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# Bioactivity and In Silico Study of Hinokinin from Phyllanthus Amarus Isopropanol Leaf Extract against Some Pests of Stored Grains

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## Abstract

The study was carried out to determine the antifungal activity of *Phyllanthus amarus* on mold causing organisms of grains namely; *Aspergillus flavus*, *Fusarium and Penicillium* species. The fresh leaves of *P. amarus* were dried and grinded. The pulverized leaves were extracted with isopropanol using soxhlet apparatus. The extract was screened for the presence of phytochemicals and GCMS was utilized to determine the constituents of the leaf extract. The analysis of the phytochemical composition of the leaf crude isopropanol extract showed the presence of phenols, flavonoids, saponins, tannins, glycosides, terpenoids, coumarin, steroids and alkaloids but anthraquinones and amino acids were not detected in the extract. The GC-MS analysis of the extract revealed 9 compounds. The extract exhibited the strongest inhibition of 79.1% against *A. flavus* at 1 mg/mL. For *Fusarium* spp., the extract achieved 76.8 % inhibition. *Penicillium* spp. showed the lowest susceptibility to the extract, with a maximum inhibition of 74.3 % at 1 mg/mL. From the molecular docking study, six phytoconstituents in the isopropanol extract of *Phyllanthus amarus* leaves demonstrated stronger binding affinities to lanosterol 14α-demethylase than the standard ligand, propiconazole, with hinokinin exhibiting the highest affinity among all the compounds.

**Keywords:** Fungi, Hinokinin, Lanosterol 14α-demethylase, Molecular docking, *Phyllanthus amarus*

## 1. Introduction

*Phyllanthus amarus* is a member of the Euphorbiaceae family, famous for its traditional uses. *Phyllanthus amarus* (Schum and Thonn) is one of the most pharmacologically important species of the Phyllanthus family. The plant is known as "gerontsuntsaye" (Hausa), "eyin-olobe" (Yoruba) and "ngwu" (Igbo). The pharmacological activities of the plant have been reported to include antioxidant, antileptospiral, anticancer, antimicrobial, anti-inflammatory, antidiabetic

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and anticonvulsant activities (Bahar *et al*., 2011; Evi and Degbeku 2011; Manikkoth *et al*., 2011; Saranraj and Sivasakthivelan, 2012; Zubair *et al*., 2017). Bioactives such as alkaloids, flavonoids, lignins, phenols, tannins and terpenes have been isolated from the plant (Bahar *et al.,* 2011). Methanolic extracts of the leaf, fruit and root showed significant concentration-dependent antibacterial activity *in vitro* against a range of gram-positive and gram-negative bacteria with the leaf extract showing the highest activity (Ravikumar *et al.,* 2011). The antifungal activity and importance of botanicals in postharvest management have been reported by Ajayi *et al*. (2024). The prevalence of fungal contamination in cowpea and maize poses significant health risks due to mycotoxin production (Ouili *et al*., 2023). Aspergillus and penicillium are two fungi species responsible for the growth of mold in stored grains. Over 50 % of maize samples from Nigerian markets have been reported to be contaminated with fungi, predominantly *Aspergillus*, *Fusarium and Penicillium* species (Afolabi *et al*., 2019; Ouili *et al*., 2023). Increasing pest resistance, high pesticide residues in food and health risks associated with synthetic pesticides necessitate alternative counter measures to combat this menace. This study therefore was undertaken to evaluate the antifungal efficacy of *P. amarus* against fungal contaminants of maize and cowpea with the objective of exploring its potential as an alternative to the use of harmful pesticide and developing a biopesticide to reduce postharvest loss of stored grains.

## 2. Materials and Methods

### Sample Preparation and Extraction

The fresh leaves of *Phyllanthus amarus* were picked out from the stalk and washed with clean water to remove dirt and sand particles. They were dried in a room away from sunlight for three (3) weeks. The plant was identified with the voucher number: UILH/001/1298/2023. The dry leaves were grinded with an electronic blender. 185g of the pulverized plant was extracted with 450mL of isopropanol using soxhlet apparatus per batch extraction. Excess solvent was removed from the extract by concentrating it on a water bath at temperature of 60$^{o}$C (Bhati *et.al.,* 2014). The extract obtained was weighed and stored in a refrigerator at 4°C until needed for further analysis.

