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Insilico Investigation of Antiviral Bioactive Compounds in Ethanol Extract of *Phyllantus amarus* Leaf

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Abstract

The plant *Phyllantus amarus*, also known as "Stonebreaker" or "ewe eyin olobe," is a small herbaceous plant that has garnered significant attention in the scientific dormitory due to its wide array of medicinal properties. Native to tropical regions, including parts of Africa, Asia, and South America, this plant has been used traditionally in folk medicine for centuries, particularly for the treatment of liver and kidney disorders, malaria, and gastrointestinal issues. It was revealed that many orthodox antiviral drugs have significant side effects, thus, several traditional plants have been found to have diverse pharmacological functions with little or no toxicological study was done to confirm the side effect of *Phyllantus amarus.* On this background, the in vitro and insilico investigation on antiviral bioactive compounds of *Phyllantus amarus* were established in this study. Several analytical methods were used for in vitro identification of bioactive compounds and molecular docking was applied for the insilico study respectively. The results depict that flavonoids, saponin, phenolic compounds, terpenoid, were found in the ethanol leaf extract of *Phyllantus amarus*, the antioxidant properties; ABTS, nitric oxide, FRAP and hydrogen peroxide the ethanol leaf of *Phyllantus amarus* extract had high scavenging capacity with BHT used as control. The bioactive compounds; ellergic, quercetin, corillagin, phyllantin, hypophyllantin etc were identified in this study to confirm the already identified ones in some researches and they were docked against some antiviral drugs purported for hepatitis B. Although, the identified bioactive candidates had relatively good antiviral performances It is against this backdrop that it can be established that *Phyllantus amarus* leaf possess some antiviral bioactive compounds, upon which invivo study can be conducted for further investigation.

Keywords: Insilico, bioactive compound, in vitro, antiviral

1.0 Introduction

Phyllantus amarus, commonly known as "Stonebreaker" or "Ewe eyin olobe in Yoruba,"a small herbaceous plant that has garnered significant attention in the scientific community due to its wide array of medicinal properties. Native to tropical regions, including parts of Africa, Asia, and South America, this plant has been used traditionally in folk medicine for

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centuries, particularly for the treatment of liver and kidney disorders, malaria, and gastrointestinal issues [1]. Going by its reputation for being a versatile therapeutic plant, *Phyllantus amarus* has become the subject of various phytochemical and pharmacological investigations. The *in vitro* identification of bioactive compounds in this plant is crucial for understanding its therapeutic potential and for the development of new drugs that can be used to combat various health conditions. The therapeutic properties of the plants are largely attributed to its rich phytochemical composition, which includes phenolic compounds such as alkaloids, flavonoids, tannins, terpenoids, lignans, and others. These secondary metabolites which are produced by the plant as a defense mechanism against herbivores and pathogens, also exhibit a variety of biological activities, including antioxidant, antimicrobial, anti-inflammatory, antiviral, and anticancer properties. Understanding the specific bioactive compounds present in *Phyllantus amarus* and their effects on human health will provide valuable insights into how the plant can be utilized in modern medicine [2]. The alkaloids found in *Phyllantus amarus* are among the most well-studied bioactive compounds in the plant. These nitrogen-containing compounds have been shown to possess a wide range of pharmacological activities, including antimicrobial, antimalarial, and anticancer effects [3]. Some studies have even suggested that alkaloids from *Phyllantus amarus* could be used in the treatment of liver disorders, particularly in hepatitis B infection, due to their ability to inhibit the replication of the hepatitis B virus [4]. Furthermore, flavonoids, which are another class of bioactive compounds found in *Phyllantus amarus*, are known for their antioxidant and anti-inflammatory properties, making them beneficial for the management of oxidative stress-related diseases, such as cardiovascular disorders and diabetes [5].

The process of identifying these bioactive compounds involves advanced techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy. These techniques enable the precise confirmation and quantification of the chemical constituents in plant extracts, ensuring that the bioactive compounds responsible for therapeutic effects are accurately characterized. By using these methods, researchers can also identify the most potent compounds in *Phyllantus amarus* that could potentially be isolated and further developed for pharmaceutical use [6]. This work was carried out to affirm the identification of antiviral bioactive compounds as established by some Researchers and amplify the investigation through insilico study.

Statement of Problem

Despite advances in antiviral drug development, many viral infections continue to pose significant

public health challenges, particularly in low- and middle-income countries. Conventional antiviral drugs are often limited by **high cost**, **drug resistance**, **restricted availability**, and **adverse side effects**, making the search for safer, affordable, and more effective alternatives a global priority. Medicinal plants have gained increasing attention as potential sources of novel antiviral agents, and among them, *Phyllanthus amarus* has long been used in traditional medicine for the treatment of various infectious diseases.

Although *Phyllanthus amarus* is reported to possess antiviral activities especially against hepatitis B virus, dengue virus, and other pathogenic viruses **scientific evidence supporting these claims remains inadequate, inconsistent, or poorly standardized**. The mechanisms of action, bioactive compounds responsible for the antiviral activity, and the safety profile of the plant are also not fully understood. In addition, variations in plant chemistry due to geographical origin, extraction methods, and dosage form pose challenges to reproducibility and reliability of results.

Furthermore, **there is limited research using modern virological techniques**, such as molecular assays, cell-based antiviral screening, and viral inhibition kinetics, to validate the antiviral effectiveness of *Phyllanthus amarus*. Without robust scientific evaluation, its potential cannot be fully exploited in the development of phytomedicines or complementary antiviral therapies.

