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Efficacy of vatica oil in controlling *Aspergillus parasiticus* in maize grain by direct contact and fumigation methods

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Abstract The objectives of the study were to test and compare the efficacy of essential oils and their derivatives for control of the aflatoxin-producing fungi *Aspergillus parasiticus* on contaminated maize grain. Among the five essential oils tested, vatica oil completely inhibited the growth and conidia germination of *A. parasiticus* TISTR 3276 by both methods. Benzyl acetate was also effective against the pathogen. The minimum inhibitory concentration of vatica oil and benzyl acetate against the fungal growth by direct contact was 10 μL mL^{-1} while it was 50 μL L^{-1} for the fumigation assay. Exposure to vatica oil at 10 μL mL^{-1} for 120 min could completely kill the conidia of the aflatoxin producing fungi while benzyl acetate showed antifungal activity but not rapid killing. SEM results illustrated that the direct contact method completely inhibited the conidia germination while the fumigation assay exhibited ultrastructure alterations of the conidia and abnormal growth of the fungal strain. Fumigation using vatica oil and benzyl acetate at their effective concentrations (10 μL mL^{-1} and 50 μL L^{-1}, respectively) decreased the contamination of *A. parasiticus* TISTR 3276 on maize grain. Moreover, both vatica oil and benzyl acetate also protected and cured the contaminated maize grain. Thus, vatica essential oil and benzyl acetate have potential use in the control of aflatoxin producing fungi *A. parasiticus*.

Keywords *Aspergillus parasiticus* · Vatica oil · Benzyl acetate · Direct contact · Fumigation

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Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops worldwide. Contamination of cereal grains by spoilage fungi has been a major concern in the food industry in recent years. *Aspergillus parasiticus*, *A. flavus*, and *A. nomius* are major aflatoxin-producing fungi found in contaminated agricultural commodities (Guo 2000; Ng'ang'a et al. 2016). *A. parasiticus* is one of the contaminants found mostly on maize grain during storage (Ng'ang'a et al. 2016). An *A. parasiticus* infection not only reduces the quality and economic value of the stored products, but also culminates in deposition of toxic metabolites when the colonizing fungi are mycotoxigenic (Ng'ang'a et al. 2016). The consumption of aflatoxins in maize and other food commodities has adverse effects on human health and animals (Gong et al. 2004). It is necessary to find a method to protect maize grain from contamination by *Aspergillus* spp.

The antifungal properties of essential oils have been recognized and used for protection of food spoilage fungi by direct contact (Passone et al. 2012; Li et al. 2016; Nerilo et al. 2016; Dela Cueva and Balendres 2018) or fumigation (Avila-Sosa et al. 2012; Velázquez-Nuñez et al. 2013; Hua et al. 2014). Essential oils are screened for antimicrobial effects most often by direct contact and diffusion in a media. Control of food spoilage fungi by direct contact with essential oils from various plants has been reported. Examples included control of *A. flavus* using essential oils of *Cinnamomum jensenianum* Hand.-Mazz and *Zanthoxylum molle* Rehd. (Tian et al. 2012, 2014), *Zingiber officinale* (Nerilo et al. 2016), and *Litsea cubeba* (Li et al. 2016). Clove oil (Passone et al. 2012), and thyme oil (Passone et al. 2012; Stevic et al. 2014; Bernardos et al. 2015) were used to control *Aspergillus* spp. Fumigation is an alternative method for application to protect stored products. The assay is conducted by placing the essential oils and microorganisms separately in a sealed environment. Microbial inhibition was achieved from a distance without direct contact of the antimicrobial agent with the food (Avila-Sosa et al. 2012). Recently, numerous studies reported the antifungal effects of plant essential oils to control food spoilage fungi by fumigation assays using the essential oils of Mexican oregano (*Lippia berlandieri* Schauer), cinnamon (*Cinnamomum verum*), and lemongrass (*Cymbopogon citratus*) against *A. niger* and *Penicillium digitatum* (Avila-Sosa et al. 2012). The essential oils of orange (*Citrus sinensis* var. Valencia) peel (Velázquez-Nuñez et al. 2013), *Litsea cubeba* (Li et al. 2016), and vatica (*Vatica diospyroides* Symington) (Boukaew et al. 2017) were able to inhibit *A. flavus* while cinnamon oil, *Litsea citrate* oil, citral, eugenol, peppermint, eucalyptus, anise, and camphor oils were effective against *A. ochraceus* (Hua et al. 2014).

Benzyl acetate is the ester formed by condensation of benzyl alcohol and acetic acid. It is a major volatile constituent of the flowers of a number of plants, including jasmine (*Jasminium grandiflorum* L.), hyacinth (*Hyacinthus orientalis*), and gardenia (*Gardenia jasminoides*) (Raguso and Pichersky 1995; Guterman et al. 2006). It was reported for use against plant pathogenic fungi including *Drechslera sorokiniana*, *Phomompsis sojae, Fusarium solani*, *Colletotrichum graminicola*, *C. gloeosporioides,* and *Macrophomina phaseolina* (Dev et al. 2004; Nidiry and Babu 2005; Wenda-Piesik 2011). It was also found to be the major compound of vatica essential oil (48.8%) identified by gas chromatography-mass spectrometry (Boukaew et al. 2017).

In this study, five commercial essential oils (peppermint, cinnamon, clove, capsicum and vatica) were evaluated for control of aflatoxin producing fungi *A. parasiticus* using direct contact and fumigation assays. The five selected essential oils are known for their antifungal effect on various pathogenic fungi. After screening, the selected essential oil and associated compound would be tested to inhibit the fungal growth in media and on maize grain.

Materials and methods

Preparation of fungal inoculum

The aflatoxin-producing fungus *A. parasiticus* TISTR 3276 Thailand Institute of Scientific and Technological Research (TISTR) was provided by the Microbiology Laboratory of the Department of Industrial Biotechnology at Prince of Songkla University. The strain was maintained on potato dextrose agar (PDA 39 g L^{-1}; Difco Laboratory) medium at 4 °C. The spore suspension was prepared by harvesting the spore from 10-day-old cultures using 5 mL water and vortexed with glass beads, and then filtered through a 30 μm mesh filter. The spores were diluted with sterilized water to achieve a final concentration of 1×10^5 spores mL^{-1}.

