ORIGINAL RESEARCH

Antihyperlipidemic studies of newly synthesized phenolic derivatives: in silico and in vivo approaches

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Background: Hyperlipidemia is a worth-mentioning risk factor in quickly expanding cardiovascular diseases, including myocardial infarction and, furthermore, in stroke.

Methods: The present work describes the synthesis of phenolic derivatives **4a–e** and **6a–c** with the aim of developing antihyperlipidemic agents. The structures of the synthesized compounds were confirmed by spectroscopic data. The in silico docking studies were performed against human 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase enzyme (PDB ID: 1HWK), and it was observed that compounds **4a** and **6a** exhibited maximum binding affinity with target protein having binding energies −8.3 and −7.9 kcal, respectively.

Results: Compound **4a** interacts with amino acids Val805 with distance 1.89 Å and Met656, Thr558, and Glu559 with bonding distances 2.96, 2.70, and 2.20 Å, respectively. The in vivo antihyperlipidemic activity results revealed that compound **4a** indicated minimum weight increment, ie, 20% compared with 35% weight increment with standard drug atorvastatin during 6 weeks of treatment. Moreover, increment in high-density lipoprotein cholesterol and decrease in total cholesterol, low-density lipoprotein cholesterol, and triglyceride levels were more prominent in case of **4a** compared to atorvastatin with $P<0.05$. The synthesized compounds were nontoxic and well tolerated because none of the mice were found to suffer from any kind of morbidity and death during 6 weeks of dosing.

Conclusion: Based on our pharmacological evaluation, we may propose that compound **4a** may act as a lead structure for the design and development of more potent antihyperlipidemic drugs.

Keywords: phenolic derivatives, synthesis, antihyperlipidemic, in silico docking, HMG CoA reductase, atorvastatin

Introduction

Human beings are persistently confronting life-threatening morbidities such as cardiovascular disorders and cancer. Cardiovascular diseases account for ~17 million deaths annually worldwide. Above all, atherosclerosis is the major factor for the pathogenesis of myocardial and cerebral infarctions. Increased plasma low-density lipoproteins and triglycerides along with low levels of high-density lipoproteins are often the major root cause of atherosclerosis and ultimately myocardial and cerebral infarctions. Correspondingly, obesity has a direct linkage with atherosclerosis and myocardial infarction. Exact etiology of obesity is relatively unclear, but still the major contributors are genetic, environmental, and dietary factors.[1]

To counter these morbidities, an efficacious and safer pharmacological solution is of most extreme significance. The process of drug discovery is fastidious and expensive. The quest for new effective molecules is challenging yet the accessibility of various

docking tools and computer-aided methodologies has limited the gap to a specific level.[2,3] Antioxidants have phenolic flavonoids that scavenge oxygen radicals and are involved in the inhibition of enzyme activity. Phenolic compounds are auxiliary metabolites present in fruits and vegetables.[4,5] They possess diverse pharmacological activities including cardiovascular,[6] antioxidant,[5–9] antiatherogenic,[10,11] anti-inflammatory,[12–16] and antithrombotic.[17–19] Various studies on the experimental animals reported the effect of phenolic compounds such as quercetin and rutinosides on the lipid metabolism causing a decrease in the serum lipid levels.[20,21]

Derivatives of natural poly-phenols have shown remarkable bioactivity in various disease areas both in prevention and treatment. Among numerous classes of polyphenols, hydroxycinnamic acid derivative is the renowned group of phenols found in cereals, roasted coffee, and green vegetables and is reported as a potential antioxidant.[22–24] Sinapic acid and ferulic acid are the most studied hydroxycinnamic acid derivatives and were found to be highly effective as antioxidant,[25,26] and in recent data, they are found to be preventive and therapeutic agents in several other diseases including atherosclerosis, inflammatory injury, and cancer.[27,28] Caffeic acid derivatives are another class of biologically active phenolic derivatives as antioxidant[29,30] and anticancer.[31–33]

Recently, few studies have demonstrated polyphenolic moieties as potential antihyperlipidemic agents by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) reductase in in silico models.[34] Considering such enormous pharmacological potential, derivatives of natural phenols are exciting prospect for future drug development especially in antihyperlipidemic and cardiovascular therapeutic domains. The use of computational analysis to establish the binding affinity of the drug-like compounds with different enzymes, receptors, and proteins is categorized in in silico pharmacology.[35] The in silico methods have been frequently used in the discovery and optimization of novel molecules with affinity to a target and in the optimization of pharmacokinetic parameters.[36,37] Various studies have been reported in which the in silico assessment of the drug-like compounds showed the binding affinity with enzymes, proteins, transporter proteins, and receptors and is very well correlated with their in vitro and in vivo pharmacological effects.[38] Consequently, the in silico pharmacology paradigm is ongoing and presents a vast platform for the discovery of new drug targets as well as screening of new drug-like compounds that can be considered as drug.

In this perspective, current study is aimed to develop potent antihyperlipidimic agents. The phenolic derivatives **4a–e** and **6a–c** have been synthesized and characterized; the in vivo antihyperlipidemic evaluation was carried out.

The in silico docking studies against HMG CoA reductase enzyme (PDB ID: 1HWK) were also performed to compare the dry laboratory results with wet laboratory findings.

Materials and methods
### Drugs and chemicals
Atorvastatin was obtained from Global Pharmaceuticals (Islamabad, Pakistan), and mice were received as gift from National Institute of Health (Islamabad, Pakistan).

Synthesis of 2-methoxyphenyl chloroacetate (2)
The 2-methoxyphenol (**1**) (0.01 mol) was dissolved in anhydrous dichloromethane (25 mL), and triethylamine (0.01 mol) was added slowly. The resulting solution was then cooled in an ice salt mixture to 0 to −5°C. The chloroacetyl chloride (0.01 mol) in dry dichloromethane was added drop wise to the reaction mixture with constant stirring over a period of 1 h maintaining the temperature constant. The reaction mixture was then stirred at room temperature for further 5 h and washed with 5% HCl and 5% sodium hydroxide solution. The organic layer was washed with saturated aqueous NaCl, dried over anhydrous magnesium sulfate and filtered, and the solvent was removed under reduced pressure. The crude product was purified by silica gel column to afford the corresponding 2-methoxyphenyl chloroacetate (**2**): melting point, 56°C–58°C; reaction time, 4 h; yield, 78%; R_f, 0.62 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 2,903 (sp2 C–H), 2,863 (sp3 C–H), 1,719 (C=O ester), 1,589 (C=C aromatic), 1,164 (C–O ester).

