RESEARCH PAPER

Mechanistic In Silico and In Vitro Evaluation of Phytochemicals Targeting HIV-1 Reverse Transcriptase and Protease

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ABSTRACT

Introduction: Human immunodeficiency virus type-1 (HIV-1) replication depends on the coordinated activity of viral enzymes, particularly reverse transcriptase and protease, which are critical for genome replication and viral maturation. Targeting these enzymes remains a central strategy in antiviral drug development.

Materials and Methods: In the present study, an integrated in silico–in vitro approach was employed to investigate plant-derived phytochemicals as potential inhibitors of HIV-1 reverse transcriptase and protease. Molecular docking was performed to predict binding affinities and interaction patterns of selected phytochemicals with the catalytic regions of both enzymes. Drug-likeness and pharmacokinetic properties were evaluated using ADMET and SwissADME tools, while biological activity was predicted using PASS analysis.

Results and Discussion: Experimental validation was carried out using enzyme-based inhibition assays, complemented by cytotoxicity assessment in host cells using the MTT assay. Docking analyses revealed stable and target-specific interactions of phytochemicals with key amino acid residues involved in enzyme activity. In vitro assays confirmed inhibitory effects with IC_{50} values ranging from 2.0 to 30.0 µM, alongside acceptable cytotoxicity profiles. Several compounds demonstrated differential inhibition of reverse transcriptase and protease, indicating distinct modes of interference with HIV-1 replication machinery.

Conclusion: Overall, this study provides mechanistic insight into the inhibition of HIV-1 enzymes by structurally diverse phytochemicals and establishes a direct correlation between predicted molecular interactions and functional enzyme inhibition. The findings highlight the potential of natural compounds as lead scaffolds for structure-guided antiviral drug development targeting HIV-1 pathogenesis.

Keywords: Anti-HIV phytochemicals, HIV-1 protease, reverse transcriptase, drug-likeness, Lipinski's Rule of Five, bioinformatics, Phyto-HIV Database, natural product-based drug discovery.

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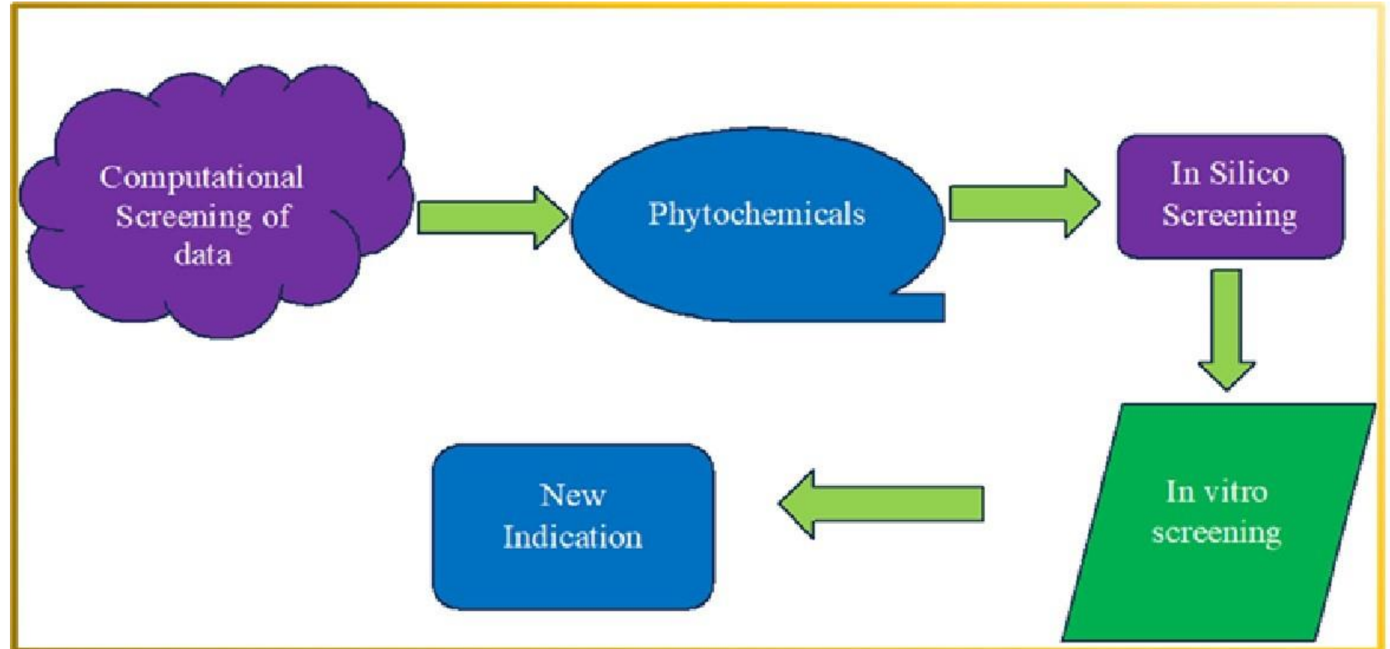
Graphical Abstract:

Schematic diagram of study

Schematic representation of the integrated *in silico–in vitro* workflow used to identify phytochemicals as inhibitors of HIV-1 reverse transcriptase and protease.



1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS). There are roughly 37 million persons infected with HIV globally. Over the previous two decades, more than two dozen novel HIV medicines have been licensed for clinical use. Combination antiretroviral treatment (cART) employs a variety of medication types that work together to inhibit HIV replication. Despite the availability of combination antiretroviral therapy, viral persistence and the emergence of drug-resistant strains continue to pose significant challenges. These limitations underscore the need for continued exploration of novel inhibitors that target essential components of the HIV-1 replication cycle. The key classes include protease inhibitors (PIs), nucleoside and nonnucleoside reverse transcriptase inhibitors (NRTIs/NNRTIs), entrance inhibitors (CCR5 coreceptor antagonists, fusion inhibitors, and post-attachment inhibitors), and integrase inhibitors (INIs) [1-3].

In 1996, a combination of antiretroviral medications was released as highly active antiretroviral treatment (HAART), transforming HIV/AIDS from a life-threatening illness to a treatable disease [4]. However, the requirement for lifetime medication, the severe side effects, and the uncertain long-term implications of this therapy remain important concerns. Furthermore, medication resistance may arise as a result of the virus's low genetic barrier, which allows for associated mutations [5]. Reverse transcriptase and protease play indispensable roles in HIV-1 pathogenesis. Reverse transcriptase catalyses the conversion of viral RNA into proviral DNA, enabling integration into the host genome, while protease mediates the cleavage of viral polyproteins, a critical step for virion maturation and infectivity. Inhibition of either enzyme disrupts viral replication; however, selective pressure on single targets often leads to resistance development. Consequently, compounds capable of interfering with multiple viral enzymes are of considerable interest for antiviral research. As a result, the discovery of new medicines for effective antiretroviral treatment remains necessary. It should also be emphasized that, despite the fact that there is minimal evidence to support this technique, alternative medicine is utilized to treat HIV all over the world [6-8].

Traditional herbal therapy is especially widespread in Africa, where it commonly serves as the sole medicinal strategy in rural areas [9]. While inefficient, non-evidence-based HIV therapy is a severe healthcare issue and a risk to the entire community, and plant secondary metabolites unquestionably merit a lot of consideration when looking for novel therapeutic options.

