Hyperlipidemia control by using the innovative association of lupin proteins and chitosan and α-cyclodextrin dietary fibres: food supplement formulation, molecular docking study and in vivo evaluation

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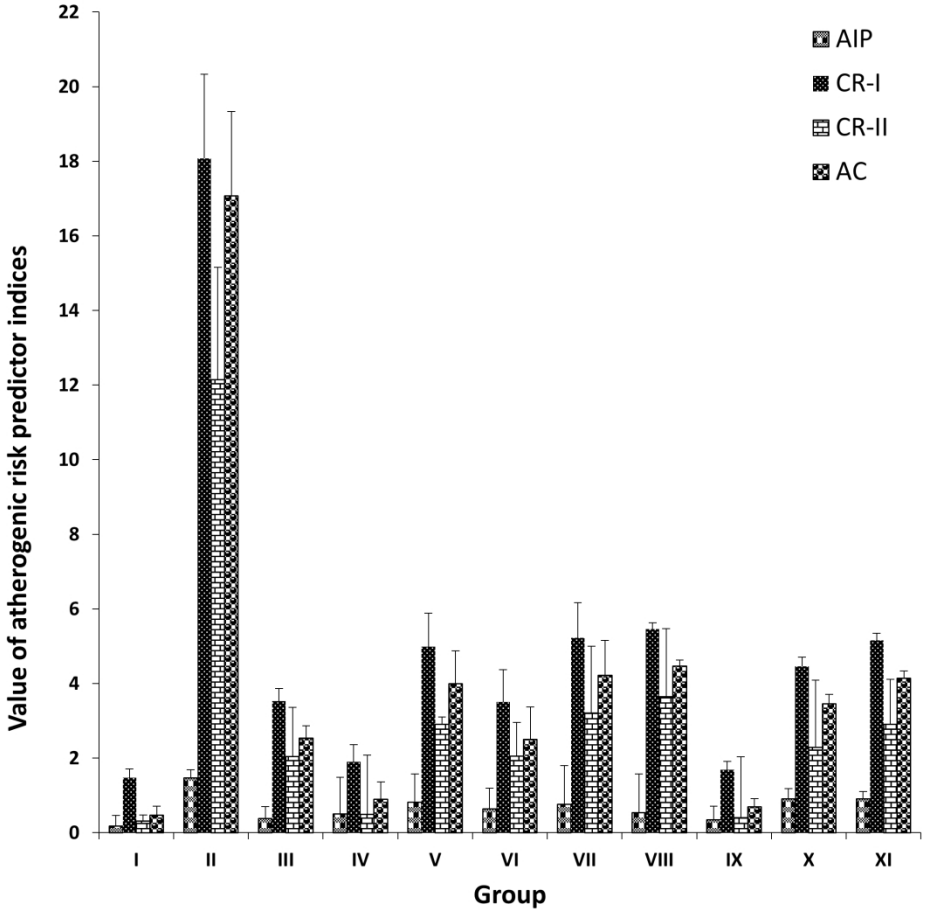

Figure 1. Atherogenic risk predictor indices of different treatments and protective groups compared to negative control (Group I) and hyperlipidemic positive control (Group II) groups.

Hyperlipidemia control by using the innovative association of lupin proteins and chitosan and α-cyclodextrin dietary fibres: food supplement formulation, molecular docking study and *in vivo* evaluation

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Abstract

A dietary supplement potentially employed for the treatment and/or prevention of hyperlipidemia was developed. The proposed product is composed of a combination of natural macromolecules as chitosan (CH), α-cyclodextrin (α-CD), and lupin proteins (LP). Firstly, the antihyperlipidemic effect of the α-CD and LP binary mixture was assessed and compared to that of the extensively utilized antihyperlipidemic CH, using a hyperlipidemicrat model. The antihyperlipidemic effect of their combination was also demonstrated. Additionally, ligand-target and protein-protein docking studies were performed. The in vivo results displayed that on intergroup comparison, blending CH, α-CD, and LP promised a superior therapeutic effect over α-CD and LP mixture, CH and the marketed atorvastatin, potentiating a considerable reduction of serum lipid profile and the calculated atherogenic risk predictor indices. Molecular docking study revealed a weak hydrophobic cholesterol-CH and cholesterol-α-CD interactions while protein-protein docking study showed a good lipase-LP interaction, involving 8 hydrogen bonds. Then, on the base of the *in vivo* and docking study results, a tablet formulation was produced aimed to overcome the negative technological effects of the antihyperlipidemic macromolecules: long disintegration time and tablets mechanical resistance. The optimized tablet formulation has a disintegration time shorter than 15 minutes and a weight loss from friability test lower than <1%, which are in line with the regulatory specifications for uncoated tablets. Overall, this antihyperlipidemic formulation is attractive for the dietary and nutraceutical market, despite further clinical studies are required to assess the efficacy, possible side effects, and product compliance.

Keywords: chitosan; cyclodextrin; lupin proteins; cholesterol; molecular docking; tablets.

1. Introduction

In recent years, atherosclerotic cardiovascular diseases are one of the main causes of death in most developing nations around the world [1]. In this respect, hyperlipidemia represents an influent risk factor for the development and progression of cardiovascular diseases[2]. Thus, cholesterol-lowering therapy could reduce the outbreak of cardiovascular diseases (*e.g.*, coronary heart disease)as well as the risk of cerebrovascular accidents in hyperlipidemic patients [3].

Although the reduction of serum cholesterol could be achieved, in most cases, by following a well-balanced diet with low fat consumption, and by doing regular physical exercise, unfortunately, most of the time, these strategies fail due to poor compliance[4].Nowadays, statins represent the treatment of choice for hyperlipidaemias [5, 6]. They act as inhibitors of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA)reductase, the enzyme that regulates the synthesis of cholesterol [7]. However, they showed also severe side effects, *i.e.*, muscle diseases as myopathy and rhabdomyolysis, diabetes mellitus, and central nervous system complaints, that are reducing their use in the prevention and/or therapy of cardiovascular diseases [8].

For this reason, the pharmacological approach has been, in part, replaced by the introduction of diet and food supplements and nutraceuticals, which are showing encouraging results on the prevention and treatment of hyperlipidaemia[9]. In the last years, the most advised supplement has been the red yeast rice, considered as a new hypolipidemic treatment [10]. Red yeast rice contains monacolin K, a metabolite comparable, in terms of structure and mechanism of action, to statins, in particular lovastatin. However, the European Food Safety Authority (EFSA) declared that exposure to monacolin K from red yeast rice could lead to severe adverse effects, similar to those of statins, on the musculoskeletal system (including rhabdomyolysis) and the liver [11].

Nevertheless, the hypolipidemic action is exerted by many other biologically active substances, such as dietary fibers, proteins, and phytosterols[12, 13]. In particular, soluble dietary fibres are one of the most used supplements to control the level of serum lipids, acting through the reduction of dietary fats and cholesterol absorption[14]. Among them, chitosan, the deacetylated form of chitin,

an organic polymeric substance, mainly extracted from the shells of crustaceans, has shown an effective hypolipidemic activity. Chitosan acts by reducing the lipids absorption from the gastrointestinal tract rather than by blocking the cholesterol synthesis, as statins do. In fact, due to its limited digestibility, chitosan behaves as a dietary fibre. Being the amino groups of chitosan (-NH_3^+)positively charged, it can bind negatively charged substrates (X-COO^-), *i.e.*, fatty acids, bile acids, and other lipids, that will be excreted through the feces. Chitosan, through the formation of ionic complexes with lipids, reduces their absorption and interrupts the enterohepatic circulation that, consequently, stimulates the consumption of endogenous cholesterol [15].

Another promising soluble dietary fibre is α-cyclodextrin (α-CD). It derives from corn and it is frequently used as a dietary supplement as well as to stabilize and improve organoleptic properties of food and beverages[16]. Its hypocholesterolemic activity is mainly due to the chemical structure. In fact, α-CD is a cyclic oligosaccharide composed of six glucose units that form a ring structure, with an internal hydrophobic cavity where lipid molecules could be bound [17]. Thus, it can complex dietary fats and serum lipids, thereby interfering with triglycerides and cholesterol absorption[18]. Thanks to its high affinity for lipids, α-CD can be considered as one of the most effective soluble fibre in reducing lipids absorption [19]. Moreover, thanks to its safety and tolerability, FDA recognised α-CDas a Generally Recognized As Safe (GRAS) compound. Referring to literature, despite their similar complexation capabilities, α-CDwas found to be more tolerable and safer relative to β-CD that was demonstrated to negatively compromise the components of cell barriers, several eukaryotic cell lines and human erythrocytes.

Plant proteins can exert a relevant antilipidemic and anticholesterolemic effect as well. In particular, a legume highly rich in proteins is lupin (*Lupinus* spp.). The efficacy of lupinin reducing lipid levels could be mainly ascribed to some bioactive peptides, that can interact directly or indirectly with the enzymes related to the lipids metabolism [20]. In particular, conglutin γ was found to be the protein responsible for the hypocholesterolemic effect of lupin, by stimulating the LDL receptor

transcription[21].Moreover, lupin proteins showed to be effective as a protective agent against atherosclerosis progression in a rabbit model of atherosclerosis, reducing the risks of cardiovascular diseases [22]. In addition, clinical studies revealed that the supplementation of lupin proteins had a hypocholesterolemic effect, affecting positively the LDL and LDL:HDL ratio serum levels on hypercholesterolemic patients. No adverse effects were highlighted at the end of the treatments[23–25].

The present study aimed to provide an effective and safe hyperlipidemia treatment able to reduce the occurrence of cardiovascular diseases, avoiding the pharmacological approach. For this purpose, we hypothesized that a nutraceutical supplement based on the combination of three different dietary products with antihyperlipidemic activity *i.e.*, chitosan (CH), α-CD, and lupin proteins (LP) could attain health benefits and enhance the therapeutic competence. At first, the antihyperlipidemic attributes of α-CD /LP blend *versus* that of the well-demonstrated hypolipidemic macromolecule; CH was assessed in hyperlipidemic rats. In our attempt to understand the mechanisms of the hypocholesterolemic effects of our supplements, binding affinity of α-CD and CH against cholesterol as a ligand (Ligand-Target interaction) and LP against lipase enzyme (Protein-Protein interaction) were investigated by molecular docking studies. Based on the *in vivo* and molecular docking results, the active ingredients were formulated into tablets, to develop a hypocholesterolemic food supplement. Emphasis on compliance of these nominated dietary supplement tablets with the regulatory specifications for uncoated tablets was set.

2. Materials and Methods

2.1 Materials

Chitosan (viscosity of 1% solution 10 cP, degree of deacetylation 95%, protein content as total amino acids ≤0.1%, ash 0.2%; ChitoClear®), α-CD (CavamaxW6 Food, cyclodextrin content min. 98% according to USP/NF method), and lupin proteins from *Lupinus albus* (protein content 40%, Protilup 450) were kindly donated by Primex (Iceland), by WackerChemie AG (Germany), and by Inveja (France), respectively. Sodium bicarbonate was provided by Sigma-Aldrich (USA) and micronized silica USP from ACEF (Italy). Kollidon®CL (Crospovidone) was purchased by BASF, Lubritab® was provided by JRS Pharma. Poloxamer 407 (P-407, Pluronic F-127) was purchased from BASF Corporation, USA. All other materials were reagents of standard grade and were supplied by Sigma-Aldrich (USA).

2.2 *In vivo* study

All the study procedures were approved by the Research Ethics Committee of the Faculty of Pharmacy Ain Shams University (Egypt). A total of 66 male Albino rats aged 6-8 weeks old, weighing from 200 to 250 g, were selected for this study. Animals were maintained in plastic cages with free access to normal fat food and water. Following 7 days of acclimatization, acute hyperlipidemia was induced in overnight fasted rats by a single intraperitoneal (IP) injection of P-407at 1 g/kg body weight. P-407 solution was prepared by cold method in normal saline [26]. Prior to IP injection, syringes containing P-407 were placed on ice to avoid the thermal gelation of P-407 when used at high concentrations (above 23% w/w) [27].

