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Short communication

Discovery of a new class of HMG-CoA reductase inhibitor from *Polyalthia longifolia* as potential lipid lowering agent☆

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ABSTRACT

Bioassay guided fractionation of the ethanolic extract of *Polyalthia longifolia* var. *pendula*, led to the discovery of the clerodane diterpene, 16α-hydroxycleroda-3, 13 (14) *Z*-dien-15, 16-olide (**1**), as a new structural class of HMG-CoA reductase inhibitor. Importantly, the *in vivo* effects of **1** corroborated well with its molecular docking analysis and also with its hamster plasma pharmacokinetics.

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1. Introduction

Atherosclerosis is the major cause of heart disease, stroke and death in both developed and developing countries [1]. It is well established that elevated blood lipid levels constitute the primary risk factor for atherosclerosis [2]. Epidemiological studies have indicated that dyslipidemia and coagulation disturbances are among the most significant risk factors of the development of atherosclerotic condition [3]. 3-hydroxy-3-methylglutaryl co-enzyme A reductase (HMGR) catalyzes the conversion of HMG-CoA to mevalonate, a key precursor of cholesterol biosynthesis [4]. Statins represent the major class of hypolipidemic drugs in the market which act through the inhibition of HMGR [5]. Atorvastatin is still the best selling branded lipid lowering drug in the world; will be left for generic versions after June 2011 [6]. However, disorders of muscles, ranging in severity from asymptomatic creatine kinase elevation to rhabdomyolysis, are among the most discussed adverse effects associated with statins [7]. Fibrate class of drugs, require high doses to show significant efficacy [8], and are also associated with primary muscle injury, especially when used in combination with a statin [9,10]. Furthermore, the withdrawal of cerivastatin from the US market in August 2001, because of high reports of rhabdomyolysis in association with its use has opened avenues for new classes of drug to combat this dreaded metabolic disorder disease without severe side effects [11].

Natural products are the most consistently successful source of drug leads. For example, Guggulsterones obtained from *Commiphora mukul*, possesses marked lipid lowering activity [12]. As a part of our drug discovery program on Indian medicinal plants [13], we have been working on the indigenous plant *Polyalthia longifolia* var. *pendula* (Annonaceae). The bark of the plant was used as folk medicine for the treatment of pyrexia [14]. Recently, it showed significant lowering in mean arterial pressure in rats [15]. Clerodane diterpenes are the major active constituents of this plant showing significant efficacy against microbes and cancer cell lines [16,17].

In the present study, the crude ethanolic extract of the plant was found to be effective when analyzed for lipid lowering activity in high fat diet (HFD) fed hamsters. HFD fed dyslipidemic hamster model has been reported as an ideal *in vivo* model for evaluating lipid lowering drugs [18]. This is the first report on the lipid lowering properties of *P. longifolia* that prompted us to identify the

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active principle and determine its possible mechanism of action. The results are being reported in the present communication.

2. Results and discussion

2.1. Lipid lowering activity of the plant

The crude ethanolic extract (A001) of the leaves of the *P. longifolia* var. *pendula* was found to be effective in lowering of plasma levels of triglyceride (TG) by 35%, cholesterol (TC) by 14% and glycerol (Gly) by 36%, accompanied by increase in high density lipoprotein-cholesterol/total cholesterol (HDL-C/TC) ratio by 12% at the dose of 500 mg/kg body-wt of hamster, which indicated the latent potential of this plant in the management of hyperlipidemia. Subsequently, it was resolved into hexane (F1), chloroform (F2), butanol (F3) and aqueous (F4) fractions, which were further subjected for their evaluation in the same hamster model at the dose of 100 mg/kg body-wt of hamster. The lipid lowering profile of the four fractions is presented in Table 1. Although, fractions F1–F3 were effective in lowering TG, TC and Gly in the blood plasma of the treated animal, the fraction F1 was found to be the locus of the activity, as it decreased serum TG by 54% and TC by 49%. Hence, it was subjected for further column chromatography resulting in several sub-fractions which will be discussed next.