Natural products represent a structurally diverse reservoir of bioactive molecules, many of which have been shown to interact with viral proteins at the molecular level. Phytochemicals such as flavonoids and terpenoids possess chemical features conducive to enzyme binding, including hydrogen-bond donors, aromatic systems, and hydrophobic moieties. Previous studies have reported antiviral properties of these compounds; however, many investigations rely primarily on computational predictions or single-target evaluations, with limited experimental validation. Moreover, the mechanistic relationship between predicted ligand–enzyme interactions and functional inhibition of HIV-1 enzymes remain insufficiently explored. Establishing this correlation is critical for advancing pathogen-focused drug discovery beyond virtual screening. Therefore, the present study aims to systematically evaluate selected phytochemicals for their ability to inhibit HIV-1 reverse transcriptase and protease using an integrated *in silico* docking approach combined with enzyme-specific inhibition assays and cytotoxicity profiling. By linking molecular interaction patterns with functional enzyme inhibition, this work seeks to provide mechanistic insight into phytochemical

interference with HIV-1 replication machinery. Phytocompounds appear to be particularly promising in this regard, as they can inhibit a wide variety of viral and cellular enzymes participating in the HIV life cycle, such as reverse transcriptase (RT), integrase (IN), viral protease (PR), and casein kinase II, a cAMP-, cGMP-, and Ca2+/phospholipid-independent serine/threonine protein kinase [10].

Previous research has shown that various types of flavonoids, particularly certain flavonols, flavones, isoflavones, catechin derivatives, and chalcones, terpenoids can act as multitarget agents by inhibiting crucial HIV1 enzymes while also interfering with different stages of the virus's life cycle [11]. In this regard, the best researched flavonoid is quercetin, which has been shown to have strong anti-HIV action by suppressing HIV replication and lowering viral infectivity in normal peripheral blood mononuclear cells (PBMC) [12]. In pursuit of this concept, we chose 10 Phytochemicals **(Table 1.1)**, which are found in medicinal plants, to investigate their potential against HIV-1 reverse transcriptase and protease enzymes by In vitro assay, also check In vitro cytotoxicity of compounds for truthful result.

Table 1.1. Structure and nature of selected Phytocompounds

Sr. No	Name of Phytocompounds	Structure of Phytocompounds	Nature of Phytocompounds
1	Hesperidin	Hisperidin	Flavonoids
2	Isoliquiritigenin	Isoliquiritigenin	Flavonoids
3	Ellagic acid	Ellagic acid	Flavonoids
4	Diosgenin	Diosgeni	Terpanoid
5	Diosmetin	Diosmetin	Flavonoid
6	Chrysin	Chrysin	Flavonoid
7	Licoflavone B	Licoflavone B	Flavonoid
8	Capsorubin	Capsorubin	Carotenoid
9	Withaferin	Withaferin	Terpanoid
10	Alpha-Amyrin	Alpha- Amyrin	Terpanoid

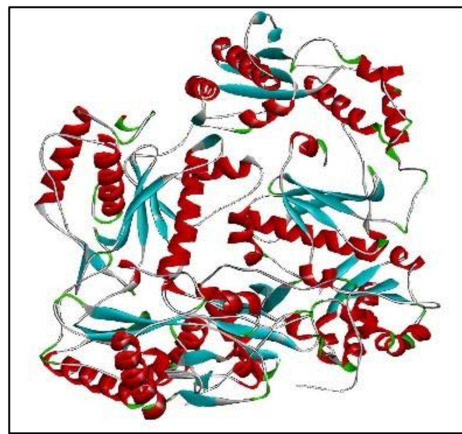
2. METHOD AND MATERIAL

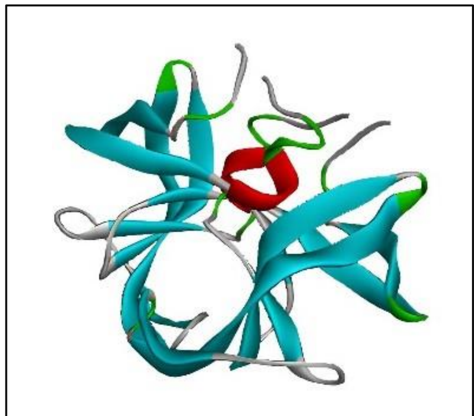
2.1 Retrieval of Phytochemicals and Preparation of Library

To pick the best of Indian plants, ancient Indian literature was examined thoroughly to develop a complete list of herbal remedies that are renowned for having power and containing renewing characteristics, which may reveal antiviral properties. Subsequently, lead compounds from plants, with promising biological activities such as antiviral, antimicrobial, antioxidant, and/or immunomodulatory potential, were searched and selected for the study based on the previous published reports available on leading science research databases like IMPPAT [13], the PubChem [14] and the ChEMBL [15] databases.

2.2 Retrieving Target Proteins

The 3D crystal structure of the receptors was retrieved from the Protein Data Bank website with PDB ID: 1REV for reverse transcriptase and 1HPV for protease enzymes. Chain A of the protein receptors was selected and cleaned from hetero atoms, then prepared for docking using Discovery Studio. Preparation and optimization of structures were done by adding polar hydrogens, deleting water molecules, fixing charges, and minimization. Once the setup procedure had been finished, they were transformed into pdbqt format. Prepared protein files were used for virtual screening of phytocompounds [16]. The schematic representation of prepared protein is given in **figure 2.1.**





Reverse transcriptase Protease

Figure 2.1. HIV Targets

2.3 Preparing Phytocompounds for Docking

Before the phytocompounds could be installed for molecular docking, they were energy minimized using various parameters—forcefield mmff94 optimization. Then phytocompounds were converted from. mol2/. mol/.sdf to. pdbqt and were then subjected to docking in Autodock Vina[20]. For multidrug docking, we used PyRx software tool[21], which works for virtual screening. After uploading the prepared target with, the prepared ligand was also uploaded. The grid size was set to 50 × 50 × 50 points with a grid spacing of 0.375 Å with exhaustiveness equal to 8 [17].

2.4 Interaction Analysis

The resulting complexes were examined for interactions using Biovia Discovery Studio Visualizer software [17].

2.5. Prediction of probable anti-viral activity

The biological spectrum of all flavonoids was analyzed using the PASS tool at the pharmacological activity (Pa) > pharmacological inactivity (Pi) stage, and an extensive dataset was acquired. Then, an entire dataset was searched for the term viral to find antiviral actions against viruses.

2.6. In Vitro Evaluation of Phytochemicals

All selected phytocompounds were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) with a purity range from 98 to 99%.

2.6.1. Instruments Used for In- Vitro Assay

Centrifuge, vortex mixer, biological safety cabinet, BMG fluostar plate

2.6.2. ELISA kit typically includes the following components [Sigma Aldrich]

A 96-well plate that's pre-coated with a capture antibody, A biotin-conjugated detection antibody, Streptavidin-HRP conjugate: A streptavidin-HRP conjugate, A lyophilized assay buffer, protein standard dilutions, A stop solution, Substrate solution: A substrate solution, wash buffer, HIV-RTs enzymes and Protease enzymes.

2.6.3. Evaluation of in vitro cytotoxicity of the Phytocompounds

2.6.3.1. MTT Assay

It is a method to evaluate cell viability by measuring mitochondrial activity. K562 cells are plated in 96-well plates. Test compounds are dissolved in DMSO and serially diluted in culture medium to create a range of concentrations. The DMSO concentration is kept below 0.1% to avoid toxicity, and the cells are incubated with these compounds for 72 hours at 37°C in 5% CO_2. After the incubation period, MTT reagent is added to each well. Viable cells reduce the MTT to form dark blue formazan crystals. The formazan product is dissolved in DMSO. Absorbance is measured at 550 nm using a plate reader to quantify the amount of formazan produced. The percentage of inhibition is calculated by comparing the absorbance of treated wells to that of untreated control wells.

IC_{50} values (the concentration at which 50% of cells are inhibited) are determined by plotting the percentage inhibition against the concentration of the test compound. This test

assesses the cytotoxic effects of substances by assessing their influence on cell viability [18].