### Phytochemical Screening of the Leaf Extract

The method of Ajuru *et al*. (2017) and Ukwubile *et al*. (2017) were adopted for determination of the qualitative phytochemical screening of the leaf extract.

### Gas Chromatography-Mass Spectrometry (GCMS) Analysis Identification of the Components

The GC-MS analysis of the extract was performed using GC-MS Clarus 500 Perkin-Elmer system comprising an AOC-20i auto-sampler. The instrument is equipped with a VF-5ms fused silica capillary column with 30 m of length, 0.25 mm of diameter and 0.25 µm of film thickness. The temperatures employed were column oven temperature 80°C, injection temperature 250°C at a pressure of 108.0 kPa with total flow and column flow of 6.20 mL/min and 1.58 mL/min respectively. The linear velocity was 46.3 cm/s and the purge flow was 3.0 mL/min. The GC program ion source and interface temperature were 200.00°C and 250.00°C respectively with solvent cut time of 2.50 min. The MS program starting time was 3.00 min which was ended at 30.00 min, with event time of 0.50 s, scan speed of 1 666 µL/s, scan range 40–800 µs and an injection volume of 1 µL of the extract (split ratio 10:1). The total running time of GC-MS was 30 min. The relative percentage of the extract was expressed as the percentage with peak area

normalization as previously reported by Lawal *et al.,* (2015). The National Institute of Standards and Technology (NIST) database was used for the interpretation of the mass spectrum and to ascertain the chemical identity of the components of the test sample.

**Antifungal Assay**
**Isolation of Fungi from Maize and Cowpea Samples**

The method of Jimoh (2020) was adopted for the isolation of fungi from maize and cowpea samples. A wire loop was used to aseptically subculture the isolates onto freshly prepared PDA (Potato Dextrose Agar) and incubated for 72 h at a room temperature of 28 ± 2°C. The resultant pure culture of the isolated growth was used for *in vitro* and *in vivo* analysis.

**Preparation of Fungal Inoculum**

The fungal inoculum was prepared using the method described by Jimoh (2020) with slight modifications. Fresh strains of fungi were subcultured at 28 ± 2°C on sterile PDA and incubated for 72 h. The spores of fungi were swept into sterile saline, vortexed, and adjusted until they reached a turbidity of $1 \times 10^6$ CFU/mL.

***In vitro* Analysis of the Extract**

The food poisoning method was used to evaluate the antifungal activity of different concentrations of the plant extract. 1mL of the plant extract at varying concentrations of 0.25, 0.5, and 0.75, from the stock solution (10 mg/mL) was added to 10 mL of sterilized potato dextrose agar in petri plates and allowed to solidify. A 48-hour-old mycelial disk of isolated fungi was then placed at the centre of the petri dishes. The plate without the treatment served as a negative control. The plates were then incubated at 28 ± 2°C for 48–72 h. Three replicates were maintained for each treatment, and mycelium radial growth was measured over six days. Percentage inhibition of fungal growth was calculated using equation 1 (Kidd *et al*., 2016)

$$L = C - T/C \times 100, \quad (1)$$

where L is the percent 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.

**3. Results and Discussion**

The analysis of the phytochemical composition of *P. amarus* leaf crude isopropanol extract showed the presence of phenols, flavonoids, saponins, tannins, glycosides, terpenoids, coumarin, steroids and alkaloids (Table 1). Anthraquinones and amino acids were not detected in the extract.

