University of Tehran Press

Pollution

Print ISSN: 2383-451X
Online ISSN: 2383-4501

https://jpoll.ut.ac.ir/

Novel Eco-Friendly Herbal Based Air Freshener Formulation as Air-Borne Fungal Repellent in Indoor Environments Through Real Time Monitoring

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Article Info

Article type:
Research Article

Article history:
Received: 25 Aug 2022
Revised: 27 Dec 2022
Accepted: 14 Jan 2023

Keywords:
Air Fresheners
Medicinal Plants
Air Borne Fungi
Indoor Air Quality
Ambient Air Quality
Green Air Fresheners

ABSTRACT

Air fresheners are the synthetic products, used to improve the quality of indoor air by removing unpleasant or disturbing odours, in addition they disinfect the air by removing allergens and in turn add pleasant odours. However, these fresheners since they contain varied chemicals, which on magnification in a closed environment may cause respiratory illness. Therefore, constant usage of these air fresheners would deteriorate the ambient quality of indoor air. Even air fresheners which claim to be "green", since these lack regulatory norms, they too emit hazardous or chemically harmful compounds. Hence there is a dire need to use alternative products that substantiate the quality of indoor air. The present study aimed at exploring the efficacy of medicinal plant extracts of *Azadirachta indica, Menta piperita* and *Aloe barbadensis* in controlling air borne fungi in indoor environments by creating a simulation of an indoor environment and checking the efficiency of these natural air fresheners. About 60-70% reduction in the vegetative structures (colony diameter) and 30% reduction in reproductive structures were observed after exposure for 11 days to environment containing Azadirachta *indica* and *Menta piperita* extracts. Thus this study has novelty in formulating herbal based air fresheners based on the proven antifungal activities of these medicinal plant extracts, thereby replacing the usage of commercial air fresheners in the near future in controlling indoor air borne fungi. Since these natural formulations undoubtedly disinfect the indoor air, has commercial prospects and are eco-friendly, cost-effective with no health implications.

Cite this article: Lakshumanan, T. & Velrajan, M. (2023). Novel Eco-Friendly Herbal Based Air Freshener Formulation as Air-Borne Fungal Repellent in Indoor Environments Through Real Time Monitoring. *Pollution*, 9 (2), 557-566. http://doi.org/10.22059/POLL.2022.347672.1591

DOI: http://doi.org/10.22059/POLL.2022.347672.1591

INTRODUCTION

Air fresheners are the synthetic products that have been used in the field of environmental sanitation for decades in different environmental settings, such as dwellings, hospitals, offices, schools, hotels, restrooms etc. (Telpner, 2016). According to Jung *et al*., (2011) air fresheners are being indiscriminately used to get rid of disturbing odours that may affect the ambient air quality of the indoor environment and thus a pleasant ambience is provided by the ingredients present in these air fresheners through fragrance. However fragrance has been associated with disastrous effects on indoor and outdoor air quality and human health such as migraine headaches, asthma attacks, neurological problems respiratory problems, skin problems,

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cognitive problems, mucosal symptoms, immune system problems etc.

(Steinemann,2020). This may be due to many chemicals that are not revealed on the product label as manufacturers are not required to disclose all ingredients (Cohen *et al*., 2007). These chemicals could be allergens, irritants, or even toxic (National Resources Defence Council, 2007). Steinemann *et al*., in the year 2011, found numerous chemicals in air fresheners, such as acetaldehyde, acetone, benzaldehyde, and limonene that were not listed on the product label. These compounds, though they have antifungal activity are considered as health or environmental hazards because of their magnification in the indoor environment thus resulting in negative consequences. Hence there is a dire need to replace these chemical fresheners with natural products that are safe and eco-friendly but still possessing strong antifungal activities. Therefore, the present study explored the efficacy of medicinal plant extracts in controlling air borne fungi in indoor environment by creating a simulation of an indoor environment and checking the efficiency of these natural air fresheners by humidification. The plants *Azadirachta indica* , *Menta piperita* and *Aloe barbadensis* were chosen for the study for their medicinal values. Real time monitoring in a natural house hold environment was attempted for the first time to convert the laboratory studies to a natural indoor setting and the efficacy of medicinal plants extracts tested with commercial air fresheners. In addition, an herbal based air freshener formulation was made from the three plant extracts to test on a real time basis in the indoor environment, in controlling air borne fungi and disinfecting the air. The objectives of the study are as follows:

· To isolate fungi from indoor air
· To measure the antifungal activity of leaf extracts under laboratory conditions.
· To assess the fungal air load reduction in a closed environment by humidification with leaf extracts
· To compare efficiency between artificial room fresheners and leaf extracts.
· Real time monitoring of the leaf extracts in the natural household environment.

