Inhibitory potential of α-Amyrin from Calotropis procera against HIV-1 reverse transcriptase: insights from in silico and in vitro assays

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Abstract

The global HIV epidemic continues to present significant public health challenges, with millions affected worldwide, necessitating the exploration of novel antiretroviral agents. This study aims to evaluate the inhibitory potential of α-amyrin, a phytochemical from *Calotropis procera*, against HIV-1 reverse transcriptase (RT) through molecular docking and *in vitro* assays. The study involved *in silico* molecular docking to assess the binding affinity of α-amyrin with HIV-1 RT (PDB ID: 1JLB), followed by pharmacokinetic analysis to predict its drug-likeness and ADMET properties. An *in vitro* HIV-1 RT inhibition assay was conducted using a colorimetric ELISA kit, with nevirapine as the reference drug. Molecular docking revealed a strong binding affinity of α-amyrin to HIV-1 RT, with a binding energy of -7.33 kcal/mol. The compound formed two hydrogen bonds with key residues (Cys181 and Gln182) in the allosteric binding site. *In vitro*assays demonstrated dose-dependent RT inhibition, with an IC_{50} of 26 µg/mL, comparable to nevirapine (IC_{50} = 21 µg/mL). At the highest concentration (200 µg/mL), α-amyrin achieved 82% inhibition, while nevirapine displayed 92% inhibition. Lower concentrations (12.5–100 µg/mL) showed gradual inhibitory effects, indicating α-amyrin's potential efficacy even at moderate doses. Pharmacokinetic predictions confirmed its favorable absorption, metabolism, and low toxicity profile. The dual approach combining *in silico* and *in vitro* analyses highlights α-amyrin as a promising candidate for HIV-1 RT inhibition. Its strong binding affinity, significant enzymatic inhibition, and favorable pharmacokinetics suggest its potential for further development as a plant-based NNRTI, addressing drug resistance concerns and contributing to improved antiretroviral therapies.

Introduction

As of 2023, the global HIV epidemic continues to pose significant public health challenges. Approximately 39.9 million people are living with HIV worldwide, with an estimated 1.3 million new infections occurring in 2023 alone (WHO 2024). Despite advancements in treatment, HIV-related mortality remains a concern, with 630,000 deaths reported globally in 2023 (UNAIDS 2024). The WHO African Region remains the most affected, accounting for more than two-thirds of the global HIV burden (Carter et al. 2021). Since HIV-1 was identified as the cause of acquired immune deficiency syndrome (AIDS), significant medical advancements have led to the development of various drugs for its clinical treatment. These therapies have improved patient outcomes by suppressing viral replication, reducing mortality rates, and enhancing the quality of life for individuals living with the infection (Alison et al. 2016). The replication cycle of HIV involves several key enzymes, including reverse transcriptase (RT), integrase, and protease (Kimberly et al. 2005). Among these, HIV-1 RT plays a pivotal role by converting single-stranded viral RNA into double-stranded DNA, facilitating its integration into the host genome—a critical step for viral replication. This multifunctional enzyme exhibits both RNA- and DNA-dependent polymerase activities, as well as ribonuclease H activity, underscoring its significance as a therapeutic target (Opar 2007). Current antiretroviral therapies (ART) primarily comprise nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors, integrase strand transfer inhibitors, and entry inhibitors (Gandhi et al. 2024; Menendez and Delgado 2022). While these treatments have significantly improved patient outcomes, they are often associated with adverse effects such as gastrointestinal disturbances, cardiovascular complications, and metabolic disorders (Gandhi et al. 2025). Additionally, the emergence of drug-resistant HIV strains necessitates the exploration of novel therapeutic agents. Phytochemicals, with their diverse bioactive properties, present a promising avenue for developing new HIV treatments, potentially offering efficacy with reduced toxicity.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a class of noncompetitive inhibitors that specifically target a hydrophobic allosteric site on HIV-1 reverse transcriptase (RT), located approximately 10–15 Å from the DNA polymerase active site (Asp110, Asp185, Asp186) and 60 Å from the RNase H active site (Glu478, Asp443, Asp498), resulting in distortion of the catalytic aspartate triad of the RT DNA polymerase active site (Yilmaz and Schiffer 2018). Potent NNRTIs, such as Efavirenz, a second-generation inhibitor, are commonly used in clinically approved anti-AIDS treatments. The allosteric binding site of HIV-1 RT consists of residues Leu100, Lys101, Lys103, Val106, Thr107, Val108, Val179, Tyr181, Tyr188, Val189, Gly190, Phe227, Trp229, Leu234, and Tyr318, located on the p66 subunit of the RT p66/p51 heterodimer (Sharaf et al. 2017). However, first-generation NNRTIs like Nevirapine, Delavirdine, and Efavirenz exhibit a significant loss of activity due to single-point mutations, including Tyr181Cys, Pro236Leu, and Lys103Asn at the allosteric site, contributing to drug resistance (Zhou et al. 2025; Gibas et al. 2022). Therefore, the development of novel NNRTIs with greater potency, broader resistance mutation profiles, and reduced toxicity is crucial for enhancing the efficacy of combination therapy. In this context, the present research explores the binding potential of phytochemicals from *Calotropis procera* against the Tyr181Cys mutant HIV-1 RT (PDB ID: 1JLB), aiming to identify promising candidates with improved pharmacological properties and reduced resistance, which could contribute to more effective HIV treatment strategies.

Calotropis procera, commonly referred to as the "Sodom apple," is a plant known for its rich variety of phytochemicals, including flavonoids, phenolics, steroids, and cardenolides, which contribute to its extensive biological activities (Ahmad et al. 2023). The aqueous leaf extract of *C. procera* has demonstrated significant antimicrobial and antidiabetic potential, indicating its relevance in managing infections and diabetes (Habeeb et al. 2024). Furthermore, recent studies have highlighted its high antioxidant, anti-inflammatory, and antimicrobial properties, supporting its therapeutic applications (Dogara 2023). For instance, the aqueous leaf extract of *C. procera* has demonstrated significant antimicrobial activity and antidiabetic potential, highlighting its therapeutic relevance (Singh et al. 2024). *In silico* approaches, including molecular docking and pharmacokinetic analyses, have become invaluable in evaluating the drug-likeness and ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiles of phytochemicals. Recent studies have employed these methods to assess *C. procera*-derived compounds, revealing several with favorable drug-like properties and potential inhibitory effects against viral targets (Devang et al. 2025). Molecular docking studies have further identified specific phytochemicals from *C. procera* that exhibit strong binding affinities to viral enzymes, suggesting their potential as antiviral agents (Swapna et al. 2024; Dubey et al. 2025). These computational analyses are crucial for predicting the efficacy and safety of phytochemicals before proceeding to experimental validations. Building upon these insights, this research aims to investigate the potential of *C. procera*-derived phytochemicals as inhibitors of HIV-1 reverse transcriptase (RT), particularly targeting the Y181C mutant associated with drug resistance. By employing molecular docking and pharmacokinetic analyses, the study seeks to identify compounds with optimal binding affinities and favorable ADMET profiles. The objective is to discover novel, plant-based inhibitors that can overcome existing drug resistance mechanisms, thereby contributing to the development of more effective and safer antiretroviral therapies.