**Table 1:** Qualitative Phytochemical Screening of *P. amarus* Extract

| Phytochemicals | Isopropanol Extract |
|---|---|
| Saponins | + |
| Tannins | + |
| Phenolics | + |
| Flavonoids | + |
| Coumarin | + |
| Glycosides | + |
| Triterpenes | + |
| Reducing Sugars | + |
| Steroids | + |
| Alkaloids | + |
| Anthraquinones | - |
| Amino acids | - |
| Terpenoids | + |
| Resins | + |

**Legend: "+" present "-" absent**

**Table 2:** GCMS Analysis of *P. amarus* Isopropanol Extract

| Peak # | Retention Time | Name | Peak Area % |
|---|---|---|---|
| **1** | 17.936 | Cyano colchicine | 2.30 |
| **2** | 18.055 | 16-Epivincamine-14-carboxylic acid,14,15-dihy | 1.10 |
| **3** | 20.150 | Phytol acetate | 0.31 |
| **4** | 21.195 | Hexadecanoic acid methylester | 0.76 |
| **5** | 21.361 | 1,1-Diphenyl-3-(methoxymethoxy)-1-butene | 2.06 |
| **6** | 22.380 | Pyrrolidine-2-carboxylic acid methyl-phenyl-amide | 2.84 |
| **7** | 22.472 | Hinokinin | 2.29 |
| **8** | 23.425 | Phytol | 1.33 |
| **9** | 25.532 | Carissanol dimethyl ether | 43.42 |

The GC-MS analysis result as shown in Table 2 revealed 9 compounds in the isopropanol extract.

**Table 3:** Percentage Inhibition of Fungal Growth by *P. amarus* Extract

| Isolate | Extract Concentration (mg/mL) | Isopropanol Extract (%) |
|---|---|---|
| ***A. flavus*** | 0.25 | 38.7 ± 1.1 |
| | 0.50 | 52.1 ± 1.3 |
| | 0.75 | 65.4 ± 1.6 |
| | 1.00 | 79.1 ± 1.7 |
| **POS Control** | | 49.1 ± 1.2 |
| ***Fusarium*** | 0.25 | 35.8 ± 1.0 |
| | 0.50 | 49.6 ± 1.2 |
| | 0.75 | 63.2 ± 0.5 |
| | 1.00 | 76.8 ± 0.7 |
| **POS Control** | | 46.1 ± 2.2 |
| ***Penicillium*** | 0.25 | 34.2 ± 0.4 |
| | 0.50 | 47.8 ± 1.1 |
| | 0.75 | 61.7 ± 1.4 |
| | 1.00 | 74.3 ± 1.6 |
| **POS Control** | | 33.1 ± 0.9 |

***Mean values (n = 3) ± standard error (SE); Positive control (solvents alone): POS Control (1mg/mL)***

The antifungal activity of *Phyllanthus amarus* isopropanol extract against *Aspergillus flavus, Fusarium* spp., and *Penicillium* spp. is shown in Table 3. The extract exhibited the strongest inhibition, with 79.1% against *A. flavus* at 1 mg/mL. The result for the positive control, which consisted of solvent alone, further emphasizes the antifungal properties of *P. amarus* extract. The positive control exhibited lower inhibition across all fungi, with only 49.1 % inhibition against *A. flavus*, 46.1 % against Fusarium spp., and 33.1 % against Penicillium spp. at 1 mg/mL. For *Fusarium* spp., the extract achieved 76.8 % inhibition, slightly lower than for *A. flavus.* The higher resistance of *Fusarium* may be due to its robust cell wall and its capability to generate protective secondary metabolites (Al-Hindi *et al.*, 2020). *Penicillium* spp. exhibited the lowest susceptibility to the plant extracts, with a maximum inhibition of 74.3 % 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 49.1% highest inhibition, indicating that while the solvent alone had some inhibitory effect, it was not as potent as the plant extract.

## Molecular Interactions of Phytochemicals of Isopropanol Extract of *Phyllanthus amarus* Leaves Docked against Lanosterol 14-alpha Demethylase (4LXJ)

Phytochemicals from the isopropanol extract of *Phyllanthus amarus* leaves were docked against lanosterol 14-alpha demethylase (4LXJ). Four of the compounds namely cyano colchicine, carissanol dimethyl ether, 16-epivincamine and hinokinin exhibited higher binding

affinities than the standard ligand, propiconazole. Notably, hinokinin demonstrated the highest binding affinity. However, compounds such as phytol acetate, phytol, and pyrrolidine-2-carboxylic acid methyl-phenyl-amide, exhibited lower binding affinities than propiconazole (Figure 1).

