Target specificity of different phytochemicals in preventing the binding of Ox-LDL To LOX-1: An in silico approach

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A thesis submitted to the Department of Mathematics and Natural Sciences (MNS) in partial fulfillment of the requirements for the degree of BSc. In Biotechnology

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Declaration

It is hereby declared that

1. The thesis submitted is our own original work while completing our degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted or submitted, for any otherdegree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Abstract

With an estimated 17.9 million deaths each year, cardiovascular disease (CVD), particularly myocardial infarction (MI), is the leading cause of death globally. Most MIs are caused by thrombi that form over ruptured atherosclerotic plaques obstructing coronary arteries. Despite the outstanding benefits of lipid-lowering medications, 50% to 70% of patients who achieve their lipid-lowering targets still have a high CVD risk. LOX-1 (lectin-type oxLDL [oxidized lowdensity lipoprotein] receptor 1), a membrane receptor of oxLDL, has lately emerged as a viable target for CVD treatments. Various studies suggest that LOX-1 is involved in all the main steps in the pathogenesis of atherosclerosis. The aim of this research is to study the target specificity of various phytochemicals for the LOX-1 receptor and to assess their therapeutic potentialities depending on the stability of ligand-protein complexes. This study utilizes various tools and software to carry out pharmacokinetic analysis, molecular docking and molecular dynamics simulation to determine the drug likeness of the ligands, the strength of the binding affinity, hydrogen bonding and hydrophobic interactions and the various measures of dynamics simulation to predict the stability of the complexes. Among the initial 40 compounds chosen, 10 best compounds were picked based upon their drug-likeness, toxicity, absorption and clearance. Upon conducting the molecular docking simulation for these compounds alongside the statins, it was derived that the compounds generally showed a higher binding affinity compared to the statins. Upon completing the molecular dynamic simulation (MDS) for these complexes, it was derived that most of the compounds chosen showed promising results in terms of binding versatility and free binding energy.

Key words: Cardiovascular diseases, LOX-1, 1YXK, Binding Energy, Protein Active Sites, Protein Tunnels, Molecular docking analysis, Molecular Dynamic simulation, Phytochemicals, Statins, Statin alternatives

Dedicated to

This study in its entirety is dedicated to our beloved parents who provided us with inspiration, motivation and support when we thought of giving up.

To our Friends, Peers and Well Wishers who provided us with advice and encouragement to finish this study.

And lastly to Almighty God for your guidance,strength, protection,power of mind and most importantly giving us a happy and healthy life. All these we offer to you.

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We would also like to express gratitude towards everyone involved in the writing and researching process of this study, as without them this study would be far from possible either.

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List of Acronyms:

MW = molecular weight (g/mol)

HAC = No. of hydrogen bond acceptors

H-Do = No. of hydrogen bond donors;

LogP = Predicted octanol/water partition coefficient

RB = No. of rotatable bonds; IA = Intestinal absorption (% absorbed)

TC = Total clearance (log ml/min/kg)

LD50 = Oral rat acute toxicity

HTT = Hepatotoxicity

MTD = Maximum tolerated dose for human (log mg/kg/day)

NLV = No. of Lipinski's rule violations

DL = Drug-likeness (Lipinski's rule)

BBB P = Blood Brain Barrier Permeability

TELE: Decompose result consists of electrostatic energy

TVDW: Van Der Waals contribution

TGAS: Total gas phase energy

TGBSOL: Non-polar and polar contributions to solvation (TGBSOL), final estimated binding energy calculated from the terms above

TGBTOT: Final estimated binding energy calculated from the terms above

PUE - Puerarin

CRC - Curcumin

PIP - Piperolactam A

IVT - Isovitexin

SRP - Serpentine

DDZ - Daidzein

QRC - Quercetin

APG - Apigenin

MLV - Malvidin

LTL - Luteolin

Chapter 1: Introduction

1. Introduction

According to WHO, cardiovascular diseases (CVD) represented about 32% of all global deaths, as well as 38% of all premature deaths due to non-transmittable diseases in 2019. With a yearly death toll of an estimated 17.9 million people, CVDs are evidently the leading cause of death across the world each year. Heart attack and strokes that make up for 85% of all CVDs are only two of the many possible diseases classified as a CVD (Kim, 2021).

CVD consists of four major types of diseases; Coronary Heart Disease(CHD), Strokes and TIAs, Peripheral Arterial Disease and Aortic disease. Most of these diseases have subtle, or completely invisible symptoms initially, but they can be and usually are life-threatening when left unchecked (NHS. n.d.). The causes of cardiovascular diseases can be high blood pressure which is estimated to account for approximately 13% of CVD deaths, tobacco usage (9%), diabetes (6%), lack of exercise(6%), and obesity (5%) (Cardiovascular Diseases (CVDs), 2021).

The most common precursor for heart attack and stroke is atherosclerosis. While it has a 50% chance to occur on anyone over the age of 40, it can also occur anywhere in arteries that eventually blocks out blood supply to essential body parts. Additionally, if broken open, it can produce blood clots which can further worsen the situation (Heart Research Institute. n.d.). Atherosclerosis is a progressive disease that causes plaques to form inside blood arteries. LDL cholesterol is a wellknown risk factor for atherosclerosis. The origin of atherosclerosis, also known as atherogenesis can be caused by multiple factors. Among them, oxidative stress is considered to be the central catalyst. Through the process of oxidative stress, LDL turns into oxidized LDL, which eventually leads to endothelial cell dysfunction as well as cell injury; steps crucial towards the formation of atherosclerotic plaques (Barreto et al., 2020).

1.1. The Structure of LOX-1 Receptor

On a structural level, oxLDL binds to multiple surface scavenger receptors (SR) which can be assembled into classes such as SR-A, SR-B, SR-C, SR-D, SR-E, SR-F and SR-G. Among them LOX-1 of the SR-E class is one of the few notable receptors signaling a direct impact on atherosclerosis, usually only present in the endothelial cells (Moore & Freeman, 2006).

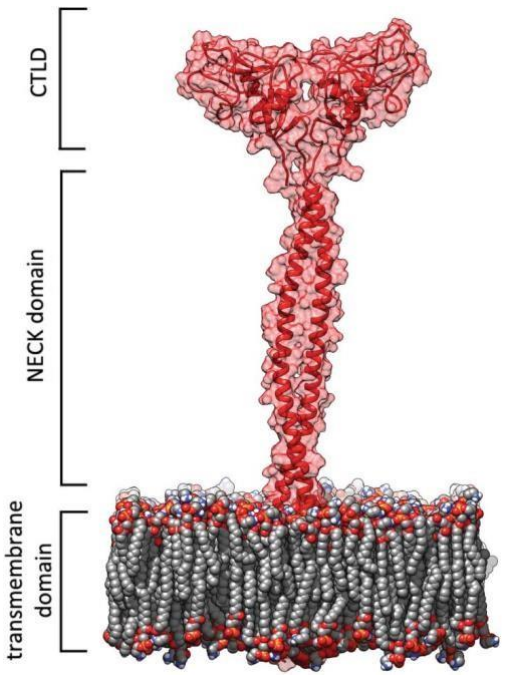


Figure 1. A depiction of LOX-1's domain structure. A transmembrane protein called LOX-1 has 273 residues and 4 domains, a cytoplasmic tail, followed by a single transmembrane domain and an extracellular region comprising of two domains, a NECK doman and C-Type Lectin like domain which is responsible for ox-LDL recognition and it exists as a disulfide-linked homodimer (Biocca et al., 2015).

LOX-1, or Lectin-like oxidized LDL receptor-1 is a transmembrane scavenger receptor that is responsible for binding, manifestation, and degradation of LDL (Sawamura et al., 1997). It is a heart shaped homodimer consisting of 4 domains. These are a single transmembrane domain, short N-terminal cytoplasmic domain, a NECK doman and C-Type Lectin like domain (CTLD) (MEHTA et al., 2006) (Figure 1). A structural analysis on the 1.4 Å LOX-1 structure shows that the monomer contains two α-helices (α-1 and 2) which surround the two β-sheets (β0-β1-β5-β1 and β2-β2-β3-β4) that make up the CTLD fold of the monomer. Moreover, Cys144-Cys155, Cys172-Cys264 and Cys244-Cys256, three conserved intrachain disulfide bonds further stabilize the aforementioned fold. However, it is seen that only human LOX-1 contains Cysteine at position 140, which generates an interchain disulfide between the monomers at the CTLD N terminus. In addition,there is a 7-8Å diameter hydrophobic tunnel going through the middle area of the structure (Park et al., 2005) (Figure 2 A and 2 B). Established literature suggests that this hydrophobic tunnel

not only plays an essential part as a binding site, but also has a major role in the identification of oxidized LDL (Francone et al., 2009). This can be further observed in tests conducted to tackle the oxidation of LDL through LOX-1, confirming the presence of residues within or around the tunnel, where if complexed with the right compound, inhibits the binding ability of OxLDL and LOX-1 receptor, hence reducing it's expression (Falconi et al., 2013, Draude et al., 1999, Guan et al., 2010).

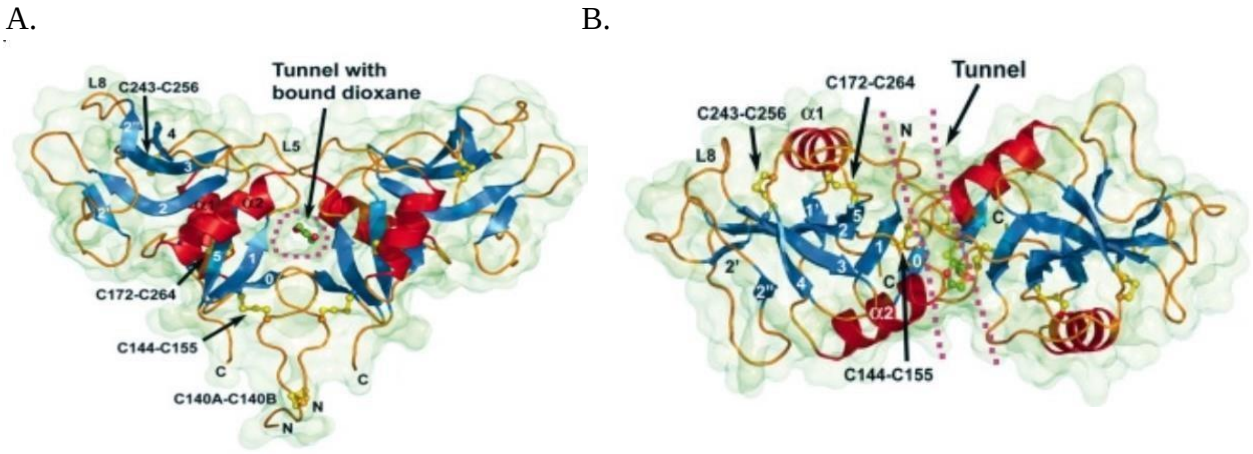


Figure 2. Depiction of the side (A) and top (B) view of LOX-1 in semi-transparent surface (green), and in ribbon form; α-helices are labeled in red, β-strands are labeled in blue, dioxane and disulfide bonds are labeled in green and yellow respectively. The tunnel is marked by dotted magenta lines(Park et al., 2005).

LOX-1 is up-regulated in atherosclerosis lesions, and its activation causes an oxidative stress response, which causes plaque vulnerability and potential rupture, ultimately leading to acute atherothrombotic vascular occlusion and tissue infarction (Vohra et al., 2006). Animal models have verified that LOX-1 plays a significant role in the atherogenic events. Intimal thickness, inflammation, and protective factor expression are all decreased in LOX-1 knockout mice (Ding et al., 2015). LOX-1-overexpressing mice, however, exhibit accelerated atherosclerotic lesion formation that is linked to increased inflammation (Xie et al., 2016). These results imply that LOX1 receptor inhibition therapies may be successful in slowing the progression of atherosclerotic and inflammatory processes. Specific LOX-1 receptor inhibitors are still unavailable. There have been

multiple established medicines in order to tackle LOX-1 upregulation. Currently, the most significant drugs established against LOX-1 are classified as statin drugs.

