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Antifungal activity of leaf extract against mycotoxin producing fungi

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ABSTRACT

Seeds are the source of food crops around the world. About 90% of the world food crops use seeds. Healthy grains are essential for the production of a healthy plant, and these seeds are also responsible for disease transmission. It happens either in the field or in post-harvest storage condition. Due to these seed-borne fungi, the seed gets deteriorated, which may cause a tremendous economic loss, as well as several types of abnormalities, occur in seeds. Dominant storage grain gets contaminated with fungi mycotoxin. Mycotoxin contaminants of fungal origin occurring worldwide and characterized by its acute and chronic toxic effects on human health. The present attempt was to study the antifungal activity of leaf extract against mycotoxin producing fungi. Fungi isolated from stored seed grains like cereals, pulses, and infected oil seeds were collected from Marathwada region of Maharashtra, India. Antifungal activity of leaf extract of Calotropis Procera, Azadirachta indica, Ocimum sanctum, Withania somnifera and Datura metel were tested against Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum by using 96-well plate method. Leaf extract showed remarkable activity against tested fungi at different concentrations as Minimum Inhibitory Concentration. The leaf extract of Calotropis procera and Azadirachta indica, have excellent potential antifungal properties against different mycotoxin producing fungi.

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INTRODUCTION

Mycotoxins are toxins or secondary metabolites produced by filamentous fungi with no apparent function. Yet, healthy metabolism of fungi, can contaminate edible crops grains or during harvest or storage. The presence of mycotoxins in feed and food investigations began in England, when in 1962 with an outbreak of turkey "X" disease where 1,00,000 of turkey poults died (Tattibayeva *et al.*, 2018). These deaths were with the presence of a toxic substance produced by Aspergillus flavus termed as aflatoxin. Till date, worldwide, more than 400 different mycotoxins have been identified. The effects of mycotoxins on health depend on various factors: the type, its concentration, and the way it is consumed, the time it takes to expose, the mode in which it acts, the animal species it affects, the gender and age and the weight of a particular organism.

Several fungal genres can synthesize mycotoxins Aspergillus, Penicillium and Fusarium. And these comprise the most significant number of mycotoxin producing fungi. Other toxogenic families include Claviceps and Alternaria (Köppen *et al.*, 2010). B1, B2, B3, B4, G1 and G2 these are the main types of mycotoxins (Aflatoxins) primarily produced by

Aspergillus flavus and A. parasiticus. Mycotoxins affect protein synthesis due to their ability to bind to genetic material DNA. Aflatoxins are incredibly potent carcinogens in all animal species investigated, exposure to mycotoxins through consumption, inhalation or dermal routes can result in a variety of health effects including mutagenicity, immunosuppression, cancer, and nephrotoxic, hepatotoxic or immunosuppressive (Goryacheva *et al.*, 2007). The International Agency for Research on Cancer classified them as Group One as they are found to be carcinogenic to the human being (IARC, 2012). Most of the researchers' work carried out the study of medicinal plants in India, its primary motivation concern of the world conservation organizations (Singh, 2017).