The rats were divided into eleven groups (each group contained 6 rats), based on the treatment administered, as reported in Table 1. In particular, CH alone at two doses (250 and 500 mg/Kg), a binary mixture of α-CD and LP at the ratio 1:1 (400 mg/Kg and 800 mg/kg), and all the three active ingredients (CH in association to the binary mixture of α-CD and LP at the ratio 1.25:1:1) were tested for their hypolipidemic and anti-atherosclerotic effects beneficial for the prevention and

treatment of hyperlipidemia. Doses of the active ingredients were selected based on previous literature and a preliminary screening study to delineate the minimum administered doses of both CH and α-CD and LP, capable of presenting anti-hyperlipidemic therapeutic outcomes [28, 29]. The treatments started one day after hyperlipidemia induction with P-407 IP injection. Groups II-IX received the treatment via oral gavage once daily over 3 days, while groups X and XI were pre-treated with pre-feeding doses of active ingredients over 3 days followed by IP injection of P-407. Group VII was treated with the marketed tablet formulation Ator® (containing atorvastatin) at the dose of 40 mg/Kg, which was triturated in a mortar and dispersed in normal saline prior to oral administration [30].

The therapeutic and protective efficacy was determined in terms of percentage reduction in serum levels of cholesterol (TC), triglyceride (TG), and low-density lipoprotein associated cholesterol (LDL-C) as well as percentage elevation in serum level of high-density lipoprotein associated cholesterol (HDL-C) with the respect to the controls.

Table 1. Experimental groups (I-XI) of Albino rats (n=6) for the *in vivo* evaluation of the anti-lipidemic effects of the active ingredients and their mixture in a P407-induced hyperlipidemic model.

Group	Hyperlipidemia induction	Treatment	Protection Pre-induction	Treatment Post-induction
I (Negative Control)	X	Saline solution	-	-
II (Positive control)	✓	Saline solution	-	✓
III	✓	CH at 250mg/kg	-	✓
IV	✓	CH at 500 mg/kg	-	✓
V	✓	α-CD+LP (1:1) at 400 mg/kg	-	✓

VI	✓	α-CD+LP (1:1) at 800 mg/kg	-	✓
VII	✓	atorvastatin tablet dispersion at 40mg/kg	-	✓
VIII	✓	CH at 250mg/kg + α-CD+LP (1:1) at 400 mg/kg	-	✓
IX	✓	CH at 500 mg/kg + α-CD+LP (1:1) at 800 mg/kg	-	✓
X	✓	CH at 500 mg/kg	✓	-
XI	✓	α-CD+LP (1:1) at 800 mg/kg	✓	-

CH:chitosan; α-CD: alpha-cyclodextrin; LP: lupin proteins

2.2.1. Serum lipid levels and ratios determination

Animals were fasted for 12 h and blood was collected from retro orbital plexus at the end of the experimental period (one day after last administered dose in post-induction treatment groups and one day after P-407 injection in pre-induction groups). Blood was centrifuged at 5000 rpm for 10 min and the serum was stored immediately at -20°C until the time of analysis. Serum cholesterol (TC), triglyceride (TG), and high-density lipoprotein associated cholesterol (HDL-C) were estimated by enzymatic colorimetric methods using Bio-diagnostic Kits (Bio-diagnostic, Egypt). They are measured by spectrophotometer (UV-1601 PC, Shimadzu, Kyoto, Japan). Low density lipoprotein associated cholesterol (LDL-C) was calculated using modified Friedewald formula (MFF) as follows:

$$LDL - C = (TC - HDL - C) * 90\% - TG * 10\%$$

Mathematical relationships between the above-mentioned lipid parameters were also determined and evaluated as markers for the development of atherosclerosis and extent of coronary heart diseases. The markers are as follows:

$$Atherogenic\ Index\ of\ Plasma\ (AIP) = \log\left(\frac{TG}{HDL - C}\right)$$

$$Castelli's\ Risk\ Index\ (CRI-I) = \frac{TC}{HDL-C}$$

$$Castelli's\ Risk\ Index\ (CRI-II) = \frac{LDL-C}{HDL-C}$$

$$Atherogenic\ Coefficient\ (AC) = \frac{(TC-HDL-C)}{HDL-C}$$

2.2.2. Histological examination

At the end of the treatment period, rats were sacrificed by neck dislocation (n = 3), and autopsy samples were taken from the liver of rats in different groups and fixed in 10% formol saline for twenty-four hours. They were dehydrated through serial dilutions of alcohols (methyl, ethyl, and absolute ethyl). Specimens were cleared in xylene and embedded in paraffin block at 56 °C in a hot air oven for 24hours. Sections of paraffin blocks were prepared at 4 µm thickness by sledge microtome. Then, the obtained sections were collected on glass slides, deparaffinized and stained by hematoxylin and eosin stain for routine examination through the light microscope [31].

2.3. Molecular Docking

2.3.1. Ligand-Target Docking

Docking studies were attempted to explore the interaction of Cholesterol as a ligand onto the 3D model of Cyclodextrins (α-CD and β-CD) and CH using AutoDock Tools (ADT, Version 1.5.6) available from Scripps Research Institute (http://autodock.scripps.edu/resources/adt). The 3D conformer of Cholesterol was downloaded from PubChem database (https://pubchem.ncbi.nlm.nih.gov/), its energy was minimized by Avogadro (https://avogadro.cc/) using MMFF94 force field and saved as PDB file. Then, their torsions were set and converted to PDBQT using AutoDock Tools.

The 3D structure of α-CD and β-CD was extracted from the protein complexes (Protein Data Bank [PDB] code: 4FEM [32] and 1JL8 [33]respectively), while a 12-mers CH molecule was obtained in PDB through the PolySac3DB polysaccharide bank (http://polysac3db.cermav.cnrs.fr/home.html).

.Hydrogenswere added for all structures and their energy was minimized by Avogadro using MMFF94 force field. Then, Kollman charges were added, Gasteiger charges were calculated and atoms were assigned to AD4 type [34]. Finally, PDB files were converted to PDBQT using AutoDock Tools.

Docking procedures: The docking simulations were carried out by AutoDockVina (http://vina.scripps.edu/) using the config files prepared according to data in (Table 2).[35] These dimensions and coordinates were determined using AutoDock Tools Grid Box. All calculations were run on a core i7 personal computer (CPU at 8 MB) with the Windows 7 operating system.

Table 2. Parameters of AutoDockv in a config files and their values.

Receptor	No. of Points			Spacing (A°)	Center Grid Box			Exhaustiveness	No. of modes	Energy Range
	x	y	z		x	y	z			
α-CD	29.758	35.697	102.833	1.0	15	15	15	10	20	3
β-CD	37.554	58.491	59.162	1.0	15	15	15	10	20	3
Chitosan	3.933	21.778	4.175	1.0	20	20	20	10	20	3

2.3.2. Protein-Protein Docking

The 3D structure for Lupin was retrieved from the crystal structure of yellow lupin LLPR-10.2B protein in complex with melatonin (Protein Data Bank [PDB] code: 5MXB[36] . The 3D structure for Lipase enzyme was retrieved from the Crystal structure of the pancreatic lipase-colipase complex inhibited by an alkyl phosphonate(Protein Data Bank [PDB] code: 1LPB . The BDB files were prepared using AutoDock Tools to be uploaded to ClusPro server to perform Protein-protein docking.

ClusProserver (https://ClusPro .bu.edu/) was introduced in 2004[37], then has been substantially modified and expanded. First, the server performs rigid body docking by sampling billions of conformations using PIPER, a docking program based on the Fast Fourier Transform (FFT) correlation approach , then the 1000 lowest energy structures are clustered based on their RMSD to

find the largest clusters that will represent the most likely models of the complex. Finally, selected structures are refined using energy minimization.[38]

ClusProserver offers an advanced option to conduct a knowledge-based docking, if experimental information indicates that certain residues are in the binding interface, by setting "Attraction" on these residues. The recommended balanced coefficients were chosen as a filtering method. The programoutput is a list of co-efficient weights of putative complexes ranked accordingto their clustering scores.

All visualization of docking results were analyzed using Autodock tools program and PyMOL molecular graphics program (https://pymol.org/2/).

2.4 Preparation and characterization of dietary supplements tablets

Different powder blends were prepared by varying the amounts of the active ingredients (CH, α-CD and lupin proteins) and excipients (sodium bicarbonate, crospovidone, silica and Lubritab®) using a V-shape mixer (Laboratori Mag DivisioneArtha, Italy) for 5 minutes. Then, 1 g tablets were obtained by direct compression of the blends by using a rotary tablet machine (AR/18N model, 15 stations, Ronchi, Italy) and a capsule-shape punch. Tablet hardness was determined through a hardness tester (TBH30, Erweka, Germany) (n = 5).Friability of tablets (n=6) was evaluated by using a USP friability test apparatus (TecnoGalenica, Italy).The disintegration time of tablets was measured in phosphate buffer pH 2.5 (F.U. XII) at 37 ± 1 °C using a disintegration test EU Ph. apparatus A (TecnoGalenica, Italy) (n = 3).

2.5 Preparation of the inclusion complexes and physical mixtures

Inclusion complexes of active ingredients with cholesterol were prepared by the co-precipitation method, as reported by dos Santos et al., with some modifications[39]. Cholesterol was dispersed in a saturated solution of α-CD (110.3 g/l) under magnetic stirring at room temperature, at three α-CD:cholesterol molar ratio, 1:1, 2:1, 4:1 (M1, M2, and M3, respectively). These solutions were

prepared in presence of CH and LP as well, added to the α-CD solution before cholesterol, keeping the same α–CD: cholesterol molar ratios, 1:1, 2:1, 4:1 (M4, M5, and M6, respectively). The three active ingredients were added at the same ratio used for the tablet formulation. These dispersions were maintained under agitation for 48h. Then, they were stored at 5°C overnight, to allow the precipitation of the complexes and filtered under vacuum by using 0.45 mm polytetrafluoroethylene (PTFE) filters. The filtrated was frozen at -20°C and then lyophilized.

Binary systems consisting of α-CD and cholesterol at molar ratio 1:1, 2:1 and 4:1(PM1, PM2, and PM3, respectively) and quaternary systems containing CH, LP, α-CD and cholesterol at molar ratios 1:1, 2:1 and 4:1 (PM4, PM5, and PM6, respectively) were obtained by physical mixing. The corresponding amounts of each system were mixed in a mortar for 5min up to obtain homogeneous blends.

2.6 Differential scanning calorimetry (DSC) analysis

DSC analysis was carried out on the inclusion complexes (M1-M6) and their relative physical mixtures (PM1-PM6) by using a PerkinElmer DSC 8500. Few milligrams of each sample (≈2-4 mg) were loaded into a closed aluminium pan. DSC analysis were carried out in the temperature range from 0 to 250 °C with a heating rate of 10°C/min.

2.7 Statistical analysis

All values of biochemical markers are presented as mean ± standard error of mean (S.E.M). Differences between groups were tested by ANOVA usingTukey–Kramer multiple comparison, GraphPadInstat® software (GraphPad Software, La Jolla, CA, USA). Statistical significance was considered at $p < 0.05$, $p < 0.01$ and $p < 0.001$.

Results and Discussion

3.1 *In vivo* studies and histological analyses

In this *in vivo* study, P-407, a non-ionic amphiphilic copolymer, was employed successfully for rapid induction of an acute hyperlipidemia model in rats as well documented in the literature[27, 40–42]. Based on the previous outcomes of Pan et al., a single injection of a high dose of P-407 can induce hyperlipidemia, showing lipid peak values 24 hours after injection, without needing treatment repetition [42].

In addition, compared to time-consuming long-term high fat feeding studies, this acute model showed reliability, reproducibility, cost-effectiveness, and wide applicability. Hypertriglyceridemia associated with IP injection of P-407 seems to be related to the direct downregulation activity of capillary lipoprotein lipase and hepatic lipase enzymes that are responsible for TG degradation[43–45][43–45]. For hypercholesterolemia, P-407 seems to inactivate cholesterol 7-α hydroxylase enzyme, which is responsible for TC elimination [44]. In this regard, P-407 possibly activates also hepatic HMG-CoA reductase enzyme, which is the rate limiting enzyme in cholesterol synthesis, leading to TC elevation through an indirect way[43, 46].

