ARTICLE

Simulation analysis reveals catechin from crude *Mentha cordifolia* has potential antiretroviral properties against HIV and suggests possible protease inhibition through flap and active site interactions

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ABSTRACT

Human Immunodeficiency Virus (HIV) still remains a significant global health concern. The reliance of developing regions on international aid and the long-term side effects associated with antiretroviral therapy (ART), pose substantial challenges in combating HIV. We investigated the antiretroviral potential of *Mentha cordifolia*, a plant with a long history of folkloric use in Southeast Asia, especially in the Philippines. Our *in vitro* experiments demonstrated that *Mentha cordifolia* extracts significantly inhibited HIV-1 virus production, as evidenced by reduced p24 antigen levels without inducing cytotoxicity. Further, our simulation analysis showed that catechin may be the potential bioactive compound in the plant extract that is responsible for the observed bioactivity. *In silico* predictions indicated that catechin possesses favorable drug-like properties, including good oral bioavailability. In addition, our molecular docking simulations revealed that catechin binds to HIV-1 protease, particularly at its flap and active site regions. These interactions are predicted to inhibit the cleavage of viral polyproteins, thus preventing viral maturation. These findings highlight the potential of *Mentha cordifolia* as a natural source of antiretroviral compounds against HIV. Further experimental validation, such as bioassay-guided fractionation, is necessary to isolate and characterize the bioactive compound and to fully elucidate its mechanism of action. While future experimental studies are warranted to confirm the inhibitory activity of catechin against viral protease function and virus production, the findings of these simulation analyses will be helpful in designing a more targeted bioassay purification approach. These efforts may lead to the development of novel, natural-product-based anti-HIV therapies.

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INTRODUCTION

Antiretroviral therapy (ART) has made HIV (human immunodeficiency virus 1) infection manageable for 39.9 million infected people worldwide, but access to treatment remains unequal. The Eastern and Southern Africa region was able to acquire ART and treat 83% of the People Living with HIV (PLHIV) because of international help. Unfortunately, the Asia-Pacific region experienced a 60% decrease in external funding (UNAIDS, 2024) to support treatment. Only 67% of PLHIV in the Asia-Pacific region have access to treatment. Furthermore, long-term use of ART is associated with neurocognitive impairments (Bertrand et al., 2021; Rudd & Toborek, 2022), mitochondrial dysfunction, and bone loss due

KEYWORDS

Mentha cordifolia, antiretroviral, HIV, molecular docking, p24 ELISA

to the increased risk of osteoporosis and fractures. All these highlight the need for innovative and sustainable treatment alternatives that can be used in developing regions.

Southeast Asia offers vast resources for drug discovery, harboring approximately 20% of the world's flora and fauna. Natural product-derived therapies offer cost-effective and accessible alternatives in resource-limited regions. *M. cordifolia*, also known as *yerba buena*, is included in the list of Ten Medicinal Plants approved by the Department of Health in the Philippines due to its history of folkloric use (Villaseñor et al., 2002). It is traditionally used for cough, toothache, fever, and migraine (Rodriguez et al., 2023). Studies also showed antihypertensive (Pakdeechote et al., 2014), antibacterial (Ragasa et al., 2001), and anti-inflammatory properties (Villaseñor & Sanchez, 2009). Moreover, de Paz-Silava (2022) demonstrated that the ammonium sulfate extract of *M. cordifolia* can inhibit HIV latently-infected cells *in vitro*. It was also observed to inhibit virus production, evidenced by a decrease in viral p24 antigen concentration, under non-cytotoxic concentrations (de Paz-Silava et al., 2022). While its mode of action remains unclear, another study demonstrated that it can inhibit aspartyl protease pepsin activity, which is similar to the HIV protease (Hunay Jr. & Sarol, 2014). This study, however, did not explore the identity of the bioactive compound and its activity on actual HIV protease. The next logical step is the identification of the bioactive compound responsible for the observed bioactivity and proposing a mechanism of action.

Bioassay fractionation of active compounds in crude extracts is a complex, time-consuming, and expensive process.

An added complication arises from the bioactive compound's obscurity (the International Natural Product Sciences Taskforce et al., 2021). Traditional methods often involve extensive compound isolation, high-throughput screening, and preclinical validation, requiring years of research. In the context of HIV-1 research, these challenges are complicated by the virus's high mutation rate, which necessitates refinement of antiviral inhibitors. HIV-1 protease inhibitors (PIs) in particular are difficult to develop because of drug resistance and metabolic instability (Yeo et al., 2020). Given these limitations, an alternative approach using computational drug discovery techniques can streamline the identification of potential bioactive compounds.

Advances in computer-aided drug design (CADD) have revolutionized drug discovery by enabling rapid screening and evaluation of potential bioactive compounds (Sadybekov & Katritch, 2023). Virtual screening, molecular docking, and molecular dynamics (MD) simulations allow prediction of molecular interactions before conducting costly laboratory experiments (Atasever, 2024). Using computational methods, it is possible to efficiently identify active compounds from natural products and propose mechanisms of action.

HIV-1 protease is one of the most common targets of ART. This enzyme, a member of the aspartate family, is responsible for the cleavage of inactive HIV polyproteins into smaller, functional proteins (Weber et al., 2021). HIV-1 protease consists of several key regions, including the flap region and the active site. The flap region is a flexible loop that undergoes conformational changes and controls the access to the active site (Badaya & Sasidhar, 2020). The active site is the catalytic center consisting of two aspartate residues and facilitates the cleavage of peptide bonds in the substrate (Baassi et al., 2023). Targeting the flap region can disrupt substrate binding and product release, while inhibiting the active site prevents catalytic cleavage of viral polyproteins, which potentially prevents HIV-1 protease from functioning properly and ultimately its inhibition.

This study builds upon the findings of de Paz-Silava *et al.*, which provided compelling evidence of *M. cordifolia*'s antiretroviral activity. While it establishes the extract's bioactivity against HIV, the specific bioactive compounds responsible are unidentified. A necessary step forward is to validate the repeatability of *in vitro* results before *in silico* analysis. It is essential to ensure that computational predictions remain grounded in experimentally verified biological activity.