2.6.3.2. Procedure for HIV Reverse Transcriptase assay [19,20,21]

2.6.3.2.1. Preparation of reagent

Place both buffers in a 37ºC water bath for 5 minutes to thaw. Prewarm the other kit components, except for the HIV-1 RT enzyme, by placing them at room temperature. The HIV RT should be stored at 20ºC except during dilution into lysis buffer. Just before usage, reaction buffers 1 and 2 are combined in equal parts. For an RT experiment, mix 500 µL of both the reaction buffer 2 to generate 1x assay reaction buffer (RB). The user will mix their test compound in the RB up to 40 µL total for each experimental well. 40 µL of RB or RB plus test compound will be added to their respective incubation tubes. Enough HIV RT is given to generate 100 wells of 1 ng standard for testing HIV RT inhibitory chemicals. The amount of 1 ng HIV RT produced in each experiment is determined by the number of samples available. To achieve a 1 ng concentration, the kit requires 399 µL of lysis buffer and 1 µL of stock HIV RT.

Once the HIV RT (standard and/or 1 ng HIV RT stock) has been produced, add 80 µL of this solution into their respective RB incubation tubes, which should contain 40 µL of RB or RB plus test article. Incubate this mixture for 20 minutes at 37 °C.

After the HIV RT reaction has incubated at 37º C for 20 minutes, remove 100 µL from each reaction incubation tube and add it to a streptavidin-coated micro well. Incubate the ELISA plate at 37º C for 20 minutes. Remove the reaction mixture from the wells and wash each well 5 times with 1x wash buffer. Add 100 µL of HRP Anti Digoxigenin Conjugate and incubate at 37º C for 45 minutes. Remove the conjugate from the wells and wash each well 5 times with 1x wash buffer. Add 100 µL of ABTS substrate solution and incubate at room temperature for 30 minutes. The plate can also be incubated at 37ºC, which will greatly speed up the reaction.

The development of colour can be monitored over time with an ELISA plate reader set to an OD of 405 nM, or the RT reaction can be terminated by adding 25 µL of Stop Solution and reading the OD at 405 nM. The plates were read on a BMG Fluostar plate reader and values were obtained in percentage inhibition.

2.6.4. Procedure for HIV protease inhibitor assay [22]

This assay allows for real-time monitoring of HIV-1 protease function and is an invaluable tool in the screening of protease inhibitors for antiretroviral drug development.

2.6.4.1. Reagent Preparation

1X Assay buffer: Prepare 1X assay buffer according to **Table 2.1.** Prepare HIV-1 protease substrate solution according to **Table 2.2.**

In an assay buffer, dilute HIV protease to the desired concentration. The recommended amount for HIV-1 protease diluent is 40 µL per well. You may change the volume according to your preferences. Dilute the test chemicals with deionized water or a suitable vehicle. The optimum volume for the diluted test substance is 10 L per experiment. You can change the volume based on your preferences.

Pour test chemicals and HIV-1 protease diluent into the microplate. The recommended total volume of HIV-1 protease diluent and test compound is 50 µL. For example, 40 µL of protease diluent and 10 µL of test compound. Incubate the plate at the proper temperature for 10-15 minutes to allow for the enzymatic reaction. Also, incubate the HIV-1 protease substrate solution at the same temperature. Add 50 µL per well of HIV-1 protease substrate solution. Shake the dish gently for 30-60 seconds to fully mix the reagents. Incubate the process at room temperature for 30-60 minutes. Keep the plate out of direct light. Optionally, add 50 µl/well of stop solution (Component E). Mix the reagents. The fluorescence intensity should be measured at Ex/Em=340 nm/490 nm. The plates were read on the BMG Fluostar.

Table 2.1. Preparation of buffer solution

Components	Volume
2X Assay Buffer (Component D)	5 Ml
1 M DTT (1000x, Component G)	10 Ml
Deionised Water	5 mL

Table 2.2. Substrate preparation

Components	Volume
HIV-1 Protease substrate Buffer (Component A)	100 Ml
1X Assay buffer (1000x, Component G)	4.9 mL
Deionised Water	5 mL

2.7. Statistical analysis

The data were expressed as mean ± Sd (standard deviation) off at least three independent experiments. Statistical significance was analyzed by one way ANOVA using excel. P value less than 0.05 was considered significantly.

3. RESULTS AND DISCUSSION

Our findings provide molecular-level insight into host–parasite relationships by demonstrating selective inhibition of HIV-1 targets that operate within the host cellular environment. In order to explore the possible inhibitory effect of these phytocompounds on the binding of the reverse transcriptase with the host cell, their interactions with the external subdomain of protein were examined with respect to the major amino acid residues. The primary metrics employed to evaluate the ligand-protein interactions were the Autodock Vina docking scores. The selected target and their PDB ID'S are shown in **Table 3.1.** Molecular docking analysis revealed that the selected phytochemicals bind favourably within functionally relevant regions of HIV-1 reverse transcriptase and protease. Several compounds demonstrated strong binding affinities, with

docking scores comparable to or exceeding those of the reference inhibitor. Interaction analysis showed that these compounds engage key amino acid residues involved in enzymatic catalysis and substrate recognition, suggesting a direct impact on enzyme function.

Experimental validation using enzyme inhibition assays confirmed the computational predictions. Phytochemicals exhibited measurable inhibitory activity against both HIV-1 reverse transcriptase and protease, with IC_{50} values ranging from low to moderate micromolar concentrations. Importantly, distinct inhibition profiles were observed among the compounds. Some phytochemicals preferentially inhibited reverse transcriptase, while others showed stronger activity against protease. This differential inhibition indicates structurally driven selectivity toward specific viral enzymes.

3.1. Reverse Transcriptase

The key contact resides in reverse transcriptase include ASN265, GLU 378, VAL381. Docking scores and amino acid interactions were obtained using Autodock Vina. Nevirapine with a binding energy of -5.9, served as the positive control, demonstrated H-bonding with ASN265, GLU378., and exhibited Pi-Alkyl hydrophobic interactions with VAL381. Each compound interacts with different sets of amino acids, indicating varied binding affinities and potentially diverse biological effects. Val A:381 and Lys B:22 are repeatedly involved across many compounds, suggesting they may play a significant role in binding. While some compounds engage in both hydrophobic and hydrophilic interactions, others may have more limited or selective interactions. Compounds that show both hydrophobic and hydrophilic interactions might have a more complex and potentially stronger binding, enhancing their efficacy as ligands [23]. The results of the molecular docking revealed that the phytocompounds tested in this study had good docking energies ranging from -10.2 to -7.9 kcal/mol (Table 3.2). The orientation of binding for each compound having the best binding affinity with the target protein was analyzed using BIOVIA Discovery Studio Visualizer 2021. **Table 3.3** presents the detailed study on the binding affinities and 2D interactions. Biological interaction analysis of phytoconstituents was carried out with respect to main contact residues. All the tested compounds had efficient binding interactions with reverse transcriptase, and it was found that every docked ligand interacted well with the identical amino acid residues. **Figure 3.1** displays the interactions between the candidate and the protein residues. Redocking was used for validation of docking result of nevirapine. The redocking protocols are valid if the RMSD value is less than 2 Å. [23] The root mean square deviation (RMSD) between the predicted conformation and the observed X-ray crystallographic conformation of nevirapine was 0.80 Å. (**Figure 3.3**]

3.2. Protease

The protease enzyme is a **cysteine protease** that at its active site includes His41, and Cys145, the catalytic dyad residues. The interaction appears to be largely mediated by Glu166, Gln189, Thr190, Phe140, and His16430. Hence, for the molecular docking computations and binding interactions, these residues were mainly targeted as the centre of attention. With a binding energy of -7.7, Nevirapine showed H-bonding with ASP29, ASP30, GLY27, ASP25 residues and hydrophobic interactions with ILE47, ILE50, ALA28, VAL82 residues for Pi-Alkyl. In the protease receptor site, the flavonoid group formed an expanded Hydrogen-bonds network with residues GLY27 and VAL82 and exhibited hydrophobic interactions with the residues ILE84, ILE47, ALA28, PRO81. The compounds indicates that those with both hydrophobic and hydrophilic interactions may exhibit more stable binding to the protease, potentially enhancing their bioactivity as inhibitors. [24]. The molecular docking results showed that the phytocompounds tested in this study had good docking energies ranged from -9.9 to -7.9 kcal/mol (Table 3.2). All the tested compounds had efficient binding interactions with reverse transcriptase and it was found that every docked ligand interacted well with the identical amino acid residues **(Figure 3.2).**

Table 3.4. has listed various phytocompounds and their interactions with a protease receptor, specifically highlighting hydrophobic and hydrophilic interactions with amino acids.