Table 3 reports serum lipid levels of the various studied experimental groups. Significant ($p < 0.001$) elevated levels of serum TC, TG, and calculated atherogenic LDL-C were exhibited in Group II (hyperlipidemic positive control), reaching a 3.59-, 6.31- and 11.86-fold increase, respectively, as compared to non-hyperlipidemic untreated animals (Group I, negative control). On the other hand, a significant ($p < 0.001$) reduction in HDL-C value was revealed in Group II as compared to Group I. Such findings suggested a well-established hyperlipidemic rat model, with an increased risk of atherosclerosis and cardiovascular diseases.

CH, α-CD, and LP represent healthy dietary choices towards the improvement of lipid profile and hence, avoidance or at least lowering diseases related to diet, including obesity, atherosclerosis, diabetes, and cardiovascular diseases[22, 28, 47, 48].

The doses of CH and LP tested was selected according to the data available from the literature for the antihyperlipidemic effect of the single dietary product. The *in vivo* hypolipidemic effect of CH was observed at a variety of doses (250-1000 mg/kg) in hyperlipidemic rats fed on a high-fat diet since the treatment succeeded in reversing the levels of serum lipids [29]. Moreover, Sewani-Rusike et al. reported that an aqueous extract of *Lupinus albus* exhibited promising competence against diabetes induced dyslipidemia as the consumption of both doses of 200 mg/kg and 400 mg/kg attenuated hyperlipidemic parameters in experimental diabetic rats[28]. Nevertheless, the hypolipidemic effect of combining α-CD, LP and CH has not been investigated yet and their co-administration can be assumed to have a complementary effect on hyperlipidemia control.

A positive impact of CH, at both doses (250 and 500 mg/Kg) on lipid profile was observed. Indeed, a significant dose-dependent marked decline in TC and LDL-C values (36.44 % and 66.60 % for TC and 45.59% and 87.10% for LDL-C, respectively) and a non-dose dependent reduction in TG levels (respective values of 75.13% and 68.33%) were obtained after treatment with CH at both doses. The slight increase in TG levels observed in the group treated with the higher dose of CH (Group IV) was not significantly different ($p=0.0676$) from those treated with the lower dose of CH (Group III). A significant similar increase in HDL-C levels was also exhibited in both groups as compared to Group II. It is worth mentioning that treatment with the higher dose of CH greatly reversed experimentally induced hyperlipidemia and normalized lipid levels.

Similarly, treatment with α-CD/LP showed a favourable effect in reducing TC, TG, and LDL-C values in a dose-dependent manner (Groups V and VI). For HDL-C, significant induction in both groups was noticed as compared to Group II. As a consequence of the different therapeutic responses, significantly lower changes in lipid values were observed in α-CD/LP treated groups (groups V and VI) compared with CH treated groups (groups III and IV). Such findings indicated a better improvement in the lipid values induced by CH with the respect to α-CD/LP.

Expectedly, treatment with marketed atorvastat in tablets dispersion in Group VII significantly lowered serum levels of TC (37.99%), TG (60.29%), and calculated LDL-C (43.51%), while a

moderate increasein HDL-C level occurred as compared to Group II. Oral administration with 40 mg/kg atorvastatin yielded similar non-significant ($p > 0.05$) results to lower doses of either CH or α-CD/LP, but significantly ($p < 0.05$) higher values in all lipids profile when compared to higher doses of CH or α-CD/LP(except HDL-C and LDL-C for the latter).

Treatment using the combination of CH and α-CD/LP, whatever their doses, revealed a non-significant difference in all lipid parameters as compared to treatment using CH alone, at both doses ($p > 0.05$). Contrarily, the mixing of low doses of CH and α-CD/LP caused a significant reduction in TG level and a significant elevation in HDL-C level in comparison to the same dose of individual α-CD/LP mixture. Rats fed combined treatment of high doses of CH and α-CD/LP showed significant reduction both in TG and LDL-C as well as a significant increment in HDL-C level as compared to the same dose of α-CD/LP.

Pre-treatment of CH, at high dose 500 mg/kg, to normolipidemic rats before induction of hyperlipidemia exerted significant protective potential against hyperlipidemia, attenuating the hyperlipidemic response as manifested by the reduced serum LC, TG, and LDL-C values by 13.07%, 11.42%, and 34.46%, respectively, and elevated HDL-C value compared with CH (groups III and IV). It should be noticed, however, that, differently from the CH-fed hyperlipidemic group, at the same dose (Group IV), the lipid profile values were still significantly higher, except for HDL-C values that showed a non-significant difference ($p > 0.05$).

Unlike the effect of pre-treatment of CH, α-CD/LP-fed normolipidemic group (Group XI), prior to induction of hyperlipidemia, showed no protective effect on lowering serum levels of TC (Table 3). However, slight, yet significant, reductions in TG and LDL-C levels were observed with respective values of 13.54% and 19.04%. Contrarily, HDL-C value increased to a greater extent compared with that detected in group X receiving pre-treatment of CH. Interestingly, pre-induction treatment of α-CD/LP significantly elevated HDL-C level, better modulating it towards normal value when

compared with the same dose administered post-induction (respective values of 39.72 and 54.66 mg/dl for Groups VI and XI).

Table 3. Effects of different treatments (III-IX groups) and protective pre-treatments (X and XI groups) on serum lipid concentration compared to negative control (Group I) and hyperlipidaemic positive control (Group II).

Group	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
I (Negative Control)	86.09 ± 4.17	87.05 ± 3.16	57.04 ± 2.18	17.43 ± 3.96
II (Positive Control)	309.10 ± 4.73^{***a}	549.64 ± 6.86^{***a}	18.25 ± 2.48^{***a}	206.80 ± 3.26^{***a}
III	196.44 ± 14.30 ^{**b} (36.44)	136.67 ± 12.43^{***b} (75.13)	56.23 ±3.80^{**b}	112.51 ± 13.55^{**b} (45.59)
IV	103.22 ± 9.02$^{***b, ++c, *g}$ (66.60)	174.04 ± 11.28^{***b,*g} (68.33)	54.26±1.13^{***b,***g}	26.65±8.57$^{***b,++c, ++g}$(87.10)
V	219.71 ± 10.19^{**b} (28.92)	284.88 ± 16.80^{***b} (48.16)	42.98 ± 3.07^{***b}	124.12 ± 7.62^{***b} (39.97)
VI	124.85 ± 20.87$^{**b, **d, *g}$ (59.60)	174.74 ± 6.35$^{***b, ***d, *g}$ (68.20)	39.72 ± 2.22^{***b}	78.32 ± 15.05^{***b,*d} (62.12)
VII	191.64 ± 14.82^{**b} (37.99)	218.25 ± 13.88 ^{***b} (60.29)	37.59 ± 2.24^{**b}	116.82 ± 16.41^{**b} (43.51)
VIII	220.32 ± 4.07^{***b} (28.72)	150.87 ± 9.95^{***b,***d} (72.55)	59.16±2.07^{***b,***d}	142.14 ± 8.91^{***b} (31.26)
IX	92.93 ± 8.63^{***b} (69.93)	124.09 ± 18.31^{***b,*f} (77.42)	57.56±1.66^{***b,***f}	22.03 ± 8.30^{***b,*f} (89.34)
X#	268.67 ± 11.10^{*b,***e} (13.07)	486.84 ± 10.34$^{*b, ***e}$ (11.42)	43.28 ± 6.79^{*b}	135.52±8.24$^{**b, ***e}$ (34.46)
XI#	301.50 ± 9.16^{**f} (2.45)	475.17 ± 15.99$^{**b, ***f}$ (13.54)	54.66±0.60$^{***b, ***f}$	167.41 ± 6.03$^{*b, **f}$ (19.04)

#pre-treatment of high doses of CS and LP for 3 days before IP injection of P-407
Data is shown as mean ±S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ^{a}*vs*.normal control, ^{b}*vs*. hyperlipidaemic control, ^{c}*vs*. CS-L, ^{d}*vs*.LP-L, ^{e}*vs*.CS-H,^{f}*vs*.LP-H, ^{g}*vs*.Ator®.Numbers in parentheses are percentages reduction of the hyperlipidaemic control value.

The measurement of atherogenic risk predictor indices for the development of atherosclerosis and cardiovascular disorders was also carried out (Figure 1). These lipid ratios were well-documented to present predictive values for cardiovascular disease risk stratifying better than isolated lipid

parameters[49, 50]; in particular TC and LDL-C. Among all the calculated markers, AIP provides the most accurate indication of the risk of atherosclerosis and myocardial infarction. Powerful sensitivity of AIP, among all indices, stems from the utilization of the logarithmical transformation in its direct calculation, proffering correction for lack of normal distribution[51].

As for AIP values, all tested groups revealed greatly significantly reduced values of AIP compared with Group II; especially Group IX that received the combination of CH and α-CD/LP at high dose, showed the highest antiatherogenic effect with an AIP value of 0.35. Among all the tested groups, CH treated groups, at both doses (groups III and IV) and combined groups with α-CD/LP (Groups VIII and IX) exhibited significantly reduced AIP values. This finding suggests the strikingly higher efficacy of CH in inhibiting atherogenesis and thus, counteracting hyperlipidemia related cardiovascular disorders.

Concerning CR-I, CR-II, and AC atherogenic predictor indices, a significant reduction in a dose-dependent manner was also observed in all groups as compared with Group II. However, it is of interest to note that there was no remarkable statistical difference observed among all groups ($p > 0.05$).

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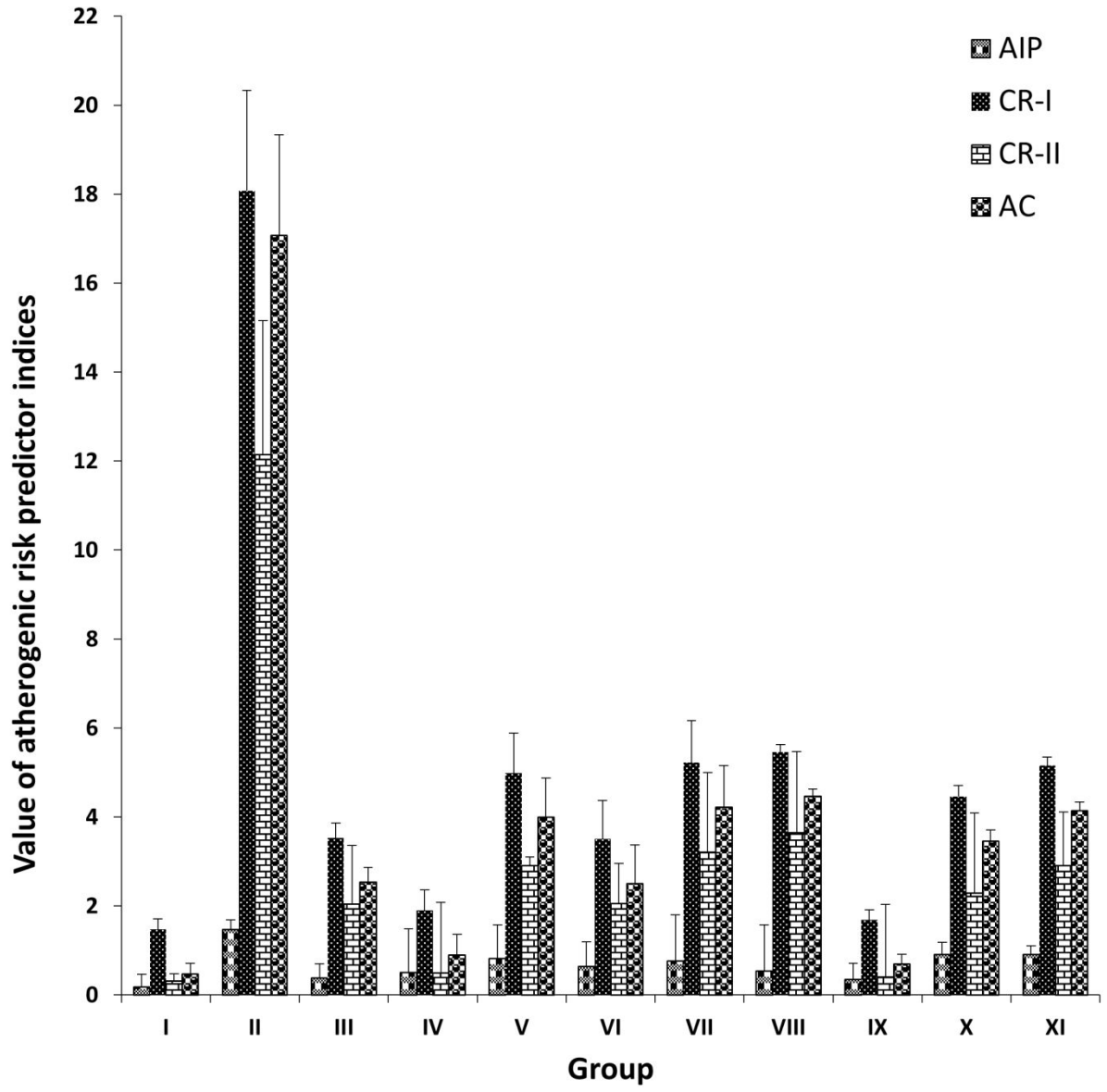

Figure 1. *Atherogenic risk predictor indices of different treatments and protective groups compared to negative control (Group I) and hyperlipidemic positive control (Group II) groups.*

Then, histological analysis was carried out to elucidate new therapeutics and preventives to reduce hyperlipidemia burden on one hand and delineate the comparative ameliorative impact of the active ingredients selected in this study on the other hand. In light, hepatic pathological features were demonstrated in different therapeutic and protective groups, compared to those following administration of the marketed tablets containing the lipid lower ingatorvastatin.

