How to Cite:
Nithyakalyani, K., Panneerselvam, P., Gandhimathi, R., Saravanakumar, A., Jeslin, D., & Padmaja, V. (2022). In-silico and in-vitro investigation of phytoconstituents present in schleichera oleosa: An enzyme based anti-hyperlipidemic activity. *International Journal of Health Sciences*, *6*(S3), 8687–8702. https://doi.org/10.53730/ijhs.v6nS3.8094

In-silico and in-vitro investigation of phytoconstituents present in schleichera oleosa: An enzyme based anti-hyperlipidemic activity

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***Abstract*---**Hyperlipidemia is a common condition among today's generation. Only a few medicines are available for treatment, but they pose a risk if used for an extended period of time. As a result, the objective of this study is to find a potential HMG-CoA reductase

inhibitor in a natural traditional plant, Schleichera oleosa. The extract was prepared through the cold maceration of the tree's bark with methanol. The preliminary phytochemical assessment was carried out in accordance with the standard test. The HMG-CoA reductase inhibition test kit was used as an in-vitro investigation to investigate the inhibitory potential of phytoconstituents at 2, 10, 20, 40, 60, 80, and 100 µg/ml. For 600 seconds, spectrophotometric scans (340 nm) were performed at 30 second intervals. The in-silico ligand-protein docking approach was used to determine the binding potential of phytoconstituents to a complex of the catalytic part of human HMG-CoA reductase (1HW8). The presence of triterpenes, phenols, tannins, glycosides, carbohydrates, and sterols was found, but flavonoids, alkaloids, and saponins were absent. The phytoconstituents were shown to be effective in the inhibition experiment, with 63.14 ± 0.34 % inhibition at 100 µg/ml, and the extract's IC50 was 54.87 µg/ml. The docking interface was comparable to that of atorvastatin, a standard chemical. Schleicher statins 1, 3, 4, 5, 7, lupeol, and betulin were discovered to bind with the residue GLU559, which is responsible for a portion of the HMG-CoA reductase inhibitory process. These findings show that the phytoconstituents may have an inhibitory effect, and more pre-clinical and clinical research is needed to determine the compounds' full potential.

***Keywords*---**S.oleosa, docking, HMG-CoA reductase, inhibition, atorvastatin, hyperlipidemia.

Introduction

Hyperlipidemia is characterised by elevated lipid (fat) levels in the blood. Primary and secondary hyperlipidemia are the two most common kinds of hyperlipidemia. Primary hyperlipidemia is caused by an underlying condition, whereas secondary hyperlipidemia is caused by an excess of lipid in the diet. Lipid profiles are frequently analysed in order to make a precise diagnosis. [1] If hyperlipidemia is present or suspected, lifestyle adjustments must be implemented in addition to medicine, such as statin therapy. Elevated blood cholesterol levels are a substantial risk factor for cardiovascular disease, heart attack, and stroke. Other factors, such as genetics, high blood pressure, smoking, sedentary lifestyle, and diabetes mellitus, significantly increase the risk [2].

According to an epidemiological study, 130 million people worldwide have excessive cholesterol levels, with 17 million dying each year. There are several forms of cholesterol, some of which are useful and others of which are harmful. [3] In several clinical investigations [4], a link has been identified between high levels of LDL and an increased risk of cardiovascular events. In general, there are three strategies for lowering cholesterol levels using medicine. Artovastatin, Rosuvastatin, and other statin-based medications are examples of HMG-CoA reductase inhibitors; similarly, fibric acid derivatives (clofibrate, gemfibrozil, and fenofibrate), bile acid sequestrants (cholestyramine, colestimide, colestipol, and colesevelam), nicotinic acid derivatives (nicacin), and cholesterol absorption

inhibitors The HMG-CoA reductase enzyme is the primary target for lowering fat levels in the body. Statins are often structural analogues of HMG-CoA reductase inhibitors that function by inhibiting HMG-Coenzyme A reductase during the production of cholesterol in the liver.

