Assessment of antifungal agent for the treatment of *Culvularia sp.* and *Lichtheimia sp.*

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Abstract. Fungal contamination in the indoor environment has become the major topic nowadays as it has been related to bad health impacts especially to children as they are more susceptible to diseases. An antifungal agent could act as the remediation to ensure indoor environment become less favorable to fungal growth. However, due to insufficient information on the use of antifungal agents in treating indoor fungal contamination, they were not widely considered for environmental application. The purposes of this study are to collect and identify the type of fungi present in indoor environment of preschool building, to obtain the pure culture of the collected indoor fungi in preschool building and lastly to access the efficacy and compare the efficiency of antifungal agents for indoor fungal remediation. The indoor fungi were collected using Anderson air sampler stage 6 at a classroom of Permata PKPS kindergarten. Wet mount slide technique was used to identify the fungal colonies collected and they were identified up to their genus level. The fungal colonies were identified as *Curvularia sp.* and *Lichtheimia sp.* Pure culture of *Curvularia sp.* and *Lichtheimia sp.* were successfully obtained. The efficacy and efficiency of the antifungal agents were assessed using disc diffusion technique through the diameter of zone of inhibition formed. The five antifungal agents tested in this study were tea tree oil, lemongrass oil, vinegar, 70% ethanol and Febreze. Four out of five tested antifungal agents exhibited antifungal properties. They were tea tree oil, lemongrass oil, vinegar and Febreze. The finding of this study shows that lemongrass oil was the most effective antifungal agents as it completely inhibited the growth of both *Curvularia sp.* and *Lichtheimia sp.* and also have the ability to inhibit the production of spore of *Curvularia sp.* and *Lichtheimia sp.* Hence, lemongrass oil should be considered as remediation for fungal contamination in indoor environment to maintain good indoor air quality.

1. Introduction
Children are believed to be susceptible to diseases. Therefore, these children do not have a strong defense system against diseases. Choo *et al.* [1] stated that children in Malaysia begin their early education at the age as early of 4 years to 6 years old by attending preschool established by the government or private agencies. A preschool is a place which provides learning space that offers an early childhood education to children. As the children are susceptible to diseases, preschool should be a place with a healthy learning indoor environment as it will affect the health and performance of the children that attend the preschool.
Department of Occupational Safety and Health (DOSH) Malaysia defines indoor air quality (IAQ) as the condition of the air within the buildings that will affect the comfort, health and performance of occupants in a building [2]. Pereira et al. [3] stated that indoor air is a complex environment as it contains both biological and non-biological contaminant. Rogawansamy et al. [4] stated that fungi are one of the lead cause of IAQ complaints in occupational settings. Fungi are commonly linked with adverse health effects such as headache, asthma, allergy, and mycoses. The main factor that enhances the growth of fungi in indoor air is sufficient moisture, nutrient and temperature [2][4]. Fungal species that is found indoor reflects those in outdoor environment as they enter the building through heating, air conditioning, windows, door and as contaminant on building materials [4]. Common fungal species found in indoor air are Aspergillus sp. and Penicillium sp.

Currently in Malaysia, there is only one guideline on indoor fungi which is Industry Code of Practice on Indoor Air Quality 2010 [2]. In this guideline, it stated that the acceptable limit of fungi in workplace is 1000 cfu/m$^3$. However, there is still no standard or guideline on indoor fungi in preschool building in Malaysia.

Hence it is important to decontaminate indoor air from continuous exposure towards fungi. Decontamination process involves the removal of visibly fungal contaminated material by the use of antifungal products. This process will help modify the condition of the indoor air and prevent future fungal growth. Antifungal can be defined as a chemical compound or biological organism used to kill or inhibit fungi and its spores [4]. The most common antifungal agents are vinegar and ethanol.

Vinegar and alcohol widely use in removing fungi on contaminated surfaces. Ethanol and vinegar are widely known for their antimicrobial properties. They are also widely used as disinfectants. Ethanol is the main ingredients in hand sanitizer. Besides ethanol, vinegar solution is also used in wiping surfaces to inhibit the growth of fungi as recommended by The Australian Mould Guideline [4].

Nowadays, there is a growing interest in another alternative of antifungal agents. For example, essential oils like tea tree oil (TTO) and lemongrass oil. TTO is a plant-derived antimicrobial agent that has the same mechanism of toxicity in killing and preventing the growth of microorganism just as 70% of ethanol and vinegar [4]. It is believed by researchers that the terpinen-4-ol component of TTO gives out its antimicrobial properties [5]. Unfortunately, TTO has not been widely considered for environmental application as there is still limited information on its use in removing indoor contamination [4].