Methodology

Phytochemicals of *C. procera*

The selected phytochemicals of *C. procera*, including 2-oxovorucharin, α-amyrin acetate, β-anhydroepidigitoxigenin, benzoyllineolone, calotropin, frugoside, and α-amyrin, were retrieved in SDF format from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (Table 1). These compounds were chosen based on their reported antiviral, anti-inflammatory, and immunomodulatory properties, making them promising candidates for HIV-1 reverse transcriptase inhibition. The SDF files were then converted into PDB format using Open Babel software to prepare them for subsequent *in silico* studies, such as molecular docking and pharmacokinetic analysis (Kumar and Mamidala 2025).

PASS prediction

PASS (Prediction of Activity Spectra for Substances) version 4.20 was used to predict the biological activity spectrum of the selected *C. procera* phytochemicals, assessing their potential antiviral effects, mechanisms of action, and specific toxicity. The prediction was performed by comparing the compounds against the source data available at PASS online (http://195.178.207.233/PASS/predict.php). The antiviral activity spectrum was analyzed for seven compounds from IMPPAT database, highlighting their activity despite variations in experimental conditions. The PASS tool calculates the Pa:Pi (probability of being active vs. inactive) ratio at different thresholds: Pa > 0.7 indicates a high likelihood of activity in experiments, but also suggests the compound may resemble known pharmaceuticals (Filimonov et al. 2014). For 0.5 < Pa < 0.7, the compound is likely to exhibit activity, but with a lower probability and less resemblance to known drugs. When Pa < 0.5, the compound is unlikely to be active; however, if activity is confirmed experimentally, it may indicate the discovery of a novel chemical entity. This *in silico* prediction aids in identifying promising antiviral candidates for further molecular docking and pharmacokinetic evaluations.

Drug- and Lead-Likeness Features

The drug- and lead-likeness properties of the selected *C. procera* phytochemicals (Table 1) were evaluated using established medicinal chemistry rules, including Lipinski's Rule of Five, Ghose filter, Veber's Rule, and Egan's Rule. This assessment was conducted through the SwissADME web service, which offers comprehensive insights into the pharmacokinetics, drug-likeness, and medicinal chemistry compatibility of small molecules (Afridi et al. 2024). These rules assess key physicochemical properties such as molecular weight, lipophilicity, hydrogen bond donors/acceptors, topological polar surface area (TPSA), and rotatable bonds. Compounds that satisfy these criteria are considered more likely to exhibit favorable oral bioavailability and therapeutic potential. This evaluation is crucial for identifying promising drug candidates with optimal pharmacokinetic profiles for further *in silico* and experimental studies.

ADMET studies

The SMILES representations of phytochemical compounds were analyzed using the pkCSM platform to predict their ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties (Pires et al. 2015). Key parameters assessed included human intestinal absorption percentage, blood-brain barrier (BBB) permeability, Ames mutagenicity, inhibition potential on hERG channels (I and II), and hepatotoxicity. Additionally, the compounds' interactions with cytochrome P450 enzymes-specifically CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4-were evaluated to determine their roles as substrates or inhibitors. This comprehensive *in silico* analysis aids in understanding the pharmacokinetic and safety profiles of the compounds, facilitating the identification of viable drug candidates for further development.

Molecular docking studies

The 3D crystal structure of HIV-1 reverse transcriptase (RT) in complex with nevirapine (PDB ID: 1JLB; Accession number: P03366) was obtained from the Protein Data Bank (PDB) (https://www.rcsb.org) in PDB format and visualized using Biovia Discovery Studio 2021 (https://discover.3ds.com) (Fig.1). Before molecular docking, the HIV-1 RT enzyme was prepared using AutoDock 4.2 (https://autodock.scripps.edu). Chain B of the enzyme, all associated water molecules, and the bound ligand nevirapine (NVP) were removed, leaving only Chain A for further docking studies. To validate the docking protocol, Chain A was redocked with nevirapine, ensuring the reliability of the docking conditions by comparing the re-docked conformation with the original crystal structure.

For docking preparation, Kollman and Gasteiger charges were assigned to the enzyme, and polar hydrogen atoms were added to enhance docking accuracy. The prepared protein structure was saved in PDBQT format. A blind docking approach was employed, covering the entire enzyme to identify potential binding sites. The grid box parameters, which defined the docking area, were saved in GPF format. This *in silico* molecular docking analysis allowed for the evaluation of the binding affinity and interaction patterns of the selected *C. procera* phytochemicals with key active and allosteric sites of HIV-1 RT, as illustrated in Fig. 1.

Molecular docking studies were carried out to assess the interactions between selected phytochemicals and the reference ligand, Nevirapine, with the HIV-1 RT enzyme using AutoDock 4.2 software. The docking simulations were executed employing the Lamarckian genetic algorithm (LGA), with parameters set to 10 runs, a population size of 150, a maximum of 27,000 generations, and up to 2,500,000 energy evaluations. The binding affinities and interactions between the phytochemicals and key active site residues of the HIV-1 RT enzyme were analyzed to identify potential inhibitors. Further examination of ligand-enzyme interactions was conducted using UCSF Chimera Version 1.19 (https://www.cgl.ucsf.edu/chimera/) and Biovia Discovery Studio (https://discover.3ds.com/) (De Azevedo et al. 2025).

Chemicals and reagents

Based on *in silico* analysis, the most promising antiviral compound was selected for *in vitro* evaluation of HIV-1 RT inhibitory activity. The chosen compound, α-amyrin, and the reference drug, Nevirapine, were procured from Biosynth Limited, India, and GlaxoSmithKline Pharmaceuticals Ltd, India, respectively. The non-radioactive HIV-RT colorimetric ELISA kit, used for assessing HIV-1 RT inhibition, was obtained from Roche Diagnostics India Private Limited. All solvents and chemicals used in the experiment were of the highest analytical grade to ensure accuracy and reliability. Both the test compound and the standard drug were prepared as stock solutions by dissolving them in DMSO at a concentration of 20 mM.

