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# *In vitro* fungitoxic efficacy of *Melaleuca leucadendra* essential oil against fungi isolated from stored finger millet

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**Abstract**

Finger millet or ragi (*Eleusine coracana* Gaertn.) is one of the ancient millets of India and known for its nutritional composition & health benefits. The study deals with the efficacy of *Melaleuca leucadendra* essential oil (MLEO), against some storage fungi associated with grains of finger millet during storage. The pH and percent moisture content of collected stored finger millet grains were ranged as 6.40-6.46 and 9.61-9.68% respectively. During mycological screening, total of 653 fungal isolates were recovered from three different stored samples. The percent occurrence frequency of sample 3 was found highest (40.73%) whereas, sample 1 exhibited the lowest (28.94%). The highest cumulative percent relative density was recorded in *Cladosporium* sp. (21.59%) followed by *Aspergillus flavus* (17.76%), *A. niger* (11.48%) and unidentified fungi (11.10%) while lowest relative density was found in *A. fumigates* (1.53%) followed by *A. nidulans* (1.68%) and *A. terreus* (1.99%). The minimum inhibitory concentration (MIC) of MLEO against toxigenic isolate *A. flavus* DDUEC-4 was recorded at 10 µlml$^{-1}$whereas, its aflatoxin $B_1$ production was completely checked at 6.0 µlml$^{-1}$. The MLEO also showed broad spectrum fungitoxicity against 10 storage fungi recovered during mycological analysis of finger millet grains. The prospects of exploitation of MLEO as acceptable plant-based preservative in qualitative as well as quantitative control of biodeterioration of stored finger millet grains have been discussed.

**Keywords:** *Melaleuca leucadendra*, essential oil, antifungal, *Aspergillus flavus,* finger millet

## Introduction

Post-harvest contamination of stored cereals by fungal species poses a significant threat to global food security and human health. Finger millet (*Eleusine coracana* L.), a nutritionally rich and climate resilient cereal [1], is highly susceptible to fungal infestation during storage, leading to qualitative and quantitative losses [2]. Storage fungi such as *Aspergillus*, *Penicillium*, and *Fusarium* species not only deteriorate grain quality but also produce harmful mycotoxins that compromise food safety and market value [3]. Conventional fungicidal treatments, though effective, often result in undesirable toxic residues, environmental hazards, and the emergence of resistant fungal strains [4]. These limitations have accelerated interest in natural, eco-friendly alternatives, particularly plant-derived essential oils, for the management of storage fungi.

Essential oils (EOs) are volatile secondary metabolites with broad spectrum antimicrobial activity, offering a promising approach for sustainable grain preservation [5]. *Melaleuca leucadendra* (L.) L. (Family Myrtaceae), commonly known as Cajeput tree, yields an essential oil rich in bioactive compounds such as 1,8-cineole, α-terpineol, and limonene, known for their potent antifungal and antioxidant properties **[6]**. Previous studies have reported the antifungal activity of *Melaleuca* species, notably *M. alternifolia,* against pathogenic and storage fungi; however, limited information is available on the fungitoxic potential of *M. leucadendra* essential oil (MLEO) against fungi contaminating stored cereals.

The present investigation aims to evaluate the *in vitro* fungitoxic efficacy of MLEO against fungal species isolated from stored finger millet. By exploring its antifungal potency and potential application as a natural grain protectant, this study contributes to the development of safe, biodegradable, and effective alternatives to synthetic fungicides in post-harvest management systems.

## Materials and Methods

### Collection of finger millet grain samples and preparation

Three different samples of stored finger millet grains (500 g) were collected from the local market of Chauri-Chaura, Gorakhpur, Uttar Pradesh, India, during October-November, 2024. The grain samples were collected separately in sterilized polythene bags to avoid further contamination. In the laboratory, grains were finely ground and powders were sieved through No. 50 mesh sieve, kept in air tight plastic containers and stored at 5ºC for further analysis [7].