The objective of this study was to perform a combination of *in vitro* and *in silico* analyses to nominate a candidate compound from the plant *M. cordifolia* that can have potential antiretroviral effects. We present in our *in vitro* results that the method of de Paz-Silava et al. is repeatable. We show through simulation that the HIV-1 protease can be inhibited by compounds from *Mentha* species. Molecular simulations nominate catechin as the most promising bioactive molecule in *M. cordifolia*. Our analysis proposes that catechin potentially interacts with the flap and active site regions of HIV-1 protease, which can induce the desired bioactivity. This provides molecular mechanistic insights into how *M. cordifolia* exerts its antiretroviral effects, which should be further validated through bioassay-guided purification steps. The information obtained from this study should help further strategize an extensive bioassay-guided fractionation and analysis.

MATERIALS AND METHOD

This study used cell culture and simulation analysis to screen potential bioactive compounds from *M. cordifolia* that have possible anti-HIV effects. No testing on live animals or humans was performed. The nominated compounds and their proposed bioactivity were purely based on *in silico* analyses.

In vitro experimental procedures were based on previously established methods of de Paz-Silava et al. (2022), with some modifications.

A. Initial Plant Processing

I. Plant Source

M. cordifolia fresh samples were purchased from Leonie Agri Corporation (LAC) in Nueva Ecija, Philippines. Plants were obtained from the same source as de Paz-Silava et al. (2022), where verification of the plant material in the Jose Vera Santos Memorial Herbarium, University of the Philippines Diliman was reported.

II. Plant Sample Preparation

To verify the previously reported bioactivity of *M. cordifolia,* we utilized the previously reported extraction method of de Paz-Silava et al. (2022) with some modifications. Aqueous extraction more closely resembles folkloric concoction preparations and was adapted in this study as previously done (de Paz-Silava et. al, 2022). *M. cordifolia* samples were washed with tap water and air-dried at room temperature for at least 24 hours. The samples were then oven-dried for 48 hours at a temperature not exceeding 35°C to prevent compound degradation. The oven-dried samples were placed in a tight-sealed container and stored at 2 to 4°C until further use.

III. Aqueous Extraction

Oven-dried samples and distilled, sterilized water at a ratio of 2:1 (v/w) were added to a 1000 mL Erlenmeyer flask and boiled for 30 minutes. After boiling, the mixture was filtered using Whatman Paper No. 1 to separate the plant

sample from the aqueous solution, and the latter was cooled down at room temperature.

IV. *Differential Ammonium Sulfate (AS) Precipitation*
This procedure was performed as an initial biopurification step. The aqueous solution was placed in a 4°C container for 30 minutes and a -4°C container for an additional 30 minutes so that the solution would reach 0°C. This is important because the concentration of the ammonium sulfate salts to reach the initial saturation level is dependent on temperature and the volume of the solution. The initial ammonium sulfate saturation that was used was 20%, and the collected supernatants from this were used to obtain precipitates from the 20-40%, 40-60%, and 60-80% saturation levels. The supernatants that were collected from each saturation level were collected and centrifuged at 15,000 x g for 20 minutes. The different ammonium sulfate (AS) extracts obtained from 40%, 60%, and 80% saturation levels were then labeled as 40% AS, 60% AS, and 80% AS.

V. *Desalting using Dialysis Tubing*
The samples collected from the supernatant were precipitated, reconstituted in water, and placed in a dialysis tube. These were submerged in sterilized water for 48 hours. Water was discarded and replaced with new sterilized water every 24 hours. The solution collected after the desalting process was collected and stored in a -80°C refrigerator and lyophilized later.

B. Cytotoxicity and Anti-retroviral Assay

I. *Cell lines*
OM10.1 cells, a promyelocytic cell line chronically infected with latent HIV-1, were used in the antiviral assays due to their well-characterized integration of the virus. In addition, it is suitable for a Biosafety Level 2, which allows for better safety while still providing an excellent model for studying HIV latency and reactivation. The OM10.1 cells were obtained from the National Institute of Health – HIV Reagent Program from the USA. OM10.1 cells were cultured using RPMI 1640 medium supplemented with 10% fetal bovine serum at 37° C and 5% CO_2.

II. *Cell viability assay*
A cell viability assay was performed to ensure that the decreased p24 production seen in antiretroviral assays is not due to cytotoxic effects on the cell lines. Cells were collected and tested for cell viability after 24 and 48 hours of incubation using the MTS Assay Kit (Cell Proliferation) according to the manufacturer's instructions. Absorbance of each well was measured at 490 nm using an ELISA plate reader. Cell viability was measured as a percentage of absorbance in a treatment well relative to a control (untreated well).

III. *Anti-retroviral activity using p24 Antigen ELISA kit*
OM10.1 cells were seeded in a 24-well plate with a cell density of (5 x 10^5 cells/mL). The following day, cells were treated with or without varying concentrations of the *M. cordifolia* fractions. To stimulate the production of HIV-1 virions from the latently-infected cells, Human Recombinant TNFα (2 ng/mL) (Roche) was added (except for the nonstimulated control) after 2 hours of treatment with the extract. After stimulation, the cells were incubated for 24 hours at 37 °C, 5% CO_2. Antiretroviral activity was measured through the extent of inhibition of p24 viral antigen expression in the cell culture supernatant. The levels of p24 were measured using a p24 ELISA system based on the manufacturer's instructions (Abcam).

IV. *Statistical analysis*
Cell viability data were analyzed using GraphPad Prism 10. ELISA data were analyzed by generating a standard curve using Google Spreadsheet. Results are presented as mean ± standard deviation. The statistical analysis of each sample was compared at a 95% confidence level using a t-test.

C. *In Silico* screening, docking, and molecular dynamics

I. *Collection of Ligands from Databases*
Seventy-nine known isolated compounds from the genus *Mentha* were collected from different databases and were used for the *in silico* analysis (Ćavar Zeljković et al., 2021; Eftekhari et al., 2021; Salehi et al., 2018; Tafrihi et al., 2021). The compounds to be screened were limited to available polar compounds that have a crystal structure from PubChem (https://pubchem.ncbi.nlm.nih.gov/), Zinc15 (https://zinc15.docking.org/), and the ChEMBL Database (https://www.ebi.ac.uk/chembl/). Amprenavir, a known protease inhibitor, was used as the positive control (Razzaghi-Asl et al., 2015).

