Combating Mucormycosis: Dual Approach using Antibiotics and Plant Extracts against *Rhizopus sp.* and *Mucor sp.*

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Abstract
In the COVID-19 era, mucormycosis (black fungus) has been extensively explored by researchers. It is relatively rare, but also very serious. In the present study, antifungal activity of plant extracts and the antibiotic susceptibility pattern of Rhizopus sp. and Mucor sp. were determined by agar well diffusion and Kirby Bauer disk diffusion method respectively.

Ketoconazole (28mm) was found to be the most effective, followed by Clotrimazole (17mm) against Rhizopus sp. The antibiotic susceptibility pattern of Mucor sp. exhibited sensitivity against Clotrimazole (18mm) followed by Amphotericin-B (12mm) Clotrimazole was found to be effective against both Rhizopus sp. and Mucor sp. Trachyspermum ammi methanolic, ethanolic and petroleum ether extracts showed antifungal activity against Rhizopus sp. with zone of inhibition ranging from 12mm to 22mm. Trachyspermum ammi petroleum ether extract showed activity against Mucor sp. The current situation of antibiotic resistance necessitates new tactics for dealing with the antibiotic resistance-related problems. It can be suggested that combination therapy is needed to cure the fungal disease and an alternative to antibiotics that is botanicals as antimicrobial also needs to be screened and studied in vitro and in vivo.

Keywords: Antibiotic susceptibility, Mucormycosis, Mucor, Rhizopus, sporangiospore.

Introduction

From ancient times to the present, the main objective of medicine and medical care has been the diagnosis and treatment of illnesses[20]. Annually, more than 1 billion people are infected with many of the diseases that are life-threatening, especially in immunocompromised patients. Immune suppression as a risk factor highlights the crucial function of the immune system in controlling opportunistic fungal infections[24]. Since the beginning of the year 2020, COVID-19 has been expanding quickly and presents serious social, economic and health problems for all. In addition to seriously harming the global economy, the COVID-19 pandemic, which was brought on by the severe acute respiratory syndrome virus-2 (SARS-CoV-2), has caused unimaginable suffering for people everywhere. During this pandemic, most countries implemented full lockdown measures to slow down the spread of the microbial disease[18,19,29,39].

Mucormycosis is an angioinvasive, rare fungal infection, also known as Zygomycosis. Mucormycosis mainly affects people who have health problems or take medicines that lower the body's ability to fight germs and sickness[28]. Sinuses or the lungs are the major organs where these fungi grow and also cause wound fungal infection. *Mucor*, *Rhizopus*, *Rhizomucor*, *Syncephalastrum*, *Cunninghamella*, *Apophysomyces species* and *Lichtheimia* (formerly *Absidia*) species are the major mucorales that cause mucormycosis[34]. Mycosis is associated with significant morbidity and mortality, especially in immunocompromised hosts such as those with poorly controlled diabetes, neutropenic patients, hematopoietic stem cell transplant (HSCT) recipients and burn and trauma victims[3].

The incidence has increased in South Asian countries when the second wave in 2021 occurred across, affecting diabetic and immunocompromised patients. Despite the use of surgery and antifungal medication, mucormycosis is a destructive and potentially fatal invasive fungal illness that frequently has poor clinical outcomes[10].

Rhizopus is a member of the *Mucoraceae* family, which is part of the Mucorales order and division of the Mucormycota. Rhizopus species are saprotrophic, aerobic, thermotolerant and common in soil, animal waste, bread and rotting vegetables. Furthermore, it grows best at 39 °C when the pH is low and the content of glucose is high[25,30]. There are 11 to 13 species described in the genus Rhizopus grouped in four complex species: *R. microsporus*, *R. stolonifer*,*R. arrhizus*(or*R. oryzae*) and *R. Delmar* (*R. Arrhizus* var. *delemar*)[15,22]. Mucor species (belonging to family *Mucoraceae*) are commonly found in cosmopolitan soil, digestive systems, plant surfaces, rotten vegetable matter and iron oxide residue in the biosorption process[35]. Mucorspores or sporangiospores can be simple or branched and form apical, globular sporangia with a lack of rhizoids[1].