3.3. Prediction of the biological spectrum

Prediction of the biological spectrum of bio-actives identified different anti-viral activities for the keyword “viral” as anti-Rhinovirus, influenza, viral entry inhibitor in which the combined action was integrated against ‘antiviral’. The antiviral activity of the combined action of selected flavonoids is presented in **Table 3.5**.

3.4. In-vitro studies

The biological source and the traditional uses of selected phytocompounds are given in **Table. 3.6**

3.4.1. MTT assay

All the selected phytocompounds decreased the cell viability in a dose-dependent manner. IC50 values (the concentration at which 50% of cells are inhibited) are determined by plotting the percentage inhibition against the concentration of the test compound. This test assesses the cytotoxic effects of substances by assessing their influence on cell viability. The cytotoxic concentrations (IC50) of phytocompounds are presented in **Table 3.7.**

3.4.2 Evaluation of Reverse transcriptase and Protease inhibitory Activity

In nontoxic concentrations, Withaferin, Isoliquiritigenin, Diosmetin reduced HIV-1 replication (Inhibition of RT), and others were ineffective. We observed that Chrysin and Licoflavone applied at a relatively high, concentration, significantly inhibited the HIV-1 reverse transcriptase. In contrast, the activity of Protease was inhibited at a lower concentration by Ellagic acid. These results suggest that Diosmetin and Licoflavone may interfere with the replication cycle of HIV at multiple stages. The IC_{50} value of

phytocompounds for reverse transcriptase, Protease are presented in **Table 3.8**. Concentration versus percentage inhibition graph for Reverse Transcriptase is given in **figure 3.4 and 3.5** for protease enzyme. Overall, the flavonoids and terpenoids showed good inhibition against HIV-1. All the phytocompounds showed the significant inhibition against both the enzymes, HIV-RTs and Protease (One-way ANOVA, $P<0.05$) [25].

Modify the epoxy and lactone groups in Withaferin A to enhance hydrogen bonding and hydrophobic interactions at the RT binding pocket. The integration of molecular docking with in vitro biological evaluation provides mechanistic insight into host–parasite relationships at the molecular level. Docking analyses predicted stable interactions between the repurposed compounds and HIV-1 target proteins that mediate viral survival within host cells. These predictions were supported by in vitro inhibition of viral activity with minimal host cytotoxicity, indicating selective disruption of parasite functions within the host cellular environment. [34, 35]

The consistency between docking-predicted interactions and observed enzyme inhibition supports a mechanistic link between ligand binding and functional disruption of HIV-1 enzymes. Compounds forming stable hydrogen bonds and hydrophobic interactions with catalytic or nearby residues exhibited stronger inhibitory activity in vitro. These findings validate the utility of structure-based docking as a predictive tool for identifying functionally relevant enzyme inhibitors.

Unlike studies limited to virtual screening, the present work integrates computational and biochemical approaches to demonstrate causal relationships between molecular interaction patterns and enzyme inhibition. This strengthens the biological relevance of the findings and supports the identification of phytochemicals as genuine inhibitors of HIV-1 replication machinery.

Cytotoxicity assessment revealed that most phytochemicals exerted enzyme inhibition at concentrations that did not significantly compromise host cell viability. This selective inhibition suggests preferential targeting of viral enzymes rather than nonspecific cellular toxicity. Such pathogen-specific selectivity is a critical consideration in antiviral research, as excessive host toxicity limits the translational potential of enzyme inhibitors.

This study is limited by the absence of in vivo validation and pharmacokinetic evaluation. Additionally, molecular docking provides a static representation of ligand–protein interactions. Future studies involving molecular dynamics simulations and animal models are warranted.

Collectively, the results advance the understanding of HIV-1 enzyme inhibition at the molecular level and contribute to pathogen-focused antiviral research by linking ligand–

Table 3.1. Selected targets for in-silico studies

Sr. No.	Selected targets	Category	PDB IDs
1	Reverse Transcriptase	HIV-1	1REV
2	Protease	HIV-1	1HPV

Table 3.2. Docking score of Phytocompounds

Sr. No.	Name of Phytocompound	Binding Energy (kcal/mol) (Reverse Transcriptase)	Binding Energy (kcal/mol) (Protease)
1	Hesperidin	-8.3	-8.1
2	Isoliquiritigenin	-9.3	-8.7
3	Ellagic acid	-8.2	-9.4
4	Diosgenin	-8.7	-9.3
5	Diosmetin	-9.3	-8.6
6	Chrysin	-8.4	-8.1
7	Licoflavone B	-8.1	-9.3
8	Capsorubin	-8.6	-8.9
9	Withaferin	-10.9	-8.3
10	Alpha- Amyrin	-8.5	-9.0

Table 3.3. Interaction of Ligands with amino acids [Reverse Transcription]

Sr. No.	Name of Phytocompound	Hydrophobic Interactions	Hydrophilic Interactions
1	Hesperidin	Pro B:25, LYS B:22	ASN B: 136, VAL A:381
2	Isoliquiritigenin	Pro B:25, VAL A:381	ASN B: 136, LYS B:22
3	Ellagic acid	Pro B:25, VAL A:381	ASN B: 136, LYS B:22
4	Diosgenin	TRP A:88, PRO B:52	THR A:165, PRO B:140
5	Diosmetin	TRP A:88, Lys B:22	VAL A:381

6	Chrysin	VAL A:381	GNL A:91, VAL A:381, TRP A:88
7	Licoflavone B	VAL A: 381,	LYS B:22, Leu B:26
8	Capsorubin	Lys B:22	LYS A:374, Val A:381
9	Withaferin	Pro B:25, LYS B:22	TRP B:414
10	Alpha- Amyrin	Pro B:25	ASN B: 136, VAL A:381

Table 3.4. Interaction of Ligands with amino acids [Protease]

Sr. No.	Name of Phytocompound	Hydrophobic Interactions	Hydrophilic Interactions
1	Hesperidin	ILE A:47, ILE A:50	GLY A:47, GLY A:48
2	Isoliquiritigenin	ILE A:50, ILE A:84, ASP A:25	GLY B:49
3	Ellagic acid	ILE A:84, ASP A:25	AAP A:25
4	Diosgenin	ILE A:50, ALA A:28, VAL A:32	GLY A:49
5	Diosmetin	ILE A:47, ALA A:28, VAL A:32	GLY A:49, ASP A:30
6	Chrysin	ILE B:50, ALA B:28, VAL A:82, ASP A:25	GLY A:49, ASP A:30
7	Licoflavone B	PRO A:81	ASP A:29
8	Capsorubin	ILE B:50, ALA B:28	GLY A:49, ASP A:30, ASP A:25
9	Withaferin	ILE A:84	GLY A:49
10	Alpha- Amyrin	ILE A:84	GLY A:49

Table 3.5. PASS Value of Phytocompounds

Sr. No.	Phytocompounds	Pa	Pi
1	Hesperidin	0,550	0,006
2	Isoliquiritigenin	0,715	0,005
3	Ellagic acid	0,486	0,003
4	Diosgenin	0,460	0,030
5	Diosmetin	0,720	0,005
6	Chrysin	0,326	0,076
7	Licoflavone B	0,426	0,025
8	Capsorubin	0,500	0,024
9	Withaferin A	0,631	0,037
10	Alpha- Amyrin	0,460	0,016

Note *: Pa- Active, Pi- Inactive, Pa should > Pi

Table 3.6. Physicochemical properties of phytocompounds

Sr. No.	Phytocompounds	Log P	Bioavailability Score
1	Hesperidin	4.01	0.17
2	Isoliquiritigenin	2.70	0.55
3	Ellagic acid	1.1	0.55
4	Diosgenin	5.7	0.54
5	Diosmetin	2.59	0.55
6	Chrysin	2.87	0.55
7	Licoflavone B	5.91	0.58
8	Capsorubin	9.07	0.17
9	Withaferin A	3.35	0.55
10	Alpha- Amyrin	9.01	0.55

Table 3.6. Selected Phytocompounds for In Vitro activity.

