Antifungal Activity and *In Silico* Study of *Phyllanthus Amarus* Ethanolic Leaf Extract against Pests of Stored Grains

Olufunmilayo A. Ajayi[1], Grace O. Otitodun[1], Taiwo O. Babatunde[2], Adepeju T. Ihum[1], Amazing Grace I. Kurrah[1], Solomon I. Ayanda[3], Temitope J. Olatunji[4], Francisca C. Oledibe[4], Saheed A. Ibrahim[4], Abigail S. Banigbe[3], Akintoye R. Olagbaju[1], and Oladipo S. Okunade[4]

[1]Durable Crops Research Department, Nigerian Stored Products Research Institute, Ilorin.
[2]Chemical Sciences Department, Ojaja University, Eiyenkorin.
[3]Perishable Crops Research Department, Nigerian Stored Products Research Institute, Ilorin.
[4]Research Operations Department, Nigerian Stored Products Research Institute, Ilorin

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CORRESPONDING AUTHOR
Olufunmilayo A. Ajayi
Durable Crops Research Department, NSPRI, Ilorin, Nigeria
ajayioa@nspri.gov.ng, funmiadeolaajayi@gmail.com
+234-803-921-5624

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Abstract

The persistence of synthetic pesticides in the environment and their toxic residues in food pose significant health risks, necessitating the pursuit of safe and eco-friendly green alternatives in postharvest management of stored grains. The antifungal efficacy of Phyllanthus amarus ethanolic leaf extract against Aspergillus flavus, Fusarium spp., and Penicillium spp. in stored maize and cowpea was evaluated in this report. The extract was screened for the presence of phytoconstituents. The results revealed the presence of phenols, tannins, flavonoids, alkaloids, and other bioactive compounds, while the GC-MS analysis identified 18 specific compounds. Antifungal activity was assessed using an in vitro food poisoning assay and in vivo storage studies. The results of the in vitro assay showed 85.3% inhibition against A. flavus, 83.7% against Fusarium spp., and 81.5% for Penicillium spp. The positive control demonstrated significantly lower inhibition, confirming the efficacy of P. amarus bioactive compounds. In vivo storage studies showed that the extract maintained fungal counts at 1.2–1.8 × 10^6 cfu/g, whereas untreated samples reached 8.5 × 10^6 cfu/g for A. flavus. Four phytoconstituents from the GC-MS result of the ethanolic extract of the Phyllanthus amarus leaves were docked against lanosterol 14-alpha demethylase (4LXJ), with Propiconazole as the standard ligand. Hinokinin was identified as the hit ligand, exhibiting the highest binding affinity compared to the other ligands. The outcome of this study suggests that P. amarus is a promising bio-preservative for grain storage, and its antifungal activity is attributed to the activity of the phytoconstituents, with hinokinin serving as the lead compound.

Keywords:
Antifungal, Hinokinin, Molecular docking, Pesticide, Phyllanthus amarus, Storage

Introduction

The persistence of pesticides in the environment and the presence of toxic residues in food are major global concerns, exacerbated by the indiscriminate use of synthetic insecticides. According to the Stockholm Convention on Persistent Organic Pollutants (POPs, 2001), ten of the twelve most hazardous and persistent chemicals are pesticides. The growing awareness of these risks has fueled interest in natural antifungal alternatives from medicinal plants. Stored grain pests cause significant postharvest losses globally, affecting food security and the economy.

Postharvest losses due to fungal contamination have a significant impact on food security, particularly in developing countries. Fungal contamination, particularly by *Aspergillus* and *Penicillium* species, leads to the production of mycotoxins, posing serious health risks and reducing grain quality. While synthetic fungicides have been effective in controlling these threats, their misuse has led to pesticide resistance, non-target toxic effects on organisms, and environmental pollution (Boateng & Kusi, 2008). This has necessitated the search for safer, plant-based alternatives.

Phyllanthus amarus (Schum. & Thonn.), a medicinal plant belonging to the Euphorbiaceae family, is traditionally used for various pharmacological purposes. It

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contains bioactive compounds, namely alkaloids, flavonoids, sterols, triterpenes, tannins, and volatile oils. Bioactive compounds, such as geraniin, phyllanthin, hypophyllanthin, and lignans, with proven antibacterial, antifungal, and antioxidant activities, have also been isolated from the plant (Calixto et al., 1998; Bagalkotkar et al., 2006). The plant has demonstrated antimicrobial, antifungal, antioxidant, insecticidal, and repellent properties (Bahar et al., 2011; Saranraj & Sivasakthivelan, 2012). Additionally, *P. amarus* has shown antifungal efficacy against Aspergillus, Fusarium, and Penicillium species, indicating its potential as a natural alternative to synthetic fungicides (Abdulkadir et al., 2024). Given its potent bioactive profile, *Phyllanthus amarus* has been explored for its capability as a natural fungicide. However, despite its promising bioactivity, research on *P. amarus* in postharvest management remains limited. Molecular docking can predict interactions between *P. amarus* phytocompounds and fungal and molecular targets, thereby aiding in the development of pesticides. *In silico* studies identify lead compounds that target specific enzymes and pathways, while computational studies enhance the understanding of structure-activity relationships and optimise the development of botanical pesticides. This study, therefore, evaluated the antifungal efficacy of *P. amarus* against fungal contaminants of maize and cowpea, as well as the *in silico* activity of the lead compound, hinokinin, against the fungal enzyme lanosterol 14-alpha-demethylase, exploring its potential as an eco-friendly biopesticide and alternative to synthetic fungicides for protecting stored maize.

Materials and Methods

Grain sourcing

Untreated maize grains (SWAN 2 maize variety) were procured from Ijaye farm settlement in Akinyele Local Government Area, Ibadan, Oyo State. The grains were sorted and cold-shocked for 72 hours before use to eliminate any pre-existing insect infestations, following the method described by Alejandro et al. (2019).

Extraction of *Phyllanthus amarus* leaf

Fresh leaves of *P. amarus* at 8 weeks old were destalked, washed with clean water, and shade dried for three (3) weeks. The dry leaves were pulverised with an electronic blender. 185 g of the powdered plant was extracted with 450 mL of ethanol using a Soxhlet apparatus. Excess solvent was removed from the extract by concentrating it in a water bath at 60 °C (Bhati et al., 2014). The green-colored paste obtained was weighed and stored in a refrigerator at 4 °C until needed for further analysis.