General procedure for the synthesis of title compounds **4a–4e** and **6a–6c**
The substituted benzoic acids (**3a–3e**) (0.01 mol), triethyl amine (0.01 mol), and potassium iodide (0.01 mol) were mixed in dimethylformamide (25 mL) and stirred at room temperature. The 2-methoxyphenyl chloroacetate intermediate (**2**) was then added to the reaction mixture slowly and at the same temperature overnight (Scheme 1). The final products (**4a–4e**) were then extracted with ethyl acetate (3×25 mL). The combined ethyl acetate layer was then washed with 5% HCl, 5% sodium carbonate, and finally brine solution. The organic layer was dried over anhydrous magnesium sulfate and filtered, and the solvent was removed under reduced pressure to afford the crude products (**4a–4e**). The title compounds (**4a–4e**) were purified by silica gel column chromatography (n-hexane:ethyl acetate 3:1). The same procedure was used for the preparation of final products (**6a–6c**) (Scheme 2).
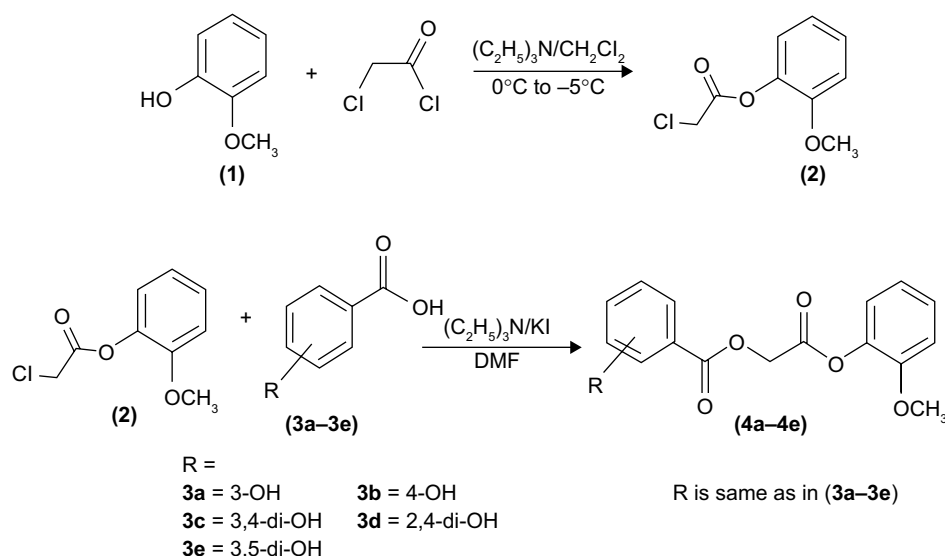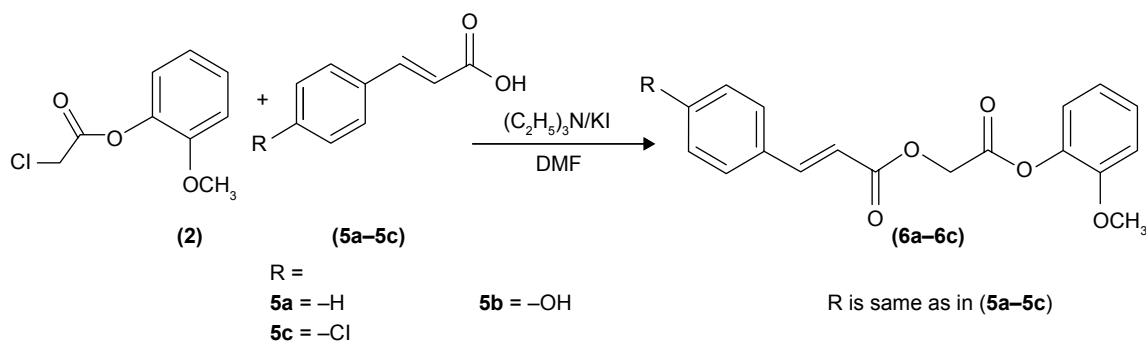
Scheme 1 Synthesis of phenolic derivatives (**4a–4e**).

2-(2-Methoxyphenoxy)-2-oxoethyl 3-hydroxybenzoate (**4a**): solid; melting point, 81°C–83°C; yield, 76%; R_f, 0.56 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,248 (O–H), 3,965 (sp2 C–H), 2,864 (sp3 C–H), 1,723 (C=O ester), 1,595 (C=C aromatic), 1,164 (C–O ester); ^{1}H NMR (CDCl$_3$, δ ppm): 7.69 (m, 1H, H-6), 7.58 (dd, J=1.6, 1.6 Hz, 1H, H-2), 7.33 (dd, J=4.8, 4.4 Hz, 1H, H-5), 7.24 (m, 1H, H-4), 7.12 (dd, J=6.4, 1.6 Hz, 1H, H-6′), 7.07 (dd, J=3.6, 1.8 Hz, 1H, H-3′), 6.95–6.99 (m, 2H, H-4′, H-5′), 5.65 (s, 1H, –OH), 5.10 (s, 2H, –CH$_2$), 3.85 (s, 3H, –OCH$_3$); ^{13}C NMR (CDCl$_3$, δ ppm); 166.1 (C=O ester), 165.7 (C=O ester), 155.8 (C-3), 150.9 (C-2′), 139.0 (C-1′), 130.4 (C-5), 129.7 (C-6), 127.3 (C-2), 122.6 (C-1), 122.3 (C-4), 121.4 (C-4′), 120.8 (C-6′), 116.6 (C-3′), 114.5 (C-5′), 61.0 (–CH$_2$), 55.8 (–OCH$_3$); anal calcd for C$_{16}$H$_{14}$O$_6$: C, 63.57; H, 4.63; found C, 63.64; H, 4.71.

2-(2-Methoxyphenoxy)-2-oxoethyl 4-hydroxybenzoate (**4b**): solid; melting point, 126°C–128°C; yield, 78%; R_f, 0.53 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,149 (O–H), 2,932 (sp2 C–H), 2,842 (sp3 C–H), 1,718 (C=O ester), 1,593 (C=C aromatic), 1,148 (C–O, ester); ^{1}H NMR

(DMSO-d_6, δ ppm): 7.90 (d, J=8.8 Hz, 2H, H-2, H-6), 7.26 (ddd, J=6.8, 1.6, 1.4 Hz, 1H, H-4′), 7.11–7.16 (m, 2H, H-3′, H-6′), 6.97 (ddd, J=6.4, 1.6, 1.21H, H-5′), 6.90 (d, J=8.8 Hz, 2H, H-3, H-5), 5.15 (s, 2H, –CH$_2$), 3.78 (s, 3H, –OCH$_3$), 3.33 (s, 1H, –OH); ^{13}C NMR (DMSO-d_6, δ ppm): 166.8 (C=O ester), 165.4 (C=O ester), 162.9 (C-4), 151.1 (C-2′), 139.0 (C-1′), 132.2 (C-2, C–), 127.7 (C-5′), 123.0 (C-1), 121.3 (C-3′), 119.6 (C-4′), 116.0 (C-3, C-5), 113.5 (C-6′), 60.9 (–CH$_2$), 56.3 (–OCH$_3$); anal calcd for C$_{16}$H$_{14}$O$_6$: C, 63.57; H, 4.63; found C, 63.66; H, 4.73.

