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Anti HTLV compounds from fungal metabolites as protease inhibitor; in silico study

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Abstract

There is no curative treatment for patients infected with HTLV. HTLV protease plays an important role in activities such as replication cycle and virus maturity. The fungal metabolites as bioactive molecules could potentially be considered as a good source of discovery of new medicines. The aim of the present study was to investigate the inhibitory effect of fungal metabolites on HTLV-1 protease. Twenty fungal secondary metabolites from five different chemical groups were selected based on the inhibitory effects on HIV protease, including Colossolactones, Lanostane type Triterpenoids, Farnesyl Hydroquinones, Steroquinones, Cytochalasins, while indinavir was considered as the control. Human T-cell lymphoma virus protease (PDB code: 3LIN) was selected as the receptor for subsequent computational docking and molecular dynamic simulation study with Autodock and Gromacs, respectively. Results of the docking and molecular dynamic simulation study suggested a favorable binding mode of Colossolactone IV, Colossolactone II, Ganoderiol F, and Schisanlactoneto the HTLV protease with respect to indinavir. Analysis of hydrogen bond pattern and RMSF plot revealed a nearly similar ligand-receptor interaction for ColossolactoneIIandGanoderiol Fin complex with HTLV protease in comparison with indinavir. Colossolactone II and IV derived from *Colossum Ganoderma* and Ganoderiol F derived from *Ganoderma Lucidum* exhibited an anti-HTLV protease effect that has been proposed to be able to be developed as a drug.

Key words: HTL-V1 protease, inhibitor, fungal metabolites

1.Introduction

HIV[1] and HTLV-1[2] are both oncoviruses from the Retroviride family, and they have a single RNA genome with positive polarity [1]. HIV causes AIDS[3] , and HTLV-1 causes a type of blood cancer called ATL[4], a chronic neural disorder in the form of paralysis called HAM/TSP[5] [2-3]. Due to the wide range of diseases that these viruses cause in the human body, it has always been important to find new effective drugs to inhibit their function.

Protease is one of the few proteins of the HTLV whose 3D structure has so far been determined that considered as aspartic acid protease and functions as a homodimer [4-5]. HTLV-1 and HIV protease enzymes are among the most important enzymes of these viruses. These enzymes belong to the aspartic acid protease family, and they have ae engaged in important activities such as replication cycle and cell maturity [6]. HTLV-1 and HIV have a homodimer activity structurally, and each monomer of these enzymes has 125 and 99 amino acids, respectively. The active site of these proteases is located in a valley located between two monomers and ASP32 in HTLV-1 protease and ASP25 in HIV protease in each monomer act as the main catalytic amino acids [6]. The sequence alignment evidence shows a 28% identity similarity between the primary sequence of HTLV-1 protease and HIV protease enzyme while they are more conserved in the active site (45%) [5]. Although both proteases have high similarity in structure and function, they exhibit a slight difference in the specific binding to the substrate and their inhibitory profile. Many different compounds are designed and synthesized to inhibit HTLV protease, including peptide-based inhibitors [5-6], statin-based inhibitors [7], and plant secondary metabolites-based inhibitors such as Indinavir inhibitor [6]. Ritonavir, Nelfinavir, Saquinavir, and Amprenavir as highly potent HIV protease inhibitors did not show any inhibitory effect on HTLV protease [8-9], while Kuhnert et al., [6] showed that indinavir, as HIV inhibitor protease, has a highly potent inhibitory effect on HTLV protease. Trying to find new compounds that are more capable of inhibiting the enzyme could pave the way to a more effective treatment of patients.

There is still no specific antiviral agent available for the treatment of HTLV-1 infections. Fungal metabolites have a good potential for antiviral treatment. The folds of HIV and HTLV protease are very similar in spite of low primary sequence identity similarity. In this study, the

[1] *human immunodeficiency viruses*
[2] *Human T-lymphotropic virus 1*
[3] *Acquired immune deficiency syndrome*
[4] *Adult T cell leukemia*
[5] *HTLV-1 associated myelopathy /tropical spastic paraparesis*

inhibitory effect of fungal metabolites, which have been introduced as an inhibitor of HIV protease, was investigated on HTLV-1 protease.

