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Rosuvastatin and atorvastatin affect terpenoid production in *Centella asiatica* (Linn.) Urban differently

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Summary: Treatment of *Centella asiatica* with two statin molecules, that are used to inhibit HMG Co-A reductase enzyme to control production of cholesterol in human, showed their contrasting effects on the production of asiatic acid and madecassic acid (triterpenes) in its leaves and petioles. This might be due the fact that the isopentenyl pyrophosphate (IPP), which is precursor of triterpenes in plants, is biosynthesized in two different ways, and these two statins affect different enzymes of the unrelated pathways as an inhibitor, and as an enhancer/modulator respectively. Atorvastatin presumably inhibits HMG CoA reductase, that plays key role in the mevalonate pathway, while rosuvastatin might probably be modulating CDP-ME synthase of the alternative non-mevalonate pathway favorably.

Key words: *Centella asiatica*, asiatic acid, asiaticoside, madecassic acid, madecassoside, rosuvastatin, atorvastatin, mevalonate pathway, non-mevalonate pathway.

INTRODUCTION

Centella asiatica (Linn.) Urban is a well-documented medicinal herb of the dicot family Apiaceae. Known for its biologically active pentacyclic triterpenes, asiatic acid and madecassic acid which usually exist as ester glycosides, asiaticoside and madecassoside, the plant has shown both morphogenetic variation and differential content of the glycosides, especially in relation to pressure conditions and tissue damage (Kumar *et al.* 2012a).

In plants, pentacyclic triterpenes are obtained from isopentenyl pyrophosphate (Isvett *et al*., 2002), the molecule that comes mainly through the classical mevalonate pathway with acetyl Co-A as the starting material (Brown, 1998). In vertebrate animals, IPP is used to produce cholesterol in the liver (Horton *et al*., 2002).

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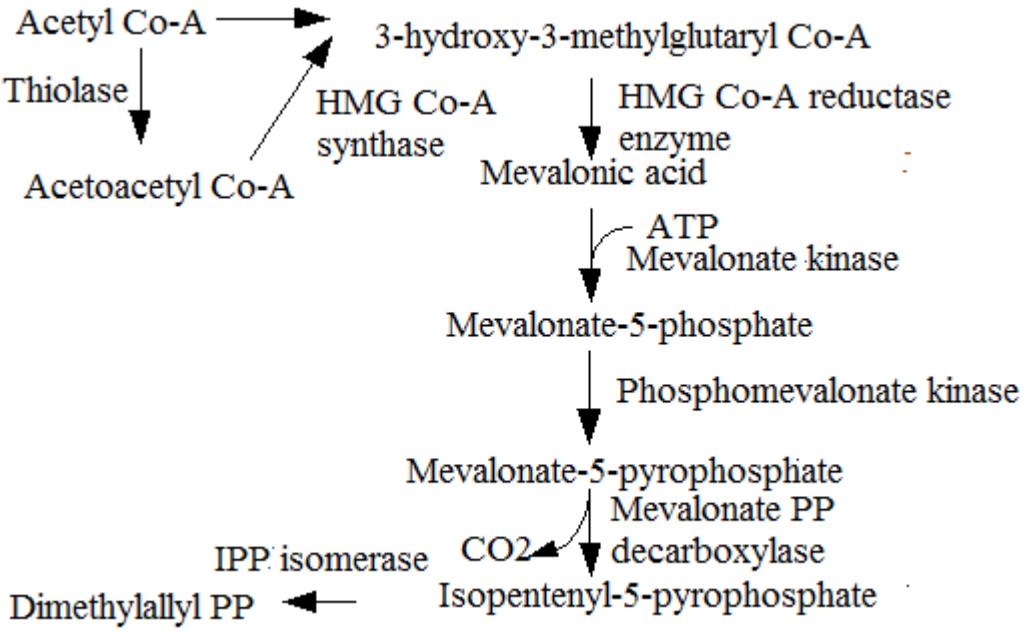
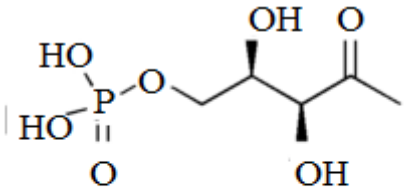


In mevalonate pathway, HMG Co-A reductase is the rate limiting enzyme that is inhibited by statin molecules to control cholesterol formation in human (Istvan and Deisenhofer, 2001).

