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Quercetin from Phyllanthus amarus Inactivates HIV-1 Expression by Interfering with Host Signaling Mechanisms

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Abstract:
Indian medicine has traditionally used *Phyllanthus amarus* to treat a variety of conditions, including hepatitis B. This study examines the potential anti-HIV-1 capabilities of the subject. The aim was to assess the effectiveness of *P. amarus* extracts and quercetin, a bioactive molecule, in suppressing HIV-1 expression. We utilised in vitro and in silico techniques to investigate the antiviral properties. We synthesised and analysed crude extracts from the plant to determine their capacity to suppress HIV-1 in MT-2 cells. We assessed the presence of the HIV-1 gag p24 protein using ELISA. Additionally, we evaluated the suppressive effects on HIV-1 reverse transcriptase (RT) using an HIV-RT assay kit. This study investigates Quercetin's binding interactions with HIV-1 p24 and RT proteins through molecular docking experiments. Our study's findings demonstrate that methanol extracts of *P. amarus* effectively suppressed HIV-1 p24 expression. Specifically, the leaf and seed extracts exhibited inhibition rates of 87.52 ± 1.96% and 88.74 ± 1.03%, respectively, at a concentration of 200 µg/ml. The HIV-RT assay showed that leaf extracts inhibited HIV by 85.55 ± 1.65%, whereas seed extracts inhibited it by 88.91 ± 1.22%. Molecular docking showed that quercetin has strong interactions with the NF-kappa B protein, which supports its antiviral activity. *P. amarus* and its bioactive part, quercetin, are very good at fighting HIV-1. They may do this by stopping the virus from replicating and stopping reverse transcriptase. The results highlight the therapeutic potential of *P. amarus* in developing innovative anti-HIV therapies. Additional research is necessary to investigate its clinical uses.

Keywords: Human Immunodeficiency virus; Quercetin; Phyllanthus amarus; Molecular docking

Introduction:

The genus Phyllanthus, comprising numerous medicinal species, has been a cornerstone in traditional oriental medicine, particularly for the treatment of chronic viral infections. Phyllanthus amarus, a prominent species within this genus, has been extensively utilized in the Indian medicinal system for its broad therapeutic properties, including its efficacy against hepatitis B virus [1]. This traditional usage has spurred scientific interest in investigating its potential antiviral properties against other viruses, notably the Human Immunodeficiency Virus type 1 (HIV-1). HIV-1 remains a global health challenge, with millions of individuals affected worldwide. Despite advances in antiretroviral therapy (ART), which has transformed HIV-1 from a fatal disease to a manageable chronic condition, there is an ongoing need for novel therapeutic agents [2]. This necessity arises due to issues such as drug resistance, side effects, and the inability of ART to eradicate latent viral reservoirs. Consequently, the exploration of alternative and complementary therapies, including those derived from medicinal plants, has garnered considerable attention [3].

P. amarus is rich in various bioactive compounds, including alkaloids, flavonoids, phenols, and glycosides. Among these, quercetin, a flavonoid, has been identified as a key component with significant biological activities. Quercetin is known for its antioxidant, anti-inflammatory, and antiviral properties, which suggest a potential role in inhibiting HIV-1 replication [4]. Previous studies have demonstrated that quercetin can interfere with multiple stages of the viral life cycle, including entry, reverse transcription, and integration.

This study aims to evaluate the anti-HIV-1 properties of *P. amarus* extracts, with a particular focus on quercetin. By employing in vitro and in silico methods, we sought to elucidate the mechanisms by which these extracts and compounds inhibit HIV-1 replication [5]. The specific objectives were to assess the inhibitory effects of *P. amarus* on HIV-1 gag p24 protein expression and reverse transcriptase activity, and to perform molecular docking studies to understand the interaction of quercetin with HIV-1 proteins. In our experimental approach, crude extracts of *P. amarus* were prepared and tested for their antiviral activity against HIV-1 in MT-2 cells. The inhibition of HIV-1 gag p24 protein was measured using enzyme-linked immunosorbent assay (ELISA) [6]. Additionally, the extracts were evaluated for their ability to inhibit HIV-1 reverse transcriptase using a specific assay kit. Molecular docking studies were conducted to visualize and analyze the binding interactions between quercetin and HIV-1 p24 and reverse transcriptase proteins. This study highlight the potential of *P. amarus* and quercetin as promising candidates for anti-HIV-1 therapy. The findings suggest that these natural products can effectively inhibit HIV-1 replication, possibly by interfering with key viral proteins. This research not only supports the traditional use of *P. amarus* but also underscores the importance of natural products in the ongoing search for new and effective antiviral agents.