II. *Prediction of ADMET properties and bioactivity*
To determine which compound/s were best for simulation, we initially predicted the potential bioactivity using Molinspiration Virtual Screening software (https://www.molinspiration.com/). Furthermore, we determined violations of Lipinski's Rule of Five, which assesses the druggability of a compound with the following parameters: MW<500, cLogP < 5, HBA <10, HBD <5, TPSA < 140, and NRTOB <10 (Benet et al., 2016; Desa et al., 2017) using available data from Pubchem and Chembl, and analyzed using Osiris DataWarrior (freeware software, DataWarrior V4.7.2, Idorsia Pharmaceuticals Ltd., Allschwil, Switzerland). The pharmacokinetic properties, namely absorption, distribution, metabolism, excretion, and toxicity (ADMET) of compounds were profiled, loaded, calculated, and determined through PreADMET. The compounds' drug score values were analyzed to determine candidate compounds for molecular docking.

III. *Preparation of Ligands and Protein*
Structures of the protein enzyme HIV-1 protease (PDB ID: 3NU3, Resolution: 1.02 Å) were obtained and inspected from the Protein Data Bank (https://www.rcsb.org/). Obtained crystal HIV-1 viral protease was processed using BIOVIA Discovery Studio to locate the active site and remove water molecules and native ligands. Polar compounds from *M. cordifolia* with crystal structure were processed using OpenBABEL GUI (https://openbabel.org/docs/current/GUI/GUI.html).

IV. *Molecular docking*
In order to validate the compound-target association and appropriate binding orientations, the molecular docking procedure was performed through MGL Tools and Auto Dock 4.1. The receptor was kept rigid while the ligand was set to explore, rotate, and be flexible to locate the most probable binding location (Paramashivam, 2015). The potential ligand interaction with the protein was then visualized using PyMOL and Protein-Ligand Interaction Profiler.

V. *Molecular dynamics simulation of HIV-1 protease*
The 3D structure of HIV-1 protease was obtained from the Protein Data Bank database (PDB ID: 3nu3). The

bound antiviral drug amprenavir and water molecules were removed from the protein structure in preparation for MD simulations. The protein was protonated using the PDB2PQR from the ADB webserver (pH = 7.0). Water molecules were added to the protein system to solvate the protein, and 0.15 M KCl was added as counter ions using the tleap module of AmberTools. Protein (FF19SB) and water (OPC) force fields were also added. The parameter (parm7) and coordinate (rst7) files were also obtained from the same software.

All-atom MD simulation of the protein system was done using NAMD2.14. Protein (FF19SB) and water (OPC) force fields were added to the protein beforehand using AMBERTools tleap. The system underwent energy minimization at 0 K for 1000 steps each (10 ps) with fixed atom constraints sequentially done for gradual system relaxation. The system then underwent gradual heating from 0 K to 300 K (50 K increments) for 50 ps at NVT. After heating, the system was then equilibrated at NPT at 50 ps, followed by NPT 100-ns production at 300 K and 1 atm. A Langevin thermostat and piston were used to maintain the temperature during the NPT run, and pressure during the NVT run.

VI. Ensemble Molecular Docking of Catechin with HIV-1 Protease

The trajectories obtained from the MD simulation of HIV-1 protease were used for the ensemble molecular docking analysis. The protein trajectories were clustered based on an RMSD cutoff of 2 A° and one representative confirmation from each cluster was used in the analysis. The binding score and the docking pose of catechin were determined using AutoDock 4. The search box of the ensemble docking experiment was centered on the alpha carbon of the 126 glycine residue with a box size of 36 A° x 55 A° x 35 $\AA$. Blind docking was performed, and the docking pose with the highest binding score was used for the analysis. The potential ligand interaction with the protein was then visualized using PyMOL and Protein-Ligand Interaction Profiler.

RESULTS

M. cordifolia extracts have shown potential antiviral and non-cytotoxic properties

To ensure the repeatability of prior findings and validate the antiretroviral potential of *M. cordifolia*, we conducted *in vitro* assays before proceeding to computational analysis. For the *in vitro* study, we replicated the experimental framework established by de Paz-Silava et al. (2022) to validate the repeatability of their methodology. This step was not only a procedural necessity but also a critical validation measure since *in silico* predictions require a strong foundation of experimental data. It was essential to first confirm that the plant extract we are studying retained its antiretroviral activity while exhibiting minimal cytotoxic activity— consistent with the findings of de Paz-Silava et al. (2022) prior to identifying candidate bioactive compounds.

The potential cytotoxicity of *M. cordifolia* crude fractions was evaluated using the WST assay in OM10.1 cells. We employed differential ammonium sulfate (AS) precipitation at saturation levels of 20–40% (40%AS), 40–60% (60% AS), and 60–80% (80% AS) to refine the precipitation range and optimize bioactive component isolation. Extraction concentrations (50 to 1600 µg/mL) were selected to match those previously tested by the group of de Paz-Silava (2022). Our results demonstrated that all fractions (40% AS, 60% AS, and 80% AS) exhibited negligible cytotoxicity concentrations up to 800 µg/mL (Figure 1a). However, at the highest concentration tested (1600 µg/mL), a slight decrease in cell viability was observed, indicating a potential threshold for cytotoxic effects. We selected 800 µg/mL as the highest non-toxic dose for subsequent antiretroviral evaluation.

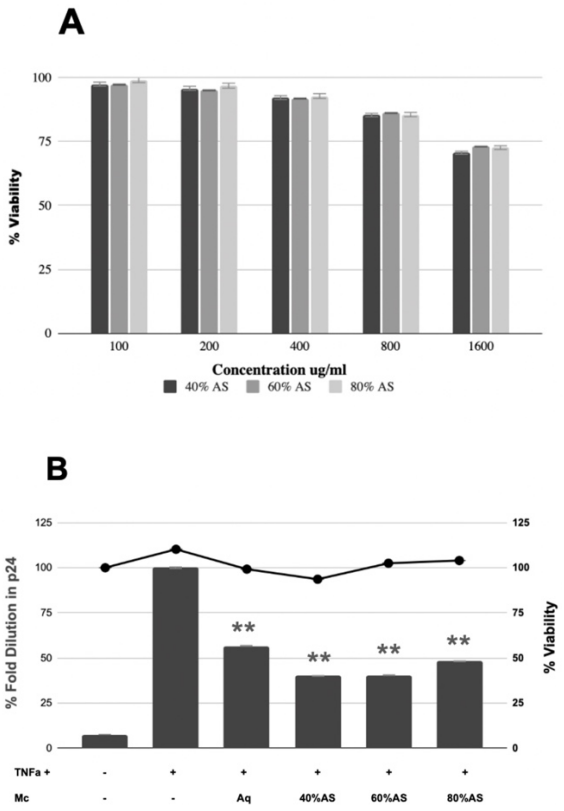

Figure 1: (**A**) *Mentha cordifolia* extracts (40% AS, 60% AS, and 80% AS) exhibited negligible cytotoxicity at concentrations ranging from 50 to 800 µg/mL, but slight cytotoxicity was observed at 1600 µg/mL. (**B**) *Mentha cordifolia* extracts (aqueous, 40% AS, 60% AS, and 80% AS) decreased viral p24 expression without inducing cytotoxicity in TNFα-induced OM10.1 cells. Samples that have ** above the columns are significantly different compared to the TNFα-induced untreated control at 95% confidence level based on t-test.

