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Contributed Paper

Antifungal Activity of the Essential Oil Extracted from *Zanthoxylum piperitum* Seeds Against *Aspergillus flavus*

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ABSTRACT

The antifungal activity of essential oil extracted from *Zanthoxylum piperitum* seeds was tested and the oil's potential as a food preservative was evaluated. The main chemical components determined in the oil were β-phelladrene (23.2%), sabinene (14.6%), and brevifolin (11.9%). The oil's antifungal activity was tested in solid and liquid cultures of aflatoxigenic *Aspergillus flavus*. The minimum inhibitory concentration of oil was 4.5 μL/mL. The possible actions against *A. flavus* are distortion in conidial heads and decrease of hyphal diameters as observed under a light microscope. A reduction in the ergosterol content in fungal plasma membrane by 34.41 % also occurred. The use of this essential oil as an antifungal coat was tested on dried bird chili. The essential oil at 4.5 and 9.0 μL/mL reduced fungal development on chili peel to 45.20 % and 89.32 %, respectively. Thus, application as a food preservative is possible.

Keywords: *Aspergillus flavus*, ergosterol, essential oil, antifungal activity, *Zanthoxylum piperitum*

1. INTRODUCTION

Protection of food commodities from spoilage caused by microorganisms during processing, transportation, and storage is challenging for food industries. Toxigenic fungi produce mycotoxins that lead to significant qualitative losses of commodities and potentially induce various health problems in consumers. Aflatoxins are a group of very hazardous and common toxic metabolites produced by *Aspergillus flavus* and *A. parasticus*. Aflatoxins are widely known for their carcinogenic, teratogenic, hepatotoxic, mutagenic, and immunosuppressive properties and they can interfere several metabolic systems [1]. The production of these toxins affects agricultural commodities, e.g., dried chili powder, a widely used favoring ingredient in Thai dishes. Also, the high humidity of tropical and subtropical countries promotes fungal growth. Aflatoxins are highly thermostable in nature and do not degrade during the cooking process [2]. Thus, control of aflatoxin-producing fungi is essential. The use of synthetic chemical preservatives to control aflatoxigenic fungi in food has led to a number of environmental and health problems because of their carcinogenicity, teratogenicity, high and acute toxicity, and long degradation periods. The concern of consumers and food

processors about these risks has led to safer alternative preservatives to replace chemical fungicides. Natural products, such as essential oils, are interesting alternative antifungal agents because they are safe for consumers and have a low risk of resistance being developed by pathogenic microorganisms [3].

Zanthoxylum piperitum (Thai name: prikhom) is a medicinal plant belonging to the *Rutaceae* family and is distributed mainly in tropical and subtropical regions, including Korea, China, Japan, and Taiwan [4]. This plant produces a diverse range of aromatic agents, including essential oils such as myrcene, octanal, limonene, linalool, citronellal, geraniol, phellandral, and geranyl acetate and is used to treat stomach ache, vomiting, diarrhea, abdominal pain, and moist dermal ulcer [5]. Its beneficial effects have been traditionally associated with antibacterial, antilipid peroxidative, and antiviral activities. The essential oil is also a cholesterol acyltransferase inhibitor and is used as a food additive to mask fishy flavor in Korean food [6-10]. In addition, essential oil derived from *Z. piperitum* (ZSEO) repels mosquitoes [11], inhibits growth of human pathogens, such as *Streptococcus mutans*, an oral pathogen [12]. However, the antifungal activity of essential oil from *Z. piperitum* seeds is not known to control *A. flavus*. Therefore, this research was conducted to develop an essential oil from *Z. piperitum* seeds that would be an eco-friendly antifungal agent. In this study, the essential oil from *Z. piperitum* was extracted and evaluated for its effects on the mycelial growth and biomass of the fungus *A. flavus* that had been isolated from dried chili powder. Also, the morphological changes in hyphae of the fungus caused by the essential oil and the oil's possible mode of action was studied. The essential oil's potential application to control post-harvest spoilage of stored chili was also assessed.

2. MATERIALS AND METHODS

2.1 Extraction of Essential oil

The seeds of *Z. piperitum* were purchased from a local market in Chiang Mai, Thailand. Essential oil (ZSEO) was extracted from 100 grams of ground seeds by hydrodistillation using a Clevenger-type apparatus for 4 h. The collected oil was dried over anhydrous sodium sulfate and then stored in a dark glass bottle at approximately 4 ^{o}C for further analysis and antifungal testing.