1.2. Statin Discovery, Pros and Cons

The journey of statin discovery started off in 1976, with the publishing of compactin or ML-236B (ENDO et al., 1976). In 1939, cholesterol and heart attacks were found to be genetically linked, leading to the growing interest of companies and eventually, a tremendous amount of effort was made to understand the pathway in which cholesterol is produced. Consequently, within 1960 a proper pathway was established, in which the rate-limiting step was discovered to be the reduction of HMG-CoA to mevalonate. Leading to the conclusion that a potent HMG-CoA reductase inhibitor would be an efficient remedy against cardiovascular diseases (ENDO, 2010).

As time went on, more and more varieties of statins started being developed and tested. Statins such as Atorvastatin, Lovastatin, Pravastatin, Simvastatin, Fluvastatin etc. have been tested very effectively against diseases such as stroke, myocardial infarction and even deaths due to CVD. Through the utilization of pathways such as eNOS (endothelium-derived nitric oxide synthase) expression, inflammatory marker inhibition, impairing NK or T cell proliferation etc. statins were constantly proven effective against these diseases (Pinal-Fernandez et al., 2018).

According to a study by Biocca et al. (2015), these statins inhibit LOX-1 function by direct interaction. As a result, these statins are chosen as a standard in this study. However, despite their positive significance, statins are tested to have very negative side effects. Derived from a collective of studies (Cai et al., 2021, Ganga et al., 2014, Björnsson et al., 2012), it is observed that statins were directly or indirectly increasing the risk for renal insufficiency, liver dysfunction, diabetes and some muscle disorders. This encourages us to seek out an alternative therapy with fewer side effects and one that is nature-derived.

1.3. Phytochemical alternatives for statins

It has been proposed for some time that naturally derived compounds or phytochemicals are both necessary as well as potent as an alternative to statins. For example, certain phytochemicals such as Chrysin or Berberine were tested in order to tackle the intolerance of statins among certain patients, showing promising results as an alternative (Yuvaraj et al., 2022, Koppen et al., 2017). This study therefore focuses on other naturally derived compounds in order to test their efficacy

compared to statins; namely Puerarin, Curcumin, Piperolactam A, Isovitexin, Serpentine, Quercetin, Luteolin, Apigenin, Malvidin and Daidzein.

Puerarin and Isovitexin are extensively used in traditional Chinese medicine, both of which have been discovered to have antioxidant and anti-inflammatory properties (Bacanlı et al., 2018, Azubuike-Osu et al., 2020). More specifically, Puerarin has the capacity to control ROS overproduction, eNOS downregulation, LOX-1 overexpression and p38 MAPK phosphorylation. (Bao et al., 2014). According to a study, isovitexin derived from *L. divaricata* extract reportedly decreased the amount of ROS by a significant amount. In addition, it was found out to be useful in the inhibition of endothelial activation (Azubuike-Osu et al., 2020).

Curcumin is a compound that gives turmeric its yellow color. Despite its poor bioavailability, it has shown to contain anti-inflammatory, antioxidant as well as kidney beneficiary properties. A test done on hepatic stellate cells (HSCs) suggests that curcumin can lower the promoter activity in LOX-1 present in HSCs, more specifically the activity of luciferase, and thus protecting the cells from oxidative stress (Kang & Chen, 2009).

Serpentine originates from the extract of Serpentina or Indian snakeroot. While the plant itself is potentially deadly in effect (WebMD. n.d.), the extracted and purified serpentine compound appears to have antioxidant properties. For example, research shows that human Hyaluronan Binding Protein1 (HABP-1) overexpressing in mice fibroblast cells, when treated with serpentine, shows signs of reduced ROS formation, while not being affected in its survivability in any way (Dutta et al., 2011).

Piperolactam-A compound is one of the few derived compounds of *P. attenuatum* methanol extract (PA-ME). PA-ME has been tested to reduce p50/NF-κB, alongside COX-2 and iNOS amounts (Kim et al., 2017).

Flavonoids and isoflavonoids are polyphenolic secondary metabolites that are typically produced by plants as a result of long-term adaptation to changing ecological environments (Hodgson et al., 1996). Flavonoid research has received special attention in recent years, following the discovery of their positive effects on the cardiovascular system. Flavonoids exert these beneficial effects primarily due to their antioxidant properties, which result from their ability to reduce the oxidation of low-density lipoproteins (Ciumărnean et al., 2020). In this study, five different flavonoids have been included as ligands, which are Quercetin, Luteolin, Malvidin, Daidzein and Apigenin.

1.4. Objectives of the Study

This research aims to analyze the efficiency of these compounds to bind with LOX-1 through molecular docking analysis, as well as compare them with some well known statin drugs such as Atorvastatin, Pravastatin, Simvastatin and Lovastatin. In order to reach the desired outcome, the objectives set for this study are:

- Analysis of pharmacokinetic properties of the chosen ligands.
- Determine the binding affinities between ligands and protein through molecular docking analysis.
- Analyze and differentiate the types of bonds being formed between the ligands and the amino acid residues within the proteins. - Analyze the molecular dynamic simulation of the protein-ligand complexes.

Chapter 2: Materials and Methods

2. Materials and Methods:

2.1. Selection of the protein and the ligands

Using RCSB Protein Data Bank (https://www.rcsb.org/), a crystal structure of human lectin-like oxidized low-density lipoprotein receptor-1 (PDB ID: 1YXK), a disulfide linked dimer, was selected as the target receptor for this study (Burley et al., 2020). The structure of said macromolecule was retrieved in a .pdb format and conserved for the optimization process. On the other hand, PubChem (authorized by National Center for Biotechnology Information, as part of National Library of Medicine) was screened to construct a library of phytochemicals depending on established literature based upon their effectiveness against LOX-1 receptors. Eventually, a total of 20 pharmacophore ligands were generated.

Afterwards, depending on the ligand source and established literature type, viability of the drug through ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling, top ten ligands were selected for the molecular docking and molecular dynamic simulation steps. Established statins namely Atorvastatin, Lovastatin, Pravastatin and Simvastatin were selected as a comparison.

2.2. Pharmacokinetic analysis and ligand validation through ADMET profiling

A batch of 10 pharmacophore ligands originating from natural sources were selected for ADMET profiling in order to illustrate the viability of the drug through its pharmacokinetic disposition as well as a measurement of the drug kinetics of the tissue. First of all, in order to get an understanding of the compound's drug-likeness, as well as the viability of the compound through Lipinski violations, SwissADME (http://www.swissadme.ch/index.php) was utilized (Daina et al., 2017). Additionally, further detailed information regarding absorption, toxicity as well as expression levels were retrieved by using "pkCSM" (http://biosig.unimelb.edu.au/pkcsm/) (Pires et al., 2015).

2.3. Ligand Optimization

The chosen ligands were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) in SMILES format. Then, their structures were simulated using USCF Chimera software (https://www.cgl.ucsf.edu/chimera/) and following the gasteiger's method their energy was minimized to zero net charge. Finally, the structures were saved in ".mol2" format (Pettersen et al., 2004).

2.4. Protein Optimization

The aforementioned protein (PDB ID: 1YXK) was retrieved from RCSB PDB as the target macromolecule. After importing the .pdb structure file in UCSF Chimera software, a sequential order was followed in order to optimize the structure. Firstly, the unwanted water molecules, ions, ligands etc. were removed from the structure. Secondly, the necessary number of hydrogens were added to facilitate a proper optimization of the protein structure. Finally, the optimized structure was saved (Pettersen et al., 2004).

2.5. Protein active site detection

Since this research relies on the docking of selected ligands with the LOX-1 active sites, it is imperative to determine the best active site to be able to acquire the best possible results. In order to carry this out, a web service CASTp 3.0 (http://sts.bioe.uic.edu/castp/index.html) was used to determine the active sites of the selected protein. The information was utilized later during the docking analysis (Tian et al., 2018).

2.6. Identification of protein tunnels

Using the CAVER Web service (https://loschmidt.chemi.muni.cz/caverweb/) the tunnel dimensions for the macromolecule were determined (Stourac et al., 2019).

2.7. Molecular docking analysis

In order to test the viability of the ligands as potent drugs against the LOX-1 receptor, a site specific docking analysis was conducted in both Chimera 1.16 (https://www.cgl.ucsf.edu/chimera/download.html) PyRx 0.8 (https://pyrx.sourceforge.io/) (Dallakyan & Olson, 2014). The sites to restrict the docking boundaries within were determined through the previously obtained information. The binding affinity (kcal/mol) as well as RMSD (root mean square deviation) were calculated from the acquired results.

2.8. Visualization of docked positions

The docked output files were retrieved, and visualized using PyMol version 2.5 (https://pymol.org/2/). The protein-ligand complexes saved in pdb format for further analysis.

2.9. Hydrogen bonds and Hydrophobic Interaction analysis

The pdb files obtained from PyMOL were analyzed using Ligplot+ 2.2.5 (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/), in order to visualize the hydrogen bonds and hydrophobic interactions within the protein-ligand complexes(Laskowski & Swindells, 2011).

2.10. Molecular Dynamics Simulation

To verify the stability of the protein-ligand complexes and to investigate the conformational changes following interactions, molecular dynamics simulations are carried out. Initially, using the default parameters, the pdb file of the unbound protein was uploaded to the CABS-flex 2.0 web server. Model created by CABS-flex 2.0 depicts how its conformation structure changed over the course of ten nanoseconds. Each chain's fluctuation plots show changes in the number of amino acid residues in relation to the RMSF in Å units. For further analysis, the protein-ligand complexes were subjected to the LARMD simulation system (Yang et al., 2020) (http://chemyang.ccnu.edu.cn/ccb/server/LARMD/index.php).

Chapter 3: Results

3. Results:

3.1. Protein and Ligand Selection

The selected protein was PDB ID: 1YXK (Lectin-like oxidized low-density lipoprotein receptor-1 or LOX-1) disulfide-linked dimer. The ligands chosen were namely Puerarin, Curcumin, Piperolactam A, Isovitexin, Serpentine, Quercetin, Luteolin, Apigenin, Malvidin and

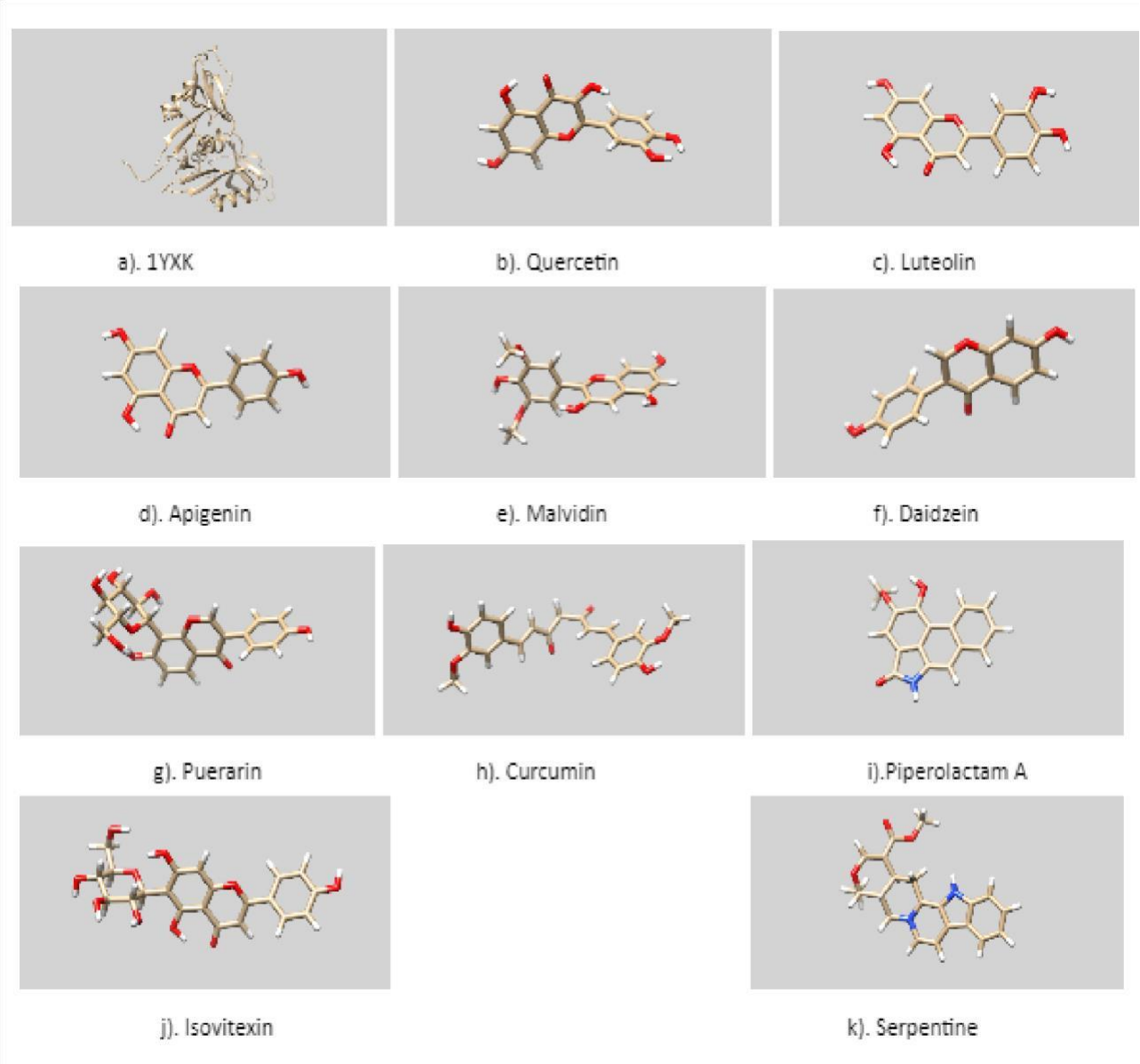


Figure 3. Depiction of (a) the selected protein/macromolecule and (b),(c),(d),(e),(f),(h),(i),(j),(k) the desired naturally extracted compounds.