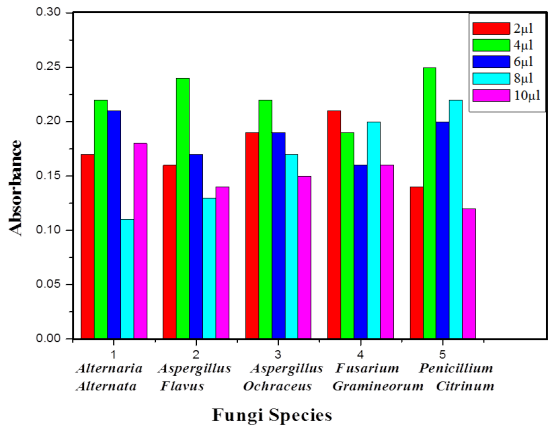
MATERIALS AND METHODS

Study Area and Collection of Seed Samples

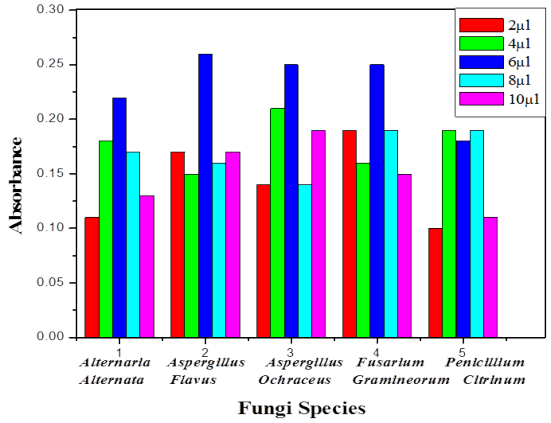
The present study was conducted at Seed Pathology Laboratory, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University Aurangabad; samples were collected from Aurangabad, Beed, Jalna, Latur, Nanded, Parbhani, Osmanabad and Hingoli district of Marathwada region. Infected, diseased plant parts, grains collected from various storehouses, market places, agriculture field, rural and urban markets etc. Samples were collected from multiple kinds of cereal, pulses, and oilseeds infected fruits and diseased plant parts. The samples were placed in cloth bags to allow air circulation that reduced condensation and limited fungal growth after sampling until and employed for isolation of pathogens, fungi associated with them. Isolated fungi were identified by using authentic literature, web facilities and monographs (Neergaard, 1973).

Fungal isolation and purification

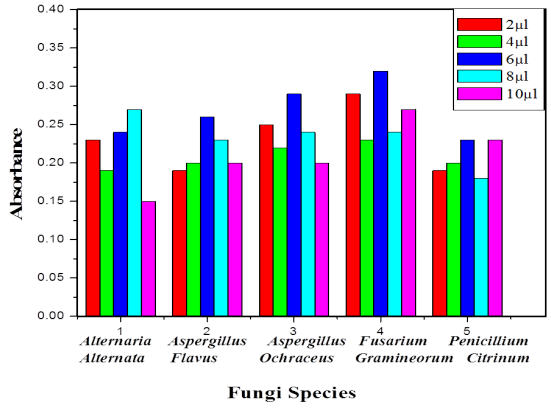
Agar plate method: selected cereals, pulses, oilseeds infected fruits and diseased plant parts were surface-sterilized with cotton wool soaked in surface sterilization with 0.1% $HgCl_2$ for two minutes and cut into small segments using sterile scalpels. Small segments were placed on potato dextrose agar (PDA), pH 5.5. Ten small segments were placed in each Petri dish, and dishes were incubated at 24±1°C under an alternating cycle of light and darkness for seven days for 24 hours. Pure cultures of filamentous fungi were identified based on cultural and morphological features such as colony growth pattern, conidial morphology and pigmentation using slide culture technique and microscopic examination (Figure 1).



Graph 1: Antifungal activity of Calotropis procera leaf extract



Graph 2: Anti fungal activity of Azadirachta indica leaf extract



Graph 3: Antifungal activity of Ocimum sanctum leaf extract

Table 1: Antifungal activity of Calotropis procera leaf extract

Sr. no	Calotropis procera	Alternaria alternata	Aspergillus flavus	Aspergillus ochraceus	Fusarium gramineorum	Penicillium citrinum
1	2 μl	0.17	0.16	0.19	0.21	0.14
2	4 μl	0.22	0.24	0.22	0.19	0.25
3	6 μl	0.21	0.17	0.19	0.16	0.20
4	8 μl	0.11	0.13	0.17	0.20	0.22
5	10 μl	0.18	0.14	0.15	0.16	0.12
	MIC	8 μl	8 μl	10 μl	6,10 μl	10 μl