2-(2-Methoxyphenoxy)-2-oxoethyl 3,4-dihydroxy-benzoate (**4c**): solid; melting point, 154°C–156°C; yield, 72%; R_f, 0.48 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,166 (O–H), 2,935 (sp2 C–H), 2,837 (sp3 C–H), 1,722 (C=O ester), 1,600 (C=C aromatic), 1,148 (C–O ester); ^{1}H NMR (DMSO-d_6, δ ppm): 7.43 (d, J=2.0 Hz, 1H, H-2), 7.39 (dd, J=6.0, 2.0 Hz, 1H, H-6), 7.25 (ddd, J=3.6, 2.0, 1.6 Hz, 1H, H-4′), 7.12–7.16 (m, 2H, H-3′, H-6′), 6.97 (ddd, J=4.0, 2.4, 1.6 Hz, 1H, H-5′), 6.84 (d, J=8.0 Hz, 1H, H-5), 5.13 (s, 2H, –CH$_2$), 3.80 (s, 3H, –OCH$_3$),

Scheme 2 Synthesis of phenolic derivatives (**6a–6c**).

3.42 (s, 2H, −OH); ^{13}C NMR (DMSO-d_6, δ ppm): 171.4 (C=O ester), 166.8 (C=O ester), 165.5 (C-4), 151.4 (C-3), 151.1 (C-2′), 148.1 (C-1′), 139.0 (C-5′), 127.9 (C-6), 123.2 (C-4′), 122.7 (C-3′), 121.1 (C-6′), 119.8 (C-2), 116.9 (C-5), 113.5 (C-1), 60.9 (−CH$_2$), 56.1 (−OCH$_3$); anal calcd for C$_{16}$H$_{14}$O$_7$: C, 60.37; H, 4.40; found C, 60.44; H, 4.46.

2-(2-Methoxyphenoxy)-2-oxoethyl 2,4-dihydroxybenzoate (**4d**): solid; melting point, 109°C–111°C; yield, 76%; R_f, 0.46 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,263 (O−H), 2,942 (sp2 C−H), 2,864 (sp3 C−H), 1,726 (C=O ester), 1,606 (C=C aromatic), 1,162 (C−O ester); ^{1}H NMR (DMSO-d_6, δ ppm): 7.73 (d, J=8.8 Hz, 1H, H-6), 7.24–7.29 (m, 2H, H-3′, H-6′), 7.15 (ddd, J=6.4, 5.6, 1.6, Hz, 1H, H-4′), 6.98 (ddd, J=5.2, 3.6, 2.0, Hz, 1H, H-5′), 6.43 (dd, J=6.4, 2.4 Hz, 1H, H-3), 6.34 (d, J=2.0 Hz, 1H, H-5), 5.20 (s, 2H, −CH$_2$), 3.78 (s, 3H, −OCH$_3$), 3.44 (s, 2H, −OH); ^{13}C NMR (DMSO-d_6, δ ppm): 168.3 (C=O ester), 166.5 (C=O ester), 165.1 (C-2), 163.2 (C-4), 151.1 (C-2′), 139.0 (C-1′), 132.5 (C-5′), 127.8 (C-6), 123.2 (C-4′), 121.3 (C-3′), 119.6 (C-6′), 116.0 (C-3), 109.0 (C-5), 103.1 (C-1), 61.1 (−CH$_2$), 56.1 (−OCH$_3$); anal calcd for C$_{16}$H$_{14}$O$_7$: C, 60.37; H, 4.40; found C, 60.41; H, 4.48.

2-(2-Methoxyphenoxy)-2-oxoethyl 3,5-dihydroxybenzoate (**4e**): solid; melting point, 173°C–175°C; yield, 84%; R_f, 0.45 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,134 (O−H), 2,919 (sp2 C−H), 2,838 (sp3 C−H), 1,729 (C=O ester), 1,608 (C=C aliphatic), 1,598 (C=C aromatic), 1,145 (C−O, ester); ^{1}H NMR (DMSO-d_6, δ ppm): 7.27 (ddd, J=5.6, 3.6, 1.6 Hz, 1H, H-4′), 7.12–7.15 (m, 2H, H-3′, H-6′), 6.96 (ddd, J=6.4, 6.4, 2.4 Hz, 1H, H-5′), 6.89 (d, J=2.4 Hz, 2H, H-2, H-6), 6.48 (dd, J=2.4, 1.6 Hz, 1H, H-4), 5.17 (s, 2H, −CH$_2$), 3.78 (s, 3H, −OCH$_3$), 3.33 (s, 2H, −OH); ^{13}C NMR (DMSO-d_6, δ ppm); 166.6 (C=O ester), 165.6 (C=O ester), 159.1 (C-3, C-5), 151.1 (C-2′), 139.0 (C-1′), 130.7 (C-5′), 127.8 (C-2, C-6), 123.0 (C-4′), 121.1 (C-3′), 113.5 (C-6′), 108.2 (C-4), 107.8 (C-1), 61.3 (−CH$_2$), 56.3 (C-1″); anal calcd for C$_{16}$H$_{14}$O$_7$: C, 60.37; H, 4.40; found C, 60.43; H, 4.47.