2. Methods

2.1. Ligand Preparation:

In order to check the effect of the inhibitors on the protease enzyme, first, the structure of fungal and plants metabolites were designed by HyperChem software [10]; then, for optimizing their structure, a solvation box was created for them, and molecular dynamic simulation was performed by amber force field under the temperature of 300˚K in 0.5 picoseconds in order to achieve the most sustainable state and the lowest energy of the structure of inhibitors to be used in the future studies. Next, these structures were saved as pdb files to be used in the docking process [11].

2.2. Protein preparation:

First, the crystallography structure of HTLV-1 and HIV protease enzymes were selected from the protein data bank with the respective PDB codes of 3LIN and 1YT9. Then, all the non-amino acid molecules (water, ion, ligand, inhibitor, et cetera) were removed to make that prepared to be used in the docking process. Then, to get more reliable results, the enzyme structure was optimized in a quasi-natural condition in terms of temperature, atmosphere pressure, pH, and the existence of water as a solvent in order to get their balanced structure. Enzyme optimization was done by Gromacs 5.1.2 software based on Linux operating system. Gromacs is a software set used for molecular dynamics simulations and minimizing the energy based on Newtonian motion equations. First, the topology of protein was built by using the GROMOS96 53a5 force field of PDB. Then, a cubic box was considered between the protein and the edge of the box with a distance of 1.0 nanometer, and the water molecules were added to the box as solvent by using the SPC[6] model. Then, four chloride ions were added for neutralizing the system load. In the following, energy minimization was done by the steepest descent algorithm. Then, NVT equilibration and NPT equilibration were done each for 100 picoseconds, and finally, molecular dynamic simulation was performed for 10000 picoseconds (10 nanoseconds). The obtained files are the balanced structure of HIV and HTLV-1 protease, which may be used in the docking process [12].

2.3. Molecular docking:

[6] *simple point charge*

Molecular docking is the process of computation by which ligands are moved in a 3D space so that the best space structures of the target and ligand positions are found by getting the highest scores in the functions. By using PDB files, the designed inhibitors, and the protein balanced structure file, the docking process was done by AutoDock 4.2 software. In the docking process, LGA[7] was used, and all the non-rotatable amide bonds were selected. To predict the connection between the ligands and proteins, two types of docking, including focused docking and blind docking, were done for all the 21 fungal metabolites. In focused docking, active position amino acids were selected, and a cubic box with the size of 70*70*70 Å was set around them. In blind docking, a cubic box with the size of 126*126*126 Å was set to cover all the proteins. The number of GA runs for every 250 docks was selected by 25000000 energy measurement [13]. Finally, four compositions were selected for molecular dynamic simulation from the ligands which are connected to the active site and those which had the least Gibbs free energy at the time of docking [13-14].

2.4. Molecular dynamic simulation:

Molecular dynamic simulation of the ligand-protein complex was done in 50 ns as mentioned in 2.2. Topology files of the ligands were built by Prodrug and ATB servers. [15-18]. All images from ligand-protein complexes were drawn by PyMol and Ligplot software [19].

3. Results

3.1. Protein and Ligand Preparation

In order to get more optimal and reliable results from the docking process, first, the 3D structure of HTLV-1 protease (PDB codes: 3LIN) and HIV protease (PDB code: 1YT9) were optimized by Gromacs in 10 nanoseconds. The RMSD results (root mean square deviation) and total energy showed that the structure of protein during running time is stable (figure 1 in supplementary material).