Phytochemical screening of the leaf extract

The qualitative phytochemical screening of the leaf extract was conducted using the method described by Ajuru et al. (2017) and Ukwubile et al. (2017).

Test for alkaloids

1 mL of 1% HCl was added to 3 mL of the extracts in a test tube. The mixture was heated for 20 minutes, cooled, and filtered. The filtrate was tested as follows: 2 drops of Wagner's reagent were added to 1 mL of the extract. A reddish brown precipitate indicates the presence of alkaloids

Test for tannins

1 mL of freshly prepared 10% KOH was added to 1 mL of the extract. A dirty white precipitate indicates the presence of tannins.

Test for phenolics

Two drops of 5% $FeCl_3$ were added to 1 mL of the extracts in a test tube. A greenish precipitate indicates the presence of phenolics.

Test for glycosides

10 mL of 50% H_2SO_4 was added to 1 mL of the extracts, and the mixture was heated in boiling water for 15 minutes. 10 mL of Fehling's solution was added, and the mixture boiled. A brick red precipitate indicates the presence of glycosides.

Test for saponins

Frothing test: 2 mL of the extract was mixed with a few drops of distilled water in a test tube and vigorously shaken for 2 minutes. Frothing indicates the presence of saponins.

Test for flavonoids

1 mL of 10% NaOH was added to 3 mL of the extracts. A yellow colouration indicates the presence of flavonoids.

Test for steroids

Salakowsti test: Five drops of concentrated H_2SO_4 were added to 1 mL of the extract. Red colouration indicates the presence of steroids.

Test for triterpenes

Five drops of acetic anhydride were added to 1 mL of the extracts. A drop of concentrated H_2SO_4 was then

added, and the mixture was heated for 1 hour. It was then neutralised with NaOH, followed by the addition of chloroform. A blue-green colour indicates the presence of triterpenes.

Test for terpenoids

5 mL of aqueous extracts of the samples were mixed with 2 mL of $CHCl_3$ in a test tube. 3 mL of concentrated H_2SO_4 was carefully added to the mixture, forming a layer. An interface with a reddish brown colouration is formed if a terpenoid constituent is present.

Test for amino acid

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 mL of acetone) are added to 2 mL of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

Test for coumarins

2 mL of 10% NaOH was added to 1 mL of the extracts. The presence of yellow shading indicates the presence of coumarins.

Test for phytosterols

Liberman-Burchar's test: 50 mg is dissolved in 2 mL of acetic anhydride. To this, 1 or 2 drops of concentrated H_2SO_4 are added slowly along the sides of the test tube. An array of colour changes shows the presence of phytosterols.

Fehling solution test

1 mL each of Fehling A and Fehling B solutions was mixed with 2 mL of extract. The mixture was boiled for 5-10 minutes in a water bath. A reddish-brown colour was obtained due to the formation of cuprous oxide, indicating the presence of reducing sugar.

Gas chromatography-mass spectrometry (GC-MS) analysis

Identification of the components

The database of the National Institute of Standards and Technology (NIST) was utilised for the interpretation of the mass spectrum and to determine the chemical identity of the test sample's components.

Antifungal assay

Isolation of fungi from maize and cowpea samples

Fungi were isolated from maize and cowpea samples using the method described by Jimoh (2020). The isolates were aseptically sub-cultured onto freshly prepared PDA (Potato Dextrose Agar) using a wire loop. Incubation was done for 72 h at a room temperature of 28 ± 2 °C. The resultant pure culture of the isolate growth was used for *in vitro* and *in vivo* analysis.

Preparation of fungal inoculum

The method of Jimoh (2020) was used for preparing the fungal inoculum, with slight modifications. Fresh strains of fungi were sub-cultured at 28 ± 2 °C on sterile PDA and incubated for 72 h. The spores of the fungus were suspended in sterile saline, vortexed, and adjusted to a turbidity of 1×10^6 cfu/mL.

***In vitro* analysis of the extracts**

The antifungal activity of different concentrations of the *P. amarus* ethanolic extract was evaluated by the food poisoning method. 1 mL of the plant extract at varying concentrations of 0.25, 0.5, and 0.75 from the stock solution (10 mg/mL) was applied to 10 mL of sterilised potato dextrose agar in petri plates and allowed to solidify. A 48-hour-old mycelial disk of isolated fungi was placed at the centre of the petri dishes; the plate with no treatment served as a negative control. Incubation of the plates was done at 28 ± 2°C for 48–72 h. Three replicates were maintained for each treatment, and the radial growth of the mycelium was assessed over a period of six days. The percentage inhibition of fungal growth was calculated using the formula described by Kidd et al. (2018).

$$L = C - T/C \times 100$$

Where: L is the percentage inhibition; C is the colony radius in the control plate, and T is the radial growth of the pathogen in the presence of the plant extract.

***In vivo* antifungal activity of maize under storage conditions**

The antifungal activity of the treatments was determined according to Zhang et al. (2013), with slight modifications. The grains were surface-sterilised with 5% sodium hypochlorite (NaOCl) for 1 minute and then rinsed twice with sterile water. 15 g of selected grains were mixed with 2 g of each treatment separately in each petri dish. The plates were shaken gently until the applied treatment was thoroughly absorbed by the grains. The grains were inoculated with a loopful of fungal mycelium and agitated until the mycelium was absorbed. The growth of fungi on the samples was visually evaluated after incubation at 28 ± 2 °C for seven days. The population growth was determined at three-day intervals throughout the incubation period. The experiments were replicated

twice, and the control setup consisted of grains that received no treatment.

***In-silico* study**

The *in silico* study of the ligands obtained from the GC-MS analysis was conducted by docking the compounds against lanosterol-14-alpha demethylase, a crucial fungal enzyme essential for the fungal cell membrane (Ajayi et al., 2024; Ajayi et al., 2025).