MATERIALS AND METHODS

The mixed fungi samples were isolated from the indoor environment of the kitchen in a common household by settle plate method. The agar plates were exposed to the kitchen environment, by placing the lidless plates on the kitchen floor, near damp walls and inside cupboards for a period of 20, 30 and 40 minutes for fungal isolation and hence 8 agar plates for different time durations in duplicates were used with two unexposed plates as control. Since kitchen has a damp environment, it is more prone to fungal contamination, therefore kitchen environment was chosen for the study.

A small portion of the desired fungus along with the agar portion was taken aseptically using flamed forceps (from settle plate mixed colonies) and was placed on sterile Rose Bengal agar plates fortified with chloramphenicol. Similar method was repeated for all the fungal cultures obtained by settle plate method. Following incubation at room temperature for 2-3 days, pure cultures were obtained and confirmed by Lacto phenol cotton blue staining (Leck, 1999).

The fresh leaves of the following flora *Azadirachta indica* (neem), *Menta piperita* (peppermint) and *Aloe barbadensis* (aloe) were obtained from Madras Christian college campus. Table 1 highlights the reported antimicrobial and other beneficial effects of these medicinal plants.

For both *Azadirachta indica* and *Mentha piperita,* the leaves were taken and dried (under direct sunlight for 3 days) and finely powdered by using a blender and 5mg of the powder was added to 100 ml of water and kept in a water bath at 50° c for 15 minutes to obtain the extracts. For *Aloe barbadensis,* the colourless parenchyma was spooned out and mixed well to a semi-liquid consistency. This crude suspension was then filtered out to obtain the extract. Different concentration of extracts like 50%, 60% and & 70% (using water as a diluent) were used to test

their efficacy in controlling fungal contamination.

The agar diffusion method is followed for determining the antimicrobial activity of any target compound. Though it produces semi-quantitative or only qualitative results, Janssen *et al*., in the year 1987 recognized this method as precise and reliable and Balouiri *et al* in 2016 reviewed this technique for invitro antimicrobial activity. Lawn cultures of *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* (obtained from the kitchen indoor environment) *were* made separately on sterile SDA media and wells of 5 mm diameter were cut into the agar by cork borer and 0.1 ml of the crude leaf extracts were added to each well. Following incubation at 28°c for 24-48 hours, the plates were assessed for antifungal activity by measuring the diameter of the inhibition zone formed around the well.

To study the effectiveness of the leaf extracts in an artificially made indoor environment by humidification of leaf extracts, chambers made up of polyethylene terephthalate (PET) of diameter (23x24x22cm) were selected and kept air tight to avoid contamination from external environment by sealing. One packet of commercial air fresheners, 100 ml of each leaf extract of *Azadirachta indica, Menta piperita* and *Aloe barbadensis* were kept in separate chambers. Therefore, six such chambers were prepared and each chamber with all the three fungal cultures taken for study in three agar plates and each chamber separately having the commercial air fresheners and the leaf extract (Figure 1). The lids were closed and sealed with petroleum jelly to get the internal environment saturated with these materials, thus attempting to make a simulation of a room. The cultures of *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* were inoculated in the centre of Rose bengal agar plates fortified with chloramphenicol (to avoid bacterial contamination) and then kept in separate chambers containing the above mentioned indoor environments with medicinal plant extracts and commercial air fresheners and were incubated at 28°c temperature. The culture plate without air purifier/ leaf extract to serve as untreated control was also kept inside one chamber. Observations were taken for periodic vegetative growth of the inoculated cultures in terms of colony diameter, changes in colony as well as morphological and microscopic features. After fifteen days of incubation, all the culture plates kept in air purifying environments as well as untreated environment were taken out from the respective chambers and observations were then recorded for fungal inhibition, sporulation and changes in colony morphology.