[7] *Lamarckian genetic algorithm*

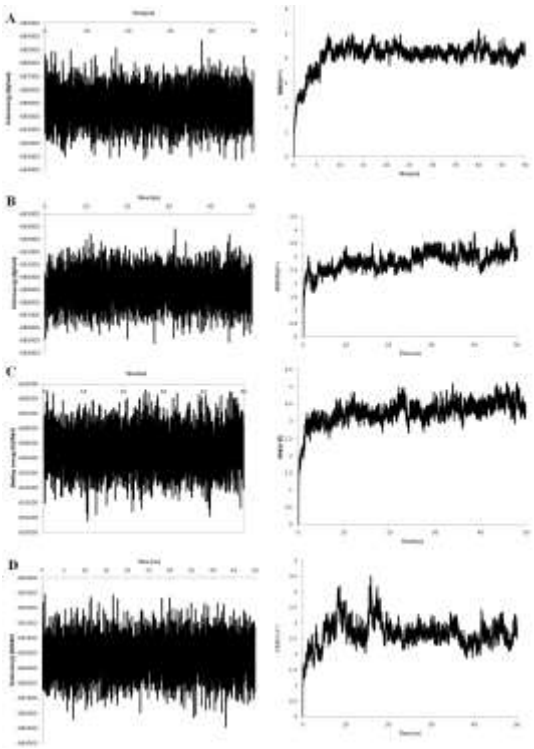

Figure 1: Total energy of systems and RMSDs during 50 ns molecular dynamics simulations for ligand- HTLV-I protease. a) Schisanlactone A, b) Colossolactone IV, c) ColossolactoneII .d) GanoderiolF.

3.2. Ligand docking analysis to predict the best binding mode

Small molecule fungal metabolites have always been good sources for new drug discovery due to their structure, wide diversity, and high biocompatibility [20]. Triterpenes are one of the most important fungal metabolites which are produced by fungus and the most common compounds derived from *Ganoderma* species which have an inhibitory effect on HIV protease enzyme (Table-1). In this study, Triterpenes are derived from *Colossum Ganoderma* which are called Colossolactones (Table 1 and Figure 2 in supplementary material) [21-22] and Lanostane type triterpenoids that derived from *Ganoderma Lucidum* species (Table 1 and Figure 2 in supplementary material) [23], and Farnesyl Hydroquinones are derived from *Ganoderma* fungus (Table 1 and figure 2-C in supplementary material) [24] and Bisalkylated2,5-dihydroxybenzoquinonesor asteriquinones (Table-1andFigure-2 in supplementary material) [25-27] and also cytochalasins (Table-1 and Figure-2 in supplementary material) [28-30] are

derived from hypoxylon fragiformeare, one of the common fungal metabolites in fungal species were docked to HTLV protease.

The HIV and HTLV proteases are functionally and structurally similar, although there is little difference in the specificity of their substrate binding. According to the results of the molecular docking shown in Table -1, all tested fungal metabolites illustrated less inhibition constant on HTLV protease in comparison with HIV protease except Semicochliodinol A. It should be noted that the binding site of only a few of these fungal compounds has been identified on the HIV protease. To investigate how these compounds bind to HIV protease, these compounds were docked onto the protease of the HIV virus (Table 1). According to previously published data, Ganomycin I and Ganomycing B are both connected to HIV protease at ASP30 [31], and Didemethylasterriquinone and Hinnuliquinone are both connected to (ASP25/ASP25', ILE50/ILE50') and DMAQ-B1 is connected to HIV protease at ASP30 and ASP 29 [32-33].

Table 1: Chemical name, formula and inhibition constant (Ki), binding energy, the number of hydrogen bond between ligand and HTLV-I and HIV protease (HB), the names of residue that participated in hydrogen bonds.

		HTLV Prptease				HIV Protease				
Name	Formula	Ki (nM)	Binding Energy (kcal/ mol)	HB	Amino Acids	Ki (nM)	Binding Energy (kcal/ mol)	HB	Amino Acids	IC50(µM)
Fungal metabolites: Group 1*										
Colossolactone I	$C_{30}H_{46}O_3$	28.58	-10.29	1	ARG10 (S1, S3)	389.56	-8.76	3	LEU10 ARG8(2)	4.1
Colossolactone II	$C_{30}H_{46}O_4$	53.18	-9.92	1	MET37' (S2', S4')	1310	-8.03	3	ASP29 ARG87' GLY27	4.4
Colossolactone IV	$C_{30}H_{44}O_5$	55.6	-9.9	1	ASP32'(active site)	1480	-7.95	2	GLN7' ARG8'	12
Colossolactone V	$C_{35}H_{54}O_9$	237.19	-9.04	4	ARG10(3)(S1,S3), TRP98(S1)	1682	-6.51	3	ARG8(2) ASP29	14.5

