

EVALUATION OF ANTIDYSLIPIDEMIC EFFECT OF MANGIFERIN AND AMAROAGENTIN FROM *SWERTIA CHIRAYITA* EXTRACT IN HFD INDUCED CHARLES FOSTER RAT MODEL AND THEIR DOCKING STUDIES

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ABSTRACT

In continuation of our research programs on bio evaluation of medicinal plants in India, we have investigated antidyslipidemic and antioxidant potential of *Swertia chirayita* (SC) plant, a potent antidiabetic and its isolated marker. The standardized hot water extracts of different batches from whole plant of *Swertia chirayita*, were tested *in vivo* for their antidyslipidemic activity in High fat diet (HFD) induced dyslipidemic Charles Foster rats. Further solvent fractionation and isolation resulted in identification of antidyslipidemic Mangiferin (MG) and Amarogentin (AM). Treatment of hyperlipidemic rats with MG and AM caused reduction in plasma levels of Low density lipoprotein (LDL) 24, 26% and very low - density lipoprotein (VLDL) 19, 25% as well as increase in levels of High density lipoprotein (HDL) 18, 22% respectively, at 200mg/kg doses. In rats with HFD, MG and AM significantly reduced levels of VLDL lipids (26% and 27%) as well as Low density lipoprotein-Total cholesterol (LDL-TC) 27% and 30% in hyperlipidemic rats at 100 and 200 mg/kg doses. This is first report of AM demonstrated as antidyslipidemic agent. In addition, both MG and AM also demonstrated more effective inhibition at 5 μ M and 10 μ M of HMGR (HMG-CoA reductase) in comparison to Pravastatin, which was further confirmed by docking studies.

Key words: *Swertia chirayita*, Cardiovascular diseases, Mangiferin, Amarogentin, Antidyslipidemic

INTRODUCTION

Cardio vascular disease (CVD) remains the leading cause of death in the developed countries and is major cause of mortality and disease in the Indian subcontinent. The World Health Organization estimates that CVD is today the largest single contributor to global mortality and will continue to dominate mortality trends in the future¹. Increase in low density lipoprotein (LDL), triglyceride (TG) and total cholesterol (TC) levels with decrease of high density lipoprotein (HDL) levels have been established as one of the risk factors for CVD². Clinical and epidemiological studies have consistently shown that low plasma levels of high-density

lipoproteins cholesterol (HDL-C) are strongly and independently associated with an elevated risk of CVD³.

Reactive oxygen species (ROS) and oxidative stress are also important features of CVD⁴. Enhanced lipid peroxidation acts as potential contributor to increase cardiovascular risk in low HDL phenotype⁵. Normal functional HDL has high antioxidant potential⁶. Therefore, agents that increase HDL cholesterol concentration in the blood and thereby ratio of HDL-C to TC (HDL-C/TC) and reduce oxidative stress would have promising therapeutic utility as antidyslipidemic and antioxidant agents.

In continuation of our research programs on bio evaluation of medicinal plants in India, we have investigated antidyslipidemic potential of *Swertia chirayita* (SC) (Roxb. ex Flem., Family: *Gentianaceae*). It is an indigenous medicinal plant of temperate Himalaya. The decoction of the plant is used traditionally for the treatment of liver disease, febrifuge, antihelminthic, laxative and for malarial fever^{7,8}. Studies on the biological activities of different extracts of SC revealed that the plant possesses antioxidant, antimicrobial, antiinflammatory, anticarcinogenic and antidiabetic properties^{9,10,11}.

The LC-ESI-MS standardized hot water extract its solvent fractions and two glycosides of Xanthone (Mangiferin- MG) and Secoiridoid (Amarogentin- AM) of SC, a potent antidiabetic¹², were studied in HFD induced hyperlipidemic rats model. Their effect on cholesterol biosynthesis was also studied in the *in vitro* system which was confirmed by molecular docking.

MATERIALS AND METHODS

Chemical and Reagents

Standard drug gemfibrozil and test kit for HMGR along with other chemicals were procured from Sigma Chemical Company St Luis, MO, (USA).