The leaf extracts of *Azadirachta indica*, *Menta piperita* and *Aloe barbadensis* were utilized in a room with passive air flow to measure their effectiveness. The extracts taken in a concentration of

Table 1. Reported antimicrobial and other beneficial effects of the medicinal plants

S.No.	Medicinal plants	Common name	Household uses
1.	*Azadirachta indica*	Veppai , vembu	Used to treat a wide range of diseases, insecticide
2.	*Menta piperita*	Pudina , Vilayati	Used to treat many ailments
3.	*Aloe barbadensis*	Kattazhi,Musambaram	Used to treat ring worms

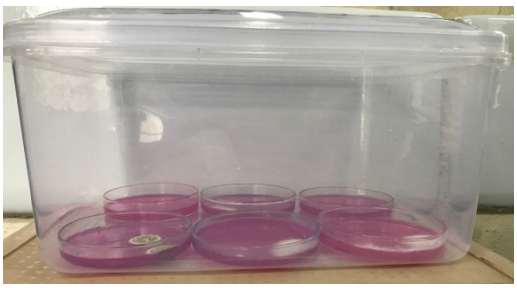

Fig. 1: Chamber set up for the study with three fungal cultures

70% (since this concentration showed effective fungal inhibition in agar well diffusion method), were sprayed nearly 8 times a day into the environment of the target room, using a sprayer (to ¾ of the height of the room) to convert them into aerosols to be suspended in the environment (to cover an area of a 300 square feet target room, 2 litres of extracts were used). This was repeated at regular intervals of every 2 days to ensure that the room was continuously humidified with the aerosols of extracts and for every 2 days' interval, the air quality was measured by settle plate method to determine the inhibition effectiveness of the plant leaf extracts.

RESULTS AND DISCUSSION

Pure cultures of fungi, *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* were isolated from the indoor kitchen environment (Figures 2 and 3). Generally the other indoor fungal contaminants are *Alternaria, Cladosporium, Penicillium, Trichoderma* and *Aureobasidium.* Moulds in indoor environments, responsible for allergies have been reported for years. Correlation exists between the air quality in indoor environment, mould load and human disease (Flanning *et al.,* 2001, Klich, 2009 Mensah-Attipoe, 2019). So there is a dire need to explore eco-friendly approaches for the management of indoor fungi. In rural areas, (especially in under developed and developing countries), mostly the people live in huts, where walls are made up of clay and the roof with paddy straw. Thus this structure serves as a favourable and susceptible environment for the luxurious growth of fungi and their multiplication. In developed countries however the humid indoor environment promotes fungi growth. The commercial air purifiers are not only costly but in addition have undisclosed ingredients such as pthalenes, volatile organic compounds, formaldehyde, benzene, toluene, etc, which may cause respiratory infections. Commercial air purifiers, though are efficient in controlling indoor fungal growth,

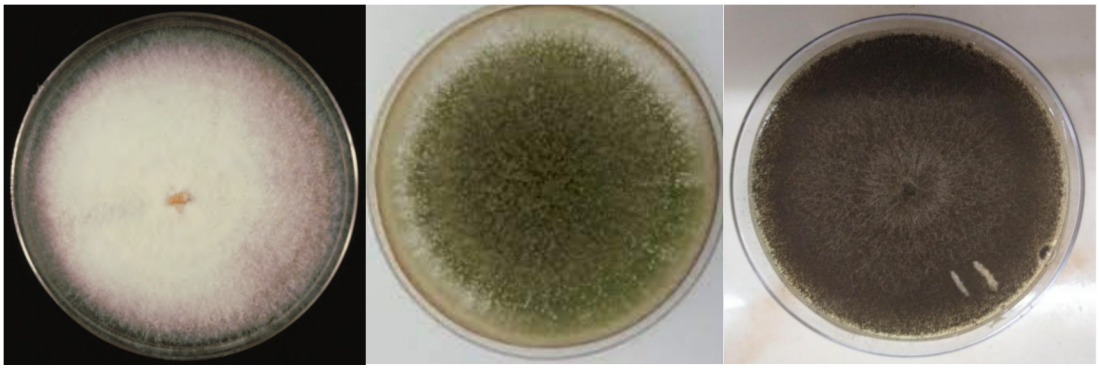

Fig. 2: Pure cultures of (a) *Fusarium oxysporum* (b) *Aspergillus flavus* (c) *Aspergillus niger*