The antiretroviral activity of *M. cordifolia* extracts was evaluated using an HIV-1 p24 ELISA-based assay of the supernatant of OM10.1 cells. We induced the viral activation in latently infected cells using TNF-α and measured p24 antigen production in the presence of *M. cordifolia* extracts following the protocols of de Paz-Silava et al. (2022).

Extracts (40% AS, 60% AS, and 80% AS) were tested at non-toxic concentrations, with TNFα-stimulated (untreated) and unstimulated (untreated) controls included for reference. Our findings revealed a significant reduction in p24 production upon treatment with *M. cordifolia* extracts, with the most pronounced inhibition observed in the 40% AS and 60% AS fractions (Figure 1b). These results not only confirm the repeatability of the bioactivity previously reported by de Paz-Silava et al. (2022) but also establish a reproducible *in vitro* framework for future investigations.

The successful replication of antiviral activity *in vitro* provided a strong foundation for our *in silico* analysis, ensuring that computational predictions were guided by experimentally validated data. Prior experimental confirmation reduces the likelihood of false-positive results, particularly in complex biological systems, and strengthens the reliability of our computational findings. We next aimed to nominate a specific compound that is responsible for the observed biological activity

using computational techniques. In addition, we leveraged molecular docking and dynamics simulations to predict the interaction between the candidate compound and a key viral target, HIV-1 protease, thereby elucidating a potential mechanism of action.

Rosmarinic acid and catechin are potential compounds responsible for the bioactivity

The next step after the *in vitro* analysis was to identify the specific compound/s responsible for the observed bioactivity in the two independent trials. However, isolating and identifying bioactive compounds from crude plant extracts through traditional fractionation and biochemical assays is often labor-intensive, costly, and time-consuming. Thus, we employed an *in silico* approach that leverages the computational screening methods to systematically evaluate candidate compounds.

A total of 79 polar compounds with known crystal structures were obtained from PubChem and subjected to initial screening. To refine this dataset, we assessed key physicochemical properties (administration, distribution, metabolism, excretion, and toxicity) of the compounds based on Lipinski's Rule of Five (Karami et al., 2022). This initial filter prioritizes compounds with favorable pharmacokinetic properties, ensuring that potential candidates would possess drug-like characteristics conducive to cellular uptake and systemic distribution.

Compounds with molecular weights greater than 500 Da were excluded, since compounds heavier than this will have difficulty entering the cell membrane (Roskoski, 2019). This eliminated 13 compounds, reducing the pool to 66 (Figure 2a). The remaining 66 compounds were further screened based on their respective hydrogen bond acceptors. Compounds with more than 10 hydrogen bond acceptors were removed, resulting in 55 compounds. Finally, compounds with more than five hydrogen bond donors were excluded, leaving 42 compounds. Compounds with excessive hydrogen bond acceptors and donors were removed because this can potentially increase the compound's water solubility and decrease membrane permeability, which can lead to poor absorption (Coimbra et al., 2021). In total, our preliminary screening removed 37 compounds, streamlining the dataset with favorable drug-like properties.

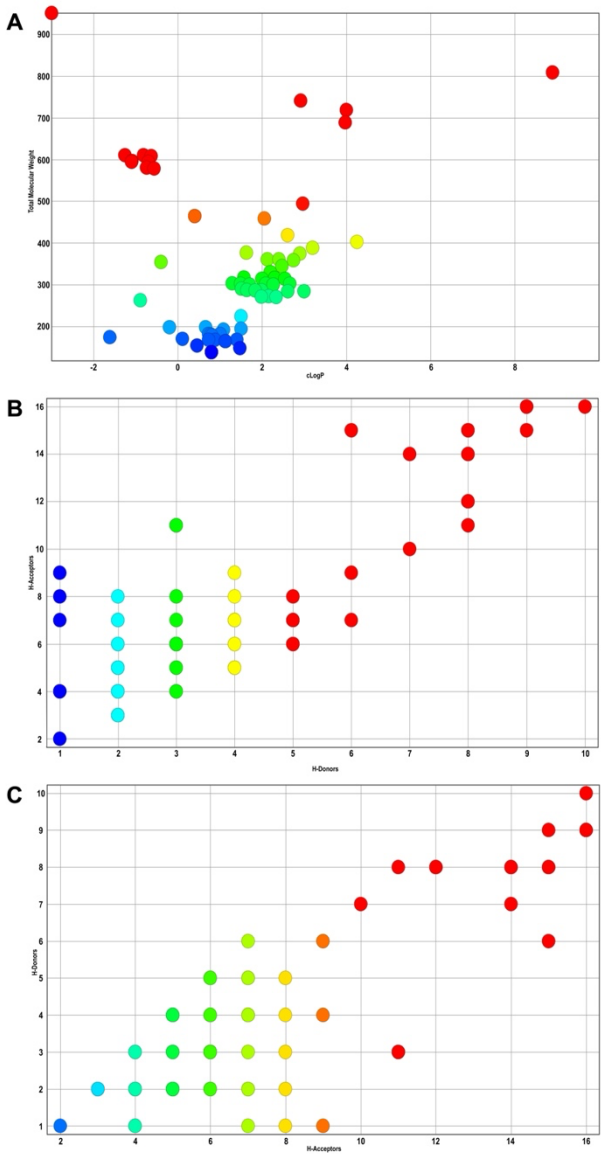

Figure 2: (**A**) Molecular distribution of the 79 polar compounds (represented in dots) collected, where the red dots have a molecular weight (MW) greater than 500 Da. (**B**) Molecular distribution of the polar compounds (represented in dots collected), where the red dots have more than 10 hydrogen bond acceptors. (**C**) Molecular distribution of the polar compounds (represented in dots collected where the red dots have more than 5 hydrogen bond donors).