Normal rats' liver section (Group I, Figure 2A) showed a normal histological structure of the central vein (CV) and surrounding intact lattice structure of hepatocytes with clearly distinct nuclei (N) in the parenchyma. There was no detectable necrosis, lesions, and inflammation of hepatocytes. Contrarily, untreated poloxamer-poisoned rat liver section (Group II; Figure 2B) demonstrated a state of hyperlipidemia induced liver steatosis. Indeed, there was severe congestion in the portal vein with periductal fibrosis and infiltration of inflammatory cells surrounding the dilated cystic bile ducts. Diffuse vacuolar degeneration (V) all over the hepatic parenchyma was also observed. This could be ascribed to poloxamer injection mediated accumulation and deposition of lipids within the liver with subsequent inflammatory changes and oxidative damage [52, 53].

Supplementation with individual CH or α-CD/LP, at both investigated doses, was found to ameliorate lipogenic induction after poloxamer injection at variable extents (Groups III-VI). Although focal necrosis in the hepatic parenchyma was still present in the liver section of rats receiving low CS dose (Group III), mild decrement of these harmful consequences was noticeable following administration of high CH dose (Group IV). Central vein congestion and diffuse Kupffer cell proliferation were demonstrated. Besides, few hepatocytes showed cytoplasmic vacuolations (Supplementary Figure S1).

Interestingly, this disturbed liver architecture was much less pronounced and effectively attenuated in groups that receivedα-CD/LP at both doses (Groups V and VI). In these groups, mild central veins congestion was observed with few cellular infiltrations. Normal hepatocytes with few cytoplasmic vacuoles were observed (Supplementary Figure S1).From a therapeutic perspective, the histopathological patterns obtained from using marketed atorvastatin (group VII) were close to those of α-CD/LP at both doses (Groups V and VI (Fig. 2C).

Using combined therapy CH and α-CD/LP gave much better results in terms of obvious regression of liver steatosis. The examined liver section of rats received the combination of CH and α-CD/LP at low doses (Group VIII) greatly lowered injurious changes manifested by only a few inflammatory cells infiltration together with few fibroblastic cell proliferation in the portal area.

More obviously, post-treatment with the combination of CH and α-CD/LP at high doses showed anormal histological structure of liver section (Group IX), indicating the strikingly synergistic impact of the tested therapeutic agents on pre-existing fatty liver and showing the highest optimal therapeutic competencyamong all investigated groups (Fig. 2D).

For elucidating the possible protective potential of the investigated CH and α-CD/L, both groups X and XI were pre-treated with individual high doses of CH and α-CD/LP respectively (supplementary Figure S1). Compared with post-treatment of high dose of CH (Group IV), pre-treatment with the same dose of CH revealed better prominent improvement of histological hepatic changes (Group X) addressed by mild cellular infiltration. The reverse was true when comparing the beneficial effects of α-CD/LP pre-treatment and post-treatment. Examination of the liver section of rats pre-treated with α-CD/LP (group XI) clarified dilatation and central vein congestion. These findings confirmed the superiority of α-CD/LP post-treatment over their pre-treatment in suppressing the severity of liver steatosis caused by poloxamer.

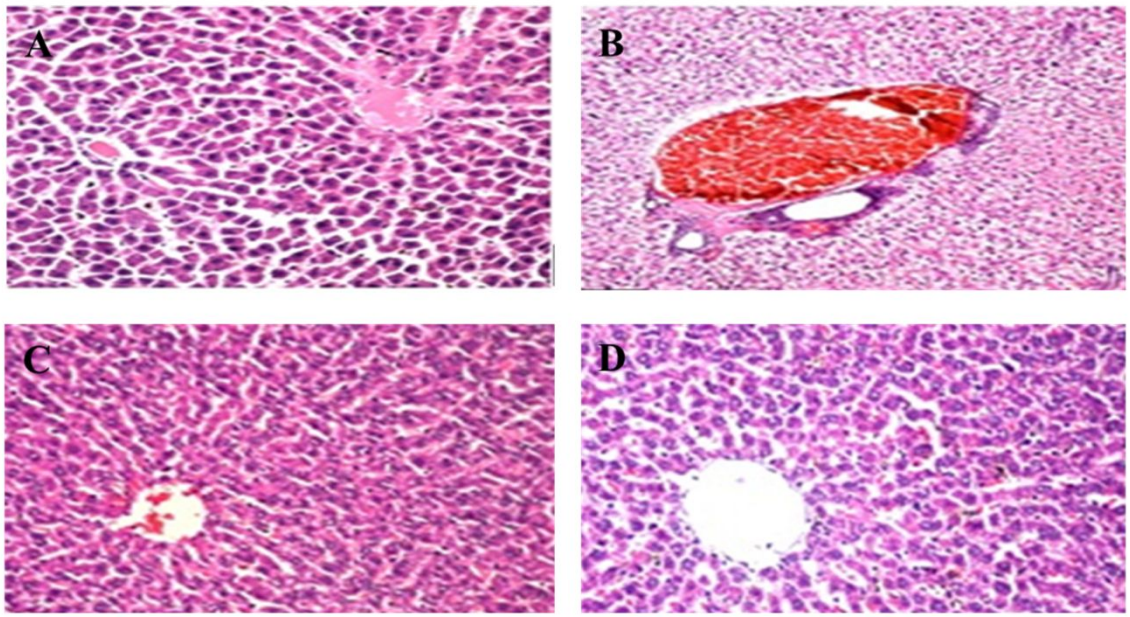

Figure 2. Photomicrography of rat liver sections of negative control (Group I) (A), of hyperlipidemic positive control (Group II) (B), of marketed atorvastatin treated group (Group VII) (C) and of the group treated with the combination of CH and a-CD/LP at high dose (Group IX) (D).

3.2 Molecular Docking

3.2.1 Ligand-Target Docking

The focal profit of implementing molecular docking is to provide an insight on ligand orientation and interaction with the guest. In our study, ligand-target docking was conducted to calculate the binding energies between Cholesterol and some oligo/polysaccharide targets, which are of the most abundant biopolymers with complex structures that can play an important role in various biological systems i.e. macrocyclic oligosaccharide α-CD and linear polysaccharide CH.

Chitosan is a linear polysaccharide while α-CD isshaped as trunked cones of six α-D-glucopyranose units expressing a hydrophobic inner cavity diameter of 5.2 A˚ and an external hydrophilic surface. Unfortunately, its cavity is not wide enough to accommodate a vast majority of chemical entities. [54]

Since the cavity size of α-CD is not optimum to accommodate the steroid skeleton of Cholesterol, we anticipated its inability to encapsulate Cholesterol. In this respect, we also studied the interaction with β-CD, which is composed of seven units with a wider cavity of 6.6 A˚ diameter, to show the difference in binding mode and interaction strength between the two cyclodextrins. This may help us to validate our docking procedure as we expect Cholesterol to fit inside the cavity of β-CD and to compare the values of binding affinity values to evaluate the binding strength.

Our molecular docking study emphasized the anticipated ability of β-CD to accommodate Cholesterol inside its cavity as shown in (Figure 3) with the least binding energy-5.7(kcal/mol), demonstrating the strongest binding with Cholesterol in comparison with α-CD that was found bound laterally with Cholesterol without encapsulation (Figure 4), and also with Chitosan (Figure 5)which both showed-3.3 kcal/mol binding affinity values. The best 10 modes of binding of each molecule and their binding affinity values are shown in (Table 4).

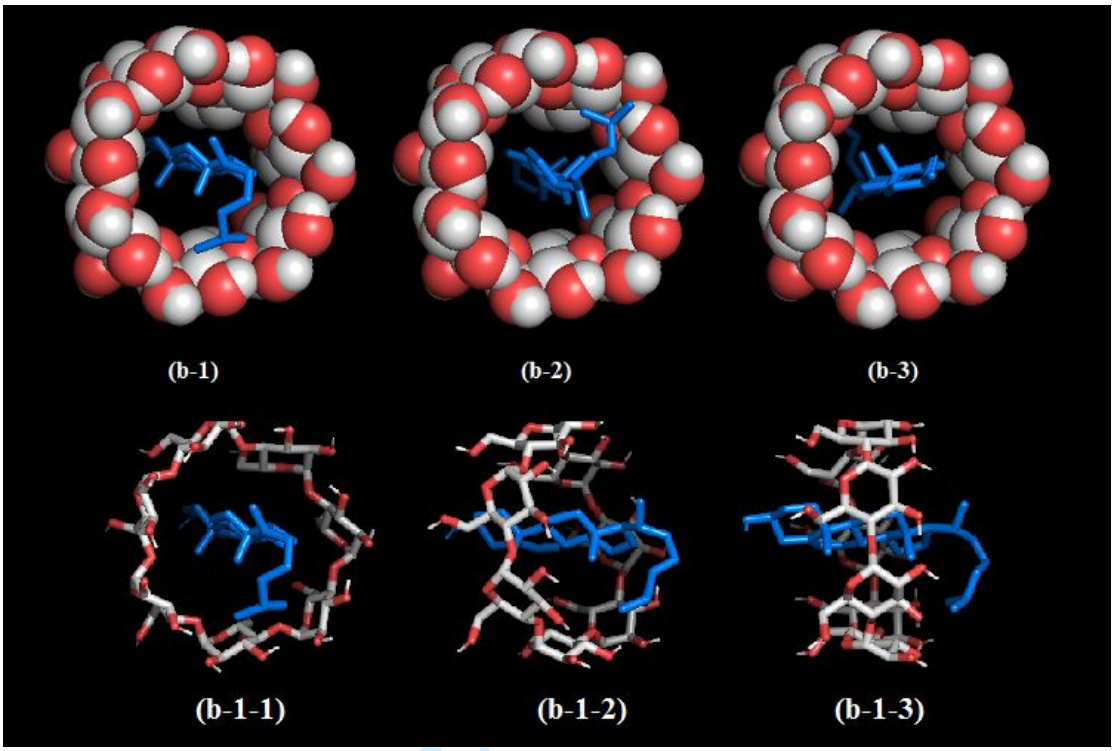

Figure 3. ***(*b-1), (b-2), and (b-3):**Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-β-CDinteractions. **(b-1-1), (b-1-2), and (b-1-3):** the best mode **(b-1)** from different angles shows the accommodation of Cholesterol insideβ-CD cavity.