3.2. Pharmacokinetic analysis

Upon observation, it is noticed that the tolerated dose of the molecules range from around -0.600 log mg/kg/day to 0.650 log mg/kg/day. Besides, intestinal absorption in the molecules range from ~65% to ~98%. Among the 10 compounds, Puerarin and Isovitexin violate one of Lipinski's rules. Additionally, Curcumin, Piperolactam A and Serpentine hold the highest LogP values at 3.3699, 3.2729 and 2.8727 respectively. Finally, although Serpentine shows signs of hepatotoxicity, it is compensated for by the high amount of total clearance level and very low amount of maximum tolerated dose (Table 1).

Table 1: Pharmacokinetic analysis of the phytochemicals

Ligand name	MW	HAC	HDO	LogP	RB	IA	TC	LD50	HTT	MLD	NLV	DL	BBBP
Puerarin	416.382	9	6	0.3861	3	67.45	-0.007	2.641	No	0.642	1	Yes	No
Curcumin	368.385	6	2	3.3699	8	82.19	-0.002	1.8333	No	0.081	0	Yes	No
Piperolactam A	265.26	3	2	3.2729	1	95.08	0.046	2.634	No	-0.128	0	Yes	Yes
Isovitexin	432.4	10	7	0.0917	3	64.73	0.442	2.558	No	0.649	1	Yes	No
Serpentine	349.4	3	1	2.8727	2	97.74	1.153	2.924	Yes	-0.594	0	Yes	Yes
Quercetin	302.24	7	5	1.23	1	77.21	0.407	2.471	No	0.499	0	Yes	No
Luteolin	286.24	8	4	2.282	1	88.18	0.495	2.455	No	0.499	0	Yes	No
Apigenin	270.24	5	3	2.11	1	93.25	0.566	2.45	No	0.328	0	Yes	No
Malvidin	331.30	7	4	0.92	3	88.79	0.687	2.345	No	0.554	0	Yes	No

Daidzein	254.24	4	2	2.87	1	94.84	0.164	2.164	No	0.187	0	Yes	Yes

MW = molecular weight (g/mol); HAC = No. of hydrogen bond acceptors; H-Do = No. of hydrogen bond donors; LogP = Predicted octanol/water partition coefficient; RB = No. of rotatable bonds; IA = Intestinal absorption (% absorbed); TC = Total clearance (log ml/min/kg); LD50 = Oral rat acute toxicity; HTT = Hepatotoxicity; MTD = Maximum tolerated dose for human (log mg/kg/day); NLV = No. of Lipinski's rule violations; DL = Drug-likeness (Lipinski's rule); BBB P = Blood Brain Barrier Permeability

3.3. Protein active site detection

Among the 50 possible obtained pocket results, the pocket containing the best binding sites; pocket 2 (Surface Area = 174.318 // Volume = 90.562) was selected. The predicted binding site residues include Asp147, Ile149, Tyr156, Leu157, Phe158, Ser159, Ser160, Ala194, Ile195, Tyr197, Ser198, Phe202.

3.4. Protein tunnel analysis

Two tunnels were discovered. Out of two tunnels, one was found to be 1.4Å and the other 16.2Å in length, with a radius of 2.0Å and 1.0Å respectively. Based on the radius and the length, the second tunnel was the suggested route for molecular docking analysis. The amino residues of the second tunnel are Asp147, Trp148, Ile149, Tyr156, Leu157, Phe158, Ser159, Ser160, Phe190, Gln193, Ala194, Ile195, Ser196, Tyr197 (Figure 4).

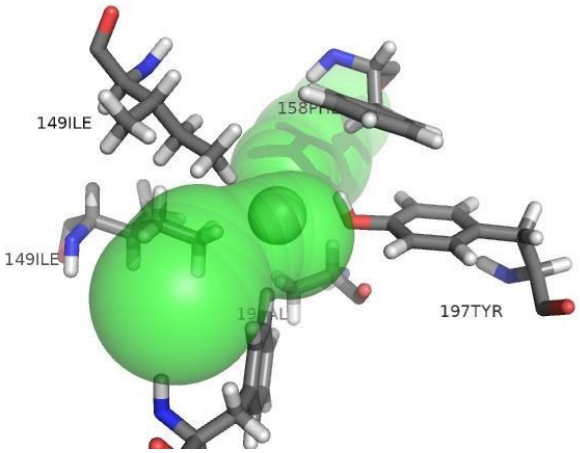


Figure 4. LOX-1 (1YXK) Protein tunnel (length: 16.2Å , radius:1.0Å) visualized through Caver Web service

3.5. Molecular docking analysis

In order to find out the preliminary binding affinities between the phytochemicals and the macromolecule, a molecular docking process was carried out. The highest binding affinity score, in other words the least amount of energy required to form bonds depicts an estimate for the best docking interaction between the protein and ligand. In this case, with a consistent grid box size of X = 58.333A; Y = 61.243A; Z = 57.678A and a Center point of X = 44.9717; Y = -48.7519, the process resulted in the following findings:

- The highest docking score seen was derived from Serpentine, at -8,9 kcal/mol.
- The lowest docking score seen was derived from Curcumin and Atorvastatin (both -6.7 kcal/mol) and Malvidin (-6.8 kcal/mol).
- Generally, the statins binding affinities ranged from -6.7 kcal/mol to -7.5kcal/mol, mostly -7.1 kcal/mol. While this is a similar range to that of the phytochemicals, most of the phytochemicals show affinities at -7.3 kcal/mol to -7.6kcal/mol.

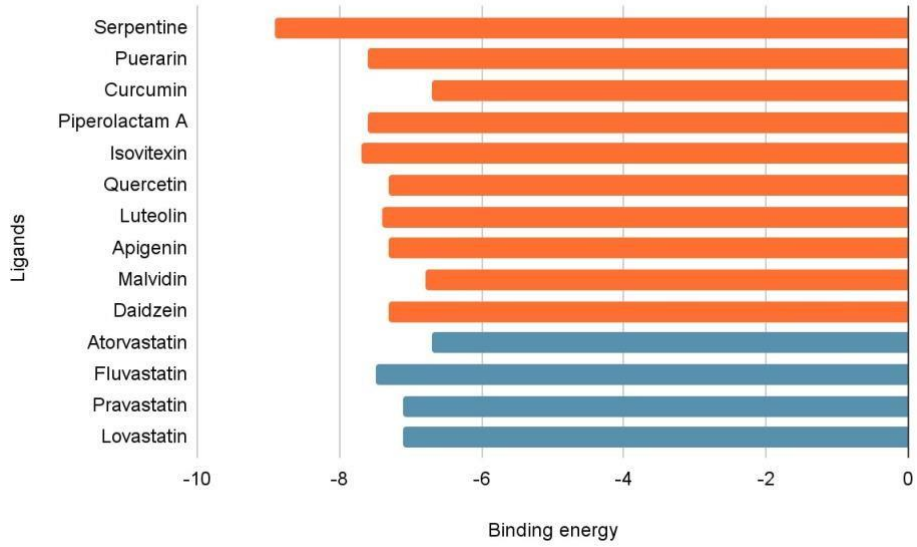


Figure 5: A bar graph comparing the binding affinities of the chosen ligands with that of the statins.

3.6. Post-molecular docking analysis

As it is noticed, among Puerarin, Curcumin, Piperolactam A, Isovitexin and Serpentine, only curcumin lacks a hydrogen bond (H-bond). On the other hand, both of the H-bond and covalent bond bound subunits appear to be mostly the same with certain variations among each other. Hbond subunits bound across the four compounds are Asp147, Phe158, Ser160 and Tyr197.

Most of these H-bonds appear to have a distance of ~2.6Å-3.8Å. On the other hand, among Quercetin, Luteolin, Apigenin, Malvidin and Daidzein, only Malvidin lacks a hydrogen bond. The amino acid residues forming H-bond at a distance of ~2.75Å-3.70Å are seen bound across the rest four compounds are Ser160, Phe158, Tyr197 and Gln193 where Luteolin and Apigenin both involve Phe158 for hydrogen bond formation. Besides, the amino acid residues forming hydrophobic interactions appear to be similar with certain variations among the different ligands.

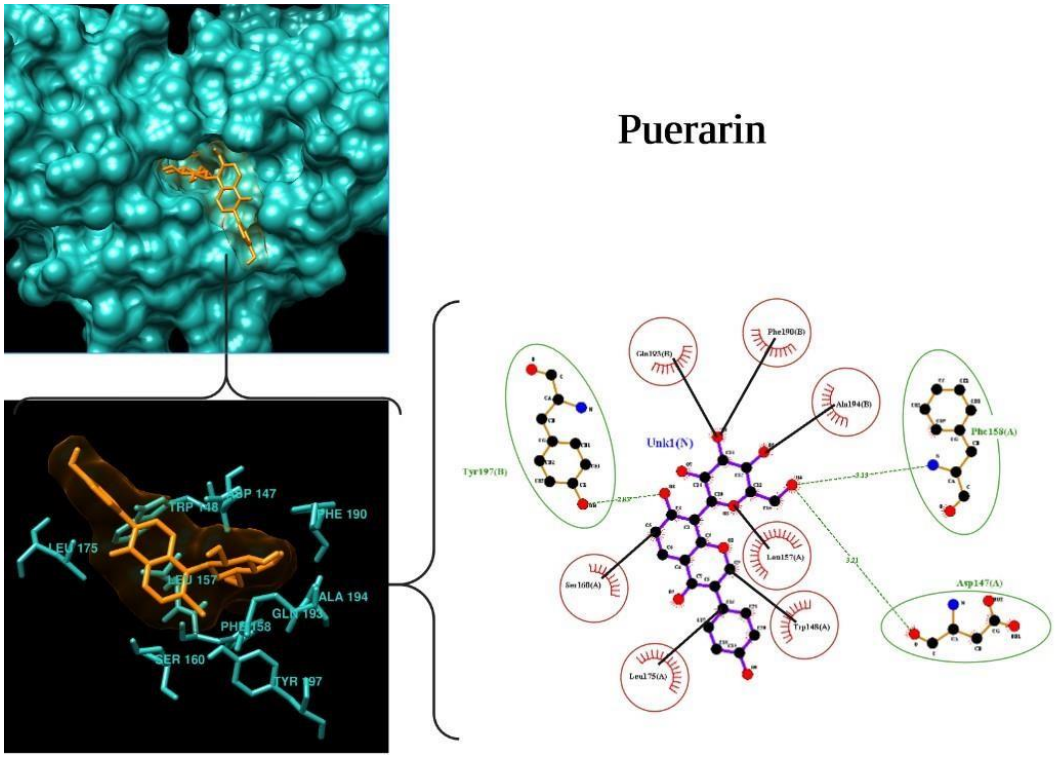
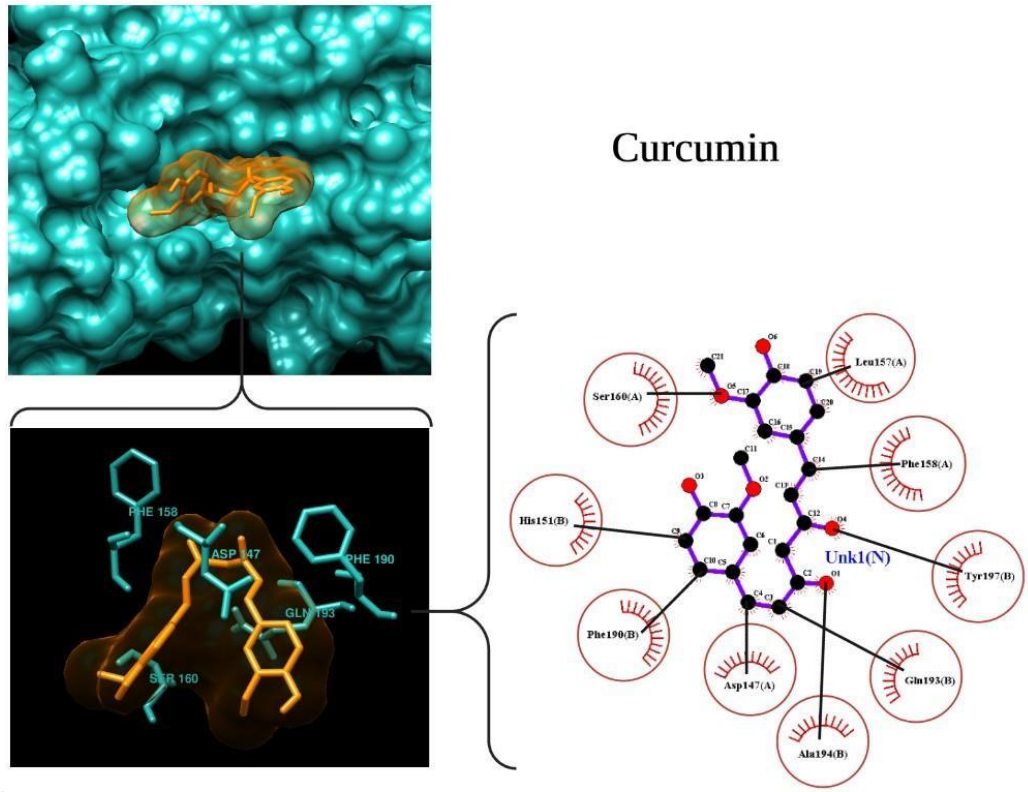