### PH and Moisture content

Aqueous suspensions (1:10; w/v) of powdered finger millet grains were prepared and stirred for 5 h, and the pH of suspension was noted using digital pH meter [7].

To determine moisture content, weighed amount (50 g) of samples were dried at 100ºC until their weights remained constant and percent moisture content was calculated following Kumar *et al*. [8].

$$\text{Moisture content (\%)} = (W_1\text{-}W_2 / W_1) \times 100$$

Where $W_1$ is the initial weight and $W_2$ is the final weight after drying.

### Mycological screening of collected finger millet grain samples

In a conical flask (250 ml), 10 grams of each powdered grain samples were suspended individually in 100 ml of sterile 0.85% saline solution. The samples were then homogenized on an electric shaker at a constant speed (120 rpm) for 15 minutes. For every sample, three-fold serial dilutions were made independently [7]. The Petri dishes having 10 ml of sterilized potato dextrose agar (PDA) medium were inoculated with 0.5 ml of the dilution ($10^{-3}$) separately. The inoculated plates were kept at 27±2°C for seven days. The process of counting the colonies started on the third day of incubation. Every mold colony with a unique morphology was recognized after being subcultured on PDA [9, 10].

### Detection of aflatoxigenic potential of isolated *Aspergillus flavus* from finger millet samples

Ten isolates of *Aspergillus flavus* were randomly selected and their aflatoxigenic potency was assessed using SMKY (sucrose, 200.0 g; magnesium sulphate, 0.5 g; potassium nitrate, 0.3 g; yeast extract, 7.0 g; distilled water, 1000 ml; pH, 5.6±0.2) as a broth nutrient medium [12]. Each *A. flavus* isolate was inoculated aseptically into 50 ml of SMKY medium with 1 mL of spore suspension (≈$10^6$ spores $\text{mL}^{-1}$) in 0.1% Tween-80, and the mixture was then incubated for ten days at 27±2 °C. Following incubation, each flask's contents were filtered (Whatman no. 1). Filtrate of each flask was separately extracted with 40 ml chloroform in a separating funnel. The chloroform extract was separated and evaporated till dryness on water bath at 70 °C. A modified thin layer chromatographic (TLC) technique of Kumar *et al.* **[7]** was used to determine the aflatoxigenic potency of *A. flavus* using following formula.

$$\text{Aflatoxin } B_1 \text{ content } (\mu g L^{-1}) = \frac{D \times M}{E \times l} \times 1000$$

Where, D-absorbance; M-molecular weight of $AFB_1$ (312); E-molar extinction coefficient of $AFB_1$ (21,800); *l*-path length (1 cm cell was used)

### *Melaleuca leucadendr*a essential oil (MLEO)

*M. leucadendra* essential oil (MLEO) was procured from Vedaoils, Gurgaon, Haryana, India to perform its fungitoxic efficacy against fungi recovered from stored finger millet grains.

### Antifungal and antiaflatoxigenic activity of MLEO

Minimum inhibitory concentration (MIC) and antiaflatoxigenic efficacy of MLEO was determined against *A. flavus* DDUEC-4 using SMKY broth medium. Different concentrations of the CCEO were prepared separately by dissolving their requisite amount in 0.5 ml 5% tween-20 followed by 49.5 ml of SMKY medium. The control sets were kept parallel to the treatment sets without MLEO. The flasks were inoculated aseptically with 1 ml spore suspension (≈$10^6$ spores/ml) of *A. flavus* DDUEC-4 and incubated at 27±2 °C for 10 days. After incubation, mycelial biomass and aflatoxin $B_1$ content in broth medium of each flask was determined [7].