Therefore, there is a critical need for **systematic, evidence-based investigation** of the antiviral properties of *Phyllanthus amarus*, including isolation of active constituents, assessment of antiviral efficacy using standardized models, and evaluation of its safety profile. This will help bridge the knowledge gap between traditional claims and modern scientific validation.

Aims of this study

The aim is to identify and characterize the antiviral bioactive compounds present in *Phyllantus amarus* through *in vitro* analysis.

Objectives

- To obtain plant extract using dilute 95% ethanol
- To identify and quantify ethanol leave extract of *Phyllantus amarus* bioactive compounds using HPLC
- To further confirm the evaluation of the antioxidant properties of the leave plant extract.
- identification and characterization of the antiviral bioactive compounds via insilico studies

2.0 Methodology

2.1 Preparation of plant extract

The plant was obtained at different locations; Shao area in Moro local government, Oja- tuntun

Ilorin, Oja-oba Ilorin and Malete, Moro local government, Kwara State, Nigeria. The plant after collection was washed with clean water and air dried for a week after 12g of the pulverized sample was macerated and soaked in 95% ethanol for 72 hours. The resulting solution was filtered and concentrated over a water bath to afford a crude ethanol extract of the leaves of *Phyllantus amarus*.

2.2 Identification of bioactive compounds in the leaf extract

This was achieved using a reverse phase C18 column with the following parameters was used :Mobile Phase: Water:Acetonitrile (Gradient, 0.1% formic acid), flow rate of 1.0mL/min,detection of UV at 254–280 nm, injection Volume: 10-20 µL and was run at time of 30–45 minutes to identify the antiviral bioacvtive compounds in the leaves extract of *Phyllantus amarus.*

2.3 Antioxidant Assay

2.3.1 Nitric oxide scavenging activity

The nitric oxide scavenging activity of the leave extract of *Phyllantus amarus* as determined according to the method of Marcocci briefly, a 2 ml of the leave extracts of *Phyllantus amarus* with varying concentrations (250–1000 µg/ml) was incubated with 0.5 ml of sodium nitroprusside (5 mM) for 2 h at 27°C. 1 ml of aliquot of each of them the incubated mixture and was mixed with 0.6 ml of Griess reagent (1.0 mL sulfanilic acid reagent [0.33%] in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthyl ethylenediamine dichloride [0.1%]). The absorbance was measured immediately at 550 nm, and synthetic antioxidant Butylated Hydroxy Toluene (BHT) used as positive control. The experiments was performed in triplicates, and scavenging activity ~~was~~ expressed as percentage scavenging, using the following formula.

$$\% \text{ Scavenging} = ([Abs_{control} - Abs_{samples}]/ Abs_{control}) \times 100$$

2.3.2 FRAP Assay

Reagents:

a) Acetate buffer 300 mM pH 3.6: A 3.1g sodium acetate trihydrate was weighed and added to 16 ml of glacial acetic acid and made the volume to 1L with distilled water

b) TPTZ (2, 4, 6-tripyridyl-s- triazine): M.W. 312.34), 0 mM in 0.1M HCl (M.W. 36.46)

c)A hydrated iron chloride with M.W of 270.30 was used

The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1w/v was used before testing. The Standard was FeSO4. 7 H2O: 0.1 in 1.5 Milimolar in methanol. All the regents were prepared from Merck (Germany) company.

Procedure

A FRAP solution (3.6 m) was added to distilled water (0.4 mL) and incubated at 37˚C for 5 min. Then this solution mixed with certain

concentration of the plant extract (80 mL) and incubated at 37˚C for 10 min. The absorbance of the reaction mixture was measured at 593 nm. For construction of the calibration curve, five concentrations of the standard $_{FeSO4.7H2O}$ in (0.1, 0.4, 0.8, 1, 1.12, 1.5 mM) were used and the absorbance values were measured as for ethanol leave extract of *Phyllantus amarus* [7].

2.3.3 ABTS Radical Cation Decolorization Assay

A 2,2′-Azino-bis radical cation ($ABTS^{\bullet+}$) decolorization was measured as described by Pellegrini et al. with minor modifications. $ABTS^{\bullet+}$ solution was prepared by mixing aqueous ABTS (7 mM) solution with 2.45 mM potassium persulfate (1:1 *v*/*v*) and incubating in darkness at 25℃ for 16 h. The working solution was then obtained by diluting 5ml of$ABTS^{\bullet+}$ solution in methanol and an absorbance of 0.70 ± 0.05 at 734 nm. In each well of a 96 well-plate, 25 µL of leave extract of Phyllantus amarus as added to 200 µL of the working solution. After a slight shake, the plate was covered with an aluminum foil and was kept at room 25℃for 30 min. Subsequently, the absorbance was recorded by a Multiskan^{TM} Microplate Spectrophotometer (Thermo Fisher Scientific, Osaka, Japan). The ABTS radical decolorizing activity of leave extract of *Phyllantus amarus* was calculated using the following formular.

ABTS radical decolorizing activity (%) = $(1 - A_{sample}/A_{control}) \times 100$

2.3.4 Free radical scavenging activity (2,2-diphenyl-1-picrylhydrazyl) DPPH

Free radical scavenging activity of leave extract of *Phyllantus amarus* as determined according to the method of [8] briefly, 500 µl of 0.3 mM alcoholic solution of DPPH (Himedia, India) was added to 2.5 ml of the leave extract to varying concentrations (250–1000 µg/ml). The mixtures was incubated in dark for 30 min, and absorbance was measured at 518 nm using UV-visible spectrophotometer (Systronics AU-2700, India). Synthetic antioxidant butylated hydroxytoluene (BHT) was used as positive control. The experiments were performed in triplicates, and scavenging activity of the leave extract as expressed as percentage inhibition, using the following formula.