2-(2-Methoxyphenoxy)-2-oxoethyl (2E)-3-phenylprop-2-enoate (**6a**): solid; melting point, 76°C–78°C; yield, 85%; R_f, 0.56 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 2,938 (sp2 C−H), 2,835 (sp3 C−H), 1,719 (C=O ester), 1,599 (C=C aromatic), 1,164 (C−O, ester); ^{1}H NMR (CDCl$_3$, δ ppm): 7.85 (d, J=16.0 Hz, 1H, H-2), 7.58 (dd, J=4.4, 1.6 Hz, 2H, H-2′, 6′), 7.41–7.43 (m, 3H, H-3′, H-4′, H-5′), 7.24 (dd, J=6.0, 2.4 Hz, 1H, H-3″), 7.13 (dd, J=6.4, 2.4 Hz, 1H, H-4″), 6.96–6.99 (m, 2H, H-4″, H-5″), 6.56 (d, J=16.0 Hz, 1H, H-1), 5.04 (s, 2H, −CH$_2$), 3.86 (s, 3H, −OCH$_3$); ^{13}C NMR

(CDCl$_3$, δ ppm); 167.7 (C=O ester), 166.1 (C=O ester), 150.9 (C-2″), 146.3 (C-2), 139.2 (C-1″), 134.1 (C-5″), 130.0 (C-2′, C-6′), 128.9 (C-3′, C-5′), 128.2 (C-4′), 127.2 (C-1′), 122.6 (C-3″), 120.7 (C-4″), 116.7 (C-6″), 112.5 (C-1), 60.5 (−CH$_2$), 55.9 (−OCH$_3$); anal calcd for C$_{18}$H$_{16}$O$_5$: C, 69.23; H, 5.12; found C, 69.29; H, 5.17.

2-(2-Methoxyphenoxy)-2-oxoethyl (2E)-3-(4-hydroxyphenyl)prop-2-enoate (**6b**): solid; melting point, 101°C–103°C; yield, 78%; R_f, 0.47 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 3,132 (−OH), 2,931 (sp2 C−H), 2,849 (sp3 C−H), 1,728 (C=O), 1,609 (C=C aromatic), 1,148 (C−O ester); ^{1}H NMR (DMSO-d_6, δ ppm): 7.70 (d, J=16.0 Hz, 1H, H-2), 7.62 (d, J=8.8 Hz, 2H, H-2′, 6′), 7.26 (ddd, J=7.6, 6.0, 1.6 Hz, 1H, H-4″), 7.11–7.16 (m, 2H, H-3″, H-6″), 6.98 (ddd, J=7.6, 6.0, 1.6 Hz, 1H, H-5″), 6.80 (d, J=8.8 Hz, 2H, H-3′, H-5′), 6.54 (d, J=16.0 Hz, 1H, H-1), 5.06 (s, 2H, −CH$_2$), 3.78 (s, 3H, −OCH$_3$), 3.33 (s, 1H, −OH); ^{13}C NMR (DMSO-d_6, δ ppm): 166.1 (C=O ester), 165.8 (C=O ester), 150.9 (C-2″), 144.8 (C-2), 139.1 (C-1″), 132.6 (C-4′), 129.5 (C-2′, C-6′), 129.3 (C-3′, C-5′), 127.2 (C-5″), 122.7 (C-1′), 117.4 (C-3″), 112.6 (C-4″), 112.5 (C-6″), 60.6 (−CH$_2$), 55.9 (−OCH$_3$); anal calcd for C$_{18}$H$_{16}$O$_6$: C, 65.85; H, 4.87; found C, 65.93; H, 4.93.

2-(2-Methoxyphenoxy)-2-oxoethyl (2E)-3-(4-chlorophenyl)prop-2-enoate (**6c**): solid; melting point, 73°C–75°C; yield, 83%; R_f, 0.55 (n-hexane:ethyl acetate 3:1); FTIR v_{max} (1/cm): 2,919 (sp2 C−H), 2,862 (sp3 C−H), 1,725 (C=O ester), 1,611 (C=C aromatic), 1,156 (C−O ester); ^{1}H NMR (CDCl$_3$, δ ppm): 7.79 (d, J=16.0 Hz, 1H, H-2), 7.50 (d, J=7.6 Hz, 2H, H-2′, 6′), 7.43 (d, J=7.6 Hz, 2H, H-3′, H-5′), 7.24 (dd, J=6.0, 1.6 Hz, 1H, H-3″), 7.11 (dd, J=6.4, 1.6 Hz, 1H, H-6″), 6.95–6.99 (m, 2H, H-4″, H-5″), 6.57 (d, J=16.0 Hz, 1H, H-1), 5.06 (s, 2H, −CH$_2$), 3.86 (s, 3H, −OCH$_3$); ^{13}C NMR (CDCl$_3$, δ ppm); 166.1 (C=O ester), 165.8 (C=O ester), 151.0 (C-2″), 144.9 (C-2), 139.1 (C-1″), 136.5 (C-4′), 132.6 (C-2′, C-6′), 129.5 (C-3′, C-5′), 127.3 (C-5″), 122.7 (C-1′), 121.6 (C-3″), 120.7 (C-4″), 117.3 (C-6″), 60.6 (−CH$_2$), 56.0 (−OCH$_3$); anal calcd for C$_{18}$H$_{15}$O$_5$Cl: C, 62.33; H, 4.32; found C, 62.41; H, 4.39.

Antihyperlipidemic studies

An aggregate of 66 mice (Balb C, male, 25–30 g, aged 4–6 weeks) was obtained, kept in National Institute of Health, and left to be acclimatized for 1 week before starting the investigation. Animal experiments were performed in accordance with the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (1996), approved by Ethical Committee

of Riphah Institute of Pharmaceutical Sciences (ref no REC/RIPS/2017/001). Mice were divided into 11 groups, each having six mice. Mice were kept in normal laboratory conditions of temperature 23°C±1°C and ambient humidity 55%±5%. Mice were housed in stainless steel cages individually and kept in an isolated room. Lights were maintained on an artificial 12 h light–dark cycle. All mice were weighed weekly during 6 weeks of experimental period. Animal care was in accordance with the guidelines established and approved by the National Institute of Health. Mice were fed with high-fat diet (HFD) mentioned in Table 1 containing cholesterol, cholic acid, peanut oil, and normal laboratory diet for 6 weeks. Standard drug (atorvastatin in this study) and test compounds from **4a–e** and **6a–c** were mixed with HFD, and the composition of the test compounds was 10 mg/kg. Experimental compounds from **4a–e** and **6a–c** were mixed with HFD, and the dose of the test compounds was 10 mg/kg. Group I received normal laboratory diet, Group II received HFD, Group III received standard drug (atorvastatin) along with HFD, and Groups IV–XI received test compounds from **4a–e** and **6a–c**, respectively, along with HFD. Mice had free access to feed and water throughout the study. The specified quantity of test compounds and standard was dissolved in 1 mL of DMSO and mixed in pellet diet. All mice were weighed weekly during 6 weeks of experimental period. The activity was continued for 6 weeks under controlled laboratory conditions (temperature 23°C±1°C and ambient humidity 55%±5%).[39]

Blood collection and analysis

At the end of the experiment, the animals were anesthetized. Blood samples were collected from the retro orbital vein. The serum was separated by centrifugation at $2,500 \times g$ for 15 min at 4°C. The amount of serum triglyceride, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were assayed automatically using an ADVIA 1650 lipid analyzer (Bayer, Wuppertal, Germany).

