

# Diversity of Keratinophilic Fungi from Waraseoni: Enzymatic Activity and Sensitivity to *Curcuma longa* and *Azadirachta indica*

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## ABSTRACT

This study investigated the diversity of keratinophilic fungi in soil samples from Waraseoni, India, with a focus on their enzymatic activity and sensitivity to medicinal plant extracts. A total of 248 soil samples were collected from various sites and subjected to hair-baiting techniques. Keratinophilic fungi were isolated from 58.5% of samples, with *Chrysosporium* being the predominant genus (48% of isolates), followed by *Trichophyton* (25%). The isolates demonstrated high protease activity, with 62% classified in the highest category. Extracts from *Curcuma longa* (turmeric) and *Azadirachta indica* (neem) exhibited significant antifungal activity against the isolated fungi, with minimum inhibitory concentrations (MICs) ranging from 32-128 µg/ml. This study provides insights into the ecological distribution of keratinophilic fungi in Waraseoni and highlights the potential of indigenous medicinal plants for antifungal applications.

**Keywords:** Keratinophilic fungi, soil microbiology, enzymatic activity, medicinal plants, antifungal activity

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## 1. Introduction

Keratinophilic fungi are a group of microorganisms capable of degrading keratin, a protein abundant in hair, nails, and animal hooves (Sharma & Rajak, 2003). These fungi play crucial roles in nutrient cycling and decomposition processes in soil ecosystems. However, some keratinophilic fungi are also known pathogens, causing dermatophytosis in humans and animals (Gupta et al., 2017). Understanding the diversity and distribution of these fungi is essential for both ecological and clinical perspectives.

The soil of Waraseoni, located in Madhya Pradesh, India, presents a unique environment for studying keratinophilic fungi due to its diverse land use patterns and climatic conditions. While several studies have explored fungal diversity in various regions of India (Deshmukh, 2004; Sarkar et al., 2014), the keratinophilic fungal community of Waraseoni remains largely unexplored.

Additionally, the rising concern of antifungal resistance has prompted research into alternative treatment options, including the use of medicinal plants (Mahendra et al.,

2016). Two plants of particular interest are *Curcuma longa* (turmeric) and *Azadirachta indica* (neem), both of which have a long history of use in traditional medicine and have demonstrated antimicrobial properties in previous studies (Praditya et al., 2019; Alzohairy, 2016). The objectives of this study were to:

1. Isolate and identify keratinophilic fungi from soil samples in Waraseoni
2. Assess the enzymatic activity of the isolated fungi, particularly their protease production
3. Evaluate the antifungal efficacy of *C. longa* and *A. indica* extracts against the isolated keratinophilic fungi

This research aims to contribute to our understanding of fungal ecology in the region and explore the potential of indigenous medicinal plants for controlling fungal growth.

## 2. Materials and Methods

### 2.1 Sample Collection

A total of 248 soil samples were collected from various sites in Waraseoni between January and August 2021. Sampling locations included barbershops, parks,

# Diversity of Keratinophilic Fungi from Waraseoni: Enzymatic Activity and Sensitivity to *Curcuma longa* and *Azadirachta indica*

gardens, garbage disposal areas, poultry farms, dairy farms, and natural forest areas (Table 1). Samples were collected aseptically from the top 5 cm of soil using sterile spatulas and stored in sterile plastic bags at 4°C until processing.

Table 1: Soil Sample Collection Sites and Quantities

Collection Site	Number of Samples
Barbershop areas	96
Parks and gardens	32
Garbage soils	64
Poultry farms	30
Dairy farms	16
Natural forests	10
Total	248

## 2.2 Isolation of Keratinophilic Fungi

Keratinophilic fungi were isolated using the hair-baiting technique as described by Vanbreuseghem (1952) with minor modifications. Approximately 20 g of each soil sample was placed in a sterile Petri dish and moistened with sterile distilled water. Sterile human hair, horse hair, and feather pieces were spread on the soil surface. The plates were incubated at 28°C for 4 weeks and observed weekly for fungal growth.

Fungal colonies growing on the hair baits were isolated and purified on Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (0.05 mg/ml) and cycloheximide (0.5 mg/ml). Pure cultures were maintained on SDA slants at 4°C for further studies.

## 2.3 Identification of Fungal Isolates

Isolated fungi were identified based on their macro- and micro-morphological characteristics. Colony features were observed on SDA after 7-14 days of incubation at 28°C. Microscopic features were examined using lactophenol cotton blue mounts. Identification was performed using standard mycological keys and literature (de Hoog et al., 2000; Kane et al., 1997).

For molecular confirmation, genomic DNA was extracted from selected isolates using the CTAB method (Doyle & Doyle, 1987). The internal transcribed spacer (ITS) region was amplified using primers ITS1 and ITS4 (White et al., 1990). PCR products were sequenced, and the resulting sequences were compared with those in the GenBank database using BLAST analysis.