Results and Discussion

The phytochemical composition of the *P. amarus* leaf extract revealed the presence of phenols, flavonoids, saponins, tannins, glycosides, terpenoids, coumarin, steroids, and alkaloids in the crude extract (Table 1). Phytochemical screening revealed that amino acids were absent in the extract. This finding aligns with the results of Arun et al. (2012), Saranraj and Sivasakthivelan (2012), and Oshomoh and Uzama-Avenbuan (2020).

The GC-MS analysis result (Table 2) revealed 18 compounds in the ethanolic extract. The GC-MS analysis of the plant extract shows various peaks of bioactive compounds, of which the activity of the plant against fungi has been attributed. These compounds act in synergy with other bioactive compounds in the plant extract to bring out the pharmacological properties of the plant. Arun et al. (2012), Mamza et al. (2012), and Ameen et al. (2021) confirmed the presence of pharmacologically active compounds in the GC-MS analysis of *Phyllanthus amarus* leaves, which may contribute to the plant's medicinal properties.

Table 1: Qualitative phytochemical screening of *P. amarus* leaf extract

Phytochemicals	Ethanol Extract
Saponins	+
Tannins	+
Phenolics	+
Flavonoids	+
Coumarins	+
Glycosides	+
Triterpenes	+
Reducing Sugars	+
Steroids	+
Alkaloids	+
Anthraquinones	+
Amino acids	-
Terpenoids	+
Resins	+

Key *+ = Present* *- = Absent*

The antifungal activity of *Phyllanthus amarus* extract against *Aspergillus flavus, Fusarium* spp., and *Penicillium* spp. is shown in Table 4. The ethanol extract exhibited strong inhibition, with 85.3% inhibition against *A. flavus* at a concentration of 1 mg/mL. This outcome aligns with Rao et al. (2020), who reported that ethanol efficiently extracts bioactive constituents, such as alkaloids, flavonoids, and tannins with strong antifungal properties, from plant materials. The ethanolic extract contains bioactive compounds responsible for its potent inhibition (Nguyen et al., 2021). The result for the positive control, which consisted of solvent alone, further emphasises the antifungal properties of *P. amarus* extract. The positive control exhibited lower inhibition across all fungi, with only 55.5% inhibition against *A. flavus*, 56.0% against *Fusarium* spp., and 49.5% against *Penicillium* spp. at 1 mg/mL. For *Fusarium* spp., the ethanol extract achieved 83.7% inhibition, which is slightly lower than that of *A. flavus.* The higher resistance of *Fusarium* may be attributed to its robust cell wall and its ability to produce protective secondary metabolites (Al-Hindi et al., 2020). *Penicillium* spp. exhibited the lowest susceptibility to the plant extracts, with a maximum inhibition of 81.5% for the extract. This aligns with previous studies indicating that *Penicillium* species exhibit structural adaptations, such as melanin production in their cell walls, which enhance resistance to antifungal compounds (Chang et al., 2022). The positive control recorded 49.5% inhibition, indicating that while the solvent alone had some inhibitory effect, it was not as potent as the plant extract. Figures 1 and 2 show the *in vivo* activity of the plant extracts against *A. flavus* and *Fusarium* on maize grains in storage. The ethanol extract exhibited strong efficacy, maintaining stable microbial counts (1.2–1.8 $\times 10^6$ cfu/g). The control samples had a fungal increase, reaching 8.5×10^6 cfu/g (*A. flavus*) and 5.3×10^6 cfu/g (*Fusarium*) by day 9. The antifungal effects of *P. amarus* are attributed to flavonoids and tannins, which inhibit fungal proliferation (Afolabi et al., 2022; Ezekiel et al., 2023). Phenolics and alkaloids also contribute by disrupting fungal cell structures (Bello et al., 2022; Okeke et al., 2023).

Binding affinities of extract of *Phyllanthus amarus* leaves docked against selected protein targets

Docking four phytochemicals from the ethanol extract of *Phyllanthus amarus* leaves, 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester, cyano colchicine, carissanol dimethyl ether, and hinokinin against

lanosterol 14-alpha demethylase (4LXJ) resulted in higher binding affinities compared to Propiconazole. Among them, hinokinin exhibited the highest binding affinity. Interestingly, the binding affinity of phytol was identical to that of Propiconazole. Conversely, phytol acetate, ethyl linolenate, pyrrolidine-2-carboxylic acid, methyl-phenyl-amide, ethyl 5-oxo-DL-prolinate, ethyl palmitate, ethyl decanoate, and ethyl octanoate displayed lower binding affinities than Propiconazole (Figure 3).

Table 2: GC-MS analysis of *P. amarus* ethanol extract

Peak #	Retention Time	Area	Area%	Height	Height %	A/H	Compound
1	12.83	2997533	0.28	1392850	1.23	2.15	Octanoic acid ethylester
2	15.35	5609304	0.52	2342048	2.07	2.40	Decanoic acid ethylester
3	16.64	13433916	1.26	2318312	2.05	5.79	2-Pyrrolidinecarboxylic acid-5-oxoethylester (Ethyl-5-oxo-DL-prolinate)
4	16.79	53502250	5.00	6511938	5.76	8.22	Cyanocolchicines
5	16.88	17205129	1.61	4399740	3.89	3.91	3-[(3,4-Dimethoxy-benzyl amino)-methyl]-8a-methyl-5-methylene-decahydro-naphtho[2,3b]furan-2-one,
6	20.15	6588515	0.62	1636018	1.45	4.03	Phytolacetate
7	20.52	76677603	7.17	7534332	6.67	10.18	5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester
8	21.40	15935949	1.49	2035831	1.80	7.83	2(3H)-Furanone-3,4-bis(1,3-benzodioxol-5-ylmethyl)dihydro (Hinokinin)
9	21.90	3164432	0.30	1062655	0.94	2.98	Hexadecanoic acid ethyl ester (ethyl palmitate)
10	22.38	6483604	0.61	1697921	1.50	3.82	Pyrrolidine-2-carboxylic acid-methyl-phenyl-amide
11	22.95	58588274	5.48	5591847	4.95	10.48	[4-[(2,4 Dimethoxyphenyl) methyl]piperazin-1-yl]-(2-methoxyphenyl)methanone
12	23.16	123998285	11.59	15102161	13.36	8.21	[4-(2,4-Dimethoxybenzyl) methyl]piperazin-1-yl]-(2-methoxyphenyl)methanone
13	23.43	12114897	1.13	3678682	3.25	3.29	Phytol
14	24.18	16544999	1.55	2639439	2.34	6.27	9,12,15-Octadecatrienoic acid ethyl ester Z (ethyl linolenate)
15	24.51	222742960	20.83	21499594	19.02	10.36	Carissanol dimethyl ether
16	25.09	194031396	18.14	16753577	14.82	11.58	[4-(2,4-Dimethoxybenzyl) piperazin-1-yl]-(2-methoxyphenyl)methanone
17	25.49	22134018	2.07	1915900	1.69	11.55	10,13-Diethoxydibenzo(a,c)Phenazine
18	26.43	217729142	20.36	14922937	13.20	14.59	Carissanol dimethyl ether