Materials and Methods:

Plant collection and extract preparation:

Fresh greenish whole plant of *P. amarus* was collected from Chennai (Fig. 1 & 2) and authenticated by Department of Botany, University of Madras, Chennai. Then it was washed with sterile distilled water until the removal of all debris. These plants were subjected to shade drying at room temperature and seed and leaf was separated. Leaf and seed were powdered by

and soaked in the aqueous and alcoholic solvents and filtered through sterile cotton wool and then with whatman no.1 filter paper and finally with 0.2µ syringe filter. Finally filtrate wasstored at -20.

Anti-HIV evaluation by HIV gag p24 inhibition Assay:

P. amarus extracts were tested for its antiviral property against human immunodeficiency virus (HIV-1). Anti-HIV-1 inhibition was measured after infecting MT-2 cells and detection of HIV-1 gag p24 by ELISA.

For the anti-HIV testing HIV-1 (Clade C), strain was used . HIV was grown on human PBMC (*Dinesh et al.,* 2016) and titrated by HIV gag p24 concentration. For the HIV p24 assays MT-2 T cell line was used. All the assays were performed in Biosafety laboratory-Level-3 (BSL-3) facilityat center for Drug Discovery and Developmetn, Sathyabama Institute of Science and Technology, Chennai. Anti-HIV testing of *P. amarus* was done as described elsewhere [5]. For anti-HIV testing, various concentrations of the *P. amarus* extracts or controls were pretreated with 15 pg of live virus and incubated for 1 hour at 37^{o}C. After this, virus/extract mixture was added to MT-2 cells ($0.3x10^{6}$ cells) and incubated at 37^{o}C for 2 hours. Cells were washed and re-suspended in 2 % RPMI and further incubated for 5 days at 37^{o}C. Cells were divided into 3 groups namely drug-positive control treated with Nonoxynol-9, another group treated with distilled water (drug-negative control) and the test group (leaf and seed extracts). After 5 days, the supernatants were collected and tested for HIV gag p24 content by ELISA (Cat. No. XB-1000). Based on the standard curve, HIV p24 concentrations in the treated cultures were calculated (Rui-Rui Wang *et al.,* 2006). All assays were repeated 3 times and the result represents the average (mean) and standard deviation (SD) of 3 experiments [3, 4].

HIV Reverse Transcriptase (RT) inhibition Assay:

In addition to HIV p24, inhibitory activity of *P. amarus* was tested for HIV-Reverse Transcriptase inhibition property , by using HIV RT assay kit (Roche USA Cat. No.11 468 120 910) was used and the assay was performed as per the manufacturers instruction and described elsewhere (Harnett *et al.,* 2005). 4 ng (20 µl) of HIV-1- RT in the reaction tube, 20 µl of leaf and seed extracts were added separately with appropriate controls and incubated for an hour at 37^{o}C. The samples were then transferred to wells of the Micro Plate module; the plate was covered and incubated for 1 hour at 37^{o}C. The plates were then washed with wash buffer for five times. To that 200 µl anti- DIG- POD working dilution was added and incubated for one hour at 37^{o}C. The plates were again washed with wash buffer five times. To that 200 µl of ABTS substrate solution was added and incubated at room temperature until a green color develops. The plate was then read at 405 nm in ELISA reader (Biotek, USA). Lysis buffer without RT was used as a negative control and Azidothymidine (AZT) with RT was used as a positive control. All assays were repeated 3 times and the result represents the average (mean) and standard deviation (SD) of 3 experiments [1-3].

Molecular Docking of HIV-1 P24 and Reverse transcriptase with chalcones:

Protein Preparation

The X-ray crystallographic structure of protein target such as NF-kappa B(PDB id: 2LE9) isolated from mus musculus was extracted from the Protein Data Bank. The protein was prepared

by removing Water molecules, ligands and other heteroatoms. Addition of hydrogen atoms to the protein was performed using CHARMm force field. Energy minimization was performed by using conjugate gradient method with an RMS gradient of 0.01kcal/Å mol on Argus lab[8-10].