Statins, like other powerful medications, have a variety of adverse effects, including gastrointestinal disorders, elevated serum transaminase, significant renal difficulties, and some studies show that statins may be linked to an increase in the development of type 2 diabetes [6, 7]. There are currently no compounds that are as effective as statins against HMG-CoA reductase. However, the substantial side effects generated by the molecules encourage industry to focus on a molecule with effective HMG-CoA reductase inhibition as well as a low level of adverse effects. It is generally known that all chemical entities have minor side effects in addition to their medicinal benefits. This difficulty has prompted the pharmaceutical industry to focus on plant-derived ingredients as a possible substitute for synthetic compounds. The only way to overcome this barrier is to understand the Indian traditional medicine systems of siddha and Ayurveda, as this is where the ability to heal has emerged. Plants and their medical effects have been studied for centuries, but we are still unable to find the perfect molecule for the correct illness since the concept of understanding the pharmacological effects varies between allopathic and traditional systems [8].

Traditional plants are not just used for primary health care in rural regions, in underdeveloped nations, but even in industrialised nations, where modern pharmaceuticals are largely used. The use of plants as a source of medication has been retained and is a major part of the medical system in India. *Schleichera oleosa* (Lou.) Oken, often known as Kusum, is a member of the Sapindaceae family. *S. oleosa* is a well-known medicinal plant in the Indian traditional medicine system; it grows naturally in Kashmir, West Bengal, as well as the tropical and subtropical regions and southern sections of the India [9]. *S. oleosa* is used in animal fodder production and acts as a good source of nectar for insects. Malaria, astringent, anti-inflammatory, ulcers, anti-microbial, and anticancer properties are all associated with the plant [10].There are only a few herbal formulations for *Schleichera oleosa* that have been reported, but kusum oil, which contains 50–60% fatty acids, is used as a topical application for itching, acne, burns, and other skin problems, as well as for joint massages and hair growth [11]. As a result, this study was designed to look into the inhibition potential of *S. oleosa* against HMG-CoA reductase using *in-silico* and *in-vitro* methods.

Methods and Materials

Preparation of extract:

The bark was freshly collected, air-dried and powdered coarsely. The coarse powder of the bark was used to prepare an extract with methanol by the cold maceration method.

Preliminary Phytochemical analysis

The preliminary phytochemical analysis was performed to determine the presence or absence of triterpenes, flavonoids, steroids, glycosides, carbohydrates, alkaloids, phenols, tannins, saponins [12-15].

- Test for triterpenes:
  A small amount of dried bark extract was mixed with 2ml of CHCL3 and 1ml of acetic anhydride. The mixture was then treated with droplets of concentrated sulfuric acid. The presence of triterpenes is indicated by the presence of a reddish violet tint.
- Test for flavonoids:
  To a small amount of extract dilute sodium hydroxide was added. The presence of flavonoids is indicated by the appearance of yellow color which disappears upon addition of dilute acids.
- Test for steroids:
  The bark extract was taken in a dry test tube and 2ml of CHCl3 was added to it and later 2ml of sulfuric acid was added to it. The mixture was shaken for a minute. The appearance of yellow-green in sulfuric acid layer and red color in CHCl3 layer indicates the presence of sterols.
- Test for glycosides:
  The extract was taken in a test tube and made alkaline by adding sodium hydroxide. Then treated with few drops of sodium nitroprusside (freshly prepared) and added to it. The presence of glycosides is identified by the appearance of blue color.
- Test for carbohydrates:
  The extract was treated with Fehling's I & II solution and warmed in a water bath. The appearance of red color indicates the presence of carbohydrates
- Test for alkaloids:
  A small amount of sample was dissolved in ammonia solution (2ml) and 8 ml of CHCl3 was added to it and shake vigorously. The CHCl3 was evaporated and mayer's reagent was added to it. Presence of alkaloids is indicated by the appearance of cream color precipitate.
- Test for phenols:
  A small amount of extract was dissolved in distilled water and few drops of 10% ferric chloride was added to it. The appearance of bluish black indicates the presence of phenols in the extract.
- Test for tannins:
  A small sample of the extract was dissolved 2 ml of CHCl3 and 1 ml of acetic anhydride was added to it. Then a few drops of sulfuric acid were added to it. The presence of tannins in the extract is indicated by the emergence of green colour.
- Test for saponins:
  10 ml of distilled water was added to the extract and hand shaken for few mins. Then the test tube was allowed to stand. The appearance of foam layer indicates the presence of saponins in the extract.

HMG-CoA reductase inhibitory assay

The inhibition assay was carried out using the HMG-CoA reductase assay kit according to the manufacturer's instructions. The positive control was atorvastatin. 4µl of NADPH and 12µl of HMG-CoA substrate were added to the 96 well plate, which was then filled to a capacity of 200µl with assay buffer. 2µl of HMG-CoA reductase was added and incubated at 37°C with the presence or absence (control) of 1µl of the drug dissolved in DMSO. The rate of NADPH was measured at 340nm at intervals of 30 seconds, beginning at 0 sec and ending at 600 sec [16].