Figure 6: Puerearin docked in LOX-1 receptor (1YXK); Top left image depicts the best binding formation within the macromolecule pocket. Bottom left image depicts the residues involved in the interaction (Ligand in orange sticks). Rightmost image depicts the binding interaction of Puerarin with LOX-1. Red circles = Hydrophobic bonds; Green ovals = Hydrogen bonds.



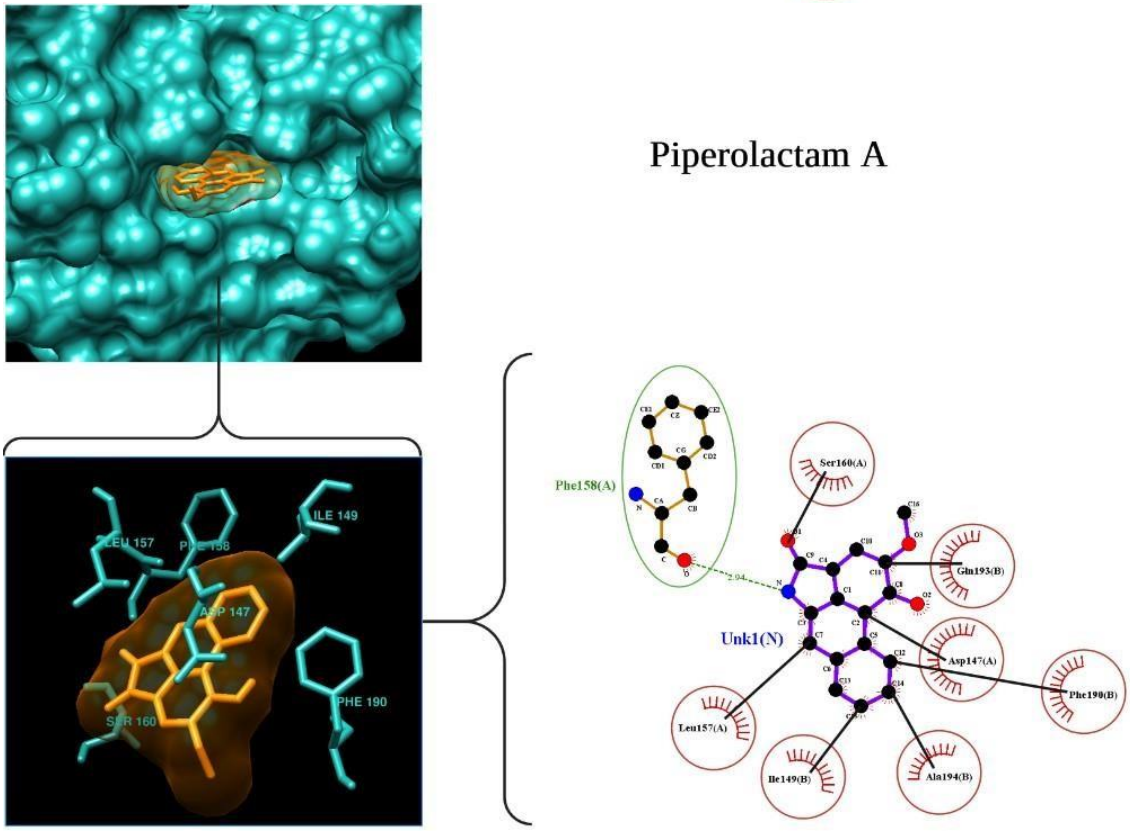


Figure 7 & 8: Curcumin and Piperolactam A docked in LOX-1 Receptor; top left images depicts the best binding formation, bottom left images depict residues involved in interactions, right most

images depict the binding interactions of Curcumin and Piperolactam A. Red circles = Hydrophobic bonds; Green ovals = Hydrogen bonds.

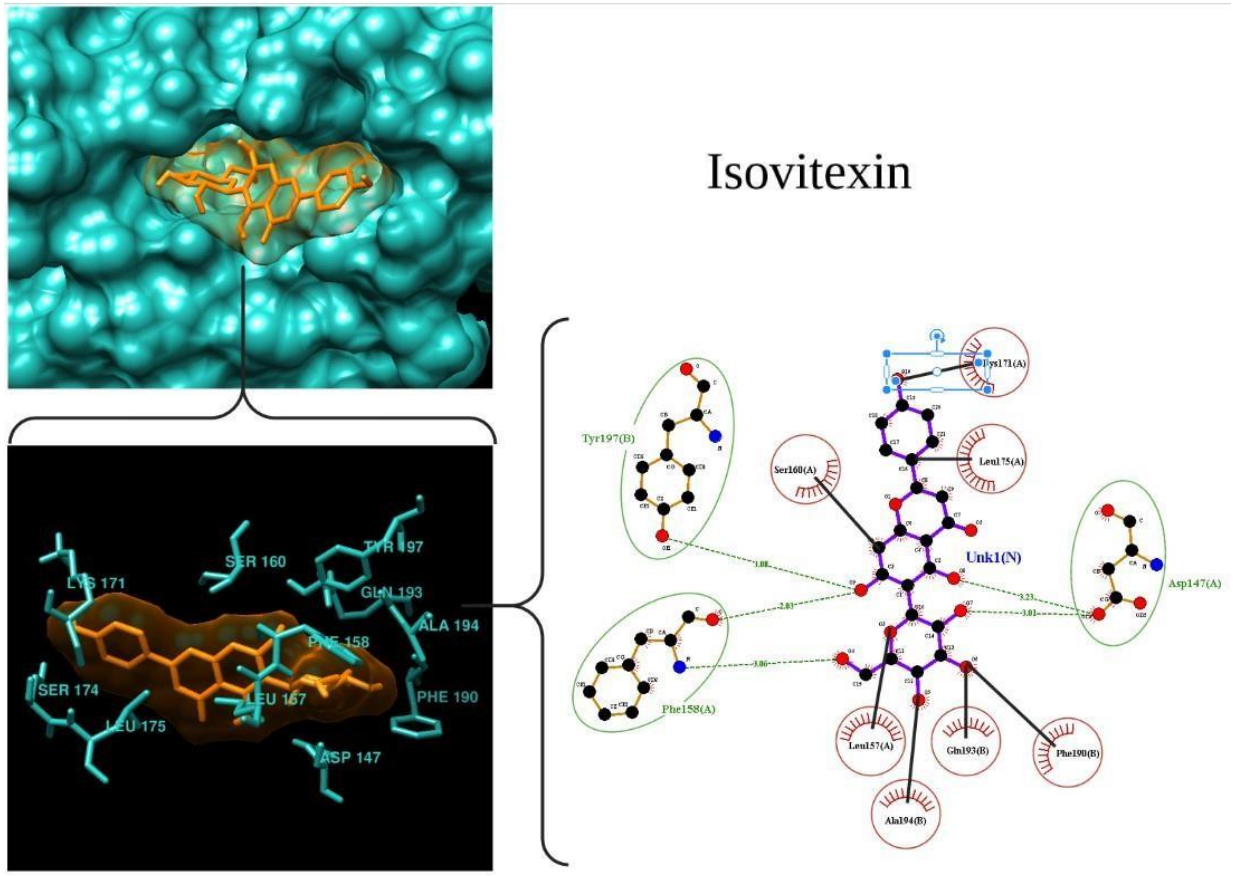


Figure 9: Isovitexin docked in LOX-1 Receptor; top left images depicts the best binding formation, bottom left images depict residues involved in interactions, right most images depict the binding interactions of Curcumin and Piperolactam A. Red circles = Hydrophobic bonds; Green ovals = Hydrogen bonds.

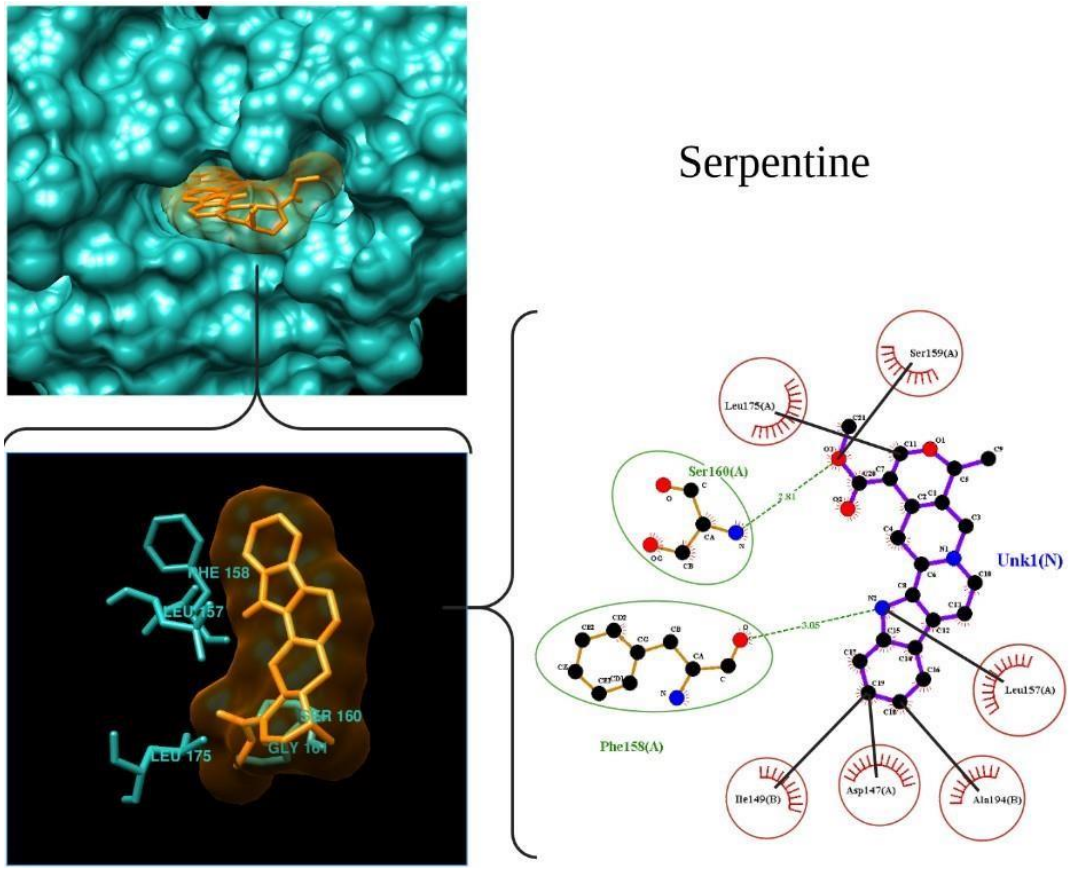


Figure 10: Serpentine docked in LOX-1 Receptor; top left images depicts the best binding formation, bottom left images depict residues involved in interactions, right most images depict the binding interactions of Isovitexin and Serpentine. Red circles = Hydrophobic bonds; Green ovals = Hydrogen bonds.

