RESEARCH ARTICLE

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Postharvest mycobial contaminants of white button mushroom (*Agaricus bisporus*) and their management using plant essential oils

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Abstract
Being highly perishable, mushrooms' quality and shelf life is affected by various factors during postharvest conditions, among which fungal contamination is the main cause. The goal of this study is to identify and manage fungal contaminants present in mushrooms during postharvest conditions. A total of 23 fungi were isolated as contaminants from the samples of *Agaricus bisporus* collected from three major vegetable markets in Kathmandu city, Nepal. *Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus stolonifer* were found to be the most frequent fungal contaminants. These were treated with various concentrations of essential oils (EOs) of *Cinnamomum tamala*, *Mentha spicata*, *Zanthoxylum armatum*, and *Eucalyptus citriodora* using poisoned food technique. Significant ($p<0.05$) inhibition of mycelial growth and spore germination was found in all testedfungi by all EOs. A strong inhibitory action of *M. spicata* oil was recorded against *A. flavus* and *R. stolonifer* while, *A. niger* was best controlled by *C. tamala* oil at the concentration of 20 µl/ml. These results suggest that EOs of three tested plants could be a good alternative to control fungal contaminants and extend the shelf life of *Agaricus bisporus* in postharvest conditions.
Keywords: *Aspergillus niger*, Botanicals, White button mushroom, Fungal contaminants, Fungitoxic effect

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INTRODUCTION

White button mushrooms i.e., *Agaricus bisporus* (J.E. Lange) Imbach belong to the class Basidiomycetes and the family Agaricaceae is commonly recognized as 'Gobre chyau' in Nepal. It is widely cultivated all over the world and plays a key economic role in the worldwide mushroom market, accounting for 15% of all mushroom production (Royse et al, 2017). It is commonly cultivated in the peri-urban area of the Kathmandu valley and the hilly region of Nepal (Shrestha, 2014). In Nepal, it is the second most cultivated mushroom that contributes 10% of total mushroom production (Raut, 2019). Additionally, this mushroom possesses a variety of therapeutic potentials including antioxidant, antibacterial, and immunomodulating activities (Ramos et al., 2019; Usman et al., 2021).

Postharvest loss refers to the quantifiable deterioration of food quantity and quality after harvest; this condition is greater for perishable crops in less developed countries (Hodges, 2011). Quantitative loss is more of an issue in these countries than qualitative loss (Humble and Reneby, 2014). The FAO estimates that 40 to 50 percent of the world's horticulture crop yield is lost in postharvest conditions (FAO, 2018). The mushrooms are highly perishable in nature and have a poor shelf life (3-5 days), so their postharvest loss is 30-35% globally (Thakur et al., 2022). Many postharvest problems such as color changes, tissue damages, cap opening,

weight loss, turgidity, bacterial contaminations, etc. occur rapidly during storage degrading its quality and quantity (Wang et al., 2017). Additionally, during the mushroom's growing and postharvest stages, different mold fungi can contaminate the crop, which harms the yield and shelf life of the mushrooms (Sharma et al., 2009; Biswas, 2014). Different researchers have conducted studies on multiple aspects of fungal contamination and diseases of *Agaricus bisporus* (Dandge, 2012; Adhikari and Jha, 2020; Wang et al., 2020) and they and they reported *Mycogone perniciosa*, *Verticillium fungicola*, *Cladobotryum dendrites*, *Trichoderma* spp., *Chaetomium* spp., *Aspergillus* spp., *Penicillium* spp., *Monilia* sp., *Geotrichum* sp., *Fusarium* spp., *Rhizopus* sp., *Mucor* sp., *Alternaria* sp., *Curvularia* sp., *Penicillium* sp., etc. were the major contaminants of *Agaricus bisporus*. These fungal contaminants produce several mycotoxins, which pose a risk to human health because they can lead to serious and incurable health problems including cancer in developing countries (Omotayo et al., 2019). Various kinds of synthetic fungicides are commonly used to reduce such microbial contamination in the field and postharvest conditions of mushrooms in Nepal (Raut, 2013; Adhikari and Jha, 2020). However, the extensive use of synthetic fungicides has, however, resulted in the emergence of infections that are resistant to them, and concerns have been expressed regarding the long-term impact on the environment and public health (Ons et al., 2020).