2.2 Essential Oil Analysis

The analysis of ZSEO was made using a 6890 Agilent Technology/MSD 5973 Hewlett Packard gas chromatograph (GC) equipped with a FID detector and an HP-5MS capillary column (bonded and cross-linked 5% phenyl-methylpolysiloxane 30 m × 0.25 mm, film thickness 0.25 μm). The injector and detector temperatures were set at 220 ^{o}C and 280 ^{o}C, respectively. The oven temperature was held at 50 ^{o}C for 3 min and then allowed to rise to 240 ^{o}C at a rate of 3 ^{o}C/min. Helium was the carrier gas at a flow rate of 1 mL/min. One μL of diluted oil (50/100 in acetone, v/v) was injected manually and in the splitless mode. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. The components were identified based on the comparison of their relative retention time and mass spectra with those of standards, W8N05ST (Wiley ver. 8.0) library data of the GC-MS system and literature data [13].

2.3 Antifungal Effect of Essential Oil on Agar Plate

Aspergillus flavus was previously isolated from dried chili powder samples purchased from a local market using the poisoned food technique [14]. Its identity was confirmed by morphological features of slide culture and 18s rRNA sequencing [15]. Antifungal activity of the essential oil was evaluated against *A. flavus*.

ZSEO was added to plates containing 0.5 mL of 5% (v/v) Tween-20 and 9.5 mL melted potato dextrose agar (PDA) was then mixed to obtain the final concentrations of 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 μL/mL. Plate without essential oil was used as a control. A 4-mm diameter disc was removed from a 3-day old culture using a cork borer and placed in the middle of all plates. The plates were then incubated at 28 ± 2 °C. The effectiveness of treatments was evaluated every day for nine days and the mycelial inhibition percentage was calculated by the formula: mycelial inhibition percentage = [(dc-dt)/dc] ×100, where dc is the mean diameter of the fungal colony in the controls and dt is the mean diameter of the fungal colony in the treatments. The lowest concentration that completely inhibited fungal growth was considered to be the minimum inhibitory concentration (MIC).

2.4 Antifungal Effect of Essential Oil in Liquid Culture

The effect of the essential oil on the wet and dry mycelial weights of *A. flavus* was determined following the method of Dikbas *et al.* [16]. Twenty mL of potato dextrose broth (PDB) medium was transferred to Erlenmeyer flasks to which requisite amounts of the essential oil were added to obtain 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 μL/mL concentrations. Then, 100 μL of the fungal inoculum containing 10^7 spores/mL was inoculated into each flask at 28 ± 2 °C in an incubator shaker. Samples without essential oil were used as controls. The flasks containing mycelia masses were harvested after nine days and washed twice with sterile distilled water. These mycelia masses were allowed to dry at 70 °C for 12 h before weighing. The percentage inhibition of the mycelial growth was calculated by the formula: inhibition percentage = [(dwc-dwt)/dwc]×100, where dwc and dwt are the mean dry mycelial weights of the controls and treatments, respectively.

2.5 Light Microscope Examination of Essential Oil-Treated *A. flavus*

The morphology of fungal hyphae was observed in slide cultures. The PDA plates with and without 1.0 μL/mL essential oils were prepared as done earlier. Agar from treatment and control plates was cut into 1×1cm squares using a sterile blade. The agar squares were transferred to sterile glass slides and inoculated with fungal mycelia using a loop on the four sides of the squares. The slide cultures were incubated at 28 ± 2° C for three days and viewed under a compound microscope at 40x magnification to study the morphology of the fungal structures.