HIV-1 Reverse transcriptase inhibition assay

The inhibitory effect of α-amyrin on HIV-1 reverse transcription was assessed using a non-radioactive HIV-RT colorimetric ELISA kit (Roche Diagnostics India Private Limited) following the manufacturer's instructions. All procedures were conducted under nuclease-free conditions to maintain assay integrity (Gujjeti and Mamidala 2014). In sterile Eppendorf tubes, 20 µL of resuspended α-amyrin (at final assay concentrations of 12.5, 25, 50, 100, and 200 µg/mL) or control solutions were mixed with 20 µL of recombinant HIV-1 RT enzyme (4 ng in lysis buffer) and 20 µL of reaction mixture containing 46 mM Tris–HCl, 266 mM potassium chloride, 27.5 mM magnesium chloride, 9.2 mM DTT, and 10 µM dUTP/dTTP. The tubes were incubated at 37°C for 1 hour. Following incubation, 60 µL of the reaction mixture was transferred into MPM wells, which were then covered with foil and

incubated at 37°C for another hour. After this, the contents were completely removed, and the wells were washed five times with 250 µL of washing buffer (solution 6), ensuring thorough removal after each wash. After the washing steps, 200 µL of anti-digoxigenin-peroxidase was added to each well, followed by another 1-hour incubation at 37°C under foil cover. Post-incubation, the solution was completely removed, and the wells were washed five more times with 250 µL of PBS-based washing buffer. Subsequently, 200 µL of ABTS substrate solution was added to each well, and the plate was incubated at room temperature for 5 minutes, leading to the development of a green color. The absorbance was measured at 405 nm (with a reference wavelength of 490 nm) using a microplate reader. The percentage of inhibition was determined using the following equation:

HIV-1 RT inhibition (%) = [(A control-A sample) / A control] x 100.

Where, A sample = Absorbance of the reaction containing α-amyrin or the positive control at 405 nm and A control = Absorbance of the reaction containing water instead of the sample at 405 nm.

Three tubes containing water instead of the test compound were used as negative controls, while Nevirapine (1.0 mg/mL) served as the positive control. Results were expressed as mean ± standard deviation from two independent experiments, each conducted in triplicate. The IC_{50} value (the concentration required to inhibit 50% of HIV-1 RT activity) was determined from a standard inhibition curve generated using Microsoft Excel.

Results

PASS prediction analysis

Based on the PASS prediction analysis of phytochemical compounds from *Calotropis procera*, both Pa (probability of activity) and Pi (probability of inactivity) values were considered to assess anti-HIV potential and are presented in Table 2. α-amyrin (Pa = 0.753, Pi = 0.004) and Frugoside (Pa = 0.728, Pi = 0.004) exhibited a high probability of activity with very low Pi values, indicating strong confidence in their antiviral potential, albeit predicted against HIV and Influenza respectively, and suggesting structural resemblance to known active pharmaceuticals. Benzoyllineolone (Pa = 0.590, Pi = 0.014) and Nevirapine (standard drug; Pa = 0.631, Pi = 0.002) fall in the moderate activity range (0.5 < Pa < 0.7) with low Pi values, supporting the likelihood of antiviral activity with moderate novelty. In contrast, Calotropin (Pa = 0.418, Pi = 0.028), 2-oxovorucharin (Pa = 0.265, Pi = 0.118), ß-Anhydroepidigitoxigenin (Pa = 0.121, Pi = 0.113), and α-amyrin acetate (Pa = 0.188, Pi = 0.039) show low Pa values (< 0.5), suggesting a lower probability of activity. However, their relatively higher Pi values, particularly in the case of 2-oxovorucharin and α-amyrin, indicate increased uncertainty or likelihood of inactivity. Nevertheless, if such compounds are experimentally validated, they could signify novel bioactive entities with unique mechanisms. Interestingly, α-amyrin acetate, though having a low Pa (0.188), also has a low Pi (0.039), which reflects a moderate level of uncertainty and supports further experimental exploration due to its prediction as an HIV-2 reverse transcriptase inhibitor.

Table 2 PASS Prediction of Antiviral and Anti-HIV Activities of Phytochemical Compounds from *Calotropis procera*

Sl.No	Compounds with PubChem ID	Pa	Pi	Activity
1	2-oxovorucharin (CID:11261800)	0,265	0,118	Antiviral (Herpes)
2	α -amyrin (CID:73170)	0,753	0,004	Antiviral (HIV)
3	α -Amyrinacetate (CID:19019008)	0,188	0,039	HIV-2 reverse transcriptase inhibitor
4	ß-Anhydroepidigitoxigenin (CID:272842500)	0,121	0,113	Antiviral (Influenza)
5	Benzoyllineolone (CID:9982084)	0,590	0,014	Antiviral (Influenza)
6	Calotropin (CID:16142)	0,418	0,028	Antiviral (Herpes)
7	Frugoside (CID:120728)	0,728	0,004	Antiviral (Influenza)
8	Nevirapine (standard)	0,631	0,002	HIV-1 reverse transcriptase inhibitor

**Pa and Pi are the probabilities of the presence and absence of activity, respectively*.

Drug-Likeness

Drug-likeness is commonly evaluated using Lipinski's Rule of Five, which considers four physicochemical parameters (MWT ≤ 500, log P ≤ 5, hydrogen bond donors ≤ 5, and hydrogen bond acceptors ≤ 10) associated with approximately 90% of orally active drugs that have progressed to Phase II clinical trials (Lipinski et al. 2001).

Based on Lipinski's Rule of Five, which evaluates drug-likeness based on molecular properties, the phytochemical compounds from *Calotropis procera* exhibit varying levels of compliance (Table 3). α-Amyrin, ß-Anhydroepidigitoxigenin, Benzoyllineolone, and the reference drug Nevirapine fully comply with all criteria, indicating good oral bioavailability potential. In contrast, 2-oxovorucharin, α-Amyrin acetate, Calotropin, and Frugoside each show one violation, primarily due to high molecular mass or Log P values exceeding 5, as seen in α-Amyrin acetate (Log P = 5.528). Notably, Frugoside exceeds the hydrogen bond donor threshold (5 donors), while 2-oxovorucharin, Calotropin, and Frugoside approach the upper limits of hydrogen bond acceptors. Despite these single violations, these compounds may still possess drug-like properties, as compounds with one violation are often still bioavailable. Overall, most phytochemicals from *C. procera* demonstrate acceptable pharmacokinetic profiles, supporting their potential as lead candidates for drug development.

Table 3 Lipinski's rule of five applied for the selected phytochemicals.

Compounds with PubChem ID	Molecular Mass (<500 Da)	Log P **(<5)**	Number of H-Bond Donors **(<5)**	Number of H-Bond Acceptors **(<10)**	Number of Violations of Rule of Five
2-oxovorucharin (CID:11261800)	588.732	2.901	3	9	1
α-amyrin (CID:73170)	426.717	4.524	1	1	0
α -Amyrinacetate (CID:19019008)	226.742	5.528	1	1	1
ß-Anhydroepidigitoxigenin (CID:272842500)	358.514	3.572	1	3	0
Benzoyllineolone (CID:9982084)	468.582	3.565	3	6	0
Calotropin (CID:16142)	533.630	1.479	4	9	1
Frugoside (CID:120728)	536.654	1.303	5	9	1
Nevirapine (standard)	266.30	2.651	1	4	0

The results generated from the medicinal chemistry filtering (Lipinski, Ghose, Veber, and Egan) are showed in Table 4. The drug-likeness evaluation of seven phytochemicals from *C. procera* and the reference drug Nevirapine based on Lipinski, Ghose, Veber, and Egan rules reveals that all compounds complied with Ghose, Veber, and Egan criteria, indicating favorable physicochemical properties related to drug absorption and oral bioavailability. However, only α-amyrin, ß-Anhydroepidigitoxigenin, Benzoyllineolone, and Nevirapine satisfied Lipinski's Rule, suggesting higher oral drug-likeness potential. 2-oxovorucharin, α-amyrin acetate, Calotropin, and Frugoside violated Lipinski's rule, likely due to high molecular weight or lipophilicity. Interestingly, Benzoyllineolone (bioavailability score: 1.07) and Frugoside (1.14) showed the highest bioavailability scores, whereas α-amyrin acetate (-0.25) and α-amyrin (0.10) showed low scores, indicating limited systemic exposure if administered orally. Despite some Lipinski violations, the overall positive compliance with the other three drug-likeness filters and favorable bioavailability scores in certain compounds highlights the potential of these phytochemicals as lead candidates for drug development.

