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Antifungal activities of azole drugs in combination with clove essential oil against *Microsporum gallinae*

Kanlaya Worawong[a], **Glenn N. Borlace**[b], **Jareerat Aiemsaard**[a,*]

[a] Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002 Thailand
[b] Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand

*Corresponding author, e-mail: jaraim@kku.ac.th

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ABSTRACT: Azole drugs are effective for the treatment of *Microsporum gallinae* infection but fungal resistance to antifungal drugs, and their adverse reactions are concerns in both veterinary and public health. This study's objective was to investigate the antifungal effects of combining clove essential oil with clotrimazole, ketoconazole, or miconazole against *M. gallinae* ATCC 90749. Antifungal activity was determined by broth microdilution, checkerboard synergy method, and time-kill tests. The minimum inhibitory concentrations (MICs) of azole drugs and clove essential oil were 0.05 and 64.00 µg/ml, respectively. Clove essential oil and the azole drugs showed partial synergistic effects with fractional inhibitory concentration (FICI) values of 0.75, and MIC values for clove essential oil (32 µg/ml) and azole drugs (0.125 µg/ml) were 2- and 4-times lower in combination, respectively. The time-kill kinetics revealed that the killing effect of the combinations was dose- and time-dependent. Formulated topical solutions of clove essential oil and azole drug combinations showed very strong antifungal activity against *M. gallinae* ATCC 9074. The results of this study indicated that clove essential oil has the potential to be used in combination with azole drugs for the control of *M. gallinae* infection.

KEYWORDS: antifungal activity, azole drugs, clove essential oil, *Microsporum gallinae*

INTRODUCTION

Azole antifungal agents have been used to treat fungal infections in humans and animals for a long time. In animals, the imidazole derivatives; i.e., clotrimazole, ketoconazole, and miconazole are available for topical and systemic applications to treat infections arising from the major animal pathogenic fungi (*Candida* spp., *Cryptococcus neoformans*, *Malassezia pachydermatis*, and *Aspergillus fumigatus*) and dermatophytes such as *Trichophyton mentagrophytes*, *Microsporum canis*, *Microsporum gypseum*, and *M. gallinae* [1, 2]. However, resistance to azole drugs is emerging in veterinary and public health cares, especially cross-resistance between animal and human pathogens [3], resulting in treatment failure, increased costs of medical treatment, and risks to animal and human health [4]. Azole drugs can also cause side effects in animals ranging from irritation of the skin at the site of application of topical agents to gastrointestinal effects, such as vomiting and diarrhea, resulting from systemic use [5].

M. gallinae is the most common cause of avian dermatophytosis. This fungus causes chronic dermatitis with hyperkeratosis and white skin scale on the bird's comb and wattle and can affect the carcass quality and productivity of farmed poultry [6, 7]. *M. gallinae* can also cause superficial mycoses and acute erythematous and painful dermatitis in mammals such as dogs, cats, squirrels, mice, monkeys, and humans [8–10]. Commercially available topical azole drug formulations, such as 2% w/w ketoconazole solution and cream, 1% w/w clotrimazole cream, and 2% miconazole cream and shampoo, are commonly employed for the treatment of *M. gallinae* infection. However, at high concentrations, these drugs can cause side effects to animals. Therefore, there have been interests in the efficacy of natural products and combinations of natural products with conventional antimicrobials to potentially reduce the amount of antimicrobial drug required to treat an infection [11].

Clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) essential oil contains terpene and terpenoid compounds, such as eugenol, caryophyllene, eugenyl acetate, and α-humulene, with outstanding antimicrobial activity as well as antioxidant and anti-inflammatory properties [12, 13]. Our previous studies demonstrated that high concentrations of clove essential oil (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) were effective against *M. gallinae in vitro* and against *M. gallinae* induced dermatitis *in vivo* [12, 14]. Therefore, the current study investigated whether solutions of clove essential oil combined with clotrimazole, ketoconazole, and miconazole showed enhanced antifungal activity against *M. gallinae* ATCC 90749 to potentially reduce the drug concentration required for treatment of *M. gallinae* infections.

MATERIALS AND METHODS

Materials

The fungus *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., Thai-

land. The essential oil was extracted from flower buds of *S. aromaticum* (L.) Merr. & L.M. Perry by steam distillation and contained 98.87% eugenol and 1.13% trans-caryophyllene as determined by gas chromatography-mass spectrometry (GC-MS) in a previous study [12]. Clotrimazole, ketoconazole and miconazole were from Sigma-Aldrich, Germany. Sabouraud dextrose agar (SDA) was from Becton Dickinson, France. RPMI-1640 medium was from Millipore, USA. All other ingredients in the topical solution formulations were pharmaceutical grade and obtained from the Union Science Trading Co., Ltd., Thailand.

