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Antifungal Assessment Of Various Plant Extracts Against Indoor Fungal Isolates Responsible For Allergies And Asthma

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Abstract:
The annual prevalence of invasive aspergillus infection in India is growing at an alarming rate. The incidence of fungal infections varies predominantly depending on geography, level of pollution, the socioeconomic characteristics of local population along with proportion of people at risk of infection and severe diseases. In clinical practice, several chemical disinfectants that are effective against wide range of pathogens used to disinfect air and surfaces particularly in environments where higher level of disinfection is required, such as in surgical areas, laboratories and areas where immunocompromised patients are housed. Furthermore, the growing concern about the development of resistance to chemical disinfectants may allow some pathogens, to develop resistance, making future infections harder to control. Emergence of cross- resistance to certain chemical disinfectants indicating an urgent need to explore natural and safe alternative solutions.
This study aims to identify the most predominant aspergillus species, a significant opportunistic pathogen associated with various diseases including aspergillosis from various indoor air samples. Several plant extract, attributed for their potent antifungal properties were selected based on previous research. The shortlisted plant species (Clove, Cinnamon, Lemongrass and Nutmeg) were subjected for in-vitro antifungal assay for evaluation of their antifungal potency against the common prevalent aspergillus species. The most promising plant extract was further evaluated to determine its Minimum inhibitory concentration (MIC) and sporicidal concentration.
The present research study identified Aspergillus niger, followed by Aspergillus flavus and Aspergillus fumigatus respectively as a predominant fungus in indoor sample areas. The in-vitro antifungal assay performed against these predominant Aspergillus spp. demonstrated that clove extract with highest potency against A. niger with average zone of inhibition(33mm), followed by A. flavus (30mm), and A. fumigatus(30mm) respectively. The second most potent extract demonstrated with antifungal assay as cinnamon with average zone of inhibition (-25mm,26mm,26mm), lemongrass –(20mm,25mm,30mm) and, Nutmeg (-10mm,19mm,12mm) respectively. The MIC concentration of clove extract was found to be (4mg/ml, 2mg/ml, and 4mg/ml) against A. niger, A. flavus, and A. fumigatus respectively. The study further confirmed clove extract with sporicidal concentration of 3mg/ml, 2mg/ml and 3mg/ml as a potent antifungal alternative.
***Background**: Invasive fungal infections becoming an important healthcare issue with an alarming prevalence rate and creating an urgency for new and effective antifungal molecules. In India , allergic bronchopulmonary aspergillosis in adults who have severe asthma with fungal sensitisation is estimated 1 million to 2.8million cases (Ray A et al, 2022) and affecting even normal wellbeing at a large extent. Aspergillus niger, A. fumigatus, and A. flavus, in same order, are among the most common aspergillus species that cause human disease. Climate and geographic conditions remain significant determinants of the prevalence and distribution of Aspergillus species in the air we breathe. Air pollution also plays a crucial role in the spread of Aspergillus spp. in the environment and its transmission to patients. Earlier studies demonstrated the small conidia size of A. fumigatus (2 to 3 um) with nasal route easily reach pulmonary alveoli as compare to that with A. flavus. This reassures A. fumigatus as the main agent of invasive pulmonary aspergillosis. Everyday exposure to aspergillus is rarely a problem for anyone with healthy immune systems however, with weakened immune system , even the mild infection may result in allergic bronchopulmonary aspergillosis (ABPA), bronchopulmonary aspergillosis. Potent antifungal plant based extracts can be used to purify the air in certain closed settings or can be used as part of a cleaning regimen within defined cleaning protocol to disinfect surfaces in environments where vulnerable individuals are.*
***Materials and Methods**: Some shortlisted plant origin-based extracts (Clove, Cumin, Cinnamon, Rosemary, Lemongrass and Nutmeg) were evaluated to assess their respective antifungal potency against the most prevalent aspergillus species with in-vitro assay. Further the potent extract was subjected for evaluation of Minimum inhibitory concentration as well as sporicidal concentration respectively.*
***Results**: The results of present undertaken research confirmed that clove extract possessed strong antifungal activity against A. niger than A. flavus and A. fumigatus with zone of inhibition as (33mm, 30mm and 30mm). The MIC concentration was found to be (4mg/ml, 2mg/ml and 4 mg/ml) whereas the respective sporicidal concentration against Aspergillus niger and A. fumigatus spore was 3 mg/ml and against A. flavus was recorded*

as 2mg/ml. The findings revealed the potency of clove extract as a promising antifungal as well as sporicidal substitute.