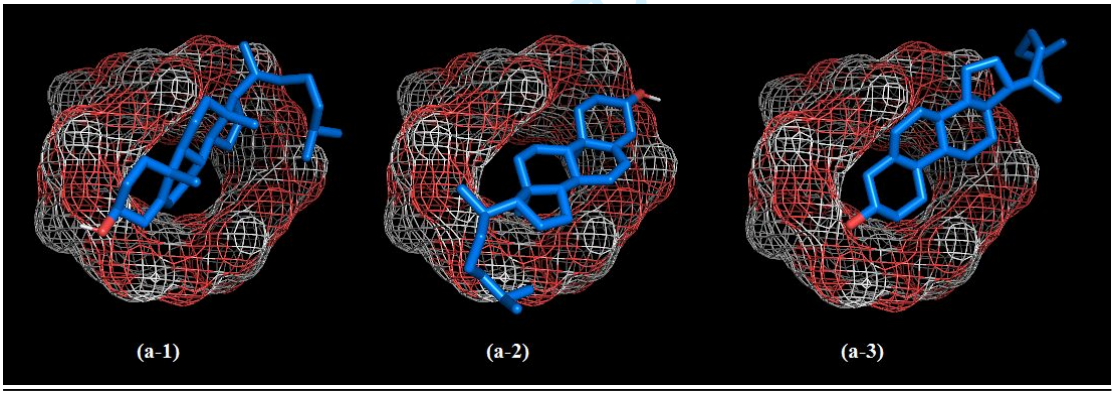

Figure 4. (a-1), (a-2), and (a-3): Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-α -CD interactions.

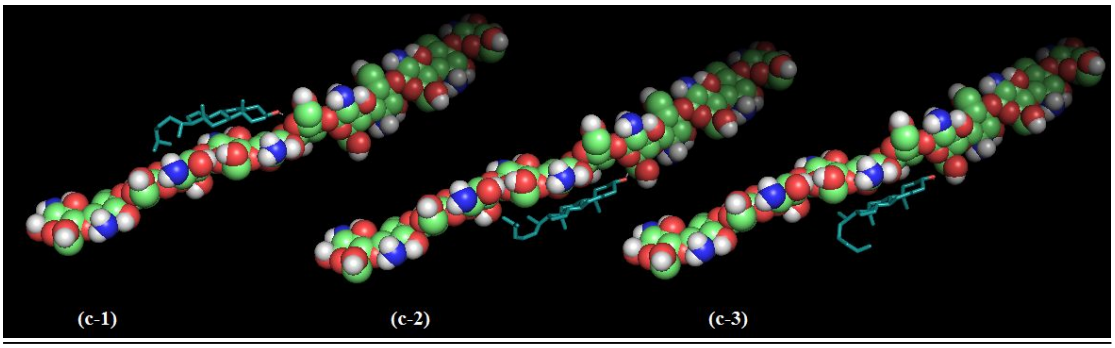

Figure 5. (c-1), (c-2), and (c-3): Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-*Chitosan interactions.*

Table 4. The binding affinity values of the best 10 modes for β-CD, α-CD and Chitosan

Mode	β-CD	α-CD	Chitosan
1	-5.7	-3.3	-3.3
2	-5.4	-3.1	-3.3
3	-5.4	-3.0	-3.2
4	-5.3	-2.9	-3.2
5	-5.3	-2.6	-3.1
6	-5.3	-2.6	-3.1
7	-5.2	-2.6	-3.0
8	-5.0	-2.5	-2.9
9	-4.9	-2.5	-2.9
10	-4.9	-2.3	-2.8

Our docking study showed that the direct interaction between cholesterol and our targets (α-CD and Chitosan) is a weak hydrophobic in comparison with that of β-CD. This finding may draw our attention to the possible involvement of our targets (α-CD and Chitosan) in additional or alternative indirect mechanisms that can reduce Cholesterol absorption, particularly given the fact that α-CD is still able to encapsulate other molecules with smaller size such as the hydrophobic chains of triglycerides.[55]

3.2.2. Protein-Protein Docking

Human Pancreatic Lipase (HPL) as shown in (Figure 6) is a glycoprotein consisting of the N region and the C region associated with a colipase.[56] The active site is located in the N region and consists of residues from 247 to 258 with the catalytic trial Ser152-Asp176-His263 .[57]

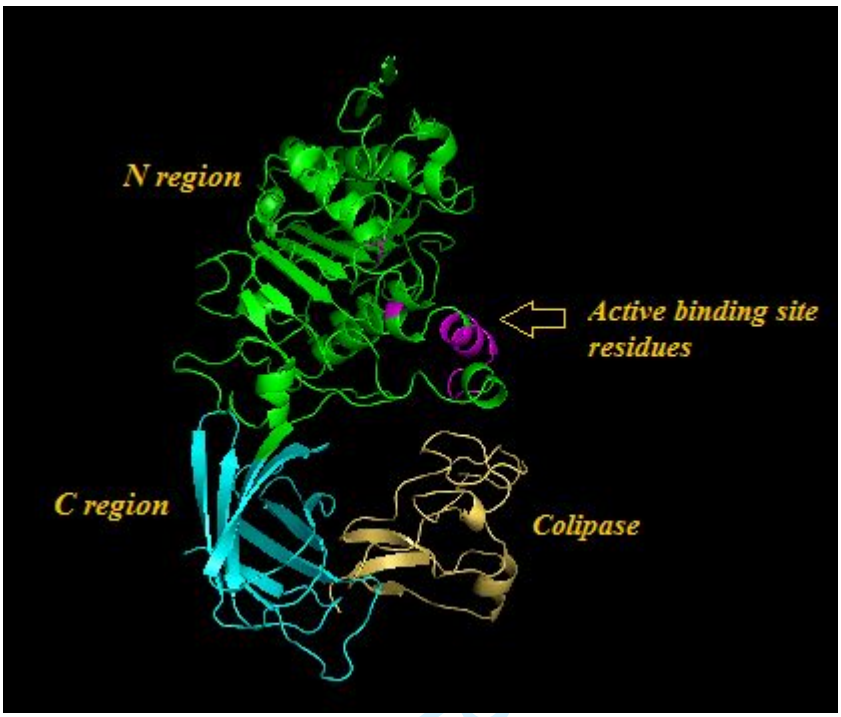

Figure 6. Pymol 3D structure for Lipase enzyme showing the N region (green), the C region (cyan), colipase (yellow) and the active residues (magneta)

In our attempt to study the interaction between HPL and Lupin, we performed two docking jobs, the first was blind allowing the two proteins to interact without any constraints and the second job was knowledge-based by specifying HPL binding site residues in the "Attraction" option to ensure their participation in the binding. The best 10 clusters for both jobs were recorded in Table 5.

Table 5. The cluster values of the best HPL-Lupin clusters of the docking jobs.

Cluster	Blind Docking			Knowledge-based Docking		
	Members	Representative	Weighted Score	Members	Representative	Weighted Score
0	160	Center	-820.2	124	Center	-886.8
		Lowest Energy	-838.1		Lowest Energy	-1022.3
1	104	Center	-692.4	97	Center	-846.9

Cluster	Blind Docking			Knowledge-based Docking		
	Members	Representative	Weighted Score	Members	Representative	Weighted Score
		Lowest Energy	-884.1		Lowest Energy	-1017.9
2	85	Center	-701.2	87	Center	-921.1
		Lowest Energy	-775.4		Lowest Energy	-1142.4
3	65	Center	-694.9	81	Center	-894.3
		Lowest Energy	-936.1		Lowest Energy	-942.5
4	48	Center	-709.1	70	Center	-885.3
		Lowest Energy	-799.3		Lowest Energy	-1032.2
5	45	Center	-704.8	68	Center	-927.5
		Lowest Energy	-802.6		Lowest Energy	-927.5
6	44	Center	-750.5	57	Center	-855.8
		Lowest Energy	-822.6		Lowest Energy	-1008.1
7	40	Center	-884.0	57	Center	-871.4
		Lowest Energy	-884.0		Lowest Energy	-929.7
8	35	Center	-776.5	56	Center	-882.5
		Lowest Energy	-776.5		Lowest Energy	-920.3
9	34	Center	-780.0	31	Center	-854.8
		Lowest Energy	-780.0		Lowest Energy	-971.4
10	31	Center	-701.3	27	Center	-856.2
		Lowest Energy	-850.6		Lowest Energy	-930.8

By investigating deeply the best HPL-Lupin docking complex (cluster No. 0) of both jobs as shown in Figure 7, strikingly, we found that Lupin came near to the binding site and was able to make a hydrogen bond with Trp252, which is one the active site residues, and Ile251 was among the interface residues between the two proteins. The knowledge-based docking gave a better interaction as the Lupin came much nearer to the binding site and was involved in 8 hydrogen bonds, two of them were with active site residues Trp252 and Thr255. In addition, active site residues Asp249, Ile251, Arg256, and Phe258 were among the interface residues (Table 6).

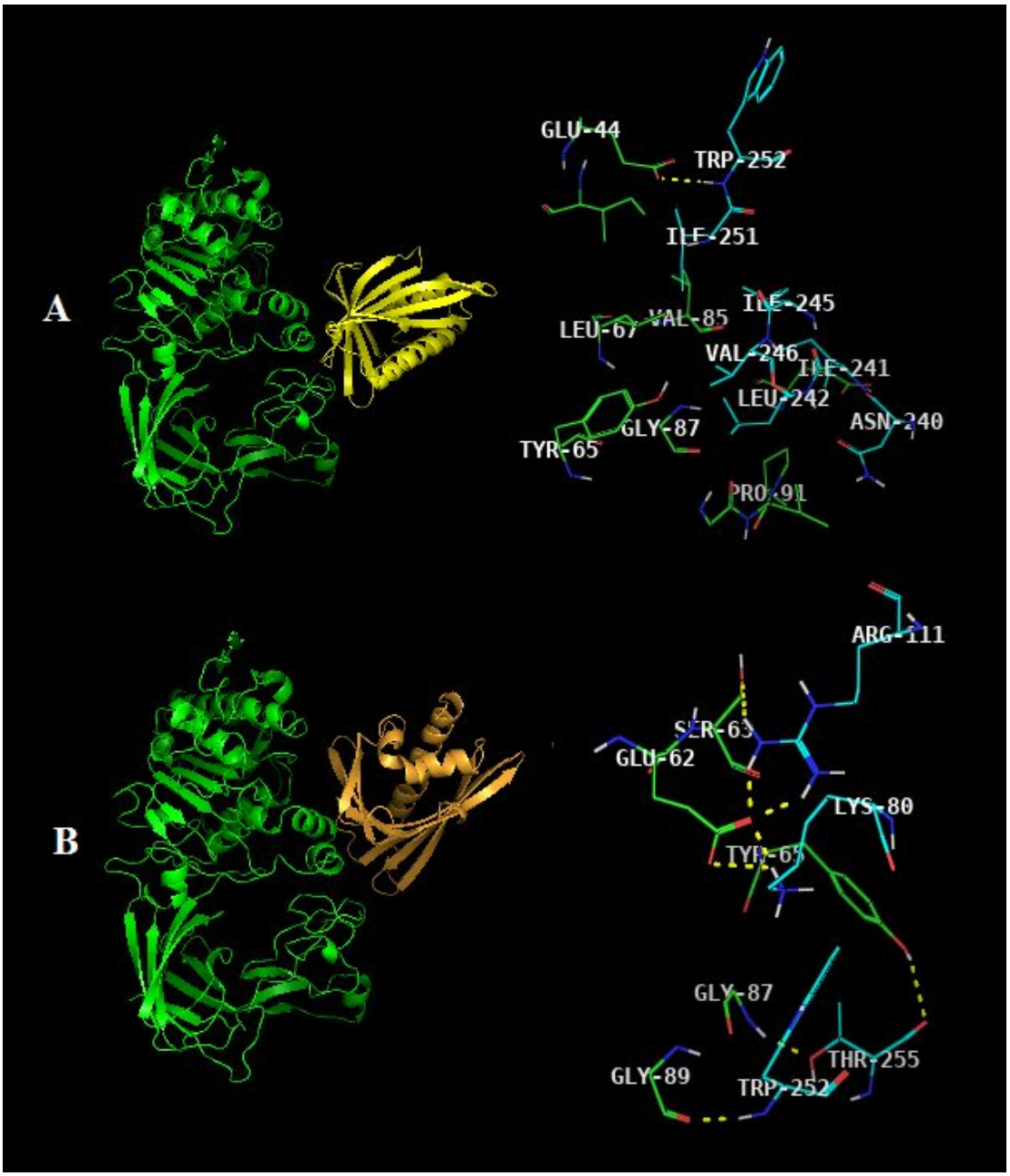

Figure 7: (A): Pymol 3D schematic diagrams of blind dockingcluster0 HPL-Lupin interaction, **(B):** diagram of knowledge-based docking cluster0 HPL-Lupin interaction. HPL residues are in cyan while Lupin residues are in green. Hydrogen bonds are indicated by yellow dashed lines between the atoms involved.