An alternative pathway, called MEP/DOXP pathway, occurs in plastids of the plants to obtain isopentenyl pyrophosphate (Eisenreich *et al.*, 2004). Here, pyruvate and glyceraldehyde 3-phosphate are the starting materials which are combined by an enzyme DOXP synthase to produce 1-deoxy-D-xylulose 5-phosphate.



1-deoxy-D-xylulose 5-phosphate

With the help of other six enzymes, namely DOXP reductase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HMB-PP synthase and HMB-PP reductase, deoxy-xylulose phosphate

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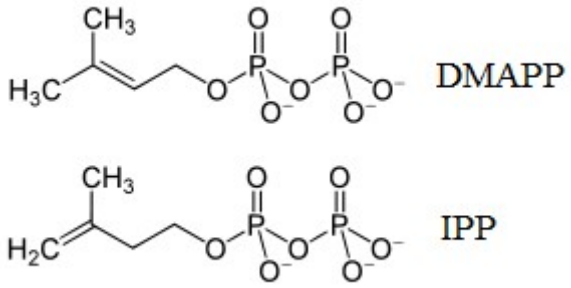
finally converts into IPP and its isomer DMAPP (Dimethylallyl pyrophosphate) which remain in balance.



Irrespective of being produced by mevalonate or non-mevalonate pathway, IPP and its isomer DMAPP are used up to synthesize triterpenes.

The present paper discusses the impact of two statin molecules i.e. atorvastain and rosuvastatin on the production of asiatic acid and madecassic acid in *Centella asiatica* under experimental conditions.

MATERIALS AND METHODS

Plants of *Centella asiatica* were grown in three separate plots of 2.0 m^2 each. Plants in Plot 1 were treated with atorvastatin (Aristo Pharmaceuticals Pvt. Ltd., Mumbai, India) while plants in Plot 2 were treated by rosuvastatin (Macleods Pharmaceuticals Ltd., Mumbai, India). Plot 3 was kept as control. Aqueous statin preparations, diluted as 1 mg/ml, were sprayed on the plants 5 times in the morning after a gap of two days each. Each time, 20 ml solution was used.

After 15 days of the last treatment, 7.5 g of leaves and petioles, air-dried for 15 hours, were powdered in mortar and pestle.

MeOh-H_2O gradient protocol was followed to isolate asiaticoside and madecassoside from the crude methanolic extract of the plant leaves and petioles through fractionation (Kumar *et al.* 2012b). Fraction 3 eluted with 60:40 MeOH-H_2O was used to obtain crystals of terpenoids at 40^0 C in a vacuum oven. 21.9 mg of crystals (0.292%) were obtained in case of control while 30.6 mg of crystals (0.408%) were obtained in case of rosuva treated

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specimens. In case of atorva treated plants, true crystals were not obtained. Instead, only 5.5 mg of black powder like material was formed. 3 mg of crystals of each sample designated respectively as CAR (Rosuva treated), CAC (Control) and CAA (Atorva treated) were sent to SAIF (CSIR-CDRI, Lucknow, India) for spectrophotometric analysis.

RESULTS AND DISCUSSION

As per the report of SAIF, the sample CAR showed distinct peaks at wavelengths 207.50 nm and 202.50 nm with absorbance 4.0 in each case which correspond to madecassic acid and asiatic acid respectively while the sample CAC (Control) had only one peak at 202.50 nm corresponding to asiatic acid. In case of the sample CAA (atorva treated), no peak was obtained. This observation clearly supports the assumption that rosuvastatin caused significant increase in the production of triterpenes (1.5 fold approx.). In contrast, atorvastatin inhibited their production.