Table 1 Fat diet composition

Ingredients	Percentage
Cholesterol	2
Cholic acid	1
Peanut oil	5
Wheat flour	27
Wheat baran	27
Dry skimmed milk	20
Fish meal	15
Common salt	0.5
Molasses	2
Mineral + vitamin mix	0.5

Histopathological evaluation

Animals were euthanized under ether anesthesia, and the liver was dissected out immediately and weighed. For histopathological analysis, the liver and adipose tissues were fixed in 10% formalin at room temperature. Adipose tissues used were collected from subcutaneous fat of the abdomen of mice. The tissue was embedded in paraffin, sectioned into 3–4 µm thickness and mounted on the glass microscope slides using standard histological techniques. The sections were stained with hematoxylin–eosin and examined using light microscopy at 200× magnitudes. These light microscopic fields were assessed by an image analyzer on each section.[40]

Docking studies

Acquisition and preparation of target protein

Crystal structure of HMG CoA reductase was acquired from Protein Data Bank as PDB format (PDB ID: 1HWK-Homosapien) (www.rcsb.org/pdb).[41] Crystal structure of target protein was validated using the MolProbity server.[42] Ligands and water molecules were removed from crystal structure using the Accelrys Discovery Studio, and the energy of protein was minimized using the UCSF Chimera.[43] Active binding site studies were viewed using the PyMOL (Python molecular visualization) software.[44]

Preparation of ligand

Structures of phenolic experimental compounds were drawn in MarvinSketch tools and were cleaned into 3D using Marvin Space and were downloaded in the PDB file extension.[45]

Docking run

Docking is a virtual screening tool used to predict in silico interaction of ligand with enzyme and its possible inhibition. Both ligand and target protein were converted from PDB to PDBQT files after the addition of hydrogen and removal of water molecules. Energies of ligand and enzyme were minimized using the UCSF Chimera. These prepared molecules were uploaded as ligand, macromolecules in PyRx, a Python-based AutoDock Vina-based scoring function screening tool, autogrid dimension were calculated for HMG CoA reductase ($X=147.451$, $Y=87.546$, and $Z=3.341$), and inhibitor and target were geometrically optimized and docked.[46,47]

Statistical analysis

For the determination of change in body weight, liver weight, plasma lipids, and HDL and LDL-cholesterol levels, individual mouse was considered as an experimental unit. All data were analyzed by IBM SPSS 20 package for one-way
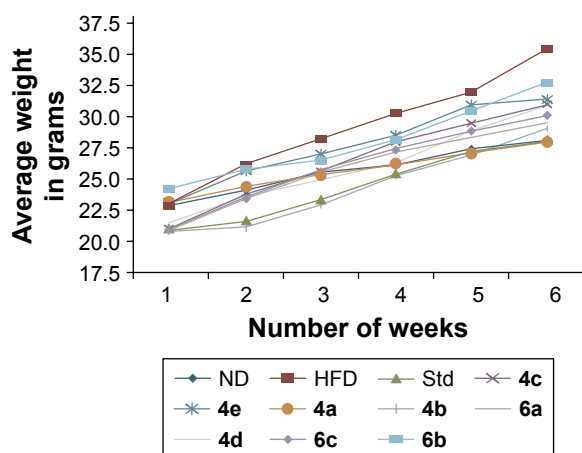
Figure 1 Impact of phenolic derivatives on body weight changes on mice supplemented with high-fat diet.
Note: Mean weight in each group is given (n=6 for each group).
Abbreviations: ND, normal diet; HFD, high-fat diet; Std, reference standard (atorvastatin).

analysis of variance (ANOVA) testing; post hoc Tukey test was used with P-value <0.05 considering as statistically significant.[48]

Results and discussion
Body weights

Among dietary factors, an increase in carbohydrate and fats in diet has been associated with obesity.[49] Similarly, few researchers have reported that the differences among strains with the ability of HFD to promote obesity have been demonstrated in both rats and mice.[32] Some strains of mice such as A/J and C57BL do develop obesity when fed on HFD. Meanwhile, SWR mice were resistant to develop obesity

when fed on similar HFD. In the present study, mice fed with a HFD demonstrated predictable weight, cholesterol, triglycerides, and LDL increment with a decrease in HDL levels after 6 weeks in the hyperlipidemic control group (HFD group) (Figure 1). After 6 weeks, mice fed with **4a** experimental compound indicated least weight increase, ie, 20% compared with 35% weight increase with atorvastatin. Even **4e**, **6a**, and **6b** showed comparable weight increment vs atorvastatin, ie, 36, and 35%, respectively (Figure 2). These results propose that weight increment in case of **4a** was significantly ($P<0.05$) less than reference standard and increase in mice weight in case of **4e**, **6a**, and **6b** was insignificant but comparable with reference standard (Figure 2).

Plasma lipid profile

As already discussed, an increase in serum cholesterol and LDL levels may lead to atherosclerosis and ultimately to myocardial infarction. Raised serum triglycerides (TGs) have also been reported to have a role in the aggravation of atherosclerosis.[50] Meanwhile various studies have shown that drugs decreasing serum TG, LDL, and cholesterol levels reduce the risk of coronary artery diseases.[51] Various phenols including gallic acid and linoleic acid have been found to be antiatherogenic especially in in vivo green tea extracts reducing serum TGs and cholesterol as well as stimulating energy expenditure, fat oxidation, and fecal lipid oxidation.[52]