Observations from the visualization analysis showed that propiconazole interacted with lanosterol 14-alpha demethylase via eleven hydrophobic bonds and one hydrogen bond (Figure 2, Table 4). Hinokinin established nine hydrophobic bonds and two hydrogen bonds with lanosterol 14-alpha demethylase (Figure 3, Table 5).

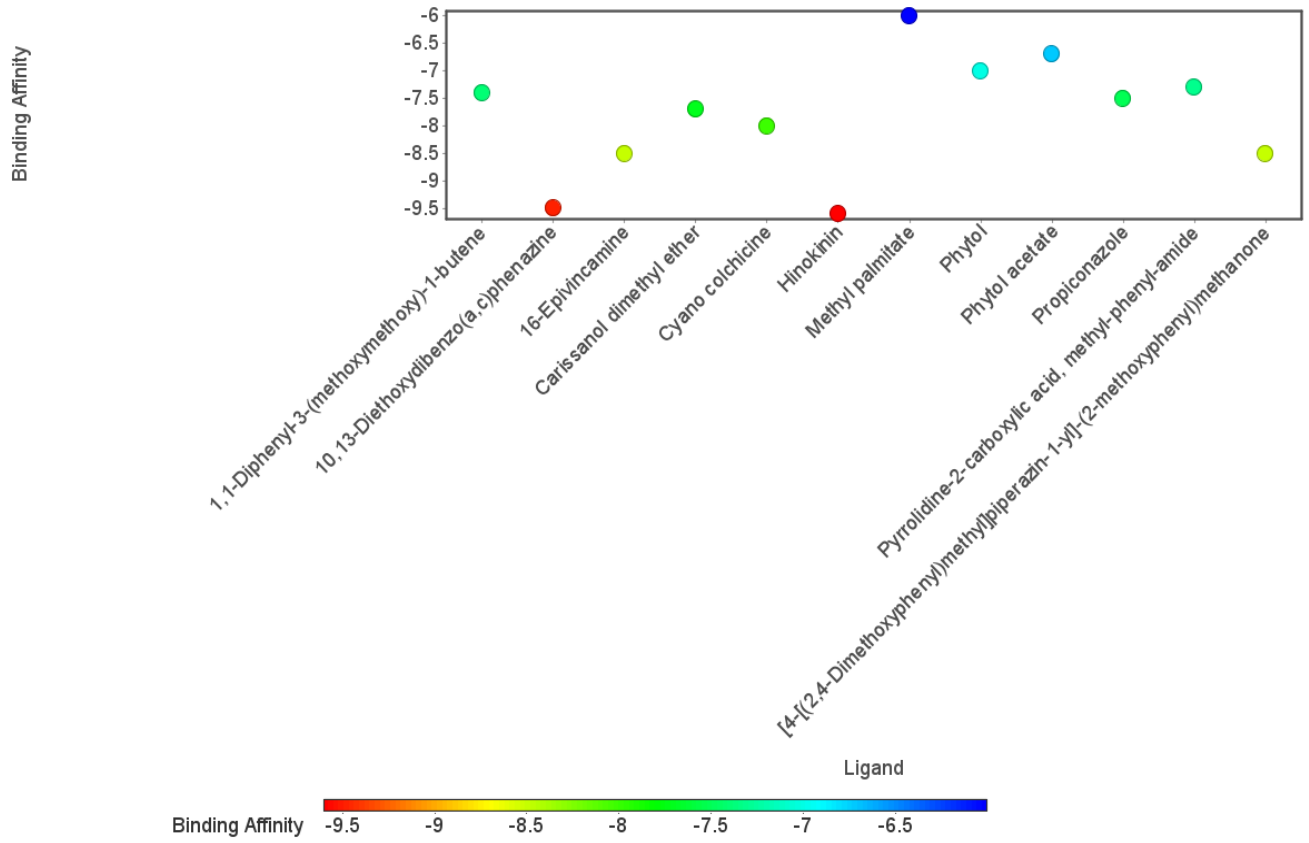



**Figure 1:** Binding Affinities of Compounds Isopropanol Extract of *P. amarus* Leaves Docked against Lanosterol 14-alpha Demethylase (4LXJ)