We next identified compounds with potential protease inhibitory activity by employing Molinspiration Cheminformatics software, which predicts bioactivity scores based on molecular structural features (Srivastava, 2021). A bioactivity score greater than 0.00 suggests potential biological activity, whereas scores between -0.50 and 0.00 indicate moderate activity, and scores below -0.50 suggest inactivity. Among the 42 remaining compounds, rosmarinic acid and catechin emerged as the strongest candidates, with protease inhibition bioactivity scores of 0.15 and 0.26, respectively (Table 1). These values indicate a moderate likelihood of protease inhibition, supporting the hypothesis that one or both compounds may contribute to the observed antiviral activity of *M. cordifolia*.

Table 1: Predicted bioactivity using the Molinspiration program.

BIOACTIVITY (0.000-1.000)	
	Protease Inhibitor
Rosmarinic acid	0.15
Catechin	0.26

Rosmarinic acid and catechin show favorable ADMET properties

We next looked at the pharmacokinetic properties of rosmarinic acid and catechin using the PreADMET software. PreADMET is a computational tool that predicts various physicochemical properties such as lipophilicity, solubility, permeability, and metabolism (Dulsat et al., 2023). We can gain insights into the potential pharmacokinetic behavior of the two compounds and identify potential challenges or limitations for drug development. The predicted absorption profiles of rosmarinic acid and catechin were evaluated by determining their lipophilicity and intestinal absorption. Lipophilicity measures a compound's affinity for a lipid-rich environment, which can play a crucial role in drug absorption (Giaginis et al., 2018). Rosmarinic acid showed a higher logP value of 3.0 compared to catechin (logP value of 1.8), as shown in Table 2. This suggests that rosmarinic acid has a higher lipid solubility, which can potentially facilitate its passive diffusion across cell membranes and enhance its intestinal absorption.

However, excessive lipophilicity can also lead to increased protein binding, reducing the amount of free drug available for systemic circulation. Catechin, with its lower lipophilicity, may have a more balanced absorption profile, avoiding excessive protein binding while still maintaining sufficient membrane permeability. On the other hand, intestinal absorption is a critical step in oral drug delivery, and both rosmarinic acid and catechin demonstrated moderate to good absorption profiles as predicted by HIA and Caco-2 permeability models. These findings suggest that these compounds have the potential to be orally administered and effectively absorbed into the bloodstream.

Table 2: Pharmacokinetics prediction using PreADMET program

	ABSORPTION					DISTRIBUTION	
	Molecular weight	Lipinski rule of 5 violation	Lipophilicity (cLogP)	Human Intestinal absorption HIA% > 30%	Colorectal adeno carcinoma Caco-2>0	(Plasma Protein Binding %)	Brain (Cbrain) and peripheral blood (Cblood) BBB > 1
Rosmarinic Acid	360.32	0	3.0	62.48	0.7246	86.24	0.1044
Catechin	290.27	0	1.8	66.70	0.6569	100	0.3439

	METABOLISM						EXCRETION	TOXICITY*		
	CYP2C19	CYP2C9	CYP2D6	CYP2D6 substrate	CYP3A4	CYP3A4 substrate	Renal OCT2 substrate	Ames	Mice	Rat
Rosmarinic Acid	non	inhibitor	non	non	Inhibitor	non	NO	+	-	+
Catechin	inhibitor	inhibitor	non	non	inhibitor	weakly	NO	+	-	-

*For toxicity, + means that the compound is toxic while - means it is non-toxic.

We next looked at the estimated distribution profiles of the two compounds by determining their respective plasma protein binding (PPB) and blood-brain barrier (BBB) values as summarized in Table 2. PPB determines the extent to which a drug binds to plasma proteins (Di, 2021). Both rosmarinic acid and catechin exhibited high PPB values, suggesting a significant portion of these compounds may be bound to plasma proteins, reducing the amount of free drug available and reducing overall efficacy. In addition, BBB is a highly selective barrier that protects the brain, especially the central nervous system (Wu et al., 2023). Rosmarinic acid demonstrated low BBB penetration, indicating limited CNS distribution, while catechin exhibited moderate BBB penetration, suggesting potential for CNS activity. It is important to note that high BBB penetration can increase the risk of central nervous system side effects. The results showed that both compounds have high PPB and low to moderate BBB, which can potentially hinder their distribution in the body.

The hypothetical metabolic fate of rosmarinic acid and catechin was also determined by analyzing their interactions with cytochrome P450 (CYP) enzymes. CYP enzymes play a crucial role in drug clearance, and their inhibition or induction can significantly impact drug pharmacokinetics and efficacy (Zhao et al., 2021). Table 2 shows that rosmarinic acid was identified as an inhibitor of CYP2C9 and CYP3A4. CYP2C9 is involved in the metabolism of various drugs, including anticoagulants, anti-inflammatories, and antidepressants. CYP3A4 is a major drug-metabolizing enzyme responsible for the metabolism of numerous drugs, including many commonly prescribed medications (Zhang et al., 2024). Catechin, on the other hand, was identified as an inhibitor of CYP2C19 and CYP2C9, and a weak substrate for CYP3A4, as shown in Table 2. CYP2C19 is involved in the metabolism of various drugs, including proton pump inhibitors, antidepressants, and antiplatelet agents (Zhao et al., 2021). Inhibition of these enzymes by rosmarinic acid and catechin may lead to increased plasma concentrations of co-administered drugs, potentially increasing the risk of adverse effects.

Lastly, we looked at the potential toxicity of rosmarinic acid and catechin by determining their mutagenicity and animal toxicity. Mutagenicity refers to the ability of a compound to induce genetic mutations (Lou et al., 2023). The results, shown in Table 2, revealed that both compounds were identified to be potential mutagens. Additionally, predicted animal toxicity in mice and rats indicated that rosmarinic acid is toxic to mice, while catechin showed no animal toxicity. These findings highlight the need for careful toxicity assessment and risk-benefit analysis before considering the clinical development of these compounds.