2.2. Lipid lowering activity of the isolated compounds

The fraction F1 was fractionated by column chromatography yielding eight sub-fractions. All the sub-fractions were again analyzed for activity and it was found that activity was concentrated in 20% ethyl acetate–hexane fraction. This active subfraction was purified which resulted in the isolation of diterpene **1**, 16-oxocleroda-3,13(14)*E*-dien-15-oic acid (**2**), (4 → 2)-abeo-16(*R*/*S*)-2,13*Z*-kolavadien-15,16-olide-3-al (**3**) and 3β, 16α-dihydroxycleroda-4(18), 13(14)*Z*-dien-15,16-olide (**4**) (Fig. 1) in pure form. The structures of all of the four diterpenes were confirmed on the basis of their spectrochemical data, mainly by, extensive 1D and 2D NMR and mass spectroscopy (please refer to supporting information) and the data were compared with those reported in the literature, thus confirming their identity [14,19,20].

It is interesting to mention that all the four isolated clerodane diterpenes (**1**–**4**) have never been investigated for their lipid lowering activity. Thus, the four pure isolates were assessed for their lipid lowering potency in the HFD hamster model and the activity compared with the reference standard drug lovastatin at the dose of 25 mg/kg body-wt. The results were remarkable and were in good accord to the preliminary screening of the plant material and its crude fractions (Fig. 2). The diterpene **1** showed pronounced activity profile, while the activities of others were not as appealing. Furthermore, another repeat experiment was performed with different group of HFD fed hamsters for **1** and lovastatin at the dose ranging between 5 and 100 mg/kg; the results are represented in Table 2. In this dose response study, 25 mg/kg was found to be the optimum dose for the diterpene **1**.

Significant increase in lipid lowering profile was observed in the diterpene **1** treated group, as compared to the lovastatin treated group. It significantly lowered the plasma TG by 45% ($p < 0.001$), while lovastatin at the same dose could lower TG by only 29%. Interestingly, a significant lowering in plasma TC by 41% ($p < 0.01$) was observed in diterpene **1** treated animals as compared to 9% in the standard drug. Furthermore, the ratio HDL-C/TC was increased by 48% after the treatment with the diterpene **1**, which is considered a beneficiary effect in the treatment of dyslipidemia condition. The ethanolic extract, its fractions and the tested compounds in hamsters did not alter significantly the food intake or weight gain between the groups, which suggested that the test compounds did not effectively inhibit the absorption of intestinal dietary cholesterol in all the above experiments and were found to be safe at higher doses without any mortality.

Though it is difficult to get an idea of structure activity relationship (SAR) based only on the four isolated diterpenes, it seems reasonable to predict that the *trans*-decalin system with C-3 olefinic bond and the lactone ring are essential features for the molecule to show lipid lowering activity, as the other related diterpenes (**2**–**4**) lacked one or more of the above structural features and they did not show appreciable activity. The exquisite potency and structural novelty of **1** prompted us to delineate its possible mechanism of action.

2.3. Mechanistic pathway analysis

Lovastatin is the representative of the statin class of drugs that in their active hydrolysed form are specific inhibitors of HMGR. The structural similarity (visual inspection) between **1** and lovastatin (Fig. 3) prompted us to check whether they both share the same mechanistic pathway. Type 1 statins, share an HMG-like moiety, which may be present in the form of an inactive lactone form that acts a prodrug and in addition they also have a rigid hydrophobic substituted decalin ring covalently bound to the HMG-like moiety. Thus, a dose response *in vitro* study was done to assess the inhibition of HMGR enzyme by **1**. The inhibitory activity was evaluated at six different concentrations ranging from 1 μM to 100 μM (Fig. 4). **1** showed 22.65% inhibition of HMG-CoA reductase at 1 μM concentration which is similar to lovastatin. Somewhat parallel results were obtained at further higher concentrations of 5, 10 and 20 μM. Maximum inhibition of 78.03% was shown by **1** at 100 μM concentration. The HMGR inhibitory activity in terms of IC_{50} value of lovastatin and diterpene **1** was found to be 20.1 μM and 30.2 μM, respectively. Furthermore, in our experiments of hypercholesterolemia regression, **1** accelerated the fall in TC level, which suggested that it inhibited the synthesis of endogenous cholesterol. These results established the idea that the hypolipidemic effect of **1** *in vivo* correlated with the inhibition of the activity of HMGR. The trial suggested that the **1** might share the same mechanism as statins. To further corroborate our findings, the molecular docking studies were carried out to find out the plausible ligand–receptor interactions with HMGR active sites, the results of which are discussed next.

