

Research Article

Antifungal Susceptibility Testing Against *Aspergillus flavus* with Herbal Extracts and Synthetic AntifungalsShajeela Iram*¹, Rafiq Khanani¹, Johar Hussain², Mehmood ul Hassan³, Mehir un Nisa Iqbal⁴, Tabinda Khawaja⁵¹Department of Pathology, Baqai Medical University, Karachi, Pakistan²Dow Institute of Biological, Biochemical & Pharmaceutical Sciences, Dow University of Health Sciences, Karachi, Pakistan³Department of Veterinary Pharmacology, Lasbela University of Agriculture, Water and Marine Sciences, Uthal, Balochistan, Pakistan⁴Department of Physiology, University of Karachi, Karachi, Pakistan⁵Aiming Change for Tomorrow, Warwickshire, England

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Abstract

The globally distributed pathogenic fungus *Aspergillus flavus* poses a threat to both animal and human health. While synthetic antifungals have shown promise in controlling this fungus, their overuse has unfortunately led to a critical problem: the development of multidrug resistance, along with various other negative consequences. Therefore, alternative therapy is mandatory. This research sought to investigate the potential of herbal extracts, known for their antifungal properties, to serve as a complementary treatment to synthetic antifungals against *A. flavus*. Antifungal susceptibility tests were employed to evaluate this possibility. To isolate and identify *A. flavus*, fifty poultry feed samples underwent conventional culture methods and microscopic examination. Subsequently, the antifungal potential of five synthetic drugs (fluconazole, itraconazole, amphotericin B, nystatin, voriconazole) and eight herbal extracts (black seed, ginger, oregano, onion, turmeric, garlic, cinnamon, basil) was evaluated through disc diffusion assays and minimum inhibitory concentration (MIC) determination. Statistical analysis revealed voriconazole as the most effectual synthetic antifungal due to its highest zone of inhibition (39.53 ± 0.72) and lowest MIC value (0.12 ± 0). Hence, garlic amongst all herbs proved the best natural remedy with the highest inhibition zone value (25.83 ± 0.24) and lowest MIC score (0.66 ± 0.16) as per Clinical and Laboratory Standard Institute. Although synthetic antifungals gave better results than herbal extracts due to inconsistent, high financial exchange rates and lethal chemical residues, most of them are considered unsafe for humans. On the contrary, conventional complementary therapy, including herbal extracts, is harmless, affordable, eco-friendly, and has a lack of residual effects. Thus, more surveillance and research for the application of natural remedies are a supreme necessity.

Keywords: Disk diffusion test, Conventional culture method, Minimum Inhibitory Concentration, Multidrug resistance, Herbal extracts

1. Introduction

The widespread saprophytic genus *Aspergillus* includes over 300 species, approximately forty of which are hazardous to public health. Notably, *Aspergillus flavus* is a highly toxigenic and

opportunistic pathogen that contaminates numerous important food crops (Melo et al. 2020). This fungus causes Aspergillosis in humans, animals, and birds. In the US, avian aspergillosis has been estimated to cause \$11

million in annual losses to the turkey industry due to mortality. Beyond poultry, it has a significant economic impact and poses a public health risk by affecting poultry workers with invasive rhinosinusitis and pulmonary forms of the disease (Hauck et al. 2020; Rudramurthy et al. 2017).