In this study, it was observed that all experimental molecules were able to decrease cholesterol, LDL, and triglycerides and increase HDL in hyperlipidemic mice, but in particular, **4a** showed highly significant reduction in serum
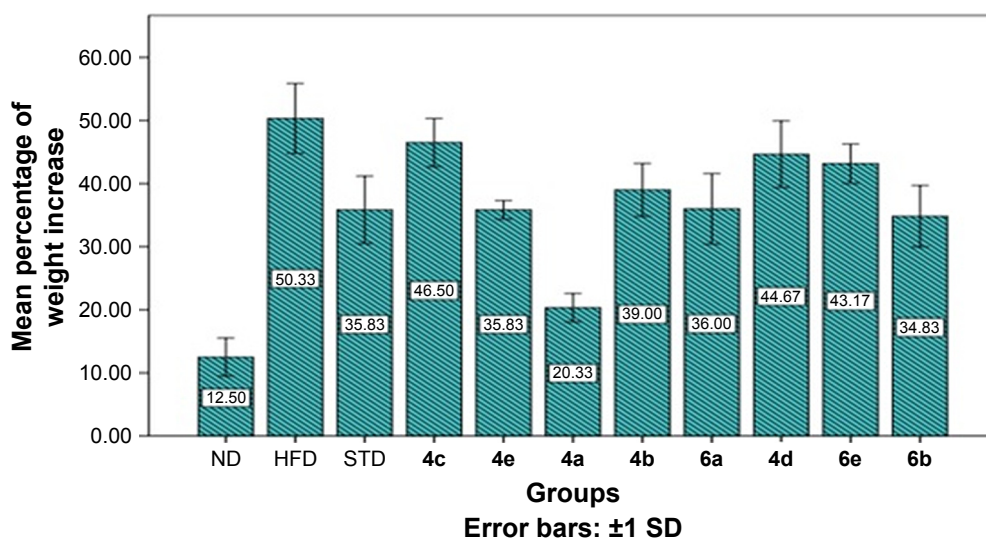

Figure 2 Effect of phenolic derivatives on body weight of mice fed on high-fat diet compared with standard (Atorvastatin) and high-fat diet alone.
Note: Mean weight ± SD (n=6 in each group).
Abbreviations: ND, normal diet; HFD, high-fat diet; STD, reference standard (atorvastatin).

Table 2 Effect of phenolic derivatives on lipid lowering efficiency in mice (n=6, mean ± SD) compared with normal control, hyperlipidemic control, and standard drug (atorvastatin)

Group	Dose (mg/kg)	Cholesterol (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
Normal control	–	260±7.45	127±5.10	128±6.90	82±4.14
Hyperlipidemic control	–	386±12.93	132±4.60	317±7.09	66±3.52
Atorvastatin	10	165±7.54	90±4.09	89±4.73	84±2.85
4a	10	150±4.79*	84±4.09	64±4.69**	96±4.35*
4b	10	171±4.47	86±5.10	54±4.56**	71±5.49
4c	10	176±6.41	86±4.43	57±4.35	69±4.64
4d	10	179±7.49	88±5.68	61±3.89	70±2.82
4e	10	236±6.80	95±4.16	88±4.02	67±4.16
6a	10	148±4.38*	80±2.48**	63±4.00	68±8.37**
6b	10	150±3.60**	85±3.68	56±4.85	77±4.04**
6c	10	156±3.65	81±3.31**	58±2.09	80±3.46**

Notes: *$P<0.01$ vs standard drug (atorvastatin). **$P<0.05$ vs standard drug (atorvastatin).
Abbreviations: TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

cholesterol compared to standard with $P<0.01$, highly significant increase in HDL compared to standard with $P<0.01$, and significant reduction in LDL compared to standard with $P<0.05$ (Table 2). In order to unveil dose-dependent effect of **4a**, it was evaluated in same hyperlipidemic mice model with three different doses, ie, high dose (10 mg/kg), medium dose (5 mg/kg) and low dose (2.5 mg/kg). Effect of **4a** reduced with decrease in dose when compared with high dose 10 mg/kg, and this decrease was statistically significant (Table 3). In vivo outcomes clearly showing the superiority of antihyperlipidemic potential of **4a** compared with other phenolic derivatives and even compared to standard atorvastatin. Furthermore, after 42 days of treatment none of the mice showed any kind of morbidity or mortality, which further advocates that all experimental phenolic compounds were well tolerated and were fairly safe during the whole experiment. These astounding outcomes establish the commendable and plausible antihyperlipidemic potential of our experimental natural phenolic derivatives.

Histopathological examination

Fatty liver is gaining importance in investigations and now is known to be most prevalent cause of abnormal liver.

Fatty liver initially starts with simple steatosis, which later may develop into advanced fibrosis and cryptogenic cirrhosis via steatohepatitis and ultimately to hepatocellular carcinoma.[53] Obesity is the single main causative factor in the development of fatty liver both in children and adults.[54] In our current investigation, **4a** fed group of mice showed significant histopathological changes in liver sections under microscopic evaluation (Figure 3). A small loss of nucleus and membrane disruption was observed when compared with the normal diet (ND) group, but no fat droplet was observed in case of **4a** group when compared with the HFD group. Histology of adipocytes showed that difference in size was evidently less in case of **4a** group compared with the HFD group (Figure 3).

Docking studies

The in silico docking studies were performed to determine the binding affinity of the synthesized compounds with target protein. It has been reported that docking is an effective predictive tool regarding possible interactions of ligands with target protein.[55] It further enables illustration of the possible atomic interactions between the particular functional group of a ligand in the active binding site of protein. To date, the

Table 3 Dose-dependent antihyperlipidemic effect of **4a** (n=6, mean ± SD)

Group	Dose (mg/kg)	Cholesterol (mg/dL)	TG (mg/dL)	LDL (mg/dL)	HDL (mg/dL)
Normal control	–	266±9.90	121±4.12	124±3.79	82±3.66
Hyperlipidemic control	–	380±10.84	129±4.60	314±7.09	68±3.52
4a	2.5 (low dose)	191±7.54*	94±4.09	71±4.73*	60±2.85
4a	5.0 (medium dose)	171±5.41**	90±4.13*	68±3.35	76±3.64*
4a	10 (high dose)	148±4.21**	87±3.89*	61±3.61**	96±4.12**