Table 1
Lipid lowering activity of the fractions of *P. longifolia* var. *pendula* in HFD fed hamsters.[a]

Plant sample	Dose (mg/kg)	TG (mM)	TC (mM)	Gly (mM)	HDL-C/TC
Vehicle		10.84 ± 3.89	10.76 ± 1.62	2.07 ± 0.80	0.19
F1	100	4.97 ± 1.49 (−54***)	5.49 ± 2.53 (−49***)	0.92 ± 0.43 (−55***)	0.15 (−30)
F2	100	7.50 ± 2.20 (−31)	6.15 ± 2.51 (−43***)	1.23 ± 0.62 (−40*)	0.15 (−30)
F3	100	7.94 ± 2.60 (−27)	6.44 ± 1.65 (−40***)	1.15 ± 0.51 (−44*)	0.22 (+16)
F4	100	11.87 ± 4.46 (+9)	10.74 ± 2.16 (−1)	1.53 ± 0.46 (−26)	0.15 (−30)

[a] Values are mean ± SEM of 8 animals, values in parentheses are % change from HFD control and $p < 0.05$ (*), $p < 0.001$ (***).

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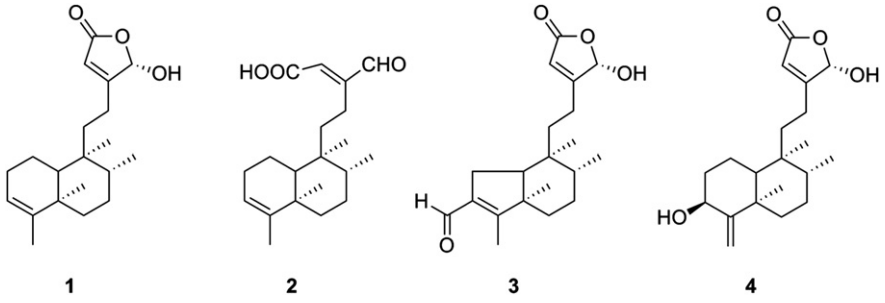

Fig. 1. Diterpene **1**–**4** isolated from the active fraction of *P. longifolia* var. *pendula*.

2.4. Binding site analysis

The docking results indicate that **1** and lovastatin both docked well into the same pocket that accommodates statin inhibitors; FlexX total score [21] for them was calculated as −14.73 and −10.67, respectively. On the basis of FlexX docking scores of docked conformations, binding of **1** was found to be superior to lovastatin. Interatomic contacts in the complexes of protein and docked conformations of ligands were thoroughly inspected to get better insight into the probable reason of higher activity of **1** as compared to lovastatin. Visual inspection of interatomic contacts between the ligands docked into the protein active site indicates that lower score of lovastatin could primarily be due to the unfavourable destabilizing hydrophobic–hydrophilic clashes between the ligand and active site residues of protein.

On thorough inspection of docked conformation of **1** (Fig. 5A), many interactions were found to be conserved as also described previously in literature [5]. Moreover, the interaction analysis shows that the hydroxyl group of Ser684 and backbone amide groups of Lys735 and Lys692 act as hydrogen bond donor for two polar O-atoms of **1** (O1 and O2). In addition, carbonyl groups of Asp690 and Ala751 were also involved in hydrogen bond interaction with hydroxyl O-atom (O3) of **1**.