A. flavus is a primary source of aflatoxin, a highly lethal and potent carcinogen that significantly compromises global food safety and poses a serious threat to human and animal health. (Tian et al. 2022). It affects meat and egg production and weakens the immune system of poultry (Guo et al. 2023). In humans, it can lead to aflatoxicosis, liver cancer, and bone disorders (Paneru et al. 2024; Persico et al. 2023). While synthetic fungicides, particularly triazoles (voriconazole, itraconazole, fluconazole, isavuconazole) and polyenes (amphotericin B (AMB), nystatin) (Ahmad 2019), are extensively used and considered primary treatments for *A. flavus* and aflatoxin contamination, and triazoles are even the preferred drugs for aspergillosis in veterinary and public health (Uppala et al. 2018), recent studies have alarmingly revealed a significant increase in *Aspergillus* isolates resistant to both triazoles and polyenes, leading to high morbidity and mortality (Khojasteh et al. 2023; Ahmad Khan et al. 2020). In 2022, the WHO released for the first time the list of life-threatening fungi for the guidance of research, development, and public health action (WHO 2022). This emergence of the resistance may be due to inadequate and irrational use of drugs, changes in gene expression, virulence factors, and complex interactions between environmental conditions (Yadav et al. 2022).

This study primarily aimed to develop alternative antimycotic agents to circumvent the concerning adverse effects of synthetic antifungals and mitigate the risks associated with mycotoxin production and the pathogenicity of *A. flavus*. Consequently, active biological compounds from natural herbs were investigated in comparison to the synthetic antifungal drugs. While synthetic drugs initially demonstrated superior results, the study concluded that herbal extracts hold greater significance due to The growing issue of multidrug resistance and the

undesirable effects of synthetic compounds, coupled with the high efficacy, easy availability, cost-effectiveness, and biodegradability of herbal alternatives.

Therefore, despite the introduction of novel synthetic antifungals, biologically active phytochemicals extracted from herbs should be prioritized for enhanced protection against fungal growth.

2. Materials & Methods

2.1. Sample Assortment

Fifty poultry feed samples were randomly collected from fifteen poultry farms across Karachi between 2021 and 2022. Specifically, three farms were chosen in Bin Qasim, five in Surjani Town, and seven in Gadap Poultry State. The herbal samples, including turmeric, ginger, basil, oregano, onion, garlic, cinnamon, and black seed, were procured from a local market. All samples were stored hygienically until further analysis.

2.2. Isolation of *A. flavus*

Each poultry feed sample was initially combined with sterile normal saline in a 1:9 ratio. Subsequently, 1ml of each diluted sample was inoculated onto Sabouraud Dextrose Agar plates using the spread plate method. The inoculated plates were then incubated at 35 °C for 3 to 7 days. Following incubation and observation of fungal colonies, pure cultures were preserved for further analysis (Osaro-Mathew et al. 2017).

2.3. Identification of *A. flavus*

Macroscopically, fungal colony features (shape, size, color) were studied, and microscopically, tiny sections of mycelium were stained by lactophenol cotton blue stain and were observed by using a digital camera with a compound microscope (Gaddeyya et al. 2012).

2.4. Herbal Extract Preparation

The plant materials were first washed and then shade-dried at room temperature. Once dried, they were ground into a fine powder using an electric grinder. For extract

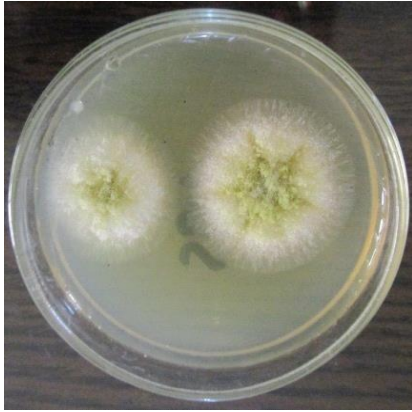


Figure 1: Macroscopic vision of *A. flavus*

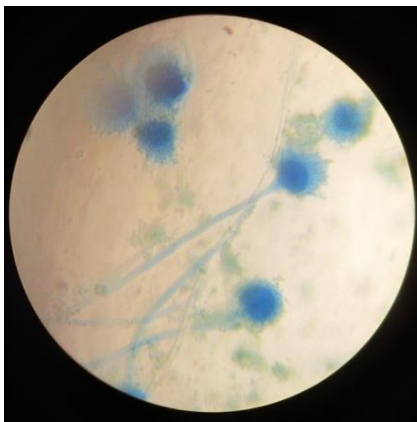


Figure 2: Microscopic vision of *A. flavus*

preparation, 50 g of each herb were individually soaked in 300 ml of ethanol for 24 hours. The resulting mixtures were filtered using Whatman No. 1 filter paper to obtain the active ingredients. The filtrates were then concentrated using a rotary vacuum evaporator under reduced pressure. The dried crude extracts were subsequently stored in a refrigerator at 4 °C until further use (Gini and Jeya 2015).