Fungal culture conditions

The fungus *M. gallinae* ATCC 90749 was cultured on SDA at 30 °C for 7 days. Fungal suspensions were prepared by adding 1 ml of sterile phosphate buffered saline (PBS) pH 7.4 to the culture plates and collecting fungal fragments with a triangle-shaped glass rod spreader [15].

Evaluation of antifungal activity by broth microdilution test

The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of clove essential oil, clotrimazole, ketoconazole, and miconazole against *M. gallinae* ATCC 90749 were determined by the broth microdilution method according to the Clinical and Laboratory Standard Institute [15]. This protocol is a reference standard for susceptibility testing of filamentous fungi (including dermatophytes) to antifungal agents by measuring MIC and/or MFC values. Lower values indicate higher fungal susceptibility to the antifungal agent. Briefly, serial two-fold dilutions of the tested substances (0.5–1,024 µg/ml for clove essential oil and 0.0156–32 µg/ml for clotrimazole, ketoconazole, and miconazole) were prepared with RPMI-1640 medium in 96-well round-bottomed microtiter plates (Corning Incorporated, USA). Then, a 2×10^3 CFU/ml fungal suspension was added into each well. The wells containing only medium and medium with fungus were used as negative and positive-growth control wells, respectively. The plates were incubated at 35 °C for 96 h. The MIC was defined as the lowest concentration of the agent that prevented visible growth after 96 h of incubation. Ten microliter samples from the wells with no visible growth were inoculated onto SDA and incubated at 30 °C for 96 h. The MFC was determined from the lowest concentration of agent that showed no growth on SDA. All tests were performed in triplicate.

Evaluation of synergistic effects by checkerboard method

Checkerboard method was performed as previously described with some modifications [16]. The method, a broth microdilution assay, tests two antifungal agents simultaneously and generates MIC values for individual agents and the combinations. Briefly, serial two-fold dilutions of clotrimazole, ketoconazole, and miconazole were prepared with RPMI-1640 medium in 96-well round-bottomed microtiter plates to give a final concentration range of 0.0039–1 µg/ml over the columns. Pre-diluted 50 µl aliquots of clove essential oil in RPMI-1640 medium were, then, added to achieve a final concentration range of 4–256 µg/ml over the rows. One hundred microliters of fungal suspension (2×10^3 CFU/ml) were added into all tested wells. Wells containing medium only and medium with fungus were used as negative- and positive-growth control wells, respectively. After incubation at 35 °C for 96 h, the MIC values of clotrimazole, ketoconazole, miconazole, and clove essential oil alone and in combination were determined. The fractional inhibitory concentration indices (FICIs) were calculated using the following formula: $\text{FICI} = (\text{MIC}_{\text{azole drug in combination}} \,/\, \text{MIC}_{\text{azole drug alone}}) + (\text{MIC}_{\text{clove essential oil in combination}} \,/\, \text{MIC}_{\text{clove essential oil alone}})$. Indicative effects of FICI values were: $\leqslant$0.5, synergistic; >0.5 – <1.0, partial synergistic; 1.0, additive; >1.0 – <4.0, indifferent; and $\geqslant$4.0 antagonistic. All tests were performed in triplicate.

Preparation of topical solutions

The ingredients of the topical solution base and azole drugs in combination with clove essential oil are shown in Table 1. Set weights of clotrimazole, ketoconazole, or miconazole (0.25 and 0.5 g) were dissolved in polyethylene glycol 40 hydrogenated castor oil at 60 °C with stirring. Then, clove essential oil (0.75 and 1.5 g) was added and mixed thoroughly (solution A). Methylparaben and propylparaben were dissolved in 96% ethyl alcohol, then distilled water was added and the solution was heated to 60 °C (solution B). Solution B was added slowly to solution A with continuous stirring until a clear solution was obtained [17].

Determination of time-kill kinetics

The time-kill kinetics of the standard and topical solutions of clotrimazole, ketoconazole, miconazole and clove essential oil alone and in combination against *M. gallinae* ATCC 90749 were performed as previously described with some modifications [12]. Briefly, 100 µl of fungal suspension (1×10^6 CFU/ml) was mixed with 900 µl of clotrimazole, ketoconazole, miconazole, or clove essential oil alone or individual azole drugs combined with clove essential oil diluted with PBS to a final concentration of 1, 4, 16, 64, and 256 times their respective MICs, or with 900 µl of each topical solution formulation (F1–F7) to determine the time-kill kinetics of the formulations. After incubation for 2, 4, 6, and 8 h at 30 °C, 100 µl of the mixture was 10-fold diluted with PBS to stop the antimicrobial activity of the agents. Then, 100 µl aliquots of dilutions 10^{-1}

Table 1 Ingredients of topical solutions A and B (100 g).