Docked lovastatin and **1** revealed the similar binding mode with the additional destabilizing hydrophobic–hydrophilic clashes between polar atoms of Arg590, Glu559, Asn658, Asp690, Ser661 residues projected towards the active site cavity and hydrophobic skeleton of lovastatin that could be responsible for lower docking score of lovastatin as observed in visual inspection of bound lovastatin to the protein (Fig. 5B). Thus, the encouraging

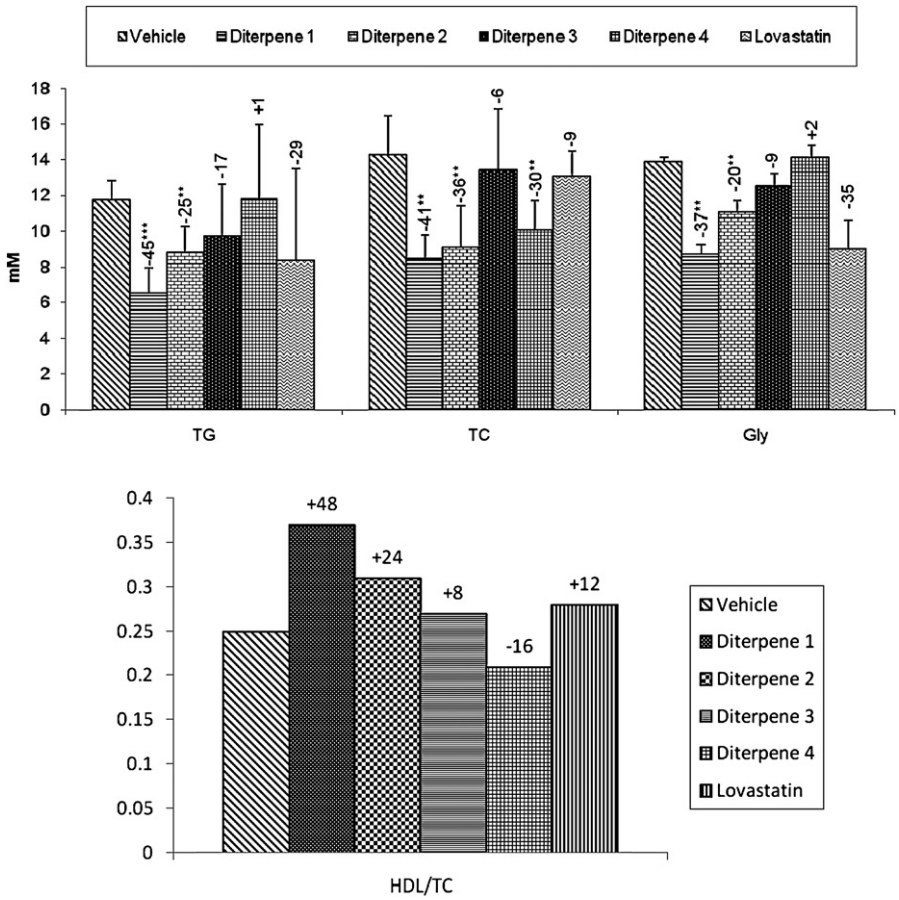

Fig. 2. Lipid lowering profile of diterpenes **1**–**4** at 25 mg/kg body-wt dose in HFD fed hamsters. The graph presented shows the % decrease/increase in TG, TC, Gly and HDL/TC ratio.

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Table 2
Lipid lowering activity of diterpene **1** at the dose ranging between 5 and 100 mg/kg body-wt in HFD fed hamsters.[a]

Group	Dose (mg/Kg)	TG (mM)	TC (mM)	Gly (mM)	HDL-C/TC
HFD Vehicle	–	–	–	–	–
1	5	−19	−27*	−10	+12
	10	−26***	−23*	−5	+12
	25	−45***	−41**	−37***	+48
	50	−46**	−55***	−48**	+54
	100	−43***	−54***	−43***	+46
lovastatin	25	−29	−9	−35	+12

[a] Values represented are % change with respect to HFD fed hamster group (group of eight animals) and $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

results of molecular docking analysis further confirmed **1** to be a new class of HMGR inhibitor, the exquisite potency and structural novelty suggest that it may serve as a valuable lead for antidyslipidemia.