Molecular docking shows catechin binds strongly to HIV-1 protease

The potential of rosmarinic acid and catechin as HIV-1 protease inhibitors was explored by molecular docking experiments using AutoDock 4.1. Molecular docking is a computational method that predicts the binding interactions between a ligand (in this case, the compounds of interest) and a protein target (HIV-1 protease) (Gao et al., 2023). During the simulations, the ligands were allowed to adopt a flexible conformation, and the protein was maintained in a rigid structure. The simulation was repeated multiple times to obtain the most favorable binding conformation and affinity. The docking results revealed that both rosmarinic acid and catechin exhibited favorable binding affinities to HIV-1 protease. Amprenavir, which served as the positive control, demonstrated a slightly higher binding affinity (-12.81 kcal/mol) compared to catechin (-12.23 kcal/mol) but higher compared to rosmarinic acid (-7.18 kcal/mol), as shown

in Figure 3. The binding score calculated from the docking simulations provides an estimate of the strength of the ligand-protein interaction; a more negative binding score indicates a stronger interaction (Agu et al., 2023). This means that catechin has a similar binding score to amprenavir, which suggests that the two compounds might have similar interactions. On the other hand, rosmarinic acid has a lower binding score compared to catechin, which means that the former forms weaker interactions compared to the latter. Catechin has better results, making it potentially a better inhibitor for HIV-1 protease.

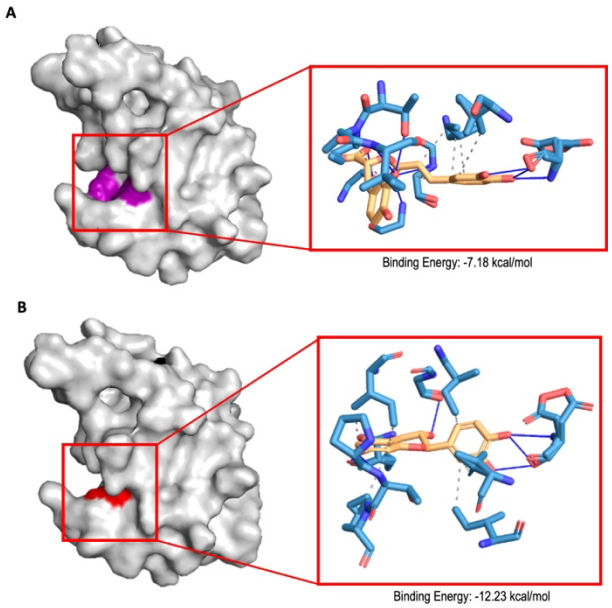

Figure 3: Molecular docking results of (A) rosmarinic acid and (B) catechin showing the hydrophobic interactions (dotted gray lines) and hydrogen bonding (solid blue line) interactions of the compounds with HIV-1 protease. Amprenavir has a binding affinity of -12.81 kcal/mol.

Ensemble molecular docking of catechin shows a potential mechanism of inhibition for HIV-1 protease

The binding interactions and potential mechanism of catechin were further explored using ensemble molecular docking experiments. Ensemble docking is a computational method that incorporates the flexibility of the protein target, allowing a more comprehensive understanding of the ligand-protein interactions. Before ensemble docking, MD simulations were performed to generate a representative ensemble of HIV-1 protease conformations. MD simulations allow the protein to undergo conformational changes under simulated conditions, providing a more realistic representation of its dynamic behavior. The MD simulations were performed for a total of 100 nanoseconds (ns), resulting in 50,000 conformations, highlighting the protein's flexibility. The Root Mean Square Deviation (RMSD) values, which indicate the average displacement of the protein's atoms compared to a reference structure, of these conformations ranged from 1-2 Å. This suggests that the protein remained relatively stable throughout the simulation, while still exploring a range of possible conformations.

The number of conformations that were used on the ensemble docking was reduced using k-means clustering on the MD trajectory. This clustering method groups similar conformations together by identifying distinct conformational states of the protein. This clustering technique grouped the conformations into 99 clusters based on their RMSD values. Clustering analysis was performed to lower the number of protein structures that will be used during molecular docking experiments while still capturing the whole dynamics of the protein. After clustering, one protein conformation was obtained from each cluster to serve as a representative. These protein conformations obtained were used in ensemble molecular docking simulations.

The ensemble molecular docking simulation (Figure 4C) was performed using a blind docking approach, and the top-ranked pose with the highest binding score was selected for further analysis. The top docking pose showed hydrophobic interactions and hydrogen bonding between catechin and HIV-1 protease. The strongest interactions occurred with ILE50 and ILE149, located in the tip of the flap region, and with ASP25, a crucial catalytic residue in the active site. The docking pose suggests that catechin binds in a position capable of influencing both flap mobility and substrate accessibility. Specifically, interactions with ILE50 and ILE149 may interfere with the dynamic motion of the flap, while hydrogen bonding with ASP25 indicates direct involvement with the catalytic machinery. Together, these interactions highlight multiple potential modes by which catechin can engage with HIV-1 protease.

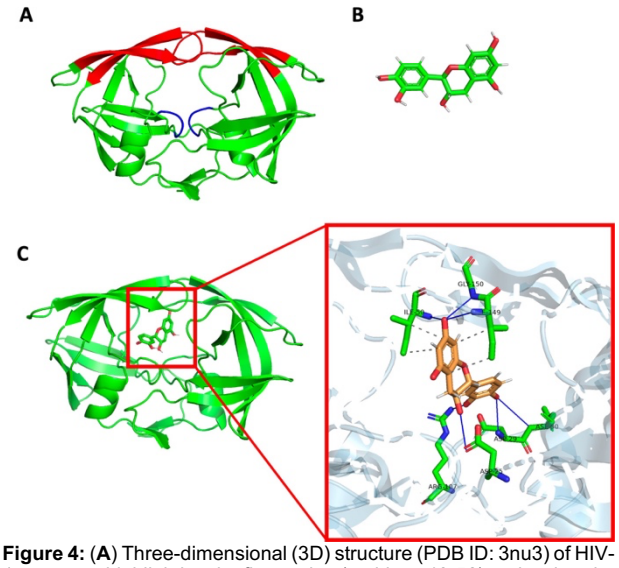

Figure 4: (**A**) Three-dimensional (3D) structure (PDB ID: 3nu3) of HIV-1 protease, highlighting the flap region (residues 43-58) and active site (residues 25-27. (**B**) Three-dimensional (3D) structure of catechin used for ensemble molecular docking. (**C**). Molecular interactions of catechin and HIV-1 protease, including hydrophobic interaction (dotted gray line) and hydrogen bonding (solid blue line) on the flap region and active site.