Table 6. Detailed data of HPL-Lupin cluster 0 of the docking jobs.

Docking Job	Cluster No. 0 Values	Interface Residues	No. of H.Bs	HPL residues involved in H.Bs
Blind Docking	Members:160 Center: -820.2 Lowest Energy: -838.1	Asn240, Ile241, Leo242, Ser243, Ile245, Val246 and **Ile251**	**1**	**Trp252**
Knowledge-based Docking	Members:124 Center: -886.8 Lowest Energy: -1022.3	Ile245, **Asp249, Ile251, Arg256, Phe258** and Ala259	**8**	Lys80, Arg111, **Trp252** and **Thr555**

HPL active site residues are bold and underline formatted.

3.3 Preparation and characterization of dietary supplements tablets

Based on the promising results obtained through the molecular docking and *in vivo* studies, a dietary supplement was developed. In particular, given the potentiation effect of CH, α-CD, and LP combination on the hyperlipidemic condition of rats, tablets containing the three active ingredients were formulated. A preliminary formulation study (data not shown) revealed that high amounts of CH and α-CH+LP are challenging for tablets formulation. Indeed, the presence of such ingredients in large amounts in the formulation produced weak tablets characterized by a long disintegration time. The last effect was related to the gelling ability of chitosan in acidic media. For these reasons, an initial screening was carried out, mainly based on the evaluation of different types and amounts of excipients, while the active ingredients were added based on the ratio tested in the *in vivo* study. The final tablets formula was the following: 42% of CH, 47% of α-CH+LP (ratio 1:1), 5% of disintegrant (crospovidone), 4.5% of sodium bicarbonate as disintegration enhancer, 1% (w/w) of glidant (silica) and 0.5% of lubricant. In particular, the choice of crospovidone as the most suitable

disintegrant was based on the fact that, from the literature, it has the best performances, independently from any external factor (*e.g.*, disintegration media, temperature) [58, 59]. Sodium bicarbonate is necessary to generate a slightly alkaline microenvironment, during the water penetration in the tablet. Such alkaline environment reduced the gelling of chitosan, thereby enhancing the disintegration process. The amount of sodium bicarbonate used as disintegration enhancer was selected after a series of preliminary tests (data not shown). Tablets were obtained bydirect compression and formulated to achieve disintegration time and friability properties in line with the specifications of F.U.XII for uncoated tablets. In particular, for the friability, a maximum mass loss of 1% (w/w) can be accepted, while the disintegration test is accepted in a time of 15 minutes, in an aqueous medium. However, in order to be close to the physiologic conditions of the human body, the disintegration test was carried out in phosphate buffer at pH 2.5, instead of water, maintaining the same disintegration time specified in the FU XII ed. In fact, pH 2.5 corresponds to the stomach pH value at the beginning of a meal, when the tablet should be ingested [60]. To modulate the performances of tablets in terms of disintegration time, tablets were prepared by changing the applied compression force, without modifying the percentage of the disintegrant (crospovidone). Different compression forces were applied to obtain tablets with a hardness of 70, 85, 100, and 130 N (Figure 8). The tablets with a hardness of 70 N showed a mean disintegration time of around 5 minutes. However, such tablets fell out the friability test because they showed a weight loss of 1.92%, approximately twice of the limit of 1% (FU). By increasing the hardness of the tablets, the measured disintegration time was longer (from 5 min up to 13 min). At the same time, weight loss (%) from the friability test decreased. Specifically, tabletsat the hardness of 130 N showed a weight loss of 0.17%, but the disintegration time was above 15 min. The adequate hardness value required for an acceptable disintegration time and friability was found at 100 N. These tablets completed the disintegration process at a mean time of 13 minutes 15 seconds witha weight loss of 0.85%.

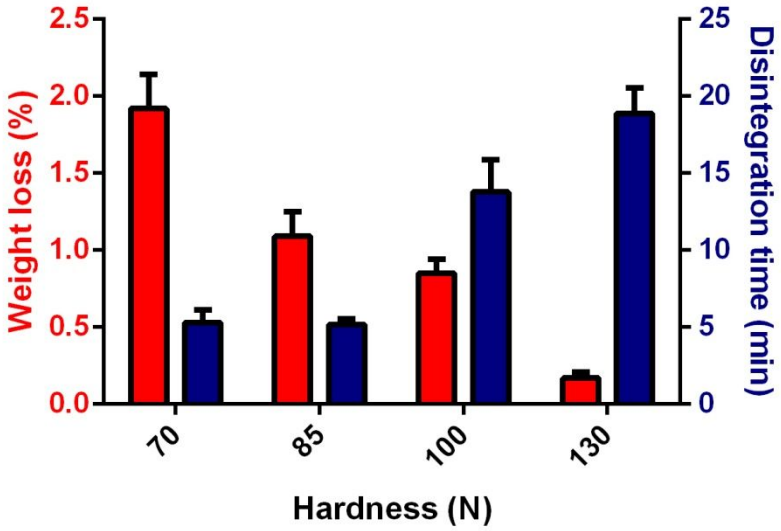

Figure 8. Effect of hardness (70-130 N) on the weight loss (%, friability) and disintegration time (min) of the formulated tablets

3.4 Complexation interaction properties of α-CD

One possible mechanism of action of the selective active ingredients in terms of reducing the cholesterol level can be related to the complexing property of α-CD. Clinical trials revealed a significant reduction of serum LDL and postprandial triglycerides in healthy subjects treated with dietary α-CD, indicating a relevant complexation of the exogenous fat introduced with diet [61, 62]. In this respect, the differential scanning calorimetry (DSC) can be usefully employed to determine the occurrence of a complexation interaction between α-CD (as host molecule) and a guest, cholesterol, at the solid state[63]. In fact, thanks to the toroidal shape, α-CD is suitable to encapsulate in its cavity lipid molecules. Through the DSC analyses, the formation of an inclusion complex should be proved by the total or partial disappearance of the guest molecule thermal transitions (*i.e.*, melting) when examined after the co-precipitation with the host molecule[64]. Thermograms obtained by DSC analysis of cholesterol (a), α-CD (b), CH (c), LP (d), M1 (e), M4 (f), PM1 (g) were reported in Figure 9. Cholesterol showed a well-defined endothermic peak centred around 150°C, which is related to the melting point reported for pure cholesterol (Figure 9a)[65]. Figure 9b revealed the typical α–CD thermogram, showing three different endothermic

peak sat 70°C, 105°C, and 130°C that are related to the loss of crystallized water from its cavity[66]. CH and LP did not show any appreciable thermal transitions in the measured range of temperature, from 0°C to 250°C, attributable to an amorphous behaviour of these two substances (Figure 9c-d). Similar results were reported by Santos et al. for DSC analysis of ChitoClear®, with no observable crystallization or melting peaks [67].

The evidence of the cholesterol - α-CD interaction can be observed in Figure 9e. In fact, the complete disappearance of cholesterol melting peak around 150°C highlighted the occurrence of interaction between the guest molecule and α-CD. The same result was also achieved for the two others investigated α–CD:cholesterol ratios, *i.e.*, 2:1 and 4:1, where the amount of the host molecule was higher (data not shown).

The presence of the two other active ingredients, CH and LP, did not affect and/or interfere with the ability of α-CD to form a complex with cholesterol; in fact, the thermogram did not reveal any thermal transition attributable to the presence of cholesterol (Figure 9f).

On the contrary, the thermograms of the physical mixture α-CD/cholesterol (Figure. 9g, 1:1 ratio), showed the presence of the typical endothermic peak of cholesterol, indicating that it was not formed complexes with α-CD by this procedure. Similarly, the physical mixtures of both the α-CD/cholesterol ratios 2:1 and 4:1 and those containing CH and LP showed the endothermic transition of the cholesterol, indicating no intercation (data not shown).

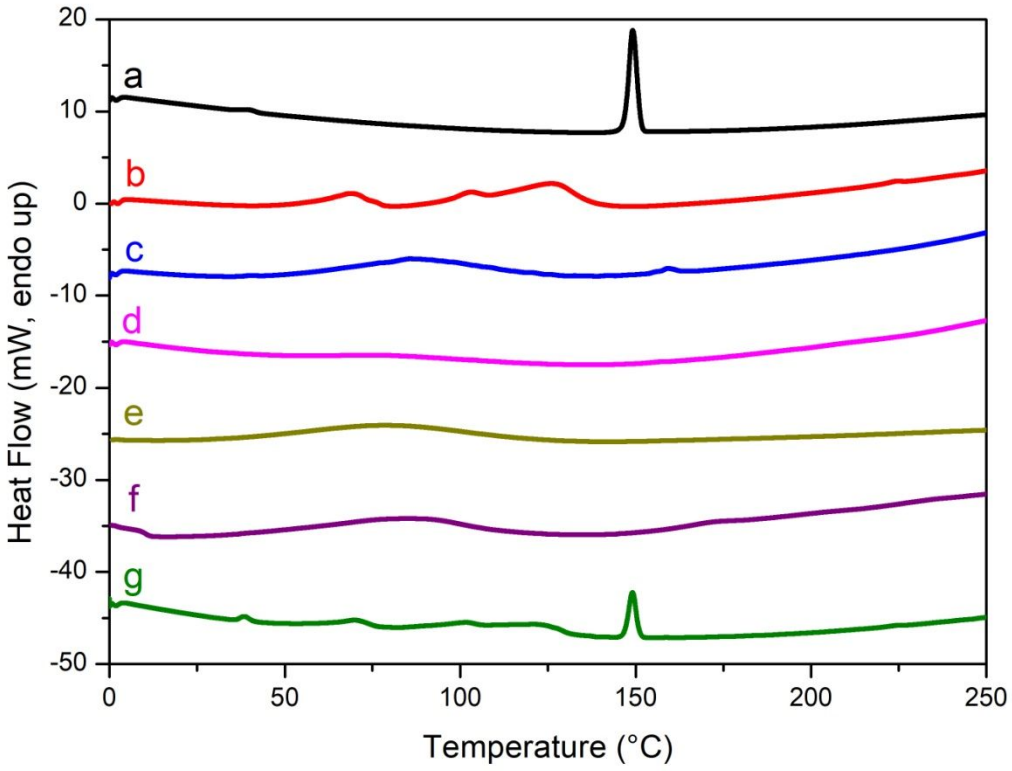

Figure 9. Thermograms of the active ingredients (a) cholesterol, (b) α-CD, (c) LP, (d) CH, the inclusion complexes (e) M1, (f) M4 and the physical mixture (g) PM1 from DSC measurements

4. Conclusions

The present work offers a comprehensive study about the development of a dietary supplement for the treatment of the hyperlipidemia, starting from the *in vivo* evaluation of the effectiveness and molecular docking studies up to the development of the final product.

The formulation developed in this work, containing CH, α-CD, and LP, could be considered an interesting alternative to the several dietary supplements today on the market. The innovative combination of the selected active ingredients showed to be very effective on an *in vivo* model of hyperlipidemia, reverting the serum lipid values to basal levels, thereby helping in reducing atherosclerosis and coronary heart disease risks. Moreover, the development of a simple pharmaceutical formulation makes this study very attractive for the dietary supplement market,

despite a clinical study is required to confirm the effectiveness of the active ingredients in human patients, to assess possible side effects, and to evaluate the product compliance.

