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EVALUATION OF ANTIFUNGAL EFFECTS OF PLANT LEAVES EXTRACTS ON ISOLATED *RHIZOPUS SPECIES* FROM SOIL

N. Jayanthi

Department of Microbiology, Mannar Thirumalai Naicker College (Autonomous), Madurai - 625004, Tamil Nadu, India.

Keywords:

Mucormycetes, *Carica papaya*, *Citrus limon*, *Psidium guajava*, *Acalypha indica* and *Rhizopus species*, disc diffusion

Correspondence to Author:

Dr. N. Jayanthi

Assistant Professor,
Department of Microbiology,
Mannar Thirumalai Naicker College
(Autonomous), Madurai - 625004,
Tamil Nadu, India.

E-mail: jayanthiashokraja2@gmail.com

ABSTRACT: Mucormycetes are a group of fungi causing a serious infection called mucormycosis. These fungi live throughout the environment, particularly in soil than in the air. This fungus is severely spread among people who have weakened immune systems; breathing in these spores can cause severe infection in the lungs or sinuses, which can spread easily to other parts of the body. In the present work, *Rhizopus species* was isolated from soil and antifungal activity of different solvent extracts of young leaves of *Carica papaya* (Papaya), *Citrus limon* (lemon), *Psidium guajava* (guava) and *Acalypha indica* (Indian nettle) were tested against *Rhizopus species* using the disc diffusion method. It was observed that all four plant leaves exhibit positive activity against *Rhizopus species*. Ethanolic extract of *Carica papaya* leaves (75mg/ml) showed more potency with an inhibition zone of 20 ± 0.2 mm. Aqueous extracts (25mg/ml) of *Acalypha indica* leaves (7 ± 0.2 mm) showed lowest zone of inhibition found in against *Rhizopus species*. Antifungal activity of aqueous, ethanol, and chloroform extracts of *Citrus limon* leaves and *Psidium guajava* leaves also were determined. The results showed that more potential antifungal activity is exhibited with ethanol extract of *Carica papaya* leaves (75mg/ml). The present study confirmed the presence of antifungal compounds in shade-dried ethanol extracts of *Carica papaya* leaves against isolated *Rhizopus species* from the soil.

INTRODUCTION: The amount and type of microorganisms in a particular section of soil are associated with several factors, such as sunlight, temperature, moisture, soil pH, nutrients, and reduction potential¹. Various types of microorganisms are present in the soil. Among them, numerous groups of fungi are widely distributed in soil, and they play important roles in the soil environment some groups of fungi act as soil-borne pathogens.

Today, it is well established that soil can be a reservoir of most pathogenic and opportunistic fungi²⁻⁵. Soil-borne pathogenic fungi directly enter the human body through direct inoculation into wounds or contaminated food by direct or indirect soil ingestion. Fungal spores may be dispersed in different environments via mud or dust particles from soil and enter the respiratory tract^{1,5}.

Mostly, mucorales is an order of saprophytic fungi growing on organic substances, such as dead plants, animal waste materials in soil, or food^{6,7}. In the soil, these mucorales may survive for a long time before infecting humans who are in contact with contaminated soil⁸. In 2007, in a taxonomic reclassification, Zygomycetes were abolished as a class and zygomycosis is now mainly attributed to

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the order Mucorales of subphylum Mucoromycotina⁹. Therefore, previously known zygomycosis is now termed as “Mucormycosis”. This term is now used in opportunistic infections caused by the order Mucorales¹⁰. Following aspergillosis and candidiasis, mucormycosis is the third most common invasive fungal infection¹¹. The importance of mucormycosis has been highlighted in recent years as a consequence of the dramatic increase in the number of patients with predisposing factors¹²⁻¹⁴. In many cases, the most common genus causing human Mucormycetes infection is *Rhizopus* followed by *Mucor* and *Lichtheimia* mucormycosis was found in 70 to 80% of all mucormycosis^{15, 16}.

Plants are a primary source of new natural medicinal products¹⁷. Of 119 drugs still extracted from plants and used globally, 74% were discovered during an attempt to identify the chemical substances amongst medicinal plants responsible for combating human diseases¹⁸. More importantly, some of the plant's natural products have various biological activities such as anti-inflammatory, anticarcinogenic and anti-atherosclerotic, antibacterial, antifungal, antiviral, antimutagenic and antiallergic activities^{19, 20}. These biological activities may be associated with their antioxidant activity²¹. The antimicrobial activities of plant extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine, and natural therapies^{22, 23}. From the above literature survey, research was carried out to investigate the use of some plant leaves extracts for the control mucormycetes species. This research was aimed to test the efficacy of the crude extracts of Young leaves of *Carica papaya* (papaya), *Citrus limon* (lemon), *Psidium guajava* (guava) and *Acalypha indica* (Indian nettle) for the control of mucormycetes as well as to determine the best plant extracts that are comparable to the efficacy of fungicide so that it can be used as an alternative to the fungicide.