Plant essential oils (EOs) are secondary metabolites made up of volatile aromatic compounds which have a significant role in the defense of the plants (Hyldgaard et al., 2012). Such EOs often possess antibacterial, antifungal, antiviral, and insecticidal properties (Falleh et al., 2020). These properties make EOs a probable substitutes for synthetic fungicides (Bassolé and Juliani, 2012). Such that essential oil can be very helpful in extending the shelf life of food and minimizing losses in postharvest conditions of agricultural products (Farzaneh et al., 2015; Prakash et al., 2015). Plant essential oils are non-toxic and biologically decomposable, they are less hazardous to public health and the environment as well. The present study aimed to isolate and identify fungal contaminants associated with *Agaricus bisporus* in postharvest conditions. Furthermore, the study also aims to evaluate the antifungal activity of EOs of four selected plant species (*Cinnamomum tamala*, *Mentha spicata*, *Zanthoxylum armatum*, and *Eucalyptus citriodora*) against some most frequent fungal contaminants.

MATERIALS AND METHODS

Isolation and Identification of Mycobial Contaminants

Fruitbodies of *Agaricus bisporus* samples were collected from three major vegetable market of Kathmandu, Nepal. The base, stipe, and pileus of the sample were cut into 3 mm pieces and plated onto potato dextrose agar (PDA) plates. A week later, each fungus was pure cultured from the numerous colonies of fungi. Fungal isolates were identified based on micromorphological and cultural characteristics by following standard literature (Barnett and Hunter, 1972; Watanabe, 2010). The three fungi (*Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus stolonifer*) among the most abundant were selected as test fungi to evaluate the extent to which EOs were inhibitory to fungi.

Extraction of Plant Essential Oils

Fresh leaves of four plant species i.e., *Cinnamomum tamala*, *Mentha spicata*, *Eucalyptus citriodora*, and *Zanthoxylum armatum*, were harvested from the garden of Central Department of Botany (Tribhuvan University) at Kirtipur and private farmland at Champadevi, Kathmandu. Fresh leaves were then dried in shade and kept in the dark until extraction of EOs. The shade-dried leaves were then hydrodistilled for 6 to 8 hours using Clevenger's equipment (Sovová and Aleksovski, 2006). The extracted EOs were then dried using anhydrous sodium sulfate (Na_2SO_4), and then kept in storage at below 10°C temperatures until use.

Antifungal Effect of Plant Essential Oils

The poisoned food technique was used to evaluate the antifungal activity of EOs on the in vitro growth and development of fungal contaminants (Grover and Moore, 1962). Five different concentrations of EOs viz 1.25, 2.5, 5, 10, and 20 µl/ml were prepared with 50% acetone. First, 1 ml of each concentration of essential oil was added to sterilized petriplates, and then 9 ml of melted PDA was added. The test fungus of 4 mm diameter, which was growing aggressively, was then inoculated into each petriplates. Instead of essential oil, distilled water and 50% acetone were utilized in control setups. On the seventh day, observations were made. Five replications were maintained and fungi toxicity of Essential oils was assessed by measuring the percentage of mycelial growth that was inhibited, which was computed as;

Inhibition of Mycelial Growth (%) = [(Mc–Mt)/ Mc] × 100

[Where; Mc= mean colony diameter in control sets and Mt= mean colony diameter in treatment sets].

The effect of EOs on the spore germination of test fungus was examined using the hanging drop technique. After a 24-hour incubation period, the data from five replicates were collected. The formula below was used to determine the percentage of spore germination (Király et al., 1974).

Spore germination (%) = (Gg/Gt) × 100

[Where; Gg = number of spores germinated per microscopic field and Gt = number of spores per microscopic field.]

Data Analysis

Microsoft office Excel 2019 was used for data entry and preparation of necessary graphs. The occurrence

frequency of fungal contaminants found in *Agaricus bisporus* was compared by using frequency rank curve. Analysis of variance (ANOVA) and a post-hoc Tukey's HSD (honestly significant difference) test with a p-value of 0.05 were used to evaluate data means using the statistical programme for social science (SPSS) version 23 software.

RESULTS AND DISCUSSION

Postharvest Mycobial Contaminants

A total of twenty-three fungi were isolated as contaminant, where two of the fungi remained unidentified. *Aspergillus niger*, *Aspergillus flavus*, and Rhizopus stolonifer were found as the most frequent mycobial contaminants (Figure 1).