Recent studies re-emphasized the need for combined treatment with surgical debridement of necrotic tissue, antifungal therapy and reversal of underlying disease for a better outcome[9]. Up to 12% of all deaths in individuals with diabetes mellitus (DM) are attributable to infectious illnesses and the worldwide rise in DM prevalence has been linked to an increase in the incidence of mucormycosis caused by

Rhizopus sp. The latest recommendations for first-line therapy are the use of liposomal amphotericin B (≥5mg/kg) combined with surgery whenever possible[7,15,29].

The over antibiotics that led to the development of resistance affects human health worldwide. An extensive increase in antibiotic resistance, owing to a sustained persistence of resistant bacteria and fungi, is becoming a serious threat to public health worldwide. One such approach is botanical agents that have the potential to be used as an alternative to antibiotics that are rich in compounds such as alkaloids, terpenoids, tannins, steroids, coumarins and flavonoids and do not normally cause resistance[14,23]. Keeping in view the above justification, present study was done to evaluate the antimycotic activity of plant extracts and commercially available antibiotics against two fungi causing mucormycosis (*Rhizopus sp*. and *Mucor sp*.).

Material and Methods

Procurement of fungal cultures: Fungal cultures *Rhizopus sp*. (MTCC 6584) and *Mucor sp*. (MTCC 157) were collected from MTCC (Microbial Type Culture Collection), Chandigarh (India). Each sample was tagged and placed in refrigerator at 4 °C in PDA slants.

Collection of plants: The parts of medicinal plants were collected from different locations. The tap water was used to wash plant parts and then dried to keep in 50˚C (oven) for 48 hrs. and thereafter mixed to make powder. The list of plants used in this study are shown in table 1 and fig. 1.

Grinding the plant parts: The plant material was grinded with the use of a mortar and pestle. After drying in the oven, powdered forms of 8 test medicinal plants are shown in fig. 2.

Soaking in the respective solvent: Plant powder (40g) was dissolved and soaked in the respective solvent (ethanol 100%, methanol 100%, petroleum ether 100% and distilled water) to make the final volume 100ml and sealed with foil paper to prevent solvent evaporation. The mixture was incubated at room temperature for 24 hrs. (Fig. 3).

Filtration process: After incubation, the extracts were filtered by sterilized filter paper of Whatmann no.1. After filtration, the extract was evaporated in a water bath. Further, the extracts were evaluated for their antimycotic activity against fungi causing mucormycosis.

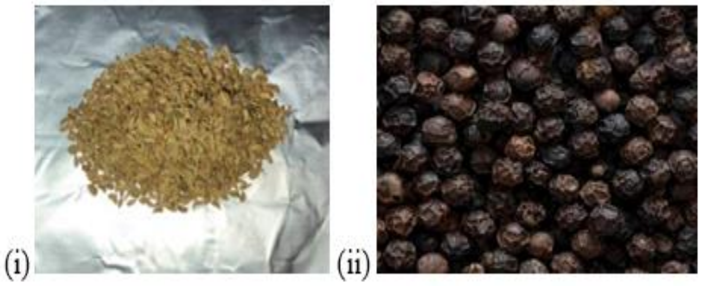

Figure 1: Medicinal plants used in the present study (i)*Trachyspermum ammi*(seeds), (ii)*Piper nigrum* (fruit)

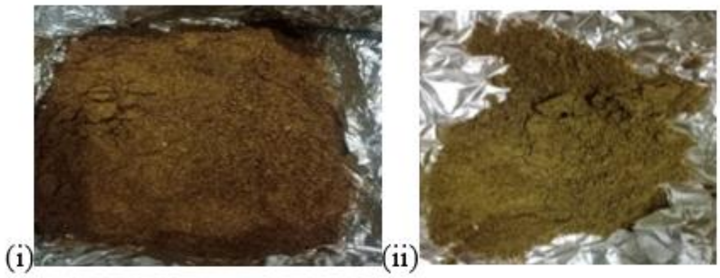

Figure 2: Grinding process of test medicinal plants: (i) *Trachyspermum ammi*(ii) *Piper nigrum*