DISCUSSION

HIV is a major public health concern in developing regions (UNAIDS, 2024). Long-term side effects, emerging drug resistance, and the dependence of developing countries on external funding threaten the sustainability of ART (Bertrand et al., 2021; Chawla et al., 2018; Rudd & Toborek, 2022; Schank et al., 2021; UNAIDS, 2023, 2024). We studied the potential of *M. cordifolia* for its antiretroviral property against HIV and proposed a possible mechanism for this. While this plant has a long history of folkloric use, it has not been fully developed as an antiretroviral agent.

Our *in vitro* results show *M. cordifolia* extracts, in crude form, exhibited antiretroviral activity on HIV-1 infected cells. We observed that the extracts significantly attenuated p24 production. This antigen is one of the products of the cleavage of gag polyprotein and a key component of the viral capsid (Anderson et al., 2018). High concentrations of p24 antigen indicate active viral replication, while the opposite indicates viral suppression (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) et al., 2018). In addition, previous studies have been using it as a biomarker to observe the progression or suppression of HIV (Passaes et al., 2021). Therefore, we employed the same marker as it has been established as a reliable indicator of virus production. We were also able to provide a concentration range for the extracts that

will attenuate p24 production without inducing cytotoxicity. The cells can survive even when treated with crude-level extracts. Our data validates the plant's bioactivity and repeatability of the findings of de Paz-Silava et al. (2022). This step minimizes the risk of misleading computational results and establishes a reliable foundation for identifying active compounds.

Purification of bioactive molecules is challenging. Natural products are complex mixtures that contain various compounds. Characterizing the bioactivity of each fraction can be a time-consuming and resource-intensive process (Popova & Bankova, 2023). This process typically takes several years. It will also require repetitive bioassays and characterization. This causes significant losses of bioactive compounds and makes it challenging to pinpoint the exact bioactive molecule. Thus, we employed *in silico* techniques to delineate which possible bioactive compound is responsible for the biological phenomenon. This approach allows us to prioritize compounds with high affinity and selectivity for the target protein, thereby reducing the time and cost associated with extraction. It should be noted, though, that *in silico* analyses performed in this study have notable limitations. In particular, the programs and databases used do not provide stringent chemical characterization or determine how much of the bioactive compound can be yielded from the crude extract of *M. cordifolia*. For this purpose, procedures like isolation, mass spectrometry, and nuclear magnetic resonance will still be necessary. However, given that all of these mentioned procedures are costly and time-consuming, the *in silico* information provided in this study can help formulate a targeted and cost-efficient bioassay-guided purification strategy.

Our computational analysis identified catechin as a candidate compound with the desired bioactivity. *In silico* screening indicates that catechin has good oral bioavailability based on Lipinski's Rule of Five. These parameters filter compounds with poor drug-like properties and retain compounds with molecular mass ≤500 Dalton, ≤5 hydrogen bond donors, and ≤10 hydrogen bond acceptors (Lipinski et al., 1997). Pharmacokinetics prediction results showed that catechin has good absorption and distribution profiles, indicating that catechin, once orally administered, can be readily absorbed from the gut into the bloodstream and reach its target site in sufficient quantities to initiate bioactivity. A study reported that PLHIV prefer to take ART orally compared to other modes of administration due to its safety, efficacy, and cost-effectiveness (Dubé et al., 2020).

Conversely, the predicted toxicology profile of catechin showed that it has potential mutagenicity, which can raise side effects. It is important to note that all drugs, including those obtained from natural products, still have potential toxicity (Gaston et al., 2020). Even some of the US-FDA-approved drugs, just like drugs that are used to treat breast cancer, elicit side effects (Chaurasia et al., 2023). Reports suggest that green tea catechins are generally safe to consume if within the recommended concentration range (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) et al., 2018). In addition, in our *in vitro* analysis, we were able to induce the therapeutic effect at a certain concentration range of *M. cordifolia* without causing significant cell death. As with other drugs, using an appropriate dosage can prevent severe adverse effects. Thus, we suggest that working on the concentration range we identified will help to ensure the balance between the therapeutic efficacy while ensuring an acceptable toxicity, just like with any other drugs. The method of drug delivery may also be critical to reduce potential risks and side effects. The use of animal models will be particularly useful.

The observed binding interactions of catechin, derived from the top-ranked docking pose obtained through blind docking, provide insights into its potential inhibitory mechanism against HIV-1 protease. HIV-1 protease is a crucial enzyme in the virus's life cycle and is responsible for the cleavage of large polyproteins into smaller functional proteins (Lockbaum et al., 2023). The interaction of catechin with ILE50 and ILE149 in the flap region is particularly noteworthy, as flap flexibility is essential for substrate entry and product release. In addition, the hydrogen bond formed by catechin with ASP25 is significant because this residue is part of the catalytic dyad responsible for proteolytic activity. By occupying this site, catechin may compete directly with viral polyproteins for binding, thereby hindering substrate cleavage. Furthermore, the positioning of catechin within the protease pocket indicates a potential steric blockade, which could limit access of substrates to the active site. The combination of impaired flap mobility, interference with catalytic residues, and physical obstruction of the binding pocket could collectively reduce the enzyme's catalytic efficiency. This, in turn, can lead to the suppression of viral maturation and ultimately a reduction in viral load (Wang et al., 2015). Based on all parameters of our simulation studies, catechin appears to be the most promising candidate for further drug development.

The findings from this study align with previous reports on the antiviral potential of catechins. For instance, tea catechins have potential antiretroviral activity against HIV-1 as seen in docking simulations (Kharisma et al., 2021). Another study reported that catechin derivatives from green tea can inhibit HIV-1 entry by blocking gp41-mediated membrane fusion (Liu et al., 2005). However, to the best of our knowledge, there are currently no published crystallographic or *in vitro* studies directly confirming catechin's inhibitory activity against HIV-1 protease, highlighting a gap that warrants further experimental investigation. Importantly, no similar studies have been conducted in *M. cordifolia*, and this work represents the first report exploring catechins from this plant as potential antiviral agents with specific activity against HIV-1.