2.6 Effect of Essential Oil on Ergosterol Content in the Plasma Membrane of *A. flavus*

The ergosterol content in *A. flavus* plasma membranes was detected following Tian *et al.* [17]. A 100 μL spore suspension of the fungus (10^7spores/mL) was inoculated into PDB medium containing 1 μL/mL of essential oil. After four days of incubation at 28 ± 2 °C, mycelia were harvested by centrifugation and then washed twice with distilled water. The weight of the wet mycelia was then measured. Five milliliters of 25% alcoholic potassium hydroxide solution was added to the cell pellets and the samples were vortexed for 2 min. This was followed by incubation at 85° C in a hot water bath for 4 h. Sterols were then extracted from samples by adding a mixture of 2 mL sterile distilled water and 5 mL *n*-heptane, followed by sufficient vortexing for 2 min. After the layers were allowed to separate for 1 h at room temperature, the *n*-heptane layer was separated and analyzed by spectrophotometry between 230 and 282 nm. The formula used to determine the ergosterol amount is:

% ergosterol + % 24(28) dehydroergosterol

$$= (A_{282}/290)/\text{pellet weight}$$

% 24(28) dehydroergosterol

$$= (A_{230}/518)/\text{pellet weight}$$

where 290 and 518 are the E values (in percentages per centimeter) determined for crystalline ergosterol and 24(28) dehydroergosterol, respectively, and pellet weight is the net wet weight in grams.

2.7 Efficacy of Essential Oil as Antifungal Coat in Dry Chili

In evaluating the dry chili model, four chili pieces per plate, 2.2 - 2.3 g each, were sterilized by autoclaving at 121° C for 15 min. These cooked chilies were allowed to dry at 70 ^{o}C for 12 h before being tested. ZSEO dissolved in 2.0 mL of 5% Tween-20 to procure final concentrations of 4.5 and 9.0 μL/mL was coated onto dry chilies using sterilized cotton bud and these chilies, after leaving air-dry, were then transferred to sterile Petri dishes. Then, 10 μL of the *A. flavus* inoculum containing 10^7 spores/mL were inoculated onto each piece. Samples without essential oil were used as controls. After incubation at 28 ± 2 ^{o}C for nine days, the fungal populations in the essential oil-coated and non-coated dry chilies were estimated by the pour plate method.

2.8 Statistical Analyses

All the measurements were replicated three times for each treatment and the data were reported as mean ± standard deviations. Significant differences between the mean values were determined by Duncan's multiple range test (p<0.05), following one-way ANOVA. The statistical analyses were performed using SPSS, 17.0 (Chicago, USA).

3. Results and Discussion

3.1 Extraction and Characterization of the Essential Oil

The essential oil yield from hydrodistillation of *Z. piperitum* seeds was 0.35 - 0.44 %w/w. Sixty-three compounds were identified in this oil, accounting for 92.6% of the total oil composition. Monoterpenes (56.5%) and oxygenated monoterpenes (31.1%) were dominant. Table 1 shows the results of the oil analyses classified as a group of compounds identified. The major compounds in the essential oil were β-phelladrene (23.2%), sabinene (14.6%), and brevifolin (11.9%). Other essential oil components were (+)-piperitone (6.4%), β-myrcene (4.6%), linalool (3.4%), α-(+)-pinene (3.1%), terpinyl acetate (2.2%), γ-terpinene (2.2%), *cis*-ocimene (2.2%), α-terpinolene (1.5%), α-terpinene (1.3%), β-fenchol (1.3%), and β-ocimene (1.1%). In general, most chemical components of essential oils are terpenoids, including monoterpenes and sesquiterpenes, and their oxygenated derivatives. Choochote *et al.* [18] reported that the major compounds of *Z. piperitum* seeds oil were (+)-limonene (37.9%), sabinene (13.3%), and β-myrcene (7.17%). Each element was different depending on species, sub-species and plant varieties, the portion of the plant used, different geographic locations where plants are grown, and weather conditions during growth and the stage of growth at harvest. Essential oils containing terpenes as major compound were reported to exhibit antimicrobial activity [19]. The different compounds in one essential oil may exhibit synergism in antibacterial and antifungal activities [20].

3.2 Antifungal Activity of the Essential Oil

Mycelial growth of *A. flavus* during nine days is shown in Figure 1. The results indicate that fungal diameter increased with incubation time but decreased with increasing concentration of ZSEO. Complete growth inhibition of *A. flavus* occurred at the MIC value of 4.5 μL/mL. The mycelial growth inhibition percentage was calculated on the ninth day. Other concentrations of the oil significantly reduced fungal growth with inhibition percentages of 46.34, 62.50, 77.83, 82.78, 86.43, 89.03, and 91.04 at 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 μL/mL concentrations, respectively. ZSEO is a comparably potent fungicide against *A. flavus* when compared its

Table 1. Chemical composition of *Z. piperitum* essential oil identified by GC-MS analysis.

