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In vitro biological activities of clove essential oil formulations against *Microsporum gallinae* ATCC 90749

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ABSTRACT: Avian dermatophytosis is a disease caused by *Microsporum gallinae*. The disease can cause economic loss in farmed chickens. We investigated the antifungal activity of clove essential oil (CO) against *M. gallinae* ATCC 90749. The main components of CO were identified by GC-MS, eugenol (98.87%) and trans-caryophyllene (1.13%) were found. The antioxidant activity of CO was determined by DPPH assay, the antioxidation index (AI) ranged from 86.55 ± 0.77 to 92.37 ± 0.25%, which was significantly higher than vitamin E and BHT ($p < 0.05$). The antifungal activity was determined by broth microdilution method and time-kill kinetics assay. The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of CO against *M. gallinae* were 0.005 and 0.01% v/v, respectively. Time-kill assay showed that the antifungal activity of CO was dose and time-dependent. The time taken to reduce the number of viable *M. gallinae* by more than 99.99% was 15 min and 3 h for 2 and 1% v/v CO, respectively. Ointments and cream formulations containing CO were tested for antifungal activity using time-kill kinetics assay. Both the 2 and 4% w/w formulations of the CO ointments and creams reduced the number of viable *M. gallinae* by more than 99.999% within 15 min. The results of this study show that CO has the potential to be developed as a drug for treating fungal skin diseases in poultry. Further studies on the stability and efficacy of these CO formulations *in vivo* are needed.

KEYWORDS: clove essential oil, antioxidant, anti-*M. gallinae*, time-kill assay, ointment and cream formulations

INTRODUCTION

Microsporum gallinae is a fungus of the genus *Microsporum* that causes avian dermatophytosis or favus. Avian dermatophytosis is a sporadic disease found throughout the world in chickens, fighting cocks and poultry farms [1]. Lesions appear on the head, wattles, and neck and have white crusts, hyperkeratosis and may include feather loss. It causes the animals distress and affects economic output by decreasing the carcass quality of the animals in the slaughterhouse [2]. Hubalek [3] reported that the prevalence of fungal skin diseases in chickens was 2.6%. Nweze [4] found the prevalence of fungal skin diseases was 18.2% in chickens and 13.6% in ducks, with *M. gallinae* isolated from 75% of infected chickens and 100% of infected ducks. *M. gallinae* has also been reported to infect dogs, monkeys, cats, squirrels, mice, canaries, pigeons, and turkeys, as well as causing ringworm in humans, and severe infections in immunocompromised hosts [5, 6].

The current treatment for avian dermatophytosis uses antifungal drugs such as miconazole and tolnaftate creams, while pentachlorophenol and 5-bromosalicyl-4-chloranilide are used as a dip [7]. These drugs can cause adverse drug effects such as irritation, redness, and itching of the skin and can be toxic to the liver, kidneys, circulatory system and nervous system. If used at high concentrations, they may be harmful to users and animals. In addition, drug use must be discontinued in livestock animals to allow for the withdrawal of drugs before sending to the slaughterhouse [8]. Therefore, there are many studies investigating natural substances as alternative antifungal drugs.

Cloves are the dried aromatic flower buds of a tree in the family Myrtaceae (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) and are used in traditional medicine as an anti-inflammatory, cough suppressant, local anaesthetic, and to reduce toothache and relieve colic. In addition, cloves have antioxidant activity, and are highly effective against bacteria, fungi, and viruses. The constituents of clove es-

sential oil are mainly eugenol, eugenol acetate, and β-caryophyllene [9]. A review of the literature indicated that clove essential oil has good antifungal activity against *Microsporum canis*, *Microsporum gypseum*, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, and *Epidermophyton floccosum* [10, 11]. However, information about the antifungal activity of clove essential oil against *M. gallinae* is limited. Therefore, we investigated the *in vitro* efficacy of clove essential oil against *M. gallinae* causing avian dermatophytosis.