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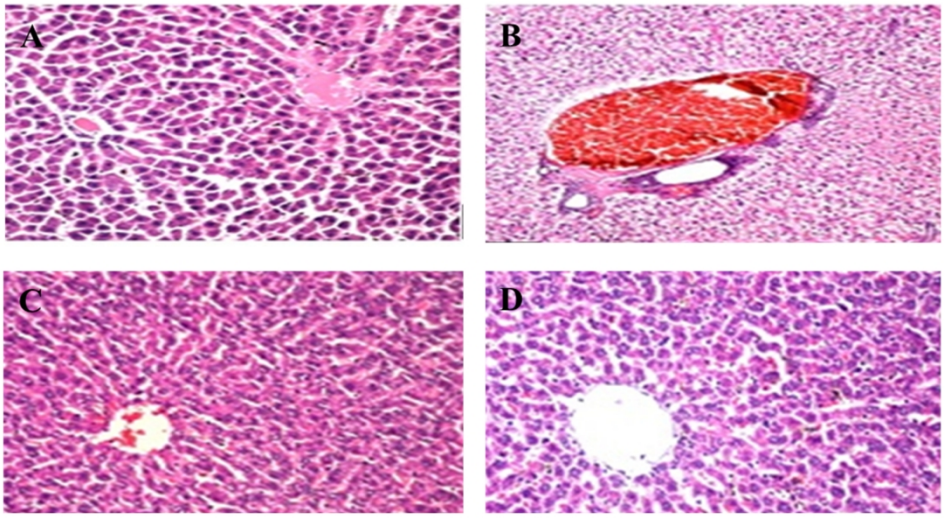

Figure 2. Photomicrography of rat liver sections of negative control (Group I) (A), of hyperlipidemic positive control (Group II) (B), of marketed atorvastatin treated group (Group VII) (C) and of the group treated with the combination of CH and a-CD/LP at high dose (Group IX) (D).

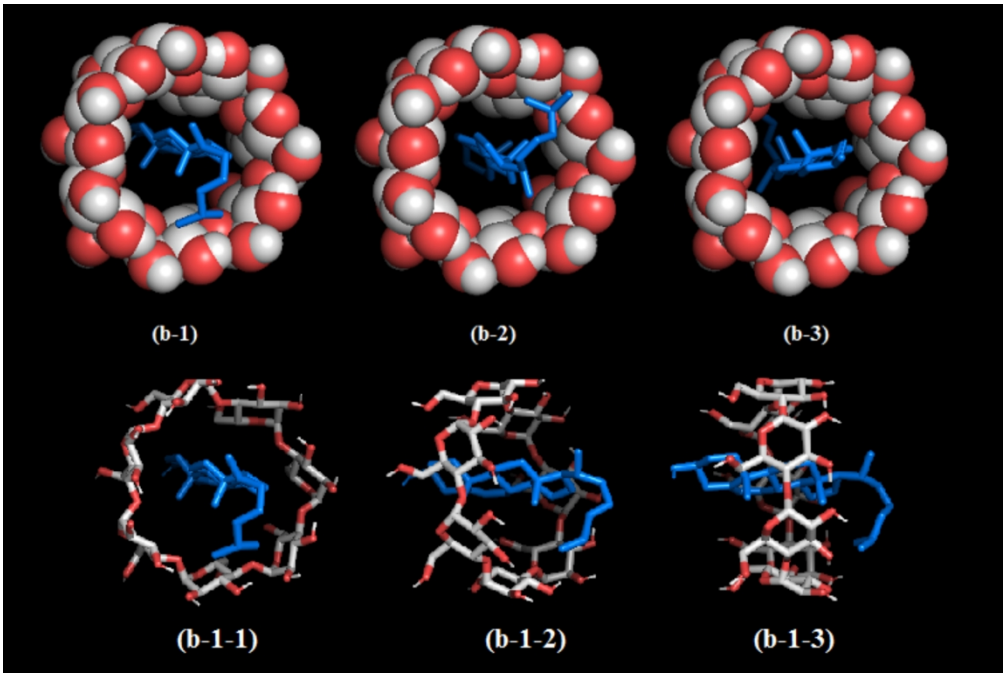

Figure 3. (b-1), (b-2), and (b-3):Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-β-CDinteractions. (b-1-1), (b-1-2), and (b-1-3): the best mode (b-1) from different angles shows the accommodation of Cholesterol insideβ-CD cavity.

503x337mm (118 x 118 DPI)

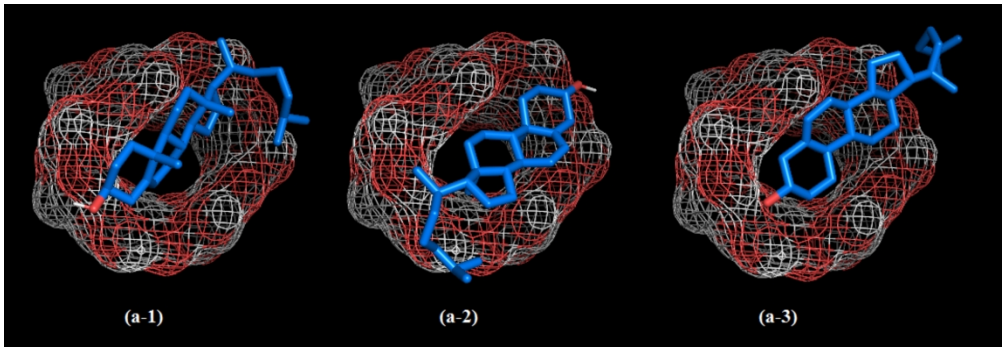

Figure 4. (a-1), (a-2), and (a-3):Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-α -CD interactions.

741x254mm (118 x 118 DPI)

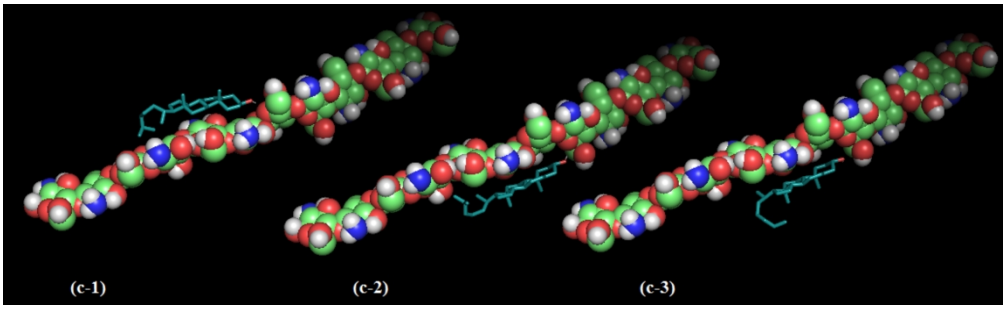

Figure 5. (c-1), (c-2), and (c-3):Pymol 3D schematic diagrams of the best 3 modes of Cholesterol-Chitosan interactions.

790x237mm (118 x 118 DPI)

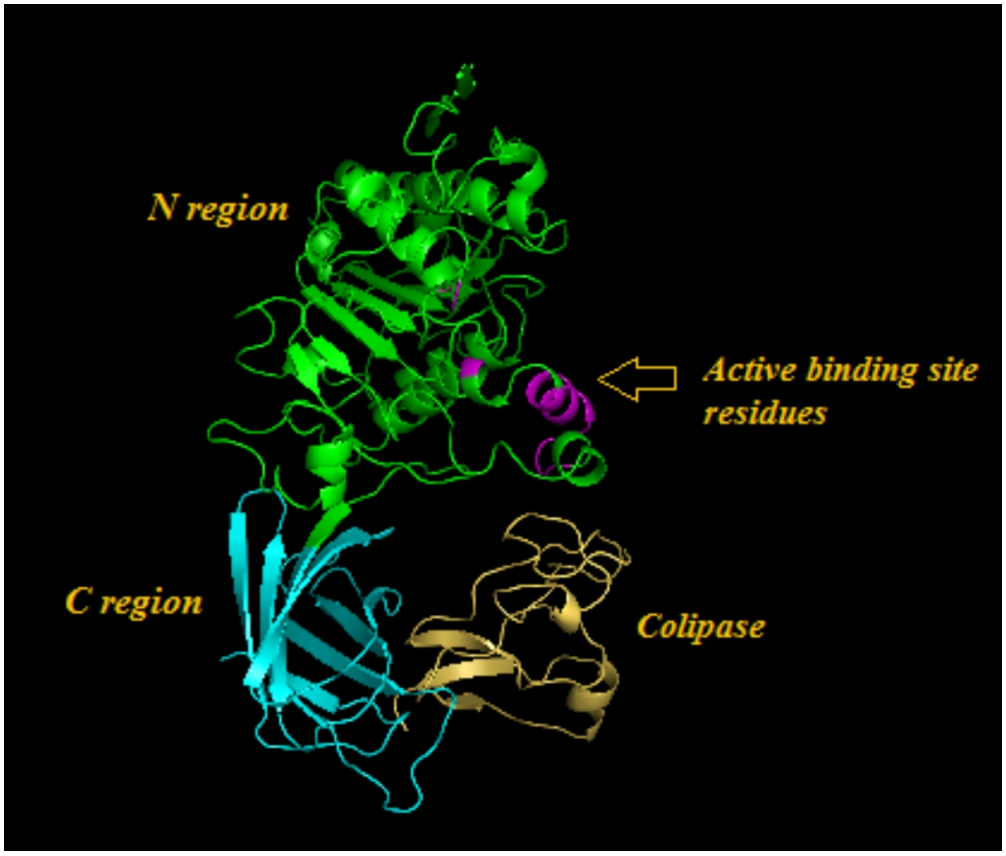

Figure 6. Pymol 3D structure for Lipase enzyme showing the N region (green), the C region (cyan), colipase (yellow) and the active residues (magneta)

280x237mm (236 x 236 DPI)

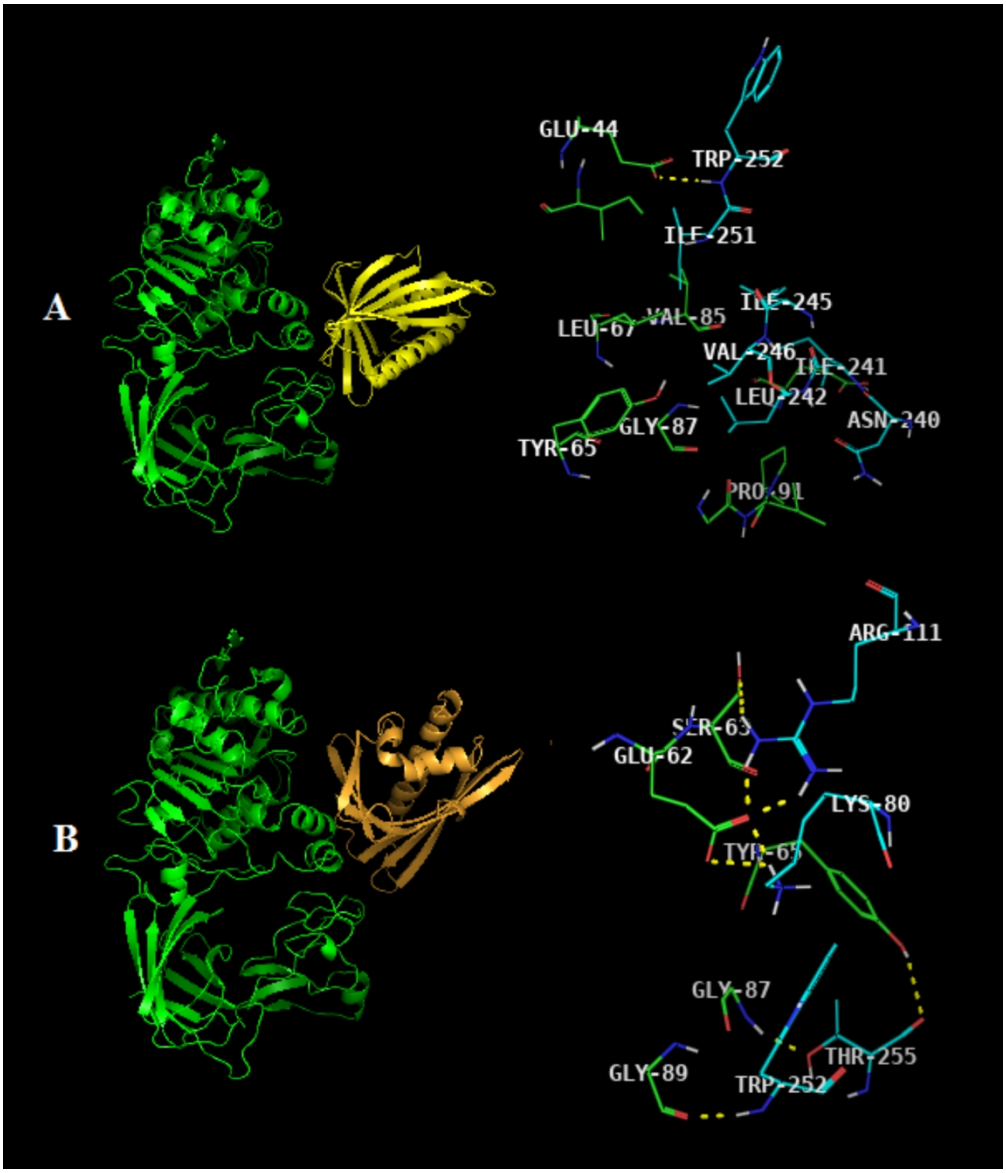

Figure 7: (A): Pymol 3D schematic diagrams of blind dockingcluster0 HPL-Lupin interaction, (B): diagram of knowledge-based docking cluster0 HPL-Lupin interaction. HPL residues are in cyan while Lupin residues are in green. Hydrogen bonds are indicated by yellow dashed lines between the atoms involved