Plant material and preparation of extract

Whole plant materials of SC were collected during Sep-Oct 2002 (Botany Reg. No. 8359, Batch No. 46 and 47 and Sep-Oct 2007 Botany Reg. No. 8563, Batch No. 48) from Simana Darjeeling (West Bengal) India and authenticated by Botany Division of CDRI, Lucknow, where the specimens are preserved. The process comprises the steps of hot extraction of powdered whole plant material (1.0 Kg) of SC for 2 h with deionized boiling water (8L x4). The combined extract was filtered with fine cloth and the filtrate was concentrated under reduced pressure at 45°C till complete dryness. These hot water extracts (*i.e.* 46/A1, 47/A1 and 48/A1) were used for the biological evaluation and separation of fractions as well as isolation of compounds.

Isolation of fractions and compounds

The extract was fractionated with hot methanol (500 mL x 4) and combined fractions were concentrated (F2). The residue (F3) was then fractionated with 10% methanol in water 200 mLx 4) which on concentration to gave (F4) and residue (F5) was dried in oven at 50 °C for 4-5 h and then used for experiment. F2 was dissolved in methanol and concentrated up to half of its volume and kept in a conical flask at 10 °C for 24 h, a yellowish amorphous compound settled at

bottom of the flask which was filtered out and passed through Sephadex LH-20 column in Water (H₂O), H₂O-methanol (8:2, 6:4, 5:5, 4:6, 2:8) and methanol and after monitoring of eluted fractions on RP-18 TLC plate in H₂O: methanol and H₂O: acetonitrile (6:4 and 5:5) and TLC plates were sprayed in 5% ceric sulphate and developed by heating at 110 °C. MG was isolated after repeated crystallization in methanol to yield (MG) in 0.4% yield. The methanol fraction was subjected to repeated column chromatography over silica gel in 5-10% methanol-chloroform mixture yielded (AM) in 0.2% yield.

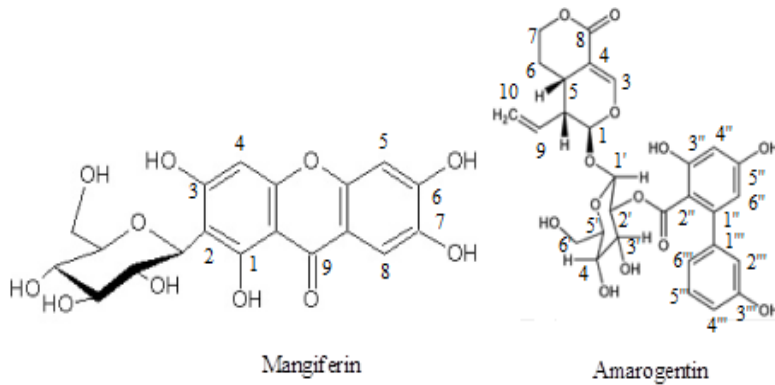


Fig. 1 Structure of Mangiferin and Amarogentin

Structure elucidation of these compounds (a) and (b) was carried out by spectroscopic techniques (see ¹H and ¹³C NMR spectra) and confirmed by comparison of its spectral data with that of reported in literature¹³.

Sample analysis LC-ESI-MS and MS/MS analysis

The LC-ESI-MS (Fig. 2) and MS/MS analysis of hot water extract (46/A001) of plant was performed on Waters TQD triple quadruple mass spectrometer (USA) equipped with Waters H-Class Acquity UPLC system and electron spray ionization source.