Quercetin

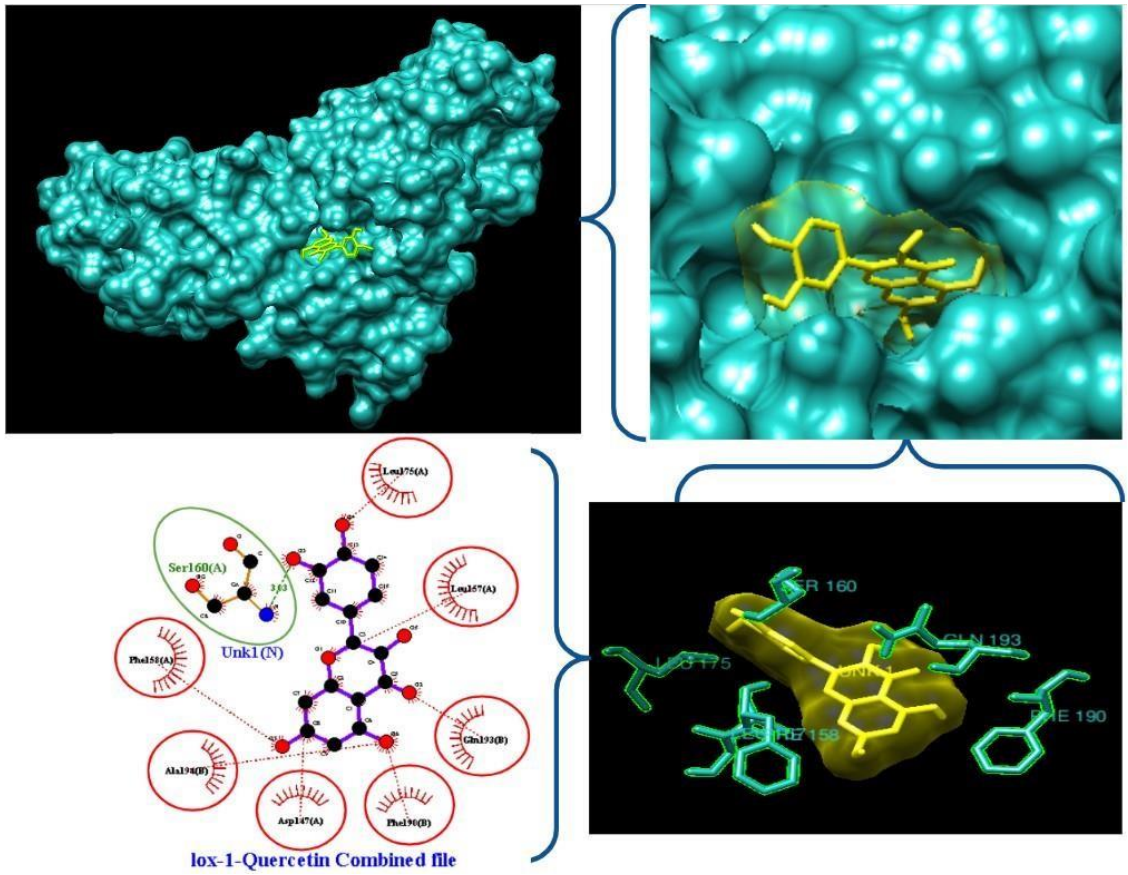


Figure 11: The image on the top left (enlarged) and right (zoomed) shows Quercetin paired with the LOX-1 receptor (1YXK); it shows the optimal binding mode inside the macromolecule compartment. The residues involved in the interaction are shown in the bottom right picture (Ligand in yellow sticks). The binding interaction of Quercetin with LOX-1 was seen in the image on the right.

Luteolin

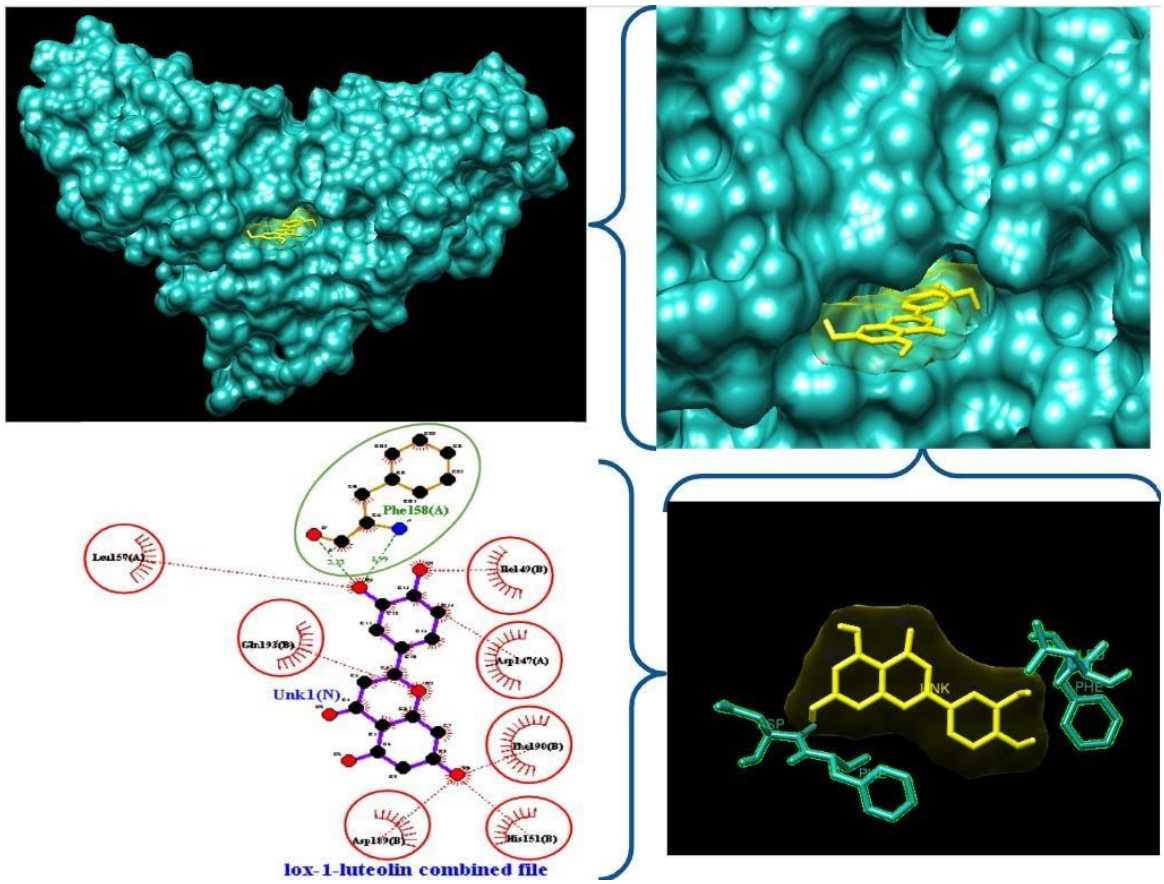


Figure 12: The image on the top left(zoomed out surface) and right(zoomed in surface) shows Luteolin paired with the LOX-1 receptor (1YXK); it shows the optimal binding mode inside the macromolecule compartment. The residues involved in the interaction are shown in the bottom right picture (Ligand in yellow sticks). The binding interaction of Luteolin with LOX-1 was seen in the image on the right.

Apigenin

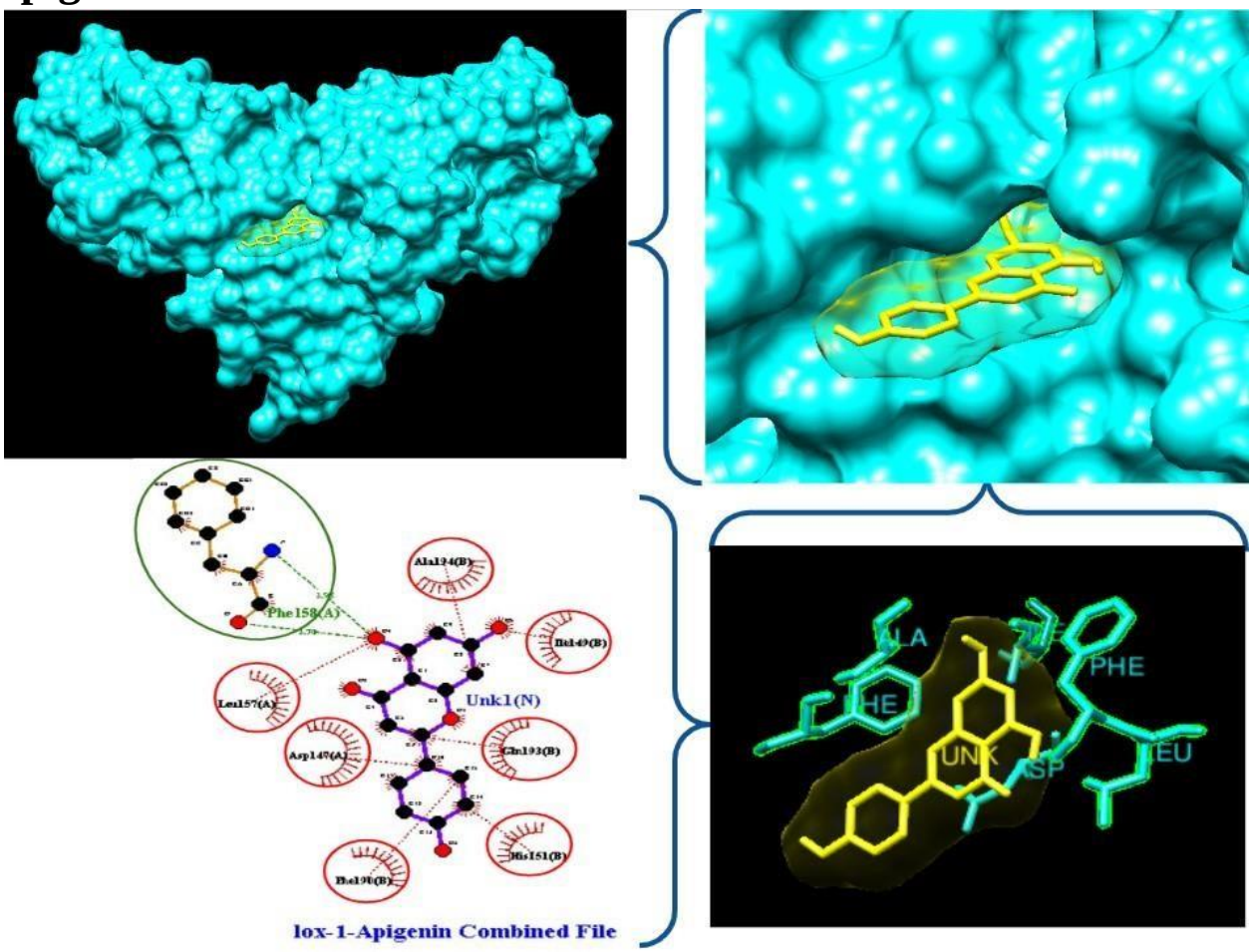


Figure 13: The image on the top left(zoomed out surface) and right(zoomed in surface) shows Apigenin paired with the LOX-1 receptor (1YXK); it shows the optimal binding mode inside the macromolecule compartment. The residues involved in the interaction are shown in the bottom right picture (Ligand in yellow sticks). The binding interaction of Apigenin with LOX-1 was seen in the image on the right.