Table 3: Morphological characteristics of the fungal isolates

S/N	Fungal isolates	Morphological Characteristics	Crop Sample
1.	*A. flavus*	Yellow-green, velvety colonies with rough-walled conidia and potential aflatoxin production.	Maize
2.	*Penicillium* spp.	Blue-green, powdery colonies with brush-like conidiophores and round conidia.	Cowpea, Maize
3.	*Fusarium* spp	Cottony white to pink colonies with banana-shaped macroconidia and mycotoxin production.	Cowpea, Maize

Table 4: Percentage inhibition of fungal growth by *P. amarus* extract

Isolate	Extract Concentration (mg/mL)	Percentage Inhibition
A. flavus	0.25	42.3 ± 1.2
	0.50	58.6 ± 1.5
	0.75	72.1 ± 1.7
	1.00	85.3 ± 1.9
POS Control		55.5 ± 1.3
Fusarium	0.25	39.5 ± 0.8
	0.50	55.2 ± 0.4
	0.75	70.4 ± 0.6
	1.00	83.7 ± 0.5
POS Control		56.0 ± 2.3
Penicillium	0.25	37.8 ± 0.5
	0.50	53.6 ± 1.3
	0.75	68.9 ± 0.5
	1.00	81.5 ± 1.8
POS Control		49.5 ± 1.5

Mean values (n = 3) ± standard error (SE) Keys: Positive control (solvent alone; 1mg/mL)

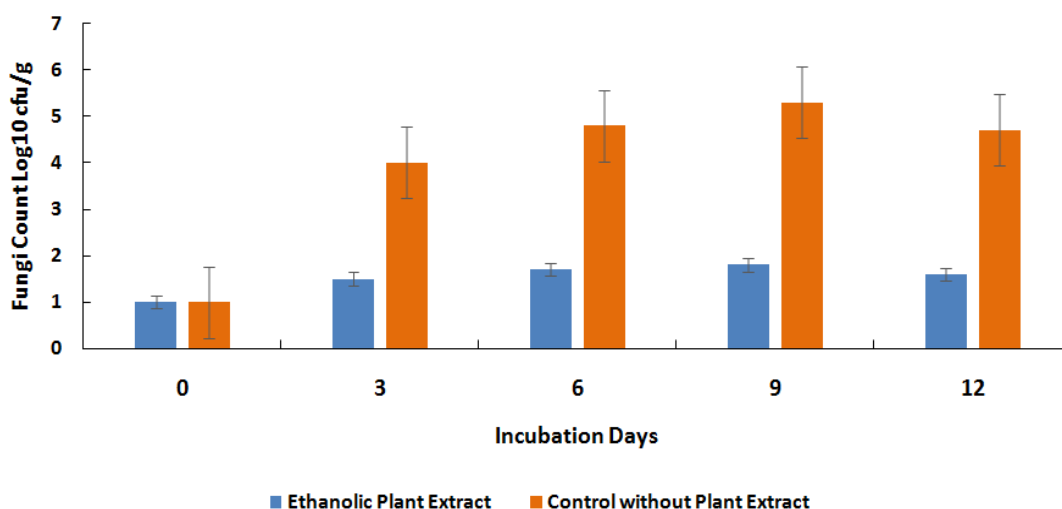

Figure 1: *In vivo* activity of plant extract against *A. flavus* on grains in storage (maize)

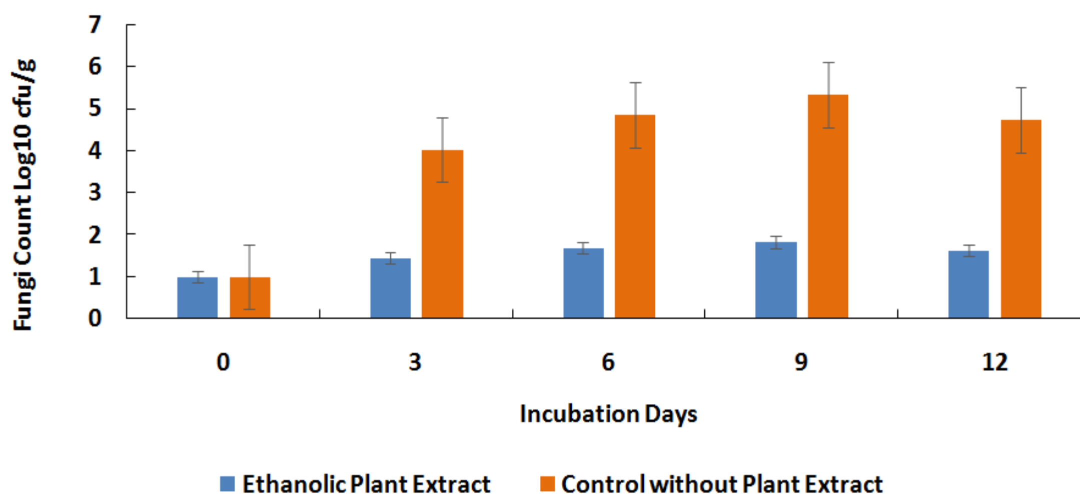

Figure 2: *In vivo* activity of plant extracts against *Fusarium* species on grains in storage (maize)