Molecular Docking

The grid-based molecular docking method is used from Argus lab. 4.0.1 ver, that employs CHARMmforcefield. The target is held rigid while the ligands are allowed to be flexible docking. Since the ligands are retrieved drug bank, Canada as per GC MS result. Then the prepared ligandssuch as Quercetinwas docked to the active site using default parameters. The results of the docking enabled the ranking of the docked conformation of the ligands according to their docking score and hydrogen binding site. Based on GC MS data the antiviral Quercetin was selected as ligand for the target protein[8-9].

Analyses and Visualization of the ligand binding sites

The docking poses were ranked according to their docking scores. The scoring function in docking score was used to predict the binding affinity of one ligand to the target molecule. In addition to the structural information, each record includes the docking score reported as negative value, where the higher value indicates a more favourable binding. This enables the energy to be used like a score. This score includes internal ligand strain energy and receptor-ligand interaction energy, and is used to sort the poses of each input ligand. The molecular visualizations of the docked complexes were analyzed using the Argus lab version 4.0.1 [8-10].

Results

Anti HIV screening:

Anti-HIV evaluation by HIV gag p24 inhibition Assay:

The anti HIV activity was evaluated after pre-treating the leaf and seed extracts of *P. amarus* with known concentration of virus. Pre-treated virus then added on to MT2 cells and incubated for 5 days and the supernatants were collected and tested for HIV gag p24 activity. *P. amarus* methanol leaf extract showed maximum inhibition at 200µg/ml to HIV P24 of 87.52± 1.96 % and seed extract showed 88.74± 1.03% of inhibition. it was compared with standard drug control Nonoxynol-9. The data are statistically significant p>0.05. As anticipated the negative control shows no anti HIV activity. The detailed results were shown in Table -1&2.

Table- 1: Anti HIV-1 testing of extracts from leaf of *Phyllanthus amarus*

Number of time tested	Viral concentration (Pg/ml)	Percentage of HIV-1 p24 Inhibition				Percentage of HIV-1-RT Inhibition			
			concentration of *P. amarus* extract				concentration of *P. amarus* extract		
		Positive Control	50 µg/ml	100 µg/ml	200 µg/ml	Positive Control	50	100	200
1	59.5	87.4	63.5	74.6	85.66	92.7	62.5	72.5	82.54
2	65.5	96	66.5	75.6	87.3	76.4	56.2	73.5	86.5

				4		3			6	4
				65.8	77.3	89.5			74.8	87.5
3	66.7		95.5	5	5	6	79.4	65.4	6	6
				65.2	75.8	87.5		61.3	73.6	85.5
			92.96±**4**	9±**1.**	5±**1.**	1±**1.**	82.83±**8**	6±**4.**	4±**1.**	4±**2.**
Mean±SD		63.9±3.85	**.82**	**59**	**39**	**95**	**.67**	**7**	**18**	**65**

Table -2: Anti HIV-1 testing of extracts from Seeds of *Phyllanthus amarus*

Number of time tested	**Pg/ml**	**Percentage of HIV-1 p24 Inhibition**				**Percentage of HIV-1-RT Inhibition**			
		Positive Control: Nonoxynol-9	**concentration of *P. amarus* extract**			**Positive Control: Azidothymidine**	**concentration of *P. amarus* extract**		
			50 µg/ml	**100 µg/ml**	**200 µg/ml**		**50 µg/ml**	**100 µg/ml**	**200 µg/ml**
1	63.5	90.2	68.3	80.2	87.56	93.25	70.2	83.54	88.54
2	64.5	89.56	69.5	82.65	89.45	94.56	72.12	84.56	87.91
3	65.5	91.8	69.8	83.55	89.2	98.21	73.84	84.98	90.27
Mean± SD	64.5±1	90.52±1.15	69.20 ±0.79	82.13 ±1.73	88.74 ±1.03	95.34±2.57	72.05 ±1.82	84.36 ±0.74	88.91 ±1.22

HIV Reverse Transcriptase (RT) inhibition Assay

In this study we screened that the methanol extract of leaf and seed *P. amarus* showed 85.55 ± 65% and 88.91± 1.22% activity against RT the testing respectively. The results were statistically significant when compared with positive control. These values are statistically significant when compared to extract group (Table -1 & 2). From this study we conclude that *P. amarus* mediated HIV inhibition could be due to interfering with RT which in turn affects the cDNA synthesis and viral replication

Phytochemical analysis of *P. amarus* leaf and seed extract by GC-MS:

Phytochemical characterization of *P. amarus* leaf and seed extracts. Initial screening of phytochemical showed the presence of alkaloids, flavonoids, carbohydrates, phenols, steroids, and glycosides.