Molecular Docking

- Ligand Preparation:
  Atorvastatin, eicosadienoic acid, erucic acid, schleicherastatins 1-7, schleicheols 1 and 2, taraxerone, lupeol, lupeol acetate, betulin, and betulinic acid were downloaded in.sdf format from the pubchem database. The ligands were subsequently prepared for docking using the suite's Ligprep tool. The OPLS4 field force was chosen since it has been shown to provide better accuracy than the prior field for protein-ligand docking [17]. The procedure was designed in such a way that each ligand returned with a pose of 10. Following the processing of the ligands, the final ligands for docking were chosen based on the state penalty ratings. The lower the state penalty score, the more probable the ligands are stable.
- Protein Preparation:
  The RSCB website provided the protein structure of HMG-CoA reductase (1HW8) with the catalytic region bound to statins, which was processed for protein preparation [18]. Initially, the PRIME option was used to add all hydrogen, zero-order bonds, disuphide groups, missing atoms, and chains. The protein was then adjusted with the PROPKA option while the sample water orientation was kept. Finally, the protein was reduced and incorporated into the suite's workspace [19].
- Receptor grid generation
  T The fundamental phase in the docking process is receptor generation, which specifies the active site of the protein. The binding site mapper and the receptor grid generator are the two choices for producing the grid in the suite. The mapper is often used when the active site of a protein is unknown or difficult to identify, whereas the grid generator is typically used to find the active site if a ligand is already attached to the protein. A statin group is already connected to a catalytic region of the protein 1HW8, which has now been chosen as the active site in this investigation [20].
- Docking
  The docking procedure was initiated using the ligand docking panel. The previously constructed receptor grid was chosen, and the prepared ligands were chosen from the workspace. The scaling factor was adjusted to 0.8 and a partial cut off charge of 0.15 to make the ligands soft for the non-polar sections of the ligand. The extra precision approach was chosen, and the docking reference location was limited to a tolerance of 0.10. The docking procedure was then initiated, and the results were stored with one pose per ligand-protein interaction.

Results and Discussion

Preliminary phytochemical screening

The preliminary phytochemical screening was performed as per the mentioned procedure and the results are tabulated, Table 1.

Table 1
Preliminary Phytochemical Analysis

S.No	Phytoconstituents	Methanolic Extract
1	triterpenes	+
2	Flavonoids	-
3	Steroids	+
4	Glycosides	+
5	carbohydrates	+
6	alkaloids	-
7	phenols	+
8	tannins	+
9	saponins	-

The presence of steroids could indicate the presence of sterols such as schleicherastatins 1-7 and schleicheols 1-2 as reported by *Pettit GR et,al (2000)*[21]. *Khan MJ et,al (2017*) reported the presence of triterpenes such as betulin, betulinic acid, lupeol and lupeol acetate in the barks and the test also indicated the presence of triterpenes [22]. The presence of glycosides and carbohydrates was also identified in the methanolic extract. The extract was also positive for the presence of tannins and phenols. The phytochemical test showed the absence of flavonoid, alkaloids and saponins.

HMG-CoA reductase inhibition assay

The inhibition assay was performed as per the kit's manufacturing instructions. The results of the assay expressed the effect of the methanolic extract against the HMG-CoA reductase. For this assay, atorvastatin was used in the preliminary screening in the inhibition of HMG-CoA reductase activity. The IC_{50} of the atorvastatin was found to be 51 nM, which is in accordance with the previous studies [23].

Table 2
Non-linear fit of S.oleosa extract

Nonlinear fit	Values
Best –fit value	
LogIC50	1.739
HillSlope	0.7047
IC50	54.87
95% CI (profile likelihood)	
LogIC50	1.660 to 1.829

HillSlope	0.5545 to 0.8819
IC50	45.72 to 67.43
Goodness of fit	
Degrees of Freedom	5
R squared	0.9802
Sum of Squares	41.04
Sy.x	2.865