In *Agaricus bisporus*, seven species of the genus *Aspergillus* (*A. niger, A. flavus, A. brevipes, A. fumigatus, A. clavatus, A. versicolor, Aspergillus* sp), 3 species of genus *Chaetomium* (*C. globosum, C. spirale, Chaetomium* sp.), 2 species of *Trichoderma* (*T. harzianum, T. viride*) *Mucor hiemalis, Penicillium notatum, Rhizopus stolonifer, Alternaria alternata, Geotrichum* sp., *Gliocladium* sp., *Fusarium oxysporum, Nigrospora sphaerica, Cladosporium* sp., and two unidentified species were isolated. Among these fungal contaminants, the frequency rank curve (Fig 1) reveals that the most frequent fungal species were *Aspergillus niger* (50.00%) followed by *Aspergillus flavus, Rhizopus stolonifer, Aspergillus brevipes,* and *Aspergillus fumigatus* with the frequency value 40.00 %, 36.67%, 33.33%, and 26.67% respectively. This result thus shows that *Agaricus bisporus* is vulnerable to fungal contaminations, in particular, that of *Aspergillus*. These results confirm the work in previous literatures (Dandge, 2012; Kertesz and Thai, 2018; Adhikari and Jha, 2020; Wang et al., 2020; Mishra, 2022) wherein reported that these fungal species are the major contaminants of *Agaricus bisporus*.

Antifungal Effect of Plant Essential Oils

The results of the antifungal bioassay of essential oil revealed that all four essential oils significantly ($p<0.05$) inhibited the mycelial growth and spore germination of all test fungi. Since the mycelial growth of *A. niger* was 0.70±0.08 cm at a concentration of 20 µl/ml, *C. tamala* oil had the best effects on *A. niger* compared to *A. flavus* and *R. stolonifer*. *C. tamala* oil has the best effects over *A. niger* than *A. flavus* and *R. stolonifer* as the mycelial growth of *A. niger* was 0.70±0.08cm at 20 µl/ml concentration (Table 1). Meanwhile, *M. spicata* oil had shown better antifungal effects over *A. flavus* (0.81±0.06cm) and *R. stolonifer* (0.85±0.05cm) than *A. niger* (0.93±0.01cm) at a concentration of 20µl/ml. But, at the lowest concentration i.e, 1.25µl/ml, *E. citriodora* showed the best effect on the *A. niger* (3.40±0.10cm) and *A. flavus* (4.24±0.32cm), while *M. spicata* oil showed the better effect on *R. stolonifer* (4.81±0.01cm).

In most of the concentrations of *C. tamala* oil, a better effect was found in *A. niger* than *R. stolonifer*. *E. citriodora* oil had shown greater inhihitory effects over *A. niger* than *R. stolonifer*. Similarly, *Z. armatum* oil had shown better inhihitory effects over *A. flavus* (0.99±0.09cm) than *A. niger* (1.13±0.20cm) and *R. stolonifer* (1.13±0.15cm) at 20µl/ml concentration. whileamong all EOs, *Z. armatum*