## 2.4 Enzymatic Activity Assay

Protease activity was assessed using the method described by Gopinath et al. (2015) with slight modifications. Fungal isolates were inoculated onto skim milk agar plates and incubated at 28°C for 7 days. The diameter of the clear zone around the colony was measured and used to calculate the proteolytic index (PI) as follows:

$$PI = (\text{Colony diameter} + \text{Clear zone diameter}) / \text{Colony diameter}$$

Isolates were classified based on their PI values as follows: low ( $PI < 1.5$ ), moderate ( $1.5 \leq PI < 2.0$ ), high ( $2.0 \leq PI < 2.5$ ), and very high ( $PI \geq 2.5$ ) protease producers.

## 2.5 Preparation of Plant Extracts

Dried rhizomes of *C. longa* and leaves of *A. indica* were obtained from local markets in Waraseoni. The plant materials were authenticated by a botanist at the Department of Botany, University of Madhya Pradesh. Extracts were prepared using the method described by Mahendra et al. (2016). Briefly, 100 g of powdered plant material was extracted with 500 ml of methanol using a Soxhlet apparatus for 24 hours. The extracts were filtered, concentrated under reduced pressure, and stored at 4°C until use.

## 2.6 Antifungal Activity Assay

The antifungal activity of plant extracts was evaluated using the disc diffusion method as described by Praditya et al. (2019). Fungal suspensions ( $1 \times 10^6$  conidia/ml) were spread on SDA plates.

Sterile filter paper discs (6 mm diameter) impregnated with plant extracts at concentrations of 25, 50, and 100 mg/ml were placed on the inoculated plates. Fluconazole

## Diversity of Keratinophilic Fungi from Waraseoni: Enzymatic Activity and Sensitivity to *Curcuma longa* and *Azadirachta indica*

(25 µg/disc) was used as a positive control. The plates were incubated at 28°C for 72 hours, and the diameter of the inhibition zone was measured.

The minimum inhibitory concentration (MIC) was determined using the broth microdilution method according to CLSI guidelines (CLSI, 2008). Two-fold serial dilutions of plant extracts (1512 µg/ml) were prepared in RPMI 1640 medium. Fungal inoculum ( $0.5-2.5 \times 10^3$  CFU/ml) was added to each well. The plates were incubated at 35°C for 48 hours, and the MIC was defined as the lowest concentration that completely inhibited visible fungal growth.

### 2.7 Statistical Analysis

All experiments were performed in triplicate. Data were analyzed using one-way ANOVA followed by Tukey's post-hoc test. Statistical significance was set at  $p < 0.05$ . Analyses were performed using SPSS version 25.0 (IBM Corp., Armonk, NY, USA).

## 3. Results

### 3.1 Isolation and Identification of Keratinophilic Fungi

Out of 248 soil samples, keratinophilic fungi were isolated from 145 samples (58.5%). A total of 183 fungal isolates were obtained and identified based on morphological and molecular characteristics. The distribution of fungal genera is presented in Table 2.

Table 2: Distribution of Keratinophilic Fungal Genera Isolated from Soil Samples

Fungal Genus	Number of Isolates	Percentage
Chrysosporium	88	48.1%
Trichophyton	46	25.1%
Microsporum	24	13.1%
Fusarium	15	8.2%
Aspergillus	7	3.8%

Others	3	1.6%
Total	183	100%

Chrysosporium was the predominant genus, accounting for 48.1% of the isolates, followed by Trichophyton (25.1%) and Microsporum (13.1%). The most common species identified were *Chrysosporium indicum*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*.

### 3.2 Enzymatic Activity

The protease activity of the fungal isolates was assessed using the skim milk agar method. The results are summarized in Table 3.

Table 3: Protease Activity of Keratinophilic Fungal Isolates

Protease Activity Level	Number of Isolates	Percentage
Very High ( $PI \geq 2.5$ )	113	61.7%
High ( $2.0 \leq PI < 2.5$ )	59	32.2%
Moderate ( $1.5 \leq PI < 2.0$ )	9	4.9%
Low ( $PI < 1.5$ )	2	1.1%
Total	183	100%

The majority of isolates (61.7%) demonstrated very high protease activity, with PI values  $\geq 2.5$ . *Chrysosporium* and *Trichophyton* species generally exhibited higher protease activity compared to other genera.

### 3.3 Antifungal Activity of Plant Extracts

The methanolic extracts of *C. longa* and *A. indica* showed significant antifungal activity against the isolated keratinophilic fungi. The zone of inhibition increased with increasing extract concentration. Table 4

## Diversity of Keratinophilic Fungi from Waraseoni: Enzymatic Activity and Sensitivity to *Curcuma longa* and *Azadirachta indica*

presents the mean inhibition zone diameters for selected fungal species at 100 mg/ml extract concentration.