% Scavenging $=([Abs_{control} - Abs_{samples}]/ Abs_{control}) \times 100$

2.4 In-silico identification of antiviral bioactive compounds in ethanol leave extract *Phyllantus amarus*

A Molecular docking of antiviral bioactive compounds in ethanol leave extract *Phyllantus marus* was carried out as described by Morris with slight modifications. Based on ligand-protein interaction, the binding affinity of the bioactive compounds in *Phyllantus amarus*, a

ligand for the binding site of the marker enzyme was elucidated using AutoDock software version 4.2. The *Phyllantus amarus* bioactive compounds -binding pocket applying standard procedure.

Based on Lipinski rule of 5, four antiretroviral drugs were docked as reference drugs in comparison to the docked identified *Phyllantus amarus* bioactive compounds. The structures of adefovir diphosphate, tenofovir phosphate and diphosphate and Lamivudine were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov). Tertiary and 3D structures of target proteins were obtained from the Protein Data Bank (PDB) and the molecular dynamics simulation software Gromacs version 5.1.2 [9].

The results in terms of binding affinity were expressed as binding energy in kcal/mol. Each drug reference was docked into the enzyme-binding pocket to determine the protein-ligand interactions between each of the reference drug. Protein-ligand interactions between each drug and target enzyme was analysed using PyMOL software version.

3.0 Results

3.1 Bioactive compounds identified in ethanol extract of *P. amarus* leaf

Six bioactive Compounds were identified and quantified in the ethanol leave extract of *Phyllanthus amarus*. These compounds include Gallic Acid, Ellagic Acid, Phyllanthin, Corrillagin, Quercetin and Hypophyllantin respectively which contribute to the medicinal properties of the plant collectively. The following chromatogram represents the retention times and intensities of identified compounds.

Corilagin

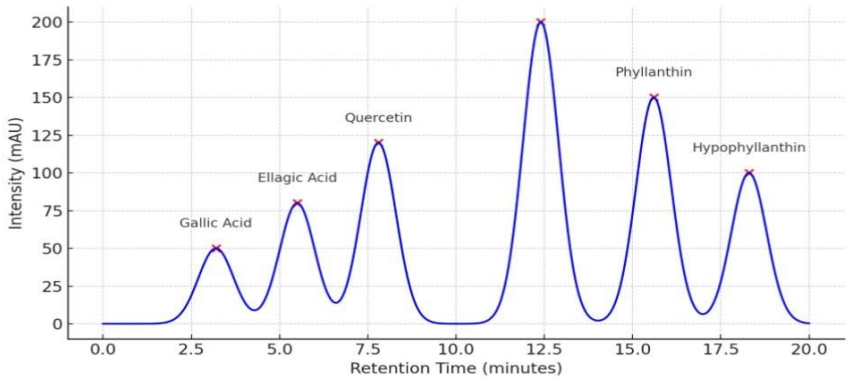


Figure 1: HPLC Chromatogram of Ethanolic *Phyllantus amarus* Extract

3.2 *In vitro* antioxidant Activity of ethanol *Phyllanthus amarus* leave extract

3.2.1 Nitric Oxide scavenging assay

The scavenging effect of extract on nitric oxide was concentration dependent as shown in table below:

Table 1: Nitric Oxide (NO) scavenging assay of extract

Concentration (µg/ml)	Percentage Inhibition (%)
1000	66.90±0.56
500	59.40±0.31
250	51.95±0.27
125	43.44±0.20
62.5	37.17±0.78
31.25	31.10±0.10
15.625	25.01±0.64
7.8125	20.33±0.26

Values are expressed as Mean ± SEM of three replicates.

3.2.2 Table 2: Free radical scavenging potential of ethanol extract of P. amarus in comparison with BHT

Samples	FRAP (Mmol Fe 2^{+} /g)
Ethanol extract of *Phyllantus amarus* leaves	1.34±0.03
BHT	2.08±0.07

Values are expressed as Mean ± SEM of three replicates.

3.2.3 ABTS scavenging assay

The oxidation of ABTS with potassium persulfate generates ABTS radical cation (17). This radical gets reduced in the presence of hydrogen donating antioxidants (35).Table 3: The ABTS % scavenging activity of Phyllanthus amarus leave extract was given in table 3 below:

Table 3: Free radical scavenging activities of of the ethanol leave extract of Phyllantus amarus in ABTS assay

Concentration (µg/ml)	Percentage Inhibition (%)
1000	51.13±0.66
500	40.17±4.19
250	34.17±2.95
125	26.35±4.08
62.5	23.90±1.04
31.25	21.13±0.37
15.625	21.27±1.89
7.8125	17.45±0.67

Values are expressed as Mean ± SEM of three replicates was also dose-dependent

3.2.4 DPPH Free Radical Scavenging Assay

The free radical scavenging effect of extract on DPPH was concentration dependent as shown in table below:

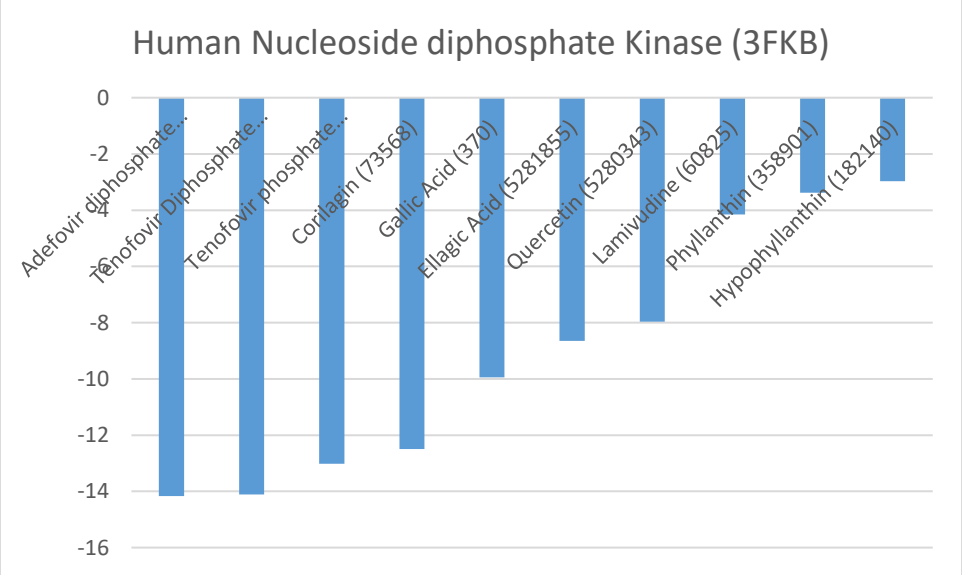
Table 4: DPPH Free Radical Scavenging Assay

Concentration (µg/ml)	Percentage Inhibition (%)
1000	60.22±0.48
500	49.96±0.33
250	45.12±0.43

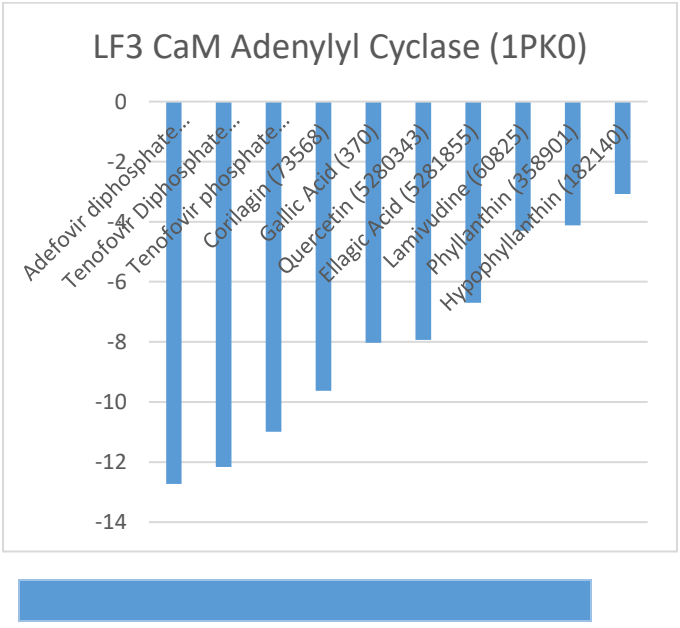
125	39.19±0.23
62.5	32.40±0.33
31.25	26.57±0.13
15.625	24.57±0.13
7.8125	19.91±0.23

Values are expressed as Mean ± SEM of three replicates was also dose-dependent

3.3 Insilico analysis on antiviral compounds in ethanol extract of *Phyllantus amarus* leaves

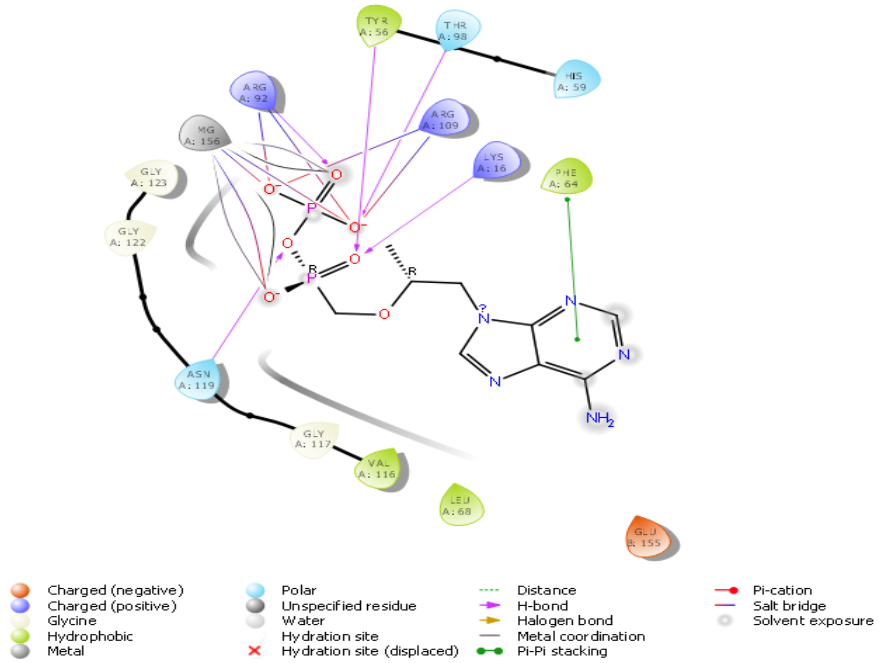


Human Nucleoside diphosphate Kinase (3FKB)	
Adefovir diphosphate (447863)	-14.169
Tenofovir Diphosphate (5481180)	-14.115
Tenofovir phosphate (6320284)	-13.018
Corilagin (73568)	-12.496
Gallic Acid (370)	-9.943
Ellagic Acid (5281855)	-8.652
Quercetin (5280343)	-7.967
Lamivudine (60825)	-4.148
Phyllanthin (358901)	-3.381
Hypophyllanthin (182140)	-2.971