2.5. Antifungal Susceptibility Test

Antifungal susceptibility of synthetic antifungal and natural herbs was evaluated by disc diffusion test and Minimum Inhibitory Concentration (MIC).

2.5.1. Disc -diffusion Test

Disk-diffusion test, as described by Clinical and Laboratory Standards Institute (CLSI 2010) with slight amendments were performed to test the antifungal susceptibility of isolates.

2.5.2. Disc Preparation for Herbs

For the disc diffusion assay of plant extracts, 6 mm diameter discs were prepared from Whatman filter paper and sterilized by autoclaving. To prepare the test solution, 8 mg of each extract was dissolved in 10 ml of DMSO, resulting in a suspension containing 800 µg/ml. It was the initial concentration, used to check anti-aspergillus activities. Each disc was impregnated with (20µl) of DMSO and used for tests (Kporou et al. 2016; Epsinel 2007).

2.5.3. Synthetic Antifungal Discs

Commercially available antifungal discs for synthetic antifungals such as Nystatin (100 units, oxoid), Itraconazole (8 µg, oxoid), Amphotericin B (20 µg, oxoid), Fluconazole (25µg, oxoid) Voriconazole (1µg, oxoid) were used.

2.5.4. Protocol

Fungal growth was supported using Mueller-Hinton agar. This sterile medium was uniformly streaked with 100 µL of a prepared inoculum of the test mold. The inoculum was prepared by using the 3-4 colonies of mature and fresh (5-7 days old) culture of *A. flavus* grown on Sabouraud Dextrose agar in saline water and was adjusted equivalent of 0.5 McFarland standard (1.5×10^6 cfu/ml). A sterile cotton swab dipped into the inoculum and used to streak on Mueller-Hinton agar three times. After drying, discs were gently pressed onto the agar surface at a proper distance and incubated for 48 hours. The diameter of the zone of inhibition for each disc was measured to determine the antifungal activity of that drug (Kporou et al. 2016).

2.5.5. Minimum Inhibitory Concentration MIC

The broth microdilution method, as given by the CLSI document (CLSI 2008), was implemented to determine the MIC of antifungal agents.

2.5.6. Protocol

To determine the Minimum Inhibitory Concentration (MIC), a 96-well microtitre plate was utilized. Each well, except the first, received 100 µl of RPMI 1640 medium. The first well was loaded with 200 µl of the antifungal drug at 800 µg/ml. Serial two-fold dilutions were then created by transferring 100 µl from each well to the next, establishing a concentration gradient from 800

Table 1(a): The disc diffusion test.

Antifungal	Mean±SEM	95% Confidence Interval		Minimum	Maximum
		Lower Bound	Upper Bound		
Variconazole	39.53 ± 0.72	36.42	42.63	38.3	40.8
Itraconazole	35.4 ± 0.47	33.36	37.43	34.7	36.3
Nystatin	26.06 ± 0.14	25.44	26.69	25.8	26.3
Amphotericin B	17.46 ± 1.53	10.85	24.08	15.2	20.4
Fluconazole	10.1 ± 0.47	8.06	12.13	9.2	10.8
Total	25.71 ± 2.93	19.41	32.01	9.2	40.8

Table 1(b): Analysis of variance (ANOVA).