Table 4 The drug-likeness features of phytochemicals of *C. procera* according to Lipinski, Veber, Ghose, and Egan rules

Compounds with PubChem ID	Lipinski	Ghose	Veber	Egan	Bioavailability Score
2-oxovorucharin (CID:11261800)	No	Yes	Yes	Yes	0.39
α-amyrin (CID:73170)	Yes	Yes	Yes	Yes	0.10
α -Amyrinacetate (CID:19019008)	No	Yes	Yes	Yes	-0.25
ß-Anhydroepidigitoxigenin (CID:272842500)	Yes	Yes	Yes	Yes	0.39
Benzoyllineolone (CID:9982084)	Yes	Yes	Yes	Yes	1.07
Calotropin (CID:16142)	No	Yes	Yes	Yes	0.47
Frugoside (CID:120728)	No	Yes	Yes	Yes	1.14
Nevirapine (standard)	Yes	Yes	Yes	Yes	0.12

Pharmacokinetic evaluation

The ADME-Tox predictable properties of the compounds were evaluated in Table 5 with an emphasis on (i) intestinal absorption, (ii) BBB permeability, and (iii) items of toxicity, expressed as AMES, hepatotoxicity, cardiotoxicity, and skin sensitization.

The computational pharmacokinetic and toxicity profiles of the seven phytochemicals from *C. procera* compared with the reference drug Nevirapine reveal both promising and variable outcomes. Nevirapine exhibits the highest intestinal absorption (97%) and is capable of crossing the blood-brain barrier (BBB), with no AMES toxicity, hERG inhibition, or skin sensitization, although it shows hepatotoxicity. Among the phytochemicals, Benzoyllineolone (93%), ß-Anhydroepidigitoxigenin (87%), and α-amyrin (90%) display high intestinal absorption and BBB permeability, similar to Nevirapine, and show no genotoxicity (AMES negative) or hERG inhibition. However, Benzoyllineolone and ß-Anhydroepidigitoxigenin share hepatotoxicity with Nevirapine. 2-oxovorucharin, Calotropin, and Frugoside showed relatively lower absorption (69%, 63%, and 57% respectively) and lack of BBB permeability, with hERG inhibition noted in all three, potentially indicating cardiotoxic risks. While α-amyrin acetate demonstrated moderate absorption (80%) and no major toxicities except for skin sensitization, 2-oxovorucharin also displayed skin sensitization and hERG inhibition, reducing its safety profile. Overall, α-amyrin, ß-Anhydroepidigitoxigenin, and Benzoyllineolone emerge as the most comparable to Nevirapine in terms of favorable pharmacokinetics and safety parameters, supporting their potential for further drug development.

Table 5 Computational pharmacokinetic and toxicity profiles for compounds.

Compounds with PubChem ID	Intestinal Absorption (%)	BBB Permeability	AMES	hERG 1 Inhibitor	Hepatotoxicity	Skin Sensitization
2-oxovorucharin (CID:11261800)	69%	No	No	Yes	No	Yes
α-amyrin (CID:73170)	90%	Yes	No	No	No	No
α -Amyrinacetate (CID:19019008)	80%	Yes	No	No	No	Yes
ß-Anhydroepidigitoxigenin (CID:272842500)	87%	Yes	No	No	Yes	No
Benzoyllineolone (CID:9982084)	93%	Yes	No	No	Yes	No
Calotropin (CID:16142)	63%	No	No	Yes	Yes	No
Frugoside (CID:120728)	57%	No	No	Yes	Yes	No
Nevirapine (standard)	97%	Yes	No	No	Yes	No

Pharmacogenomic evaluation

Furthermore, the four phytochemical compounds and reference drug appeared to induce hepatotoxicity. Therefore, a pharmacogenomic profile of these compounds was generated (Table 6). The results regarding metabolic pathways showed that all compounds had interactions with CYP3A4, CYP2C19, and CYP2C9, but not with CYP2D6.

The CYP450 enzyme interaction profiles of seven phytochemicals from *C. procera* compared to the reference drug Nevirapine provide insights into their potential drug metabolism behavior and drug-drug interaction risks. Nevirapine was identified as an inhibitor of CYP1A2, but not a substrate or inhibitor for other major CYP enzymes, indicating a relatively low likelihood of broader CYP-mediated interactions. In contrast, most of the phytochemicals—2-oxovorucharin, α-amyrin, ß-Anhydroepidigitoxigenin, Benzoyllineolone, Calotropin, and Frugoside-were predicted to be CYP3A4 substrates, suggesting they may undergo metabolism via this pathway. Notably, 2-oxovorucharin also acts as a CYP3A4 inhibitor, indicating a dual role that could potentially lead to CYP3A4-mediated drug interactions. Additionally, α-amyrin and Calotropin were identified as CYP2D6 inhibitors, raising possible concerns for interference with drugs metabolized via this enzyme. α-Amyrin acetate stood out as the only compound acting as a CYP2C9 inhibitor, while all compounds showed no inhibitory effect on CYP1A2 and CYP2C19, except Nevirapine. Overall, while most phytochemicals demonstrate limited inhibitory interactions with major CYP enzymes, the CYP3A4 substrate and inhibitor roles, particularly of 2-oxovorucharin, α-amyrin, and Calotropin, highlight the need for further assessment of potential CYP-mediated drug-drug interaction risks during pharmacokinetic evaluations.

Table 6 Pharmacogenomic profile of phytochemical compounds of *C. procera* for CYP2D6, CYP3A4, CYP1A2, CYP2C19, and CYP2C9.

Compounds with PubChem ID	CYP3A4 Substrate	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Substrate/Inhibitor	CYP3A4 Inhibitor
2-oxovorucharin (CID:11261800)	Yes	No	No	No	No / No	Yes
α-amyrin (CID:73170)	Yes	No	No	No	No / Yes	No
α -Amyrinacetate (CID:19019008)	No	No	No	Yes	No / No	No
ß-Anhydroepidigitoxigenin (CID:272842500)	Yes	No	No	No	No / No	No
Benzoyllineolone (CID:9982084)	Yes	No	No	No	No / No	No
Calotropin (CID:16142)	Yes	No	No	No	No / Yes	No
Frugoside (CID:120728)	Yes	No	No	No	No / No	No
Nevirapine (standard)	No	Yes	No	No	No / No	No

Molecular docking analysis

The molecular docking studies were performed using AutoDock software. The list of intermolecular interactions between the phytochemical compounds of *C. procera* and HIV-1 reverse transcriptase (RT) is presented in Table 7. Compounds exhibiting a binding energy lower than -6 kcal/mol are considered to have a higher binding affinity for target enzymes (Ikpa et al. 2024). Notably, α-amyrin demonstrated interaction with HIV-1 reverse transcriptase (RT) with a binding energy of -7.33 kcal/mol. The interaction modes, represented in both surface, 3D and 2D, are illustrated in Fig. 2.