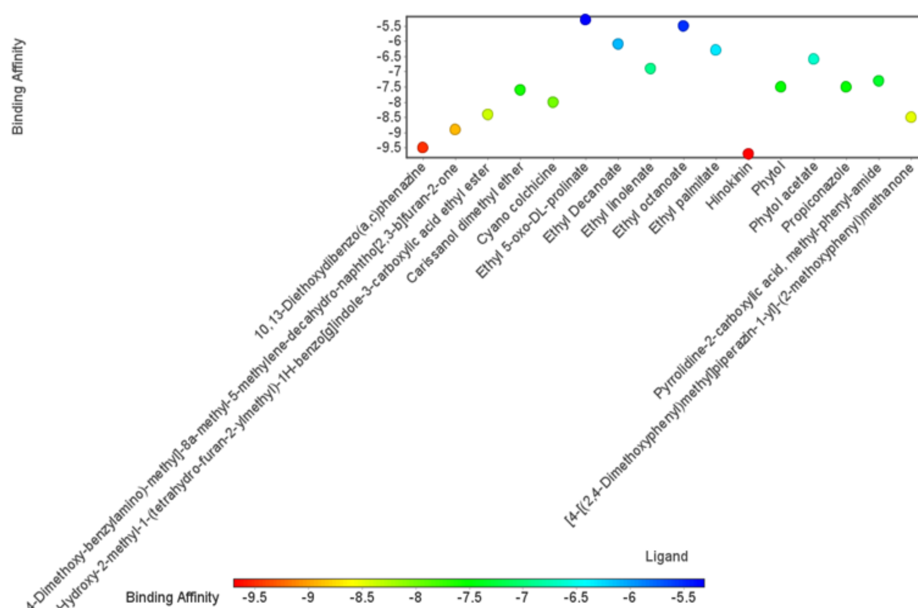

Figure 3: Binding Affinities of Ethanol Extract of *P. amarus* Leaves Docked against Lanosterol 14-alpha Demethylase (4LXJ)

Molecular interactions of phytochemicals of ethanol extract of *Phyllanthus amarus* leaves docked against lanosterol 14-alpha demethylase (4LXJ)

The visualisation study demonstrated that Propiconazole interacted with lanosterol-14-alpha demethylase through eleven hydrophobic bonds and one hydrogen bond (Figure 4, Table 5). Specifically, the key amino acid residues involved in these interactions included Tyr 140, Ile 139, Leu 147, Lys 151, Val 154, Leu 307, Val 311, Ile 471, and Phe 236. Similarly, hinokinin formed nine hydrophobic bonds and two hydrogen bonds with lanosterol 14-alpha demethylase (Figure 5, Table 6), with key interacting residues such as Met 313, Gly 314, Phe 236, His 381, Leu 380, Leu 383, Tyr 126, and Met 509. In contrast, cyano colchicine established two hydrogen bonds (Figure 6, Table 7), specifically interacting with residues TYR 126 and HIS 468 in lanosterol 14-alpha demethylase. Moreover, 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester interacted with lanosterol 14-alpha demethylase via eight hydrophobic bonds and two hydrogen bonds (Figure 9, Table 10). The residues involved included Gly 310, Tyr 140, Tyr 126, Leu 383, Met 313, Phe 134, Phe 236, and Leu 380.

Molecular interactions of ethanol extract of *P. amarus* leaves docked against lanosterol 14-alpha demethylase (4LXJ)

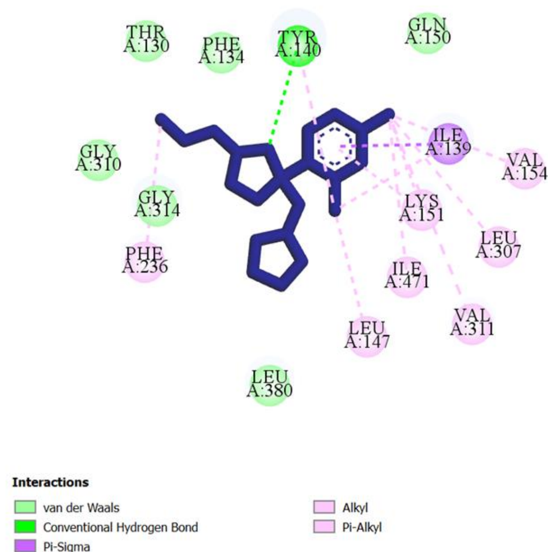

Figure 4: Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with Propiconazole

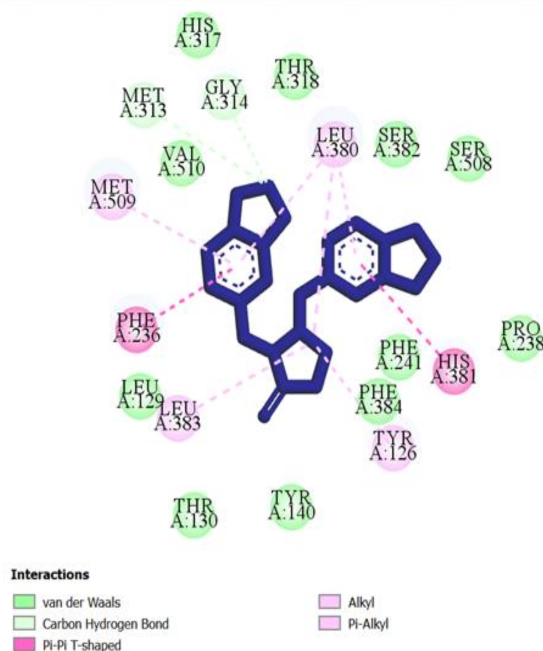

Figure 5: Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with Hinokinin

Table 5: Analysis of the interaction of propiconazole with the binding pocket of Lanosterol 14-alpha Demethylase (4LXJ)