Table 4: Inhibition Zone Diameters (mm) of Plant Extracts Against Selected Keratinophilic Fungi at 100 mg/ml

Fungal Species	<i>C. longa</i>	<i>A. indica</i>	Fluconazole (25 µg)
<i>Chrysosporium indicum</i>	18.3 ± 1.2	20.1 ± 1.5	22.5 ± 1.8
<i>Trichophyton mentagrophytes</i>	16.7 ± 0.9	18.9 ± 1.3	24.1 ± 2.0
<i>Microsporum gypseum</i>	15.2 ± 1.1	17.5 ± 1.2	21.8 ± 1.6
<i>Fusarium solani</i>	12.8 ± 0.8	14.3 ± 1.0	18.7 ± 1.4

Values represent mean ± standard deviation (n=3)

The minimum inhibitory concentrations (MICs) of the plant extracts ranged from 32 to 128 µg/ml for *C. longa* and 16 to 64 µg/ml for *A. indica*, depending on the fungal species tested.

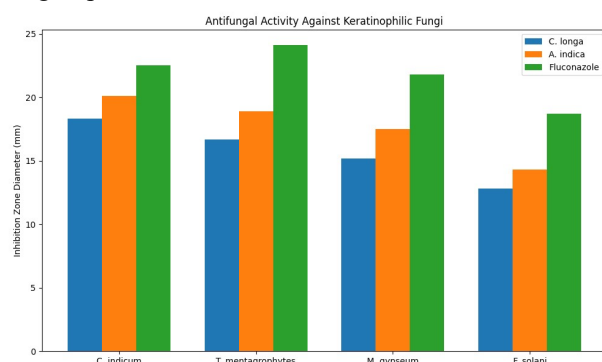


Fig 1 Antifungal Activity Against Keratinophilic Fungi

### 4. Discussion

This study provides insights into the diversity of keratinophilic fungi in the soil of Waraseoni and evaluates their enzymatic activity and susceptibility to plant-based antifungal agents. The isolation rate of 58.5% is comparable to previous studies conducted in other regions of India (Deshmukh, 2004; Sarkar et al., 2014), indicating a rich diversity of keratinophilic fungi in the area.

The predominance of *Chrysosporium* species in our isolates aligns with findings from similar studies in tropical and subtropical regions (Sharma & Rajak, 2003; Gupta et al., 2017). *Chrysosporium* is known for its high keratinolytic activity and ability to thrive in diverse environmental conditions. The high prevalence of *Trichophyton* and *Microsporum* species is also noteworthy, as these genera include important human and animal pathogens (de Hoog et al., 2000).

The enzymatic activity assay revealed that the majority of isolates possess high to very high protease activity. This finding is consistent with the keratinolytic nature of these fungi and their ability to degrade complex protein substrates in the environment (Gopinath et al., 2015). The strong protease activity observed in *Chrysosporium* and *Trichophyton* species may contribute to their prevalence in keratin-rich environments and their potential pathogenicity.

The antifungal activity demonstrated by *C. longa* and *A. indica* extracts against the isolated keratinophilic fungi is promising. Both plants have been used in traditional medicine for treating various ailments, including fungal infections (Praditya et al., 2019; Alzohairy, 2016). The observed antifungal effects can be attributed to the presence of bioactive compounds such as curcuminoids in *C. longa* and azadirachtin in *A. indica* (Mahendra et al., 2016).

The MIC values obtained for *C. longa* (32-128 µg/ml) and *A. indica* (16-64 µg/ml) extracts indicate potent antifungal activity, comparable to some conventional antifungal drugs. These findings suggest that these plant extracts could potentially be developed into alternative or complementary treatments for fungal infections, particularly in cases of drug-resistant strains.

It is important to note that while *A. indica* extract showed slightly higher antifungal activity compared to *C. longa*, both demonstrated significant inhibition of fungal growth. The variability in susceptibility among different fungal species highlights the need for targeted approaches when developing plant-based antifungal treatments.

### 5. Conclusion

This study provides valuable information on the diversity and distribution of keratinophilic fungi in the soil of Waraseoni, India. The high prevalence of *Chrysosporium* and dermatophyte species, coupled with their strong enzymatic activity, underscores the potential ecological and clinical significance of these fungi in the region.

## Diversity of Keratinophilic Fungi from Waraseoni: Enzymatic Activity and Sensitivity to *Curcuma longa* and *Azadirachta indica*

The demonstrated antifungal efficacy of *C. longa* and *A. indica* extracts against the isolated keratinophilic fungi opens up possibilities for developing novel, plant-based antifungal treatments. Further research is needed to isolate and characterize the active compounds responsible for the observed antifungal activity and to evaluate their efficacy and safety in in vivo models.

Future studies should also focus on understanding the factors influencing the distribution of keratinophilic fungi in different ecosystems and their potential impact on human and animal health. Additionally, investigating the mechanisms of antifungal resistance in these fungi and exploring synergistic effects between plant extracts and conventional antifungal drugs could lead to more effective treatment strategies.

In conclusion, this study contributes to our understanding of fungal ecology in Waraseoni and highlights the potential of indigenous medicinal plants as sources of antifungal agents. The findings have implications for both environmental management and the development of novel antifungal therapies.

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