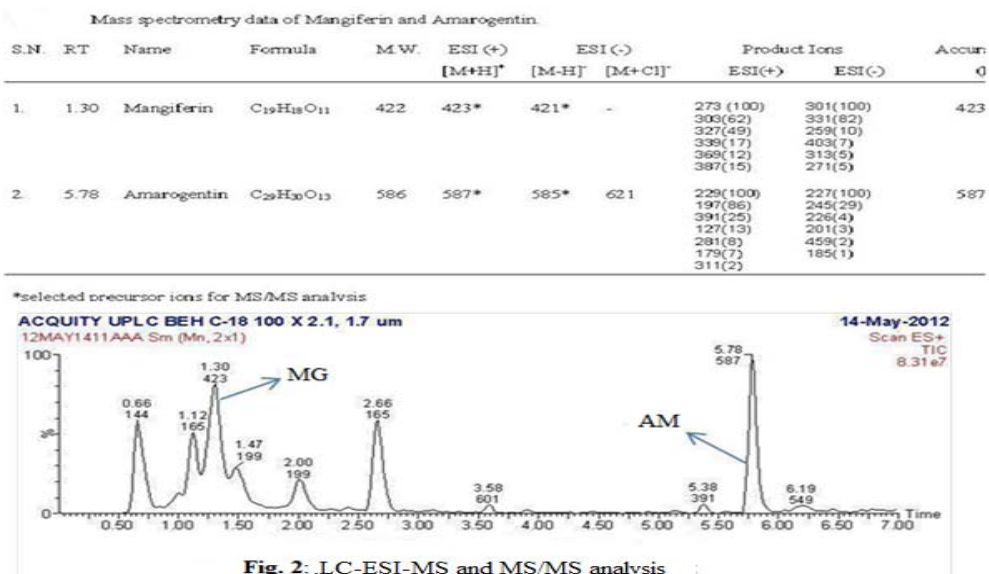


Fig. 2: LC-ESI-MS and MS/MS analysis

Mobile phase (A) acetonitrile and (B) 5 mM ammonium acetate buffer was used and linear gradient elution performed at the flow rate of 0.350 ml min⁻¹ (as 90-80% B in 0-4 min; 80-50% B in 4-6 min; 50-20% B in 6-8 min.). Waters BEH C-18 100 x 2.1 mm, 1.7µm column was used and ESI source potentials were capillary voltage 3.5 kV; cone potential at 30 V for LC-ESI-MS experiments. Nitrogen was used as the nebulizing and drying gas at flow rates of 50 and 650 L h⁻¹ respectively. Online collision induced dissociation (CID) experiments were performed at 25 eV and argon used as collision gas. Data acquisition and processing were carried out using Mass Lynx V4.1 SCN 714 software. The spectra were accumulated from the top of EIC (Extracted ion chromatogram) peak.

Experimental animals

Male adult rats of Charles Foster (CF) stain (100-150g) bred in the animal house of the institute were used after approval of Animal Ethics Committee (IAEC/2010/149). The animals were kept in controlled conditions of temperature (25-26 °C), relative humidity (60-80%) and 12/12h light/dark cycle (light from 8:00 am to 8:00pm) and provided with standard pellet diet (Lipton India Ltd) and water *ad libitum*.

Experiment design for antidyslipidemic activity

High fat diet induced (HFD) hyperlipidemia

The hyperlipidemia was produced by feeding with HFD once a day for 30 days. Drugs were administered (100 mg/kg body- wt.) orally simultaneously with HFD in drug treated groups. Control animals received the same amount of natural saline or ground nut oil. At the end of experiments rats were fasted overnight and blood was withdrawn. The animals were killed and the liver was excised immediately. The plasma was analyzed for Total cholesterol (TC), Triglyceride (TG), Phospholipids (PL) and LCAT (lecithin cholesterol acyl-transferase activity) and post heparin lipolytic activity (PHLA) activity.

Biochemical analysis of plasma/serum

LCAT activity¹⁴ and PHLA were assayed¹⁵. Serum was fractionated into VLDL, LDL and HDL by poly anionic precipitation methods¹⁶. Serums as well as lipoproteins were analyzed for their TC, PL, and TG by standard procedures reported earlier¹⁷.

Biochemical analysis of liver

Liver was homogenized (10% w/v) in cold 100mM phosphate buffer pH 7.2 and used for the assay of total lipolytic activity¹⁵. The lipid extract of each homogenates was used for estimation of TC, PL, TG and TP (total protein).

Faecal bile acids

The rat faeces were collected from all groups throughout 30 days and processed for estimation of cholic and deoxycholic acid^{18,19}.

Cholesterol biosynthesis

The effect of MG and AM on cholesterol biosynthesis was estimated as their effect on HMGR, in an *in vitro* experiment using protocol and reagents provided with standard test kit from Sigma Chemical Company.

Statistical analysis

Data were analyzed using student ‘t’ test. Hyperlipidemic groups were compared with control, hyperlipidemic and drug treated with hyperlipidemic group P<0.05 was considered as significance.

In vitro HMGR inhibitory activity

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

Molecular modeling

Three dimensional coordinates of MG and AM were extracted from PUBCHEM compound data base (Pub chem compound Id. 5464041 and 115149 respectively) for docking purposes against Human HMGR (pdb id: 1Hw9) using Flex X implemented in Sybyl 7.1²⁰. Flex X applies incremental building method for flexible docking of small molecules. Gasteiger-Huckel charges were assigned to all the ligands prior to docking calculation. All the values were kept default for docking studies. Docking parameters were validated through re docking of crystallized Simvastatin.