Column Chromatography: P. amarus leaf and seed methanol extracts were separated fraction wise for subject to GCMS analysis.

Gas Chromatography-Mass spectroscopy (GC-MS)

GC MS data fraction were summarized in table 3 and 4 based on molecular weight and average peak. It contain complex chemical composition. Some of the GC-MS peaks may not be identified due to lack of standards in our library. Eight compounds from leaf and twenty compounds from seed were identified. The methanol extract of the seed and leaf showed four compounds have major percentage compared to other compunds such as stigmasterol, Quercetin, gamma sitosterol

Stigmastanols inhibits biosynthesis of cholesterol and also prevent the cholesterol absorption by liver. Stigmastanol has the chemical formula of $C_{29}H_{52}O$ and chemical structure as follows

Table-3: GC- MS of *P. amarus* leaf extract

Retention time	Name of the compound	Molecular Formula	Molecular weight	Area Percentage
5.423	1,6-octadien-3-ol, 3,7-dimethyl-	$C_{10}H_{18}O$	154.24	1.92
6.236	Bicyclo[2.2.1]Heptan-2-one 1,7,7-trimethyl-	$C_{10}H_{16}O$	152.23	0.62
6.969	Benzene,1-methoxy-4-(2-propenyl)-	$C_{10}H_{12}O$	148.20	14.08
14.786	2,6,10-trimethyl,14-ethylene-14-pentacene	$C_{20}H_{38}$	278.51	0.53
17.510	6-octen-1-ol, 3,7-dimethyl-	$C_{10}H_{20}O$	156.26	0.81
23.860	Quercetin	$C_{15}H_{10}O_7$	302.23	33.91
24.398	5-(9H-Fluoren-2-Ylimino)-2-Methyl-5H-Indeno[1,2-B]Pyridine-3-carboxylate	$C_{29}H_{22}N_2O_2$	430.50	13.60
24.920	1-(3',4'-Dimethoxyphenyl)Icosan-5-one	$C_{26}H_{45}O_3$	405.63	27.11

Table 4. GC- MS of methanol seed extract

Retention time	**Name of the compound**	**Molecular Formula**	**Molecular weight**	**Area Percentage**
5.411	1,6-octadien-3-ol, 3,7-dimethyl-	$C_{10}H_{18}O$	154.24	7.61
6.225	Bicyclo[2.2.1]Heptan-2-one 1,7,7-trimethyl-	$C_{10}H_{16}O$	148.20	2.82
6.958	Benzene,1-methoxy-4-(2-propenyl)-	$C_{10}H_{12}O$	148.20	8.94
7.133	Y-Hydroxy Friedelan-3-one	$C_{30}H_{50}O$	426.72	0.35
7.544	2,6-octadienal, 3,7-dimethyl-	$C_{10}H_{16}O$	152.23	0.17
7.974	Alpha.-citral(E)-citral	$C_{10}H_{16}O$	152.23	0.17
10.048	Caryophyllene	$C_{15}H_{24}$	204.36	0.32
10.169	Bicyclo[3.1.1]Hept-2-ene, 2,6 Dimethyl-6-(4-methyl-3-pentenyl)	$C_{15}H_{24}$	204.35	0.29
10.392	1,6,10-Dodecatriene, 7,11-Dimethyl-3-Methylene	$C_{15}H_{24}$	204.35	0.09
10.520	1,4,8-Cycloundecatriene, 2,6,6,9-Tetramethyl-	$C_{15}H_{24}$	204.39	0.10
10.844	1,6-Cyclodecadiene, 1-methyl-5-methylene-8-(1-methylethyl)	$C_{15}H_{24}$	204.35	0.22
10.958	Napthalene, decahydro-4a-methyl-1-	$C_{15}H_{24}$	204.35	0.09