In higher plants, the cytosolic compartment contains all of the MVA pathway enzymes (Bach *et al.*, 1999) and IPP may be derived either from mevalonate pathway (Facchini and Chappell, 1992) or from DOXP pathway (McCaskill and Croteau, 1995) or from both (Lange *et al*., 2000).

It may be conveniently assumed that atorvastatin inhibited the HMG CoA reductase enzyme of the mevalonate pathway as it does in case of human HMGR (Istvan and Deisenhofer, 2001). On the other hand, rosuvastatin modulated some enzyme of the MEP/DOXP pathway in a positive way.

This enzyme might be 4- diphosphocytidyl-2-C-methylerythritol (CDP-ME) synthetase which commits the formation of IPP in the MEP/DOXP pathway as it converts 2-C-methylerythritol 4-phosphate (MEP) to 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME) by joining CMP to the substrate from a CTP (Estevez *et al*., 2001).

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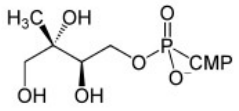

CDP-ME

It has been found that the catalytic rates of 4-diphosphocytidyl-2C-methylerythritol synthases from a plant (*A. Thaliana*) and *E. coli* are similar (Rohdich *et al.*, 2000). Hex 6.3 (Ritchie and Kemp, 2000) was used to dock rosuvastatin into the crystal structure of the chain A of the bacterial 4-diphosphocytidyl-2-C-methylerythritol synthetase (PDB code: 1I52) (Richard *et al.*, 2001) with its CTP removed, using both shape and electrostatic mode to see how the ligand (rosuva) could interact with the enzyme. Fig. 1 presents the image of the UCSF Chimera (Pettersen *et al.*, 2004) rendered docked Enzyme ChainA-rosuvastatin-CTP-MEP complex. MEP failed to dock anywhere near the enzyme-rosuva-CTP complex apparently due to the absence of the complete active pocket that is partially contributed by Chain B in the functional homodimer enzyme (Hunter, 2007). However, rosuvastatin always came to dock with the enzyme at its extended loop and bound with its salfonamide group to Ala152 through H_2O molecules (Fig. 2). Additionally, His 153 was also stimulated (Fig. 3). In the original structure (PDB code: 1I52), the CTP was held up by the residues pro13, ala15, arg19, arg20, lys27, arg85, ser88, asp106 and ala107 near its active site (Fig. 5) but when it was attempted to dock into the rosuva bound enzyme, it interacted at a different location (Fig. 4) and got involved with Ile132, Trp161, Ala163, Thr189, Ala192, Glu196, His201, Pro202 and Leu204 (Fig. 6).

Conversion of 2-C-methylerythritol 4-phosphate to 4-diphosphocytidyl-2-C-methylerythritol is an ordered reaction, with CTP binding required before 2-C-methyl-D-erythritol-4-phosphate can bind (Richard *et al.*, 2004). Both Lys27 and Lys213 have been shown to be essential to the enzyme's catalytic activity, probably playing a definitive role in stabilization of a pentacoordinate phosphate transition state resulting from in-line attack of the MEP phosphate on

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the alpha-phosphate of CTP. Thr140, Arg109, Asp106, and Thr165 were also shown to play their critical roles in the binding and proper orientation of the MEP substrate.

Figs. 7-9 show the electrostatic potential color maps of the original enzyme-CTP complex with Mg+ and CA ions (PDB code: 1I52), enzyme with rosuvastatin bound to Ala152 (with no CTP) and enzyme-rosuva-docked CTP complex respectively, rendered by Swiss-PdbViewer 4.0.4 (http://www.expasy.org/spdbv/) (Guex and Peitsch, 1997) by Coulomb computation method. Potentials equal to and less than -1.800, neutral and equal to +1.800 or above are displayed in red, white and blue shading grades. Binding of rosuvastatin to the enzyme induces approximately the same positive electrostatic potential in the molecule due to binding of the CTP. CTP docking into the complex renders it electropositively disposed for CTP's reaction with MEP.