MATERIALS AND METHODS:

Sample Collection: In December 2021, twenty soil samples were collected from twenty different cultivated lands in and around Periyakulam, Theni District. All the dust and debris were first removed from the soil surface. Then, approximately 400 gm

of soil at a depth of 5-10 cm was collected from the surface layer and stored in sterile bags using a sterile stainless steel spoon²⁴. All the soil samples were transferred immediately to a microbiology laboratory for the next processing stage.

Isolation of Mucormycetes Species: The soil samples were crushed in a mortar and then homogenized in sterile conditions. Afterward, 5 gm of each soil sample was suspended in 20 ml of sterile double-distilled water and shaken for 5 min to prepare a soil suspension^{25, 26}. Sterilized and cooled agar medium of Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol (50mg/l), was added to the sterile Petri plates. 0.5 ml of the suspension was poured approximately into the bottom of a Petri dish and spread using a sterile L Rod. All the plates were incubated at 27°C for 2-4 days, and fungal colony growth was observed daily. The primary identification of mucormycetes species was performed based on macroscopic by colony morphology and microscopic by lactophenol cotton blue mount²⁷.

Microscopic Examination: A 1 cm square of adhesive tape was cut, and with the help of sterile forceps, it was placed on the surface of mycelia and lifted off slowly. A few drops of lactophenol cotton blue (LPCB) were placed on another slide. An adhesive tape containing the fungal mycelia was placed on lactophenol cotton blue and then mounted was observed under low power 10X and high power 45X objective lens of the light microscope^{28, 29}.

Preparation of Fungal Inoculum: Fungal culture was scraped with a sterile loop to prepare inoculum suspension for mucormycetes species. Spores were collected in Sabouraud Dextrose broth. The fungal suspension was passed through sterile cheesecloth to clear residual hyphae. The inoculum was adjusted to 1 to 5×10⁶ spores/ml by adjusting the optical density at 625 nm to 0.08 and 0.1³⁰.

Collection and Identification of Plant Materials: 10 Young leaves of *Carica papaya* (papaya), *Citrus limon* (lemon), *Psidium guajava* (guava), and *Acalypha indica* (Indian nettle) were collected from ARK Organic farm, Periyakulam Taluk, Theni district, Tamilnadu, India. The plant samples were authenticated by Dr. P. Prabakaran, Assistant

Professor, Department of Botany, M. R. Government Arts College, Mannargudi. With running tap water, the plant materials were washed to remove soil and dust materials on plant leaves and washed with distilled water to remove any contamination. After that, plant materials are washed with 95% ethanol to make them sterile. All the plant leaves were dried in the shade to evaporate all the water molecules, and after that, dried plant leaves were grinding. With the help of mortar and pestle, leaves of *Carica papaya* (Papaya), *Citrus limon* (Lemon), *Psidium guajava* (Guava) and *Acalypha indica* (Indian nettle) ground well into fine powder form and stored in polythene bags with proper labeling³¹.

Preparation of Plant Leaves Extracts: For the preparation of aqueous, ethanol, and chloroform extract, One gram (1gm) of dried powder of experimental material of all plants leaves were soaked in 20 ml of water, ethanol, and chloroform respectively for 24hrs and kept in a shaking incubator at 50-60 rpm & 40°C.

The mixture was then filtered through the Whatman No.1 filter paper to ensure that no particles were present in the solution, and the extract was collected and evaporated to dryness with a rotary evaporator. For antifungal activity assays, a stock solution was made for each extract with 50 mg/ml in dimethyl sulfoxide (DMSO) and mixed well by vortexing. The stock was stored at 40°C, until used³².

Antifungal Activity of Plant Extract on Isolated Mucormycetes:

Antifungal Assay: Antifungal activity tests against isolated mucormycetes were performed by using the disk diffusion method³³. Test plates were prepared with 20 ml of sterile Sabouraud Dextrose Agar. The standardized isolated fungal inoculum was applied to the solidified culture medium by using sterile cotton swabs and allowed to dry for 5

min. A sterile paper disk (6mm) was impregnated with different concentrations (25, 50, and 75 mg/ml) of each plant leaves extract. The disks were aseptically transferred on the inoculated agar plates and incubated for 2 to 4 days. Antifungal activity was determined by measuring clear zones around discs. The clear zones indicated that the fungicidal effect of plant leaf extracts isolated mucormycetes species³⁴. Terbinafine (250µg) disks were used as a standard reference or positive controls, and disks impregnated with 1% DMSO were used as negative controls. All tests were performed in triplicate.