	Sum of Squares	Df	Mean Square	F	P value
Between Groups	1790.19	4	447.549	222.44	0.00
Within Groups	20.12	10	2.012		
Total	1810.31	14			

µg/ml down to 3.125 µg/ml. A standardized inoculum of 100 µL (1.5x10⁶ CFU) was added to each well except the medium sterility control well (12). The 11th well served as fungal growth control. After incubation, the MIC as the lowest concentration that inhibits fungal growth with no visual disturbance in the well, was recorded.

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was subjected to obtain the statistical association of antifungals and plant extracts against fungal growth by using SPSS version 22.0.

3. Results

3.1 Macroscopic and Microscopic Identification of *A. flavus*

On Sabouraud Dextrose agar, the *A. flavus* colonies initially appeared flat and granular with a yellow hue, which deepened to a dark yellowish-green as they matured (Figure 1). Microscopically, thick, and rough conidiophores, radiated conidial heads, and phialides on the complete surface of vesicles were observed. Both Uniseriate and biseriate cells were recorded (Figure 2).

3.2. Antifungal Susceptibility Tests

The results of the synthetic antifungals by using the disc diffusion test and minimum inhibitory concentration test are as follows.

3.2.1. Disc-diffusion Test for Synthetic Antifungals

Susceptibility order against *A. flavus* revealed that voriconazole is extremely efficient against *A. flavus*, while fluconazole is slightest one (Table 1a & b), (Figure 3).

3.2.2. Minimum Inhibition Concentration Test for Synthetic Antifungals

The considerable difference of susceptibility ($P \leq 0.05$) amongst all antifungal drugs demonstrated that fluconazole, the least effective drug with the highest MIC scores, and voriconazole, with the lowest MIC scores the highly effective antifungal (Table 2a & b).

3.2.3. Disk Diffusion Test for Herbal Extracts

Susceptibility of herbal extracts against fungal isolates are in the following order: garlic > ginger > basil, while rest of all the herbs proved ineffective as indicated in Table 3a & b.

3.2.4. Minimum Inhibition Concentration Test for Plant Extracts

MIC score revealed the highest fungicidal efficacy of garlic extract against *A. flavus*. On the other hand, ineffectiveness of other herbs is proved by their zero value (Table 4a & b).

Overall, voriconazole demonstrated the most potent antifungal activity among the tested synthetic drugs, while garlic extract exhibited

Table 2(a): Minimum Inhibition Concentration Test.

Antifungal	Mean ± SEM	95% Confidence Interval		Minimum	Maximum
		Lower Bound	Upper Bound		
Variconazole	0.12 ± 0	0.12	0.12	0.13	0.13
Itraconazole	0.58 ± 0.22	-0.36	1.53	0.25	1
Nystatin	2.33 ± 1.52	-1.46	6.12	1	4
Amphotericin B	5.33 ± 2.3	-0.4	11.07	4	8
Fluconazole	9.33 ± 3.52	-5.84	24.51	4	16
Total	3.54 ± 1.12	1.12	5.95	0.13	16

Table 2(b): Disk diffusion test for Herbal extracts (ANOVA).

Groups	Sum of Squares	Df	Mean Square	F	P value
Between Groups	175.917	4	43.979	4.871	0.019
Within Groups	90.292	10	9.029		
Total	266.208	14			

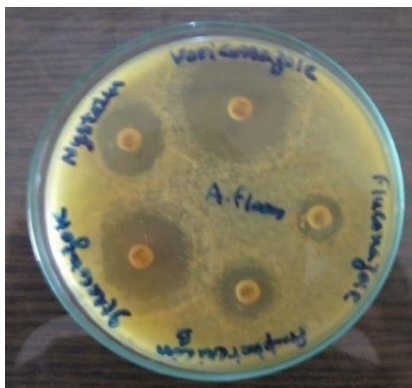


Figure 3: Disc diffusion test of synthetic antifungal against *A. flavus*, Where:1= Voriconazole, 2= Fluconazole, 3 = Amphotericin B, 4 = Itraconazole, 5 = Nystatin

the highest fungicidal potential among the evaluated herbal extracts.