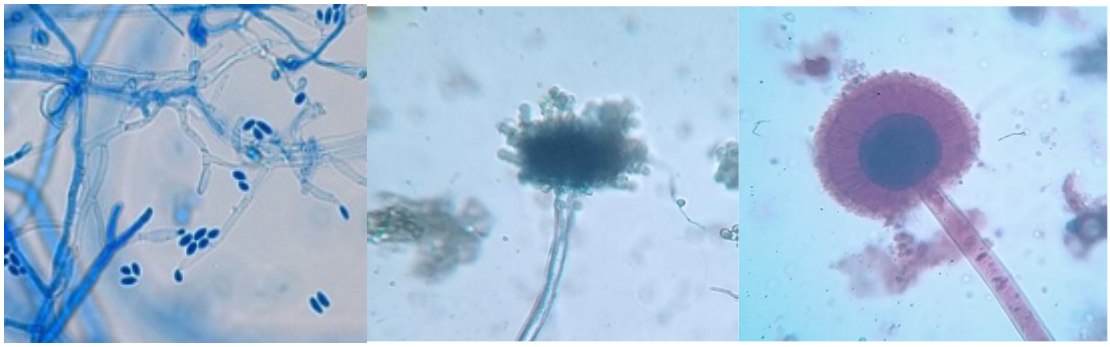

Fig. 3: Microscopic examination of (a) *Fusarium oxysporum* (b) *Aspergillus flavus* (c) *Aspergillus niger*

continued inhalation of the chemicals present in them may lead to certain allergic reactions and lung disorders such as asthma and breathing sickness (Steinemann.,2016). Hence, the current study emphasised the usefulness of some of the common natural air purifiers like *Azadirachta indica, Menta piperita* and *Aloe barbadensis* for the management of toxigenic and indoor airborne fungi, which are quite affordable. The fungi chosen for study were *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum,* since Jain, in 2010 reported that exposure to a variety of fungi such as *Aspergillus spp*. and *Fusarium spp*. may result in serious respiratory infections in immunocompromised persons. In addition, these fungi are reported to be predominant in indoor environment in all climatic conditions. People with impaired immune system who spend most of their time in indoor environments contaminated by fungi may develop serious fungal infections as investigated by Marcoux *et al*., (2009) & Wang *et al*., (2010 a,b). Chronic obstructive pulmonary disease, asthma, cystic fibrosis are disorders among persons potentially infected with *Aspergillus* (Baxter *et al.*,2011). *Fusarium oxysporum* is mostly found in soil but is also found suspended in air in an indoor environment especially on water damped walls. According to Aboul-Nasr *et al*. (2014) and Abbasi and Samaei (2019), fungi belonging to the genus *Fusarium* found commonly in soil, are plant pathogens, and are present worldwide. In humans, they have been recognized as opportunistic infectious agents, and the most frequent infections are keratitis, skin infections, and onychomycosis. These organisms produce several mycotoxins and also have broad resistance to fungicidal drugs (Aboul-Nasr et al., 2014). Therefore, effective control of these potent fungi using traditional plant extracts was attempted.

The antifungal activities of leaf extracts as measured by zone of inhibition against the three isolated fungal cultures were tabulated (Table 2) (Figure 4). Thus the efficient concentration of extract was found to be 70% in inhibiting fungal contamination.

Singh *et al.* (2010) reported that foliar spray of aqueous extract of neem cake showed antifungal efficacy against powdery mildew of balsam. Further, they explored that the neem cake has the ability to interfere in the production of secondary metabolites in soil borne phytopathogenic fungus, *Sclerotium rolfsii*. Hence in the present study, the leaf extracts of the plants *Azadirachta indica , Menta piperita* and *Aloe barbadensis* were used for the study of fungal inhibition. These plants are commonly used in Indian medicine and are found to have proven antifungal activity.

Table 2. Antifungal activity test by agar well diffusion (Mean zone of inhibition recorded for 60 and 70% concentrations).