Compounds	Retention time (min.)	Match Quality	Molecule Weight	%
Aldehyde				
Hexanal	4.187	95	100	0.1
2-Methyl-3-phenyl-propanal	22.142	98	148	0.2
				0.3 %
Monoterpenes				
α-Thujene	8.053	94	136	0.8
α-(+)-Pinene	8.329	96	136	3.1
Camphene	8.852	96	136	0.1
(+)-Sabinene	10.097	96	136	14.6
β-Pinene	10.144	94	136	0.8
β-Myrcene	10.832	95	136	4.6
α-Phellandrene	11.267	94	136	0.9
3-Carene	11.484	97	136	Tr
α-Terpinene	11.819	98	136	1.3
β-Phelladrene	12.612	96	136	23.2
cis-Ocimene	12.929	97	136	2.2
β-Ocimene	13.352	98	136	1.1
γ -Terpinene	13.799	96	136	2.2
α-Terpinolene	15.115	98	136	1.5
p-Cymenene	15.175	97	132	0.1
Neo-Allo-Ocimene	17.047	97	136	Tr
				56.5 %
Oxygenated monoterpenes				
4-Thujanol	14.234	96	154	0.9
cis-β-Terpineol	15.673	91	154	0.9
Linalool	15.926	97	154	3.4
(*E*)-*p*-Menth-2-en-1-ol	16.760	91	154	1.0
α-Campholenal	16.878	94	152	Tr
Terpinen-4-ol	17.600	96	154	0.7
(+)-*p*-Menth-1-en-4-ol	19.445	97	154	0.2
β-Fenchol	20.062	91	154	1.3
cis-Piperitol	20.785	90	154	0.5
(*E*)-Carveol	21.396	98	152	Tr
(*Z*)-Geraniol	21.807	97	154	0.1
(+)-Carvone	22.336	94	150	0.1
(+)-Piperitone	22.994	96	152	6.4
Phellandral	23.699	95	152	0.2
Bornyl acetic ether	24.169	99	196	0.3
(*E*)-Anethol	24.628	98	148	0.1
Carvacrol	25.344	97	150	0.1
Terpinyl acetate	27.054	91	196	2.2
Chavibetol	27.430	98	164	Tr
Neryl acetate	27.683	91	196	0.2
Geranyl acetate	28.523	91	196	0.6
Brevifolin	40.215	94	196	11.9
				31.1 %

Table 1. Continued.

Compounds	Retention time (min.)	Match Quality	Molecule Weight	%
Ketone				
4-Isopropyl cyclohexanone	18.352	96	140	0.1
L-Cryptone	19.727	95	138	0.5
				0.6 %
Sesquiterpenes				
Copaene	28.035	99	204	Tr
β-Elemene	28.770	99	204	0.2
Caryophyllene	29.868	99	204	0.5
α-Caryophyllene	31.267	98	204	0.1
Germacrene D	32.424	98	204	0.6
Germacrene B	33.029	95	204	0.2
α-Amorphene	33.217	98	204	0.1
α-Farnesene	33.582	98	204	0.1
δ-cadinene	34.146	99	204	0.5
				2.3 %
Oxygenated sesquiterpenes				
trans-Nerolidol	35.797	91	222	0.1
(+)-Spathulenol	36.296	99	220	0.3
β-(-)-Caryophyllene epoxide	36.431	99	220	0.4
Cedren-8-ol	37.166	99	222	0.4
Isospathulenol	38.588	99	220	0.1
Farnesol	41.690	97	222	0.1
Farnesyl acetate	45.744	97	264	0.2
				1.5 %
Phenylpropanoid				
Methyl eugenol	29.445	98	178	0.1
Aromatic compounds				
1,4-Dimethoxybenzene	18.687	96	138	Tr
Naphthalene	32.254	99	204	0.1
			Total	**92.6 %**

Tr.: trace amount <0.05%

MIC with those of essential oils extracted from other *Zanthoxylum* species. Essential oils from *Z. alatum, Z. xanthoxyloides*, *Z. leprieurii* and *Z. molle* could completely inhibit *A. flavus* growth at the concentrations of 1.25, 3, > 5 and 8 μL/mL, respectively [21-23]. Furthermore, the essential oils from the seeds of *Zanthoxylum* species have been reported in suppressing growth of bacteria and other fungi [12,24,25].