RESULTS:

Macroscopic and Microscopic Identifications: Typically, floccose and dense colonies were observed on seven Petri plates. These colonies rapidly filled the entire Petri plates with existing large intertwined aerial mycelium, resembling grey cotton candy.

The colonies were subjected to lactophenol cotton blue mount staining. Microscopic appearances are oval shape spores, non-septate mycelium, and straight sporangiophores that terminate with black sporangium containing a columella and root-like hyphae. Isolated colonies were identified as *Rhizopus* species from the macroscopic and microscopic characteristics.

Antifungal Activities of Leaves Extracts:

Extracts from plant leaves were investigated for their antifungal effect against isolated *Rhizopus species*. Among the plant leaves extracts the best activity found in the investigation was observed with the ethanol extract (75mg/ml) of *Carica papaya* leaves (20 ±0.2mm), which inhibited the growth of isolated *Rhizopus species*. The lowest zone of inhibition was found in aqueous extracts (25mg/ml) of *Acalypha indica* leaves (7±0.2mm) against *Rhizopus species*. The results were tabulated in **Table 1, 2, and 3** and **Fig. 1, 2, and 3**.

TABLE 1: ANTIFUNGAL ACTIVITY OF AQUEOUS EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

S. no.	Plant leaves extract	Zone of inhibition (nm)				
		Aqueous			Positive control	Negative control
		25mg	50mg	75mg		
1	<i>Carica papaya</i>	10± 0.2	12± 0.1	15± 0.2	28±0.1	Not detected
2	<i>Citrus limon</i>	8 ±0.1	15±0.1	12± 0.2		
3	<i>Psidium guajava</i>	11 ±0.2	12±0.2	14± 0.1		
4	<i>Acalypha indica</i>	7± 0.2	9± 0.1	11 ±0.2		

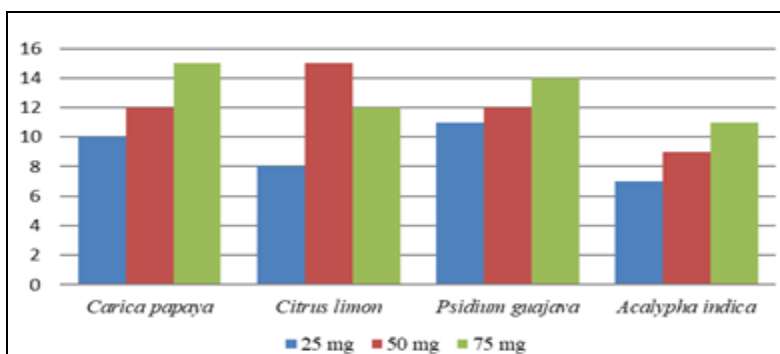


FIG. 1: ANTIFUNGAL ACTIVITY OF AQUEOUS EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

TABLE 2: ANTIFUNGAL ACTIVITY OF ETHANOL EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

S. no.	Plant leaves extract	Zone of inhibition (nm)				
		Ethanol			Positive control	Negative control
		25mg	50mg	75mg		
1	Carica papaya	10± 0.2	12± 0.1	15± 0.2	28±0.1	Not detected
2	Citrus limon	8 ±0.1	15±0.1	12± 0.2		
3	Psidium guajava	11 ±0.2	12±0.2	14± 0.1		
4	Acalypha indica	7± 0.2	9± 0.1	11 ±0.2		

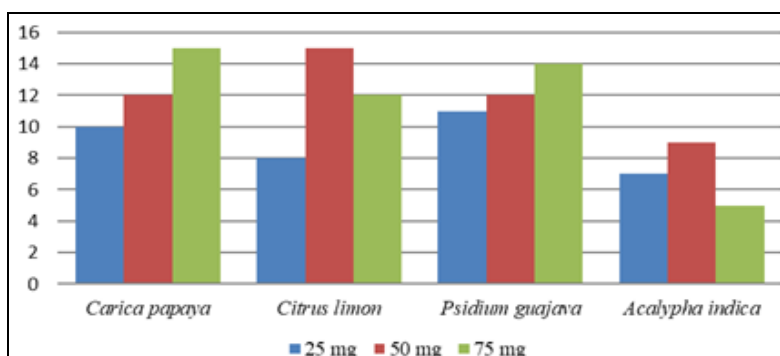


FIG. 2: ANTIFUNGAL ACTIVITY OF ETHANOL EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

TABLE 3: ANTIFUNGAL ACTIVITY OF CHLOROFORM EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

S. no.	Plant leaves extract	Zone of inhibition (nm)				
		Chloroform			Positive control	Negative control
		25mg	50mg	75mg		
1	Carica papaya	10± 0.2	12± 0.1	15± 0.2	28±0.1	Not detected
2	Citrus limon	8 ±0.1	15±0.1	12± 0.2		
3	Psidium guajava	11 ±0.2	12±0.2	14± 0.1		
4	Acalypha indica	7± 0.2	9± 0.1	11 ±0.2		