Notes: *$P<0.05$ vs hyperlipidemic group. **$P<0.01$ vs hyperlipidemic group.
Abbreviations: TG, triglyceride; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
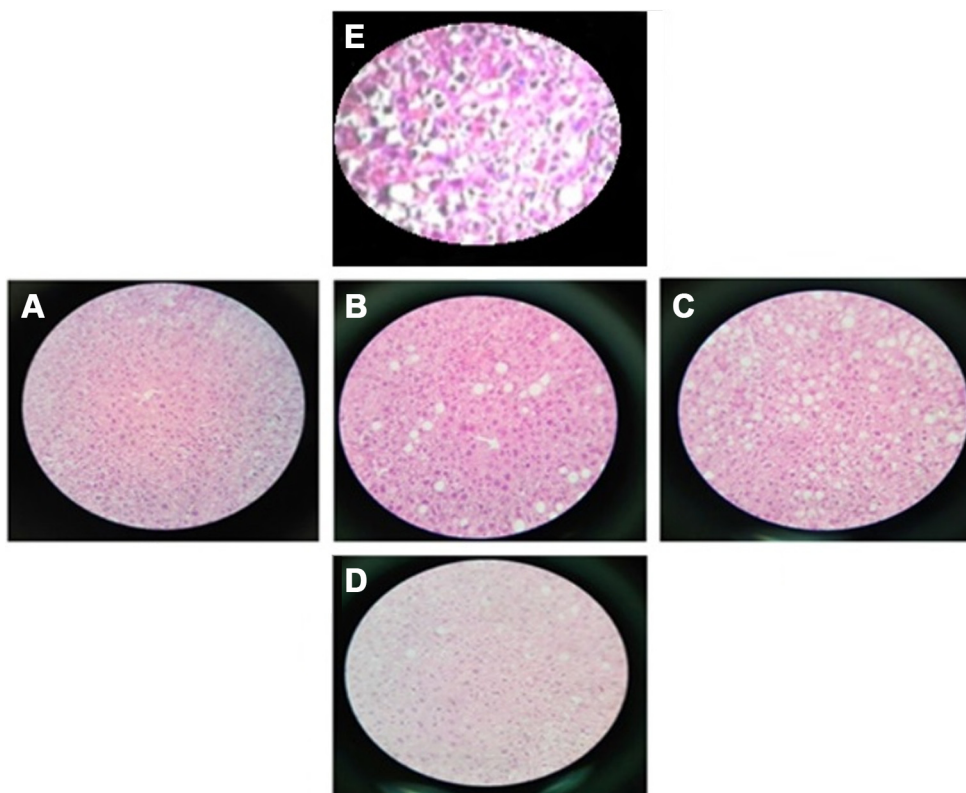
Figure 3 Histopathological evaluation of liver section slides: (**A**) **4a** group; (**B**) **6a** group; (**C**) **6b** group; (**D**) standard drug group; and (**E**) hyperlipidemic control.

most successful strategy to control hyperlipidemic disorders has been to inhibit HMG CoA reductase. This enzyme catalyzes the conversion of HMG CoA to mevalonic acid, ultimately producing cholesterol.[56] HMG CoA reductase model characterized with X-ray diffraction at a resolution of 2.22 Å was downloaded from Protein Data Bank (Figure 4). The structure was validated by using the Ramachandran plot, which demonstrated that >95.7% residues were lying in favorable region and 99.4% residues were lying in allowed region of plot (Figure 5). The experimental compounds (phenolic derivatives) tightly entrapped in active binding sites of HMG CoA reductase enzyme were visualized through Discovery Studio 4.1 (Figure 6). In the present study, we compared docking outcomes of our experimental phenolic compounds with atorvastatin (most widely used US Food and Drug Administration-approved statin), **4a** and **6a** showed even better binding affinity (−8.3 and −7.9 kcal/mol, respectively) energy values compared with atorvastatin (−7.8 kcal/mol) (Table 4). Analysis of residual interaction between inhibitor and target enzyme in active binding site revealed that the compound **4a** interacted with Val805 (at a distance of 1.89 Å), Met656 (at a distance of 2.96 Å), Thr558 (at a distance of 2.70 Å), and Glu559 (at a distance of 2.20 Å) via hydrogen bonding in the active binding site

of HMG CoA reductase (Figure 7). In contrast, **6a** interacts with Asn658 via hydrogen bond at a distance of 3.06 Å and with aspartate 767 at a distance of 4.33 Å (Figure 8). These interaction patterns give a strong impression that synthesized phenolic derivatives possess good inhibitory potential against HMG CoA reductase.

Conclusion

The synthesis of some phenolic derivatives **4a–e** and **6a–c** was successfully accomplished starting with simplest precursors. The results of in silico docking studies showed that
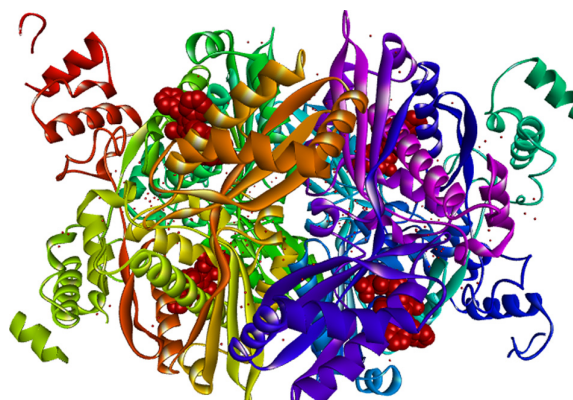

Figure 4 Three dimensional structure of homosapien HMG CoA reductase downloaded from Protein Data Bank with PDB ID 1HWK bound to ligands.
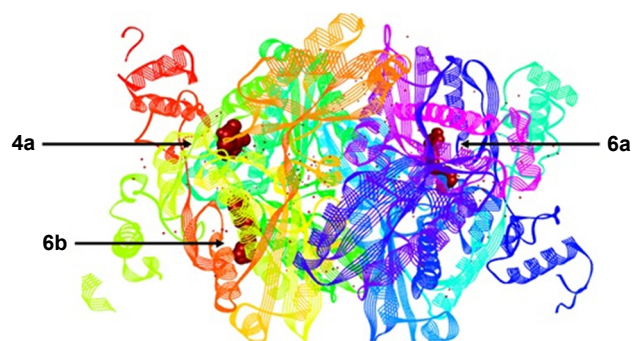
Figure 5 The phenolic derivatives **4a**, **6a**, and **6b** tightly entrapped in active binding sites of HMG COA reductase enzyme visualized through Discovery Studio 4.1.

Table 4 Binding affinity/energy values against HMG CoA reductase (PDB ID: 1HWK)

Ligand	Binding affinity	RMSD/UB	RMSD/LB
4a	−8.3	0	0
4b	−7.1	0	0
4c	−7.1	0	0
4d	−6.5	0	0
4e	−6.4	0	0
6a	−7.9	0	0
6b	−7.4	0	0
6c	−6.3	0	0
Atorvastatin	−7.8	0	0

Note: Lesser binding affinity energy means less free energy of the interaction complex, ultimately illuminating that ligand–target complex is more stable.
Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; RMSD, root mean square deviation; UB, upper bound; LB, lower bound.