### Fungitoxic spectrum of MLEO

The spectrum of fungitoxicity of the MLEO was determined at 10.0 µl$\text{mL}^{-1}$ (MIC against *A. flavus* DDUEC-4) by the poisoned food technique using PDA against 10 fungi *viz. Alternaria* sp., *A. fumigatus*, *A. nidulans*, *A. niger*, *A. terreus*, *Bipolaris* sp., *Cladosporium* sp., *Curvularia* sp., *Fusarium* sp. and *Penicillium* sp. isolated from stored finger millet grain samples during mycological analysis [7].

### Statistical analysis

The data were presented as mean ± standard error (SE), and each experiment was carried out in triplicate. SPSS software was used to conduct the statistical analysis (SPSS 16.0; IBM, NY, USA). The one-way analysis of variance (ANOVA) and Tukey's post-hoc test was used to assess treatment differences. P-values below 0.05 were regarded as statistically significant.

## Results and Discussion

### pH and Moisture content

The collected cumin seed samples showed variation in their pH and moisture content, indicating variability in storage duration and environmental conditions. The pH of cumin samples ranged from 6.40±0.06 to 6.46±0.01 (Table 1), showing a slightly acidic nature favorable for fungal colonization. Moisture content of the samples ranged from 9.61±0.30% in sample 2 to 9.68±0.29% in sample 3 (Table 1), indicating sufficient moisture to support fungal growth under prolonged storage. In stored millet grains, especially under conducive conditions, a slightly acidic pH and increased moisture content provide ideal conditions for fungal growth and mycotoxin synthesis [11]. More than 8% moisture content encourages the growth of storage fungus, including *Aspergillus, Penicillium, Fusarium* and others **[12]**. By increasing fungal enzyme activity and decreasing defense activity, these storage fungi thrive at slightly lower pH levels which promote colonization [13]. High temperature and humidity enhance respiration and lipid peroxidation, which further degrades seed quality and promotes the production of mycotoxin by *Aspergillus* species [14]. Therefore, to prevent fungal and mycotoxin contamination in finger millet grains when they are being stored under hot and humid condition, it is essential to maintain an ideal moisture content (less than 8%) and a neutral pH.

**Table 1:** pH and moisture content (%) of collected stored finger millet samples

| Finger millet samples | pH | Moisture content (%) |
|---|---|---|
| Sample 1 | 6.40±0.06$^a$ | 9.62±0.31$^a$ |
| Sample 2 | 6.45±0.03$^a$ | 9.61±0.30$^a$ |
| Sample 3 | 6.46±0.01$^a$ | 9.68±0.29$^a$ |

Values are mean (n = 3) ± SE; $P < 0.05$. The means followed by same letter in the same column are not significantly different according to One-Way ANOVA and Tukey's multiple comparison tests

### Mycological analysis of finger millet grain samples

Mycological investigation of the collected stored finger millet grain samples revealed the occurrence of diverse fungal flora. Sample 1 showed the lowest occurrence frequency (28.94%) with a total of 189 isolates while highest (40.73%) in sample 3 with 266 isolates (Table 2). The fluctuation in frequency of occurrence across samples indicates that fungal colonization was influenced by variations in storage conditions, including temperature, moisture, and aeration **[15]**. A total of 12 identified fungal species belonging to seven genera were consistently isolated on Potato Dextrose Agar (PDA). The mycological analysis reflects the susceptibility of finger millet grains to colonization by a wide range of storage fungi, particularly under suboptimal storage environments. The sample 1 exhibited the highest relative density of *Cladosporium* sp (20.63%) followed by *Aspergillus flavus* (17.46%) among 189 isolates. Sample 2 showed a total of 216 isolates in which *Cladosporium* sp. again showed highest relative density (20.37%) followed by *A. flavus* (19.44%) whereas, sample 3 showed again highest relative density in *Cladosporium* sp. (21.80%) next to *A. flavus* (15.41%) and *A. niger* (12.03%) (Table 2, Figure 1). Collectively *Cladosporium* sp. exhibited highest relative density (21.59%) followed by *A. flavus* (17.76%) and *A. niger* (11.48%) (Table 2, Figure 2). The predominance of *Aspergillus* sp. and *Cladosporium* sp. are consistent with its role as a common airborne and surface contaminant thriving under high humidity [16]. Such dominancy indicates their adaptability prevailing during storage in hot and humid conditions [13]. A total 10.10% fungal isolates recovered during study were unidentified (Table 2) and point to potential novel or less-characterized fungal species that may require molecular identification for confirmation.