Literature suggests catechin, while present in *Mentha* species, is less commonly reported as a dominant compound, and its specific concentrations vary depending on species and extraction methods (Eftekhari et al., 2021). Despite catechin being present in potentially lower concentrations, it remains a relevant bioactive compound based on our *in silico* analysis. Our molecular docking results and predicted bioactivity scores suggest that catechin exhibits strong interactions with HIV-1 protease, making it a potential candidate for the observed biological activity. Computational predictions prioritize binding affinity over compound abundance, meaning even a minor component can have a significant functional role (Pantsar & Poso, 2018). Moreover, even at lower concentrations, catechin can contribute significantly since it can exhibit strong biological effects due to its high binding affinity to target proteins. Finally, many bioactive compounds function at micromolar or even nanomolar concentrations, meaning absolute abundance is not always the deciding factor.

It should be noted that we examined the semi-purified extract, and there's a possibility that the observed bioactivity may not be solely attributed to a single compound. Catechin was identified as the potential ligand responsible for the bioactivity, but it is also possible that it interacts synergistically with other components. This aspect was not explored in the present work and could be addressed in future studies.

Thus, while catechin may not be the most abundant component in *M. cordifolia,* our *in silico* results indicate that it remains a relevant candidate compound. The combination of molecular docking results, bioactivity score predictions, and the possibility of synergistic effects supports its role in the observed bioactivity of the extract. While exact quantification in *M. cordifolia* would strengthen the correlation between *in silico* and *in vitro* findings, the available literature supports the presence of these

compounds in quantities that could contribute to the observed biological activity.

The identification of a putative candidate significantly refines the drug discovery process by reducing the trial-and-error approach traditionally required in bioactive compound isolation (the International Natural Product Sciences Taskforce et al., 2021). Rather than employing exhaustive and indiscriminate extraction techniques, future studies can now focus on targeted extraction methodologies optimized specifically for catechin isolation from *M. cordifolia.* This shift enables a more efficient and cost-effective workflow, bypassing the need for repetitive bioassays to screen crude fractions and instead directing efforts toward the development of selective assays confirming catechin's presence and its protease inhibition activity.

Moreover, our study used OM10.1 cells to create our cell model, which are generally used to study HIV-1 latent infection. One advantage of using this cell model is that it can be used even at Biosafety Level (2,) which makes it safer to handle throughout the experiment. Additionally, despite its limitations, it is an established model for studying HIV-1 latency. Taken together, this would still offer a strong foundation that can be used for subsequent investigations in other cellular models. With catechin as a lead candidate, future studies can now explore its potential antiretroviral activity in higher-risk cell models that would require stringent biosafety conditions. The stepwise progression aligns with the best practices in antiretroviral drug discovery, ensuring both reproducibility and translational relevance of our findings.

We presented in this study that *M. cordifolia* extracts can significantly attenuate p24 production based on our *in vitro* results. We then nominate catechin as a potential compound responsible for the bioactivity of the said plant from our *in silico* analysis. We were able to predict that catechin can be a potential HIV-1 protease inhibitor by binding to the flap and active site region preventing the cleavage of viral polyproteins. This simulatory prediction should be immediately validated with an *in vitro* protease inhibition analysis. In addition, we were able to show that catechin has good oral bioavailability and drug-like properties based on our *in silico* screening. Given the compelling results from our integrated *in vitro* and *in silico* analyses, our study strongly warrants further targeted investigations into catechin as a novel antiviral candidate. Future research should focus on refining its extraction, validating its inhibitory mechanism against HIV-1 protease, and evaluating its therapeutic potential in preclinical models.

RECOMMENDATION

Based on the findings, we can now strongly propose that future studies should focus on targeted extraction and isolation of catechin from the crude extract of *M. cordifolia*. By nominating catechin, a more specific bioassay-guided fractionation for the said compound should be performed, which can potentially reduce the typical time in the extraction process. Additionally, the isolated compound can be tested on T-cell models, which could better reflect the physiological environment of HIV-1 infection. *In vitro* assays that assess HIV-1 protease inhibition, which allow a direct evaluation of catechin's mechanism of action, should be an immediate experiment to be performed. Finally, further in vivo studies are recommended to assess the overall safety of the two compounds, reduce side-effect risks, and guide the development of optimal drug-delivery methods. By refining the experimental approach, future research can strengthen the foundation for developing *M. cordifolia*-derived compounds as potential antiretroviral agents.

CONCLUSION

Our study successfully reproduced the findings of de Paz-Silava et al. (2022), confirming the repeatability of *M. cordifolia*'s antiretroviral activity. We demonstrated its ability to attenuate HIV-1 production at varying concentrations while maintaining cell viability, validating its bioactivity in two independent experiments.

On the other hand, our *in silico* analysis identified catechin as a strong candidate responsible for the observed bioactivity of the plant. Molecular docking simulations suggest that the mode of action of catechin is through HIV-1 protease inhibition. Notably, this study highlights a downstream mechanism, which may be particularly effective in targeting latent HIV reservoirs. Although all the predicted biological effects against HIV-1 appear to be promising, it would still be necessary to further assess its mutagenic potential as well as its overall potential to cause side effects in appropriate preclinical and clinical studies.

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CONFLICT OF INTEREST

Most of the authors declare that the research was conducted independently, without any commercial or financial ties that could be interpreted as a potential conflict of interest. Moreover, the authors declare that Dr. de Paz-Silava holds a patent related to the research described in this paper, specifically the methods used in the extraction process. This potential conflict of interest has been managed by doing independent verification of results among the different investigators to ensure that there is no bias in the interpretation of the findings.

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CONTRIBUTIONS OF INDIVIDUAL AUTHORS

JB, VB, RA, SdPS, and AM conceptualized and designed the experiments. JB and VB performed the experiments and generated the data. JB and VB wrote the manuscript. All authors edited and approved the submitted version.

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SUPPLEMENTAL DATA

Dock Cluster Number	Binding Score (kcal/mol)
1	-8.1
2	-8.8
3	-9.3
4	-7.9
5	-8.4
6	-7.4
7	-7.8
8	-8.0
9	-8.6
10	-8.2
11	-7.8
12	-11.3
13	-8.3
14	-7.9
15	-8.5
16	-7.7
17	-7.9
18	-7.5
19	-7.6
20	-7.8
21	-8.0
22	-8.4
23	-7.6
24	-7.9
25	-9.2
26	-7.7
27	-7.4
28	-8.0
29	-7.8
30	-7.8
31	-7.8
32	-8.5
33	-8.4
34	-7.8
35	-7.7
36	-8.2
37	-7.8
38	-8.4
39	-8.5
40	-7.5
41	-7.6