The inhibitory effect of ZSEO on mycelial dry weight was determined in PDB (Table 2). All concentrations of the essential oil were observed to be effective in inhibiting the biomass production of *A. flavus*. Mycelial growth was decreased by approximate 50% of the control at 2.0 μL/mL. ZSEO was found most effective (no observed mycelia) at 4.5 μL/mL (3.94 mg/mL) concentration. The essential oil showed a significant effect in reduction of the mycelial biomass. A biomass reduction in *Aspergillus* spp. and other fungi by other essential oils also occurred in previous researches [16,17,21,26]. These results indicate that the essential oil of *Z. piperitum* significantly restricts mycelial growth and the biomass of mycelia of *A. flavus* in a dosage-responsive manner.

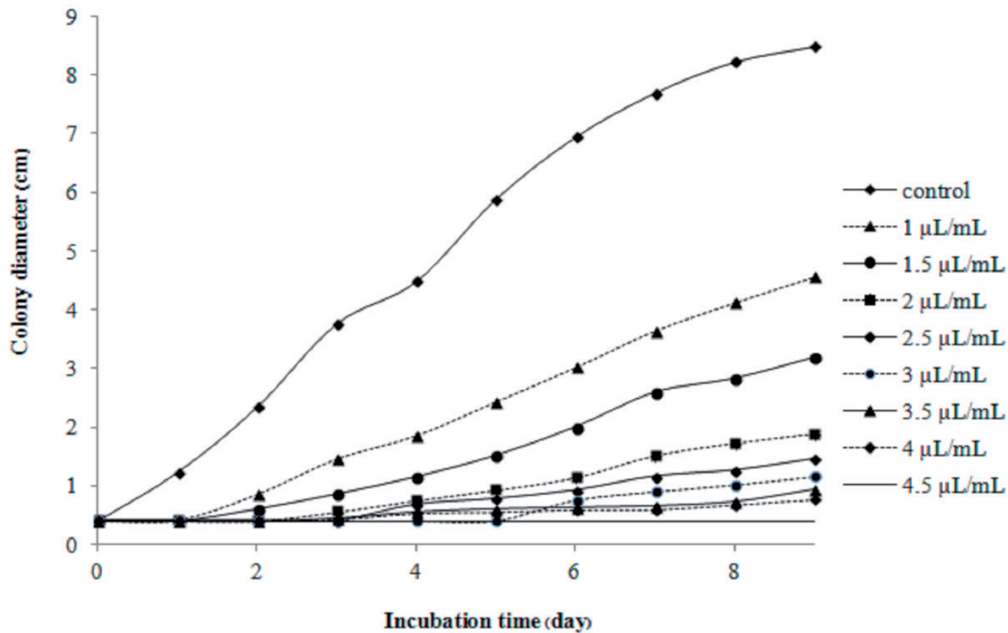

Figure 1. Effects of the different concentrations of essential oil from *Z. piperitum* seeds on the colony diameter (cm) growth of *A. flavus* in PDA. The plates were incubated at a temperature of 28 ± 2 °C for 9 days.

Table 2. Mean dry mycelium weight (g) of 9-day old *A. flavus* in liquid medium added with the essential oil from *Z. piperitum* seeds at eight different concentrations.

Concentration of *Z. piperitum* oil (µL/mL)	Dry mycelium weight* (g)	% Inhibition
Control	0.1346±0.0027[a]	-
1.0	0.1159±0.0056[b]	13.89
1.5	0.0935±0.0117[c]	30.53
2.0	0.0656±0.0076[d]	51.26
2.5	0.0376±0.0023[e]	72.06
3.0	0.0332±0.0011[e]	75.33
3.5	0.0296±0.0006[e,f]	78.00
4.0	0.0251±0.0026[f]	81.35
4.5	0.0047±0.0047[g]	96.51

*Values are mean (n = 3) ± standard deviations. Values followed by the same letter in each column are not significantly different in ANOVA and Duncan Multiple Range Test ($p < 0.05$).