4. Discussion

The agriculture sector is critically important to Pakistan, serving as the primary source of income for a huge portion of its population. Studies have established a clear correlation between agricultural progress and the country's national economy (Ullah et al. 2023). Plants, known for their powerful antimicrobial characteristics, can provide a natural defense for food against diverse toxins. In addition, natural compounds extracted from plants show promise as effective antifungal

agents (Abid et al. 2022).

Over the past two decades, *Aspergillus* infections have evolved into serious and life-threatening mycotic diseases. Epidemiological data indicates a high rate of fungal diseases, with 150 million cases resulting in 1.7 million deaths annually (Kainz et al. 2020). The rising rate of these incidences is linked to the overuse of chemotherapeutic drugs, irradiation, and immunosuppressants. Current studies have shown a higher frequency of occurrence in immunocompromised persons, including up to 30% of the intubated COVID-19 patients (Bartoletti et al. 2021). Antifungal susceptibility tests, such as disc diffusion test and Minimum Inhibitory Concentration, have become vital tools for fungal infections treatment as well as for resistance detection against antifungals (Alastruey-Izquierdo et al. 2015). The treatment landscape relies on a limited number of antifungal agents, including broad-spectrum triazoles (e.g., voriconazole, itraconazole) and polyenes (e.g., amphotericin B, nystatin). Among these, triazoles are globally recognized and extensively utilized as a preferred remedy (Maertens et al. 2021; Berkow et al. 2020). These findings agree with our results that revealed triazoles with high mean log values like voriconazole (39.53 ± 0.72), followed by

Table 3(a): Disk diffusion test.

Plants Extract	Mean ± SEM	95% Confidence Interval		Minimum	Maximum
		Lower Bound	Upper Bound		
Garlic	25.83 ± 0.24	24.79	26.86	25.5	26.3
Ginger	20.2 ± 0.26	19.06	21.33	19.7	20.6
Basil	11.83 ± 0.78	8.47	15.18	10.5	13.2
Onion	0	0	0	0	0
Black seed	0	0	0	0	0
Turmeric	0	0	0	0	0
Cinnamon	0	0	0	0	0
Oregano	0	0	0	0	0

Table 3(b): ANOVA for Herbal extracts.

Groups	Sum of Squares	Df	Mean Square	F	P value
Between Groups	2390.58	7	341.51	1238.108	0.00
Within Groups	4.41	16	0.27		
Total	2394.99	23			

itraconazole (35.4 ± 0.47), nystatin (26.06 ± 0.14), amphotericin B (17.46 ± 1.530) and fluconazole (10.1 ± 0.47). Voriconazole demonstrated the highest efficacy among the tested synthetic antifungals, exhibiting the lowest MIC score (0.12 ± 0) against invasive aspergillosis, a finding consistent with the recommendations of (Douglas et al. 2021) and (Rudramurthy et al. 2017), who also advocate for its use as a first-line treatment. However higher MIC score (9.33 ± 3.52) of fluconazole showed the least susceptibility against *A. flavus*, which is in accordance with previous reports (Govindarajan et al. 2022; Murray et al. 2020; Sarrafha et al. 2018) who demonstrated fluconazole as a poor antifungal agent.

Unfortunately, the constant use of synthetic antifungals, coupled with the ability to mutate in response to environmental conditions, has led to the emergence of new pathogenic resistant strains. This resistance is reflected in an increase in the value of MIC of drugs during treatment (Rabaan et al. 2023; Kaur et al. 2023). The increased resistances of synthetic drugs encourage the search for a complementary remedy based on natural products without any