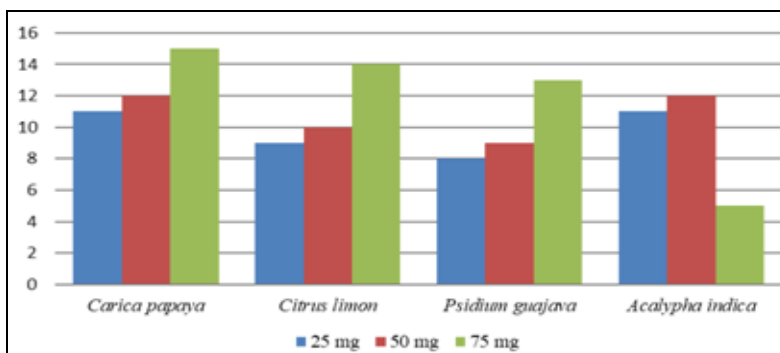


FIG. 3: ANTIFUNGAL ACTIVITY OF CHLOROFORM EXTRACTS OF PLANT LEAVES ON ISOLATED RHIZOPUS SPECIES

DISCUSSION: Mucormycetes are called thermotolerant molds, widely found on soil and other organic substances. High humidity and temperature of 27°C are the favorable environmental conditions required for the growth and sporulation of mucorales on these substrates. According to the literature survey, most mucormycetes are thermophilic species have been isolated from composting plant materials³⁵.

Today, mucormycosis is identified as an emergent disease, owing to the increasing incidence and mortality over the past two decades³⁶. Diagnosis and control of mucormycosis remain challenging tasks in worldwide and no radiological signs or clinical specific have been identified for mucormycosis. Moreover, standardized antigen or serological detection tests are not currently available³⁷. In previous studies, researchers have attempted to compare the identification of mucorales fungi, using molecular and phenotypic methods³⁸. From the present study, all the macroscopic and microscopic characteristics of isolated colonies were observed, and isolated colonies were identified as *Rhizopus species*. From the clinical mucormycosis sample, *Rhizopus* species were frequently isolated. Additionally, members of the genus *Mucor* are second to *Rhizopus* in frequency³⁹.

Previous studies have shown the antifungal effect of organic extracts derived from plant sources and also has been shown that the effects of phytochemicals may vary depending on the type of solvent used. In accordance with the last, antifungal activity has been reported in polar compounds such as glycosylated flavonoids and saponins isolated from polar extracts^{40, 41} and in non-polar compounds, like terpenoids^{42, 43}. In the present study, antifungal activity of leaves of *Carica papaya* (papaya), *Citrus limon* (lemon), *Psidium guajava* (guava) and *Acalypha indica* (Indian nettle) were investigated by the disc diffusion method. Among these four extracts, the Ethanol extract of *Carica papaya* leaves (75mg/ml) was showed the highest zone of inhibition (20 ±0.2 mm) against isolated *Rhizopus species*. Aqueous extracts (25mg/ml) of *Acalypha indica* leaves exhibit the lowest zone of inhibition (7±0.2 mm) against *Rhizopus species*. Aqueous, ethanol and chloroform extracts of leaves *Carica papaya* and

Psidium guajava leaves also showed antifungal activity against *Rhizopus species*. One study by⁴⁴ suggested that the antimicrobial activity of *Acalypha indica* leaves extract could be attributed to the active compounds of alkaloids and tannins. According to a study conducted by⁴⁵ the presence of bioactive compounds such as alkaloids, tannins, steroids, saponins, flavonoids, glycosides, and phenolic compounds was also detected during it phytochemical testing. Antibacterial activity leaves extract of *Carica papaya*, *Psidium guava*, and *Citrus limon* showed varying degrees of antibacterial and antifungal activity against all microorganisms tested. There are many reports of plants in the family Euphorbiaceae possessing antimicrobial activity and antifungal activity⁴⁶.

CONCLUSION: The result of this work suggests that the leaves extract of *Carica papaya* (papaya), *Citrus limon* (lemon), *Psidium guajava* (guava), and *Acalypha indica* (Indian nettle) have various medicinal properties, which may be attributed to the compounds present in these plant leaves. This study shows that all the four leaves extract has antifungal effects against isolated *Rhizopus* species from the soil. Even though the best antifungal activity was observed with the ethanol extract of *Carica papaya* leaves, ethanol extract of *Carica papaya* leaves can be used for future references for mucormycetes.

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CONFLICTS OF INTEREST: Nil.

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