Screening of five essential oils for control of aflatoxin producing fungi by direct contact and fumigation assays

Direct contact assay

The effects of peppermint (*Mentha balsamea*), cinnamon (*Cinnamomum bejolghota*), clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry), capsicum (*Capsicum annuum* var. acuminatum Fingerh), and vatica (*Vatica diospyroides* Symington) oils (purchased from Saiburi Samoonprai Ordinary Partnerships Company in Hat Yai, Thailand) on mycelial growth of *A. parasiticus* TISTR 3276 on PDA were studied using direct contact assay (Omidbeygi et al. 2007; Matusinsky et al. 2015). Each essential oil at 50 μL mL^{-1} was mixed with 10 mL melted sterile PDA and poured into a 9 cm diameter culture plate, while melted sterile PDA at an equivalent amount was used as a control. A 5 mm diameter plug of mycelia was cut from a 3-day old *A. parasiticus* TISTR 3276 colony and transferred onto the center of the test

agar plates. After 5 days of incubation at ambient temperature (28 °C), the diameters of the colonies on the three plates were recorded. For each treatment, three replicates were realized. The inhibition percentage was calculated using the following formula.

$$\text{Inhibition}\ (\%) = [(\text{R1}–\text{R2})/\text{R1}] \times 100$$

where R1 = colony radius of *A. parasiticus* TISTR 3276 alone on PDA (control) and R2 = colony radius of *A. parasiticus* TISTR 3276 incorporated with essential oil.

Fumigation assay

The fumigation effects of the five essential oils on the radial growth of *A. parasiticus* TISTR 3276 on PDA were studied using an antifungal bioassay (Boukaew et al. 2017). Large Petri dishes (140 mm diameter × 20 mm height with 0.5 L inner volume) contained four small Petri dishes (50 mm diameter × 15 mm height). Three of the smaller dishes each contained 5 mL of PDA inoculated with a 0.5 cm diameter fungal plug from the periphery of an actively growing culture of pathogenic fungi, while the fourth dish contained a piece of autoclaved filter paper (Whatman®) to which one of the essential oils was added at 50 μL L^{-1} of airspace in treatment containers (droplet to filter paper in an enclosed Petri dish system). Equivalent amounts of sterile distilled water were used as a control. The Petri dishes were covered with a lid, sealed with Parafilm M®, and incubated at ambient temperature (28 °C). After 5 days of incubation at ambient temperature (28 °C), the diameters of the colonies in the smaller plates were recorded. For each treatment, there were three replicates. The percentage inhibition of hyphal growth was calculated as described above.

Effects of selected essential oil and benzyl acetate concentrations on fungal growth inhibition based on direct contact assay

Mycelial growth of A. parasiticus TISTR 3276

Direct contact assay was determined by both solid culture (Matusinsky et al. 2015) and liquid culture (Li et al. 2011). For the solid culture, different amounts of the promising commercial essential oil and benzyl acetate (the major compound of vatica oil) (Boukaew et al. 2017) at 0, 1, 10, 50, and 100 μL were each mixed with 10 mL melted sterile PDA at a final concentration of 10 mL and poured into a 9 cm diameter culture plate, while melted sterile PDA at an equivalent amount was used as a control. A 5 mm diameter plug of mycelia colony of *A. parasiticus* TISTR 3276 was transferred onto the center of the test agar plates. After 5 days of incubation at ambient temperature (28 °C), the diameters of the colonies in the three plates were recorded. For each treatment, three replicates were realized. The inhibition percentage was calculated as described above. The effective dose was defined as the minimum dose that completely inhibited the mycelial growth of the aflatoxin producing fungal stain.

For liquid culture, the promising commercial essential oil and benzyl acetate at 0, 1, 10, 50, and 100 μL were incorporated into 100 mL potato dextrose broth (PDB) in 250-mL flasks, while sterile PDB at an equivalent amount was used as a control. A 5 mm diameter plug of mycelia colony of *A. parasiticus* TISTR 3276 was transferred into each flask and incubated at ambient temperature (28 °C) on a rotary shaker at 150 rpm for 5 days. There were three replicates for each treatment. After incubation, the mycelial mats were passed through dried pre-weighed filter paper and dried at 60 °C for 3 days, and weighed (Li et al. 2011). For each treatment, three replicates were realized. The inhibition percentage was calculated as described above. The effective dose of both compounds was also selected.

Conidia germination of A. parasiticus TISTR 3276

The promising commercial essential oil and benzyl acetate at 0, 1, 10, 50, and 100 μL mL^{-1} were each mixed with 10 mL melted sterile PDA and poured into a 9 cm diameter culture plate, while melted sterile PDA at an equivalent amount was used as a control. A volume of 50 μL of a spore suspension of *A. parasiticus* TISTR 3276 dosed at 1×10^4 spore mL^{-1} was spread onto the test agar plates. After 24 h of incubation at ambient temperature (28 °C), the percentage of conidia germination was calculated. For each treatment, three replicates were realized.

Fungicidal kinetics of vatica oil and benzyl acetate based on direct contact application

The antifungal activity of the promising commercial essential oil and benzyl acetate on conidia germination of *A. parasiticus* TISTR 3276 were investigated. The two compounds at the effective dose of 10 μL mL^{-1}

were each mixed with 9940 μL melted sterile PDB in the 125-mL flasks. Then, the homogenous suspension (1×10^4 spore mL^{-1}) at 50 μL mL^{-1} of *A. parasiticus* TISTR 3276 was mixed vigorously by vortexing before adding into the flasks at the final volume of 10 mL. The cultures with and without (control) the two compounds were incubated at ambient temperature (28 °C) on a rotary shaker at 150 rpm. Samples were taken after incubating for 0, 30, 60, 90, 120, 150, 180, 210, and 240 min. The fungicidal kinetics of the promising commercial essential oil and benzyl acetate to inhibit conidia formation of *A. parasiticus* TISTR 3276 were determined following the procedure as described by Li et al. (2013). For each treatment, three replicates were conducted and the experiment was repeated three times.

Effects of selected essential oil and benzyl acetate concentration on fungal growth inhibition on PDA plate based on fumigation assay

An antifungal bioassay was conducted to study the effect of the concentrations of vatica oil and benzyl acetate on mycelial growth of *A. parasiticus* TISTR 3276 following the procedure described above. The concentrations of the two compounds were varied at 0, 1, 10, 50, and 100 μL L^{-1} of culture per liter of airspace in treatment containers, while a Petri plate without vatica oil and benzyl acetate treatment was set as a control. After 5 days of incubation at ambient temperature (28 °C), the diameters of the colonies in the smaller plates were recorded, and the total number of the conidia per plate was assessed. For each treatment, three replicates were realized.