Colossolactone VII	$C_{33}H_{50}O_7$	91.08	-9.61	3	ARG10(2)(S1,S3), TRP98(S1)	2980	-7.54	3	GLY48 ASP29 ILE50	24.7
Schisanlactone A	$C_{30}H_{40}O_4$	83.94	-9.65	1	MET37' (S2', S4')	131.14	-9.39	2	THR4 ARG8	10.8
Fungal metabolites: Group 2**										
Ganoderic acid GS-1	$C_{30}H_{42}O_6$	204.73	-9.13	2	GLY60, ASP36' (S2',S3',S4')	1380	-8	2	ARG8(2)	58
Ganoderic acid GS-2	$C_{30}H_{44}O_6$	80.04	-9.68	1	ARG10 (S1,S1'; S3,S3')	225.59	-9.07	4	ARG8(2) GLN7(2)	30
GanodericacidDM	$C_{30}H_{44}O_4$	33.45	-10.2	2	ARG10(S1,S3), MET37 (S2,S4)	454.61	-8.65	2	ASP29 LYS45	38
Ganoderic acid b	$C_{30}H_{44}O_7$	38.7	-10.11	2	ARG10(S1,S1'; S3,S3'),TRP98(S1)	4070	-7.35	3	ARG8(3)	116
Ganoderiol F	$C_{30}H_{46}O_3$	236.85	-9.04	3	ASP32 (active site), ARG10 (S1,S3), GLY34'	3430	-7.45	3	THR4(2) ARG8	22
Ganodermadiol	$C_{30}H_{48}O_2$	208.51	-9.11	3	ARG10 (S1, S3), MET37 (S2, S4),GLY34'	4440	-7.3	2	ASP25 GLY27	29
Fungal metabolites:Group3***										
Ganomycin I	$C_{21}H_{26}O_4$	456.39	-8.65	3	GLY58', ASN96 (S1,S3), HIS66	10980	-6.77	3	GLN7(2) TRP6	7.5
Ganomycin B	$C_{21}H_{28}O_4$	653.93	-8.44	3	ARG10 (S1,S3), ALA59, ASN96 (S1, S3,)	20220	-6.4	4	THR4(2) TRP6 GLN7	1
Fungal metabolites:Group4****										
Didemethylasterriquinone	$C_{22}H_{14}N_2O_4$	186.03	-9.18	2	GLY58' ALA59	1630	-7.9	1	ARG8'	0.18

Semicochliodinol A	$C_{27}H_{22}N_2O_4$	23.35	-10.41	2	ALA59 GLY58'	2.99	-7.54	1	ARG8'	0.37
Hinnuliquinone	$C_{32}H_{30}N_2O_4$	487.78	-8.61	1	GLY60	11690	-6.73	2	ASP25 ASP25'	2.5
DMAQ-B1	$C_{32}H_{30}N_2O_4$	94.91	-9.58	0	-	2110	-7.74	1	ASP29	57
Fungal metabolites:Group5[*****]										
L-696,474	$C_{30}H_{39}NO_4$	659.95	-8.43	1	LEU57'	3740	-7.4	1	LEU10	3
Cytochalasin A	$C_{29}H_{35}NO_5$	208.99	-9.11	1	GLY60'	900.59	-8.25	1	ASP25	5

[*]Fungal metabolites-Group1: Triterpenes are derived from *Colossum Ganoderma* which are called Colossolactones.

[**]Fungal metabolites-Group2:Lanostane type Triterpenoids are another type of Triterpenes derived from *Ganoderma Lucidum*specise.

[***] Fungal metabolites-Group3: Farnesyl Hydroquinones are derived from *Ganoderma* fungus.

[****] Fungal metabolites:Group4:Asteroquinones are common metabolites in fungal species.

[*****] Fungal metabolites:Group5 :Cytochalasins are derived from *Hypoxylon Fragiforme*.

3.3. Molecular Dynamics Simulation to validate Ligand-Protein Complexe's stability

The overall stability of the protease-ligand complex structures for all compounds listed in Table-1 was estimated by considering the Root Mean Square Deviation (RMSDs) of Cα atoms and total energy during 50 nanoseconds of the MD simulations (Figure 3). The best results were obtained for Colossolactone IV, Schisanlactone A, ColossolactoneII, Ganoderiol.