side effects. Several antifungal studies have evaluated that herbal medications due to lower occurrence of adverse reactions and having antimicrobial bioactive compounds for both humans and plant pathogens are considered the best replacement of the chemotherapeutic agents (Socaciu et al. 2020; Natu et al. 2019; Sitara and Hassan 2011). In this study, we evaluated the antifungal activity of eight herbal extracts: garlic, cinnamon, ginger, basil, onion, turmeric, black seed, and oregano. Among these, garlic (MIC: 0.66 ± 0.16), ginger (MIC: 3 ± 1), and basil (MIC: 5.33 ± 1.33) demonstrated the most promising MIC values, suggesting their potential as substitutes for synthetic chemicals. This study is consistent with (Silva-Beltrán et al. 2023; Medalcho et al. 2023; Espinoza Tellez et al. 2020). Garlic with the lowest MIC score acts as the most effective antifungal, which is due to its sulfur compound content, such as allicin and ajoene, for their anti- microbial, anti-inflammatory, and antioxidant properties. These results are in line with (Rahman, et al. 2022; Zhang et al. 2020; Varga-Visi et al. 2019). Ginger is identified as the Second most herbal medicinal product, containing caprylic acid as the

Table 4(a): Minimum Inhibition Concentration Test.

Plant extracts	Mean ± SEM	95% Confidence Interval		Minimum	Maximum
		Lower Bound	Upper Bound		
Garlic	0.66 ± 0.16	-0.0504	1.3838	0.5	1
Ginger	3 ± 1	-1.3027	7.3027	1	4
Basils	5.33 ± 1.33	-0.4035	11.0702	4	8
Onion	0	0	0	0	0
Black seed	0	0	0	0	0
Turmeric	0	0	0	0	0
Cinnamon	0	0	0	0	0
Oregano	0	0	0	0	0

Table 4(b): ANOVA for Herbal extracts.

	Sum of Squares	Df	Mean Square	F	P value
Between Groups	83.292	7	11.899	11.31	0
Within Groups	16.833	16	1.052		
Total	100.125	23			

against fungal growth. Likewise, (Kalhor et al. 2022; Vivian. et al. 2019) also reported ginger with a pharmacological effect. Basil showed moderate antifungal effects as it consists of linalool, a phytochemical of average antimicrobial activity. Similar results were recorded by (Nugroho et al. 2019).

In contrast, oregano, turmeric, onion, cinnamon, and black seed, when tested at the same concentrations, did not exhibit any noticeable fungistatic or fungicidal activity. This finding aligns with the observations of (Chalfoun et al. 2004), who also reported poor antifungal effects for oregano, onion, thyme, mint, and cinnamon. As a result, some herbal compounds can be utilized as preservatives, which may successfully replace synthetic antifungals and provide an alternative method for the protection of food from the toxic fungal contamination likewise (Hussain and Shafqatullah 2012).

5. Conclusion

It is concluded from the existing study that although some synthetic antifungals have clinical significance against fungal infections but their

irrational use and the emergence of new resistant strains of microbes have enhanced the multidrug resistance of these drugs. Whereas complementary therapy consisting of natural herbal extracts has proved its best antifungal efficacy without any adverse effects, plus, affordability and easy availability are additional characteristics. Therefore, further research is recommended for the implementation of this alternative therapy in the food and pharmaceutical industries. Moreover, public awareness programs regarding the prevention of fungal contamination of food with these natural herbs should also be emphasized.

Conflict of Interest

The authors declared no conflict of interest.

Funding

No Funding is provided by any institute or company.

Ethical Approval

This study was conducted on poultry feed samples, which were obtained from commercial

poultry farms. Hence, this project needs no live animal or bird, so no ethical committee approval was required for this study.

Consent Forms

NA

Author Contributions

Study concept and design, SI and RK; acquisition of the data, SI and MI; analysis and interpretation of data, JH and TK; drafting of the manuscript, MH and SI; critical revision of the manuscript for important, intellectual content, MH, JH and TK; statistical analysis, MI; study supervision, SI and RK.

Data Availability

The dataset presented in the study is available on request from the authors.

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