Malvidin

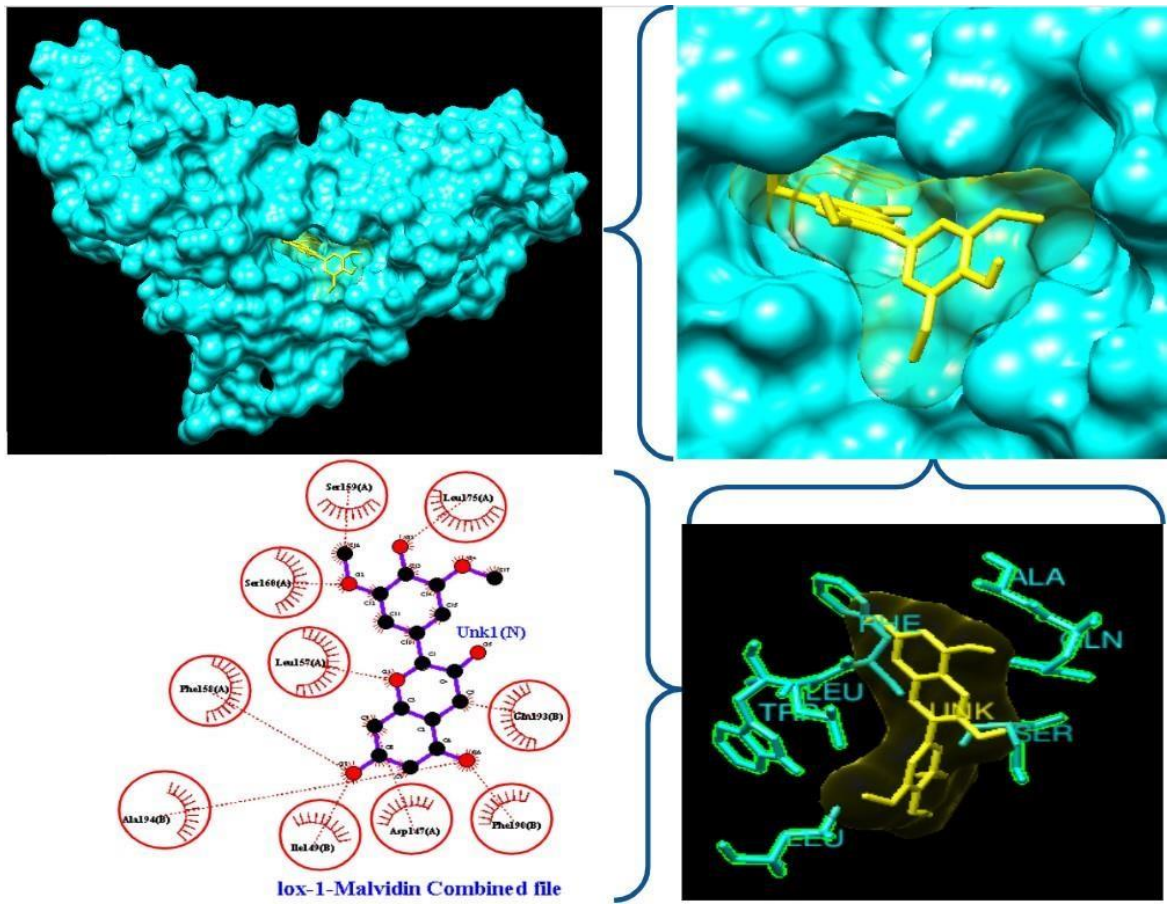


Figure 14: The image on the top left(zoomed out surface) and right(zoomed in surface) shows Malvidin paired with the LOX-1 receptor (1YXK); it shows the optimal binding mode inside the macromolecule compartment. The residues involved in the interaction are shown in the bottom right picture (Ligand in yellow sticks). The binding interaction of Malvidin with LOX-1 was seen in the image on the right.

Daidzein

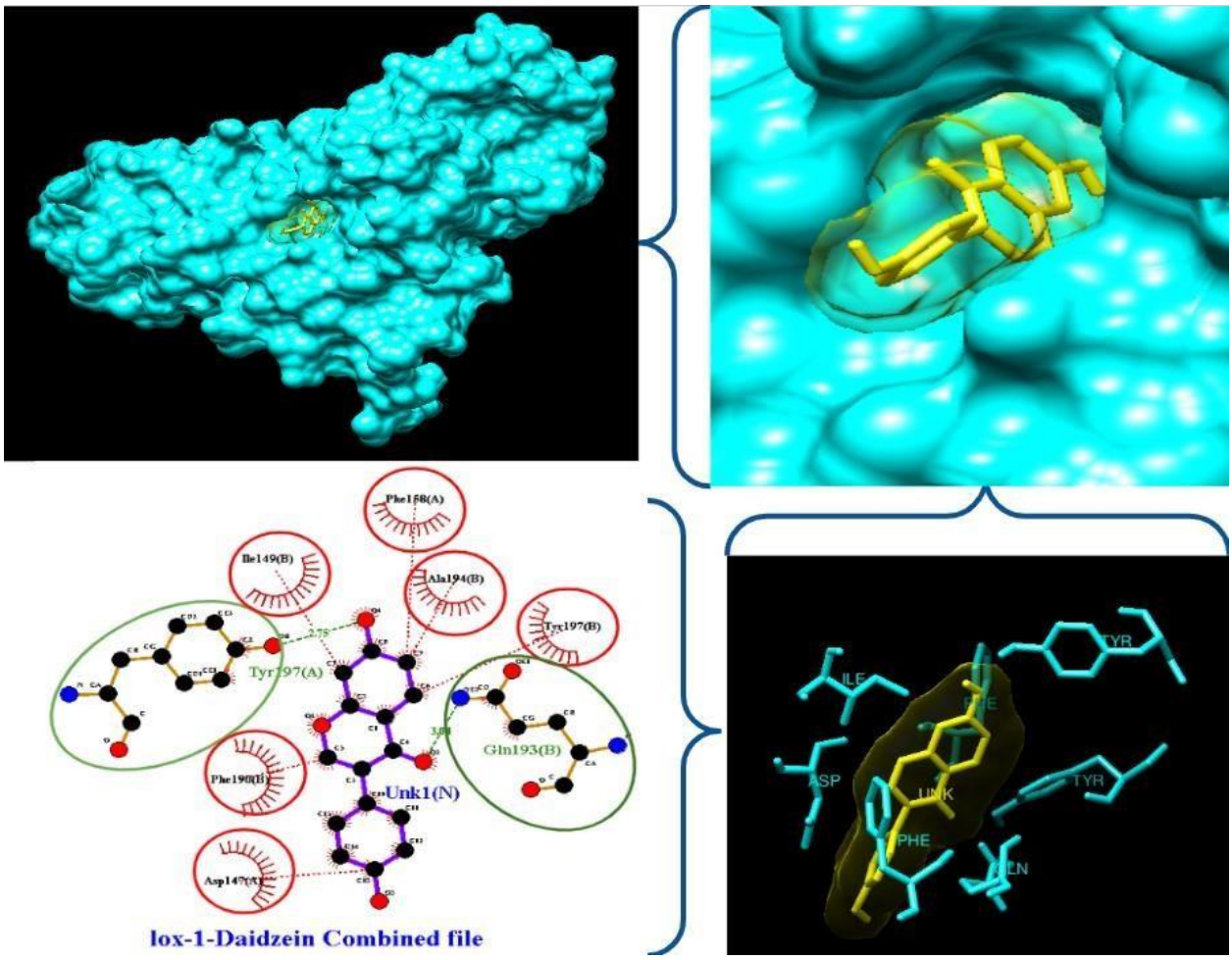


Figure 15: The image on the top left(zoomed out surface) and right(zoomed in surface) shows Daidzein paired with the LOX-1 receptor (1YXK); it shows the optimal binding mode inside the macromolecule compartment. The residues involved in the interaction are shown in the bottom right picture (Ligand in yellow sticks). The binding interaction of Daidzein with LOX-1 was seen in the image on the right.

Table 2: Depiction of the Hydrogen bonds and Hydrophobic Interactions found between the compounds and the amino acid residues of the LOX-1 receptor.

Compound Type	Compound Name	Hydrogen bonds	Hydrophobic Interactions

Statins	Atorvastatin	Leu175(3.17A), Lys266(2.94A)	Pro145, Gln146, Asp147, Trp148, Leu157, Phe158, Asp189, Phe190, Gln193, Ala194, Tyr197
	Fluvastatin	Phe158(3.34Å), Ser160(3.34Å and 2.85Å)	Asp147, His151, Leu157, Ser159, Lys171, Leu175, Phe190, Gln193, Ala194, Tyr197
	Pravastatin	Gln146(3.09Å), Asp147(2.70A), Trp148(3.19Å), Phe158(2.91Å), Tyr197(2.87Å)	Pro145, Leu157, Ser160, Phe190, Gln193
	Lovastatin	Asp189(3.21Å), Gln193(2.98Å)	Asp147, Leu 157, Phe158, Ser160, Phe190, Ala194, Tyr197
Chosen Ligands	Curcumin	None	Asp147, His151, Leu157, Phe158, Ser160, Phe190, Gln193, Ala194, Tyr197
	Puerarin	Asp147(3.21 Å), Phe158 (3.13 Å), Tyr197(2.83Å)	Trp148, Leu157, Ser160, Leu175, Phe190, Gln193, Ala194
	Piperolactam A	Phe158(2.94Å)	Asp147, Ile149, Leu157, Ser160, Phe190.Gln193, Ala194
	Isovitexin	Asp147(3.23Å and 3.01Å), Phe158(2.83Å and 3.06Å), Tyr197(3.08Å)	Leu157, Ser160, Lys171, Leu175, Phe190, Gln193, Ala194
	Serpentine	Phe158(3.05Å), Ser160(2.81)	Asp148. Ile149, Leu157, Ser159, Leu175, Ala194
	Quercetin	Ser160(3.03)Å	Leu175(A), Leu157(A), GIn193(B), Phe190(B), Asp147(A), Phe158(A), Ala194(B)

	Luteolin	Phe158(3.23Å) and(2.99Å)	Leu157(A), Ile149(B), GIn193(B), Asp147(A), Phe190(B), Asp189(B), His151(B)
	Apigenin	Phe158(3.70Å) and (2.98Å)	Leu157(A), Asp147(A), Phe190(B), His151(B), GIn193(B), Ile149(B), Ala149(B)
	Malvidin	None	Ser159(A), Leu175(A), Ser169(A), Leu157(A), Phe158(A), Aln194(B), Ile149(B), Asp147(A), Phe190(B), Gln193(B)
	Daidzein	Tyr197(2.75Å), GIn193(3.04Å)	Asp147(A), Phe190(B), Ile149(B), Phe158(A), Ala194(B), Tyr197(B)

3.7. Molecular Dynamics Simulation

3.7.1. Binding Energy fluctuation results:

The molecular dynamics simulation (MDS) was performed using the CABS-flex 2.0 web server, applying the default parameters. Fluctuations in the amino acid residues in the unbound LOX-1 and bound structure of LOX-1 with the different ligands during a 10 ns molecular dynamics simulation are shown in Figure 16 & 17.

At a 0.05 significance level, two-tailed paired T tests were carried out for all of the ligands to determine the statistical differences in the fluctuation between the unbound LOX-1 structure and the bound LOX-1 structure (Table 3). It was derived that two of the 10 ligands show signs of instability in the A chain, however, only 3 of the 10 ligands show signs of stability in the B chain. This is supported by the fluctuation differences visible in the chart as well. In the A chain, there's some signs of differences overall, however mostly the lines follow a similar path. Likewise in the B chain, there appears to be quite a bit of fluctuation differences within the initial 8 residues as well as residues B207-B247.

Table 3: Results derived from conducting a paired two-tailed T test. Results obtained from separate chains are separately shown. p value <0.05 indicates significant differences in fluctuation, p >0.05 score indicates no significant difference in fluctuation.

Chain	PUE	CRC	PIP	IVT	SRP	DDZ	QRC	APG	MLV	LTL

A	p>0.05	p>0.05	p<0.05	p>0.05	p>0.05	p>0.05	p<0.05	p>0.05	p>0.05	p>0.05
B	p<0.05	p>0.05	p<0.05	p<0.05	p>0.05	p<0.05	p<0.05	p<0.05	p<0.05	p>0.05

PUE - Puerarin; CRC - Curcumin; PIP - Piperolactam A; IVT - Isovitexin; SRP - Serpentine; DDZ - Daidzein; QRC - Quercetin; APG - Apigenin; MLV - Malvidin; LTL - Luteolin.