Conclusion: *In conclusion, clove, cinnamon and lemongrass extract exhibits a promising antifungal potency against predominant fungal pathogen species of Aspergillus present in indoor air samples. Instead of relying on hazardous chemical or synthetic disinfectants, alternative natural plant-based disinfectant may offer an eco-friendly solution in healthcare and similar settings, reducing associated risks while still effectively control the spread of infection caused by airborne pathogens like aspergillosis.*

Key Word: *Allergies and asthma, Aspergillosis, Plant derived Antifungals*

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I. Introduction

The genus *Aspergillus*, which includes almost 200 species, has a tremendous impact on public health both beneficially as industrial applications and negatively as human pathogens. Fungal sensitization is very common in bronchial asthmatic cases, and the connection with airway colonization by fungi remains uncertain and is still an area of active research. Climate and geographic conditions play a significant role and also determinants of the prevalence and distribution of Aspergillus species in the air we breathe. Infections caused by Aspergillus species have grown in importance in recent years. This probably results from a higher number of patients being at risk, including transplant recipients, neutropenic individuals, allergic patients and those treated with corticosteroids or other immunosuppressive regimens. To overcome limitations associated with chemical and synthetic disinfectants along with an emergence of cross- resistance, use of natural plant extract as an antifungal agent has gained importance in recent years. Herbal medications can mistakenly be perceived as safe because they are natural products. They may produce negative effects such as allergic reactions, rashes. Plant based extract contains several components: alkaloids, glycosides, phenolics, terpenoids, and flavonoids which reduces the toxicity of plant material.

II. Material And Methods

Study area - Mumbai (19.07 '60N, 72.87’ 77E, month Feb-march, and humidity 32%) is one of the populated cities in India.

Preparation of Media -Temperature 25°C & 37°C. Morphological features of colonies on above culture media as well as microscopically characteristics for the major strains were studied and then compared with those of standard Aspergillus strains.

Standard Test organisms-

The most frequent fungal isolates are drawn from the earlier studies. *A. Flavus, A. niger, A. fumigatus.*

Sample collection, Isolation and Identification of fungal strain-

Total of 20 samples obtained from indoor sampling by exposure of media plates at different sites. Total number 60 sterile potato dextrose agar (PDA) media plates exposed at 20 different sites in the indoor environment for 15 mins for consecutive three days at same time. The expose plates were incubated at room 25^0C for 48 hrs. After 48 hrs the total number of colonies forming units were calculated for each sample and continue further incubation for 7days. After incubation period of almost seven days, the morphological characteristics of the predominant fungal colonies were observed and documented. Results obtained were compared with the standard aspergillus species on potato dextrose agar.

Selection and Extraction of plant material-

Based on earlier research assessment few plant materials as Clove - *Syzygium aromaticum*, Cinnamon - *Cinnamomum zeylanicum*, Lemongrass- *Cymbopogon citratus*, Nutmeg- *Myristica fragrans Houtt*, were selected for the present research assessment. Different parts of the test plants are collected from authentic ayurvedic suppliers from Mumbai region.

Pure powder of plant material Lemongrass leaves, Nutmeg, Clove and Cinnamon was obtained using mortar and pestle. 40 gm. of powder of each plant material was soaked in 500 ml methanol and extracted with Soxhlet extraction. Final residual extract was collected in a screw cap tube After complete extraction, resulted plants extract of *Cinnamomum zeylanicum* (Cinnamon*), Syzygium aromaticum (*Clove*), Cymbopogon citratus (*Lemongrass*), Myristica fragrans Houtt (*Nutmeg*),* labelled as test sample 1,2,3,4 respectively and refrigerated till final assay was performed.