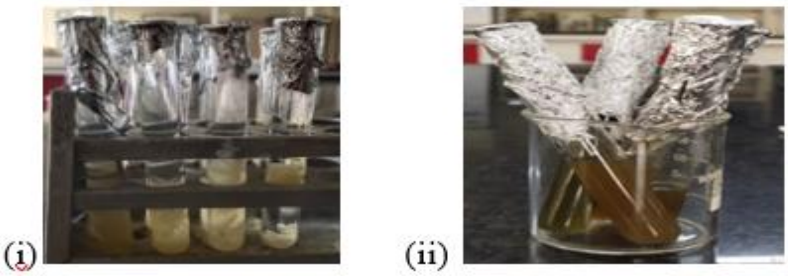

Figure 3: Soaking process of 7 medicinal plants in all 4 solvents (ethanol 100%, methanol 100%, petroleum ether 100% and distilled water): (i)*Trachyspermum ammi*(ii) *Piper nigr*

Table 1
Medicinal plants used in this study

S.N.	Common Name	Botanical Name	Parts Used
1.	Carom seeds (Ajwain)	*Trachyspermum ammi*	Seeds
2.	Black pepper	*Piper nigrum*	Fruit

Inoculum preparation: Fresh fungal cultures (3-4 days old) were used to prepare fungal inoculum suspension on potato dextrose agar plates. 5 ml of distilled sterile water was flooded on fungal culture plates. Tween 20 (5%) was added to facilitate the preparation of fungal strains. For fungal strains, rub the colonies with a sterile loop to prepare the fungal suspension/inoculum. The fungal culture was shaken for 15 seconds and transferred to a sterile tube. After filtering, the suspension was gathered in a sterile tube. By removing most of the hyphae, this process created an inoculum that was mostly made up of spores[12,32].

Screening of antifungal activity of medicinal plant extracts by agar well diffusion method: In this method, fungal inoculum was spread with a sterile glass spreader. A 6mm well was made with a sterile borer. 100µL volume of the plant extract was poured into the well of the inoculated agar plates. The plates were incubated for 3-5 days at 27°C. The zone of inhibition on plates was observed and measured in mm[17].

Qualitative phytochemical Analysis: Qualitative screening of phytochemicals such as alkaloids, flavonoids, saponins, tannin, glycoside and phenol was analyzed[4].

Fungal Inoculum preparation: The fresh fungal culture (3-4 days old) on potato dextrose agar plates was used to prepare inoculum suspensions (10^6cells/ml). 5 ml of distilled sterile water was flooded on fungal culture plates. Tween 20 (5%) was added to facilitate the preparation of *Mucor sp*. and *Rhizopus sp*. The fungal culture was shaken for 15 seconds and transferred to a sterile tube. The suspension was collected in a sterile tube, having an inoculum mainly composed of spores[12,32].

Antibiotic susceptibility pattern of fungal cultures: The antibiotic susceptibility pattern of fungal cultures was tested by following Kirby Bauer disc diffusion method. The inoculum (10^6spores/ml) was spread with a sterile glass spreader on PDA Plates. The hexa amtimyco-01 (HIMEDIA Laboratory Pvt. Ltd., Mumbai) disc was used. Hexa antimycotic-01 contains amphotericin-B, flucanozole, itraconazole, ketaconazole, clotrimazole and nystatin. Hexa antimycotic-01 was placed on the standardized fungal culture-seeded agar plates. The plates were incubated at 30°C for 3-5 days for fungal growth. After incubation, the diameter of the zone of inhibition was observed and measured in mm[17].

Results and Discussion

Out of 8 extracts of two medicinal plants, *Trachyspermum ammi* petroleum ether extract showed antimycotic activity against *Mucor sp*. with a zone of inhibition of 12mm. No other extracts of *Piper nigrum* and *Trachyspermumammi* showed any inhibitory activity against *Mucor sp*. *Trachyspermum ammi* (Omam) methanolic, ethanolic and petroleum ether extracts showed inhibitory activity against *Rhizopus sp*. with zone of inhibition ranging from 12mm to 22mm. No activity was observed in *Trachyspermum ammi* aqueous extract. *Trachyspermum ammi* (ajwain) extract has more potential effect against the fungi and the results coincides with earlier demonstration[31] which showed that Ajwain methanolic extract was more effective against *Aspergillus niger*, *A. lavatus*, *Epidermophyton flocossum* and *Trichophyton rubrum* with an inhibition zone 23 mm in diameter at a concentration of 1 mg/ml.