Sr. No	Name of Phytocompounds	Biological Source	Traditional Uses	References
1	Withaferin A	Withania Somnifera	Anti-inflammatory, Anticancer, Antiviral	25
2	Alpha-Amyrin	Camellia Sinensis	Anti-inflammatory, Anticancer	26
3	Capsorubin	Capsicum Annuum	Antioxidant	27
4	Licoflavone B	Glycyrrhiza Glabra,	Anticancer	28
5	Diosmetin	Lepisorus Ussuriensis,	Anticancer, Antimicrobial, Antioxidant	29

6	Chrysin	Oroxylum Indicum	Antineoplastic, Antioxidant, Hepatoprotective	30
7	Isoliquiritigenin	Glycyrrhiza Pallidiflora,	Antineoplastic Agent and Geroprotector	31
8	Hesperidin	Citrus Aurantium	Antihypertensive, Anticancer, Antifertility, Antioxidant, Analgesic,	32
9	Diosgenin	Trigonella Foenum Graecum.	Antidiabetic, Anticancer	33
10	Ellagic Acid	Punica Granatum L.	Antioxidant, Anti-Inflammatory, Antimutagenic	34

Table 3.7. IC_{50} Value for cytotoxicity determination of Phytocompounds

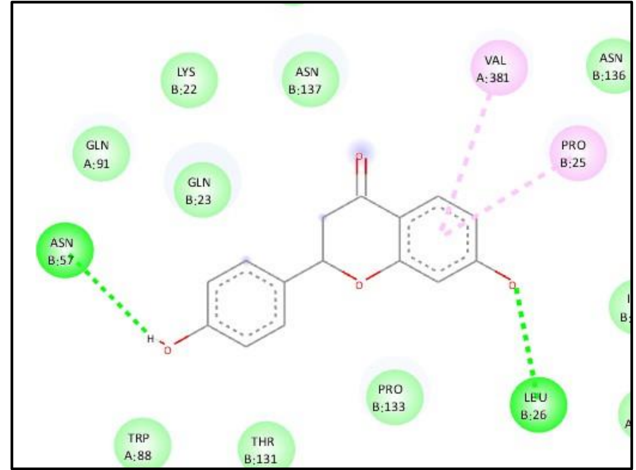
Sr. No.	Name of Phytocompound	IC_{50} µM
1	Hesperidin	**>10**
2	Isoliquiritigenin	**>10**
3	Ellagic acid	**5.0**
4	Diosgenin	**>10**
5	Diosmetin	**>10**
6	Chrysin	**>10**
7	Withaferin	**5.50**
8	Alpha- Amyrin	**>10**
9	Licoflavone B	**>10**
10	Capsorubin	**>10**

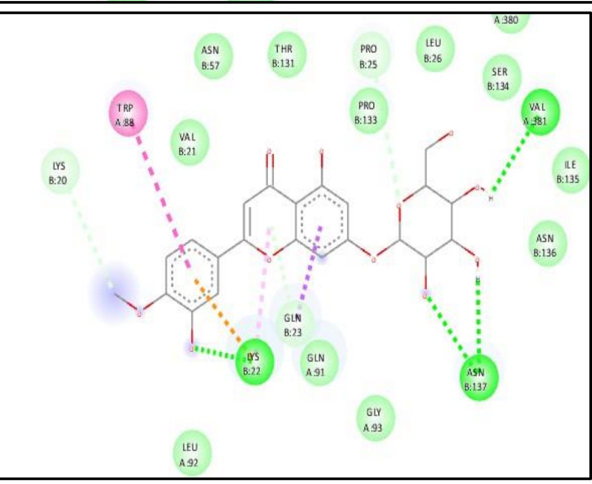
***Note: IC_{50}: >10 – Weak or Inactive**

Table 3.8. IC_{50} value of Phytocompounds for Reverse transcriptase and Protease enzyme.

Sr. No.	Name of Phytocompound	IC50 [RTs] [µM]	IC50 [Protease] [µM]
1	Hesperidin	28.13± 0.21	18.70 ± 0.094
2	Isoliquiritigenin	5.42± 0.043	27± 0.092
3	Ellagic acid	18.39± 0.16	4.20± 0.25
4	Diosgenin	22.50± 0.24	8.50± 0.20
5	Diosmetin	7.52± 0.23	18.5± 0.04
6	Chrysin	18.07± 0.20	11.5± 0.14
7	Withaferin	2.65± 0.21	22.6± 0.20
8	Alpha- Amyrin	20.01± 0.08	10± 0.21
9	Licoflavone B	30.01± 0.44	10± 0.091
10	Capsorubin	20.12± 0.21	10.5± 0.085

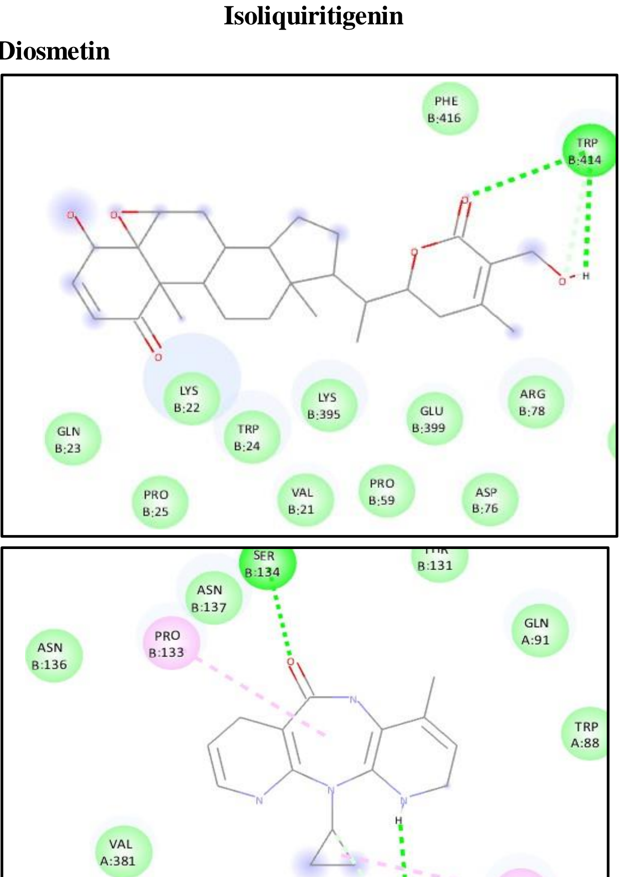
***Note: IC_{50}: 1-10- Strongly active, 10-15- Moderately Active, >15- Weakly active, Data were presented as mean ±Sd values, n=3, (One- way Anova, P < 0.05). All the phytocompounds significantly inhibited both HIV-RTs and Protease enzyme.**