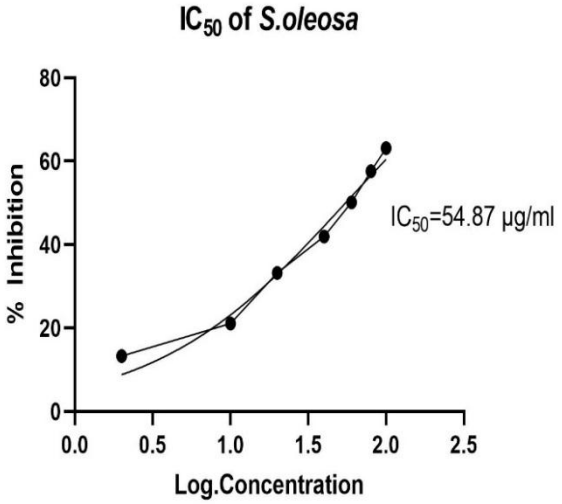

Figure 1. IC_{50} of S.oleosa

According to the findings, the methanolic extract of *S.oleosa* inhibited the HMG-CoA reductase with an IC_{50} of 54.87 g/ml (Figure 1) (Table 2), indicating a concentration-dependent inhibition. The results of the experiment indicate that the phytoconstituents in the extract inhibit the enzyme. The spectrometric scans were taken at 30 second intervals for 600 seconds. The readings are represented as graphs. (Figure 2)

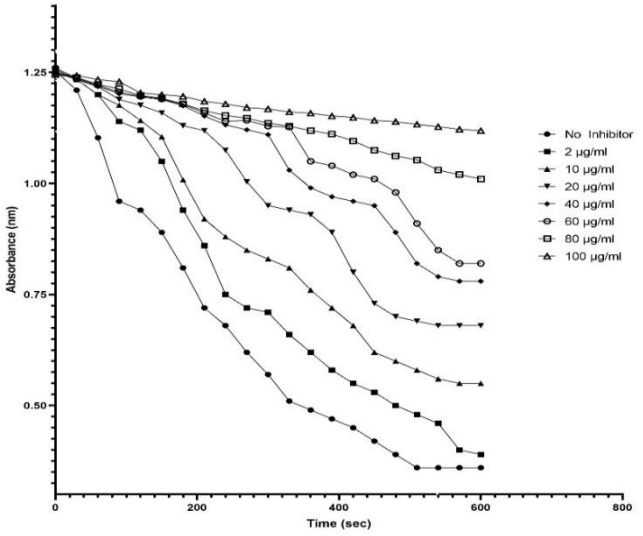

Figure 2. Spectrometric time scans at different concentration

Docking studies

Table 3
Docking scores of Phytoconstituents and atorvastating agaisnt 1HW8

S.No	Compounds	Docking Score	XP docking score	Glide Score	Residue Interacted
1.	Atorvastatin	-10.049	-10.051	-10.051	ASN755, ARG590, LYS691, ASP690, LYS735, SER684, GLU 599
2.	Schleicherastatin 4	-8.594	-8.598	-8.598	GLU665, GLU559, ASN755, LYS691, ASN658, SER661
3.	Schleicherastatin 3	-7.204	-7.204	-7.204	ALA856, LYS691, GLU559, ASN755
4.	Schleicherastatin 7	-5.050	-5.050	-5.050	LYS691, GLU559, ASN755
5.	Schleicherastatin 5	-3.889	-3.889	-3.889	GLU559, ASN755, LYS691
6.	Eicosadienoic acid	-3.842	-3.842	-3.842	LYS735, LYS692, ASP690
7.	Schleicheol 2	-3.833	-3.833	-3.833	GLU559,ASN755
8.	Betulinic acid	-3.631	-3.637	-3.637	LYS691, ARG590
9.	Schleicherastatin 6	-3.554	-3.554	-3.554	ARG571
10.	Schleicherastatin 1	-3.486	-3.486	-3.486	ASN755, GLU559
11.	Erucic acid	-3.457	-3.457	-3.457	LYS692, LYS735
12.	Lupeol	-3.407	-3.407	-3.407	ASN755, GLU559
13.	Schleicherastatin 2	-3.225	-3.225	-3.225	ARG590, LYS691

14.	Schleicheol 1	-3.080	-3.080	-3.080	ASN755, LYS691, GLU559
15.	Betulin	-2.924	-2.924	-2.924	LYS691, GLU559
16.	Taraxerone	-2.759	-2.759	-2.759	-
17.	Lupeol acetate	-1.522	-1.522	-1.522	ARG590