Another study reported that ajwain extract was effective against *C. albicans* with an inhibition zone of 21 mm in diameter. In a previous investigation[36], the ethanolic and methanolic extracts of Ajwain showed an effective zone of inhibition against *C. albicans*, *A. niger*, *A. fumigatus* and *Trichophyton rubrum*. *Piper nigrum* extracts did not show antimycotic activity against *Rhizopus* and *Mucor sp*. The results are shown in tables 2 and 3 and figures 4 and 5. The activity may be due to the presence of phytochemicals including alkaloids, carbohydrates, flavonoids, resins, steroids, tannins, inorganic acids, organic acids, phenolic compounds, amino acids, protein and coumarins[2].

Piper nigrum (black pepper), did not show antimycotic activity against *Rhizopus sp.* in evaluating antimicrobial and antifungal activities of leaf extracts and essential oils extracted from black pepper (*P. nigrum* L.). Significant action has been found against different microorganisms, namely, bacteria and yeast strains (*Pseudomonas aeruginosa*, *Candida albicans*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli*). Activity may be due to the presence of phyto-molecules such as monoterpenes and sesquiterpenes, which correspond to some of the chemical constituents elucidated in the fruits and the volatile oil of black pepper[11,13]. Literature search revealed that no more data are available on the antifungal activity of *Trachyspermum ammi* and *Piper nigrum* fungi causing mucormycosis. In the present study, the antibiotic susceptibility pattern of *Rhizopus sp*. and *Mucor sp*. was screened by agar disc diffusion assay. The antibiotic susceptibility pattern of *Rhizopus sp*. exhibited high sensitivity against ketoconazole (28mm) followed by clotrimazole (17mm) and it was found to be resistant against itraconazole, fluconazole, nystatin and amphotericin-B. Ketoconazole exhibited greater *in vitro* activity than the other five antibiotics against *Rhizopus sp.* The results are shown in table 4 and figure 6.

Table 2
Antimycotic activity of medicinal plant extracts against *Mucor sp.*

Solvents	Antimycotic microbial activity of medicinal plant extracts against *Mucor sp.*			
Medicinal plants	**Methanol**	**Ethanol**	**Petroleum ether**	**Distilled water**
Trachyspermum ammi	NA	NA	12mm	NA
Piper nigrum	NA	NA	NA	NA

NA: No activity

Table 3
Antimycotic activity of plant extracts against *Rhizopus sp.*

Solvents	Antimycotic microbial activity of plant extracts against *Rhizopus sp.*			
Medicinal plants	**Methanol**	**Ethanol**	**Petroleum ether**	**Distilled water**
Trachyspermum ammi (Omam)	12mm	15mm	22mm	NA
Piper nigrum	NA	NA	NA	NA

NA: No activity

Table 4
Antibiotic susceptibility pattern of *Mucor sp.* and *Rhizopus* sp.

Zone of Inhibition in mm						
Antibiotics / Fungi	**Amphotericin-B AP**	**Clotrimazole CC**	**Fluconazole FLC**	**Itraconazole IT**	**Ketoconazole KT**	**Nystatin NS**
Rhizopus sp.	R	17mm	R	R	28mm	R
Mucor sp.	12mm	18mm	R	R	R	R

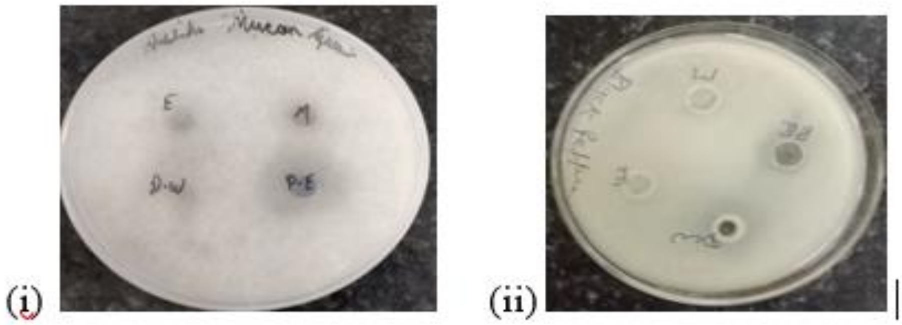

Figure 4: Antimycotic activity of plant extracts (i)*Trachyspermumammi (Omam)* (ii) *Piper nigrum* against *Mucor sp*.