MATERIALS AND METHODS

Materials

M. gallinae ATCC 90749 was obtained from the American Type Culture Collection (ATCC), Virginia, USA. Clove essential oil was purchased from Thai-China Flavors and Fragrances Industry Co., Ltd., Ayutthaya, Thailand, batch no. 6010334-1. The essential oil was extracted from flower buds of *S. aromaticum* (L.) Merr. & L.M. Perry) by steam distillation. Ketoconazole and clotrimazole were from Sigma-Aldrich, Germany. Other chemicals were purchased from: dimethyl sulfoxide (DMSO) from V.S. Chem House, Ayutthaya, Thailand; absolute ethanol and hexane from Merck, Germany; tocopheryl acetate (vitamin E) from Namsiang Co., Ltd., Bangkok, Thailand; butylated hydroxytoluene (BHT) from Riedel de Haen, Lower Saxony, Germany; 2,2-diphenyl-l-1-picrylhydrazil (DPPH) from Fluka, Germany; Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) from Becton Dickinson, Grenoble, France. All of ingredients of the ointment and cream formulations were obtained from the Chemipan Corporation Co., Ltd., Bangkok, Thailand.

Determination of chemical composition by gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed according to the method previously described by Aiemsaard et al [12] and Amelia et al [13] with modifications for the Agilent CN10402086 gas chromatograph (separating vaporized mixtures instrument) interfaced with the Agilent US35120381 mass spectrometer (detector). The column used was a DB-5ms fused silica capillary column (30 m × 25 mm, film thickness 0.25 μm). The carrier gas was helium with a flow rate of 1 ml/min. The oven temperature was increased from 70–120 °C at a rate of 3 °C/min, then from 120–270 °C at 5 °C/min. The chemical constituents of clove essential oil were identified by comparing the results of the chromatogram and reference retention times.

Determination of antioxidant activity by DPPH assay

The DPPH assay was performed according to the method previously described by Lertsatitthanakorn et al [14]. Briefly, clove essential oil was diluted with DMSO to give a final concentration of 16% v/v and 50 μl was added to 50 μl of absolute ethanol in 96-well round-bottomed microtiter plates (Corning Incorporated, USA). Serial two-fold dilutions were done. Then 50 μl of 0.0004 M DPPH solution was added into all test wells and mixed for 5 min. The mixtures were incubated in the dark for 25 min at room temperature and their absorbance at 517 nm was recorded by Vis-spectrophotometer (Genesys 10 VIS, Thermo Scientific, USA). BHT and vitamin E were used as positive antioxidant controls. The percentage of DPPH radical inhibition (% antioxidation index; %AI) was calculated according to the following equation:

$$\%\mathrm{AI} = \frac{\mathrm{Abs_{ct}} - (\mathrm{Abs_s} - \mathrm{Abs_b})}{\mathrm{Abs_{ct}}} \times 100,$$

where $\mathrm{Abs_{ct}}$ represents the absorbance of the sample containing all reagents except the antioxidant, $\mathrm{Abs_s}$ is the absorbance of the sample containing an antioxidant, and $\mathrm{Abs_b}$ is the absorbance of the sample containing all reagents except antioxidant and DPPH.

Determination of antifungal activity by broth microdilution method

The MICs and MFCs of clove essential oil were determined by the broth microdilution method according to Chamdit et al [15] with modification. Briefly, 50 μl of SDB was added to all wells of a 96-well round-bottomed microtiter plate. Clove essential oil was diluted with DMSO to give a final concentration of 5% and added to each well of the first column. Serial two-fold dilutions were done from the first to the tenth columns. Fifty microliters of *M. gallinae* suspension (10^4–10^5 CFU/ml) was added into wells from the first to eleventh columns. The wells of the eleventh and twelfth columns were used as positive and negative growth controls. The plate was incubated at room temperature (25–30 °C) for 72–96 h. Ketoconazole and clotrimazole were used as antifungal controls. DMSO was used as solvent control. The MIC was determined from the lowest

Table 1 Ingredient of ointment and cream formulations (100 g).

Formulation	Phase	Ingredient	Amount (g)
Ointment	Oil	Stearyl alcohol	25
		White soft paraffin	25
	Water	Sodium lauryl sulfate	1
		Propylene glycol	12
		Methylparaben	0.025
		Propylparaben	0.015
		Distilled water	Add to 100
Cream	Oil	Mineral oil	10
		White beeswax	5
		Cetyl alcohol	5
		Span 60	2.5
	Water	Tween 60	7.5

concentration of antifungal agent inhibiting visible growth after 72–96 h of incubation. Ten microliters from the wells with no visible growth was inoculated onto SDA plates and incubated at room temperature for 72–96 h. The MFC was determined from the lowest concentration of antifungal agent that inhibited growth on SDA. All tests were performed in eight duplicates.