Table 2: Antifungal activity of Azadirachta indica leaf extract

Sr. no	Azadirachta indica	Alternaria alternata	Aspergillus flavus	Aspergillus ochraceus	Fusarium gramineorum	Penicillium citrinum
1	2 μl	0.11	0.17	0.14	0.19	0.10
2	4 μl	0.18	0.15	0.21	0.16	0.19
3	6 μl	0.22	0.26	0.25	0.25	0.18
4	8 μl	0.17	0.16	0.14	0.19	0.19
5	10 μl	0.13	0.17	0.19	0.15	0.11
	MIC	2 μl	4 μl	2,8 μl	10 μl	2 μl

Table 3: Antifungal activity of Ocimum sanctum leaf extract

Sr. no	Ocimum sanctum	Alternaria alternata	Aspergillus flavus	Aspergillus ochraceus	Fusarium gramineorum	Penicillium citrinum
1	2 μl	0.23	0.19	0.25	0.29	0.19
2	4 μl	0.19	0.20	0.22	0.23	0.20
3	6 μl	0.24	0.26	0.29	0.32	0.23
4	8 μl	0.27	0.23	0.24	0.24	0.18
5	10 μl	0.15	0.20	0.20	0.27	0.23
	MIC	10μl	2 μl	10 μl	4μl	8 μl

Table 4: Antifungal activity of Withania somnifera leaf extract

Sr. no	Withania somnifera	Alternaria alternata	Aspergillus flavus	Aspergillus ochraceus	Fusarium gramineorum	Penicillium citrinum
1	2 μl	0.13	0.17	0.14	0.10	0.17
2	4 μl	0.18	0.14	0.15	0.15	0.19
3	6 μl	0.17	0.16	0.18	0.13	0.15
4	8 μl	0.21	0.19	0.21	0.21	0.28
5	10 μl	0.17	0.15	0.21	0.19	0.13
	MIC	2 μl	4 μl	2 μl	2 μl	10 μl