The effects of the concentrations (0, 1, 10, 50, and 100 μL L^{-1}) of vatica oil and benzyl acetate on the inhibition of conidial germination were studied. Aliquots (50 μL) of conidial suspension (1×10^4 spore mL^{-1}) were spread onto PDA in smaller plates. Each concentration of vatica oil and benzyl acetate was placed alongside the conidial suspensions inside larger plates and sealed as described above. The percentage of inhibition on conidia germination was examined after 24 h and calculated as described above. For each treatment, three replicates were realized.

SEM of the structure of A. parasiticus TISTR 3276 after treatment by the promising commercial essential oil using direct contact and fumigation assays

Since vatica oil exhibited stronger inhibition on the mycelial growth of *A. parasiticus* TISTR 3276 than benzyl acetate, it was selected to study the mode of action. Fungal biomass obtained from 5-day-old cultures grown on PDB from direct contact and the fungi grown on PDA from fumigation assay treated with and without vatica oil were used for scanning electron microscopy (SEM) to illustrate the possible mode of action. The fungal biomass preparation and the SEM performed employed the procedure described by Boukaew and Prasertsan (2014).

Effect of selected essential oil and benzyl acetate concentrations on fungal growth inhibition on maize grain using fumigation assay

Maize (*Zea mays* L.) grains were soaked in 100 mL of distilled water for 5 h and then autoclaved at 121 °C for 15 min (Yang and Chang 2010) before use. The activity of vatica oil and benzyl acetate on the growth of *A. parasiticus* TISTR 3276 on contaminated maize grain was studied. Five maize grains were transferred into three of the smaller dishes (five grain/dishes), then 50 μL of spore inoculum of *A. parasiticus* TISTR 3276 at 1×10^5 spore mL^{-1} was spread on each maize grain. For the fourth dish, various concentrations of vatica oil and benzyl acetate at 0, 1, 10, 50, and 100 μL L^{-1} of airspace in treatment containers were added to each Petri dish and the same concentrations of sterile distilled water were used as the control. All Petri dishes were covered with a lid, sealed with Parafilm M® and incubated at ambient temperature (28 °C) for 5 days. Then, the maize grains in the dishes were individually analyzed for the percentage of grain contaminated under a stereo-binocular microscope (Sumalan et al. 2013). The number of contaminated grains was used in order to estimate the grain contamination index (SCI) according to the procedure described by Doolotkeldieva (2010) using the formula:

$$\text{SCI}\ (\%) = [(\text{Number of contaminated maize grain}/\text{Total number of maizegrain}) \times 100].$$

Preventive and curative effects of the selected essential oil and benzyl acetate fumigation to protect maize grain contaminated by A. parasiticus TISTR 3276

Preventive application of vatica oil and benzyl acetate against *A. parasiticus* TISTR 3276 on maize grain was conducted. Fifteen maize grains were fumigated with the minimum dose to prevent the maize grain from contamination of vatica oil and benzyl acetate at 0, 6, 12, and 24 h and transferred to three of the smaller dishes (five grain/dish), and then 50 μL of spore suspension of *A. flavus* at 1×10^5 spore mL^{-1} was spread on each maize grain and incubated at ambient temperature (28 °C) for 5 days. Similarly, the Petri dish without the two compounds treatment was set as a control.

The curative application of vatica oil and benzyl acetate against *A. parasiticus* TISTR 3276 on maize grain was studied. Fifteen maize grains were inoculated with 50 μL of a spore suspension of *A. parasiticus* TISTR 3276 at 1×10^5 spore $mL^{-1}$and transferred into three of the smaller dishes (five grains/dish), and then fumigated with the minimum effective dose of the two compounds at 0, 6, 12, and 24 h after inoculation with *A. parasiticus* TISTR 3276. All treatments were incubated at ambient temperature (28 °C) for 5 days. Similarly, the Petri plate without the two compounds was set as a control. After 5 days of incubation, the maize grains in the dishes were analyzed for the percentage of grain contamination under a stereo-binocular microscope (Sumalan et al. 2013). The percentages of both preventive and curative protection were determined using the following formula: Protection percentage = [{(Control treatment)/Control} × 100]. For each treatment, three replicates were conducted and the experiment was repeated three times.

Statistical analysis

Statistical Package for the Social Science (SPSS) for Windows was used to analyze the data. The levels of antifungal activity of essential oils against *A. parasiticus* TISTR 3276 both in *in vitro* and on maize grains were compared using the Tukey HSD of Statistica software and a $P < 0.05$ was considered to demonstrate a significant difference.

Results

Screening of five essential oils for control of aflatoxin producing fungi by direct contact and fumigation assays

The efficiency of five essential oils against *A. parasiticus* TISTR 3276 by both fumigation and direct contact assays is presented in Fig. 1. By the fumigation assay (Fig. 1A), cinnamon, peppermint, clove, and vatica oil completely inhibited (100%) the mycelial growth of *A. parasiticus* TISTR 3276, while capsicum oil was less active (32.3% inhibition). In the direct contact method (Fig. 1B), vatica oil proved to be the most effective as it completely inhibited (100%) *A. parasiticus* TISTR 3276 followed by clove, peppermint, cinnamon, and capsicum oil, respectively. Therefore, vatica oil was selected as it strongly inhibited the mycelial growth of *A. parasiticus* TISTR 3276 by both direct contact and fumigation assays.

Effect of vatica oil and benzyl acetate concentrations on fungal growth inhibition based on direct contact assay

Since benzyl acetate was previously found to be the major compound of vatica oil (Boukaew et al. 2017), commercial benzyl acetate (Sigma–Aldrich, for GC,

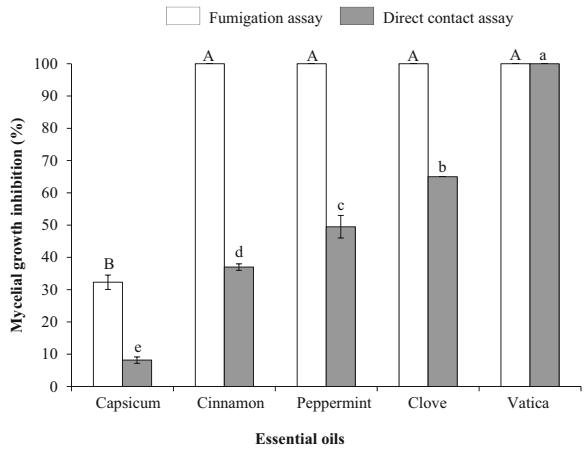

Fig. 1 Effect of five essential oils on mycelial growth of *A. parasiticus* TISTR 3276 tested using fumigation assay at 50 μL L^{-1} of airspace in treatment containers and on PDA plate by direct contact at 50 μL mL^{-1} after five days incubation at ambient temperature (28 °C). Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 3$) ± standard error

≥99.7% pure by GC) was used to compare with vatica oil on their inhibitory activities against *A. parasiticus* TISTR 3276 on solid and in liquid cultures (Table 1). Five concentrations (0 to 100 μL mL^{-1}) of vatica oil and benzyl acetate efficiently inhibited mycelial growth ($P < 0.05$) of *A. parasiticus* TISTR 3276 with 100% inhibition at 10 μL mL^{-1} on solid culture (Table 1A). However, complete inhibition (100%) in liquid culture by vatica oil was at 50 μL mL^{-1} while benzyl acetate was at 100 μL mL^{-1} (Table 1B). Therefore, the effective doses of the two compounds in liquid culture required 5 and 10 times higher concentrations than in the solid cultures.