2.5. In vivo pharmacokinetic studies

Oral pharmacokinetic studies were carried out in hamster as similar to *in vivo* activity model. The pharmacokinetic studies revealed that the animals tolerated the treatment as no peculiarities in the animal's behaviour were observed. Plasma concentration–time curve were smooth enough to estimate pharmacokinetic parameters and the elimination phase was well fitted to first order one compartment pharmacokinetic model. The parameter T_{max} and C_{max} after 25 mg/kg oral administration of diterpene **1** were 0.5 ± 0.0 h and 2486.66 ± 800.0 ng/mL, respectively, indicating rapid gastro-intestinal absorption. **1** was observed and quatitated in the systemic circulation for 48 h following single oral administration (Fig. 6).

3. Conclusion

Diterpene **1** emerged as the lead lipid lowering molecule from natural source which is safe and easy to access, whose pathway of action seems to proceed through HMGR inhibition. The pharmacokinetic study in hamster model indicated rapid gastro-intestinal absorption of **1** maintaining prolonged levels in systemic circulation. This is the first study that shows clerodane diterpenes as the new class of lipid lowering agent. It is interesting to note that our further mechanistic studies on this "privileged structure" revealed that it is capable of binding to multiple receptor targets, as it also inhibited the DNA topoisomerase I of *L. donovani* [22]. Although drugs are indented to be selective, at least some bind to several physiological targets, explaining their polypharmacology which is common in natural leads [23].

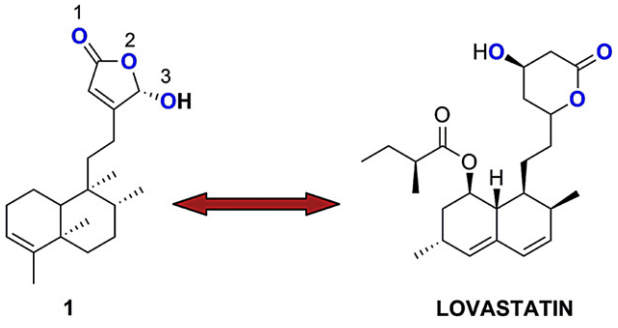

Fig. 3. Structural resemblance of diterpene **1** with standard drug lovastatin.

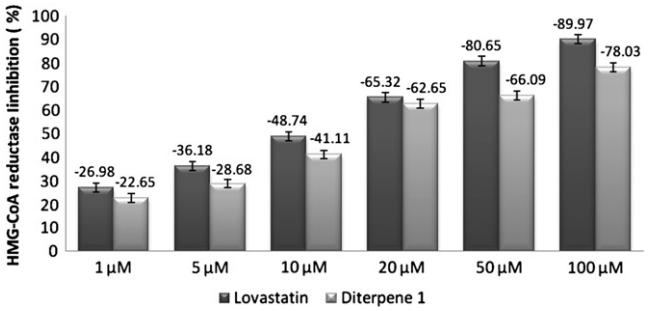

Fig. 4. *In vitro* dose response study of diterpene **1** and lovastatin for HMG-CoA inhibitory activity.

4. Experimental

4.1. General experimental information

IR spectra were recorded on Perkin Elmer 399B spectrophotometer. Optical rotation were measured on Autopol III serial no. 30166 Polarimeter (Rudolph Research) with 1 dm cell. All NMR spectra were obtained from Bruker Avance DRX 300 MHz spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz, respectively), in $CDCl_3$ and CD_3OD (chemical shift δ in ppm, J in Hz) using TMS as an internal standard. ESI mass spectra were recorded on Thermo Lcq Advantage Max-IT. Elemental analyses were performed on Carlo Erba EA-1108 micro analyzer/Vario EL-III C H N S analyzer. Purity of the isolated compounds was found to be greater than 95%. Organic solvents were distilled prior use. Column chromatography was carried out on Si gel (60–120 and 230–400 mesh, Merck). Thin layer chromatography was performed on silica gel $60F_{254}$ aluminium plates (Merck).