	methylene-7-(1-methylethenyl)			
11.039	Alpha Selinene	$C_{15}H_{24}$	204.35	0.09
11.128	2,5-Heptadiene, 2-Methyl-6-(4-methyl-3-Cyclohexen-1-yl)	$C_{15}H_{22}O$	218.33	0.04
11.523	1,4,8-Cycloundecatriene, 2,6,6,9-Tetramethyl-	$C_{15}H_{24}$	204.39	0.67
11.983	1,1,4,7-Tetramethyldecahydro-1H-Cyclopropa[E]Azulen-4-ol	$C_{15}H_{26}O$	222.36	0.05
23.863	Carbaryl-d7	$C_{12}H_4D_7NO_2$	208.26	0.22
26.163	Quercetin	$C_{15}H_{10}O_7$	302.23	5.12
26.964	Gamma-Sitosterol, Stigmast-5-en-3-ol, (3 beta., 24S)	$C_{29}H_{50}O$	414.70	62.87
27.053	Stigmasterol	$C_{29}H_{52}O$	464.38	9.45

Quercetin present in both seed and leaf extract average peak length was 5.12, Based on the GC MS data the Quercetin underwent anti HIV activity. Commercially available Quercetin (purchased from Sigma aldrish) was tested for anti HIV activity including P24 and RT assay with appropriate positive control, it showed the maximum number (>90%) of percentage of HIV-1 inhibition (table-5).

Table -5: Anti HIV-1 testing of known Quercetin

Number of repeats	**Viral Concentration Pg/ml**	**Positive control**		**Percentage of HIV-1 p24 Inhibition**	**Percentage of HIV-1-RT Inhibition**
		Nonoxynol-9	**Azidothymidine**		
1	63.5	91.2	94.85	90.35	93.2
2	64.5	92.5	94.25	91.25	94.01
3	65.5	92.8	95.25	94.56	94.5
Mean±SD	64.5±1	92.17±0.8	94.78±0.5	92.05±2.2	93.90±0.66

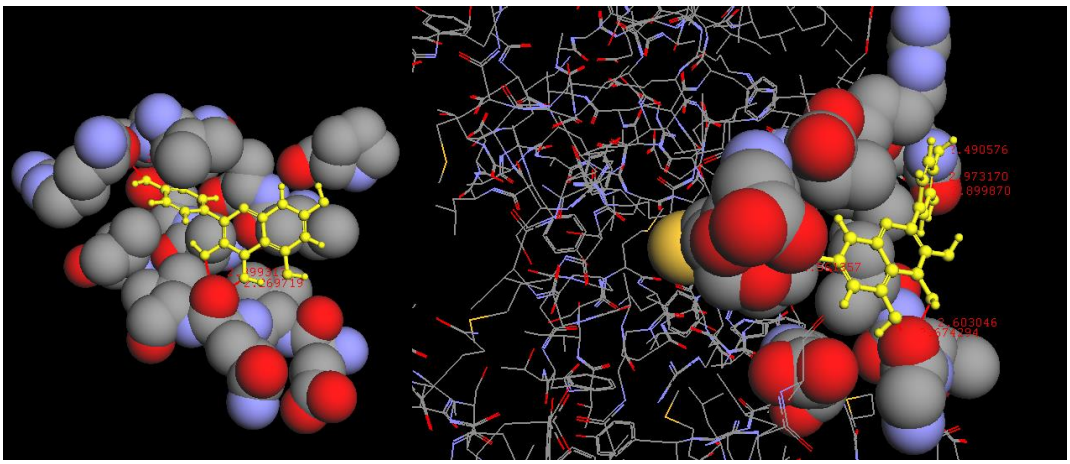
Docking studies:

As per data obtained from GC-MS, Quercetin compound was downloaded in .mol format from drug bank. Stigmanosterol, gamma sisosterol and Quercetin were docked with HIV P24 (Pdb id: 1SJE) and HIV RT (PDB id: 2JLE) protein targets, the above three ligands were not bind with HIV proteins. Based on the literature survey. Quercetin, stigmansterol and gamma sitosterol docked with regulatory proteins. Based on the result Quercetin considered docked with NF-Kappa B (PDB id: 2LE9) protein support the observation of the above proteins docked with Quercetin (Table -6; Figure -1). Ligands in the docking score in the range of -8 to -11 kcal/mol in

Table-6: Docking scores of Quercetin compound isolated from *P. amarus*against NF-kappa B