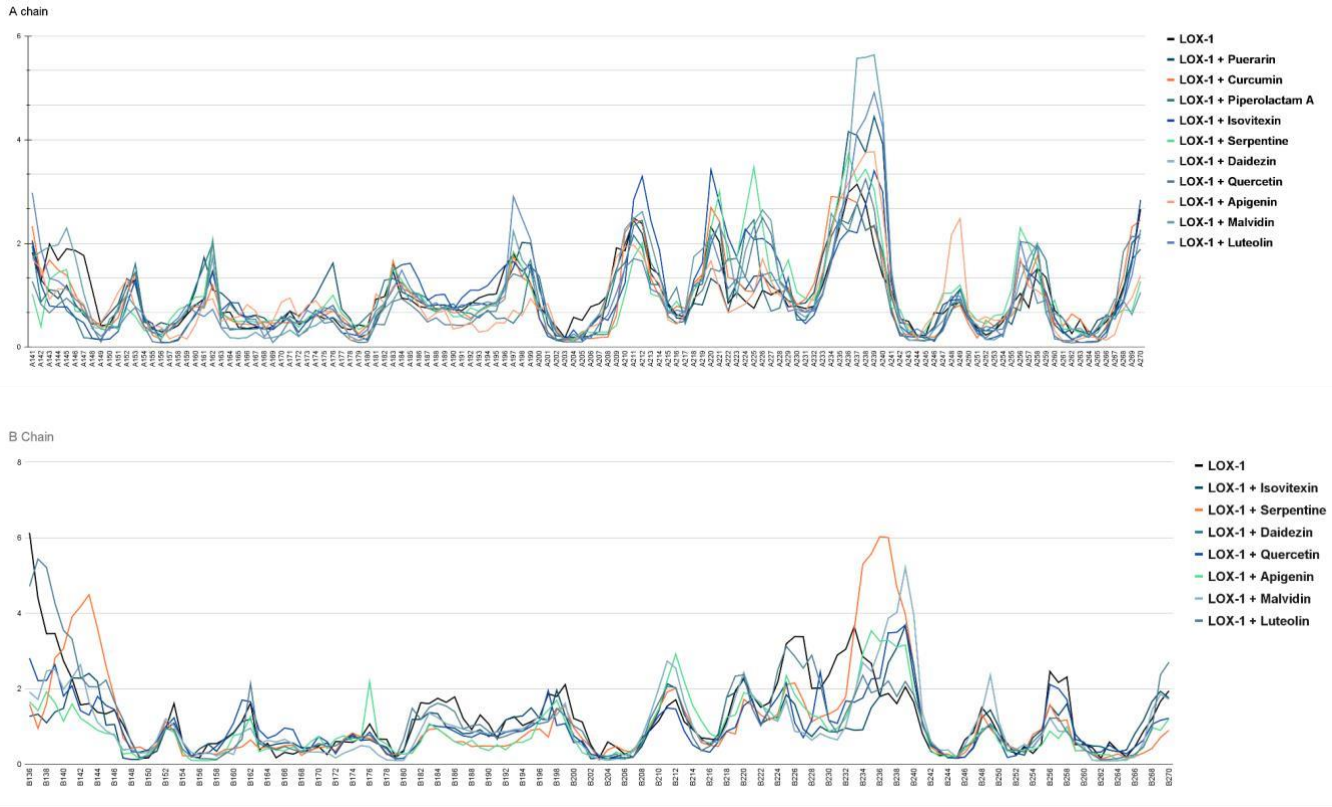


Figure 16 & 17: Visual comparison between the fluctuation points observed in both of the chains observed in unbound LOX-1 (black line) as well as the bound LOX-1 complexes.

3.7.2. Binding free energy analysis

Molecular mechanics Poisson–Boltzmann surface area (MMPBSA) and molecular mechanics generalized Born surface area (MM-GBSA) methods were used to evaluate the binding free energies of the compounds (Table 4).

Lox-1 receptor residues that contribute to the decompose results consisting of the electrostatic energy (TELE) as calculated by the MM force field, van der Waals contribution (TVDW) from

MM, total gas phase energy (TGAS), the sum of non-polar and polar contributions to solvation (TGBSOL), final estimated binding energy calculated from the terms above (TGBTOT), are displayed in a heatmap. The unit of them is kcal/mol. The residues with contribution ranked top 10 in decompose calculation are arranged from top to bottom according to the energy ('TGBTOT'), and values are centered and scaled in the row direction shown with heatmap. In all 10 protein-ligand complexes, the major contribution to the binding energy is the van der Waals energy component.

Some of the residues are more common than others among these 10 protein-ligand complexes. A157, B193, A147, B190, and B194 are the top contributors to all of these 10 complexes.

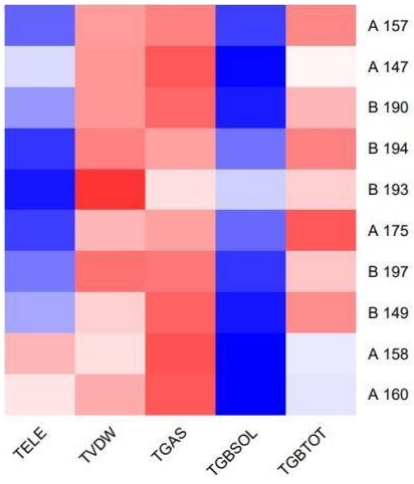
Curcumin and Apigenin has the most suitable result both having the best binding energy, Apigenin having the most binding energy with ASP(A)147(-3.24 kcal/mol) and Curcumin ASP(A)147 (-3.19 kcal/mol) . Isovitexin has the second most binding energy with LEU(A)157 (2.95 kcal/mol). Malvidin and Luteolin have the third most binding energy both similar at ASP(A)147 (-2.67 kcal/mol). Piperolactam A and Serpentine have the fourth and fifth most binding energy with GLN(B)193 (-2.44 kcal/mol) and GLN(B)193 (-2.52 kcal/mol), . Puerarin and Daidzein have the lowest binding energy LEU(A)157 (-2.41kcal/mol) and B193 (-2.24 kcal/mol).

Table 4: MM-PB(GB)SA binding free energy (kcal/mol) calculations for the protein-ligand complexes.

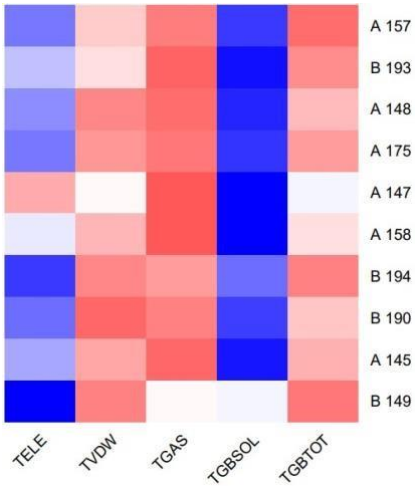
Protein-ligand complexes	**ELE**	**VDW**	**GAS**	**PBSOL**	**PBTOT**	**GBSOL**	**GBTOT**	**TS**	**delGPB**	**delGGB**
LOX1-Serpentine	-6.96	-36.44	-43.39	13.81	-29.58	8.34	-35.05	14.82	-14.76	-20.23
LOX1-PUERARIN	-16.23	-46.65	-62.88	31.88	-31	24.15	-38.73	18.11	-12.89	-20.62
LOX1-Piperolactam A	-2.47	-34.45	-36.92	13.14	-23.78	7.92	-29	8.6	-15.18	-20.4
LOX1-Isovitexin	-21.59	-42.53	-64.12	33.18	-30.95	28.35	-35.78	18.01	-12.94	-17.77
LOX1-Curcumin	-5.18	-36.45	-41.63	20.42	-21.21	15.68	-25.95	17.52	-3.69	-8.43
LOX1-Quercetin	-4.76	-34.52	-39.281	9.17	-20.12	11.84	-27.44	11.33	-8.79	-16.11

LOX1-Luteolin	-6.03	-33.8	-39.84	19.01	-20.83	11.13	-28.71	14.97	-5.86	-13.74
LOX1-Apigenin	-2.77	-34.38	-37.15	14.72	-22.42	8.66	-28.49	8.94	-13.48	-19.55
LOX1-Malvidin	-5.78	-35.42	-41.2	16.69	-24.51	11.48	-29.72	16.38	-8.13	-13.34
LOX1-Daidzein	-8.64	-35.26	-43.89	17.26	-26.64	12.52	-31.37	10.11	-16.53	-21.26

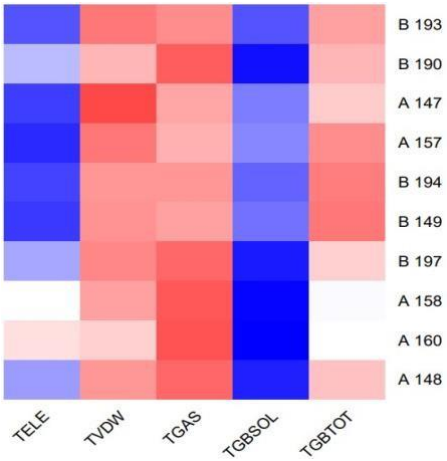
ELE: Electrostatic energy; VDW: van der Waals energy; GAS: Gas phase energy; PBSOL, GBSOL: Non-polar and polar solvation energy; GBTOT: Summation of electrostatic energy, van der Waals energy and non-polar and polar solvation energy; TS: Entropic energy; delGPB, delGGB: Final estimated binding free energy.