4.2. Isolation of active compounds

The leaves of the plant were collected from Lucknow in April 2005. The identity of the plant was confirmed and a voucher specimen (No. 6381) was deposited in the herbarium of the Botany Division, Central Drug Research Institute, Lucknow, India. Air dried and grounded leaves (16 kg) of *P. longifolia* var. *pendula* were extracted with ethanol for three consecutive days at room temperature. Evaporation of the solvent under reduced pressure yielded ethanolic extract A001 (2 kg). It was macerated with hexane and then remaining residue dissolved in water and partitioned with chloroform and butanol to yield another three fractions, which on concentration under reduced pressure at water bath temperature 20–50 °C; yielded hexane fraction F1 (1.2 kg), chloroform fraction F2 (50.2 g), butanol fraction F3 (300 g) and aqueous fraction F4 (300 g).

F1 (1.2 kg) was subjected to silica gel (60–120 mesh) column chromatography using ethyl acetate: hexane gradient (0:100 to 100:0) to give major eight fractions. A part of active 20% ethyl acetate: hexane fraction (30 g) was taken up for the separation of diterpenes. It was further fractionated into eight sub-fractions over flash silica gel (230–400 mesh) column chromatography with the gradient of ethyl acetate in hexane. Subsequent purification of fraction IV (6% ethyl acetate: hexane, 6.5 g) gave **1** (3.56 g), fraction III (4% ethyl acetate: hexane, 0.85 g) gave **2** (0.29 g) and fraction VIII (15% ethyl acetate: hexane, 4.2 g) on repeated chromatographic separations gave **3** (0.27 g) and **4** (0.35 g), in quantitative yields.

4.3. In vivo lipid lowering activity

Golden Syrian Hamsters, males, 12 week old, 110 ± 5 g, were used in the present study. Animals were housed in groups of 8

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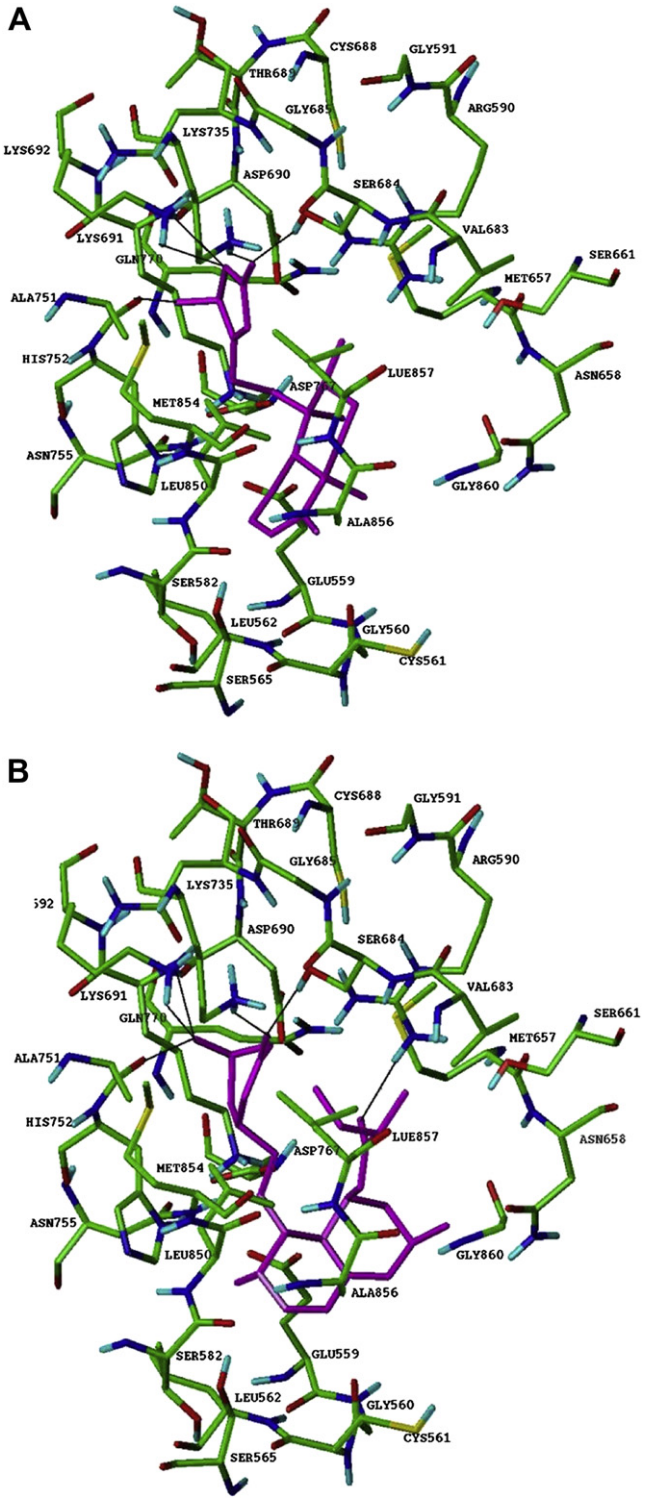