The efficacies of both vatica oil and benzyl acetate on inhibition of the conidia germination of *A. parasiticus* TISTR 3276 on PDA are shown in Fig. 2. The percentage of conidia germination was significantly ($P < 0.05$) inhibited (100%) by vatica oil and benzyl acetate at concentrations of 1.0 and 10 μL mL^{-1}, respectively. Therefore, the effective dose of vatica oil to inhibit conidia germination (1.0 μL mL^{-1}) was 10 times lower than required to inhibit mycelial growth (10 μL mL^{-1}). On the other hand, the effective dose of benzyl acetate in both cases was at the same concentration (10 μL mL^{-1}).

Fungicidal kinetics of vatica oil and benzyl acetate based on direct contact application

The inhibition kinetics on conidia germination of *A. parasiticus* TISTR 3276 by vatica oil and benzyl acetate is presented in Fig. 3. Exposure of the *A. parasiticus* TISTR 3276 spores to the two compounds for a period of 0–240 min caused varying degrees of conidia germination inhibition. The results indicated that conidia germination decreased as the exposure time increased. Vatica oil totally inhibited (100%) spore germination at 210 min of exposure, while the inhibitory effect of benzyl acetate occurred at 240 min.

Effects of vatica oil and benzyl acetate concentrations on fungal growth inhibition on PDA plate by fumigation assay

The antifungal activities of the concentrations of vatica oil and benzyl acetate (0–100 μL L^{-1}) on the inhibition of growth and conidial germination of *A. parasiticus* TISTR 3276 during 5 days of incubation are presented in Fig. 4. Mycelial growth was significantly ($P < 0.05$) inhibited (70–100%) by 1–100 μL L^{-1} of vatica oil, while benzyl acetate in the same concentration range inhibited mycelial growth to a lesser degree (5–100%). Complete inhibition

Table 1 Effect of vatica oil and benzyl acetate concentrations on growth of *A. parasiticus* TISTR 3276 (A) in solid culture (PDA) and (B) in liquid culture (PDB) using direct contact

(A)

Amount of vatica oil (μL mL^{-1})	Mycelial growth (cm)	Mycelial growth inhibition (%)	Amount of benzyl acetate (μL mL^{-1})	Mycelial growth (cm)	Mycelial growth inhibition (%)
0	3.70 ± 0.22^{a}	0^{c}	0	3.70 ± 0.22^{a}	0^{c}
1	1.93 ± 0.10^{b}	47.97^{b}	1	2.63 ± 0.17^{b}	29.05^{b}
10	0 ± 0.00^{c}	100^{a}	10	0 ± 0.00^{c}	100^{a}
50	0 ± 0.00^{c}	100^{a}	50	0 ± 0.00^{c}	100^{a}
100	0 ± 0.00^{c}	100^{a}	100	0 ± 0.00^{c}	100^{a}

(B)

Amount of vatica oil (μL mL^{-1})	Mycelial dry weight (mg)	Mycelial growth inhibition (%)	Amount of benzyl acetate (μL mL^{-1})	Mycelial dry weight (mg)	Mycelial growth inhibition (%)
0	146.30 ± 0.38^{a}	0^{d}	0	146.30 ± 0.38^{a}	0^{e}
1	68.10 ± 4.70^{b}	53.45^{c}	1	142.47 ± 1.74^{b}	2.62^{d}
10	48.50 ± 0.80^{c}	66.85^{b}	10	138.90 ± 0.17^{c}	5.06^{c}
50	0 ± 0.00^{d}	100^{a}	50	38.00 ± 0.18^{d}	74.03^{b}
100	0 ± 0.00^{d}	100^{a}	100	0 ± 0.00^{e}	100^{a}

Data are the mean of three replicates ± standard errors (SE). Data followed by same letter within each column are not significantly different using ANOVA after Tukey HSD at $P < 0.05$

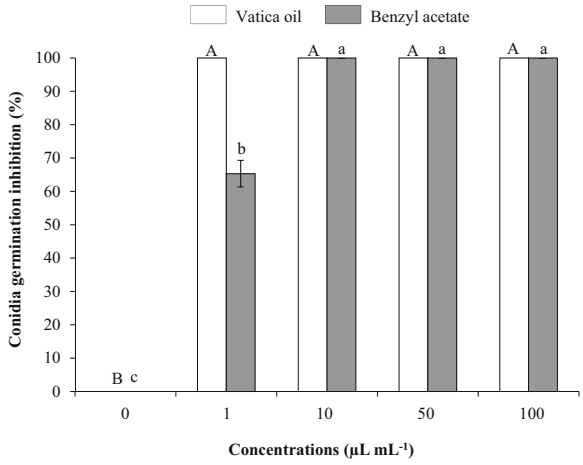

Fig. 2 Effect of vatica oil and benzyl acetate on the conidia germination of *A. parasiticus* TISTR 3276 after 24 h incubation at ambient temperature (28 °C) by direct contact. Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 3$) ± standard error

(100%) was achieved at 50 µL L^{-1} fumigation by both compounds (Fig. 4a). In addition, spore germination inhibition of *A. parasiticus* TISTR 3276 was significantly ($P < 0.05$) inhibited by 10–100 µL L^{-1} of vatica oil (90–100% inhibition) and benzyl acetate (55–100% inhibition). The effective dose of both compounds (100% inhibition) was at 50 µL L^{-1} (Fig. 4b).