**Molecular Interactions of Isopropanol Extract of *P. amarus* Leaves Docked against Lanosterol 14-alpha Demethylase (4LXJ)**

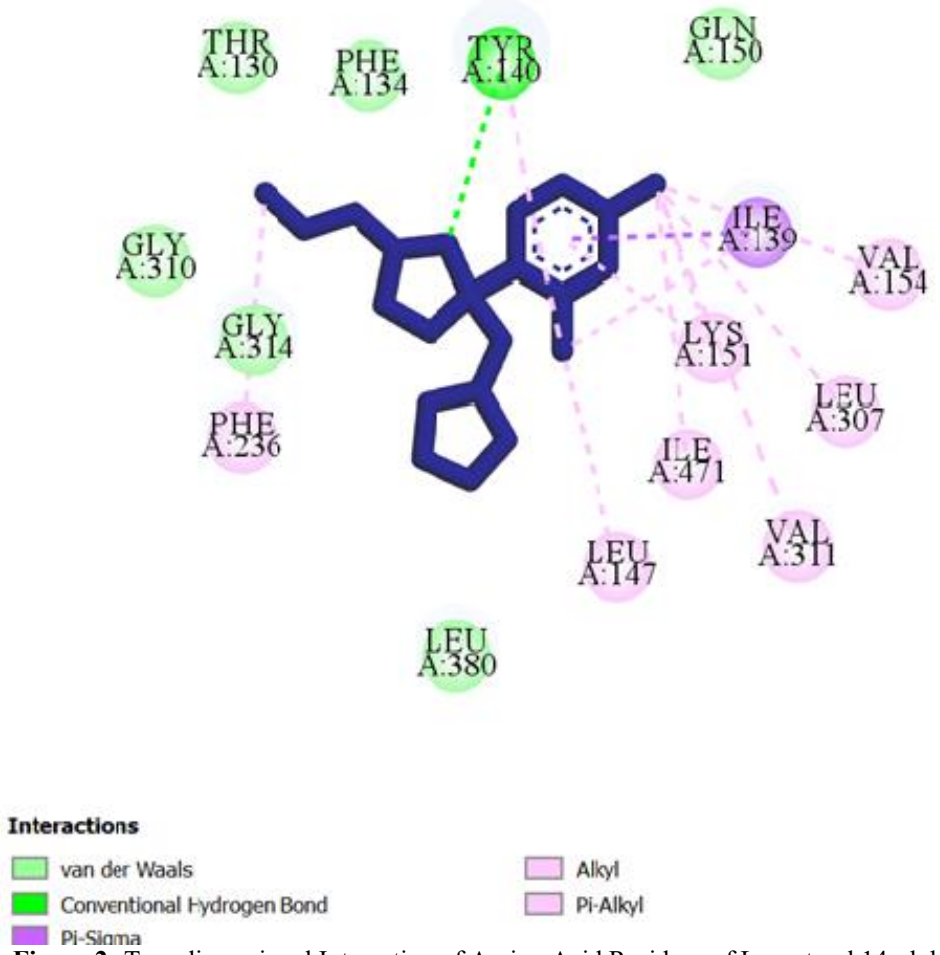



**Figure 2:** Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with Propiconazole

**Table 4:** 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 | 3.00863 |
| A:ILE139:CD1 – Propiconazole | Hydrophobic | Pi-Sigma | 3.72589 |
| Propiconazole- A:ILE139 | Hydrophobic | Alkyl | 4.80231 |
| Propiconazole- A:LEU147 | Hydrophobic | Alkyl | 4.53999 |
| Propiconazole- A:LYS151 | Hydrophobic | Alkyl | 4.16419 |
| Propiconazole- A:VAL154 | Hydrophobic | Alkyl | 4.01026 |
| Propiconazole- A:LEU307 | Hydrophobic | Alkyl | 4.84693 |
| Propiconazole- A:VAL311 | Hydrophobic | Alkyl | 5.24531 |
| Propiconazole- A:ILE471 | Hydrophobic | Alkyl | 5.19925 |
| A:TYR140 – Propiconazole | Hydrophobic | Pi-Alkyl | 4.97056 |
| A:PHE236 – Propiconazole | Hydrophobic | Pi-Alkyl | 5.16002 |
| Propiconazole- A:LYS151 | Hydrophobic | Pi-Alkyl | 5.26892 |