The molecular docking was carried out, and the findings are presented in Table 3. As a control, atorvastatin was used against the target protein. The docking score represents the ligand's affinity for the protein's active region. *Holdgate GA et al. (2003)* discovered that GLU559 is involved in the inhibition of HMG-CoA reductase. As a result, the docking study focused on both the docking score and the binding of the ligands to the GLU559 residues. Atorvastatin had the highest score of all ligands with -10.049 Kcal/mol, followed by Schleicherastatin 4,3,7 with scores of -8.594, -7.204 Kcal/mol, and -5.050 Kcal/mol. The other ligands had very low scores, i.e., less than 50% of the reference compound. Only a few chemicals exhibited no interaction with the targeted protein. Because the residue GLU559 was discovered as being involved in the inhibition process, ligands that bind to this residue were evaluated for potential [24]. Ten compounds were discovered that interact with GLU559 and have the ability to inhibit HMG-CoA reductase (Figure 3-13).

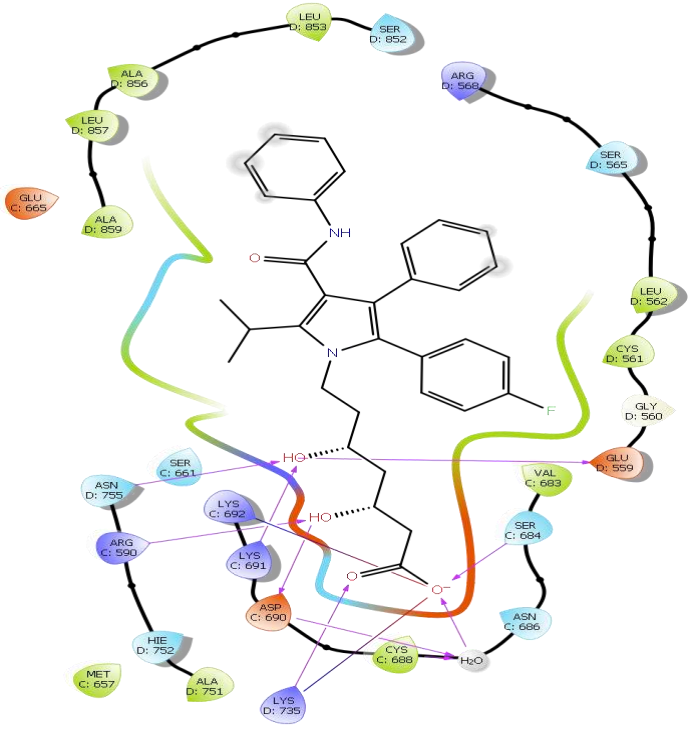

Figure 3. Interaction of Atorvastatin with active site of 1HW8

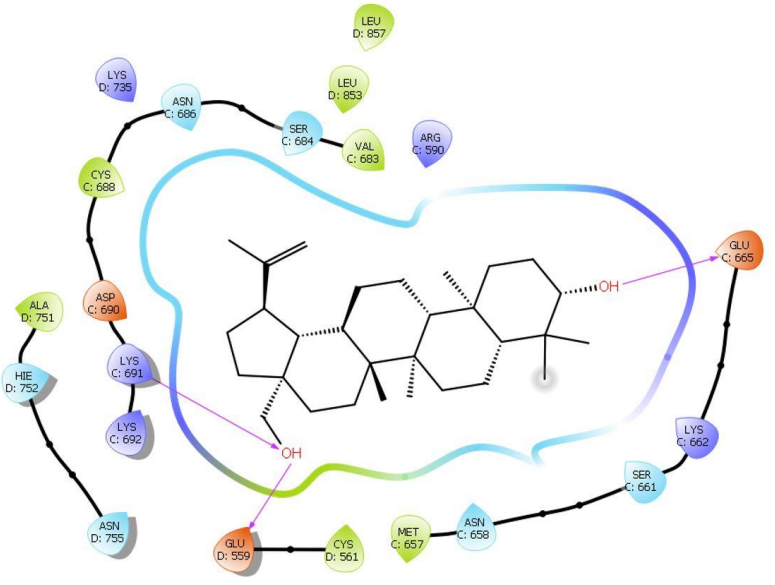

Figure 4. Interaction of Betulin with active site of 1HW8

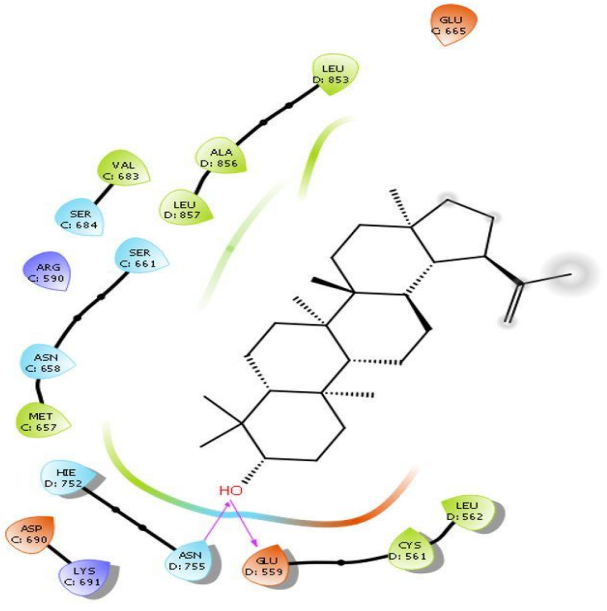

Figure 5. Interaction of Lupeol with active site of 1HW8

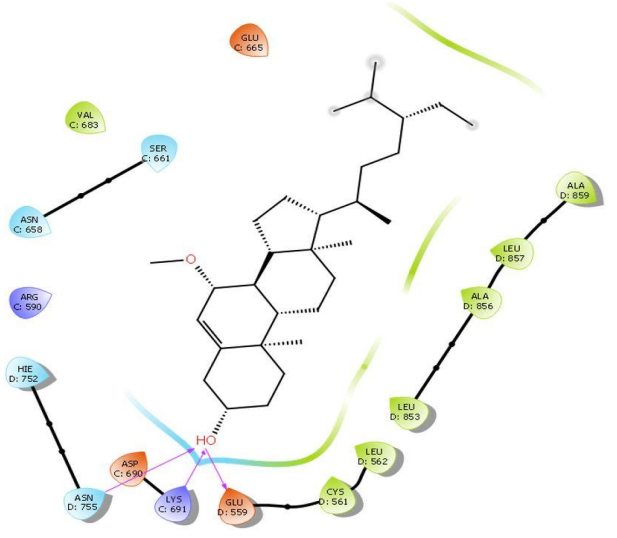

Figure 6. Interaction of Schleicheol 1 with active site of 1HW8

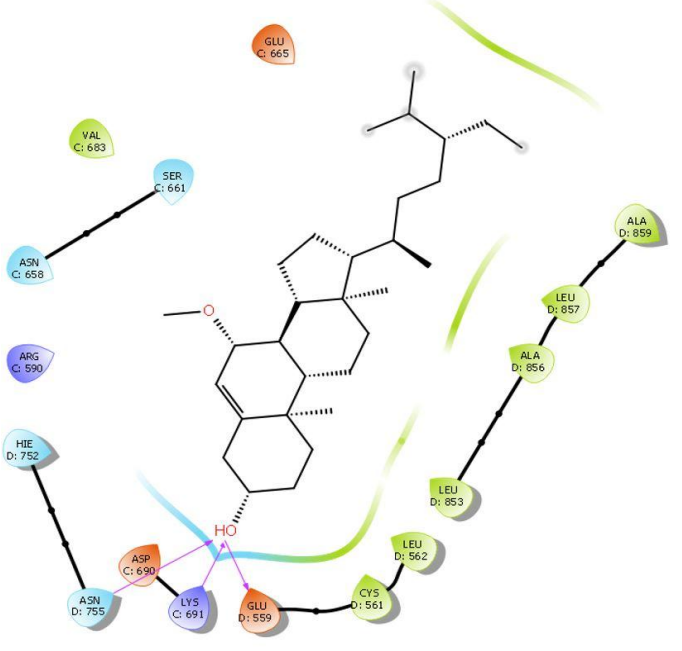