Name	Category	Types	Distance
A:TYR140:HH - Propiconazole	Hydrogen Bond	Conventional Hydrogen Bond	3.01362
A:ILE139:CD1 - Propiconazole	Hydrophobic	Pi-Sigma	3.72806
Propiconazole - A: ILE139	Hydrophobic	Alkyl	4.80063
Propiconazole - A: LEU147	Hydrophobic	Alkyl	4.54438
Propiconazole - A: LYS151	Hydrophobic	Alkyl	4.16256
Propiconazole - A: VAL154	Hydrophobic	Alkyl	4.00551
Propiconazole - A: LEU307	Hydrophobic	Alkyl	4.85425
Propiconazole - A: VAL311	Hydrophobic	Alkyl	5.24191
Propiconazole - A: ILE471	Hydrophobic	Alkyl	5.18941
A:TYR140 - Propiconazole	Hydrophobic	Pi-Alkyl	4.97012
A:PHE236 - Propiconazole	Hydrophobic	Pi-Alkyl	5.1868
Propiconazole - A: LYS151	Hydrophobic	Pi-Alkyl	5.27279

Table 6: Analysis of the interaction of hinokinin with the binding pocket of Lanosterol 14-alpha Demethylase (4LXJ)

Name	Category	Types	Distance
Hinokinin - A: MET313:O	Hydrogen Bond	Carbon Hydrogen Bond	3.70625
Hinokinin - A: GLY314:O	Hydrogen Bond	Carbon Hydrogen Bond	3.38391
Hinokinin – Hinokinin	Hydrophobic	Pi-Pi Stacked	5.52549
A:PHE236 – Hinokinin	Hydrophobic	Pi-Pi T-shaped	4.60212
A:HIS381 – Hinokinin	Hydrophobic	Pi-Pi T-shaped	5.44787
Hinokinin - A: LEU380	Hydrophobic	Alkyl	4.61412
Hinokinin - A: LEU383	Hydrophobic	Alkyl	5.3243
A:TYR126 – Hinokinin	Hydrophobic	Pi-Alkyl	4.34606
Hinokinin - A: LEU380	Hydrophobic	Pi-Alkyl	4.92063
Hinokinin - A: MET509	Hydrophobic	Pi-Alkyl	4.99735
Hinokinin - A: LEU380	Hydrophobic	Pi-Alkyl	5.18129

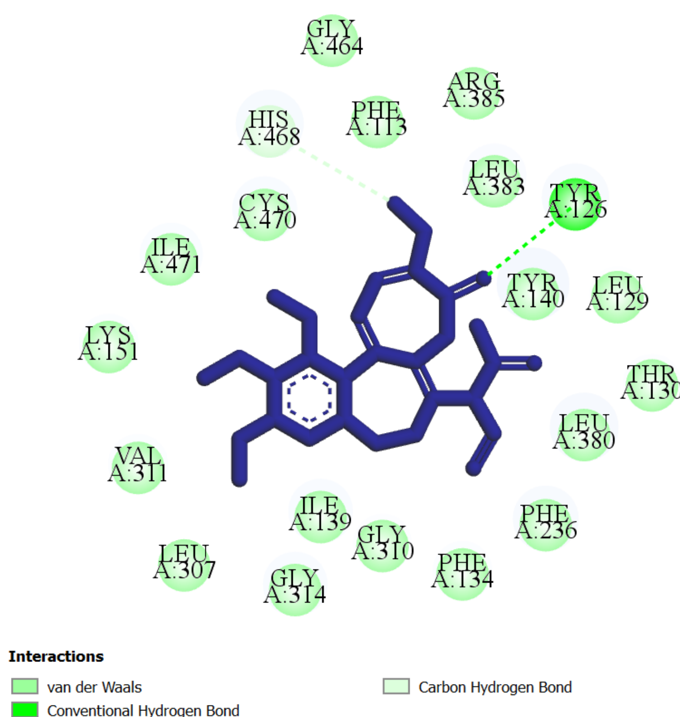

Figure 6: Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with Cyano Colchicine

Table 7: Analysis of the Interaction of Cyano Colchicine with the Binding Pocket of Lanosterol 14-alpha Demethylase (4LXJ)

Name	Category	Types	Distance
A:TYR126:HH – Cyano Colchincine	Hydrogen Bond	Conventional Hydrogen Bond	2.54809
Cyano Colchicine - A: HIS468:O	Hydrogen Bond	Carbon Hydrogen Bond	3.41718

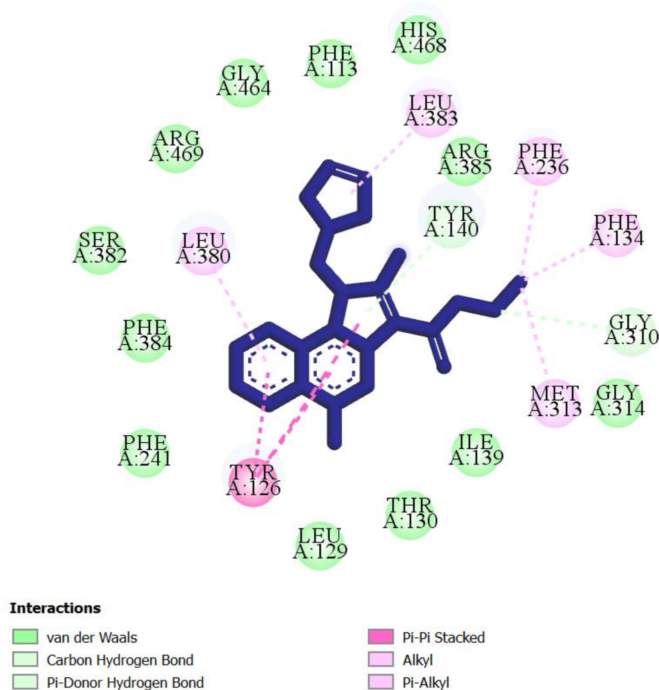

Figure 7: Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester

Table 8: Analysis of the interaction of 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester with the binding pocket of Lanosterol 14-alpha Demethylase (4LXJ)