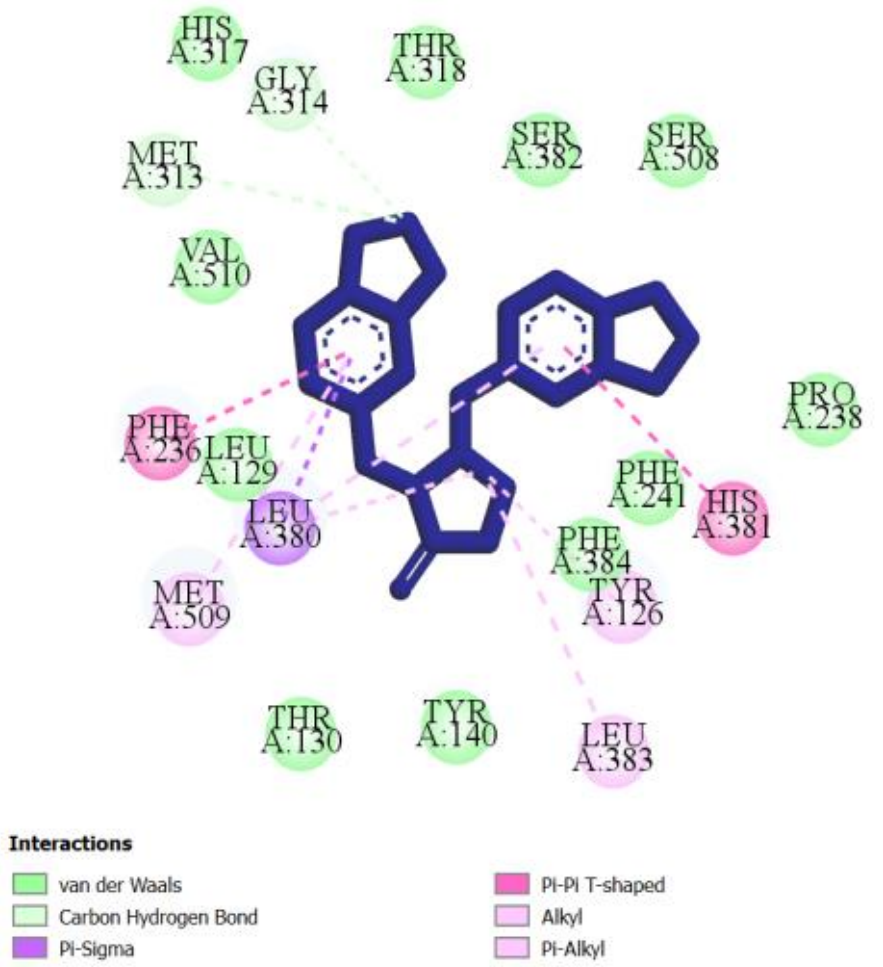



**Figure 3:** Two-dimensional Interaction of Amino Acid Residues of Lanosterol 14-alpha Demethylase (4LXJ) with Hinokinin