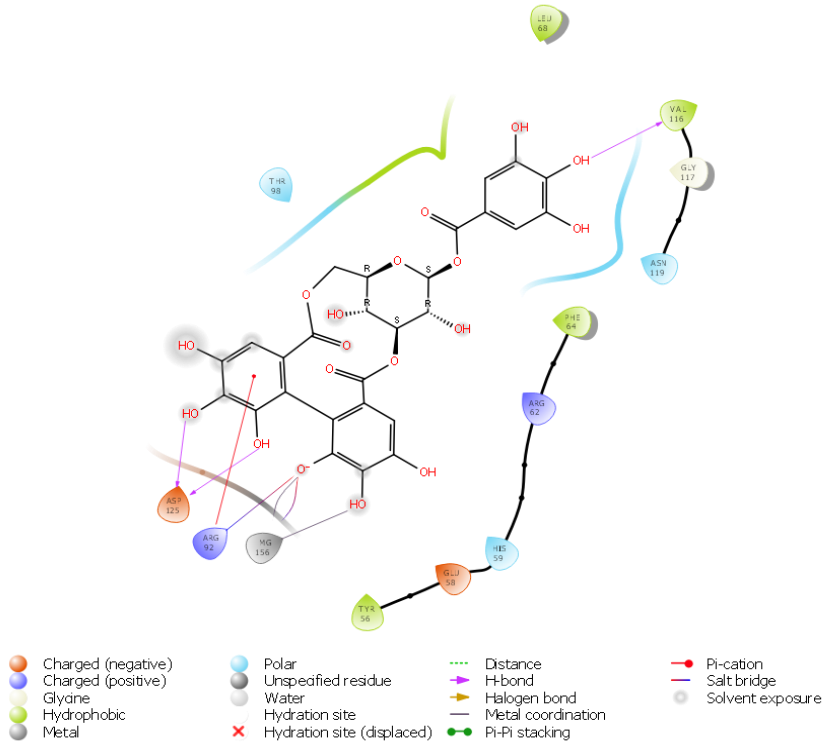


LF3 CaM Adenylyl Cyclase (1PK0)	
Adefovir diphosphate (447863)	-12.724
Tenofovir Diphosphate (5481180)	-12.166
Tenofovir phosphate (6320284)	-10.99
Corilagin (73568)	-9.628

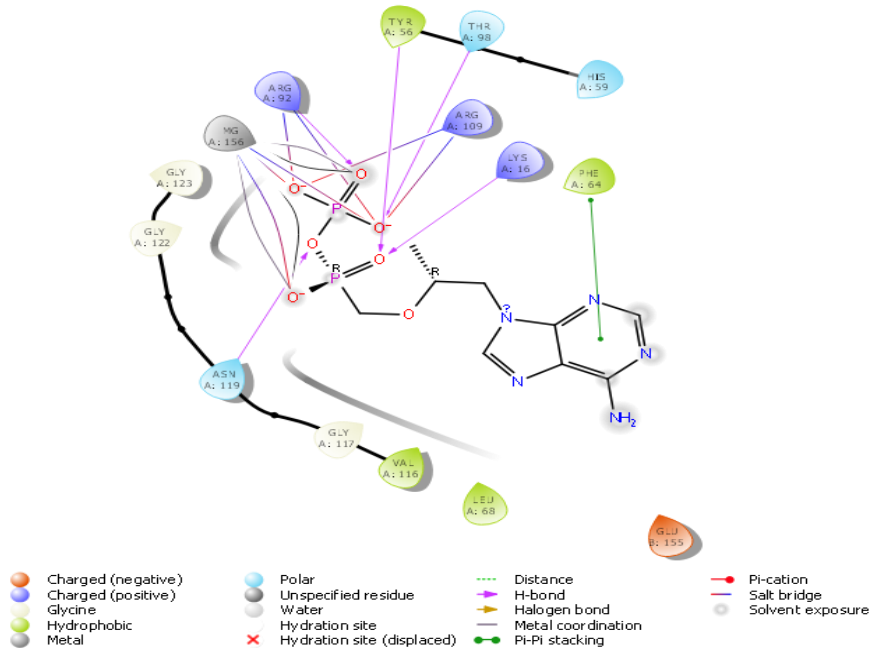
Gallic Acid (370)	-8.031
Quercetin (5280343)	-7.934
Ellagic Acid (5281855)	-6.693
Lamivudine (60825)	-4.302
Phyllanthin (358901)	-4.116
Hypophyllanthin (182140)	-3.081