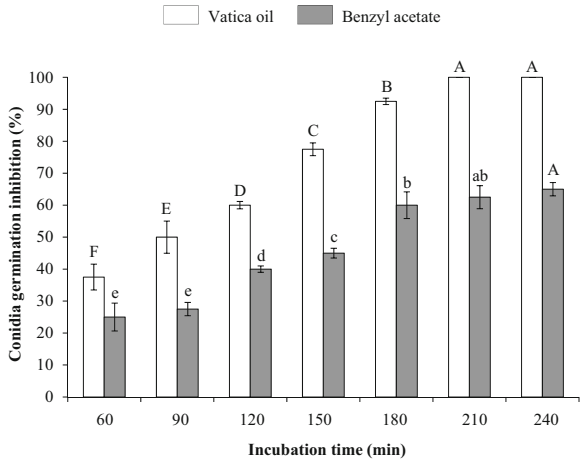

Fig. 3 Fungicidal kinetics of vatica oil and benzyl acetate on inhibition of the conidia germination of *A. parasiticus* TISTR 3276 after incubation at ambient temperature (28 °C). Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 9$) ± standard error

SEM of the structure of *A. parasiticus* TISTR 3276 after treatment by vatica oil using direct contact and fumigation assays

The effects of vatica oil on the ultrastructure alterations of the growing *A. parasiticus* TISTR 3276 mycelia by both direct contact and fumigation assays after 5 days treatment is illustrated by SEM in Fig. 5. SEM provided evidence of the morphological changes due to exposure to vatica oil by both methods. There were no morphological changes in the control samples. The conidiophores appeared as globular and formed spherical heads containing swollen conidia (Fig. 5a, b) while the hyphae retained complete tubular shapes with the normal appearance of smooth cell walls (Fig. 5c). Exposure to vatica oil at 10 µL mL^{-1} completely inhibited conidia germination by direct contact (Fig. 5d, e, and f), while fumigation assay exhibited morphological changes of conidia with the abnormal growth of the fungi (Fig. 5g, h). Also evident from the SEM were the markedly shriveled, crinkled cell walls, and flattened hyphae of the fungi (Fig. 5e, f, and i).

Effects of vatica oil and benzyl acetate concentration on fungal growth inhibition on maize grain using fumigation assay

Vatica oil and benzyl acetate fumigation treatment at different concentrations could reduce the growth of *A. parasiticus* TISTR 3276 in the inoculated maize grain (Fig. 6). The percentages of the maize grain contamination index were significantly reduced ($P < 0.05$) in the treatment group compared with the control group and also significantly reduced ($P < 0.05$) with increasing concentrations of the two compounds after 5 days incubation at ambient temperature (28 °C). The most effective fumigation concentration of vatica oil was 10 µL L^{-1} with complete reduction (100%) of *A. parasiticus* TISTR 3276 on contaminated maize grain, while complete reduction (100%) required 50 µL L^{-1} of benzyl acetate.

Preventive and curative effects of vatica oil and benzyl acetate fumigation to protect maize grain contaminated by *A. parasiticus* TISTR 3276

The minimum fumigation period of vatica oil and benzyl acetate (50 µL L^{-1}) on maize grain to prevent the infection by *A. parasiticus* TISTR 3276 was studied. The results showed that fumigation with vatica oil for

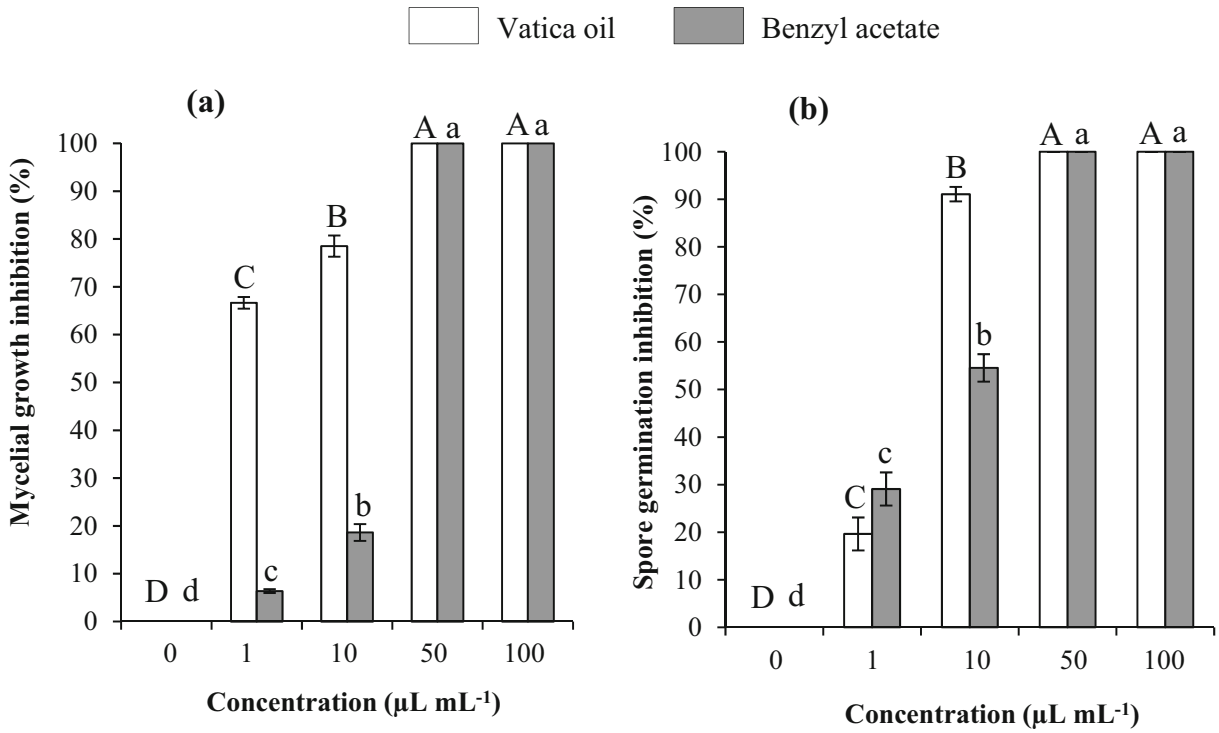

Fig. 4 Effects of different concentrations of vatica oil and benzyl acetate on the mycelial growth (**a**) and spore germination (**b**) of *A. parasiticus* TISTR 3276 in PDA media by fumigation assay and incubated at ambient temperature (28 °C) for 5 day on mycelial growth and 24 h on spore germination. Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 3$) ± standard error

6 h and benzyl acetate for 24 h (before inoculation of the pathogen) gave the highest protection (100%) of maize grain infected by the aflatoxigenic fungi (Fig. 7a). For the curative effect, fumigation on the inoculated (*A. parasiticus* TISTR 3276 maize grain) with the two compounds for 6 h showed 100% curative effect of the infected maize grain (Fig. 7b). Therefore, the minimum fumigation periods of vatica oil and benzyl acetate to control maize grain infected with *A. parasiticus* TISTR 3276 were 6 and 24 h, respectively.