Fig. 5. A. Docking of **1** into the active site of HMG-CoA reductase (PDB ID: 1hw9). B. Docking of lovastatin into the active site of HMG-CoA reductase (PDB ID: 1hw9).

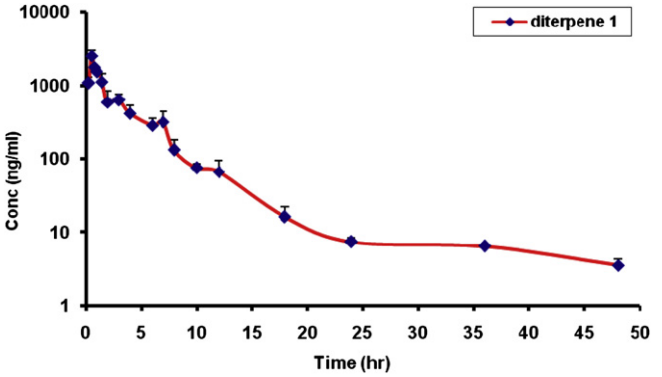

Fig. 6. Plasma concentration–time profile of diterpene **1** in hamsters at 25 mg/kg oral administration.

individuals in a cage in a room controlled for temperature (25 ± 2.0°c) and 12/12 h light/dark cycle. Dyslipidemia was produced by feeding the animals with HFD. The composition of the high fat diet (HFD) in combination with fructose was chosen from previous studies in hamsters and rats, and is now produced and procured by Research Diets, Inc., New Brunswick, USA. The combination of oil, cholesterol, and fructose is found to give a very severe and rapidly induced dyslipidemia with hypercholesterolemia and hypertriglyceridemia. The animals had free access to the HFD and water *ad-lib* throughout the experiment for both the experiment. Body weight and food intake was measured daily. The animals were allowed to acclimatize for at least one week before introducing the high fat diet. Experimental protocols were approved by our institutional ethical committee, which follows guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), which complies with international norms of INSA. Biochemical analysis of plasma samples was performed on Synchron CX-5, Clinical Systems, Beckmann Coulter Instrument, using enzymatic diagnostic kits. Data was analyzed using Graph Pad Prism version 3.02 data templates. Significance (p values) was calculated using one way analysis of variance of ANOVA programme.

4.4. *In vitro HMG-CoA reductase inhibitory activity*

The HMG-CoA reductase assay was performed using the HMG-CoA reductase assay kit from Sigma–Aldrich. HMG-CoA (substrate), NADPH, assay buffer and enzyme HMGR were supplied with the assay kit.