Next, lemongrass oil (LGO) has been widely used in traditional medicine in countries around the world due to their antibacterial and antifungal properties [6]. There is an urgent need for the use of natural antifungal substances, especially with high efficiency and less toxic compared to currently used drugs. However, there is still limited information on antifungal properties of lemongrass oil.

Last but not least is a widely used commercial household product, Febreze. It has been used since 1996 in killing microorganism. However, there is no study or information on the efficiency of the product in killing or slowing down the activity of these microorganisms. Thus, this offers a unique setting to investigate the potential of these antifungal agents in killing or inhibiting the growth of fungi in indoor environment.

2. Methodology

2.1 Indoor Airborne Fungi Sampling

Indoor fungi samples were collected on Malt Extract Agar (MEA) plates by using Anderson Sampler. Each sample was collected for 10 minutes. After the sampling process, the plates were removed from the sampler and was immediately sealed using parafilm before being transport to the laboratory for the cultivation and incubation process [4]. Before sampling procedure was conducted, the Anderson Sampler was sterilized to kill the microorganisms that might attach during the journey from the laboratory to the preschool. The sampler was sterilize using cotton swab that is soaked in alcohol to kill any unwanted microorganism.
2.2 Identification and Isolation of Fungi
The collected samples were incubated at 25°C for 7 days. The growth of the fungus on the agar plate was observed naked eyes after 7 days. The fungi observation was done by using phase-contrast microscopy. The fungi genera present in the medium were recorded. Two genera representing the most commonly isolated fungi from the samples were selected for the use of this study. To obtain pure culture of the fungi, 6 mm in diameter of three agar plugs were cut from the edge of individual colony. The plugs were aseptically transferred to a fresh MEA plate and evenly place them apart. The plates will be wrapped with parafilm and incubated at 25°C for 7 days [4].

2.3 Antifungal Agent Preparation
Five agents were used in this study which was 70% ethanol, vinegar, tea tree oil (TTO), lemongrass oil (LGO) and commercially available product Febreze. These agents were tested in direct contact onto the agar plates.

2.4 Antifungal Efficacy using Disc Diffusion Assay
To test the inhibitory effect of the antifungal agents on the growth of the two selected fungi genera, disc diffusion assay method was conducted. The spore suspension was prepared by flooding the fungal culture plates with 3 ml of distilled water. Then, sterile loop will be used to agitate the colonies. 100 ml of each spore suspension were pipette to inoculate MEA plate. The plates were allowed to dry at room temperature for 15 minutes [4]. 20 ml of each agent were pipette onto an autoclaved 9 mm diameter Whatman TM filter paper which is placed in the middle of the plate. Phenol (88% solution) and sterile distilled water were used as positive and negative control for fungal growth inhibition [4]. All the plates were sealed with parafilm and will be incubated at 25°C for 7 days. After 7 days, the fungal growth and formation of zone of inhibition around the disc were observed. Each test was repeated 3 times for each agent. This was done to avoid any bias and to achieve accuracy.

2.5 Data Analysis
The diameter of inhibition zones formed by the antifungal agents selected was compared by using ONE-WAY ANOVA. The assumption of data normality was checked to meet the parametric analysis. Tukey Multiple Comparisons Test were used to perform post-hoc analysis. The significance of all test was set at p ≤ 0.05. Statistical analysis was performed using IBM-SPSS [4].

3. Results and Discussion
The zone of inhibition of Curvularia sp. and Lichtheimia sp. against the five tested antifungal agents were obtained in this study. Table 1 and Figure 1 shown the mean diameter of zone of inhibition for each antifungal agents. The capability of each antifungal agents seems to differ on both fungal genera. LGO show successfully inhibited the growth of both Curvularia sp. and Lichtheimia sp. The LGO was the most effective antifungal agent compared to the other assessed antifungal agents in this study.

The first tested antifungal agent was TTO successfully inhibited the growth of both fungi. However, the effect of TTO on Curvularia sp. (75.0 mm) was greater compared to Lichtheimia sp. (57.7 mm). The fungal inhibitory effect of TTO on Curvularia sp. was comparable to the result of positive control of Curvularia sp. which was Phenol (88% solutions). The result obtained was in agreement with the literature as reported that TTO exhibit antifungal properties on wide range of filamentous fungi [4],[5],[7].
Table 1: Mean diameter of zone of inhibition (mm) of *Curvularia* sp. and *Lichtheimia* sp.

| Antifungal Agents | Mean Diameter of Zone of Inhibition (mm) |
|-------------------|----------------------------------------|
|                   | *Curvularia* sp. | *Lichtheimia* sp. |
| Tea Tree Oil      | 75.0          | 57.7               |
| Lemongrass Oil    | 75.0          | 75.0               |
| Vinegar           | 16.3          | 23.3               |
| 70% Ethanol       | 0.0           | 0.0                |
| Febreze           | 18.7          | 26.7               |

Figure 1: Mean diameter of zone of inhibition (mm) of *Curvularia* sp. and *Lichtheimia* sp.