Determination of antifungal activity by time-kill kinetics assay

The antifungal activity of clove essential oil was studied according to Chamdit et al [15] with modifications. Briefly, 100 µl of *M. gallinae* suspension (10^4–10^5 CFU/ml) was mixed with clove essential oil and diluents in microcentrifuge tubes (1.5 ml) to give final concentrations of 10, 100, 200 and 400 times of MIC in 1000 µl, then mixed by vortex mixer for 1 min. After incubation for 15 and 30 min, 3, 6 and 24 h at room temperature, 100 µl from each microcentrifuge tube was inoculated onto SDA. Each experiment was performed in triplicate.

Formulation preparation, and antifungal activity evaluation

Clove essential oil was prepared as two formulations: ointment and cream. The composition and amounts of the formulation ingredients are shown in Table 1. Both the oil phase and water phase were separately heated up to 75–80 °C. Then, the water phase was added to the oil phase with continuous stirring. The appropriate amount of clove essential oil was added to give final concentrations of 2% and 4% v/w, and then stirring continued until ointment and cream were formed. The antifungal activity was performed by the time-kill kinetics assay as described above.

Table 2 Chemical composition of *S. aromaticum* (clove essential oil) as determined by GC-MS.

Component	Molecular formula	Retention time (min)	Area (%)
Eugenol	$C_{10}H_{12}O_2$	23.37	98.87
Trans-caryophyllene	$C_{15}H_{24}$	26.35	1.13

Statistical analysis

Each experiment was performed in either triplicate or eight duplicates and the data were reported as mean ± standard deviation. One-way ANOVA was used to compare means ($\alpha = 0.05$). All analyses were performed using SPSS for Windows version 19.0 (SPSS Inc., USA), KKU license.

RESULTS AND DISCUSSION

Chemical composition and antioxidant activity of clove essential oil

GC-MS analysis showed that the tested CO consisted of 2 main constituents: eugenol, which had the highest concentration (98.87%), and trans-caryophyllene with a concentration of 1.13% (Table 2). This result was in accordance with the previous studies of Pumnuan et al [16], which showed 97.1% eugenol and 1.685% trans-caryophyllene, and Asawapattanakul et al [17], which showed the concentration of eugenol and trans-caryophyllene as 98.87 % and 1.13 %, respectively. The results of multiple studies have shown the main constituents of CO are more than 70% eugenol, followed by 5–15% of eugenyl acetate and β-caryophyllene [18–20]. Differences in the chemical composition of CO depend on the environment and geography of the cultivated area, plant part used, as well as the storage conditions and extraction methods used [21, 22].

The antioxidant activities of the tested CO, as determined by DPPH assay, are shown in Table 3. All concentrations of CO (0.125–8%) had a strong antioxidant effect (range 86.55–92.37%AI), which was higher than the %AI of 1–8% BHT and all concentrations of vitamin E ($p < 0.05$). Multiple previous studies have shown that CO has a high antioxidant capacity tested by DPPH assay [23, 24], which is consistent with the findings of our study. Hemalatha et al [22] reported that 0.02% CO had %AI ranging from 85.3–92.2%, which was greater than that for vitamin E (84.1%AI). Moreover, the

Table 3 Antioxidation indices of tested concentrations (0.125–8%) of clove essential oil (CO), vitamin E and butylated hydroxytoluene (BHT).

Substance	Antioxidation index (%)						
	0.125%	0.25%	0.5%	1%	2%	4%	8%
CO	86.55 ± 0.77^{a}	87.88 ± 0.66^{a}	89.65 ± 0.58^{a}	91.41 ± 0.22^{a}	91.12 ± 0.34^{a}	92.30 ± 0.22^{a}	92.37 ± 0.25^{a}
Vitamin E	20.25 ± 0.43^{b}	32.84 ± 2.17^{b}	48.95 ± 1.91^{b}	62.50 ± 1.80^{c}	88.58 ± 0.43^{b}	89.07 ± 0.32^{b}	89.41 ± 0.12^{b}
BHT	87.76 ± 1.24^{a}	88.38 ± 0.83^{a}	89.69 ± 1.06^{a}	87.97 ± 0.83^{b}	88.24 ± 0.48^{b}	88.10 ± 0.72^{b}	88.10 ± 0.55^{b}

Values represent the means of triplicate experiments ± SD. Different superscript letters within a column indicate statistically significant differences between the means ($p < 0.05$).

result of Nassar et al [25] showed 83%AI for 0.04% CO, which was similar to BHT.