Target name	Polypeptide	'H' Bond	Score	Kcal/mol	Amino acid positions	Distance
NF-kappa B (musmusculus; PDB id: 2LE9)	Chain A	3	-9.53254	-9.9845	125ASP	2.99
					124ARG	0.76
					128GLN	2.03
	Chain B	6	-8.53395	-8.74145	332ARG	2.41
					1065GLN	2.66
					296GLY	2.85
					297ASP	2.74
					298PHE	2.56
	Chain E	4	-9.12806	-9.40705	1065gln	2.66
					1211TYR	2.95
					1095TYR	2.87
					1182LYS	2.63
					1179cys	2.94
	Chain F	5	-7.3555	-7.38762	1473ARG	2.5
					1432VAL	2.8
					1434THR	2.89
					1475TYR	2.78
					1473ARG	2.26

Fig-1: Molecular interactions of the targetsNF-kappa B(a) amino acid interaction of Chain A with Quercetin and b.DDL (b) residue with the Quercetin



Discussion

The intricate nature of HIV infection presents substantial obstacles to the creation of efficacious treatment strategies. According to Erickson and Burt (1996), the complex structure and functions of the virus require a comprehensive approach to treatment. Although highly active antiretroviral therapy (HAART) has significantly transformed the treatment of HIV/AIDS by specifically targeting viruses in the bloodstream, it frequently does not completely eradicate viruses that are hiding inside cells. This results in the virus's continued presence and the development of strains

that are resistant to the drugs used in therapy.Within this particular framework, natural compounds have emerged as highly potential reservoirs of innovative antiviral medicines.

Phyllanthus amarus, a herbal remedy commonly employed in Indian medical practices, has attracted interest due to its potential as an anti-HIV agent. Rangasamy Balamurugan et al. (year) conducted a study that focused on the antiviral properties of *P. amarus* extracts, particularly their capacity to inhibit HIV-1 replication.The assessment of its effectiveness against HIV entailed conducting in vitro experiments on raw extracts derived from the leaves and seeds of *P. amarus*. Table 1 and Table 2 clearly show that *P. amarus* extracts can stop HIV-1 p24 antigen and reverse transcriptase (RT) from working. For p24, the inhibition percentages range from 87.4% to 92.96%, and for RT, they range from 82.83% to 95.34%. The data indicate that *P. amarus* has strong anti-HIV activity, which may be due to its bioactive components.We subjected the *P. amarus* extracts to phytochemical examination using gas chromatography-mass spectrometry (GC-MS) [7], which revealed the presence of several substances such as quercetin, stigmasterol, and gamma-sitosterol.

Quercetin, specifically, emerged as a prominent constituent with well-established antiviral characteristics. More research into the isolated quercetin showed that it was very good at fighting HIV, with inhibition percentages similar to those seen with nonoxynol-9 and azidothymidine (Table 5) [6]. Molecular docking experiments further elucidated Quercetin's method of action against HIV-1. Quercetin had good interactions with NF-kappa B, which is a key transcription factor for HIV-1 replication. By blocking NF-kappa B activity, quercetin can stop viruses from copying and tranforming. This is one of the ways it fights viruses.The reported antiretroviral action of *P. amarus* and its component, quercetin, is consistent with earlier studies on the therapeutic benefits of this plant genus. Several Researchstudies has shown that Phyllanthus species exhibit anti-HIV properties [8-10].

These effects are believed to be the result of different processes, such as the suppression of viral binding and reverse transcriptase activity.Moreover, the use of natural compounds such as *P. amarus* presents prospective benefits, such as fewer adverse effects and decreased expenses in medication development when compared to synthetic pharmaceuticals. This study emphasises the need to investigate traditional medicinal plants as potential sources of new antiretroviral substances. It also emphasizes quercetin's therapeutic potential in treating HIV/AIDS.To summarize, the study provides strong evidence of the anti-HIV effects of *P. amarus* extracts, specifically quercetin. These findings suggest the need for additional research on the precise mechanisms of action and therapeutic effectiveness of *P. amarus* and its components. Furthermore, the progress in developing quercetin-based therapies shows potential for addressing the persistent challenges presented by HIV/AIDS and propelling the field of drug discovery using natural products.

Conclusion:

The study showcases the strong antiviral effects of Phyllanthus amarus extracts, specifically quercetin, against HIV. The results emphasize the therapeutic capacity of natural substances in fighting against HIV/AIDS, as well as the importance of further investigation into their mechanisms of action and clinical uses. Quercetin has great potential as a candidate for developing new antiretroviral treatments. By investigating traditional medicinal plants such as *P.*

amarus, we can gain useful knowledge to tackle the intricate problems associated with HIV infection and enhance the progress of drug discovery endeavours.

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