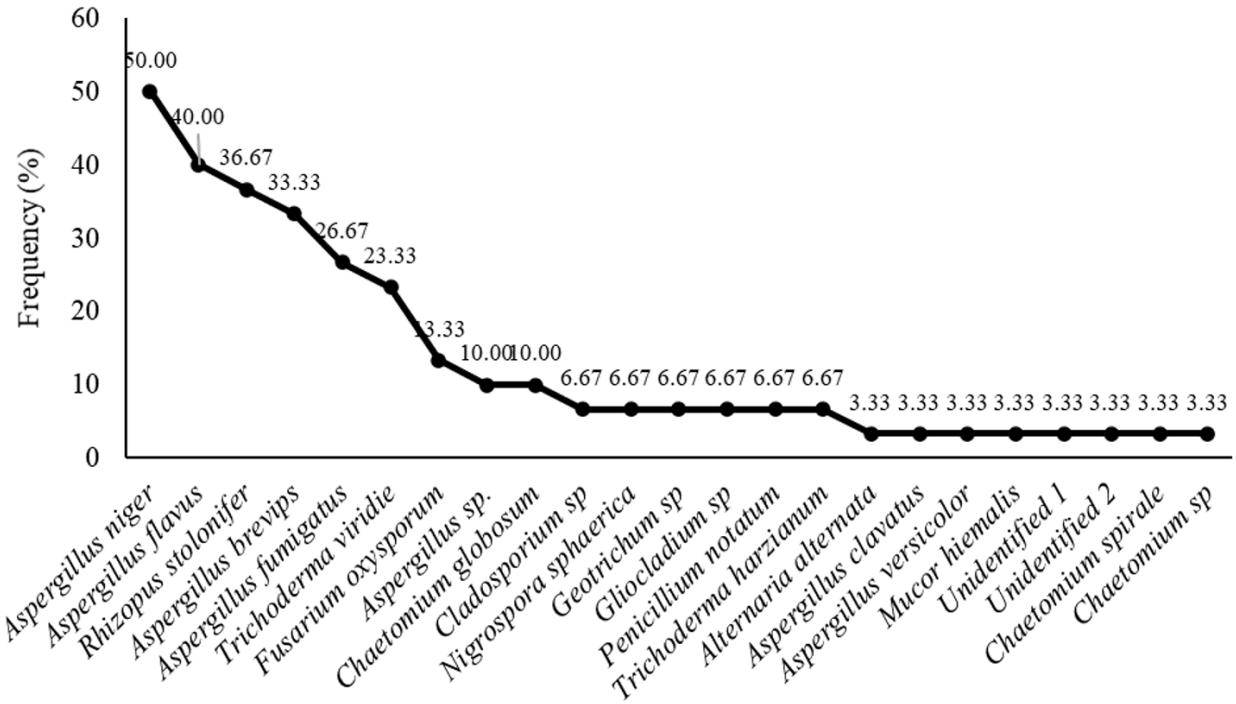

Figure 1. Frequency rank curve and rank of most commonly found fungal species in Agaricus bisporus.

Table 1. Antifungal effect of different EOs against radial mycelial growth (Mean±SD) of mycobial contaminants

Fungi	Concentrations	*E. citriodora*	*M. spicata*	*C. tamala*	*Z. armatum*
Aspergillus niger	20 µl/ml	0.95±0.05^{A}	0.93±0.01^{A}	0.70±0.08^{A}	1.13±0.20^{A}
	10 µl/ml	1.42±0.13^{B}	1.27±0.10^{B}	1.54±0.15^{B}	1.66±0.31^{B}
	5 µl/ml	2.24±0.08^{C}	1.79±0.12^{C}	2.74±0.09^{C}	2.01±0.09^{B}
	2.5 µl/ml	2.68±0.11^{C}	2.39±0.16^{D}	3.35±0.06^{C}	3.19±0.23^{C}
	1.25 µl/ml	3.40±0.10^{D}	4.57±0.08^{E}	4.16±0.25^{D}	5.21±0.03^{D}
	Negative Control	7.35±0.17^{E}	5.76±0.06^{F}	4.80±0.10^{E}	5.80±0.37^{E}
	Control	7.54±0.07^{E}	6.39±0.05^{G}	7.32±0.16^{E}	6.52±0.12^{E}
Aspergillus flavus	20 µl/ml	0.94±0.08^{a}	0.81±0.06^{a}	1.66±0.07^{a}	0.99±0.09^{a}
	10 µl/ml	1.64±0.14^{b}	1.18±0.08^{b}	2.34±0.13^{b}	1.28±0.12^{ab}
	5 µl/ml	3.03±0.11^{c}	2.01±0.08^{c}	2.52±0.10^{b}	1.62±0.10^{b}
	2.5 µl/ml	3.44±0.07^{d}	3.88±0.18^{d}	3.37±0.22^{c}	2.31±0.22^{c}
	1.25 µl/ml	4.24±0.32^{e}	4.63±0.12^{e}	4.46±0.17^{d}	4.46±0.27^{d}
	Negative Control	5.09±0.09^{f}	6.06±0.18^{f}	5.13±0.12^{e}	5.97±0.29^{e}
	Control	6.23±0.23^{g}	6.76±0.17^{g}	6.87±0.11^{f}	6.48±0.50^{e}
Rhizopus stolonifer	20 µl/ml	0.99±0.04$^{\alpha}$	0.85±0.05$^{\alpha}$	1.07±0.02$^{\alpha}$	1.13±0.15$^{\alpha}$
	10 µl/ml	2.52±0.04$^{\beta}$	2.47±0.04$^{\beta}$	2.03±0.05$^{\beta}$	1.85±0.08$^{\beta}$
	5 µl/ml	4.50±0.06$^{\gamma}$	3.13±0.12$^{\gamma}$	5.15±0.02$^{\gamma}$	2.36±0.16$^{\beta}$
	2.5 µl/ml	4.25±0.14$^{\delta}$	3.46±0.05$^{\delta}$	5.15±0.35$^{\delta}$	3.66±0.02$^{\gamma}$
	1.25 µl/ml	6.59±0.08$^{\eta}$	4.81±0.01$^{\eta}$	6.12±0.11$^{\eta}$	5.23±0.16$^{\delta}$
	Negative Control	7.82±0.05$^{\theta}$	6.51±0.06$^{\theta}$	7.21±0.03$^{\theta}$	6.89±0.03$^{\eta}$
	Control	8.05±0.12$^{\theta}$	7.24±0.10$^{\mu}$	7.56±0.09$^{\mu}$	7.44±0.15$^{\theta}$