Discussion

This study demonstrated the control of aflatoxin producing fungi *A. parasiticus* TISTR 3276 both *in vitro* and *in vivo* using vatica oil and its major component benzyl acetate by direct contact and fumigation assays. The two compounds demonstrated a strong antifungal effect to control the colonization and contamination of maize grain by *A. parasiticus*. However, the presence of other microbial species in non-autoclaved maize may affect the efficacy, but this was not tested. *Fusarium*, *Alternaria*, *Penicillium* and *Aspergillus*, especially *A. parasiticus*, were frequently found to contaminate maize grain during storage (Ng'ang'a et al. 2016).

The efficiency of various essential oils for controlling *Aspergillus* spp. was reported such as *Thymus eriocalyx* essential oil (at the dilution of 1/2) exhibited >90 mm inhibition zones of *A. parasiticus* (Rasooli and Abyaneh 2004), *T. vulgaris* essential oil (at 1000 µg mL^{-1}) inhibited 100% inhibition on growth of *A. parasiticus* NRRL 2999 (Razzaghi-Abyaneh et al. 2009), and *Eucalyptus globulus* essential oil (at concentration of 1500 µL) inhibited 100% inhibition on growth of *A. flavus* and *A. parasiticus* (Vilela et al. 2009), but they were applied only by either direct contact assay (Li et al. 2013; Pinto et al. 2013; Stevic et al. 2014; Matusinsky et al. 2015) or fumigation assay (Avila-Sosa et al. 2012; Cardiet et al. 2012; Tian et al. 2014; Li et al. 2016; Boukaew et al. 2017). Both methods were used in this study against *A. parasiticus* TISTR 3276. In the present work, the results of the antifungal screening showed that vatica oil exhibited the highest efficiency (100%

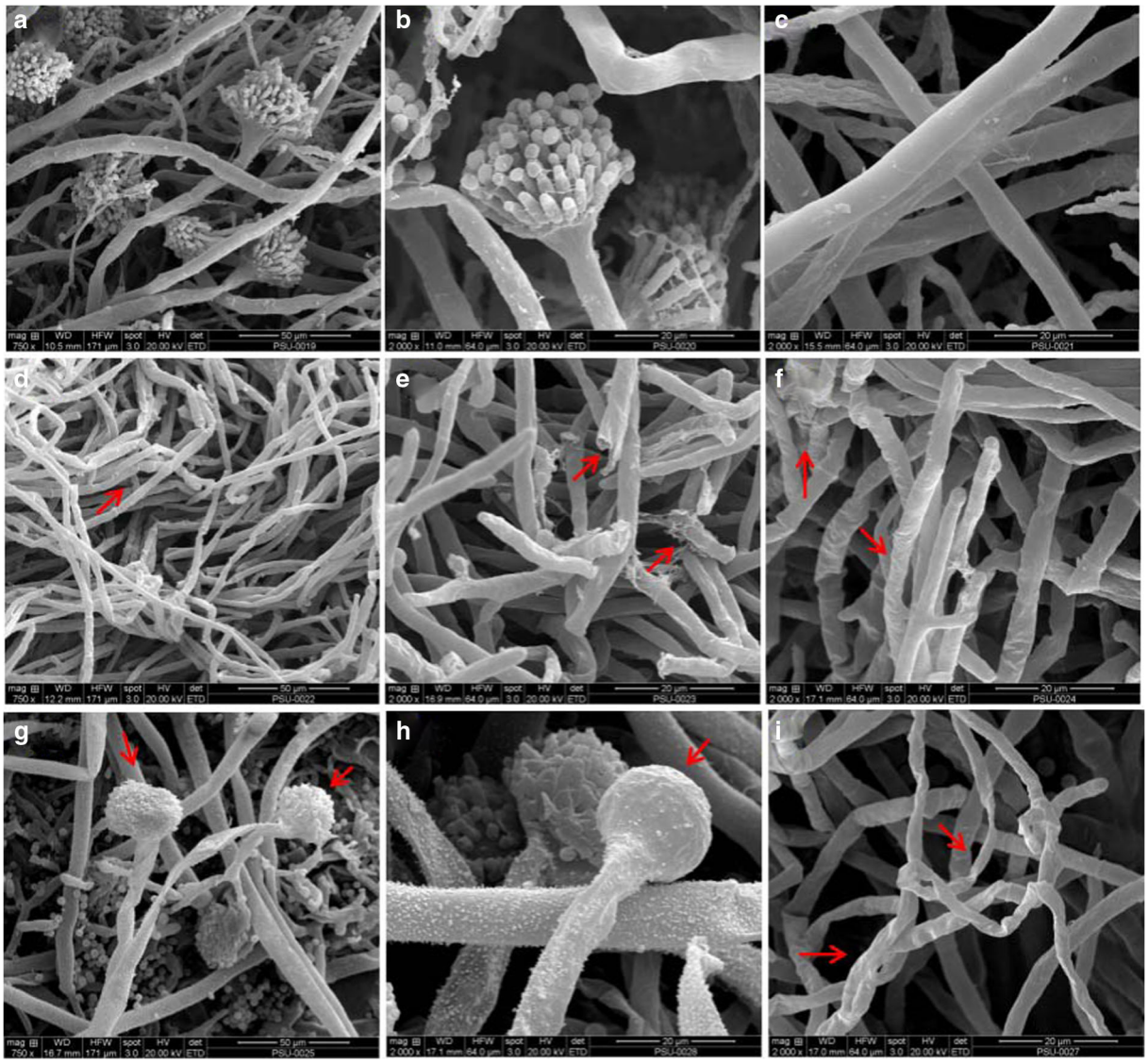

Fig. 5 Scanning electron micrographs (SEM) of normal hypha, conidiophore, and spore of *A. parasiticus* TISTR 3276 without exposure to vatica oil (the control) after incubation at ambient temperature (28 °C) for 5 days. **a**, **b**, and **c**; mycelia treated with vatica oil at 10 μL mL^{-1} by direct contact after incubation (**d**, **e**, and **f**) and mycelia treated with vatica oil at 10 μL L^{-1} by fumigation assay after incubation (**g** **h**, and **i**). Arrows indicate *A. parasiticus* TISTR 3276 hyphal cell wall and vesicle damaged by vatica oil

inhibition) against the mycelial growth of *A. parasiticus* TISTR 3276 by both methods compared to cinnamon, clove, capsicum, and peppermint oil. The efficacy of fumigation assay (32.30–100% inhibition) on the five essential oils was higher than that of direct contact assay (8.16–100% inhibition). This result agreed with previous reports that fumigation using an essential oil gave higher inhibition than the direct contact assay (Soylu et al. 2006; Vilela et al. 2009; Tian et al. 2014). The major compound of vatica oil was found to be benzyl acetate (48.8%) (Boukaew et al. 2017) which is also present in several plants (Raguso and Pichersky 1995; Guterman et al. 2006). It is the ester formed by condensation of benzyl alcohol and acetic acid which are known to be antimicrobial agents (Dev et al. 2004; Nidiry and Babu 2005). The *in vitro* efficacy of vatica oil and benzyl acetate revealed that the colony diameter of the mycelial growth or dry mycelium weight of *A. parasiticus* TISTR 3276 decreased with increased concentrations of the two compounds. In the direct