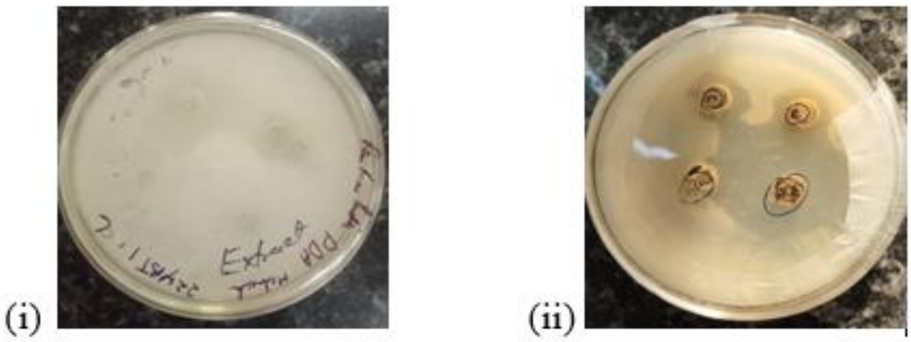

Figure 5: Antimycotic activity of plant extracts (i) *Trachyspermum ammi* (ii) *Piper nigrum* against *Rhizopus sp.*

The antibiotic susceptibility pattern of *Mucor sp*. exhibited sensitivity against clotrimazole (18mm) followed by amphotericin-B (12mm) and was found to be resistant against ketoconazole, fluconazole, nystatin and itraconazole (Table 4 and figure 7). Histopathologic and cultural analyses of the afflicted tissue are used to make additional diagnoses.

Since the disease is progressing quickly and has a frequently deadly prognosis, a prompt and accurate diagnosis is essential. A multimodal approach is used to address the disease which includes early medication delivery and surgical excision of affected tissues. Among the antifungals, azoles and amphotericin- B remain the gold standard drugs of choice for initial treatment[37].

The effectiveness of the existing systemic antifungal medications is still below par and the order Mucorales is becoming increasingly resistant to them. Understanding the molecular processes behind the antifungal resistance in mucormycosis can help us develop new treatments and offer critical knowledge to our arsenal of antifungals. The current situation of antibiotic resistance necessitates new tactics for dealing with the antibiotic resistance-related problems. Reassessing the natural products obtained from plants and microorganisms (bacteria, actinomycetes and fungi) is one such potential subject[25].

The present work is an overview of the antibiotic susceptibility pattern of *Rhizopus sp.* and *Mucor sp.* against six different antibiotics screened by using Kirby- Bauer Disc Diffusion Assay. The antibiotic susceptibility pattern of *Rhizopus sp.* exhibited highly sensitivity against Ketoconazole (28mm) followed by Clotrimazole (18mm) and it was found to be resistant against Itraconazole, Amphotericin-B, Fluconazole, Nystatin.

According to Viecceliet al[38], Ketoconazole, a synthetic imidazole is an antifungal drug, considered as a second-line drug. The antibiotic susceptibility pattern of *Mucor sp.* exhibited sensitivity against Clotrimazole (18 mm) followed by Amphotericin-B (12mm) and found to be resistant against Ketoconazole, Fluconazole, Nystatin, Itraconazole.

Among the six tested antibiotics, clotrimazole was found to be the most effective antifungal drug against both fungi. Clotrimazole is an imidazole and a topical broad-spectrum antifungal agent used for the treatment of dermatophyte infections and candidiasis and other fungal infections like mucormycosis[26].