4.5. *Molecular docking*

Molecular docking study was performed using FlexX from SYBYL Molecular Modelling System Version 7.1. 2005, Tripos Inc, St. Louis, MO. FlexX is a docking program that takes into consideration the flexibility of the ligand but not that of the receptor. The docking method is based on an incremental construction algorithm which automatically selects the base fragment using a pattern recognition technique called pose clustering and place into an active site followed by incremental building of the remaining of the portion onto the active site. The conformational flexibility of the ligand is included by generating multiple conformations for each fragment and placement of the ligand is scored to estimate the free energy of binding of protein–ligand interactions [21]. Reference protein coordinates for docking were obtained from the X-ray structure of catalytic portion of human HMG-CoA reductase complexed with simvastatin (PDB ID: 1HW9). First, water molecules of the receptor molecule were removed and hydrogens were added. A binding pocket of the receptor was selected as the 6.5 Å around the bound simvastatin for the study. Thirty conformations were generated for each ligand using flexible fit method with FlexX. The default parameters were used to dock the ligands and the conformations were re-ranked on the basis of Cscore. Top-ranked ligands were

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analyzed for protein ligand interaction studies based on the comparison of the hydrogen bond interaction among the top-ranked docked poses with the protein and secondly, the analysis of binding score between the docked ligand and the enzyme using CScore.

To ensure that the ligand orientation and position obtained from the docking studies were likely to represent valid and reasonable binding modes of the inhibitors, the FlexX program was first validated for the crystal structure used (1HW9). The ligand, simvastatin was extracted and docked back to the corresponding binding pocket, to determine the ability of FlexX to reproduce the position orientation of the ligand observed in the crystal structure. It was observed that FlexX generated the optimal orientation of the docked simvastatin, close to that of the original orientation found in the crystal. The low r.m.s. of 0.85 Å deviation between the docked and crystal ligand coordinates indicate fairly good alignment of the experimental and calculated orientations (refer to the supplementary data).

4.6. In vivo pharmacokinetic studies

In vivo pharmacokinetic experiments were performed in healthy male golden Syrian hamster weighing 150 ± 20 gm, obtained from Laboratory Animal Division, CDRI, Lucknow, India. The study was conducted in $n = 3$ per time point. All experiments were carried out as per the guidelines of CPCSEA. Hamsters were treated with test compound **1** orally at 25 mg/kg. Blood samples were collected from the retro-orbital plexus of hamster under light ether anaesthesia into microfuge tubes containing heparin as an anti-coagulant at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 7.0, 8.0, 10.0, 12.0, 18.0, 24.0 30.0 and 48.0 h post dosing. Pharmacokinetic parameters were calculated by fitting the plasma concentration–time profile to a non compartmental model with WinNonlin program, version 1.5 (Scientific Consulting Inc.). Samples were analyzed via electrospray ionization (ESI) on an API-4000 (Applied Biosystems, MDS Sciex Toronto, Canada) triple-quadrupole instrument that was coupled with Shimadzu LC-10AD pumps (Columbia, MD). Analyte were chromatographically separated on a Symmetry-Shield C_{18} (5μ, 4.6 × 150 mm) column, using acetonitrile : 0.1% aqueous formic acid (92:08, v/v) as the mobile phase. The total run time was 5 min, and the HPLC flow rate was 0.6 mL/min. The mass spectral analyses were performed using multiple reaction monitoring (MRM), with transitions for **1** (*m/z* 317.4/273) and internal standard (rosuvastatin) (*m/z* 483.0/418.0) utilizing a Turbo-Ion spray source in negative ionization mode (5.0 kV spray voltage). All data were analyzed using AB Sciex Analyst 1.4.1 software. The method was validated as per FDA guideline (Guidance for Industry, Bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration center for Drug Evaluation And Research Center, Center for Veterinary Medicine, 2001).

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.08.012.

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