Based on the RMSD values, the system equilibrated after three nanoseconds. The RMSD values of ligand Schisanalactone A, Colossolactone IV, Colossolactone II and are stable at a total trajectory, and the backbone *RMSD values* are distributed around 1 Å, 1.5 Å, and 1.5 Å, respectively. Ganoderiol F showed two *RMSD values range* in the period of 3 nanoseconds to 20 nanoseconds between 2- 4.5Å, and then it fluctuated in the range of 2 to 3Å in a range of 20 to 50 nanoseconds which suggests the stability of the complex in the final 30 nanoseconds of calculation (Figure 1).

3.4. Molecular dynamic results of fungal metabolites –HTLV protease complex

HTLV-1 and HIV protease enzymes as aspartic acid protease, have a role in important virus activities such as replication cycle and cell maturity [11-12]. HTLV-1 and HIV have a homodimer activity structurally, and each monomer of these enzymes has 125 and 99 amino acids, respectively. The active site of these proteases is located in a valley located between two monomers and ASP32 in HTLV-1 protease and ASP25 in HIV protease in each monomer act as the main catalytic amino acids [13]. The sequence alignment evidence shows a 28% identity similarity between the primary sequence of HTLV-1 protease and HIV protease enzyme while they are more conserved in the active site (45%) [8]. Although both proteases have high similarity in structure and function, they exhibit a slight difference in the specific binding to the substrate and their inhibitory profile. So far, many compositions specifically have been synthesized and recognized to inhibit HIV proteases, such as Daronavir, Indinavir, Neflinaavir, Amprenavir, Tipranavir, Herbal terpenoids, and Fungal metabolites. [33-34] Ritonavir, Nelfinavir, Saquinavir, and Amprenavir, as highly potent HIV protease inhibitors, did not show any inhibitory effect on HTLV protease [20-21] while Kuhnert et al., [11] showed that indinavir, as HIV inhibitor protease, has a highly potent inhibitory effect on HTLV protease and is the only drug to inhibit HTLV protease [35]. Today, fungal metabolites have received much attention for viral therapies.

HTLV Protease homodimer has eight binding pockets that are called S1 to S4 in the first monomer and S1’ to S4’ in the second monomer. The HTLV protease substrates were surrounded by amino acids located on S1 to S4 and S1’ to S4’ subsites. The subsites S1’ and S1 are hydrophobic, and they are composed of the residues of Arg10, Leu30, Gly34, Val56, Leu57, Asn96, Gln97, Trp98, and Ile100. The subsites S2 and S2’ are big hydrophobic pockets which are mainly composed of the residues of Ala35, Asp36, Met37, Val39, Thr54, Val56, Leu57, Val92, and Ile100. The subsites S3 and S3’ are composed of Arg10, Asp36, Lys95, Asn96, and Asn97. The subsites S4 and S4’ are surface-exposed pockets that are composed of residues of Asn53, Met37, Asp36, Val92, Cys90, Ser55, and Thr54 [36].

Based on results of 50 ns molecular dynamics simulation, Colossolacton II and IV, Schisanlactone A, and Ganoderiol F are located in a similar site with indinavir (figure 2). Ruker et al. [37] investigated similarities and differences in HTLV and HIV protease-ligand interactions at the molecular level. They showed the main protease-ligand interactions occurs in similar regions of proteases: The N-terminal (HIV-PR R8; HTLV-PR R10); the active site (HIV-PR L23-D30; HTLV-PR L30-D36), the flap region (HIV-PR M46-I50; HTLV-PR S55-A59), and the lateral loops of the ligand-binding site (HIV-PR P81-I84; HTLV-PR N97-I100). Also, they showed, based on interaction energy profile, although some well-conserved residues