Agar disc diffusion antifungal assay

Fungal inoculum prepared in Tween 80 saline suspension. Each of the fungal suspensions containing inoculum (10µL) poured into Petri plates and mix through with rotation and allowed solidification. With disc diffusion technique 20µL of each plant extract was added to 10 mm sterile blank filter disks and placed on the petri plate. All samples were placed in triplicate and incubated at 25°C for 48 hrs. After Incubation zone of inhibition was observed and documented for each test respectively. As each test was performed in triplicate, the average of the three zone of inhibition was documented.

Minimum Inhibitory concentration -

Minimum inhibitory concentration (MIC) is the lowest concentration of a test sample that inhibits microbial growth. On the basis of observed maximum zone of inhibition, the most potent and promising test sample only was selected for assessment of MIC. Potato dextrose agar media plate with seed inoculum of respective test organism was incubated with various concentrations of test sample as 2mg/ml, 4 mg/ml, 6mg/ml, 8mg/ml, 10mg/ml prepared.

Sporicidal activity

Determination of sporicidal activity of selected test sample was performed using Czapek yeast extract broth. Organisms grown on PDA media for sporulation and spores are harvested. when the cultures are fully sporulated which was achieved after 10 days incubation. spores are collected by adding 5 ml sterile water containing 0.1% (v/v) tween 80 to each petri plate and rubbing the surface with sterile spreader. The suspension was collected and centrifuged at room temperature at 2000 r/min for 5 min. 1 ml of spore suspension was prepared against each concentration of test sample. Test samples prepared in 5 ml Czapek yeast broth with various concentration of 2mg/ml, 4 mg/ml, 6mg/ml, 8mg/ml, 10mg/ml respectively and mix the with 1ml of spore suspensionin 100 ml flask. Incubated the test at 25°C for 48 hr. on a rotary shaker.

III. Result

Plant Material Collection and isolation

40 gm of powder in 500 ml methanol extracted by Soxhlet extraction. This mixture was heated to a temperature approximately between 70° C. to 80° C. for approximately two hours. The observed final colour, pH, and volume of each plant extract was documented. obtained. The respective obtained amount of each dried plant extract calculated after solvents extraction and was dissolved in DMSO to make stock test sample with concentration as 2mg/ml.

Isolation and Identification of fungal strain-

Total of 60 PDA plates with indoor sampling after incubation period with isolated colonies on PDA agar were analysed for identification of *Aspergillus species.* The observed specific colony characteristics were documented as illustrated in table 1. Based on the observed morphological colony characteristics the most frequently observed *Aspergillus species* was concluded as most prevalent one.

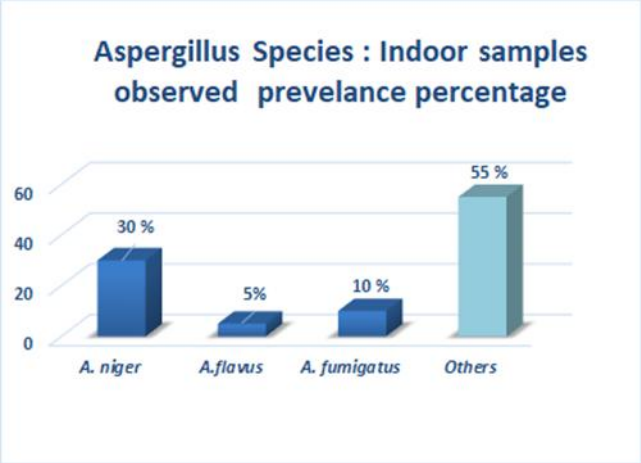
Table: 1 Observed morphological colony characteristics

Colony characteristics	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*
Colour	Slightly brown	Pale brown	Greyish near apex
Shape	Glubose	Glubose	Glubose
Size	400-3000 um	400-800 um	200-400 um
Conidia surface	Very rough	Smooth finely	Smooth /spinose

Table 2 represents the presence of aspergillus species in indoor samples analyzed. Exposure of the PDA media plate at various indoor environments such as kitchen space, refrigerator, home air at ceilings and office space, the results confirmed that *Aspergillus niger was* present at almost 30% samples as a more prevalent species, followed by Aspergillus *fumigatus* 15% and Aspergillus *flavus* 5% respectively as represented below in (Graph 1).