Extracts	Control	70% conc.	60% conc.	50%
Azadirachta indica	_	16 mm	12 mm	_
Menta piperita	_	15 mm	12.5 mm	_
Aloe barbadensis	_	_	_	_

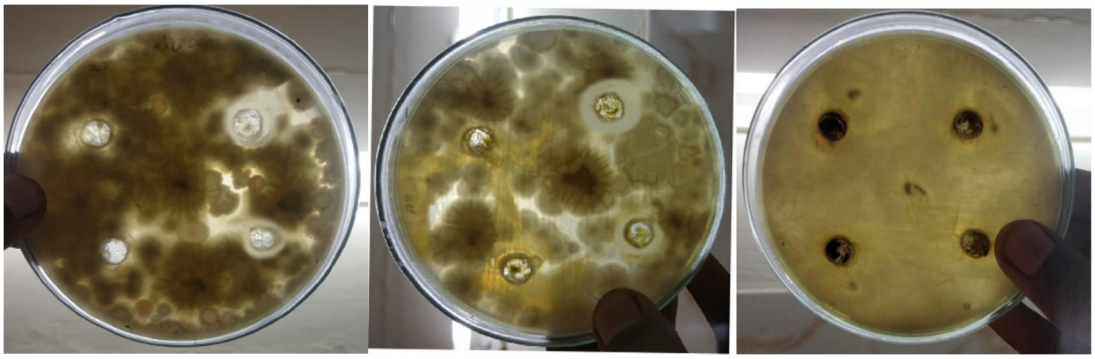

Fig. 4: Zone of inhibition by Agar Well diffusion method *Aspergillus flavus* (a & b zone of inhibition for *Azadirachta indica* and *Menta piperita* extracts at 60% concentration and (c) No zone of inhibition for *Aloe barbadensis* extract.

They are found to possess the phytochemical agents such as Azadirachtin, nimbin, nimbanene, salanins, menthol, menthone, limonene, emodin, barbaloin and isobarbaloin, thus showing antimicrobial activity. Therefore, the study focussed on the usage of medicinal plant extracts as foliar sprays in controlling indoor airborne fungi. The leaf extracts of *Azadirachta indica* , *Menta piperita* showed antifungal activity and inhibited fungal growth at 60 & 70% concentration, however *Aloe barbadensis* extract did not show promising results for it has shown inhibition only against selected pathogenic fungi as reported by Saniasiaya *et al* in the year 2017. Bansod and Rai (2008), studied antifungal activity of essential oils from Indian medicinal plants against *Aspergillus niger* and *Aspergillus fumigatus*. Oils extracted from fifteen medicinal plants were screened for their activity against *A.fumigatus* and *A.niger* by disc diffusion method. Minimum inhibitory concentrations (MICs) of oils (%v/v) against *Aspergillus fumigatus* and *Aspergillus niger* done by agar dilution method and minimum inhibitory concentration (MIC) and minimum cidal concentration (MCCs) data (%v/v) obtained by the broth micro dilution method. The results showed that the maximum antimycotic activity was demonstrated by oils of *Cymbopogon martini, Eucalyptus globulus* and *Cinnamomum zylenicum* as compared to control, followed by *Cymbopogon citratus* which showed activity similar to control (miconazole nitrate). The oils of *Mentha spicata, Azadirachta indica, Eugenia caryophyllata, Withania somnifera* and *Zingiber officinale* exhibited moderate activity. Mahmoud *et al*., in the year 2011 did a study to evaluate the effect of aqueous, ethanolic and ethyl acetate extracts from neem leaves on growth of some human pathogens (*Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus terreus, Candida albicans* and *Microsporum gypseum*) in vitro. Different concentrations (5, 10, 15 and 20%) prepared from these extracts inhibited the growth of the test pathogens and the effect gradually increased with concentration. Witkowska *et al* in 2016 found that the use of peppermint oil (46%) also reduced the population of air, wall, surface and litter fungi by fogging broiler houses. In line with the previous works, fungal cultures were subjected to agar diffusion assay method against plant extracts at different concentration to study the inhibition concentration and analyse the zone of inhibition. Both *Azadirachta indica* and *Menta piperita* extracts showed a mean zone of inhibition between 12 mm - 16 mm diameter, but the *Aloe* extracts showed no zone of inhibition.