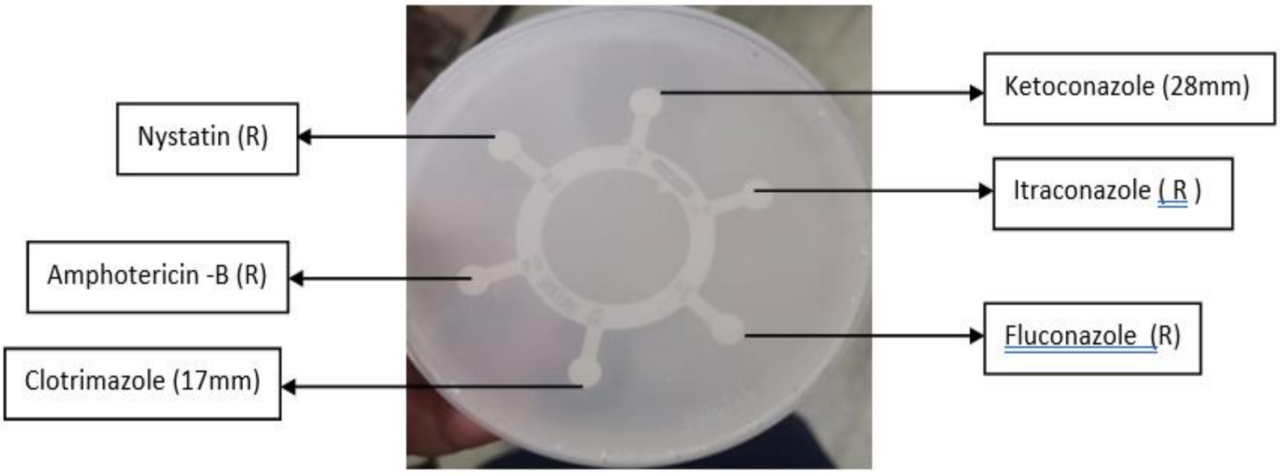

Figure 6: Antibiotic Susceptibility pattern of *Rhizopus* sp.

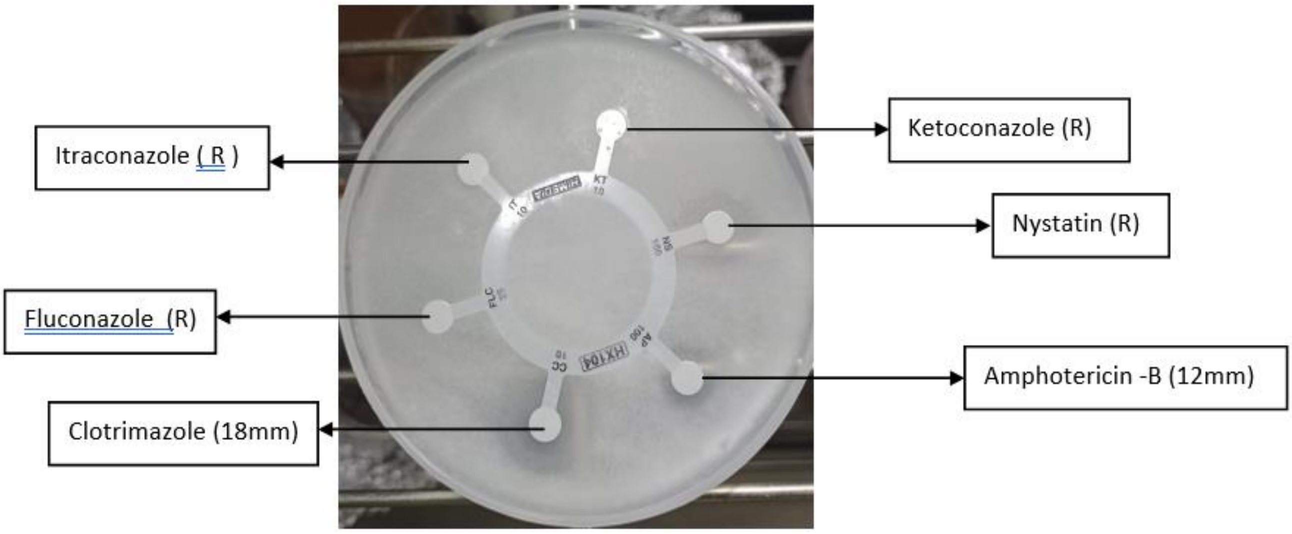

Figure 7: Antibiotic Susceptibility pattern of *Mucor* sp.

Clotrimazole as a positive control showed maximum antifungal activity against *Mucor sp.* (18mm) and minimum in *Rhizopus sp.* (17mm). The first-line treatment for this life-threatening fungal infection is liposomal AmB. Due to residual concerns, isavuconazole and new posaconazole formulations have been recommended for clinical use, but only as a second-line treatment after liposomal AmB[8]. Posaconazole and Terbinafine have exhibited in vitro activity against a few species of Mucorales. According to Khatter and Khan[21] clotrimazole is an antifungal medication in the imidazole class used to manage and treat fungal infections.