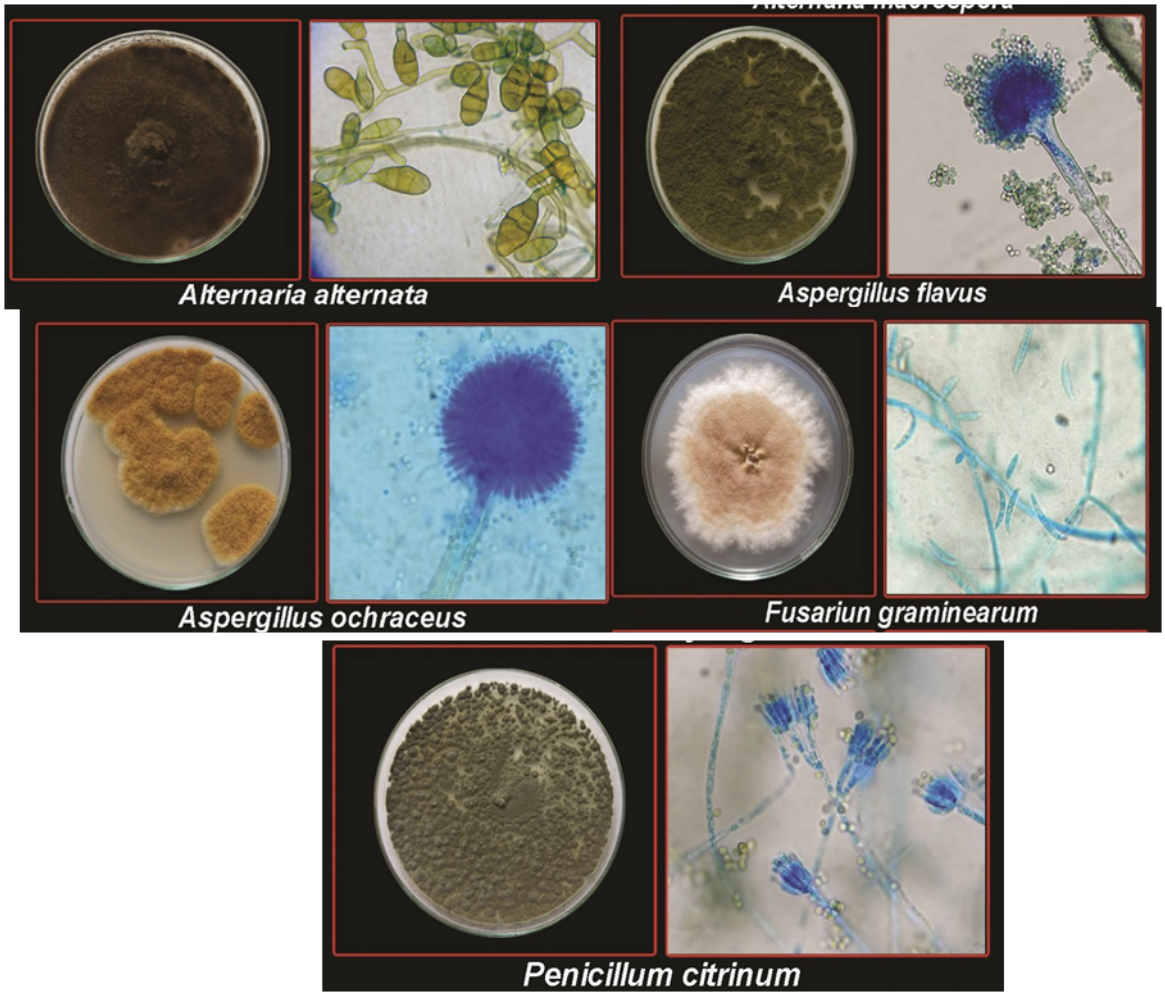


Figure 1: Pure Culture & microphotographs of selectedfungi

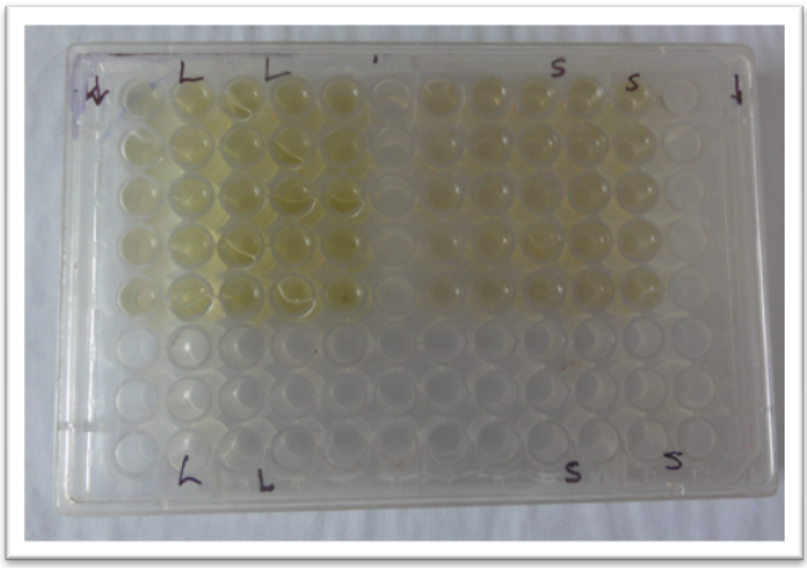
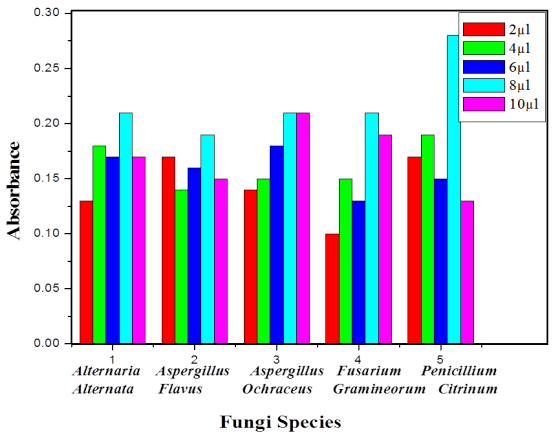