compounds **4a** and **6a** exhibited maximum binding affinity with target protein having binding energies −8.3 and −7.9 kcal, respectively. Compound **4a** interacts with amino acids Val805 having distance 1.89 Å and Met656, Thr558, and Glu559 having bonding distances 2.96, 2.70, and 2.20 Å, respectively. Compound **4a** exhibited excellent in vivo antihyperlipidemic

activity with minimum weight increment (20%) compared to standard atorvastatin (35% weight increment). The synthesized compounds were nontoxic and well tolerated because none of the mice were found to suffer from any kind of
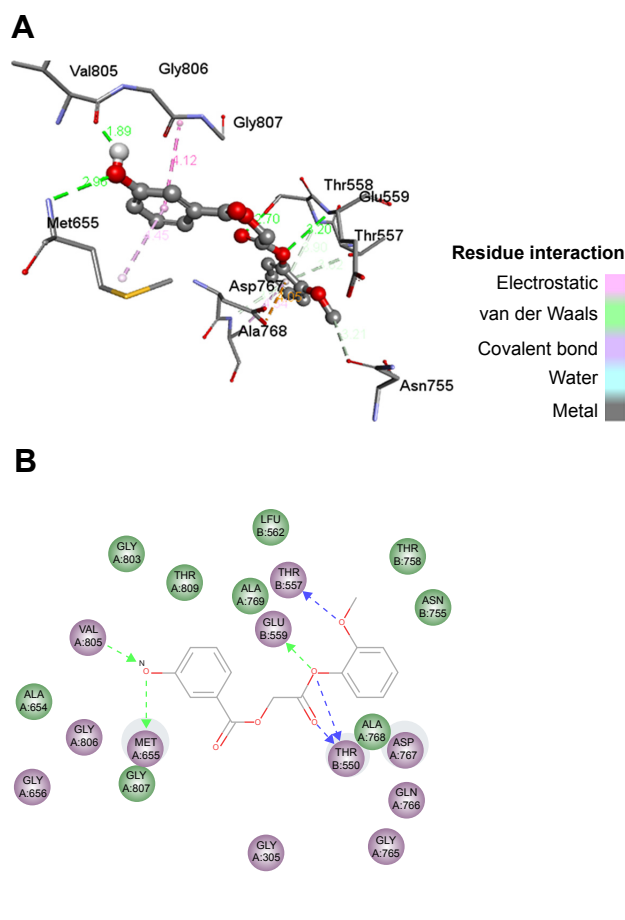

Figure 6 Residual interaction of 4a in binding pocket of HMG CO A reductase.
Notes: (**A**) Shows three dimensional docking in active binding site. (**B**) Shows two dimensional interaction patterns. The legend inset represents the type of interaction between the ligand atoms and the amino acid residues of the protein. Compound **4a** interacts via hydrogen bonding with Val 805 (at a distance of 1.89A°), Met 656 (at a distance of 2.96A°), Thr 558 (at a distance of 2.70A°), and Glu 559 (at a distance of 2.20A°) amino acids.
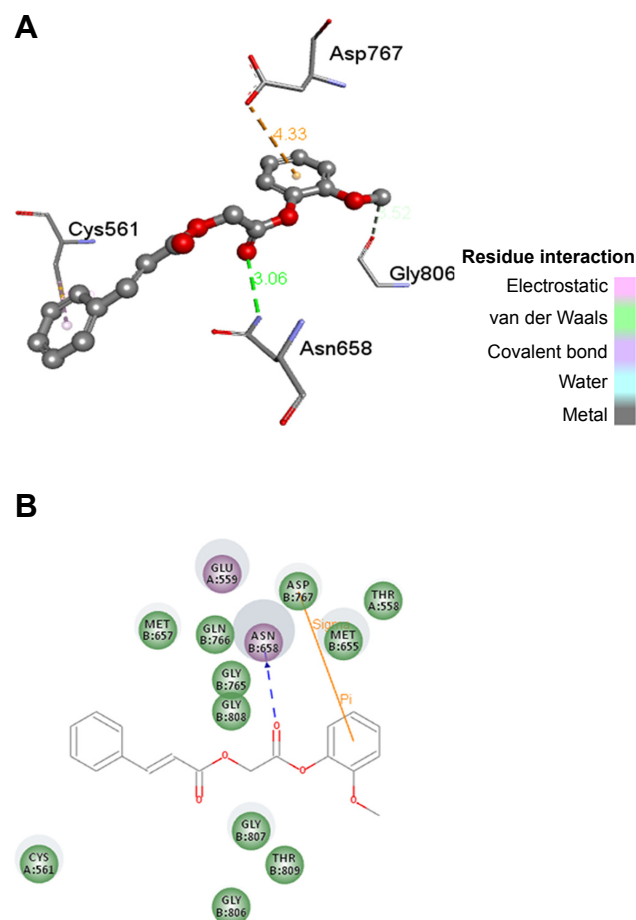

Figure 7 Residual interaction of **6a** in binding pocket of HMG CO A reductase.
Notes: (**A**) Shows three dimensional docking in active binding site. (**B**) Shows two dimensional interaction patterns. The legend inset represents the type of interaction between the ligand atoms and the amino acid residues of the protein. Compound **6a** interacts via hydrogen bonding with ASN 658 (at a distance of 3.06A°) and with ASP 767 (at a distance of 4.33A°) amino acids.
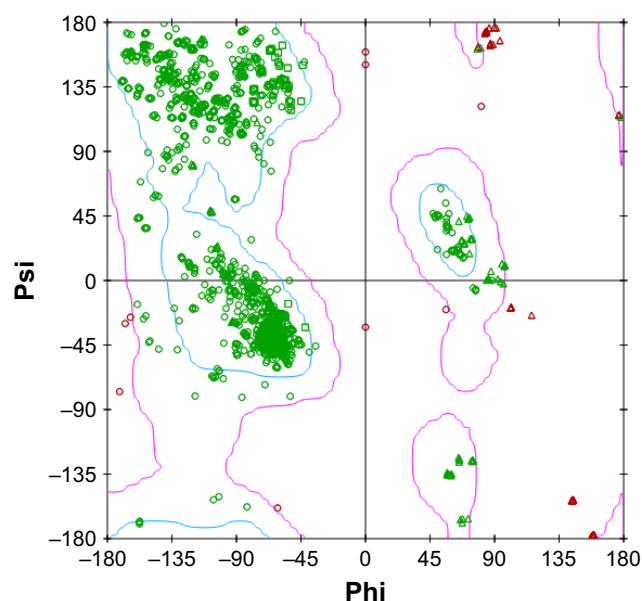
Figure 8 Ramachandran plot of protein structure of target protein (HMG COA reductase).
Notes: Total residues =1573; residues in favored region =98%; residues in allowed region =99.4%; total outliers =9.

morbidity and death during 6 weeks of dosing. Based on our pharmacological evaluation, we may propose that compound **4a** may act as a lead structure for the design and development of more potent antihyperlipidemic drugs.

Disclosure

The authors report no conflicts of interest in this work.

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