Table: 2 Predominant *Aspergillus species* in indoor air samples

Samples used	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*
Office Space	Positive	Negative	Negative
Refrigerator	Positive	Negative	Negative
Home air	Positive	Negative	Positive
Ceilings	Negative	Negative	Positive



Graph1: Aspergillus species: prevalence of aspergillus species in indoor environment

Agar disc diffusion antifungal assay

The agar disc diffusion technique with 20µL of each test sample incubated with test pathogens were placed incubated at 25°C for 48 hrs. After Incubation zone of inhibition was observed for each test sample is as represented in below table no. 3. The results of an antifungal assay confirmed that clove extract has highest antifungal activity against *Aspergillus niger* with zone of inhibition of 33mm, followed by *Aspergillus flavus* with zone of inhibition of 30mm and *Aspergillus fumigatus* with zone of inhibition of 30mm respectively. The observed results also revealed that minimum antifungal activity of nutmeg extract, whereas lemongrass .cinnamon extract had demonstrated moderate antifungal activity against aspergillus species as represented in graph 2.

Table: 3 Antifungal activity *of Plant extracts against Aspergillus species*

Test Samples	Zone of inhibition in mm (Average)		
	A. niger	*A.flavus*	*A. fumigatus*
Cinnamomum zeylanicum	12	16	12
Syzygium aromaticum	33	30	30
Cymbopogon citratus	25	26	26
Myristica fragrans Houtt	10	19	12

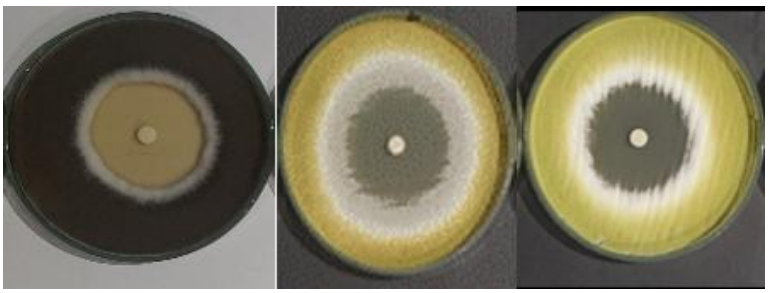
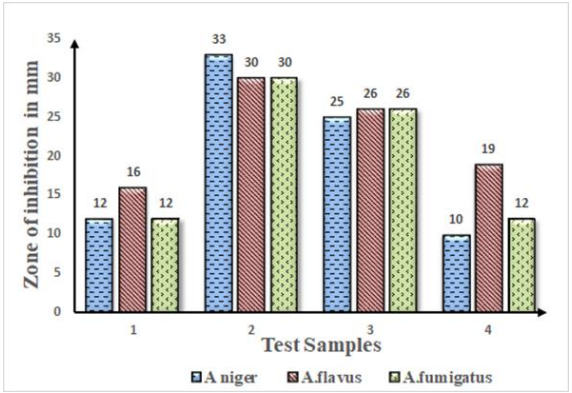


Fig 1 Antifungal Activity Of Test Samples 1,2 ,3



Graph 2: Agar Disc Diffusion Antifungal Assay With Zone Of Inhibition (In Mm)

Minimum Inhibitory Concentration and Sporicidal Assay

Minimum inhibitory concentration (MIC) assay with various concentrations of the most potent test sample of *Syzygium aromaticum* used as 2mg/ml, 4 mg/ml, 6mg/ml, 8mg/ml, 10mg/ml respectively on a Potato dextrose agar media plate. The MIC assay confirmed lowest inhibitory concentration as 4mg/ml for *Aspergillus niger and Aspergillus fumigatus whereas the recorded MIC against Aspergillus flavus species is 2mg*/ml as represented in table 4.

Table 4: Minimum Inhibitory concentration

Test Sample	Inhibition conc. in mg/ml (average)		
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus fumigatus*
Syzygium aromaticum	4	2	4

Sporicidal activity *Syzygium aromaticum* extract reveals that *Aspergillus niger and Aspergillus fumigatus* exhibits a highest sporicidal concentration of 3 mg/ml , whereas *Aspergillus flavus* it was observed to be 2mg/ml to inhibit the spores completely as represented in table no.