Table 7. Molecular docking score and interaction amino acid residues of selected phytochemical compounds docked against HIV-1 RT enzyme (PDB code: 1JLB)

S. No	Compound	Binding Energy (kcal/mol)	Residue involving hydrophobic interaction	No. of H bonds	Interaction of residues forming H_2 bonds	Bond Length (A^0)
1	2-oxovorucharin	-6.52	Trp229, Ile94, Met230, Trp266, Gly231, Gln242, Val245, Tyr232, Ile244, Pro243	0	-	-
2	α-amyrin	-7.33	Gln91, Leu92, Glu89, Val90, Trp88, Phe87, Ala158, Ser162, Thr165, Gln161	2	Cys181, Gln182	2.13 2.49
3	α-Amyrinacetate	-5.99	Lys101, Lys102, Leu100, Pro236, Leu234	1	His235	1.38
4	ß-Anhydroepidigitoxigenin	-6.08	Gly316, Asn348, Val317, Val314, His315, Lys347, Lys238, Phe346	0	-	-
5	Benzoyllineolone	-5.79	Thr27, Ile31, Pro133, Gln23, Pro25, Lys22, Trp24, Gly141, Pro140, Ser134, Asn137, Ile135	2	Glu28, Leu26	1.84 2.06
6	Calotropin	-6.11	Tyr181, Thr165, Ser162, Gln91, Leu92, Glu89, Gln161, Ala158, Val90, Phe87, Gln85	1	Trp88	2.38
7	Frugoside	-3.55	Gln500, Gly504, Tyr405, Tyr501, Trp406, Lys366, Ala508, Ile505, Gly504, Thr362, Gly359	2	Arg358, His361	2.21 2.24
8	Nevirapine (Reference drug)	-5.28	Gly99, Pro95, Ile382, Gly384, Val381, Leu92, Gly93	2	His96, Ile94	1.88 1.95

α-amyrin binding interaction with HIV-1 RT

The molecular docking analysis of selected phytochemicals from *C. procera* against HIV-1 reverse transcriptase (PDB ID: 1JLB) revealed diverse binding affinities and molecular interactions with key residues at or near the allosteric and active sites. Among the compounds, α-amyrin showed the highest binding affinity (-7.33 kcal/mol), interacting with residues Gln91, Leu92, Glu89, Val90, Trp88, Phe87, Ala158, Ser162, Thr165, and Gln161, and forming two hydrogen bonds with Cys181 and Gln182 at bond lengths 2.13 Å and 2.49 Å, respectively. These interactions directly involve residues of the NNRTI allosteric binding pocket, suggesting strong inhibitory potential.

The 2D interaction diagram of the ligand with the HIV-1 reverse transcriptase (RT) enzyme illustrates a strong binding profile, highlighting both hydrogen bonding and hydrophobic interactions with key amino acid residues in the NNRTI allosteric binding pocket. The α-amyrin forms two conventional hydrogen bonds: one between the hydroxyl oxygen atom of the α-amyrin and the sulfur atom of Cys181, and another with the side chain of Gln182, likely involving its oxygen. These hydrogen bonds are significant as Cys181 is the site of the common Y181C mutation associated with resistance to first-generation NNRTIs like Nevirapine. The presence of direct interaction with Cys181 suggests the compound may retain activity even in the presence of such mutations, enhancing its potential as a more robust inhibitor.

In addition to hydrogen bonding, the ligand engages in multiple alkyl interactions with hydrophobic residues such as Leu92, Ala158, Val90, and Trp88. These residues, forming part of the hydrophobic core of the allosteric binding pocket, help anchor the ligand through van der Waals and alkyl contacts, which further stabilizes the binding orientation. Other surrounding residues involved in van der Waals interactions include Phe87, Glu89, Gln91, Gln161, Ser162, and Thr165, which contribute to the overall molecular complementarity and binding affinity. Notably, residues like Leu92, Val90, and Trp88 are well-known contributors to NNRTI binding, and their involvement indicates that the ligand binds efficiently within the classic non-nucleoside allosteric site of the HIV-1 RT enzyme.

The dual hydrogen bonding combined with extensive hydrophobic contacts and van der Waals interactions establishes a strong binding conformation, aligning well with critical residues that define NNRTI efficacy. This binding pattern suggests a potential to overcome resistance mutations, especially Y181C, by forming direct interactions with the mutated site and maintaining additional stabilizing contacts. The observed bond lengths for hydrogen bonding interactions, approximately 2.13 Å and 2.49 Å, indicate optimal bond distances for stable interactions. Overall, the α-amyrin exhibits a promising profile for further development as a potent and resistance-evading HIV-1 reverse transcriptase inhibitor.

Interactions of other phytochemicals with HIV-1 RT

Binding modes (3D and 2D) for 2-oxovorucharin, α-Amyrinacetate, ß-Anhydroepidigitoxigenin, Benzoyllineolone, Calotropin and Frugoside with HIV-1 RT (PDB ID 1JLB) provided in Supplementary Figures S1-S6. 2-oxovorucharin showed a good binding affinity of -6.52 kcal/mol, forming non-covalent interactions with Trp229, Ile94, Met230, Trp266, Gly231, Gln242, Val245, Tyr232, Ile244, and Pro243, though it did not form hydrogen bonds; its proximity to functionally important regions may still support effective binding (Supplementary Fig. 1). Calotropin, with a binding score of -6.11 kcal/mol, interacted with Tyr181, Thr165, Ser162, Gln91, Leu92, Glu89, Gln161, Ala158, Val90, Phe87, and Gln85, forming a hydrogen bond with Trp88 (2.38 Å), reinforcing its engagement with allosteric site residues (Supplementary Fig. 2). α-Amyrinacetate demonstrated a moderate binding energy of -5.99 kcal/mol, binding to Lys101, Lys102, Leu100, Pro236, and Leu234, and formed a hydrogen bond with His235 (1.38 Å), implicating its interaction with critical NNRTI-associated residues (Supplementary Fig. 3).