Terpinene-4-ol and 1,8-cineole were believed to be responsible for altering the cell membrane and deterioration of the fungal cellular functions [5]. Inouye *et al.* [8] reported that the mechanism of antifungal agents of TTO was by the alteration of the cell membrane. Soylu *et al.* [9] stated that TTO vapor was directly absorbed on the aerial hyphae and inhibited spore production. This alteration causes the cell membrane to become permeable. Hence, causes leakage of cellular material and disruption to the cellular functions. It was observed that TTO not only inhibited the growth of wide range of filamentous fungi but also inhibited the production of spore of the fungi.

The second tested antifungal agent was lemongrass oil (LGO) successfully inhibited the growth of both *Curvularia* sp. and *Lichtheimia* sp. (75.0 mm). The fungal inhibitory effect of LGO on both *Curvularia* sp. and *Lichtheimia* sp. were comparable to the result of positive controls (Phenol, 88% solutions). The result obtained in this study was consistent with previously published data on the antifungal properties of LGO [10],[11],[12]. The citral, geraniol, terpenes and phenolic compounds were a main active compound of LGO that give out the antifungal properties of LGO. The terpene compound was believed to destruct the cell membrane which affects the cell permeability, it leads to major surface alteration and morphological structures. While citral and geraniol compound might help in inhibiting the mycelial growth [13],[14],[15]. Previous study also reported that LGO also successfully inhibited...
another type of fungal spore production up to 70% for B. cinerea, 58% for C. coccodes and 41% for A. niger [10].

The third tested antifungal agent was vinegar. After seven days period of incubation, vinegar successfully inhibited the growth of both Curvularia sp. (16.30 mm) and Lichtheimia sp. (23.30 mm). It shows that vinegar has selective capability in inhibiting the growth of fungi. The phenolic compound present in vinegar was believed to be responsible to exhibit the antifungal properties. The previous study was reported that due to the presence of phenolic compound in the vinegar, it brings out the antimicrobial properties of vinegar [16]. In addition, acetic acid present also plays a small role in antimicrobial activity of vinegar [17]. The mechanism of action of vinegar as antifungal agents was believed to involved various targets. A study reported that vinegar interferes with the synthesis of the fungal cell wall and cell permeability. Vinegar was also disrupted the transport of electron and nutrients absorption of the fungi. Vinegar may also cause denaturation of cellular protein and deactivation of various cellular enzyme [18].

The fourth antifungal agents tested in this study was 70% ethanol. Table 1, the mean diameter of zone of inhibition for both Curvularia sp. and Lichtheimia sp. on 70% ethanol were 0.00 mm, which indicated negative antifungal properties on both fungi. The result obtained in this study were comparable to the negative controls which were sterile water. The similar finding also is reported inefficient of 70% ethanol as an anti-fungal agent [4],[19].

The last tested antifungal agent was Febreze. The effect of Febreze as an antifungal agent on Curvularia sp. (18.70 mm) was lower compared to Lichtheimia sp. (26.70 mm). Three main ingredients in Febreze which were Didecyl Dimethyl Ammonium Chloride (salt), ethyl alcohol and Benzisothiazolinone compound that responsible for the antifungal properties of Febreze. Comparable study on the efficiency of antimicrobial agents among common household products using disc diffusion method was conducted by Kelly [20] using Febreze and Clorox. Both product show capability as antimicrobial agents, however results show Clorox was a greater antimicrobial compared to Febreze. Deepu et. al. [21] state Benzisothiazolinone compound has wide range of antifungal activity due to its mechanism of antifungal action by disrupting the mitochondrial function.

4. Conclusion
The efficacy of the assessed antifungal agents was tested on both Curvularia sp. and Lichtheimia sp. This study revealed that four out of five tested antifungal agents exhibited the antifungal properties. The four tested antifungal agents were tea tree oil, lemongrass oil, vinegar and Febreze. They were considered as an antifungal agents as they successfully inhibited the growth of both Curvularia sp. and Lichtheimia sp. and they also have the ability to inhibit the production of spore of both Curvularia sp. and Lichtheimia sp. 70% ethanol were not considered as antifungal agents as it failed to inhibit the growth of Curvularia sp. and Lichtheimia sp. The most effective antifungal agents tested was lemongrass oil as it completely inhibited the growth of Curvularia sp. and Lichtheimia sp. and inhibited the production of spore of Curvularia sp. and Lichtheimia sp. Lemongrass oil was the most effective followed by tea tree oil, then Febreze and lastly vinegar.

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