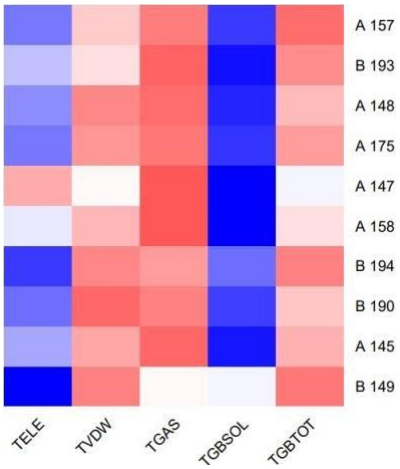
a). Curcumin



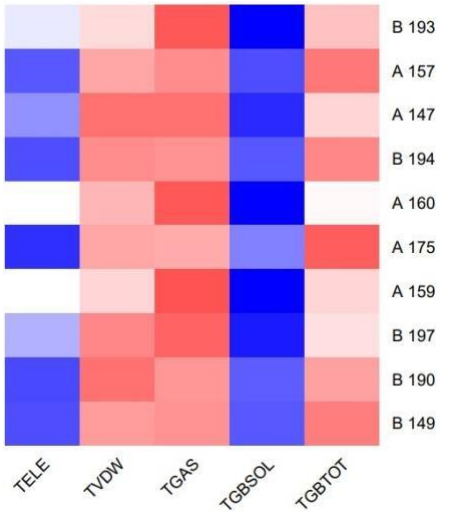
b). Puerarin



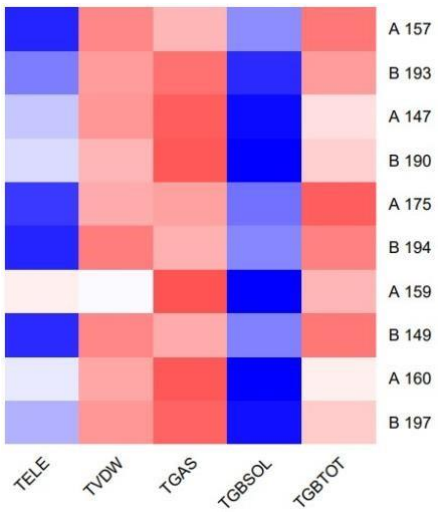
c). Piperolactam A



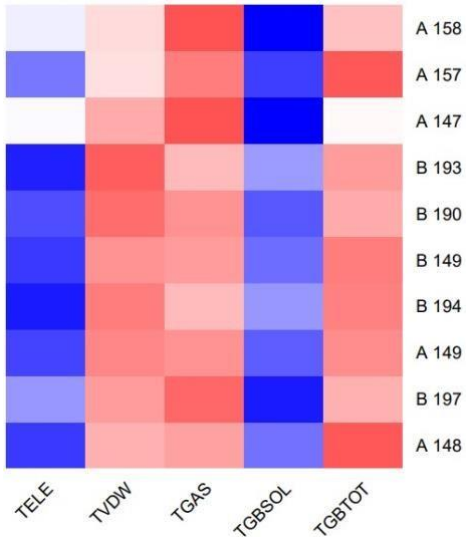
d). Isovitexin



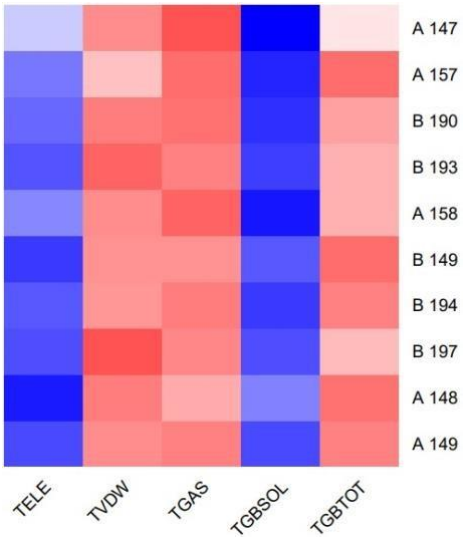
e). Serpentine



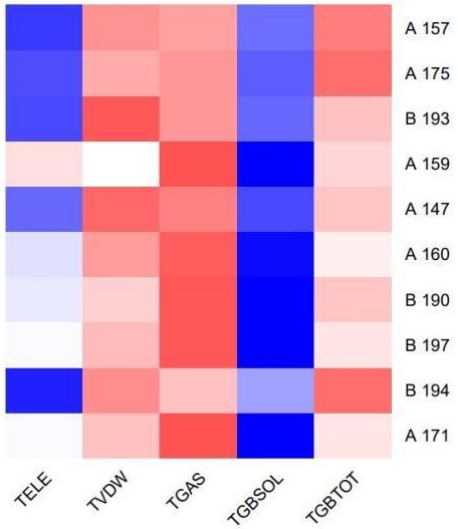
f). Quercetin



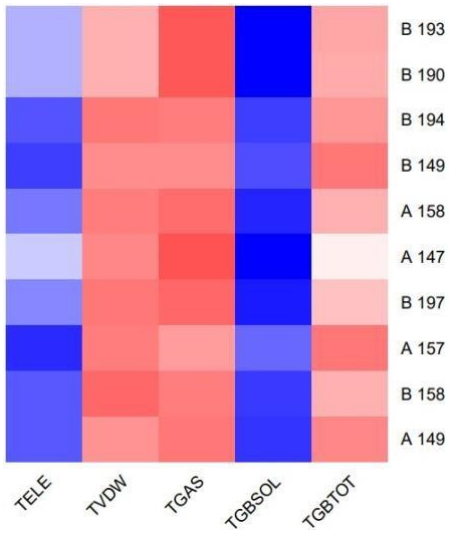
g). Luteolin



h). Apigenin



i). Malvidin



j). Daidzein

Figure 18: Heatmap showing the energy decomposition for top 10 residues (a) Lox-1_Curcumin (b) Lox-1_Puerarin (c) Lox-1_Piperolactam A (d) Lox1_Isovitexin (e) Lox-1_Serpentine (f) LOX-1_Quercetin (g) LOX1_Liteolin (h) LOX1_Apigenin (i) LOX1_Malvidin (j) LOX1_Daidzein

Chapter 4: Discussion

4. Discussion:

4.1. Initial selection and pharmacokinetic analysis

Due to the severity of cardiovascular diseases, and especially atherosclerosis, the need for effective and potent medicine is always there. Additionally, despite the presence and the effectiveness of already established statin drugs, the side-effects mentioned before leaves their versatility upto debate. In order to address and possibly tackle this situation, 5 pharmacophore compounds alongside 4 statin drugs were tested against LOX-1, a transmembrane scavenger receptor responsible for the oxidation of endothelial cells (Sawamura et al., 1997). With the ultimate goal being the validation for the efficacy of naturally found (plant extract) compounds against the formation of either oxidized cells, reactive oxygen species or inhibition of the receptor altogether, the statin drugs were used as control in order to set a standard to compare the potency of the compounds. However, in order to carry this process out, there were a number of screening methods put forward first.

First of all, 10 pharmacophore ligands were selected based upon the study of “Lipinski rule of 5”. This suggested that for a compound to be considered an efficient drug, it has to meet a certain set of criterias, such as H-bond donors being less or equals to 5, H-bond acceptors being less or equals to 10, molecular mass being less than 500 g/mol and logP being less than 5. However, a compound can still be used as a drug if only one of these criterias aren’t met. In order to carry out this process, two web tools known as SwissADME, and pkCSM were used to retrieve pharmacokinetic results. From the initial 20 compounds chosen for the screening, 10 different phytochemicals; namely Puerarin, Curcumin, Isovitexin, Piperolactam A, Serpentine, Apigenin,

Quercetin, Luteolin, Malvidin and Daizedin were found to show the most promising results. Alongside the drug-likeness of the compounds, certain important pharmacokinetic information were derived from the tests as well.

Serpentine is the only compound among them to show hepatotoxicity, however it is also the compound with the highest amount of intestinal absorption (97.74%) and total clearance rate (1.153 log ml/mm/kg). Besides that, Puerarin, Isovitexin show intestinal absorption of 65+-2%,

Quercetin, Curcumin, Malvidin and Luteolin show intestinal absorption ranging from ~77% to ~89%. Only Piperolactam A, Serpentine, Apigenin and Daidzein show intestinal absorption over 90%. Additionally, Curcumin and Piperolactam A show the highest LogP/Lipophilicity values at over 3.2. On the other hand, Puerarin and Malvidin and Isovitexin have the lowest LogP values at 0.3861, 0.92 and 0.0917 respectively.The general consensus is that a LogP value between 1-4 indicates stable properties for oral drugs (Gleeson, 2008), it can be said that Puerarin, malvidin and Isovitexin will show poor ADMET properties, while Curcumin and Piperolactam A despite having a good LogP value can run into the risk of poor absorption and solubility (Arnott & Planey, 2012). This can be seen in established literature regarding Curcumin's poor absorption and solubility already (Zheng & McClements, 2020). Finally, Aside from Piperolactam A,
Serpentine and Daidzein, no other molecule showed signs of blood-brain barrier permeability.

4.2. Tunnel and amino acid prediction

Before carrying out the docking process, it is imperative to understand which subunits the desired ligands are most likely to bind, as well as to locate the area at which the subunits are situated. For LOX-1, the best binding points were suggested to be within the hydrophobic tunnel based upon previous studies (Falconi et al., 2013, Draude et al., 1999, Guan et al., 2010). To verify, the protein chain was analyzed through both Caver web service as well as CASTp web service in order to obtain the hydrophobic tunnel's information as well as the best binding sites available within the protein itself. As mentioned before, the Caver web analysis indicates Asp14, the tunnel contains almost all of the residues that are predicted to be potent binding sites by CASTp. Additionally, the binding patterns observable from Figure 6 - Figure 15 and residues listed in Table 2 also support the initially mentioned findings; including the ones suggesting LOX-1 receptors tunnel to be a critical binding point for OxLDL.

4.3. Molecular docking analysis:

The molecular docking analysis, a process to understand the ability of a compound to act as a drug when attached to the desired receptor was carried out among all 10 compounds. Conducted both in Chimera and PyRX software in order to cross reference docking scores, the results represented a potential for the compounds to act as drugs compared to the statins. (Some extra info about other stuff maybe). However, from softwares such as Chimera or PyRX it was possible to only understand the amount and position of the subunits binding with the ligand; a tool such as Ligplot (info) was utilized to further analyze the specific types of bonds that the ligand had taken part in, which are hydrogen bonds and the hydrophobic interactions. This made possible a visual analysis of the possible bindings; in terms of the specific subunits and their binding type, the amount and distance of derived Hydrogen bonds as well as the amount and strength of hydrophobic bonds. Upon observation, the H-bonds appear to have the most visible pattern among all of them; Asp147, Phe158, Ser160, Gln193 and Tyr197 appear to have the most common binding patterns across both the statins as well as the selected compounds in terms of hydrogen bonds.

4.4. Molecular dynamics simulation

Molecular dynamics simulation(MDS) is perhaps the most important step during drugdetermination due to the fact that it indicates the stability of a compound when complexed with the desired macromolecule. There are a wide variety of tools available ready to be utilized in order to determine the binding viability of the compound as a drug. For this study, CABS-flex 2.0 and LARMD web services were utilized in order to have a thorough understanding of the process. CABS-flex web service is an efficient method in the determination of protein flexibility. Applying the default parameters (10ns), the observed fluctuations for LOX-1 unbound as well as bound to all 10 of the molecules are observed at figure 16 and figure 17. However, in order to understand whether or not the differences visible in the fluctuation points are significant or not, a statistical paired two-tailed T test; significance (α) of 0.05 has to be conducted between unbound LOX-1 and all of its complexes. According to the obtained data, it can be seen that piperolactam A and Quercetin show significant differences in fluctuation in both chains; Curcumin, Serpentine and Luteolin show no significant fluctuation differences in both chains; rest of the molecules appear to show no significant fluctuation differences in A chain, although the opposite can be observed in B chain. This leads to the verdict that Curcumin, Serpentine and Luteolin would potentially prove to

be the most versatile options to inhibit LOX-1 with, while Piperolactam A and Quercetin would potentially prove to be the least versatile options to inhibit LOX-1 with. Besides that, the rest of the molecules could prove to be potent drugs if expected to bind exclusively to the A chain of the LOX-1 receptor.

In order to further analyze the binding energy and efficacy of compounds as drugs, the LARMD web service was utilized, and heat maps depicting the binding energy between the compounds and LOX-1 were observed. LARMD website helps in Molecular dynamics utilizing bioinformatic tools through third parties. Utilizing the "nor" mode(this mode provides the quickest possible result) of LARMD the free binding energy of protein ligand complex were calculated where it was derived that Apigenin and Curcumin had the most amount of free binding energy with ASP(A)147 at (3.24kcal/mol) and (-3.19 kcal/mol) respectively followed by Isovitexin with LEU(A)157 at (-2.95 kcal/mol) while Serpentine,Puerarin and Daidzein has the least amount of free binding energy at GLN(B)193 (-2.44 kcal/mol), LEU(A)157 (-2.41kcal/mol) and GLN(B)193 (-2.24 kcal/mol) respectively.

Due to the versatility observed in LOX-1 and Curcumin complex, alongside its depicted release of the most amount of binding energy during the binding process, it can be said that Curcumin is a very promising compound for Lox-1 inhibition. On the other hand, while Serpentine showed stability in both chains, it also appeared to show the third least free binding energy among the 10 molecules which puts its overall quality among the 10 to question. However, Daidzein not only shows exclusive stability to A chain, but also the best binding energy it frees is within a B chain residue. Therefore, it can be said that Daidzein has one of the least amount of potential in terms of acting as a proper drug against LOX-1 receptors. For the rest of the molecules, since Piperolactam A and Quercetin appear to show significant fluctuation differences among both of the chains, it can be said that they aren't reliable in terms of forming a proper complex. Finally, as mentioned before the rest of the molecules appear to be stable and promising when complexed with the A chain, leading to the verdict that they have potential in terms of being a proper drug, although only Luteolin and Serpentine shows the versatility in terms of stability.

5. Conclusion

Cardiovascular diseases are indeed an extremely dangerous form of precursor for human fatalities every year, and atherosclerosis is the main precursor for the most prevalent form of cardiovascular

diseases. And although there have been statins established to help a suffering patient alongside the necessary moderation of eating and exercise habits, there can be some instances where either a statin might have an equally worse side-effect, or some where the patient might be allergic or incompatible to the statins entirely. This opens up the demand for natural alternatives for statins that might not suffer from the same drawbacks as statins while acting as a potent drug against atherosclerosis. This study attempts to utilize 10 promising naturally-derived compounds in a molecular dynamic analysis in order to compare their efficiency as a drug to that of a few established statins. Utilizing the benefits of multiple web services and offline tools, a number of tests detailing the information of ligands, desired protein, the binding affinity, the viability and stability of the phytochemicals as drugs were determined. In the end, it was determined that most of the compounds; Curcumin and Serpentine appearing to be the most versatile; Apigein and Isovitexin having the best observed free binding energies show a promising result against LOX-1 receptor. On the other hand, Daidzein, Piperolactam A and Quercetin show the least amount of potential as a drug against the LOX-1 receptor.

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