ß-Anhydroepidigitoxigenin interacted with Gly316, Asn348, Val317, Val314, His315, Lys347, Lys238, and Phe346, though it formed no hydrogen bonds (Supplementary Fig. 4) despite a modest binding score of -6.08 kcal/mol. Benzoyllineolone, with -5.79 kcal/mol, engaged residues Thr27, Ile31, Pro133, Gln23, Pro25, Lys22, Trp24, Gly141, Pro140, Ser134, Asn137, and Ile135, forming two hydrogen bonds with Glu28 and Leu26 (Supplementary Fig. 5) at bond lengths of 1.84 Å and 2.06 Å, respectively. Frugoside, despite a lower binding score (-3.55 kcal/mol), formed two hydrogen bonds with Arg358 and His361 (bond lengths 2.21 Å and 2.24 Å) and interacted with

Gln500, Gly504, Tyr405, Tyr501, Trp406, Lys366, Ala508, Ile505, Thr362, and Gly359 (Supplementary Fig. 6) suggesting some affinity at distal or alternative binding regions. The reference drug Nevirapine showed a binding energy of -5.28 kcal/mol, forming hydrogen bonds with His96 (1.88 Å) and Ile94 (1.95 Å), while interacting with Gly99, Pro95, Ile382, Gly384, Val381, Leu92, and Gly93 (Supplementary Fig. 7) consistent with known NNRTI binding modes. Overall, α-amyrin, Calotropin, and 2-oxovorucharin exhibit strong binding affinities and interact with critical residues at or near the NNRTI allosteric site, highlighting their potential as promising HIV-1 RT inhibitors.

When compared to the reference drug Nevirapine (Fig. 3), the α-amyrin exhibits a stronger and more diverse interaction profile with the HIV-1 reverse transcriptase (RT) enzyme. Nevirapine primarily interacts with residues such as Gly99, Pro95, Ile382, Gly384, Val381, Leu92, and Gly93, forming two hydrogen bonds with His96 and Ile94, with bond lengths of 1.88 Å and 1.95 Å respectively (Table 7).

These interactions are mainly confined to residues around the entrance of the NNRTI binding pocket and lack direct contact with the critical Cys181 residue, which is commonly mutated (Y181C) in drug-resistant HIV-1 strains. In contrast, the selected phytochemical, α-amyrin forms direct hydrogen bonds with Cys181 and Gln182, which are crucial residues in the allosteric pocket and particularly significant in the context of resistance. Moreover, the α-amyrin establishes multiple alkyl and van der Waals interactions with key residues like Leu92, Ala158, Val90, Trp88, Phe87, and Glu89, contributing to enhanced binding stability. These additional interactions provide a more robust binding network compared to Nevirapine, indicating that the α-amyrin may offer greater binding affinity and potentially improved efficacy against drug-resistant HIV-1 RT variants, especially those harboring the Y181C mutation.

In vitro HIV-1 reverse transcriptase (RT) inhibitory Assay

The effect of the α–amyrin on reverse transcription was evaluated using a non-radioactive HIV-RT colorimetric ELISA kit. The HIV-1 reverse transcriptase (RT) inhibition assay was conducted using different concentrations of α-amyrin (test sample) and nevirapine (positive control), and the results were evaluated in terms of the percentage inhibition and IC_{50} values (Table 8).

At the lowest concentration of 12.5 µg/mL, α-amyrin exhibited 37% inhibition, while nevirapine showed 45% inhibition, indicating a stronger initial effect by the standard drug. As the concentration increased, both compounds displayed a dose-dependent increase in inhibition. At 25 µg/mL, α-amyrin achieved 51% inhibition, whereas nevirapine displayed 58% inhibition, maintaining its superior potency. At 50 µg/mL, the inhibition values were relatively close, with α-amyrin and nevirapine showing 62% and 65% inhibition, respectively (Table 8).

At 100 µg/mL, α-amyrin inhibited 68% of HIV-1 RT activity, while nevirapine showed 71% inhibition, demonstrating a marginal difference. At the highest concentration of 200 µg/mL, α-amyrin achieved a notable inhibition of 82%, whereas nevirapine reached 92%, confirming its stronger inhibitory effect even at elevated doses.

Table 8 HIV-1 RT inhibition of α-Amyrin and Nevirapine (Positive Control)

Different concentrations in (µg/mL)	α-Amyrin (Test Sample)		Nevirapine (Positive Control)	
	Percentage of HIV-1 RT inhibition	IC_{50} (µg/mL)	Percentage of HIV-1 RT inhibition	IC_{50} (µg/mL)
12.5	37	26	45	21
25	51		58	
50	62		65	
100	68		71	
200	82		92	

The IC_{50} value, which represents the concentration required to inhibit 50% of the enzyme activity, was calculated for both compounds. α-Amyrin exhibited an IC_{50} of 26 µg/mL, while nevirapine demonstrated a slightly lower IC_{50} of 21 µg/mL, confirming the higher potency of the standard drug. However, the relatively close IC_{50} values indicate that α-amyrin exhibits significant inhibitory potential, suggesting it could be a promising candidate for further development as an HIV-1 RT inhibitor.

The results highlight the potential of α-amyrin as a natural compound with notable anti-HIV-1 RT activity. Although nevirapine remains more potent, the moderate inhibitory activity of α-amyrin, coupled with its phytochemical origin and potentially lower toxicity profile, makes it a valuable lead compound for further optimization. These findings support the potential use of α-amyrin in combination therapy or as a structural template for the development of novel non-nucleoside reverse transcriptase inhibitors (NNRTIs).

Discussion

The results of the study on α-amyrin from *Calotropis procera* align with recent findings in the field of HIV-1 research, particularly concerning the efficacy of natural compounds as reverse transcriptase inhibitors. For instance, a study by Swapna et al. (2024) demonstrated that several phytochemicals, including cardenolides derived from *C. procera*, exhibited significant inhibitory effects against HIV-1 RT, with binding affinities comparable to established NNRTIs (Swapna et al. 2024). Similarly, their research emphasized the importance of molecular docking studies in predicting the binding interactions of these compounds with critical residues of the enzyme, corroborating the findings of α-amyrin's strong binding affinity and the formation of hydrogen bonds with residues associated with drug resistance, such as Cys181. This suggests that phytochemicals could serve as valuable alternatives or adjuncts to conventional antiretroviral therapies, particularly in light of the growing concern over drug-resistant HIV strains.

Moreover, the pharmacokinetic properties of α-amyrin, which indicate favorable absorption and low toxicity, resonate with findings from other studies that have explored the therapeutic potential of plant-derived compounds. For example, research conducted by Dogara et al. (2023) highlighted the promising ADMET profiles of various phytochemicals, suggesting their viability in drug development pipelines (Dogara 2023). The dual approach of *in silico* and *in vitro* evaluations, as demonstrated in the study of α-amyrin, is crucial for identifying compounds that can effectively target HIV-1 RT while minimizing adverse effects. This integrated methodology not only reinforces the potential of α-amyrin but also underscores the broader applicability of natural products in addressing the challenges posed by HIV, particularly in the context of resistance and toxicity.

Recent studies have increasingly highlighted the potential of phytochemicals, particularly α-amyrin from *C. procera*, as promising candidates for inhibiting HIV-1 reverse transcriptase (RT). In a comprehensive study, molecular docking revealed that α-amyrin exhibits a strong binding affinity to the allosteric site of HIV-1 RT, with a binding energy of -7.33 kcal/mol. This interaction was characterized by the formation of two significant hydrogen bonds with crucial residues (Cys181 and Gln182), which are known to be associated with drug resistance mutations. The in vitro assays demonstrated that α-amyrin effectively inhibited HIV-1 RT activity with an IC_{50} of 26 µg/mL, comparable to the standard drug nevirapine (IC_{50} = 21 µg/mL). These findings suggest that α-amyrin not only possesses significant antiviral activity but also offers a potentially lower toxicity profile due to its natural origin.