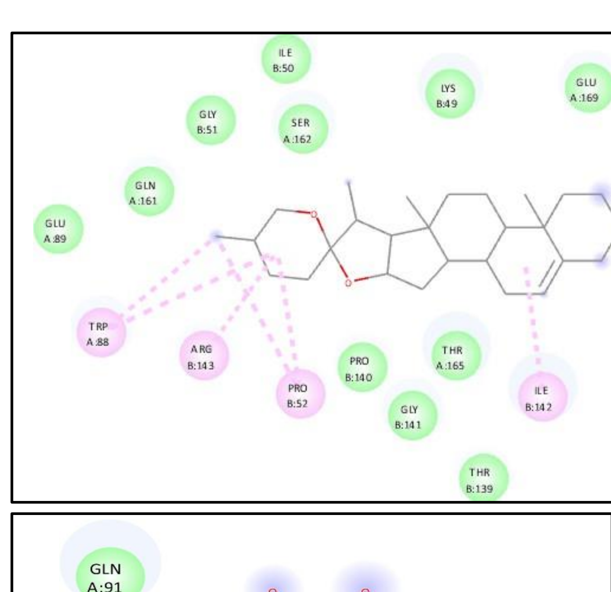
Isoliquiritigenin

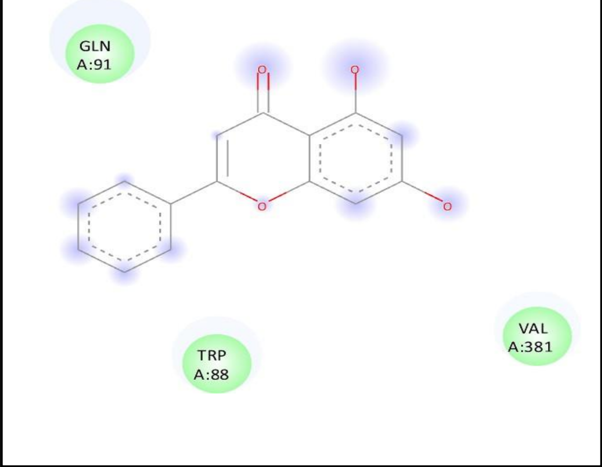
Diosmetin



Withaferin

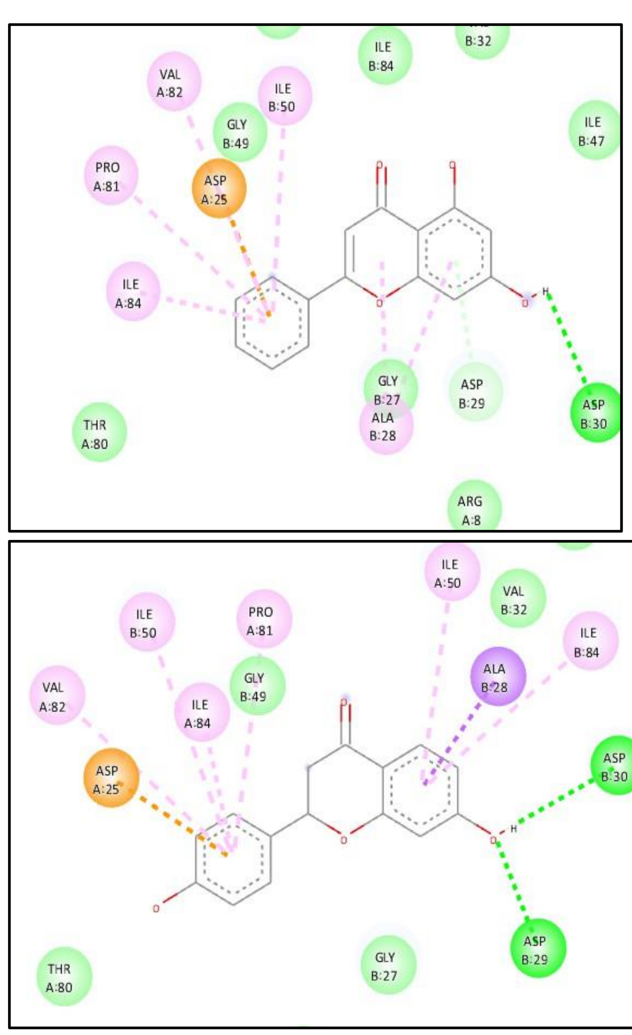
Nevirapine





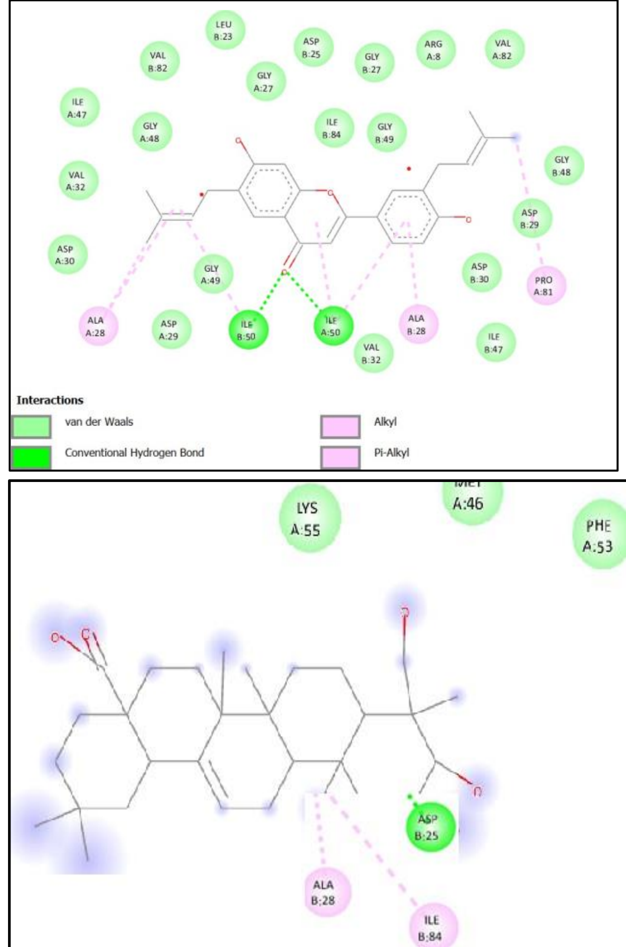
Diosgenin Chrysin

Figure 3.1. Interactions between protein amino acids with the Phytocompounds (Reverse Transcriptase).



Chrysin

Isoliquiritigenin



Licoflavone

Ellagic acid

Figure 3.2. Interactions between protein amino acids with the Phytocompounds (Protease)