Table 5 Sporicidal Assay

Test Sample	Sporicidal concentration. in *mg* /ml		
	Aspergillus niger	*Aspergillus flavus*	*Aspergillus fumigatus*
Syzygium aromaticum	3	2	3

IV. Discussion

Fungal allergens, both indoors and outdoors are an emerging concern in rhinitis and asthma complications Exposure to fungi and their spores in home environment is an important factor for fungal allergy. Limiting the exposure of vulnerable populations to allergenic fungal spores is crucial to preventing severe respiratory conditions(Dharmage S. st al 2002). Few recent research studies documented rise in the percentage of children suffering from various respiratory disorders. Often several patients suffering with respiratory allergic experience acute asthmatic attacks more frequently during the changing season and weather conditions (Priftis KN, et al ,2006).

Findings of the present study *Aspergillus niger* and *Aspergillus fumigatus* being significant prevalent was also corelate with the previous studies undertaken in various regions of India, including Mumbai (Singh R et al, 2015), Delhi (Das S. et al, 2008), which has mainly focused on incidence of Aspergillus infections in occupational settings.

Also, the aerobiological indoor as well as outdoor fungal assessment study within India, inclusive of both the qualitative and quantitative prevalence of clinically significant fungal isolates confirmed Aspergillus species and their spores being significant among observed fungal concentrations. From the 35 fungal genera isolated, the prevalent fungal groups from all of the indoor settings were different species of *Aspergillus*, *Penicillium*, *Alternaria*, and *Cladosporium.* Nonsporing isolates and yeast were also frequently isolated but they were grouped into "others." Some of these fungi have been reported as the most common airborne fungi in different environments and have been considered to be the potent allergens in indoor air samples of many indoor and outdoor environments contributing to the increase in respiratory diseases in children as well as adults. (Rashmi Sharma et al, 2011)

More focused need to be put forward towards implementing strategies for monitoring sources and preventing seasonal fungal spore concentrations. The prevalence of *Aspergillus species* is become more common in indoor settings and several studies confirmed their association with several allergies and asthma cases predominantly with patients with pulmonary diseases, weakened immune systems, or those undergoing prolonged corticosteroid therapy. Aerobiological monitoring of asthma studies also revealed growth of *Aspergillus* fungus from indoor air sampling more common within residential areas. Aspergillus spores pose a significant risk to respiratory health and also need to consider seriously for their allergenic potential.

The results of an antifungal assay shows that clove extract has highest antifungal activity against *Aspergillus niger* followed by *Aspergillus flavus* and *Aspergillus fumigatus.* Present study findings data was found to be similar with earlier findings (Lee fang tan et.al., 2019), even few (Laila Muñoz Castellanos et.al. 2020) demonstrated antifungal activities of essential oils of clove, cinnamon, cumin and many more are considered more potent than plant extracts. The effectiveness of antifungal several plant extracts is defined by their chemical compositions. The most potent antifungal extract of clove demonstrated in the present study confirm with presence of Eugenol as its main bioactive component contributing for its effective antimicrobial attribute. Antifungal activity of this chemical component can be more precisely due to the presence of an aromatic nucleus

and a phenolic OH group that are known to be reactive and can form hydrogen bonds with –SH groups in the active sites of target fungal enzymes, resulting in the deactivation of enzymes in fungi. The main constituent of another extract of cinnamon is due to the presence of cinnamaldehyde, the compound containing an aldehyde group and conjugated double bond outside the ring. This compound possesses much stronger antifungal activity (Wang et al., 2005) and it may be a potential lead compound for the development of antifungal drugs through the control β-(1,3) glucan and chitin synthesis in yeasts and molds.

V. Conclusion

Allergy to several fungi and their spores has been linked to a wide range of illnesses, including rhinitis and asthma. Aspergillus spp. poses a life threat to society, and particularly immunocompromised patients, since many strains have shown resistance towards the existing synthetic and chemical antifungal compounds. Present study confirmed the antifungal potency of plant derived bioactive components which may serve as potential antimicrobial resource to combat with challenges emerged with cross- resistance. However, the development and implementation of strategies for plant derived bio active component as a part of the regular regimen in various antimicrobial treatments and their standardization is still challenging.

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