RESULTS AND DISCUSSION

Treatment of hyperlipidemic animals with MG and AM caused reduction in plasma levels of TC (20, 23%), TG (19, 25%), PL (21, 21%), TP (23, 20%), LDL (24, 26%), VLDL (19, 25%) and atherogenic index (35, 39%) as well as increase in levels of HDL (18, 22%), HDL/LDL ratio

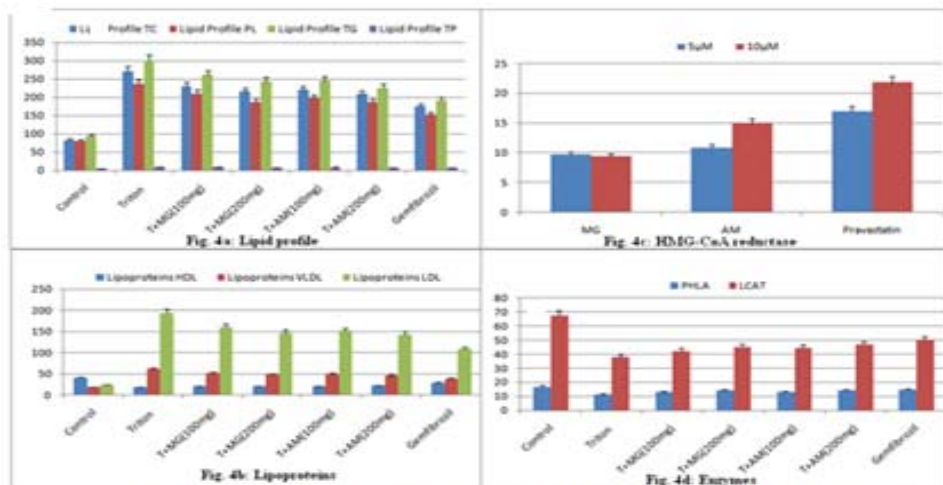


Fig 3 a:Effect of MG and AM on lipid, Fig 3 b:Effect of MG and AM on lipoprotein level of hyperlipidemic rats, Fig 3 c:Effect of MG and AM on HMG-Coa reductase Fig 3 d:Effect of MG and AM on lipolytic enzyme of plasma

respectively, at 200mg/kg doses (Fig. 3a, b). MG and AM have demonstrated better effect on HMGR inhibition at 5µM and at 10µM concentration in comparison to pravastatin, a potent inhibitor of HMGR (Fig. 3c) and activated LCAT (20, 24%) and PHLA (22, 21%), (Fig. 3d).

Effect of SC extract on high fat diet-induced dyslipidemia

Studies on MG and AM were carried out using HFD-induced hyperlipidemic rats. The HFD induced hyperlipidemic rats model is more suitable for study, because the rats consume the diet and water *ad libitum*, similar to human eating patterns, rather than being force-fed. The results of administration of HFD in rats (Table: A1) suggested an increased in their serum levels of TC, PL, and TG by 131%, 91% and 89% respectively.

Table A1: Effect of MG and AM on lipid composition in serum lipoproteins in HFD induced hyperlipidemic rats:

Parameters	Control	HFD treated	HFD+MG1	HFD+MG2	HFD+AM1	HFD+AM2	HFD + Gemfibrozil
(A1)Serum							
TG ^a	86.66±5.10	200.58±15.12***(+2.31F)	160.00±13.00**(-20)	169.00±11.82*(18)	150.00±15.00***(-25)	138.00±10.00(-31)	195.20±13.48***(-35)
PL ^a	86.00±7.70	168.80±12.30***(+1.91F)	130.00±8.90**(-22)	135.00±11.00**(-20)	125.00±12.00***(-25)	120.00±10.82(-29)	142.22±11.00***(-33)
TC ^a	106.88±9.0	201.93±14.44***(+1.88F)	145.20±12.11***(-28)	158.00±14.00**(-21)	143.00±14.00***(-29)	140.00±10.77(-31)	152.60±11.66***(-34)
Protein ^b	6.05±0.17	10.80±0.33***(+1.79F)	8.60±0.17**(-21)	8.70±0.74**(-20)	8.40±0.89***(-23)	7.60±0.40(-30)	8.48±0.32***(-30)
(A2) VLDL							
TC ^c	6.30±0.38	10.87±1.00***(+1.72F)	9.49±0.10*(-13)	9.43±0.35*(-13)	9.50±0.41*(-14)	9.10±0.27*(-16)	8.70±0.18*(-20)
PL ^c	10.00±0.70	18.14±1.03***(+1.81F)	14.97±1.30*(-17)	14.84±1.00*(-17)	14.62±0.88*(-19)	14.50±0.92**(-20)	14.20±0.78**(-22)
TG ^c	45.60±4.00	90.30±7.37***(+1.98F)	73.37±5.12*(-19)	70.76±6.00**(-21)	70.29±5.00**	69.61±4.12***(-18)	67.81±2.84***(-25)
AP ^d	6.30±0.50	12.12±1.80***(+1.92F)	9.56±0.60**(-21)	9.30±0.70(-23)	9.15±0.25***(-26)	9.00±0.38***(-26)	8.80±0.31***(-27)
(A3) LDL							
TC ^c	8.23±0.60	17.23±1.48***(+2.09F)	13.28±1.00**(-22)	13.00±0.79***(-24)	12.80±1.10***(-26)	12.50±1.18***(-27)	12.00±1.00***(-30)
PL ^c	8.14±0.47	15.73±1.77***(+1.93F)	13.20±1.18*(-16)	13.00±1.00*(-17)	12.83±0.79*(-18)	12.63±1.08**(-20)	12.50±0.69***(-21)
TG ^c	18.47±0.56	30.23±2.12***(+1.63F)	25.60±2.00*(-15)	25.40±1.87*(-16)	25.10±1.66*(-17)	24.85±1.99*(-17)	24.40±1.64***(-19)
AP ^d	17.56±1.07	28.62±2.24***(+1.62F)	24.00±2.00*(-16)	23.37±1.90*(-18)	23.10±1.79*(-17)	22.80±0.98**(-25)	22.20±1.43*(-22)
(A4) HDL							
TC ^c	45.38±4.00	38.14±2.80*(-16)	44.00±3.87*(+13)	39.88±3.00 ^{NS} (+4)	40.10±3.33 ^{NS} (+5)	40.33±3.00 ^{NS} (+5)	41.60±3.60 ^{NS} (+8)
PL ^c	37.40±3.14	28.60±2.00***(-24)	33.17±2.60*(+14)	34.10±2.70*(+16)	35.21±2.81*(+19)	35.28±2.17*(+19)	36.00±2.81**(+21)
TG ^c	15.10±1.00	12.10±0.17*(-20)	15.10±1.10*(+20)	15.00±1.15*(+19)	15.27±1.00**(+21)	15.30±1.08**(+21)	15.68±1.30***(+23)
AP ^d	168.20±12.20	120.30±13.00***(-28)	140.37±13.00*(+14)	143.88±13.70*(+16)	144.17±12.14*(+17)	144.20±12.44*(+1)	145.00±14.00*(+17)
(A5)Plasma							
LCAT ^e Activity	68.00±6.00	38.81±3.00***(-42)	48.40±4.00*(+20)	49.16±3.87**(+21)	49.66±3.18**(+22)	50.00±3.97**(+22)	52.88±4.39***(+26)
PHLA ^e	17.80±1.00	10.00±0.81***(-44)	13.85±0.87**(+28)	14.00±1.00***(+29)	14.17±0.79***(+29)	15.00±1.48***(+3)	15.79±1.30***(+37)

Units-(a) mg/dl serum, (b) g/dl, (c) n mol cholesterol released/h/L plasma. (d) n mol free fatty acid formed/hours/ml plasmid value are mean± SD of six ratio P<0.005; P<0.01; P<0.05; HFD compound with control, cholesterol and drug treated with cholesterol. MG 1(100mg/kg),MG 2 (200mg/kg), AM1(100mg/kg), AM2(200mg/kg).