We hypothesize that rosuvastatin induces some stereo-chemical changes in the enzyme after hydrogen bonding with the alanine residue at its position 152 that result into creation of a separate binding pocket for CTP. Histidine153 might be playing some role to orientate CTP at a nearby location. The residues Lys27 and Lys213 along with the other assisting residues like Thr140, Arg109, Asp106, and Thr165 get more positively oriented to carry out CTP and MEP reaction at all the three active sites of the homotrimeric enzyme (Richard *et al*., 2002) enhancing the production of 4-diphosphocytidyl-2-C-methylerythritol.

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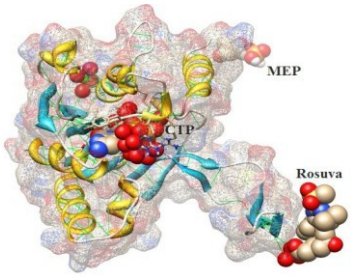

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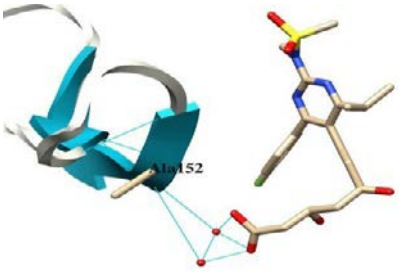

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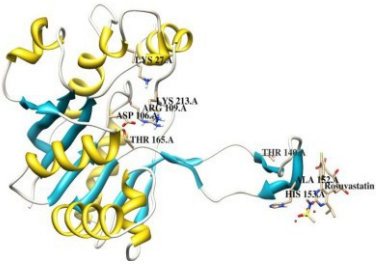

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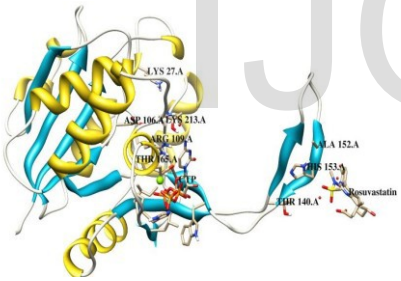

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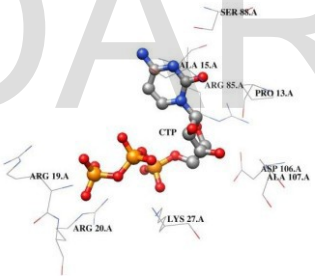

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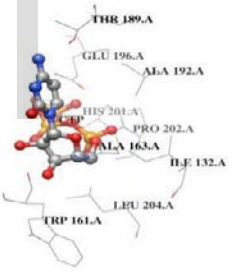

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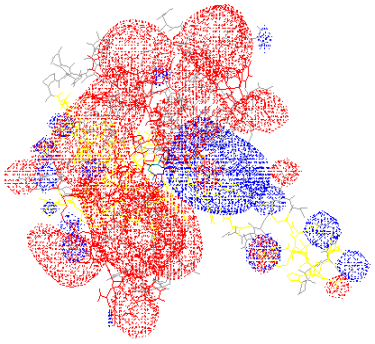

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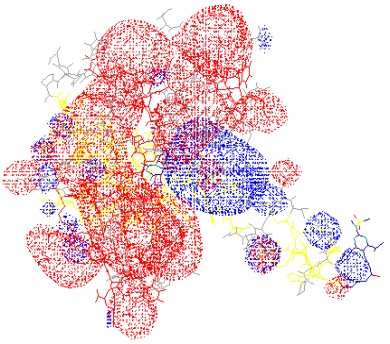

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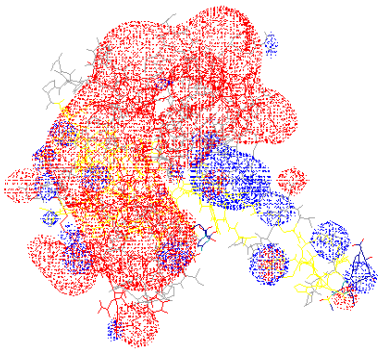

Fig. 9