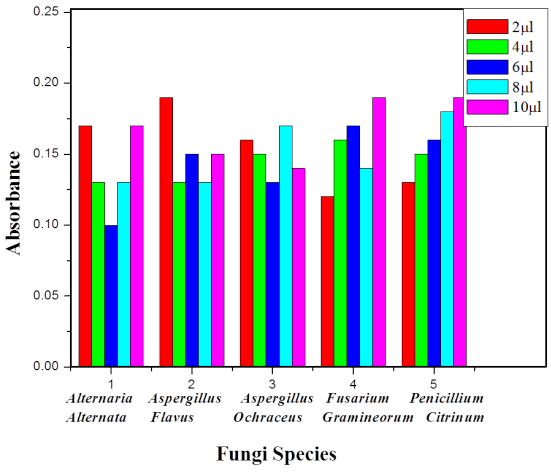
Figure 2: Photo Plates of 96-microtitre plate micro-broth dilution assay technique.

Table 5: Antifungal activity of Datura metel leaf extract

Sr. no	Datura metel	Alternaria alternata	Aspergillus flavus	Aspergillus ochraceus	Fusarium gramineorum	Penicillium citrinum
1	2 μl	0.17	0.19	0.16	0.12	0.13
2	4 μl	0.13	0.13	0.15	0.16	0.15
3	6 μl	0.10	0.15	0.13	0.17	0.16
4	8 μl	0.13	0.13	0.17	0.14	0.18
5	10 μl	0.17	0.15	0.14	0.19	0.19
	MIC	4 μl	4,8 μl	6 μl	2 μl	2 μl



Graph 4: Antifungal activity of Withania somnifera leaf extract



Graph 5: Antifungal activity of Datura metel leaf extract

Identification of isolated fungi

Identification of isolated fungi was made according to (Fawole and Oso, 1995). The various fungi were isolated from different seeds samples. They were preliminarily identified based on colony characters, morphological and sporulation features such as sexual or asexual spores by using a stereoscopic binocular microscope. Identification and confirmation of seed-borne fungi were made by preparing slides of fungal culture and observing them under a compound microscope. These seed-borne fungi were identified with the help of authentic pieces of literature, manuals and books earlier studied (Kakde and Chavan, 2011).

Detection of Seed Mycoflora

The standard protocol for isolation of seed mycoflora suggested by the International Seed Testing Association (ISTA, 1966); as well as (Agarwal, 1976) were followed.

Preparation of plant extract

The prepared leaf powder of Calotropis procera, Azadirachta indica, Ocimum sanctum, Withania somnifera and Datura metel was filtered through a muslin cloth. 10gm of fine powder was extracted with the help of Soxhlet extractor for 18-24 hours or till the sample become colourless at 65ºC, methanol used as a solvent and concentrated at 40ºC by using an evaporator and stored in an amber coloured bottle at 4ºC. These extracts were used for antifungal activity against selected fungi.

Antifungal activity

Antifungal activity of leaf extract of tested against Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum in Mueller-Hinton broth by using 96-microtitre plate micro-broth dilution assay technique. These techniques were implicated for antibacterial study Curcuma pseudomontana J. Grahm and rhizomes and leaf (Shriram and Shripatrao, 2017; Rs *et al.*, 2017). Extracts of selected plant materials were dissolved in DMSO to produce stock

solutions at 100μg/ml. Initially, pathogenic fungal cultures were dispensed in 100 μL of Mueller-Hinton broth medium into 96-well plate culture, then 1μL of control and different concentrations as 2, 4, 6, 8 and 10μL of extract were added in duplicate wells (Figure 2). The prepared well plates sealed with parafilm and kept for incubation at 37^{o}C for 24 hours, and absorbance was measured on a spectrophotometer at 570 nm (Matainaho and Barrows, 2013).