Exposing the fungal samples to different air purifiers significantly affected the radial growth. About 60-70% reduction in the colony diameter was observed after exposure for 11 days to environment containing *Azadirachta indica* and *Menta piperita* extracts (Figure 5, 6) whereas 0% -10% inhibitions were observed for *Aloe* extract (Figure 7). *Aloe barbadensis* extract did not

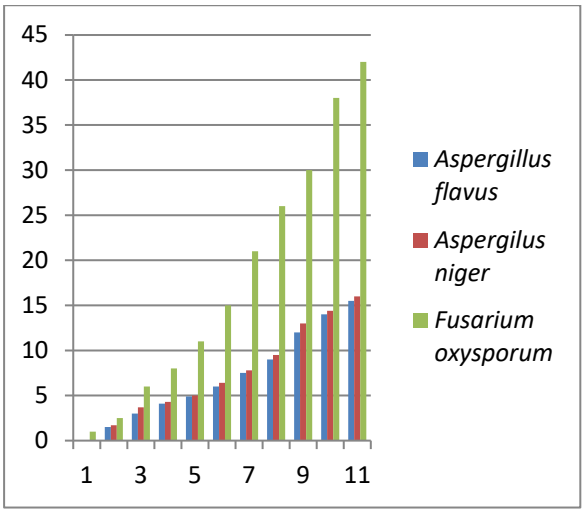

Fig. 5: Radial diameter of three fungal colonies in an environment of *Azadirachta indica* extract

show promising results for any of target fungal isolates, since it shows inhibition only against selected fungi as reported by Saniasiaya *et al* in the year 2017. Commercial air purifiers showed 99 % inhibition of the fungal structures for the same period (Figure8). Variations in microscopic characters like size of the head of conidiophores were also observed during incubation or exposure to the environment containing air purifiers. Maximum of 30 % reduction in head of conidiophores was observed when *Aspergillus* cultures were treated with *Azadirachta indica* and *Menta piperita* environment and the conidiophores were absent after 11 days of incubation when kept in *Menta piperita* environment. This character was not observed for *Azadirachta indica* and *Aloe* in indoor environments. No notable changes were observed for *Fusarium oxysporum* in any environments.

Nayak, et al., (2020), used desiccator vessels to study the inhibition of *A.flavus* A28 by saturating the chamber with traditional air purifiers. In this study too, the fungal samples were subjected to humidification studies using closed chambers as simulation of a closed environment with leaf extracts and agar plates with fungal cultures. The radial colony diameter of the fungal cultures was analysed for a brief period of 15 days and the difference in the growth characteristics were

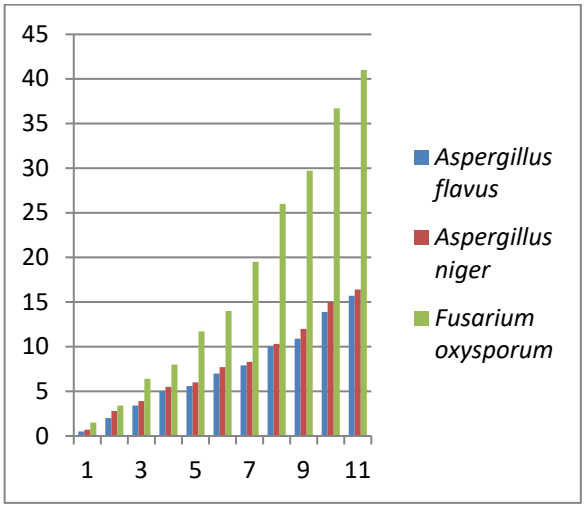

Fig. 6: Radial diameter of three fungal colonies in an environment of *Menta piperita* extract

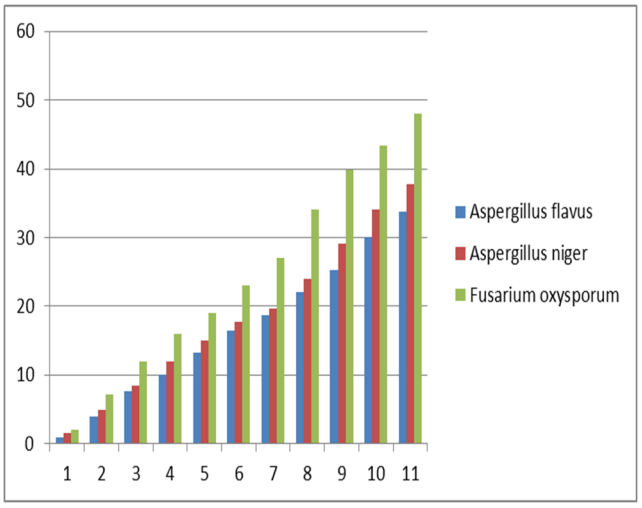

Fig. 7: Radial diameter of three fungal colonies in an environment of *Aloe* extract (X axis- number of days,Y axis – radial diameter in mm)