Feeding with these extracts, fractions, MG, AM and gemfibrozil reversed the level of these serum lipids (18-31%) in HFD and drug treated animals. The analysis of hyperlipidemic serum showed a marked increase in the levels of lipids and Apo proteins constituting β -lipoproteins and those effects were pronounced for VLDL-TG (198%) and LDL-TC (209%) and increased in level. Treatment with these test samples and gemfibrozil significantly reduced these levels of VLDL lipids (17-26% and 20-27%) as well as LDL-TC (22-27% and 30%), PL (16-20% and 27%), TG (15-17% and 19%) and Apo-LDL (16-20% and 22%) respectively (Table A2, A3), and at the same time also recovered in level of HDL and Apo-HDL in hyperlipidemic rats. HFD feeding caused the inhibition of plasma LCAT (44%), PHLA (41%) (Table A5), total lipolytic activity (45%) in liver and caused a significant decrease in faecal excretion of cholic acid (42%) and deoxycholic acid (56%). Treatment with compounds lowered the level of TC, PL, TG and protein in liver (52%, 55%, 52% and 45%) and simultaneously also showed to recovered the level of cholic and deoxycholic acid by MG and AM (21-23% and 33-38%) and gemfibrozil (26 and 41%) in HFD and drug fed animals.

Docking studies

It proposes numerous conserved interactions between MG and AM and receptor. Both the MG and AM bind in same active site with several polar as well as hydrophobic interactions with the active site residues that are also consistent with the binding of co-crystallized ligand simvastatin. Polar side chains of Glu559, Arg590, Asp690 and Asn658 are found to be steadily involved in polar interactions with both MG and AM molecules while major hydrophobic interactions are contributed by Met657, Leu853, Ala751, Ala856 (Fig. 6a).

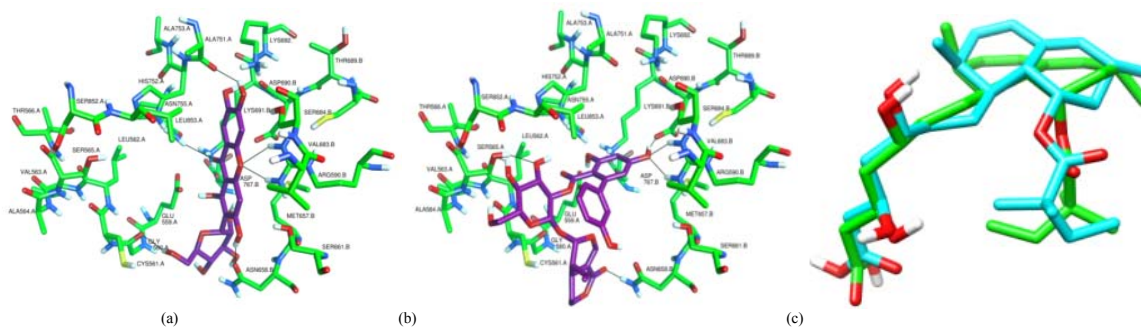


Fig. 6 Intermolecular interactions of active site residues of HMG- CoA reductase (PDB id 1HW9) with docked conformation of (a) Mangiferin and (b) Amarogentin (c) Superimposition of docked simvastatin (in cyan) over co-crystallized simvastatin (in green) with HMG- CoA reductase (1HW9).

Several polar interactions are found with residues Asp690, Lys691 and Ser684 which belongs to cis-loop, which is essential for the formation of HMG-binding site (Fig. 6b). Flex X docking score (Energy) obtained was MG (-30.589) and AM (-15.708).

CONCLUSION

In conclusion, we observed that standardized hot water extracts from different collection and two major markers (MG) and (AM) of SC was effective *in-vivo* antidyslipidemic activity in both Triton and HFD-induced hyperlipidemic rat model. Treatment of hyperlipidemic rats with MG and AM caused reduction in plasma levels of LDL and VLDL as well as increase in levels of HDL was also observed. In rats with HFD, MG and AM significantly reduced levels of VLDL lipids as well as LDL-TC in hyperlipidemic rats at 100 and 200 mg/kg doses. We report Amarogentin has demonstrated new property of antidyslipidemic agent. MG and AM also showed significant inhibition of superoxide anion, hydroxyl free radicals and microsomal lipid peroxidation. In comparison to Pravastatin, MG and AM are found to be potential cholesterol lowering agents as they demonstrated much better inhibition of HMGR. The docking results suggests, modifications in MG and AM structure to improve its HMGR activity, which will be useful to the structure -based design of new chemical entities of MG and AM. Thus, our results support that *Swertia chirayita* hot water extract has a therapeutic potential for treatment of cardiovascular disease and its related disorders.

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