**Table 5: 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 | 3.61421 |
| Hinokinin- A:GLY314:O | Hydrogen Bond | Carbon-Hydrogen Bond | 3.41582 |
| A:LEU380:CD2 – Hinokinin | Hydrophobic | Pi-Sigma | 3.93961 |
| Hinokinin- Hinokinin | Hydrophobic | Pi-Pi Stacked | 5.47986 |
| A:PHE236 – Hinokinin | Hydrophobic | Pi-Pi T-shaped | 4.62395 |
| A:HIS381 – Hinokinin | Hydrophobic | Pi-Pi T-shaped | 5.42258 |
| Hinokinin- A:LEU380 | Hydrophobic | Alkyl | 4.44727 |
| Hinokinin- A:LEU383 | Hydrophobic | Alkyl | 5.23219 |
| A:TYR126 – Hinokinin | Hydrophobic | Pi-Alkyl | 4.48518 |
| Hinokinin- A:MET509 | Hydrophobic | Pi-Alkyl | 4.96137 |
| Hinokinin- A:LEU380 | Hydrophobic | Pi-Alkyl | 5.11074 |

**Bioavailability Assessment of Propiconazole and Hinokinin from Isopropanol Extract of *Phyllanthus amarus* Leaves Docked against Selected Protein Targets**