Figure 7. Interaction of Schleicheol 1with active site of 1HW8

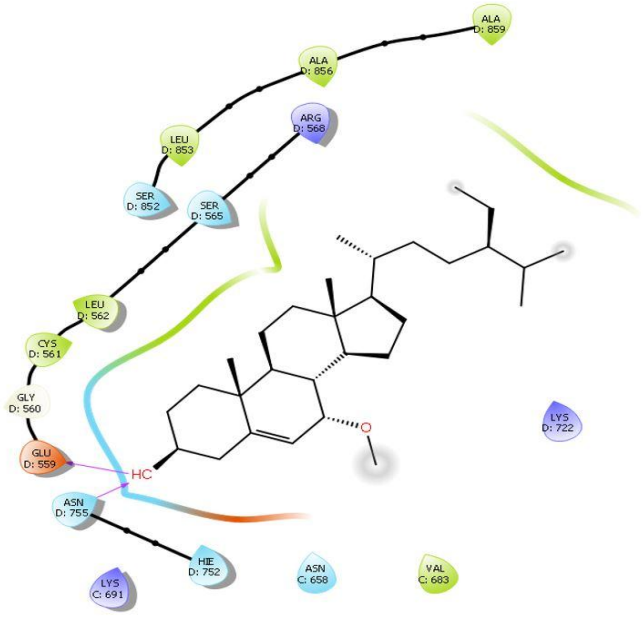

Figure 8. Interaction of Schleicheol 2 with active site of 1HW8

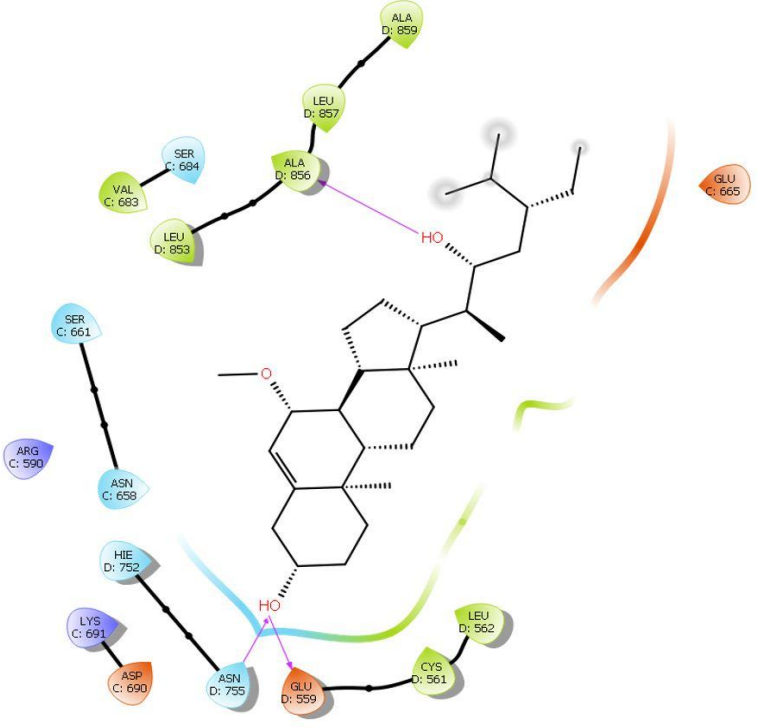

Figure 9. Interaction of Schleicherastatin 1 with active site of 1HW8

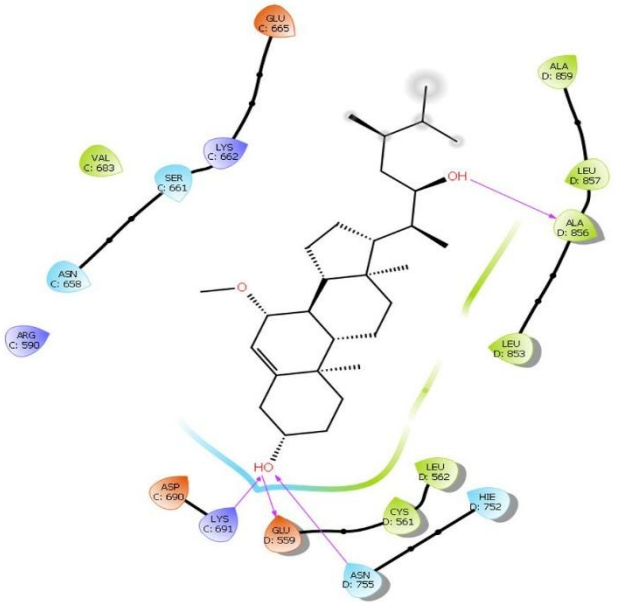

Figure 10. Interaction of Schleicherastatin 3 with active site of 1HW8

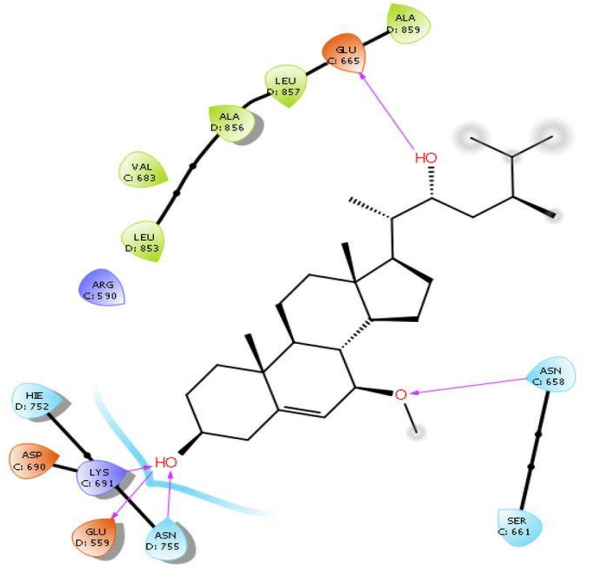

Figure 11. Interaction of Schleicherastatin 4 with active site of 1HW8

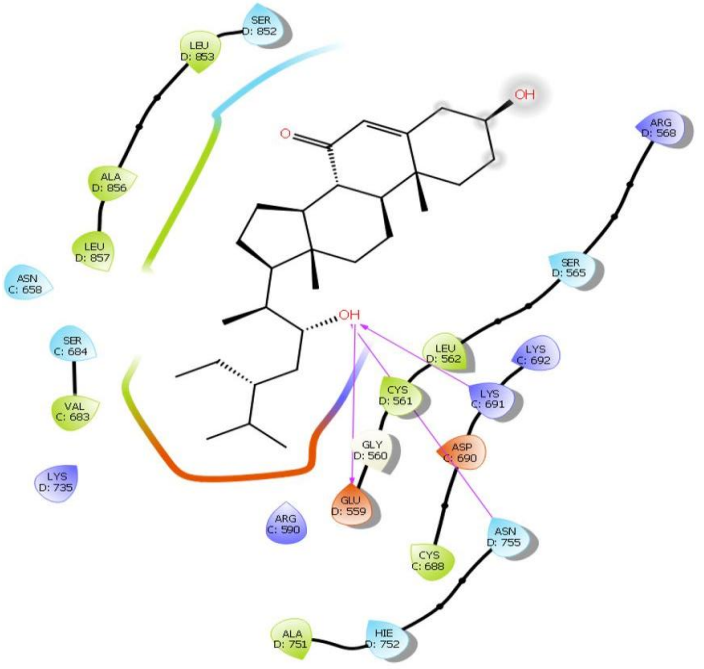

Figure 12. Interaction of Schleicherastatin 5 with active site of 1HW8

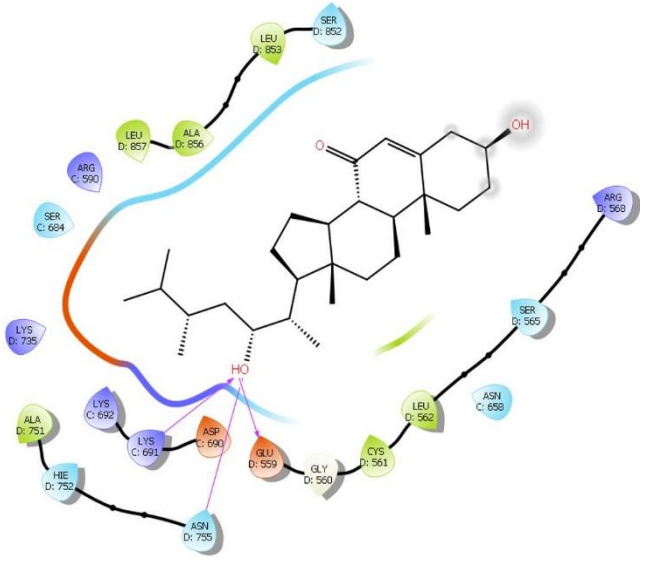

Figure 13. Interaction of Schleicherastatin 7 with active site of 1HW8

Conclusion

Based on the findings, it is inferred that *S.oleosa* phytoconstituents have an anti-hyperlipidemic effect via inhibiting the HMG-CoA reductase enzyme at the GLU559 residue, in a manner similar to statins. Schleicherastatin 4 has a docking score of -8.598 Kcal/mol, indicating high affinity for the active site and

comparable binding to the reference medication, atorvastatin. Few compound did not show any interaction with the site. Also, the inhibition assay shows a concentration dependent inhibition with the extract. These findings imply that phytoconstituents should be studied further in preclinical and clinical settings to determine their full potential for decreasing cholesterol levels.

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