**Table 2:** Mycological analysis of collected finger millet grain samples

| Isolated Fungi | Millet Sample 1 | Millet Sample 2 | Millet Sample 3 | Total isolates | Relative density (%) |
|---|---|---|---|---|---|
| *Alternaria* sp. | 05 | 06 | 05 | 16 | 2.45 |
| *Aspergillus candidus* | 13 | 10 | 20 | 43 | 6.58 |
| *Aspergillus flavus* | 33 | 42 | 41 | 116 | 17.76 |
| *Aspergillus fumigatus* | 03 | 03 | 04 | 10 | 1.53 |
| *Aspergillus niger* | 18 | 25 | 32 | 75 | 11.48 |
| *Aspergillus nidulans* | 02 | 03 | 06 | 11 | 1.68 |
| *Aspergillus terreus* | 04 | 05 | 04 | 13 | 1.99 |
| *Bipolaris* sp. | 08 | 13 | 16 | 37 | 5.66 |
| *Cladosporium* sp. | 39 | 44 | 58 | 141 | 21.59 |
| *Culvularia* sp. | 09 | 12 | 13 | 34 | 5.20 |
| *Fusarium* sp. | 14 | 18 | 18 | 50 | 7.65 |
| *Penicillium* sp. | 16 | 9 | 16 | 41 | 6.27 |
| Unidentified | 25 | 29 | 15 | 69 | 10.10 |
| Mucorales* | 06 | 03 | 02 | | |
| Total isolates | 189 | 216 | 266 | 653 | |
| Occurrence frequency (%) | 28.94% | 33.07% | 40.73% | | |