(Note: The values of each EO in each test fungi sharing the same letters within a group in column are not significantly different in Tukey multiple range tests, $p < 0.05$.)

oil had shown less inhihitory effect on the mycelial growth of both fungal contaminants. Overall, these findings are more or less supported by findings reported by many researchers (Prakash et al., 2012; Zaidi and Dahiya, 2015; Barbosa et al., 2016; Kedia et al., 2016; Adhikari and Jha, 2017; Xiang et al., 2020; Piras et al., 2021).

At a concentration of 20 µl/ml of oil, the inhibitory effect of EOs on the spore germination of a few distinct fungal contaminants was found to be similar to the effect on mycelial growth, but at higher concentrations, the result was different. Of the four oils tested, *M. spicata* had the least antifungal impact, whereas *C. tamala* had the strongest inhibitory effect against *A. niger* (Fig. 2A). *C. tamala* oil demonstrated the greatest inhibition (79.30%) at a 20 µl/ml oil concentration, followed by *Z. armatum* (76.94%), *E. citriodora* (76.60%), and *M. spicata* (76.56%) against the *A. flavus* (Figure 2B). However, *M. spicata* oil demonstrated the maximum suppression (84.48%) of the spore germination of *Rhizopus stolonifer* Figure 2C). followed by *Z. armatum* (78.80%), *E. citriodora* (71.02%), and *C. tamala* (69.80%) at a 20 l/ml oil concentration. Eugenol, cinnamaldehyde, cinnamyl alcohol, cinnamyl acetate, cinnamic acid, and many other phytochemical constituents found in it might be the substances that give it its antifungal effects (Haddi et al., 2017).

Mentha oil had shown the best inhibitory effect (49.25%) at the lowest concentration (1.25µl/ml) too. Similarly, spore germination of *A. flavus* was also best controlled by *M. spicata* oil (77%) at the concentration of 20µl/ml whereas the effect of other EOs is 71.87-67.64%. These results are more or less supported by results from previous literatures (Thompson, 1986; Caccioni and Guizzardi, 1994; Bluma et al., 2008; Guerra et al., 2015; Adhikari and Jha, 2017; Teia et al., 2018; Hu et al., 2019). The different chemical compositions of the essential oils may be the cause of the variation in antifungal activity at the same concentration (Nazzaro et al., 2017). Thus, essential oils are among the significant plant-based substance that have a strong inhibitory effect on the mycelial growth and spore germination of fungal contaminants (Kalemba and Kunicka, 2003; Prakash et al., 2012; Ghalem, 2016; Hu et al., 2019). So, to preserve the quality and extend the shelf life of mushrooms in postharvest conditions, EOs can be used as a good alternative (Ju et al., 2019) to synthetic fungicides (Nuvan, Bavistin DF and Dicholrophus 76 EC, Mancozeb and Carbendazim etc.) used at vegetable markets in Kathmandu. The Food and Drug Administration (FDA) has categorized EOs as "Generally Recognized as Safe" (GRAS) and has determined that they are safe for human health due to their natural origin and widespread market acceptance (Edris, 2007). For instance, cinnamaldehyde and eugenol from C. tamala essential oil were approved by the Food and Drug Administration (FDA) to be used as GRAS compounds in human food (Gomes et al., 2011).

