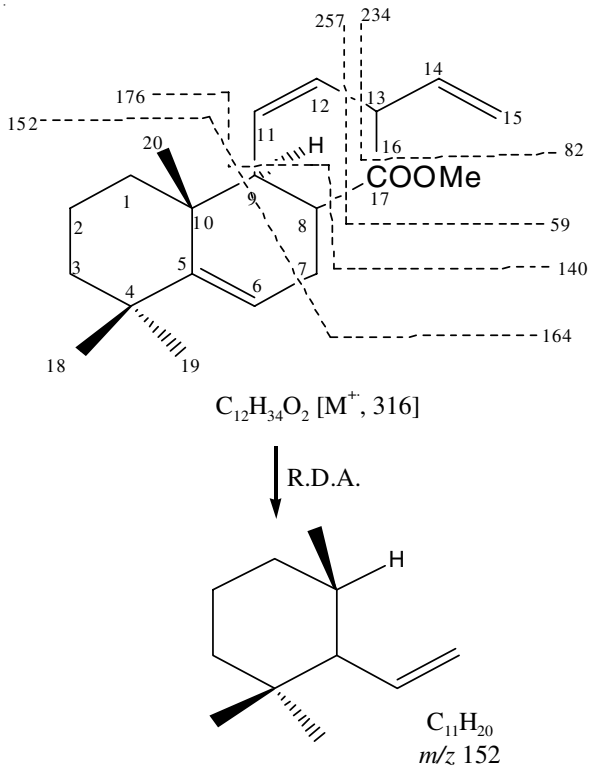

Fig. 2. Mass fragmentation scheme of compound **1**

The spectral data were compared with the reported values of similar diterpenes of this class [11].

The molecular and pharmacokinetic properties of the isolated diterpene as predicted by Molinspiration software are presented in Table-2. The compound obeyed Lipinski rule of five for molecular weight (< 500), hydrogen bond donor (< 5) and hydrogen bond acceptor (< 10) but showed violation for partition coefficient of log P. The diterpene is expected to be hydrophobic because of its log P value, which is calculated as 5.13. Thus, it can be suggested that the compound will not have good oral bioavailability.

TABLE-2
DRUG LIKENESS SCORE FOR ISOLATED DITERPENE BY MOLINSPIRATION SOFTWARE

Compound	mi log P[a]	TPSA[b]	*n* Atoms	n ON[c]	*n* OHNH[d]	*n* violation	*n* rotb[e]	MW[f]
1	5.13	26.30	23	2	0	1	5	316.49

[a]Logarithm of partition coefficient between *n*-octanol and water (mi log P); [b]topological polar surface area (TPSA); [c]number of hydrogen bond acceptors (*n*-ON); [d]number of hydrogen bond donors (*n*-OHNH); [e]number of rotatable bonds (*n*-rotb); [f]molecular weight (MW).

TABLE-3
BIOACTIVITY SCORE OF THE COMPOUND ACCORDING TO MOLINSPIRATION SOFTWARE

Compound	GPCR ligand	Ion channel modulator	Kinase inhibitor	Nuclear receptor ligand	Protease inhibitor	Enzyme inhibitor
1	-0.10	-0.23	-0.59	0.35	-0.15	0.21

As a general rule, larger is the bioactivity score, higher is the probability that investigated compound will be active. Therefore, a molecule having bioactivity score more than 0.00 is most likely to possess considerable biological activities, while values -0.50 to 0.00 are expected to be moderately active and if score is less than -0.50 it is presumed to be inactive [12]. The results of the present study indicated that the diterpene is a biologically active molecule and will produce the physiological actions by multiple mechanisms by acting as GPCR ligand, nuclear receptor ligand and will inhibit protease and other enzymes. Though bioactivity score was observed in the following order; nuclear receptor ligand > enzyme inhibitor > GPCR ligand > protease inhibitor > ion channel modulator > kinase inhibitor (Table-3). Thus, it can be proposed that the isolated molecule will produce its effect by acting as a nuclear receptor ligand.

PASS computer program predicts the activity based on structural activity relationship (SAR) analysis of the training set containing more than 205,000 compounds in its data base. The mean accuracy of prediction is approximately above 90 %. The predicted activity spectrum of a compound estimates as probable activity (Pa) and probable inactivity (Pi) [13] which varies from 0.00 to 1.00. Usual interpretation of prediction results is based on the Pa values. If Pa > 0.7 the chance to find the activity in experiment is high, but in many cases the compound may occur to be the close analogue of known pharmaceutical agents. If 0.5 < Pa < 0.7 the chance to find the activity in experiment is less, but the compound is not so similar to known pharmaceutical agents. If Pa < 0.5 the chance to find the activity in experiment is even less [13]. The diterpene showed Pa values above 0.5 for only possible antifungal activity (Table-4).

TABLE-4
PASS PREDICTED BIOLOGICAL ACTIVITIES OF ISOLATED DITERPENE (COMPOUND **1**)

Pa	Pi	Predicted biological activity
0.502	0.030	Antifungal
0.411	0.037	Antihypertensive
0.343	0.030	Antinociceptive
0.310	0.175	Antidiabetic
0.309	0.061	Hepatoprotectant
0.297	0.112	Analeptic
0.293	0.040	Contraceptive
0.173	0.063	Insecticide
0.152	0.119	Antioxidant

Conclusion

The present phytochemical investigation of the whole plant of *Phyllanthus amarus* resulted in isolation and characterization of a new labdane type diterpene. To the best of our knowledge, this is the first report of its occurrence in *Phyllanthus* species. The compound is expected to exhibit antifungal activity by PASS software.

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