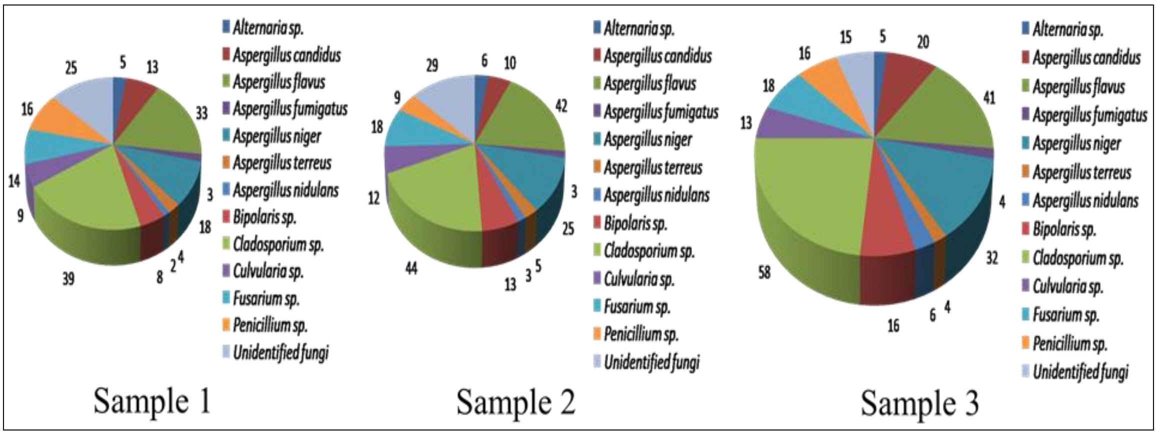

**Fig 1:** Number of fungal isolates from individual millet grain samples

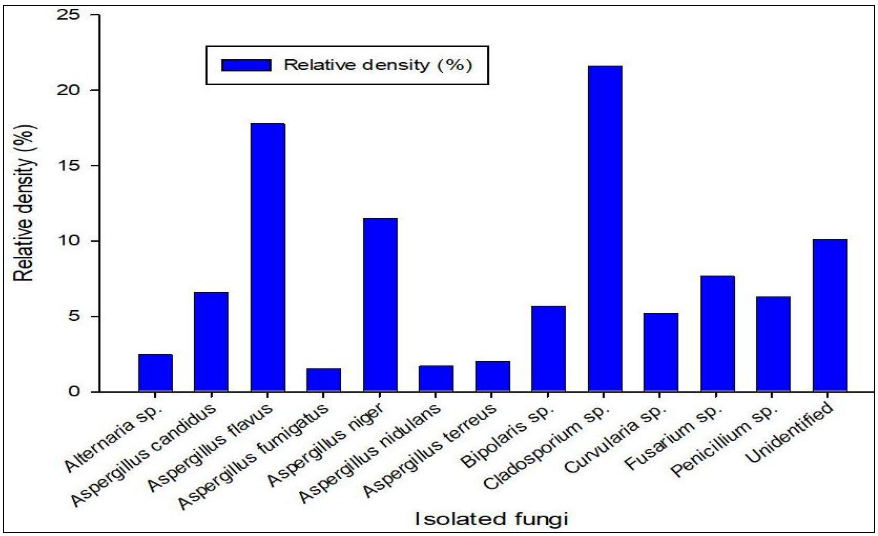

**Fig 2:** Cumulative relative densities of isolated fungal species from cumin seed samples

## Aflatoxigenic potential of isolated *Aspergillus flavus*

Ten isolates of *A. flavus* were randomly selected to determine their aflatoxigenic potential from finger millet samples using TLC method revealed a significant toxigenic potential among the fungal populations associated with stored millet grains. Out of 10 isolates, 3 (30%) exhibited toxigenic potential. The highest aflatoxin B$_1$ production (812.917 µgl$^{-1}$) was reported from isolate *A. flavus* DDUEC-4 (Table 3) highlights comparatively its superior toxigenic capacity and selected as test fungus for further study. The predominance of *A. flavus* as both a frequent and toxigenic species emphasizes its ecological suitability and preference for substrates of finger millet grains, particularly under hot and humid storage conditions conducive to aflatoxin biosynthesis [13].

**Table 3:** Toxigenicity of *Aspergillus flavus* isolated from stored finger millet grains.

| Fungal isolates | AFB$_1$ (µgl$^{-1}$) |
|---|---|
| *A. flavus* DDUEC-1 | - |
| *A. flavus* DDUEC-2 | - |
| *A. flavus* DDUEC-3 | 326.312 |
| *A. flavus* DDUEC-4* | 812.917 |
| *A. flavus* DDUEC-5 | - |
| *A. flavus* DDUEC-6 | - |
| *A. flavus* DDUEC-7 | - |
| *A. flavus* DDUEC-8 | - |
| *A. flavus* DDUEC-9 | - |
| *A. flavus* DDUEC-10 | 598.239 |

* Fungal isolate *A. flavus* DDUCC-8 from cumin seeds exhibited the highest aflatoxin B$_1$ producing potential

## Antifungal and antiaflatoxigenic activity of MLEO

MLEO exhibited potent fungitoxicity against *A. flavus* DDUEC-4 and its minimum inhibitory concentration (MIC) was recorded at 10 µlml$^{-1}$. In addition, MLEO was also found efficient to inhibit the AFB$_1$ production and completely checked at 6.0 µlml$^{-1}$ (Table 5). A direct relation was found between fungal growth and AFB$_1$ production i.e. decreases in mycelial biomass resulted low AFB$_1$ production and vice versa. The observed inverse relationship between fungal biomass and AFB$_1$ production supports earlier findings that toxin biosynthesis is growth dependent and can be significantly reduced by disrupting cellular and metabolic processes **[13]**. One of the most widely recognized mechanisms is disruption of fungal cell membrane and cell wall integrity. Lipophilic EO components such as limonene, thymol, carvacrol, and citral penetrate the lipid bilayer, increasing membrane permeability, causing leakage of vital cellular contents (ions, proteins, nucleic acids), and leading to cell lysis **[5, 17]**.