The effect of ZSEO on *A. flavus* mycelial structure was examined using a light microscope at 40x magnification. Figure 2 shows the results of fungus treated with 1.0 μL/mL of essential oil and of non-treated fungus. The hyphal structure of the control was regular and homogeneous, with cylindrical principal axes (Figure 2A). Large and radiate conidial heads and the conidiophore were clearly visible (Figure 2C). On the other hand, *A. flavus* treated with ZSEO exhibited degenerative alterations, including decreased diameters of hyphae and distortion of conidial heads (Figure 2B and 2D). These results are similar to those from some previous work in which the microstructure of *Aspergillus* spp. treated with essential oils was studied [15,27,28]. The components of essential oil may interfere with the enzymatic synthesis of cell walls. Also, some alteration induced by essential oil may cause a lack of cytoplasm, damage of integrity, and, ultimately, mycelial death due to the cell wall structure [29].

Ergosterol, the end-product of the biosynthetic pathway and the main sterol in eukaryotic cells, is responsible for structural membrane characters, such as fluidity and permeability. This is similar to the way cholesterol functions in mammalian cells. Some studies have shown that essential oil can cause a significant reduction in the quantity of ergosterol [26]. In this study, the ergosterol content was determined by previously described methods [17]. This

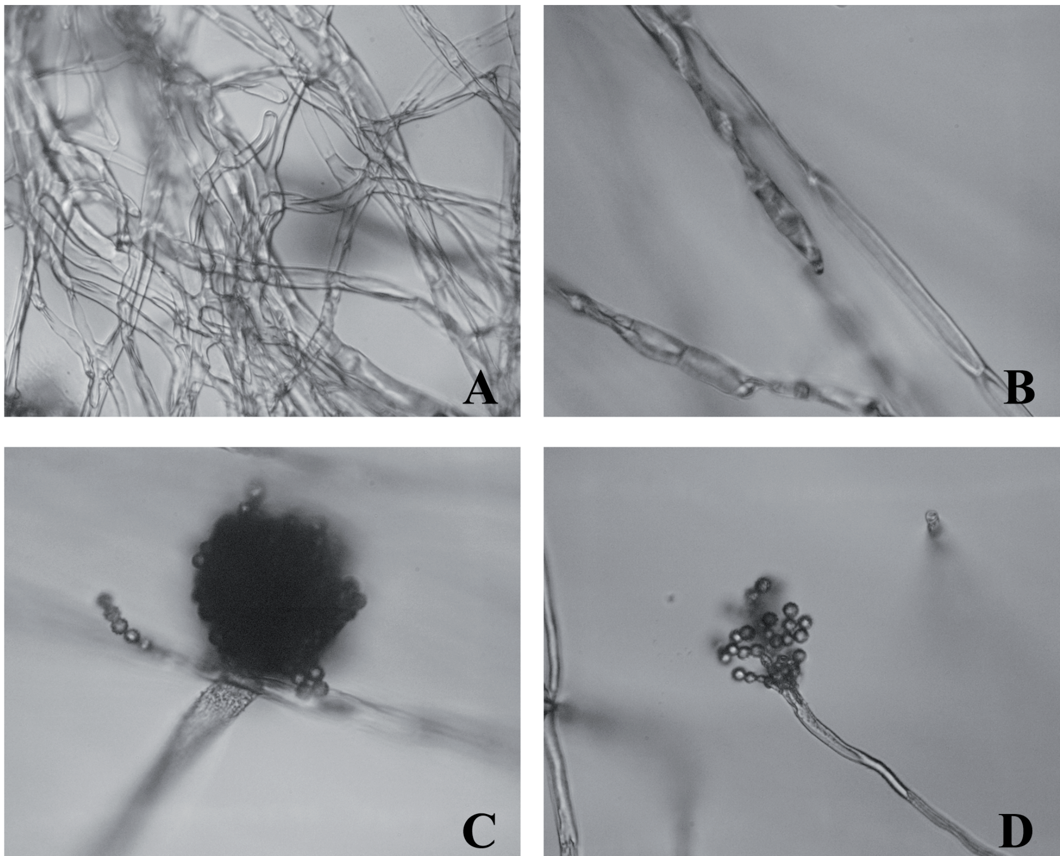

Figure 2. Morphology of *A. flavus* under 40X microscope on PDA without (A, C) and with the essential oil from *Z. piperitum* seeds (B, D) at 1 μL/mL, indicating hyphae (A, B) and conidial head(C, D).