Name	Category	Types	Distance
5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester - A:GLY310:O	Hydrogen Bond	Carbon Hydrogen Bond	3.56914
A:TYR140:HH - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrogen Bond	Pi-Donor Hydrogen Bond	2.65448
A:TYR126 - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrophobic	Pi-Pi Stacked	4.33403
A:TYR126 - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrophobic	Pi-Pi Stacked	4.01995
5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester - A:TYR126	Hydrophobic	Pi-Pi Stacked	5.26148
A:LEU383 - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrophobic	Alkyl	4.0062
5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester - A:MET313	Hydrophobic	Alkyl	5.23164
A:PHE134 - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrophobic	Pi-Alkyl	4.94421
A:PHE236 - 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester	Hydrophobic	Pi-Alkyl	4.7804
5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester - A:LEU380	Hydrophobic	Pi-Alkyl	5.27245

Bioavailability assessment of standard compound (Propiconazole) and Lead compound (Hinokinin) from ethanolic extract of *Phyllanthus amarus* leaves and propiconazole docked against Lanosterol 14-alpha Demethylase (4LXJ)

Neither the standard compound (Propiconazole) nor the lead compound (hinokinin) failed any of the six bioavailability criteria- lipophilicity, flexibility, unsaturation, insolubility, polarity, and molecular weight (size)-as illustrated in Figures 8 and 9.

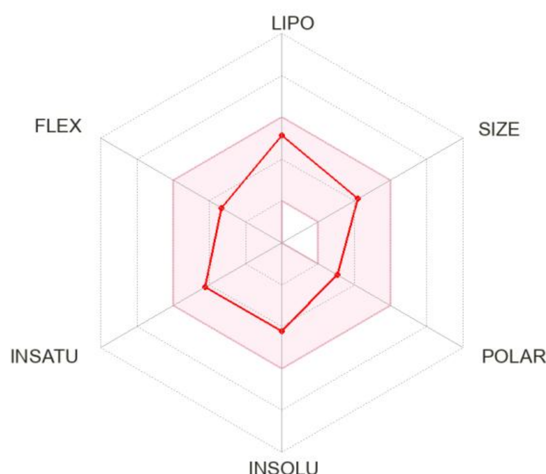

Figure 8: Bioavailability Properties of Propiconazole

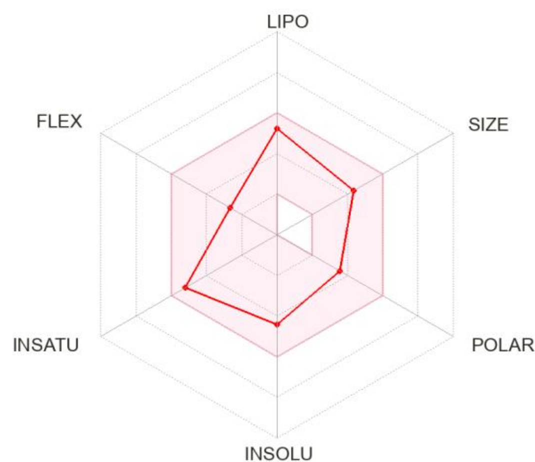

Figure 9: Bioavailability Properties of Hinokinin

Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties of Propiconazole and Hinokinin docked against Lanosterol 14-alpha Demethylase

Enzyme inhibition plays a crucial role in fungal drug discovery and treatment, particularly through targeting lanosterol 14-alpha demethylase (CYP51), a key enzyme in the ergosterol biosynthesis pathway of fungi. This enzyme converts lanosterol to ergosterol, a crucial component of fungal cell membranes that affects membrane permeability and fluidity (Korkut et al., 2017; Istiqomah et al., 2023). Inhibiting CYP51 leads to the depletion of ergosterol and accumulation of lanosterol, resulting in impaired fungal cell growth and viability (Korkut et al., 2017*)*. Azole antifungals, which inhibit CYP51, have been a successful strategy in combating fungal infections, although their clinical use is sometimes limited by toxicity, resistance, and pharmacokinetic issues (Saravana Kumar & Purnima, 2011). Therefore, the search for novel CYP51 inhibitors is a rational research endeavour. Indeed, recent studies have focused on identifying new inhibitors from natural sources, which show strong binding affinity to CYP51 and potential as antifungal agents (Sama-ae et al., 2023). In particular, compounds from traditional medicinal plants have demonstrated antifungal activity by inhibiting CYP51, with terpenoids, flavonoids, and phenols showing promise in *in-silico* studies (Istiqomah et al., 2023).

In this study, specific phytochemicals from the ethanol extract of *Phyllanthus amarus* leaves were docked against lanosterol 14-alpha demethylase (CYP51). Compounds such as 5-Hydroxy-2-methyl-1-(tetrahydro-furan-2-ylmethyl)-1H-benzo[g]indole-3-carboxylic acid ethyl ester, cyano colchicine, carissanol dimethyl ether, and hinokinin—identified in the ethanol extract, demonstrated stronger binding affinities for CYP51 than propiconazole, the reference inhibitor (Figures 3 and 4).

Notably, hinokinin, which exhibited the strongest binding affinity among all docked compounds, has previously been confirmed to possess antifungal activity (Zhou et al., 2015). Moreover, it has been shown to synergise with conventional fungicides, such as tebuconazole, against *Fusarium graminearum* (Sanchez Matías et al., 2024), thereby reinforcing its therapeutic potential. Binding affinities, typically expressed as negative values in molecular docking studies, reflect the strength of interactions between ligands and their target proteins (Ajiboye et al., 2024). Higher affinities (more negative binding energies) suggest a more stable and effective ligand-target interaction, which is a valuable predictor of drug potency. Such findings are crucial in antifungal drug discovery, as strong ligand-target interactions indicate promising therapeutic potential (Godge et al., 2023; Mani Chandrika and Prathyusha, 2024; Kırboğa et al., 2024; Raghav et al., 2024). Therefore, the strong binding affinities observed for hinokinin and other phytochemicals in this study (Figures 3 and 4) underscore their plausibility as effective agents for inhibiting fungal growth.