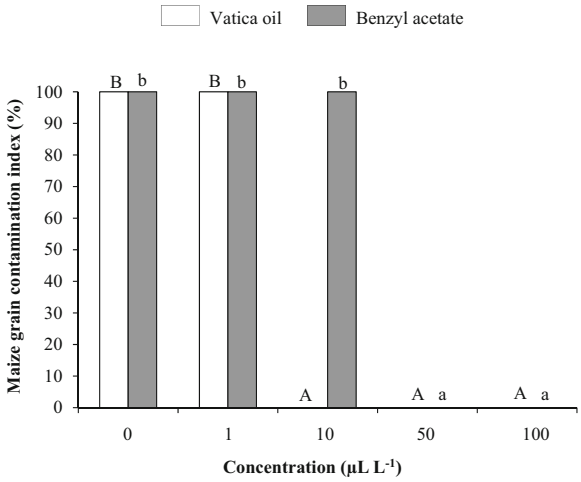

Fig. 6 Effect of concentrations of vatica oil and benzyl acetate against *A. parasiticus* TISTR 3276 contaminated on maize grain after incubated for 5 days at ambient temperature (28 °C) by fumigation assay. Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 3$) ± standard error

contact assay, the effective dose of the two compounds was at 10 μL mL^{-1} which showed complete inhibition (100%) of mycelial growth and spore germination of *A. parasiticus* TISTR 3276 while the effective dose in the fumigation assay was at 50 μL L^{-1}. Therefore, the fumigation method was more efficient than direct contact as it required 200 times lower concentration of the two substances (50 μL L^{-1} or 0.05 μL mL^{-1} vs 10 μL mL^{-1}). Based on the effective concentrations of both compounds by direct contact assay, vatica oil (10 μL mL^{-1}) was more effective than many plant essential oils such as oregano essential oil (2500 μL mL^{-1}) against *A. wenti* (Kocic-Tanackov et al. 2012), ginger essential oil against growth and conidial germination of *A. flavus* (150 μL mL^{-1} and 100 μL mL^{-1}, respectively) (Nerilo et al. 2016) but less effective than the essential oil from *Cicuta virosa* L. var. *latisecta* Celak (5.0 μL mL^{-1}) against the mycelial growth and aflatoxin B1 synthesis of *A. flavus* (Tian et al. 2011). Vatica oil was 500 times more effective than thyme oil (Özcan and Boyraz 2000) against *A. parasiticus* (10 and 5000 μL mL^{-1}, respectively) and 100 times more effective than the major compound (1,8-cineole) of *Eucalyptus globules* Labill essential oil (1000 μL mL^{-1}) against *A. flavus* and *A. parasiticus* (Vilela et al. 2009).

The spore is an important structure for the survival and spread of aflatoxin-producing fungi (Rabea et al. 2003). A significant decrease in the percentage of spore germination was observed on *A. parasiticus* TISTR 3276 with increasing exposure time. The kinetic study revealed that the effective exposure time of 210 min of vatica oil (10 μL mL^{-1}) completely (100%) inhibited the conidia of *A. parasiticus* TISTR 3276. This was slightly higher than the effective exposure time of 180 min for both *Cinnamomum jensenianum* Hand.-Mazz essential oil and *Zanthoxylum molle* Rehd. essential oil (at 8 μL mL^{-1}) against *A. flavus* (Tian et al. 2012, 2014). Moreover, some studies focused on the effects of the essential oils on fungal conidia germination such as *Cicuta virosa L. var. latisecta* Celak essential oil (Tian et al. 2011), citronella oil (Li et al. 2013), and *Zingiber officinale* essential oil (Nerilo et al. 2016). Benzyl acetate could not completely inhibit (65.0%) at an exposure time less than 210 min. Therefore, the vatica oil exhibited greater antifungal activity than its derivative, benzyl acetate. This may be attributed to some minor components that has a synergistic effect with the major components. These observations supported the hypothesis that the minor compounds also played a very important role in antifungal activity of vatica oil, and benzyl acetate was not solely responsible for fungal destruction.

Application of various essential oils for controlling *Aspergillus* spp. using fumigation method was reported such as *Chromolaena odorata* essential oil (2 μL mL^{-1}) exhibited pronounced efficacy >75% protection of chickpea seeds from *A. flavus* infestation (Prakash et al. 2012a), *Cuminum cyminum* seed essential oil (0.6 μL mL^{-1}) showed remarkable efficacy in protecting wheat (65.85%) and chickpea (75.00%), from fungal contamination during storage up to 12 months (Kedia et al. 2014), *Zanthoxylum alatum* Roxb. essential oil (1.25 μL mL^{-1}) showed efficacy in protecting *Piper nigrum* L. fruits (100%) from *A. flavus* infestation (Prakash et al. 2012b), and *Vatica diospyroides* essential oil (50 μL L^{-1}) exhibited strong antifungal activity as it completely inhibited growth, sporulation, conidial germination, and disease infection of *A. flavus* PSRDC-2 both *in vitro* and on maize seeds (Boukaew et al. 2017).