sterol quantifying method is indicative of the ergosterol and 24(28) dehydroergosterol contents based on the exclusive spectral absorption at 230 and 282 nm of extracted sterols. The results indicated that the ergosterol content in the plasma membrane of *A. flavus* was greatly reduced by ZSEO. After incubation of *A. flavus* with 1.0 μL/mL ZSEO, a reduction of the ergosterol content to 34.41% in the plasma membrane was observed compared to the control. In previous works, the plasma membrane was the target of essential oils supported by the damage seen under SEM or TEM [17,28]. Thus, the plasma membrane is an important antifungal target of ZSEO, which could lead to structure and metabolic changes in the fungal cell, inhibition of extra cellular enzyme reaction, the electron transport chain, H^+-ATPase inhibition, the disruption of the cell wall structure involved in leakage of cytoplasmic contents, and even cell necrosis, as in other essential oils [29]. The mode of action of this class of compounds is not fully understood, but it is regarded as involving membrane destruction due to its lipophilic characteristic [30]. Essential oil passes freely through cell membranes because of its low molecular weight and highly lipophilic components [31]. ZSEO exhibited high antifungal activities, which may be attributed to its high content of β-phelladrene, sabinene, and brevifolin. However, its antifungal activity may be attributed to some minor components that have a synergistic effect with the major components. Thus, the essential oil of *Z. piperitum* has potential for development as a food preservative.

3.3 The Essential Oil as an Antifungal Coat on Dried Bird Chili

Dried bird chili, a model product, coated with ZSEO was tested for its susceptibility for *A. flavus* infection. The results of the pour plate culture at 10^{-5} dilution of infected chili are shown in Figure 3. After incubation at 28 °C for nine days, the essential oil at concentrations of 4.5 and 9.0 μL/mL (3.94 and 7.88 mg/mL) resulted in reduction of fungal development by 45.20 % and 89.32 %, respectively (Figure 3B and 3C), compared with the control (Figure 3A). In summary, the essential oil of *Z. piperitum* has potential as an ideal antifungal agent against food spoilage fungi and as a safe preservative in foods.

The model product experiment showed lower resistance to fungal infection because humidity conditions and nutrition in food favor spoilage fungi growth more than in laboratory media [32]. Thus, the model products are required higher concentrations of essential oils than in laboratory media in order to completely inhibit fungal growth. The use of essential oils can safely inhibit microorganisms in food without residues after storage. However, the essential oils have limitation of use because they have strong

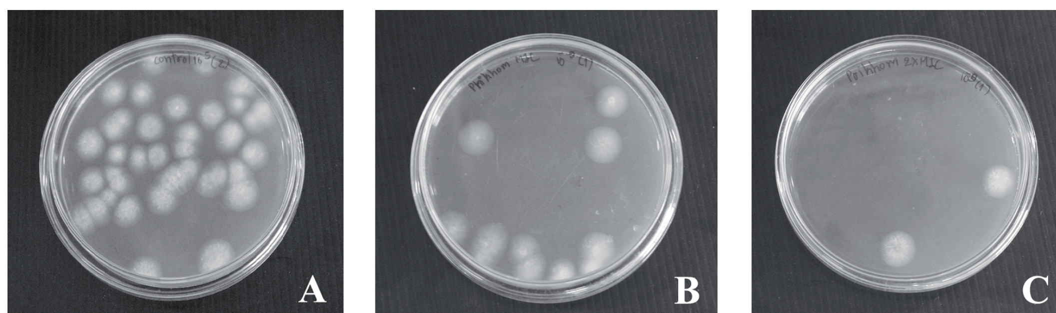

Figure 3. Pour plates cultures after 9 d of incubation of dried bird chili non-coated (A), coated with 4.5 μL/mL (B) and 9.0 μL/mL essential oil from *Z. piperitum* seeds (C).

flavors and have applicability only in products with compatible flavor. In addition, the use of essential oil in food has to meet the standard safety limit values and sensory characteristics such as color, aroma, and firmness [14].

4. Conclusion

The present study has demonstrated an antifungal activity of the essential oil extracted from *Zanthoxylum piperitum* seeds both in solid and liquid culture. The destructive action in *Aspergillus flavus* has been confirmed by light microscope and reduced content of ergosterol, an important lipid in membrane integrity. The essential oil is a promising food preservative as tested in dried chili model. The practical use to control postharvest spoilage, though, has to be further investigated.

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