have strong interactions with the substrate in two proteases (R8/R10, A28/A35, D29/D36, G49, G58, and I84/I100), there are many not-conserved residues that have strong interactions in the similar ligand binding pockets in HIV and HTLV protease (D30/M37, I47/V56, G48/L57, I50/A59, and V82/W98). According to their data HIV D30/D30' and HTLV-PR M37/M37' also strongly affect ligand-protease binding in both proteases. Ruker et al. showed that the active site in HIV PR: L23-29 and HTLV-P; L30-D36 is entirely conserved, though Singh et al. reported that MET37 in the binding pocket of HTLV protease shows a repulsive role with known HIV inhibitors. Functional analysis of M37A mutation clearly shows that Met37 is highly important for protease function [38-39]. The Colossolactone IV and II (figure 2c and d), Ganoderiol F (figure 2e) at the same place with indinavir and Schisanlactone with a little difference (figure 2f), stay in the cavity that is formed between active site, flap, and c-terminal regions of HTLV protease dimmers during 50 ns molecular dynamic simulation. Interestingly, Met37 did not show a repulsive effect on them during the simulation, which could increase the likelihood of developing a suitable inhibitor of HTLV protease.

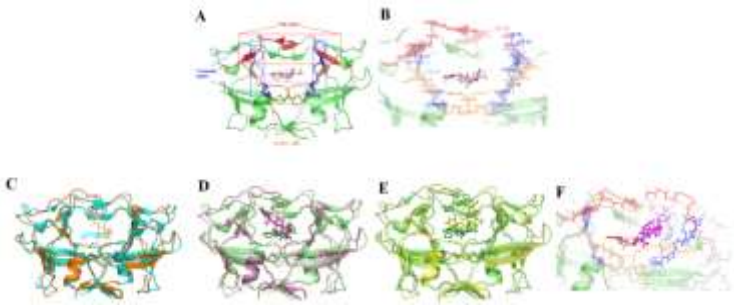

Figure 2: The location of the ligands on the protease cavityafter 50 ns molecular dynamics simulations. The a and b)the location of the indinavir on HTLV protease cavity(PDB code: 3WSJ), c) Colossolactone IV, d) Colossolactone II, e) Ganoderiol F, and f) Schisanlactonecomplexed with HTLV protease. Intheb, c, d and f parts of figure 4, the ligands (Colossolactone IV (orange color), Colossolactone II (purpule color), ganodeiol F (yellow color) and Schisanlactone (pink color)-protease complex superimposed on indinavir-HTLV protease complex (green color).

4. Discussion

4.1. H bond and hydrophobic interaction

Hydrogen bonds numbers play a critical role in the formation and stabilization of the drug-receptor complex. To gain more insight into the mechanism of complex formation and its stability, the number of H-bonds between active site, flap, and c-terminal residues of HTLV

protease and the ligands was analyzed (figure 3, only residues with h-bonds are shown in the figure). As shown in figure 3, the median number of H-bonds in the Ganoderiol F-HTLV protease complex is greater than the Colossolactone II- HTLV protease Colossolactone IV-HTLV protease, and Schisanlactone-HTLV protease complexes. Keikha [40] showed that the most interesting siting site for ligands on HTLV-protease is near Asp32 and Asp 36 residues at the enzyme active site. According to our results, all tested ligands interact with Asp36, though the number of H-bonds between active site residues and Ganoderiol F is higher than the other.

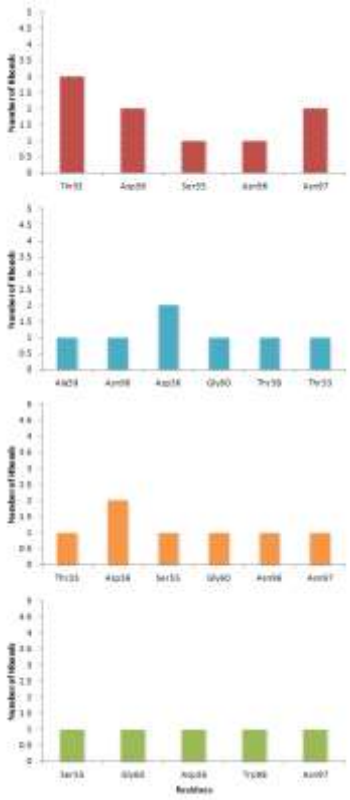

Figure 3: The number of hydrogen bonds between HTLV protease and the a) Ganoderiol F, b) Schisanlactone, c)ColossolactoneII, d) ColossolactoneIV during 50ns trajectory.