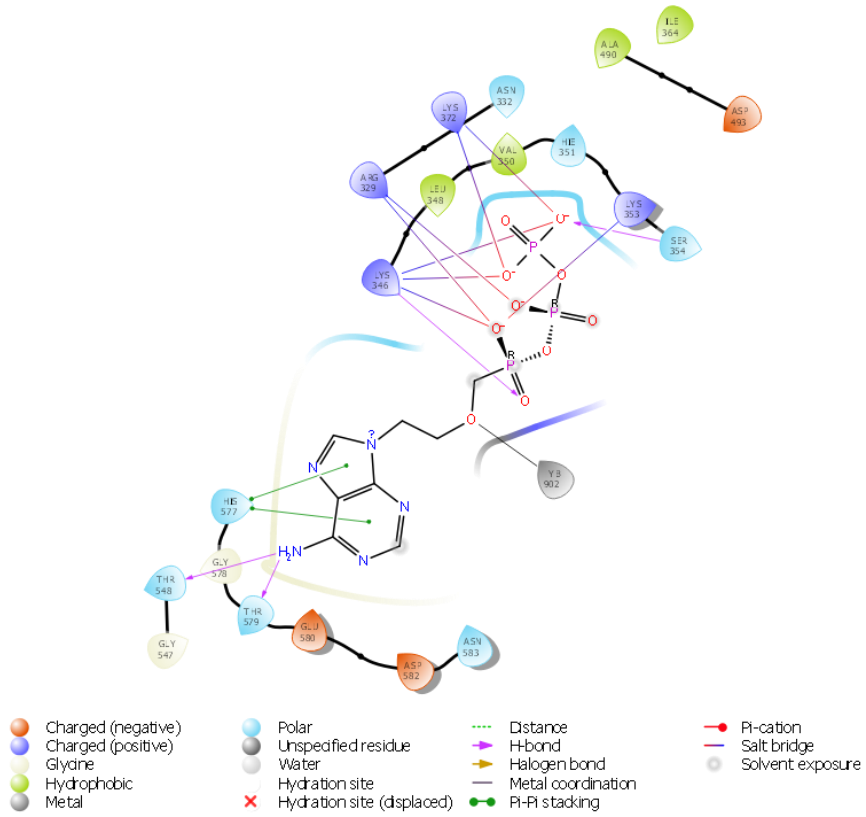
3FKB- Adefovir diphosphate



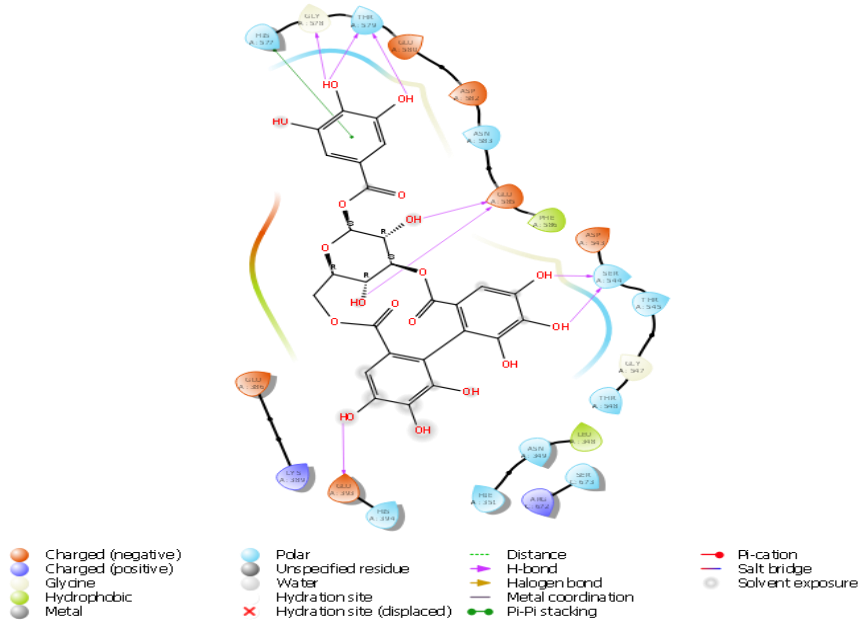
3FKB- Corilagin



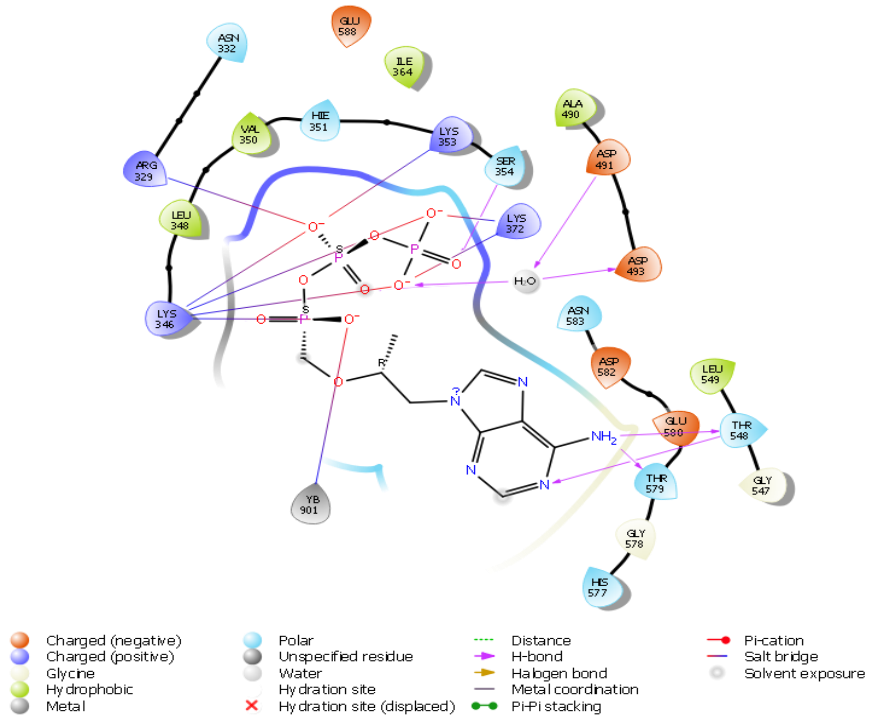
3FKB- Tenofovir diphosphate



1PKO- Adevofir diphosphate



1PKO- Corilagin



1PKO- Tenofovir diphosphate

Results interpretation

- **Binding trends are highly consistent across both targets.** The same four compounds lead the pack in the same order for both proteins: adefovir diphosphate ≈ tenofovir diphosphate > tenofovir phosphate > corilagin. The rank order across targets is **near-identical** (Spearman ≈ 0.99; i.e., almost perfect agreement).

- **Nucleotide di-/phosphates dominate.** The diphosphate forms (adefovir, tenofovir) dock much more strongly than the monophosphate (tenofovir phosphate) and far beyond the aglycones/polyphenols.