405x471mm (118 x 118 DPI)

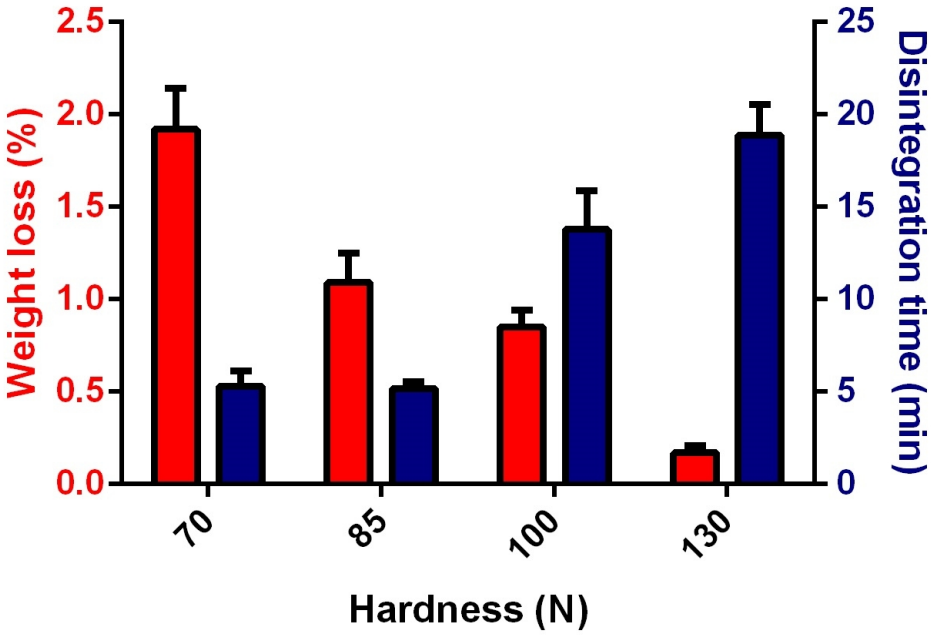

Figure 8. Effect of hardness (70-130 N) on the weight loss (%, friability) and disintegration time (min) of the formulated tablets

109x77mm (300 x 300 DPI)

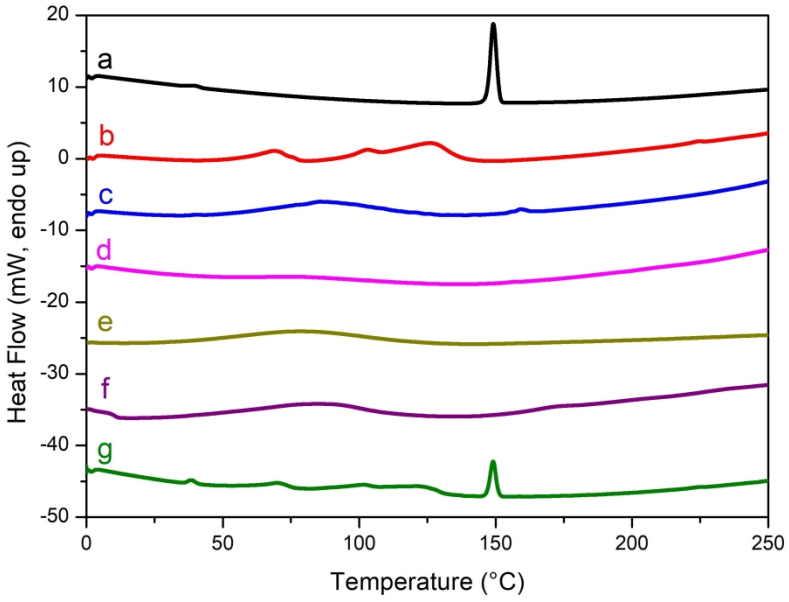

Figure 9. Thermograms of the active ingredients (a) cholesterol, (b) α-CD, (c) LP, (d) CH, the inclusion complexes (e) M1, (f) M4 and the physical mixture (g) PM1 from DSC measurements

289x202mm (300 x 300 DPI)

Group	Hyperlipidemia induction	Treatment	Protection Pre-induction	Treatment Post-induction
I (Negative Control)	X	Saline solution	-	-
II (Positive control)	✓	Saline solution	-	✓
III	✓	CH at 250mg/kg	-	✓
IV	✓	CH at 500 mg/kg	-	✓
V	✓	α-CD+LP (1:1) at 400 mg/kg	-	✓
VI	✓	α-CD+LP (1:1) at 800 mg/kg	-	✓
VII	✓	atorvastatin tablet dispersion at 40mg/kg	-	✓
VIII	✓	CH at 250mg/kg + α-CD+LP (1:1) at 400 mg/kg	-	✓
IX	✓	CH at 500 mg/kg + α-CD+LP (1:1) at 800 mg/kg	-	✓
X	✓	CH at 500 mg/kg	✓	-
XI	✓	α-CD+LP (1:1) at 800 mg/kg	✓	-

CH:chitosan; α-CD: alpha-cyclodextrin; LP: lupin proteins

Table 2. Parameters of AutoDockv in a config files and their values.

Receptor	No. of Points			Spacing (A°)	Center Grid Box			Exhaustiveness	No. of modes	Energy Range
	x	y	z		x	y	z			
α-CD	29.758	35.697	102.833	1.0	15	15	15	10	20	3
β-CD	37.554	58.491	59.162	1.0	15	15	15	10	20	3
Chitosan	3.933	21.778	4.175	1.0	20	20	20	10	20	3

Table 3. Effects of different treatments (III-IX groups) and protective pre-treatments (X and XI groups) on serum lipid concentration compared to negative control (Group I) and hyperlipidaemic positive control (Group II).

Group	TC (mg/dl)	TG (mg/dl)	HDL-C (mg/dl)	LDL-C (mg/dl)
I (Negative Control)	86.09 ± 4.17	87.05 ± 3.16	57.04 ± 2.18	17.43 ± 3.96
II (Positive Control)	309.10 ± 4.73^{***a}	549.64 ± 6.86^{***a}	18.25 ± 2.48^{***a}	206.80 ± 3.26^{***a}
III	196.44 ± 14.30 ^{**b} (36.44)	136.67 ± 12.43^{***b} (75.13)	56.23 ±3.80^{**b}	112.51 ± 13.55^{**b} (45.59)
IV	103.22 ± 9.02$^{***b, ++c, *g}$ (66.60)	174.04 ± 11.28^{***b,*g} (68.33)	54.26±1.13^{***b,***g}	26.65±8.57$^{***b,++c,}$ $^{++g}$(87.10)
V	219.71 ± 10.19^{**b} (28.92)	284.88 ± 16.80^{***b} (48.16)	42.98 ± 3.07^{***b}	124.12 ± 7.62^{***b} (39.97)
VI	124.85 ± 20.87$^{**b, **d, *g}$ (59.60)	174.74 ± 6.35$^{***b, ***d, *g}$ (68.20)	39.72 ± 2.22^{***b}	78.32 ± 15.05^{***b,*d} (62.12)
VII	191.64 ± 14.82^{**b} (37.99)	218.25 ± 13.88 ^{***b} (60.29)	37.59 ± 2.24^{**b}	116.82 ± 16.41^{**b} (43.51)
VIII	220.32 ± 4.07^{***b} (28.72)	150.87 ± 9.95^{***b,***d} (72.55)	59.16±2.07^{***b,***d}	142.14 ± 8.91^{***b} (31.26)
IX	92.93 ± 8.63^{***b} (69.93)	124.09 ± 18.31^{***b,*f} (77.42)	57.56±1.66^{***b,***f}	22.03 ± 8.30^{***b,*f} (89.34)
X#	268.67 ± 11.10^{*b,***e} (13.07)	486.84 ± 10.34$^{*b, ***e}$ (11.42)	43.28 ± 6.79^{*b}	135.52±8.24$^{**b, ***e}$ (34.46)
XI#	301.50 ± 9.16^{**f} (2.45)	475.17 ± 15.99$^{**b, ***f}$ (13.54)	54.66±0.60$^{***b, ***f}$	167.41 ± 6.03$^{*b, **f}$ (19.04)

#pre-treatment of high doses of CS and LP for 3 days before IP injection of P-407
Data is shown as mean ±S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ^{a}*vs*.normal control, ^{b}*vs*. hyperlipidaemic control, ^{c}*vs*. CS-L, ^{d}*vs*.LP-L, ^{e}*vs*.CS-H,^{f}*vs*.LP-H, ^{g}*vs*.Ator®.Numbers in parentheses are percentages reduction of the hyperlipidaemic control value.

Table 4. The binding affinity values of the best 10 modes for β-CD, α-CD and Chitosan

Mode	β-CD	α-CD	Chitosan
1	-5.7	-3.3	-3.3
2	-5.4	-3.1	-3.3
3	-5.4	-3.0	-3.2
4	-5.3	-2.9	-3.2
5	-5.3	-2.6	-3.1
6	-5.3	-2.6	-3.1
7	-5.2	-2.6	-3.0
8	-5.0	-2.5	-2.9
9	-4.9	-2.5	-2.9
10	-4.9	-2.3	-2.8

Table 5. The cluster values of the best HPL-Lupin clusters of the docking jobs.

Cluster	Blind Docking			Knowledge-based Docking		
	Members	Representative	Weighted Score	Members	Representative	Weighted Score
0	160	Center	-820.2	124	Center	-886.8
		Lowest Energy	-838.1		Lowest Energy	-1022.3
1	104	Center	-692.4	97	Center	-846.9
		Lowest Energy	-884.1		Lowest Energy	-1017.9
2	85	Center	-701.2	87	Center	-921.1
		Lowest Energy	-775.4		Lowest Energy	-1142.4
3	65	Center	-694.9	81	Center	-894.3
		Lowest Energy	-936.1		Lowest Energy	-942.5
4	48	Center	-709.1	70	Center	-885.3
		Lowest Energy	-799.3		Lowest Energy	-1032.2
5	45	Center	-704.8	68	Center	-927.5
		Lowest Energy	-802.6		Lowest Energy	-927.5
6	44	Center	-750.5	57	Center	-855.8
		Lowest Energy	-822.6		Lowest Energy	-1008.1
7	40	Center	-884.0	57	Center	-871.4
		Lowest Energy	-884.0		Lowest Energy	-929.7
8	35	Center	-776.5	56	Center	-882.5
		Lowest Energy	-776.5		Lowest Energy	-920.3
9	34	Center	-780.0	31	Center	-854.8
		Lowest Energy	-780.0		Lowest Energy	-971.4
10	31	Center	-701.3	27	Center	-856.2
		Lowest Energy	-850.6		Lowest Energy	-930.8

Table 6. Detailed data of HPL-Lupin cluster 0 of the docking jobs.

Docking Job	Cluster No. 0 Values	Interface Residues	No. of H.Bs	HPL residues involved in H.Bs
Blind Docking	Members:160 Center: -820.2 Lowest Energy: -838.1	Asn240, Ile241, Leo242, Ser243, Ile245, Val246 and **Ile251**	**1**	**Trp252**
Knowledge-based Docking	Members:124 Center: -886.8 Lowest Energy: -1022.3	Ile245, **Asp249, Ile251, Arg256, Phe258** and Ala259	**8**	Lys80, Arg111, **Trp252** and **Thr555**

HPL active site residues are bold and underline formatted.