4.2. Dynamical behavior of complex

The dynamic behavior of the protein may play a role in the interaction between the substrate and the enzyme. HIV protease region for interaction with substrate is divided into functionally different regions including (1) Elbow (residues 36–46) or epitope region, (2) Catalytic site (residues 25–27), (3) Cantilever (residues 59–75), (4) Dimer interface including residues 4 to

10 and c-terminal regions (residues 90 to 99), and (5) Flaps (residues 43–58). Theoretical studies represented the flexibility of the flap region in HIV protease functionality is important for the substrate to access the active site of protease [41], and the flap region in free protease has two open and semi-open forms [42]. The protease flap region changes to the semi-open form to allow substrate cleavage when a ligand binds it [41-42]. Therefore, the dynamics of the flaps regions play a critical role in protease's ability to bind its substrates since the same role is expected for HTLV protease functional regions as HIV protease due to the high percent conserved sequence and similar folding and function between them.

Analysis of the flexible regions based on root-mean-squared fluctuations (RMSF) of the protein (Figure-4) showed the average position of fluctuations of all the Cα atoms of the residues with respect to the ligand and free-protease structures. Due to the high functional and structural similarity between HIV and HTLV, different functional regions in the structure of HTLV were named according to HIV virus, including (1) Fulcrum (residues 10-32), (2) Elbow (residues 45-55) or epitope region, (3) Catalytic site (residues 32-42), (4) Cantilever (residues 70-90), (5) Dimer interface including residues 4 to 10 and c-terminal regions (residues 90 to 99), and (6) Flaps (residues 55-65) (Figure-4). The pattern of the dynamical behavior of HTLV protease in binding to Ganoderiol F, ColossolactoneII, IV in A and B chains are similar to the indinavir-HTLV protease B-factor diagram (Figure-4). The overall flexibility of the flap and c-terminal regions is reduced except for Schisanlactone (Figure 4). Bayada *et al.* [42] reported in free HIV-protease, flap opening is accompanied by the concerted downward motion of the elbow (residues 36–46), fulcrum (residues 10–23), and cantilever (residues 59–75). It is expected that by ligand binding to the HTLV protease, the fluctuations of the mentioned regions will be affected as well (Figure-4). By ligand binding, elbow fluctuations in A and B chains are reduced with the exception of the A chain elbow region in binding to Schisanlactone A.

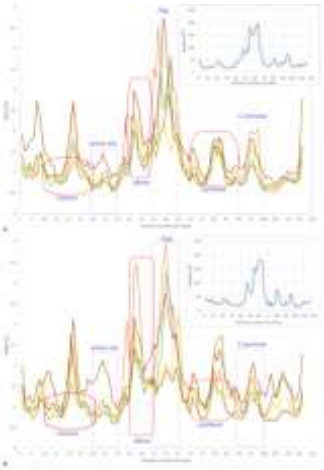

Figure 4: RMSFs during 50 ns molecular dynamics simulations for ligand-free (orange color) and ligand-binded HTLV-I protease: Schisanlactone A (brown color), Colossolactone IV (lightgreen color), Colossolactone II (yellow color), Ganoderiol F (dark green color). The B-factor digram for Indinavir –HTLV protease (PDB code: 3WSJ).

5. Conclusion:

A number of patients who are infected with the virus face some problems throughout their lives, for which no definitive cure has yet been found. Among the various viral proteins, protease is an important therapeutic target whose inhibition can largely stop the reproduction and growth of the virus. In this study, the inhibitory potential of different groups of fungal secondary metabolites was investigated. Despite binding of HTLV proteases to the Schisanlactone composition during trajectory, it showed a different dynamical behavior compared with indinavir and at times showed more fluctuations than free protease. Inhibitory constants, hydrogen bonding pattern, and their dynamic behavior showed that the two compounds, the Ganodriol F and Colossolactone II and Colossolactone IV, can be introduced as targets with the suitable potential to inhibit HTLV protease.

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