- **Human NDPK (3FKB) is predicted to bind these ligands more tightly than CaM-activated adenylyl cyclase (1PK0).** For most ligands, 3FKB scores are ~1–3 kcal/mol more favorable (i.e., stronger) than 1PK0.

- **Polyphenols (corilagin ≫ gallic > quercetin ≈ ellagic)** are mid-tier binders; classic nucleoside analog **lamivudine** is weak in its base form (triphosphate not tested), and the lipophilic **phyllanthin/hypophyllanthin** are very weak—unsurprising for a phosphate-rich ATP site.

9. Phyllanthin −3.381
10. Hypophyllanthin −2.971

Quick ranking (more negative = stronger predicted binding)

3FKB – Human Nucleoside Diphosphate Kinase

1. Adefovir diphosphate −14.169
2. Tenofovir diphosphate −14.115
3. Tenofovir phosphate −13.018
4. Corilagin −12.496
5. Gallic acid −9.943
6. Ellagic acid −8.652
7. Quercetin −7.967
8. Lamivudine −4.148

1PK0 – CaM-activated Adenylyl Cyclase

1. Adefovir diphosphate −12.724
2. Tenofovir diphosphate −12.166
3. Tenofovir phosphate −10.990
4. Corilagin −9.628
5. Gallic acid −8.031
6. Quercetin −7.934
7. Ellagic acid −6.693
8. Lamivudine −4.302
9. Phyllanthin −4.116
10. Hypophyllanthin −3.081

How strong is "strong"? (very rough affinity back-calculation)

If we (cautiously) translate ΔGdock to Kd at 298 K via ΔG ≈ RT ln Kd, you'd get **ballpark** Kd estimates like:

Ligand	**3FKB Kd (approx)**	**1PK0 Kd (approx)**
Adefovir diphosphate	**0.04 nM**	**0.47 nM**
Tenofovir diphosphate	**0.04 nM**	**1.21 nM**
Tenofovir phosphate	**0.29 nM**	**8.78 nM**
Corilagin	**0.69 nM**	**87.5 nM**
Gallic acid	51 nM	1.30 μM
Ellagic acid	454 nM	12.4 μM
Quercetin	1.44 μM	1.53 μM

4.0 Discussion

The use of ethanol extract of *Phyllanthus amarus* leaves as a source of antioxidant and antiviral chemicals was supported by the ethanol extract with notable phytochemical richness and bioactivity potential. The extract passed the qualitative phytochemical screening for phenols, saponins, tannins, flavonoids, and alkaloids and protein. The crude extract revealed the antioxidant properties as shown in the result of phytochemical results obtained in the previous research. Similar chemicals, especially polyphenols and alkaloids, were found to be abundant in extracts made with polar solvents like ethanol by [10], who conducted a thorough evaluation of *Phyllanthus amarus* and reported similar results to the phytochemical profile seen in this study. This consistency in phytochemical content provides a foundation for verification of the biological effects of the exract.

The antioxidant activity of the *Phyllanthus amarus* extract was assessed using multiple standard assays to ensure high validation. In the nitric oxide free radical scavenging assay, the extract exhibited a maximum inhibition of 66.90% at the highest tested concentration of 1000 µg/ml. This result is similar to that obtained by [11], who found nitric oxide scavenging activity around 62% at the same concentration indicating a radical scavenging potential.

The FRAP assay showed a minimum inhibition 1.3% compared to the standard which contractdicts the nitric oxide findings and the antioxidant capacity of the extract. In the ABTS scavenging assay, the extract demonstrated its strongest inhibition at 51.13% at 1000 µg/ml, suggesting an anti-oxidant mechanism potentially mediated by the phenolic content. his agrees with the report by [12], who emphasized that nitric oxide scavenging is one of the major pathways by which *Phyllanthus amarus* exerts therapeutic action, particularly in inflammatory conditions. Additionally, the DPPH assay quantified the reducing power of the extract , which, while lower than the standard butylated hydroxytoluene (BHT), still signifies considerable antioxidant capacity. [13] recorded a comparable FRAP value (1.36 mmol Fe^{2+}/g) for zinc oxide nanoparticles synthesized using *Phyllanthus amar*us leaf extract, further confirming the plant's reducing potential. These antioxidant assays collectively shows the presence of compounds capable of neutralizing free radicals and oxidative stress, which are often involved in the pathogenesis of viral diseases and chronic inflammation.

High-performance liquid chromatography (HPLC) analysis provided detailed insight into the specific bioactive molecules present in the extract. Identified compounds included gallic acid, ellagic acid, quercetin, corilagin, phyllanthin, and hypophyllanthin, all of which are known for their pharmacological importance. Gallic acid and ellagic acid, both hydrolysable tannins, have well-established antioxidant and anti-inflammatory activities, contributing significantly to the observed radical scavenging effects. These phenolics have been shown to modulate inflammatory cytokines and inhibit viral gene expression. [14] documented the presence of these compounds in *Phyllanthus amarus* and attributed the plant's antimicrobial and antioxidant properties to their actions. Quercetin, a flavonoid, has been recognized for its potent antiviral activity which acts by blocking viral entry, replication, and protease activity, particularly in viruses such as influenza, dengue, and certain strains of coronavirus. It is considered a promising lead compound for natural antiviral therapies due to its broad spectrum of action and relatively low toxicity. Studies including those by [12] and [14] confirm its consistent presence in Phyllanthus extracts, reinforcing its relevance.