The molecular docking analyses of the ethanol extract of *Phyllanthus amarus* revealed significant interactions between various phytochemicals and CYP51. Propiconazole, the standard antifungal agent, exhibited a strong binding profile with 11 hydrophobic interactions and one hydrogen bond (Tables 5 and 9), serving as a benchmark for evaluating the binding potential of the phytochemicals. Hydrophobic

interactions promote the association between non-polar regions of a drug candidate and hydrophobic amino acid residues within the target protein. This stabilises the drug-protein complex, enhancing both binding affinity and specificity (Lou & Martin, 2021). Similarly, hydrogen bonds play a crucial role in facilitating molecular interactions within the cell, thereby supporting the binding affinity and therapeutic potential of compounds (Pairas & Tsoungas, 2016). However, Patil et al. (2010) clearly asserted that stronger protein-ligand binding is achieved when hydrophobic interactions predominate over hydrogen bonding (Patil et al., 2010). Therefore, the hydrophobic interactions and hydrogen bonds formed between the lead compounds and CYP51 likely played a significant role in enhancing their binding affinities.

Table 9: Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) Properties of Propiconazole and Hinokinin

Property	Propiconazole	Hinokinin
Physicochemical Properties		
Lipinski's Rule Violations	0	0
Molecular Weight	342.23	354.36
Hydrogen Bond Donor	0	0
Hydrogen Bond Acceptor	5	6
LogP	3.65	2.72
Absorption		
Pgp-inhibitor	Inhibitor (0.66)	Non-Inhibitor (0.44)
Pgp-substrate	Non-substrate (0.07)	Non-substrate (0.56)
HIA (Human Intestinal Absorption)	HIA+ (0.74)	HIA + (0.58)
Distribution		
BBB (Blood–Brain Barrier)	BBB+ (0.89)	BBB + (0.97)
Metabolism		
CYP450 1A2 inhibitor	Non-inhibitor (0.39)	Inhibitor (0.54)
CYP450 1A2 substrate	Substrate (0.57)	Substrate (0.56)
Excretion		
$T_{1/2}$ (Half Life)	1.38 (Low)	1.66 (Low)
Toxicity		
H-HT (Human Hepatotoxicity)	Hepatotoxic (0.79)	Hepatotoxic (0.67)
AMES (Ames Mutagenicity)	Non-Ames toxic (0.31)	Non-Ames toxic (0.41)

The bioavailability profile provides a quick assessment of the drug-likeness of a compound by summarising key properties, including molecular size, solubility, lipophilicity, polarity, saturation, and flexibility (Daina et al., 2017). Each property is plotted along a separate axis, with the ideal range for drug-likeness shown as a pink zone. When a compound falls within this zone, it indicates promising bioavailability features (Daina et al., 2017). Hinokinin met all the evaluated criteria, just like propiconazole, the reference drug (Figures 8 and 9). In particular, lipophilicity reflects a compound's ability to cross cell membranes, with optimal values favouring absorption. Molecular size plays a role in permeability, as smaller molecules tend to be absorbed more efficiently (Daina et al., 2017). Polarity, often measured by TPSA, needs to be moderate to balance

solubility and permeability. Adequate solubility is crucial for a compound to dissolve in gastrointestinal fluids and facilitate absorption. Lastly, lower molecular flexibility and higher saturation are generally associated with better metabolic stability and improved bioavailability (Daina et al., 2017). Therefore, hinokinin shows promising ability to be effectively produced as a druggable antifungal agent, as did propiconazole.

ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) evaluations are crucial in computational biology for drug development, as they help establish a compound's pharmacokinetic properties and safety profile (Jia &Gao, 2022). ADMET result, as a preliminary evaluation of a compound's specific properties, can help avoid expensive setbacks in the later phases of drug development (Jia & Gao, 2022; Lin et al., 2003).

In the physicochemical assessment, propiconazole and the lead compound, hinokinin, adhered to all of Lipinski's parameters without any violations (Table 9). Indeed, satisfying Lipinski's parameters significantly influences drug development by guiding the design of orally bioavailable compounds. In addition, propiconazole and hinokinin were identified as permeable to the human intestine. Compounds capable of penetrating the human intestinal lining are more efficiently absorbed into the bloodstream, resulting in improved bioavailability and greater overall absorption. Indeed, the pharmacokinetics of orally administered drugs are primarily influenced by how quickly they are absorbed through the intestinal wall into the systemic circulation (Azman et al., 2022). Therefore, hinokinin's ability to be absorbed through the intestine suggests a favourable pharmacokinetic profile. In the distribution evaluation, propiconazole and hinokinin were found to be permeable to the blood-brain barrier (BBB) (Table 9). Therefore, these compounds will function well as antifungal agents (Saxena et al., 2023). In the metabolism assessment, hinokinin was identified to be a substrate and an inhibitor of the CYP450 1A2 enzyme. Hinokinin is metabolised by the CYP450 1A2 enzyme when acting as a substrate, facilitating its elimination from the body (Jones et al., 2009; Wang & Zhou, 2009; Zhou et al., 2009). However, when functioning as an inhibitor, it may suppress the enzyme's activity, potentially interfering with the metabolism of other CYP1A2 substrates and increasing the risk of drug–drug interactions and associated toxicity (Jones et al., 2009; Wang & Zhou, 2009; Zhou et al., 2009). The fact that Propiconazole is a substrate of CYP450 1A2 carries the same significance as noted for hinokinin. Ames toxic (Table 9).

Conclusion

This study demonstrates the significant antifungal efficacy of *Phyllanthus amarus* leaf ethanolic extract against *Aspergillus flavus*, *Fusarium* spp., and *Penicillium* spp. The findings lend support to the use of *P. amarus* as a natural bio-preservative for maize and cowpea storage, thereby reducing reliance on synthetic fungicides and contributing to food safety. Certain phytochemicals present in the ethanolic extract of *Phyllanthus amarus* leaves demonstrated strong binding affinities to lanosterol 14α-demethylase in an *in-silico* study, with hinokinin showing the highest affinity among all compounds evaluated. Hinokinin also met all bioavailability criteria and showed highly favourable ADMET properties. Overall, the antifungal activity observed in the extract is attributed to these key phytochemicals, particularly hinokinin, which stands out as a promising lead compound. Ongoing research focuses on investigating the mechanism of antifungal action by analysing cellular leakage and microscopic structural damage in other fungi. Additionally, microbial interactions in stored grains are being explored through metagenomic sequencing and competitive exclusion tests. Furthermore, the encapsulation of the plant extract, guided by the outcome of the *in silico study,* is being pursued to enhance antifungal efficacy.

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