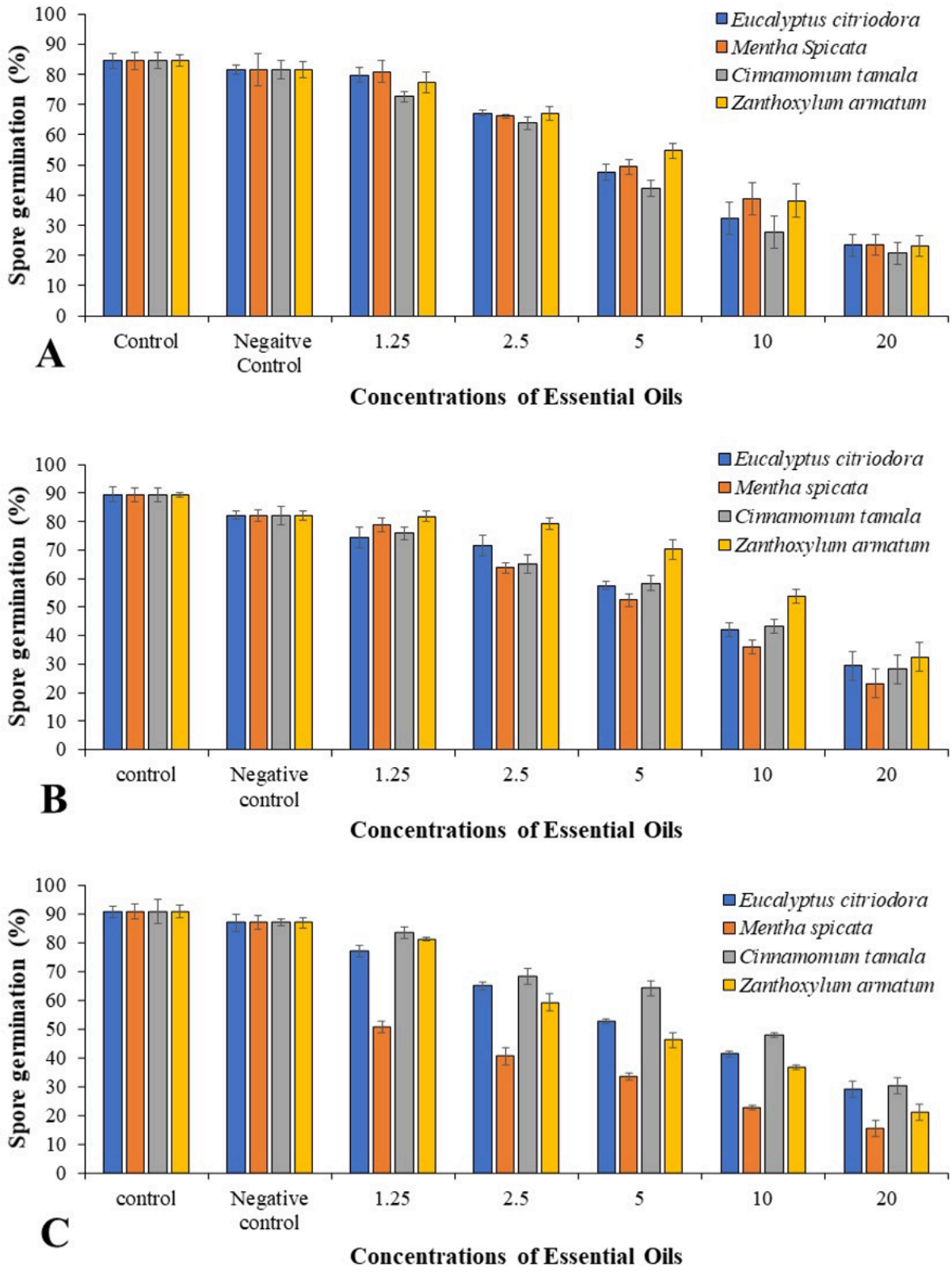

Figure 2. Antifungal effect of different EOs against spore germination of A. niger (A), A. flavus (B), and R. stolonifer (C). (Error bars represent standard errors of the means).

CONCLUSION

Mycobial contaminants are responsible for the postharvest loss of *Agaricus bisporus*, among which *A. niger, A. flavus,* and *R. stolonifer* are the most frequent. Regarding the control of these contaminants, the mycelial growth of *A. niger* and *A. flavus* were best inhibited by the EOs of *C. tamala* whereas *R. stolonifer* was best controlled by the essential oil of *E. citriodora*. The germination of all test fungal spores was also inhibited by the essential oil of *C. tamala* in comparison to that of other EOs. Therefore, the current study offers plant essential oils as a substitute input to prevent the postharvest deterioration of mushrooms. Such botanical pesticides are preferable because it minimizes the use of chemical load, minimize the cost ratio, avoid health hazards, and are eco-friendly as well.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

HSA designed the research experiment, performed the entire research and wrote the manuscript. SKJ supervised the entire research work.

Ethical approval

Ethics committee approval is not required.

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No financial support was received for this study.

Data availability

Data will be made available on request to corresponding author.

Consent for publication

All authors consented to the publication of this manuscript.

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