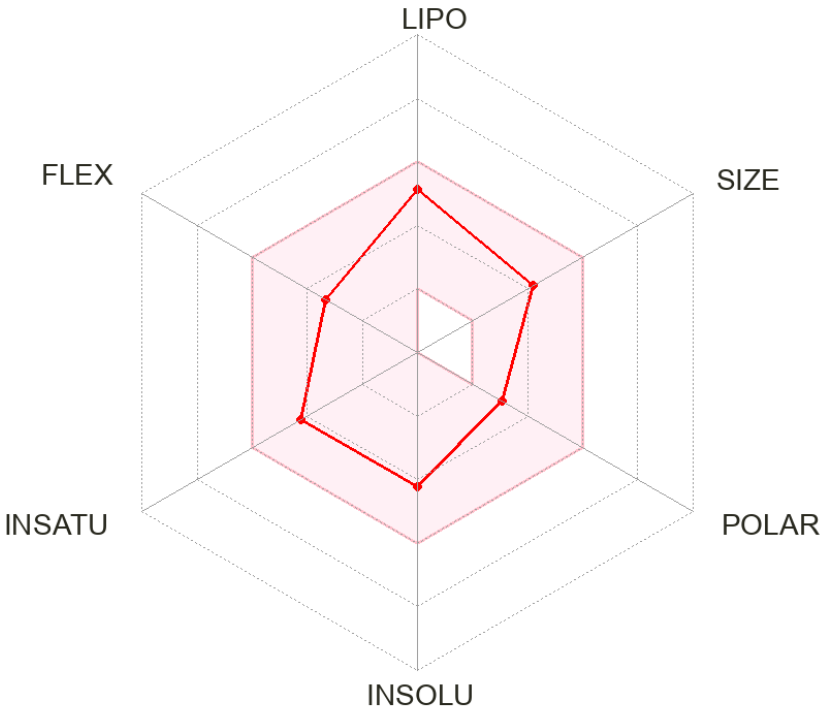



**Figure 4:** Bioavailability Properties of Propiconazole

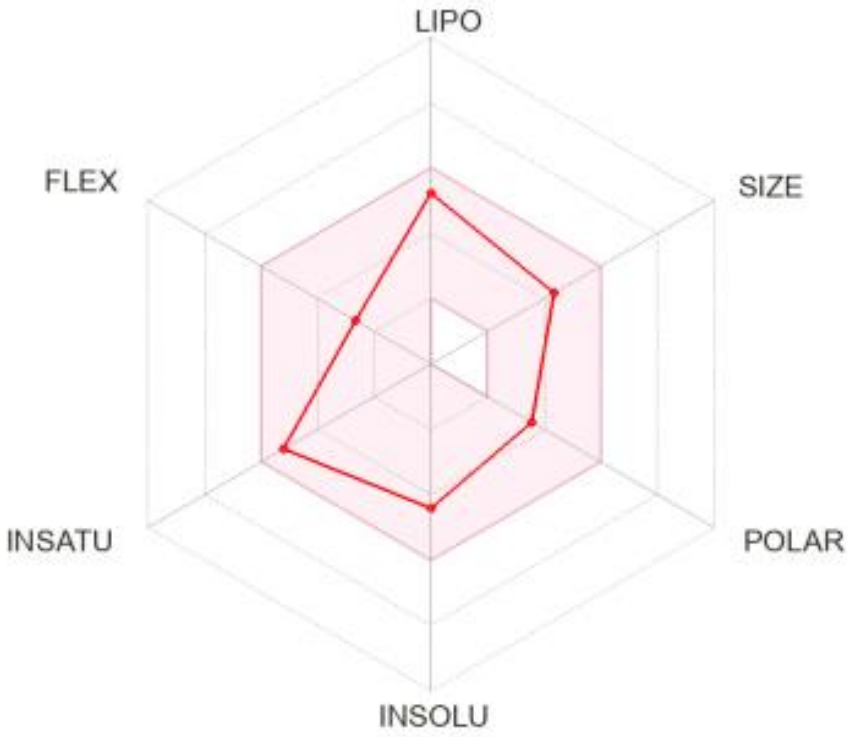



**Figure 5:** Bioavailability Properties of Hinokinin

Hinokinin and propiconazole passed the bioavailability criteria (Figures 4 and 5). Enzyme inhibition targets lanosterol 14-alpha demethylase (CYP51), a key enzyme in the ergosterol biosynthesis pathway of fungi. It plays a crucial role in fungal drug discovery and treatment, is essential for converting lanosterol to ergosterol, a vital component of fungal cell membranes that influences membrane permeability and fluidity (Istiqomah *et al*., 2023; Korkut *et al*., 2017). Inhibiting CYP51 leads to the depletion of ergosterol and accumulation of lanosterol. This results in impaired fungal cell growth and viability (Becher and Wirsel 2012; Korkut *et al., 2017)*. Compounds from traditional medicinal plants have shown antifungal activity through inhibition of CYP51, with terpenoids, flavonoids, and phenols showing promise in *in-silico* studies (Istiqomah *et al*., 2023).

Zhou *et al*., 2015, had reported the antifungal activity of hinokinin, which exhibited the strongest binding affinity among all docked compounds. It has also been reported to synergize with conventional fungicides like tebuconazole against *Fusarium graminearum* (Sanchez Matías *et al*., 2024).

From the results obtained, the strong binding affinities displayed by hinokinin and the other phytoconstituents of the plant extract signify their potential as effective agents for inhibiting fungal growth (Figure 1).

The standard antifungal agent, propiconazole exhibited a strong binding profile with 11 hydrophobic interactions and one hydrogen bond (Table 4). The association between non-polar regions of a drug candidate and hydrophobic amino acid residues within the target protein are promoted by hydrophobic interactions, contributing to the stability of the drug-protein complex and improving both binding affinity and specificity (Lou & Martin, 2021). The presence of hydrogen bonds plays an important role in facilitating molecular interactions within the cell, supporting the binding affinity and therapeutic potential of compounds (Pairas & Tsoungas, 2016). The hydrophobic interactions and hydrogen bonds formed between the lead compounds and CYP51 likely played a significant role in enhancing their binding affinities.

According to Daina *et al*., 2017, the evaluation of a compound's drug-likeness is shown by the bioavailability radar which highlights key properties such as lipophilicity, molecular size, polarity, solubility, flexibility, and saturation. Hinokinin, like propiconazole the reference drug met all the criteria (Figures 4 and 5). Hinokinin therefore has the potential to be developed as an antifungal agent.

## 4. Conclusion

From the results of this study, *Phyllanthus amarus* isopropanol extract shows very promising potential in the postharvest management of stored grains against *Aspergillus flavus, Fusarium* spp., and *Penicillium* spp. The outcome of the molecular docking study also highlights six phytoconstituents in the isopropanol extract of *Phyllanthus amarus* leaf demonstrating stronger binding affinities to lanosterol 14α-demethylase than the standard ligand, propiconazole. Hinokinin exhibited the highest affinity among all the compounds and also met all bioavailability criteria. The antifungal activity observed in the extract therefore, is due to the presence of hinokinin which stands out as a promising lead. Further *in-vitro* and *in-vivo* investigations are warranted to fully explore its antifungal potential.

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