The antioxidant effect of CO is presumably due to the main component eugenol, which belongs to the group of phenolic volatile oils. Further antioxidant effects may come from other phenolic compounds such as eugenyl acetate, flavonoids, hydroxybenzoic acids, hydroxycinnamic acids, hydroxyphenylpropenes, and others [26]. Eugenol has radical scavenging and metal ion chelating properties, acting as a reducing agent, hydrogen atom donator and singlet oxygen scavenger. When the hydrogen atom from the phenolic hydroxyl group is given to the DPPH phenoxyl radical (DPPH·), the radical is changed to its reduced form (DPPH-H), leading to increased stability and stopping the chain reaction of oxidation [27, 28]. Excessive production of reactive oxygen species is called oxidative stress, which is commonly found in inflamed tissues due to infection, allergies, poisoning, and autoimmune diseases. Excessive release of proinflammatory cytokines during the immune response causes cell and tissue damage, and oxidative stress consequently follows. While inflammation is producing oxidative stress, the reactive oxygen species and other oxidative agents increase the severity of inflammation. For these reasons, inhibiting the chain reaction of oxidation can reduce the inflammatory process in affected tissues [29, 30].

Antifungal activity of clove essential oil against *M. gallinae* ATCC 90749

The antifungal activities of CO and two antifungal drugs, clotrimazole and ketoconazole, against *M. gallinae* ATCC 90749 are shown in Table 4. The CO and both antifungal drugs showed high effectiveness against *M. gallinae*. The MIC of CO (0.005% v/v) was 2 times lower than its MFC (0.01% v/v), while MICs of ketoconazole and clotrimazole (0.000048 and 0.000024% w/v) were 4 and 83 times lower than their MFCs (0.0002 and 0.002% w/v), respectively. Although ketoconazole and clotrimazole had higher antifungal efficacies than CO, CO provided good results in this study when compared to the disadvantages of antifungal drugs such as side effects, fungal-resistance, and high price. Although no previous study has reported activity of CO against *M. gallinae*, many studies have reported antifungal activity of CO against other *Microsporum* spp. and dermatophytes [31, 32]. The report of Pinto et al [33] showed that CO had MICs and MFCs against *M. canis* and *M. gypseum* (standard strains) in the range of 0.00625–0.05% w/v. Aiemsaard et al [34] reported MIC and MFC of CO against *M. canis* and *T. mentagrophytes* were 0.00002% v/v and 0.00004% v/v, and the MIC and MFC against *M. gypseum* were 0.00004% v/v. Our results for the standard antifungal drug activities against *M. gallinae* corresponded to the study of Fernandez-Torres et al [35] who reported ketoconazole and clotrimazole MICs for *M. gallinae* of 0.000025 and 0.000001% w/v, respectively.

Table 4 Minimum inhibitory concentrations and minimum fungicidal concentrations of clove essential oil (% v/v) and antifungal drugs (% w/v) against *M. gallinae* ATCC 90749 ($n = 8$).

Antifungal agent	MIC	MFC
Clove essential oil	0.005 ± 0.00^{b}	0.01 ± 0.00^{b}
Clotrimazole	0.000024 ± 0.00^{d}	0.002 ± 0.00^{c}
Ketoconazole	0.000048 ± 0.00^{c}	0.0002 ± 0.00^{d}

Values represent the means of eight fungal samples ± SD. Different superscript letters within a column indicate statistically significant differences between the means ($p < 0.05$).

Time kill assay of clove essential oil on *M. gallinae* ATCC 90749

The time-kill kinetics of antifungal activity of CO against *M. gallinae* ATCC 90749 is given in Fig. 1.