Most importantly, corilagin and phyllanthin, two lignans prominently found in *Phyllanthus amarus,* have shown specific antiviral effects. Corilagin was shown by [1] to significantly inhibit hepatitis C virus (HCV) replication *in-vitro*, acting by blocking viral RNA replication and reducing pro-inflammatory cytokine release. This supports the idea that *Phyllanthus amarus* extract could optimized for use against hepatitis-related viral infections. Similarly, phyllanthin and hypophyllanthin have demonstrated inhibitory effects against hepatitis B virus (HBV) surface antigen production and replication. (50) highlighted their mechanism as involving inhibition of DNA polymerase activity required for HBV genome replication. Thus, the presence of these compounds in the extract directly aligns

with previous pharmacological findings, validating the selection of Phyllanthus amarus for antiviral exploration.

Both targets have **phosphate-rich nucleotide pockets** (NDPK binds NTP/NDP; adenylyl cyclase binds ATP). Adding **extra phosphate groups** dramatically increases:

- **Electrostatic complementarity** to Lys/Arg-rich P-loops and metal-coordinated regions.
- **Hydrogen-bonding density** with backbone amides and polar side chains.
  That's why **adefovir/tenofovir diphosphates ≫ monophosphate ≫ neutral bases**.

Corilagin and other polyphenols

Corilagin's high H-bond donor/acceptor count lets it "plug" into polar pockets and form multi-dentate networks, explaining its strong scores (especially in 3FKB). However, polyphenolic **PAINS** liability (redox activity, metal chelation, aggregation, fluorescence) can **inflate docking/assay signals**. Treat as **tentative** until counterscreened.

Weak binders (lamivudine base; phyllanthin/hypophyllanthin)

- **Lamivudine** docks poorly as a neutral base (−4 kcal/mol). Its **active triphosphate** metabolite would be the relevant comparator; without the triphosphate tail it simply won't compete well in ATP/NDP pockets.

Phyllanthin/hypophyllanthin

- are lipophilic lignans with few H-bonding handles; they're mismatched to polar phosphate pockets—hence weak scores.

Cross-target selectivity & off-target risk

Compute ΔΔG = ΔG(3FKB) − ΔG(1PK0):

- Diphosphates and corilagin are **~1.4–2.9 kcal/mol more favorable** for **3FKB** than **1PK0**. That implies **higher risk of off-target engagement of human NDPK** if you pursue them as adenylyl cyclase inhibitors.
- Quercetin and the lignans show minimal selectivity (all weak anyway).

Practical implication: If the therapeutic goal is to inhibit a pathogen's or toxin's **calmodulin-activated adenylyl cyclase**, these nucleotide analog diphosphates *may* bind—but they also look **even stickier to human NDPK**, which could perturb host nucleotide homeostasis (undesirable). You'd need to **engineer selectivity** away from NDPK.

Pocket/pose logic you can check in your figures

For the poses you mention (e.g., "3FKB–Adefovir diphosphate," "1PK0–Tenofovir diphosphate"):

- Look for **bidentate/bridged interactions** of the β/γ phosphates with **Mg^{2+}/Mn^{2+}** (if present) and nearby Lys/Arg residues in the P-loop; the **nucleobase** should stack or H-bond near conserved polar residues that recognize adenine/guanine analogs.
- In **NDPK**, the **catalytic His** (e.g., His118 in many NDPKs) and surrounding Lys/Arg often cradle the phosphate chain; very strong

diphosphate scores suggest the ligand is sitting where the **γ-phosphate transfer** chemistry occurs.

- In **adenylyl cyclase**, check orientation toward the **ATP α/β/γ site** and any **calmodulin-induced gating residues**; if the polyphenols sit shallow or occlude the entry channel, expect **non-competitive** behavior; if they overlay ATP, expect **competitive** inhibition.

SAR signals to carry forward

- **Phosphorylation state drives affinity** (DP > MP ≫ base). If cell entry is a concern, consider **prodrug masks** (as with adefovir dipivoxil, TDF/TAF for tenofovir) while designing **intracellularly unmasked DP/TP** forms.
- **Scaffold polarity vs. pocket polarity.** Highly polar, anionic ligands fit; hydrophobic lignans don't.
- **Polyphenol caution.** Corilagin is a potent in-silico binder but a *classic* interference risk. Derivatize to reduce redox/aggregation while preserving H-bond geometry—or down-prioritize after early counterscreens.

What to validate next (prioritized)

1. **Enzyme assays with ATP/NDP present at physiological levels.**
    - **NDPK:** measure NDP↔NTP exchange; check **competitive inhibition vs. NDP/NTP**.
    - **Adenylyl cyclase:** measure **cAMP production** ± calmodulin; determine **IC_{50} & Ki** vs. **ATP**.
2. **Selectivity panel.** Counter-screen top hits against **human NDPK** if your target is AC, and against **other kinases/phosphotransferases** to detect phosphate-site promiscuity.
3. **PAINS/aggregation controls** for corilagin/polyphenols: include **detergent**, test **redox activity**, run **orthogonal readouts** (e.g., mass-spec product formation, not just fluorescence).
4. **Metal dependence.** Repeat docking/assays with correct **Mg^{2+}/Mn^{2+} coordination** and **key waters**; metal placement and protonation states can swing scores by kilocalories.
5. **MD + MM-GBSA/IFP** on the top 3–4 complexes to check **pose stability**, **water networks**, and **interaction fingerprints** over time.
6. **Cellular readouts.** If AC is the intended target, test **intracellular cAMP**; if NDPK engagement is a liability, monitor **nucleotide pools** and **cell viability**.
7. **Medicinal chemistry directions.**
    - For **selectivity vs. NDPK**, explore vectors that clash with NDPK's catalytic histidine region but are tolerated in AC (or vice-versa).

For **cell entry**, consider **prodrugging** (phosphate masking) while preserving the diphosphate geometry upon unmasking.

Competing Interests

The authors have declared that no competing interest exists.

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