**Table 5:** Antifungal and antiaflatoxigenic activity of MLEO against *A. flavus* DDUEC-4

| Concentration (µlml$^{-1}$) | Biomass (g) | AFB$_1$ (µgl$^{-1}$) |
|---|---|---|
| Control | 0.438±0.074$^{c}$ | 872.264±122.013$^{c}$ |
| 2 | 0.116±0.026$^{b}$ | 218.568±64.346$^{b}$ |
| 4 | 0.042±0.016$^{a}$ | 47.218±24.136$^{ab}$ |
| 6 | 0.023±0.009$^{a}$ | 0.000±0.000$^{a}$ |
| 8 | 0.014±0.004$^{a}$ | 0.000±0.000$^{a}$ |
| 10 | 0.000±0.000$^{a}$ | 0.000±0.000$^{a}$ |

Values are mean (n = 3) ± SE; $P < 0.05$. The means followed by same letter in the same column are not significantly different according to One-Way ANOVA and Tukey's multiple comparison tests

MLEO also exhibited broad fungitoxic spectrum against some other storage fungi recovered from stored millet grains during mycological analysis. It completely checked the proliferation of all the tested fungal species at 10 µlml$^{-1}$ (MIC against *A. flavus* DDUEC-4) except *Bipolaris* sp. (91.67±4.68%), *Cladosporium* sp. (84.16±6.28%) and *Curvularia lunata* (93.94±2.64%) (Table 6). The broad-spectrum fungitoxic activity of MLEO against various storage fungi suggests that its bioactive constituents, particularly 1,8-cineole, α-terpinene and linalool, may interfere with membrane integrity and enzyme systems essential for fungal growth [18, 19].

**Table 6:** Fungitoxic spectrum of MLEO against other storage fungi

| Fungal species | Percent inhibition at 10 µlml$^{-1}$ |
|---|---|
| *Alternaria* sp. | 100.00±0.00$^{c}$ |
| *Aspergillus fumigatus* | 100.00±0.00$^{c}$ |
| *Aspergillus niger* | 100.00±0.00$^{c}$ |
| *Aspergillus terreus* | 100.00±0.00$^{c}$ |
| *Aspergillus nidulans* | 100.00±0.00$^{c}$ |
| *Bipolaris* sp. | 91.67±4.68$^{b}$ |
| *Cladosporium* sp. | 84.16±6.28$^{a}$ |
| *Curvularia* sp. | 93.94±2.64$^{b}$ |
| *Fusarium* sp. | 100.00±0.00$^{c}$ |
| *Penicillium* sp. | 100.00±0.00$^{c}$ |

Values are mean (n = 3) ± SE; $P < 0.05$. The means followed by same letter in the same column are not significantly different according to One-Way ANOVA and Tukey's multiple comparison tests

## Conclusion

The results of this study offer a solid foundation for using MLEO as a multipurpose, natural preservative for stored grains and other goods that are kept in storage. Due to presence of various bioactive compounds, MLEO showed strong antifungal, antiaflatoxigenic, and antioxidant properties. It can also reduce losses from fungi and aflatoxin contamination in storage systems. The EO's promise as an ecofriendly phytopreservative for the safe storage of grains and other millets is highlighted by its broad-spectrum fungitoxicity and efficacy. In postharvest management systems, these results provide credence to the use of MLEO into botanical fungicide formulations as environment friendly substitutes for synthetic pesticides.

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