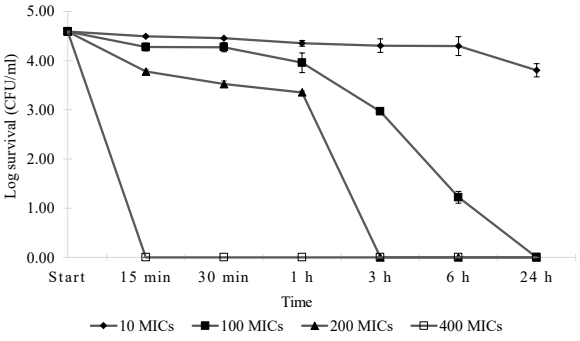

Fig. 1 The time-kill assay of clove essential oil against *M. gallinae* ATCC 90749. Values represent the means of triplicate experiments with error bars (SD). 10 MICs = 0.05% v/v, 100 MICs = 0.5% v/v, 200 MICs = 1% v/v, 400 MICs = 2% v/v.

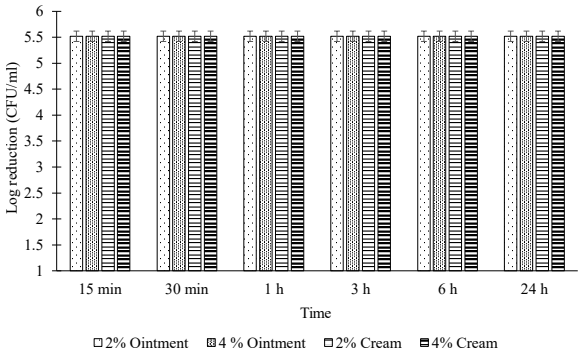

Fig. 2 The time-kill assay of clove essential oil ointment and cream against *M. gallinae* ATCC 90749. Y-axis shows log reduction of viable fungal cells. Values represent the means of triplicate experiments with error bars (SD).

The results demonstrated that the effectiveness of CO is dose and time-dependent. The solution of CO at 400 times its MIC (20 µl/ml; 2% v/v) possessed the most potent anti-*M. gallinae* effect, eradicating more than 99.99% (4-log reduction) of the inoculum within 15 min. The second most effective concentration of CO was 200 times MIC (10 µl/ml; 1% v/v), which gave a 90% reduction in viable fungi (1-log reduction) at 30 min and 1 h, and more than 99.99% reduction at 3 h. This was followed by 100 times MIC (5 µl/ml; 0.5% v/v), which required at least 3 h to reduce the number of viable fungi by 90%, and at least 6–24 h for 99.99% reduction. The CO concentration at 10 times MIC (0.5 µl/ml; 0.05% v/v) did not have any antifungal activity against *M. gallinae* up to 6 h, but did reduce the number of fungi slightly after 24 h. Previous studies have suggested that the antifungal effect of CO results from eugenol, the main constituent, which destroys the fungal cell wall and cell membrane, and causes deformation of molecules within the cells [10, 36].

Antifungal activity of clove essential oil ointment and cream against *M. gallinae* ATCC 90749

Based on the results of the time-kill kinetic experiments, ointments and creams containing 2 and 4% v/w CO were formulated. The time-kill assays of the formulations (Fig. 2) show that both 2 and 4% CO formulations of ointment and cream decreased the number of viable fungal cells by more than 99.999% (5-log reduction) within 15 min. These results indicated that the CO contained in both formulations retained its efficacy against *M. gallinae* when used at concentrations of at least 2% of CO with 15 min of contact time. There are many studies supporting that CO in emulsion and nanoemulsion formulations has strong antifungal activity [37, 38]. The ointment developed in our study was a hydrophilic emulsion of water and oil. The cream was an oil-in-water formulation, which makes it less greasy and easier to wash off with water compared to oleaginous creams or water-in-oil creams. The emulsion formulas are suitable for both oil-soluble and water-soluble active ingredients and allow the drugs to disperse and penetrate the skin. Also, the emulsion acts as a moisturizer that promotes drug contact to the skin for a longer time, which is useful for antimicrobial drugs that need extended contact time with microbes to improve their effectiveness [39, 40].

In conclusion, CO has strong antioxidant and antifungal activity, which results from its main constituent eugenol. When prepared as ointment and cream with CO at concentrations of 20–40 µl/g (2–4% v/w), the formulations were found to be active against *M. gallinae*, reducing the number of viable fungal cells by more than 99.99% within 15 min. Further studies should examine the stability, *in vivo* antifungal efficacy and potential for irritation of the formulations.

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