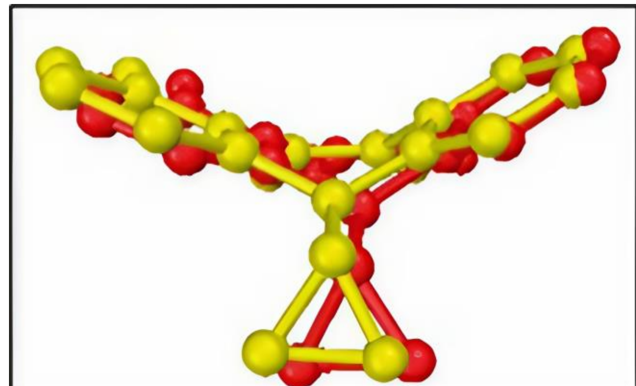
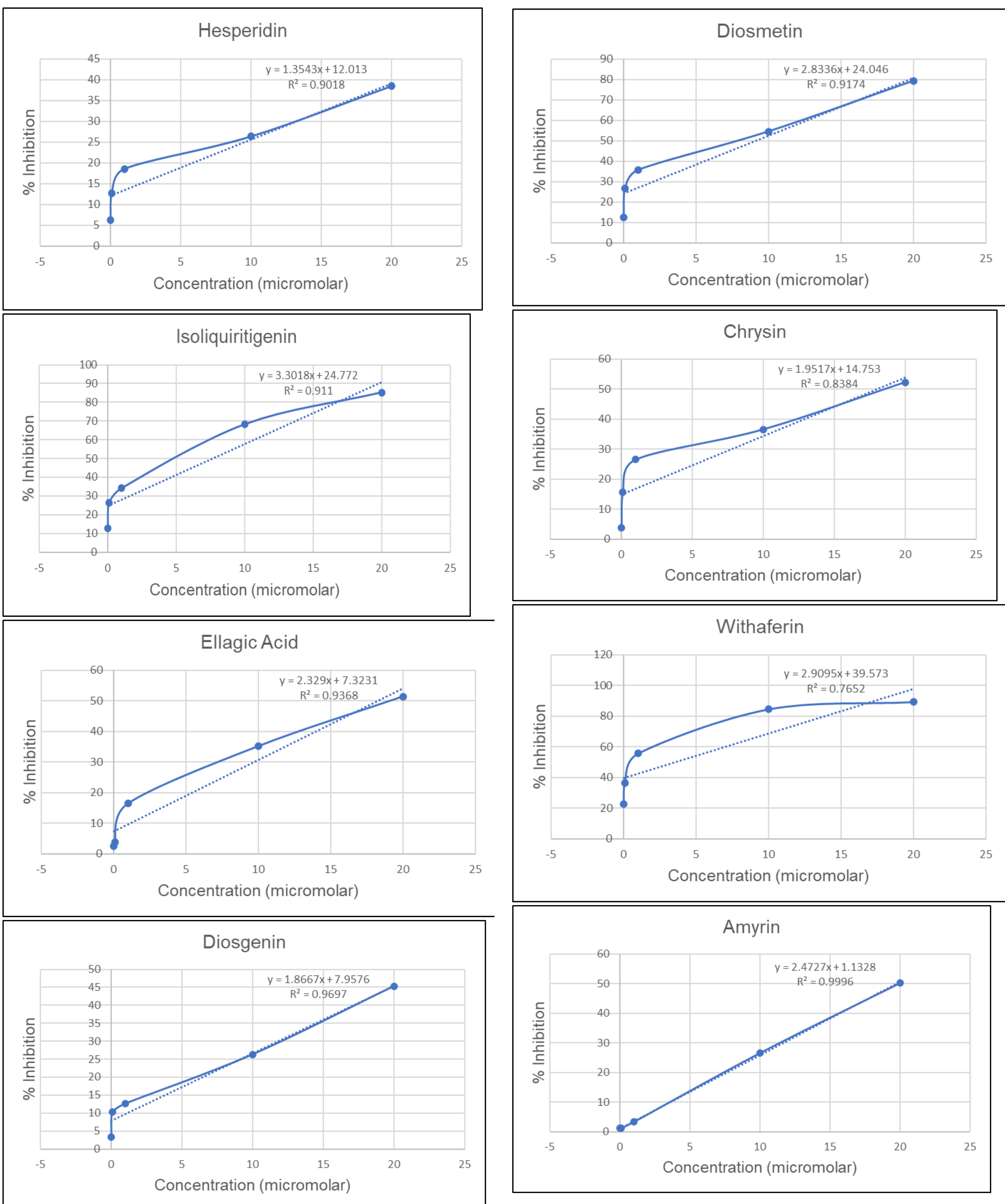


Figure 3.3. Comparison between the binding position of nevirapine found within the crystal structure (yellow) and the conformation predicted by AutoDock (red).


Hesperidin
y = 1.3543x + 12.013
R² = 0.9018
% Inhibition
Concentration (micromolar)
Diosmetin
y = 2.8336x + 24.046
R² = 0.9174
% Inhibition
Concentration (micromolar)
Isoliquiritigenin
y = 3.3018x + 24.772
R² = 0.911
% Inhibition
Concentration (micromolar)
Chrysin
y = 1.9517x + 14.753
R² = 0.8384
% Inhibition
Concentration (micromolar)
Ellagic Acid
y = 2.329x + 7.3231
R² = 0.9368
% Inhibition
Concentration (micromolar)
Withaferin
y = 2.9095x + 39.573
R² = 0.7652
% Inhibition
Concentration (micromolar)
Diosgenin
y = 1.8667x + 7.9576
R² = 0.9697
% Inhibition
Concentration (micromolar)
Amyrin
y = 2.4727x + 1.1328
R² = 0.9996
% Inhibition
Concentration (micromolar)

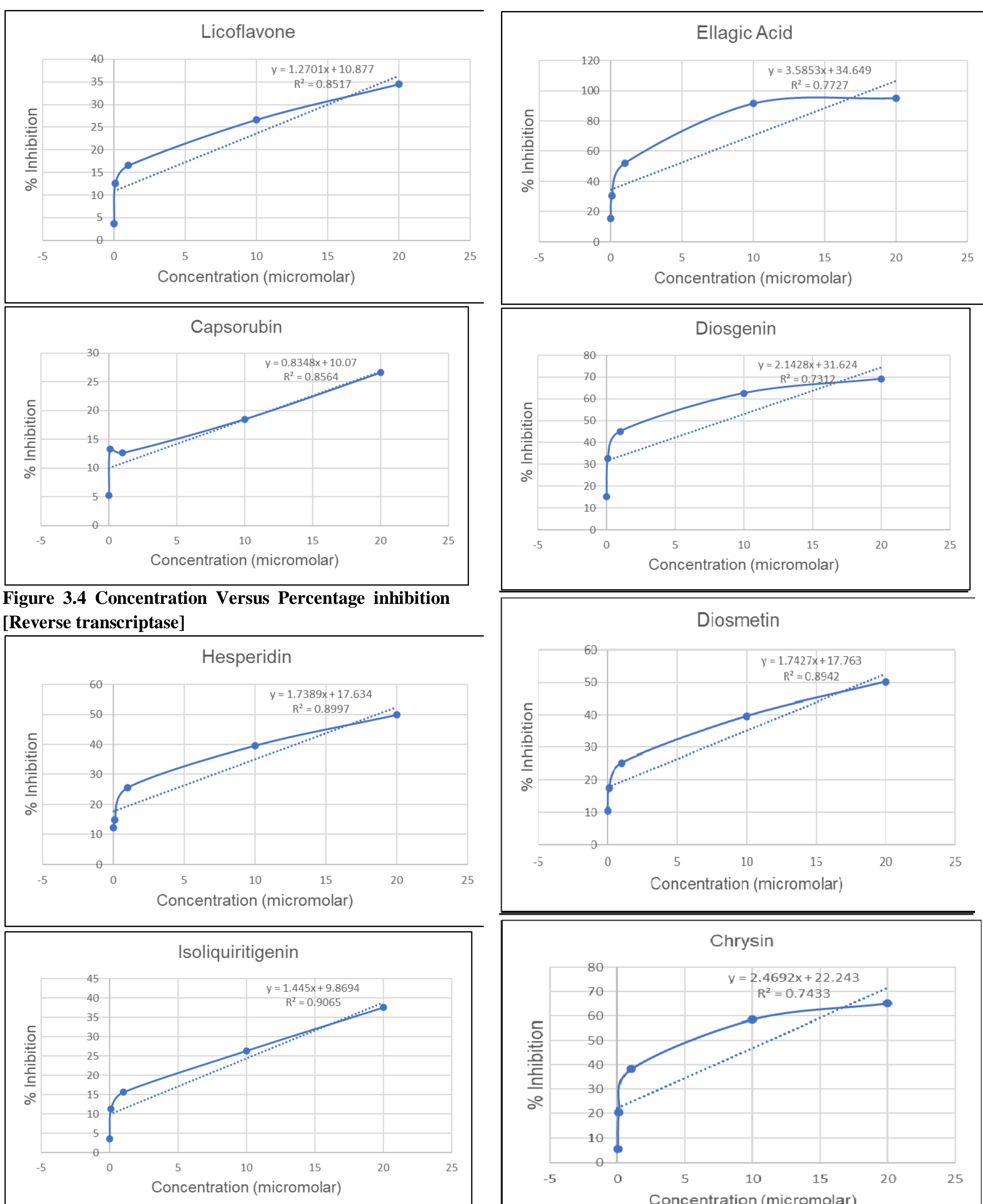
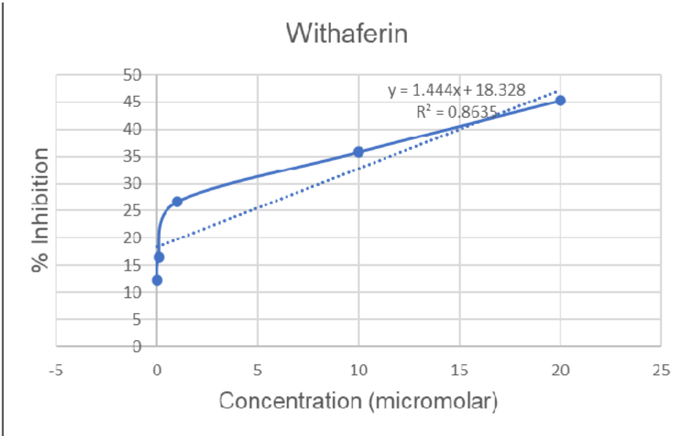
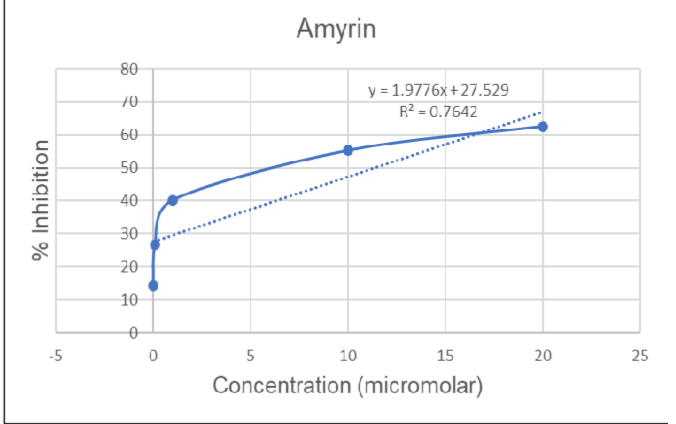
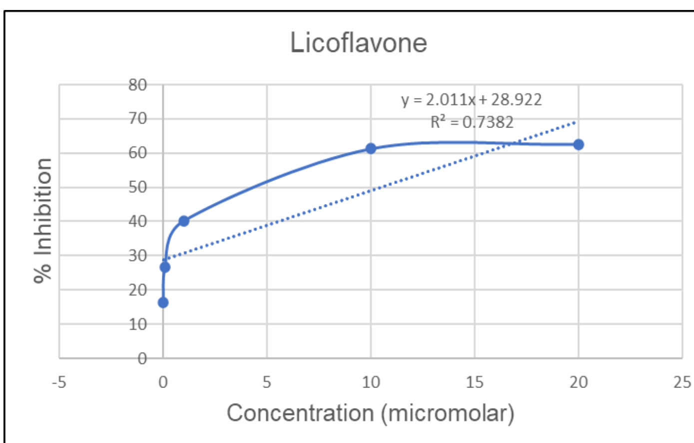