Moreover, the pharmacokinetic evaluations of α-amyrin indicated favorable absorption and drug-likeness properties, reinforcing its viability as a lead compound for further development. Recent literature corroborates these findings, emphasizing the role of natural compounds in overcoming challenges posed by drug-resistant HIV strains. For instance, studies have identified other phytochemicals that similarly target the allosteric sites of HIV-1 RT, suggesting a broader class of plant-derived compounds could be harnessed for antiretroviral therapy. This trend underscores the importance of integrating natural products into HIV treatment strategies, particularly as researchers continue to explore innovative approaches to combat drug resistance and improve therapeutic outcomes.

Conclusion

This study highlights the potential of α-amyrin from *Calotropis procera* as a promising HIV-1 reverse transcriptase (RT) inhibitor. Molecular docking revealed a strong binding affinity of α-amyrin to the allosteric site of HIV-1 RT, with a binding energy of -7.33 kcal/mol and the formation of two hydrogen bonds with key residues (Cys181 and Gln182). The *in vitro* HIV-1 RT inhibition assay demonstrated dose-dependent activity, with an IC_{50} of 26 µg/mL, comparable to the standard drug nevirapine (IC_{50} = 21 µg/mL). At the highest concentration (200 µg/mL), α-amyrin achieved 82% inhibition, confirming its significant antiviral potential. Additionally, pharmacokinetic predictions indicated favorable absorption, low toxicity, and drug-likeness properties, supporting its viability as a lead compound for further development. The study also underscores the therapeutic value of plant-derived compounds in addressing drug resistance challenges. With its strong inhibitory effect and favorable pharmacokinetics, α-amyrin holds promise as a complementary or alternative agent in antiretroviral therapy, warranting further clinical investigations and optimization.

Declarations

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Author contribution EM conceived the research idea, formulated the research questions and designed the study, Supervision. PM contributed to the formal analysis, Data curation, Software and Methodology

Availability of data and material Data will be made available on request

Consent for publication All authors agreed to publish the manuscript

Competing Interest The authors declare that they have no competing interests

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Table

Table 1 is available in the Supplementary Files section

Figures

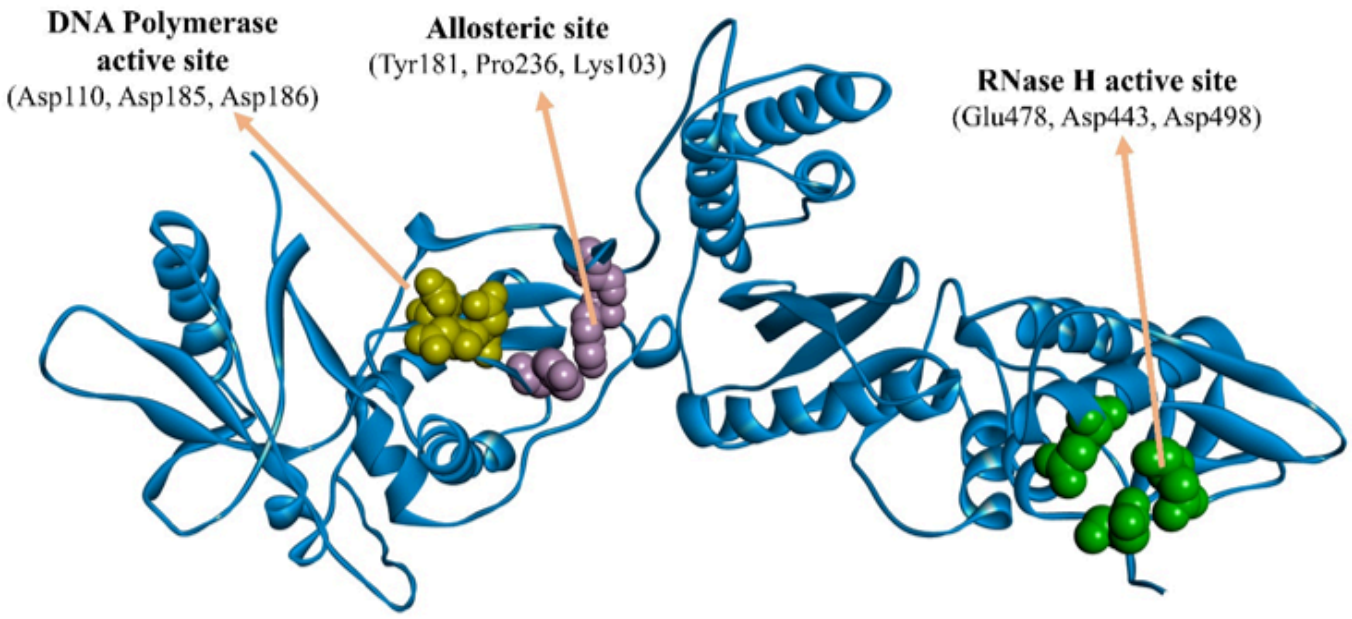

Figure 1

Ribbon representation of the HIV-1 RT (PDB code: 1JLB). The p66 subunit of HIV-1 RT are colored blue.

. Residues present in the DNA polymerase active site (D110, D185, D186) and the RNase H active site (E478, D443, D498) are represented with yellow and green spheres. Allosteric site (Y181C, P236L and K103N) is colored with violet. Single-point mutations (Y181C, P236L and K103N) in the allosteric site are represented with violet colour.