Experimental Results

Antifungal activity of Calotropis procera leaf extract was tested in different concentrations against selected pathogenic fungi viz., Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum, it was cleared from results that, maximum inhibition for A. alternata were found in 10 μl concentration, A. flavus 8 μl, A. ochraceus 10 μl, F. gramineorum 6, 10 μl and for P. citrinum it was found to be 10 μl respectively. Regarding minimum inhibitory concentration (MIC) it was 10μl for A. alternata, 8 μl for A. flavus, 10 μl for A. ochraceus, 6, 10 μl for F. gramineorum and 10 μl for P. citrinum respectively (Table 1, Graph 1).

Antifungal activity of Azadirachta indica leaf extract was tested in different concentrations against selected pathogenic fungi viz., Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum, it was cleared from results that, maximum inhibition for A. alternata were found in 2 μl concentration, A. flavus 4 μl, A. ochraceus 2, 8 μl, F. gramineorum 10 μl and for P. citrinum it was found to be 2 μl respectively. Regarding minimum inhibitory concentration (MIC) it was 2 μl for A. alternata, 4 μl for A. flavus, 2, 8 μl for A. ochraceus, 10 μl for F. gramineorum and 2 μl for P. citrinum respectively (Table 2, Graph 2).

Antifungal activity of Ocimum sanctum leaf extract was tested in different concentrations against selected pathogenic fungi viz., Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum, it was cleared from results that, maximum inhibition for A. alternata were found in 10 μl concentration, A. flavus 2 μl, A. ochraceus 10 μl, F. gramineorum 4 μl and for P. citrinum it was found to be 8 μl respectively. Regarding minimum inhibitory concentration (MIC) it was 10 μl for A. alternata, 2 μl for A. flavus, 10 μl for A. ochraceus, 4μl for F. gramineorum and 8 μl for P. citrinum respectively (Table 3, Graph 3).

Antifungal activity of Withania somnifera leaf extract were tested in different concentrations against selected pathogenic fungi viz., Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum, it was cleared from results that, maximum inhibition for A. alternata were found in 2 μl concentration, A. flavus 4 μl, A. ochraceus 2 μl, F. gramineorum 2 μl and for P. citrinum it was found to be 10 μl respectively. Regarding minimum inhibitory concentration (MIC) it was 2 μl for A. alternata, 4 μl for A. flavus, 2μl for A. ochraceus, 2μl for F. gramineorum and 10μl for P. citrinum respectively (Table 4, Graph 4).

Antifungal activity of Datura metel leaf extract was tested in different concentrations against selected pathogenic fungi viz., Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum, it was cleared from results that, maximum inhibition for A. alternata were found in 4 μl concentration, A. flavus 4, 8 μl, A. ochraceus 6μl, F. gramineorum 2μl and for P. citrinum it was found to be 2 μl respectively. Regarding minimum inhibitory concentration (MIC) it was 4 μl for A. alternata, 4, 8 μl for A. flavus, 6μl for A. ochraceus, 2μl for F. gramineorum and 2μl for P. citrinum respectively (Table 5, Graph 5).

CONCLUSIONS

The leaf extract of Calotropis procera, Azadirachta indica, Ocimum sanctum, Withania somnifera and Datura metel have potential antifungal properties against different mycotoxin producing seed born fungi such as Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus, Fusarium gramineorum and Penicillium citrinum at different concentrations. It may be due to the presence of various kinds of secondary metabolites present in the leaf extract.

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Conflict of Interests

The authors declare that there is no conflict of interests in the publication of this paper.

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