Figure 3.4 Concentration Versus Percentage inhibition [Reverse transcriptase]







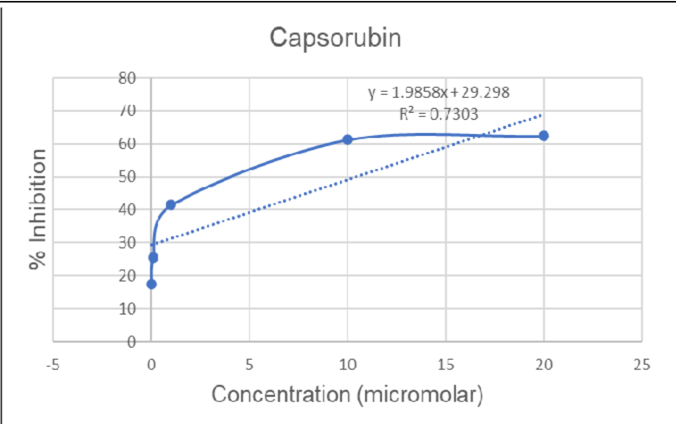


Figure 3.5. Concentration versus percentage inhibition (Protease)

CONCLUSION

The current study aimed to identify and evaluate phytochemicals with anti-HIV potential by combining computational, in vitro, and bioinformatic approaches. Key findings were drawn from molecular docking studies targeting HIV-1 protease and reverse transcriptase, as well as cytotoxicity and anti-HIV activity assays. This systematic approach highlighted the potential of several plant-derived compounds as novel candidates for HIV therapy. To achieve this goal, several key HIV target proteins were selected based on comprehensive bioinformatics analysis and literature review. Phytochemicals from various medicinal plants were then virtually screened using advanced docking techniques to assess their binding affinities with the selected HIV proteins. This process led to the identification of several promising phytochemical candidates. From the prioritized compounds, a subset was previously reported to exhibit anti-HIV activity, validating the efficacy of the employed methodology. Among the shortlisted phytochemicals, **Withaferin, ellagic acid, Diosgenin, Isoliquiritigenin, Diosmetin, Licoflavone, Capsorubin,** demonstrated inhibitory potential against HIV virus in in- vitro assays. Diosmetin, through its multi-target antiviral and host-modulatory actions, presents a strong potential to: Suppress HIV replication, Delay or overcome drug resistance, enhance outcomes when used in combination with standard Anti-retroviral therapy.

In-depth analysis of the physicochemical properties of the prioritized phytochemicals revealed that they possess favourable profiles in terms of drug-likeness and bioavailability, adhering to the 'Rule of Five' and related pharmacokinetic guidelines. These findings suggest that integrating phytochemicals with such properties into anti-HIV drug discovery pipelines may accelerate the identification of effective candidates. Phytochemicals such as Isoliquiritigenin, and Withaferin demonstrated significant interactions with active sites of HIV-1 Reverse Transcriptase, involving key hydrophobic and hydrophilic interactions. These interactions indicate their strong binding affinities and the potential to inhibit HIV-1 RTs activity effectively.

This study demonstrates the significant potential of phytochemicals in anti-HIV therapy, offering a pathway to novel, plant-derived treatments that can complement or enhance existing options.

Impact and Therapeutic implications of this study:

Bioinformatics and molecular docking approaches are effective for identifying potential anti-HIV phytochemicals by targeting key viral proteins. Several prioritized phytochemicals exhibit promising anti-HIV activity. This dual-target interference with HIV-1 replication machinery underscores the potential of natural compounds as valuable lead scaffolds for multi-target antiviral drug development.

Overall, the study advances the understanding of HIV-1 enzyme inhibition at the molecular level and demonstrates the utility of

combining structure-based prediction with biochemical validation to identify functionally relevant antiviral candidates. These findings provide a rational foundation for future structure-guided optimization and mechanistic studies aimed at developing novel inhibitors targeting HIV-1 pathogenesis.

Future studies should focus on in vivo evaluations of the prioritized phytochemicals to assess their pharmacodynamics, pharmacokinetics, and safety profiles, paving the way for translational applications. This study demonstrates the significant potential of phytochemicals in anti-HIV therapy, offering a pathway to novel, plant-derived treatments that can complement or enhance existing options. The findings encourage integrating computational and experimental approaches in natural product-based drug discovery for HIV and other infectious diseases. Future research should focus on as per below: Structural modifications to enhance the selectivity and reduce the cytotoxicity of potent anti-HIV compounds, such as Ellagic Acid, could improve their therapeutic profiles. Understanding the precise mechanisms through which these phytocompounds inhibit HIV-1 reverse transcriptase and protease could offer insights into designing more targeted derivatives. Exploring these compounds in combination with existing antiretroviral drugs might yield synergistic effects, enhance overall efficacy while potentially minimize adverse effects. Pharmacokinetics and Bioavailability Future studies should also assess the bioavailability, metabolic stability, and pharmacokinetic properties of promising phytocompounds to ensure effective in vivo performance. Overall, while several phytocompounds demonstrate potential anti-HIV-1 activity, future investigations should aim at optimizing their efficacy, safety, and delivery mechanisms to advance the development of novel plant-based antiretroviral therapies.

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