Fumigation is ideal to control the spoilage of fungi because it leaves no residual essential oils (Tian et al. 2011; Li et al. 2016). Although *in vitro* tests of essential oils is an important first step in selecting plants with antifungal potential, *in vivo* tests are needed to check whether the positive results of the *in vitro* tests can be obtained or not (Tegegne et al. 2008). The *in vivo* results indicated that vatica oil and benzyl acetate had a positive

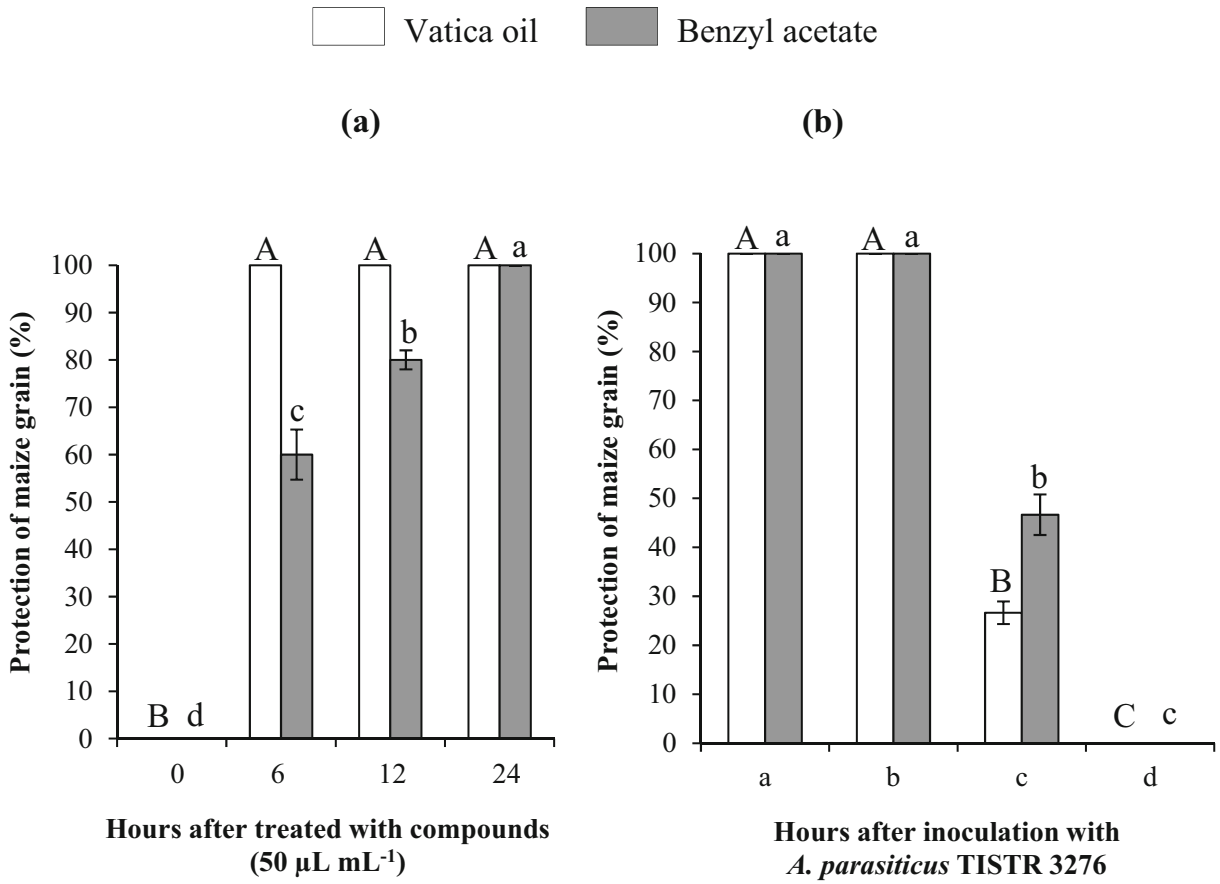

Fig. 7 Timing of preventive (**a**) or curative (**b**) measures by the vatica oil and benzyl acetate fumigation on the protection of maize grain inoculated with *A. parasiticus* TISTR 3276 after incubation for 5 days at ambient temperature (28 °C). Different letters above bars indicate significant differences ($P < 0.05$) using ANOVA after the Tukey HSD in each group. Values are mean ($n = 9$) ± standard error

effect on the decrease of contamination of *A. parasiticus* TISTR 3276 on maize grain. Fumigation using vatica oil at 10 µL L^{-1} and benzyl acetate at 50 µL L^{-1} demonstrated the greatest reduction in the percentages of maize grain contamination index for *A. parasiticus* TISTR 3276. Fumigation by *Zanthoxylum molle* Rehd. essential oil at a concentration of 0.2 µL L^{-1} had a positive effect on storage life and reduced (91.7%) cherry tomato decay caused by *A. flavus* (Tian et al. 2014). Thymol oil at a concentration of 250 µL L^{-1} and carvacrol oil at 500 µL L^{-1} showed no incidence of fungal mycelium on the surface of lemon caused by *Penicillium digitatum* and *P. italicum* (Pérez-Alfonso et al. 2012). All these data indicated that the effective dose of any plant essential oil depended on the pathogen and samples tested.

According to the preventive and curative assay, fumigation using vatica oil for at least 6 h showed complete (100%) protective and curative effects on maize grain infected by *A. parasiticus* TISTR 3276, while benzyl acetate had a protective effect but not curative effect even at 24 h. As a result, the maize grain must be fumigated by vatica oil and benzyl acetate before grain storage.

The data and SEM results clearly indicated that the suppression activity of vatica oil could damage the hyphae, conidiophores, and spores of *A. parasiticus* TISTR 3276 by either method. Many reports showed that the hyphae, conidiophores, and spores of *Aspergillus* spp. could be damaged by essential oils that included *Matricaria chamomilla* L. flower essential oil (Tolouee et al. 2000), *Origanum vulgare* L. essential oil (dos Santos et al. 2012), cinnamon essential oil (Manso et al. 2013), citronella essential oil (Li et al. 2013), *Thymus vulgaris* essential oil (Kohiyama et al. 2015), *Litsea cubeba* essential oil (Li et al. 2016), and *Zingiber officinale* essential oil (Nerilo et al. 2016). From these results, it could be stated that the vatica oil destroyed the

cell wall of *A. parasiticus* hyphae, passed through the cell membrane, penetrated into the cytoplasm, and acted on the main organelles, to kill the hyphae cells at last.

Conclusion

Usage of essential oils has been emerging as a possible alternative for the control of aflatoxin producing fungi. The results indicated that vatica essential oil and its derivative (benzyl acetate) had strong antifungal activity against aflatoxin producing fungi *A. parasiticus* TISTR 3276 both in *in vitro* and *in vivo* by direct contact and fumigation methods. Vatica oil had high potential to be used as an effective natural antifungal agent by both methods to protect maize grain from *A. parasiticus* TISTR 3276 as well as other common spoilage fungi during the storage.

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Compliance with ethical standards

Conflict of interest The authors declare having no conflict of interest.

Human and animal studies This research did not involve human and/or animal participants.

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