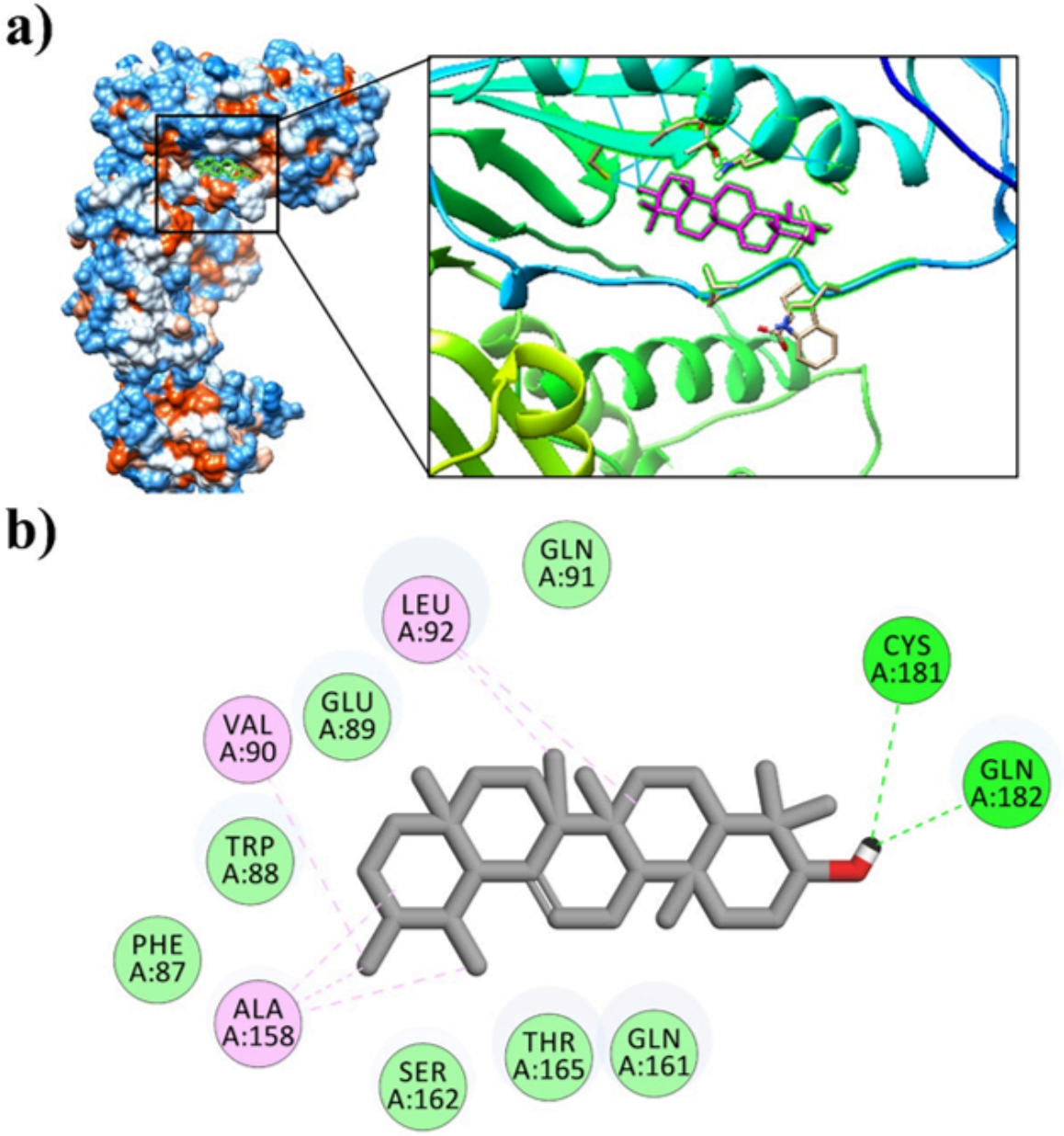

Figure 2

Binding mode for α–amyrin with HIV-1 RT (PDB ID 1JLB)

a) Ligand is shown in "Stick" format and green color whereas the HIV-1 RT is shown in "Surface" format. b) 2D image generated using discovery studio 4.1 Visualizer showing amino acid residues involved in interactions between ligand and enzyme.

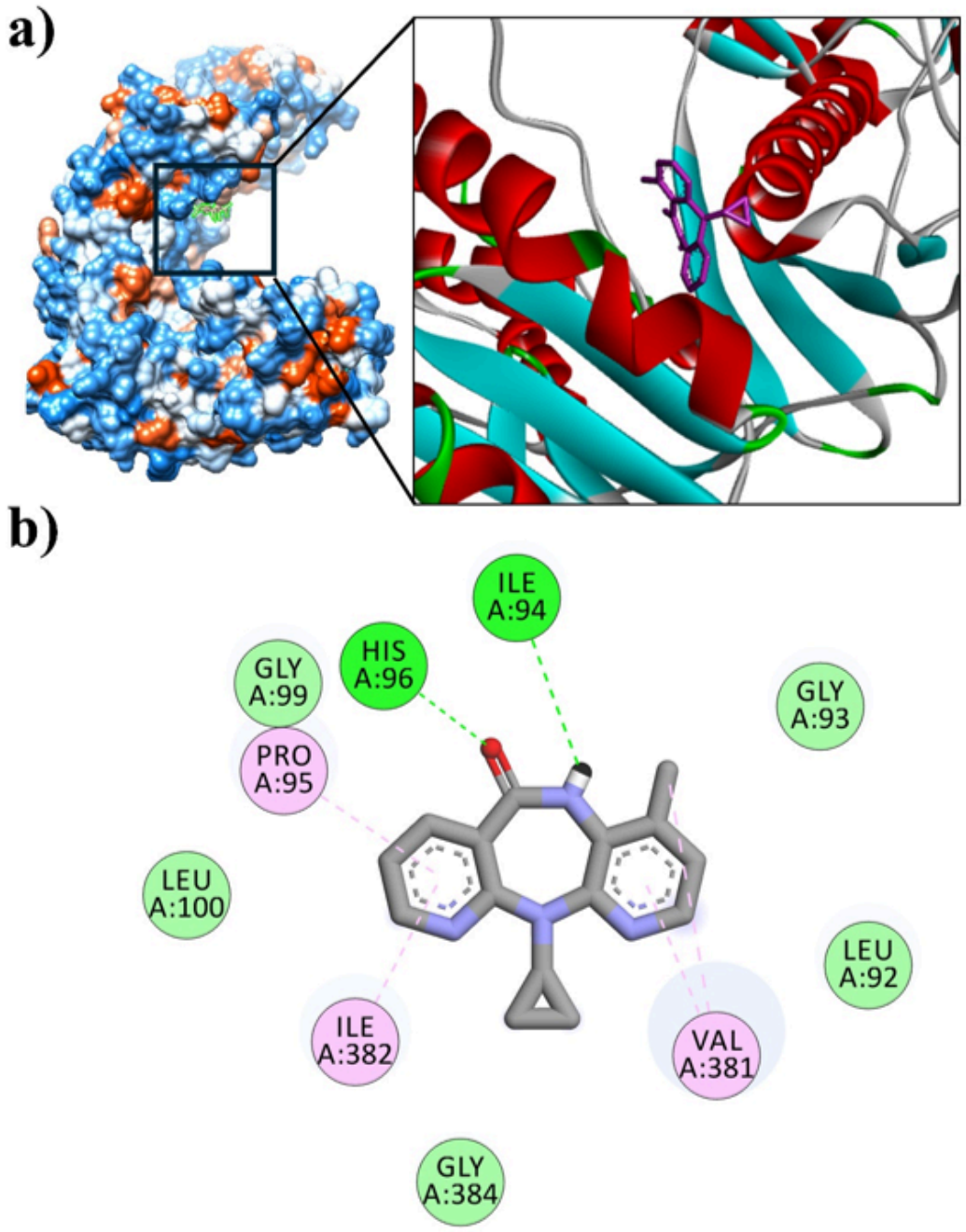

Figure 3

Binding mode for Nevirapine (reference drug) with HIV-1 RT (PDB ID 1JLB)

a) Ligand is shown in "Stick" format and green color whereas the HIV-1 RT is shown in "Surface" format. b) 2D image generated using discovery studio 4.1 Visualizer showing amino acid residues involved in interactions between ligand and enzyme.

Supplementary Files

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- 4SupplementaryFile.docx
- Table1.docx