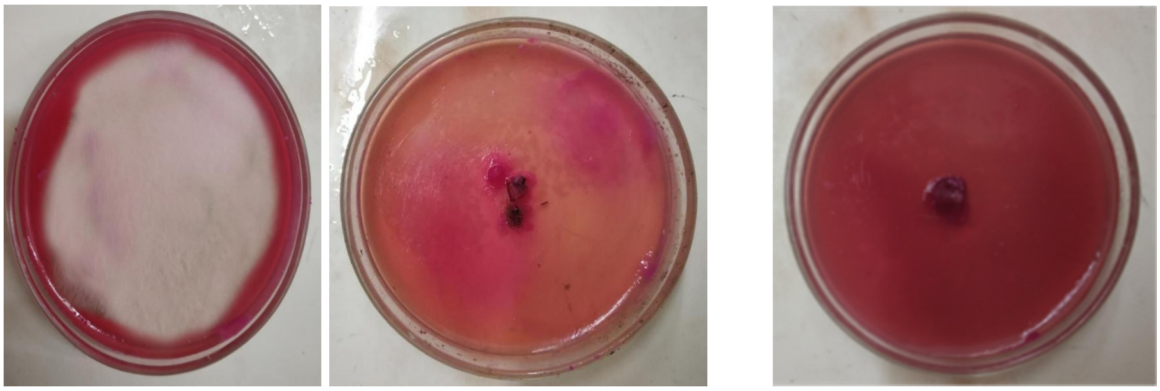

Fig. 8: Comparison of *Fusarium oxysporum* in (a) *Aloe* extract (b) & (c) commercial air fresheners environment.

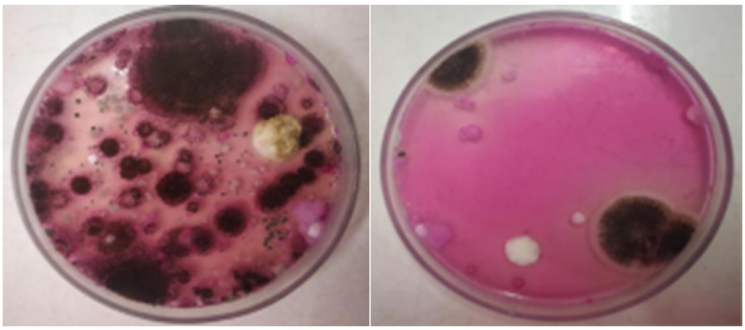

Fig. 9: (a) Before application of plant extracts (b) After application of plant extracts

recorded for comparison with commercial air purifiers.

The settle plate method (in duplicates) to determine the antifungal activity by spraying with 70% extract revealed significant reduction in the indoor fungal load, though less efficiently than commercial air purifiers. (Figure 9). The Colony Forming Units (CFU) before and after treatment of the extracts were recorded and subjected to t- test (CFU before treatment was found to be 296 and after treatment was reduced to 32). The two tailed P value was less than 0.0001, therefore this difference was considered to be extremely statistically significant.

The extracts were also used in real time monitoring to inhibit the fungal growth in an indoor environment with passive air flow and the results were found to be promising though for a limited period of 2 or 3 days.

Though the medicinal plants have proved efficient in controlling the air borne fungal contaminants, the results were promising only for a limited period. Since the inhibitory concentration may vary for different indoor fungi, the exact formulation of plant extracts to control all indoor fungal contaminants may not be feasible.

CONCLUSION

The present study aimed at improving the indoor air quality by using natural air purifiers in reducing the air borne fungal air load and the results proved the efficacy of natural leaf extracts in controlling airborne *Aspergillus flavus, Aspergillus niger* and *Fusarium oxysporum* in an indoor environment at a concentration of 60 to70%. Since the plant extracts inhibited the conidiophore formation, their potential in controlling the multiplication and fast spread was proved in an indoor environment. The medicinal plant extracts are eco-friendly, cost-effective and are easy

to formulate, with no health implications but could boost the immunity due to inhalation of the natural phytochemical agents present in their extracts. The study could be extended to other air borne fungi for inhibition and refined formulation of extracts could be worked out. Further study could be done to improve the inhibition property by blending with essential oils and formulating it into a commercial product. Thus the study has future prospects in commercial applications thereby replacing the harmful commercial air fresheners in usage.

GRANT SUPPORT DETAILS

The present research did not receive any financial support.

CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research.

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