There are treatments available, but less promising and less effective. So, researchers are focusing on the promising agents against mucormycosis. It is reported that early treatment with liposomal amphotericin B (AmB), manogepix, echinocandins isavuconazole, posacanazole and other promising therapeutic agents has overcome the burden of mucormycosis. Lipid formulations of Amphotericin-B have become the standard treatment for mucormycosis due to their greater safety and efficacy[18].

Comparing our analysis with previous studies, Janowski et al[20] reported that the mechanism of action involves inhibition of the activity of Fluconazole (2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazole-1-yl) prpan-2-ol) that are resistant to azole antifungal drugs as fluconazole, a triazole family member and stands as a measure antifungal agents[16]. This antifungal drug whose mechanism of action involves inhibiting activity not show any antifungal activity against either of the fungi[20].

In comparison to Amphotericin-B, new itraconazole formulations were linked to lower mortality rates and good responses of up to 80% in invasive mucormycosis, according to a recent review of matched cases[3]. The different zone of inhibition was observed on the plates, which show the fungi's sensitivity, resistance or intermediate. The minimum inhibitory concentrations of Amphotericin- B and other antifungal agents depend on the agents and species against which the antifungal activity[27] is measured. When taken in accordance with its primary indication, ketaconazole, a polyene produced from triazole, exhibits higher tolerance than amphotericin-B in 25 strains of Mucorales, 18 of which were of the genus Rhizopus, in roughly 63% of patients. Current literature revealed that available Ketaconazole is an effective and reliable drug[38]. *Rhizopussp.*is highly sensitive to Amphotericin B combinations as suggested by Franco et al[15] and Mammen et al[25].

All extracts of *Trachyspermum ammi* showed the presence of tannins, Phenols and glycosides. Saponins and alkaloids were present in *Trachyspermum ammi* aqueous extract. Flavonoids were not present in any of the test *Trachyspermum ammi* extracts. *Piper nigrum* showed the presence of flavonoids, glycosides and phenols in the methanolic extract. *Piper nigrum* showed the presence of alkaloids, flavonoids and glycosides in the ethanolic extract. *Piper nigrum* petroleum ether extract showed the presence of all the test phytochemicals except tannins and phenols (Table 5). Every plant has thousands of compounds and because of these compounds, every plant has medicinal values such as antimicrobial, antidiabetic, anticancer and others.

Bashyal and Guha[4] revealed the presence of flavonoids and saponins in all *Trachyspermum ammi* extracts prepared in methanol, acetone, chloroform and water. Alkaloids, flavonoids, terpenes and essential oils are all present in *Piper nigrum* L of these, piperine has been studied the most[6]. *Piper nigrum* L. is essential for culinary use and contains bioactive compounds with health potential.

Conclusion

In the present study, ketoconazole was found to be most effective followed by clotrimazole against *Rhizopus sp.* However, resistance was observed in amphotericin-B, nystatin, itraconazole and fluconazole against *Rhizopus sp.*

Table 5
Qualitative phytochemical analysis of *Trachyspermum ammi* and *Piper nigrum*

Plant Names	Solvent	Phyto-constituents analysed					
		Alkaloids	Flavonoids	Saponins	Tannins	Glycosides	Phenols
Trachyspermumammi	Methanol	-	-	-	+	-	+
	Ethanol	-	-	-	+	+	+
	Petroleum ether	-	-	-	+	+	+
	Distilled water	+	-	+	+	+	+
Piper nigrum	Methanol	-	+	-	-	+	+
	Ethanol	+	+	-	-	+	-
	Petroleum ether	-	_	-	-	-	+
	Distilled water	+	+	+	-	+	-

The antibiotic susceptibility pattern of *Mucor sp*. exhibited sensitivity against clotrimazole followed by amphotericin-B and was found to be resistant against ketoconazole, fluconazole, nystatin and itraconazole. Clotrimazole was found to be effective against both *Rhizopus sp*. and *Mucor sp*. The current situation of antibiotic resistance necessitates new tactics for dealing with the antibiotic resistance-related problems. *Trachyspermum ammi* petroleum ether extract showed good antifungal activity against both tested fungal strains. It can be suggested from the present investigation that re-assessing the natural product obtained from plants and microorganisms (bacteria, actinomycetes and fungi) is one such potential subject.

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