Ingredient		Amount (g)						
		F1	F2	F3	F4	F5	F6	F7
Solution A	Clotrimazole	0	0.25	0.5	0	0	0	0
	Ketoconazole	0	0	0	0.25	0.5	0	0
	Miconazole	0	0	0	0	0	0.25	0.5
	Clove essential oil	0	0.75	1.5	0.75	1.5	0.75	1.5
	Polyethylene glycol 40 hydrogenated castor oil	30	30	30	30	30	30	30
Solution B	Methylparaben	0.4	0.4	0.4	0.4	0.4	0.4	0.4
	Propylparaben	0.08	0.08	0.08	0.08	0.08	0.08	0.08
	96% ethyl alcohol	34	34	34	34	34	34	34
	Distilled water	Added to 100 g						

Table 2 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of azole drugs and clove essential oil against *M. gallinae* ATCC 90749.

Antifungal agent	MIC (µg/ml)	MFC (µg/ml)
Clove essential oil	64.00 ± 0.00	128.00 ± 0.00
Clotrimazole	0.50 ± 0.00	1.00 ± 0.00
Ketoconazole	0.50 ± 0.00	1.00 ± 0.00
Miconazole	0.50 ± 0.00	1.00 ± 0.00

Values represent means ± SD of triplicate experiments.

to 10^{-3} were inoculated onto SDA plates. After incubation at 30 °C for 96 h, visible colonies were counted and recorded. Each experiment was performed in triplicate.

Statistical analysis

The time-kill kinetics, MIC, MFC, and FICI results were analyzed using Microsoft Excel Spreadsheet Software (Official Microsoft 365®, Microsoft Corporation, USA) and described by descriptive statistics. The reduction in the number of viable fungal cells for each antifungal agent alone was compared to its combination with clove essential oil (at the same time point) by the Mann-Whitney U test (α=0.05) using IBM® SPSS® Statistics (International Business Machines Corporation; IBM, USA).

RESULTS

Antifungal activity of clotrimazole, ketoconazole, miconazole, and clove essential oil

The MIC and MFC results from the broth microdilution testing of the three azole drugs and clove essential oil against *M. gallinae* ATCC 90749 were shown in Table 2. MFC values were one dilution higher than MIC values for all tested antifungal compounds. The tested azole drugs had MIC and MFC values of 0.05 and 1.00 µg/ml, respectively. The clove essential oil MIC (64.00 µg/ml) and MFC (128.00 µg/ml) values were over 100 times higher than those of the azole drugs.

Antifungal synergy testing of azole drugs combined with clove essential oil

In combination, the individual MIC values for clove essential oil and the tested azole drugs against *M. gallinae* ATCC 90749 were lower than their respective MIC values alone (Table 3). For clove essential oil, the combination MIC was 32.00 µg/ml, half the MIC of clove essential oil alone (64.00 µg/ml), for all three combinations (fractional inhibitory concentration, FIC = 0.5). The combination MIC values for clotrimazole, ketoconazole, and miconazole were all 0.125 µg/ml, which was one quarter of their 0.5 µg/ml MIC values alone (FIC = 0.25). The FICI for each of the combinations was 0.75 µg/ml, indicating partial synergy between the clove essential oil and each of the tested azole drugs.

Time-kill kinetics of azole drugs and clove essential oil alone and combination

Fig. 1 shows time-kill results for clove essential oil, clotrimazole, ketoconazole, and miconazole against *M. gallinae* ATCC 90749 when tested alone and in combination at concentrations ranging from 1- to 256-times their MIC. When tested alone, clove essential oil showed a fungicidal effect (defined as a greater than 3-$\log_{10}$ reduction in the number of viable cells) against *M. gallinae* ATCC 90749 at 4,096 and 16,834 µg/ml (64- and 256-times MIC) at 2 h. Similarly, clotrimazole and miconazole were fungicidal at 128 µg/ml (256-times MIC) at 8 h when tested alone; but ketoconazole alone did not show a fungicidal effect at 256-times MIC at 8 h. In contrast, all of the clove essential oil/azole drug combinations were fungicidal at 2,048/8 and 8,192/32 µg/ml (64- and 256-times MIC) at 2 h. Thus, lower concentrations of the clove-essential oil/azole drug combinations were required for effective fungal killing than either constituent alone. When tested in combination with azole drugs, clove essential oil was fungicidal at 2 h at half the concentration required when tested alone (2,048 µg/ml versus 4,096 µg/ml, respectively). When tested alone, ketoconazole was

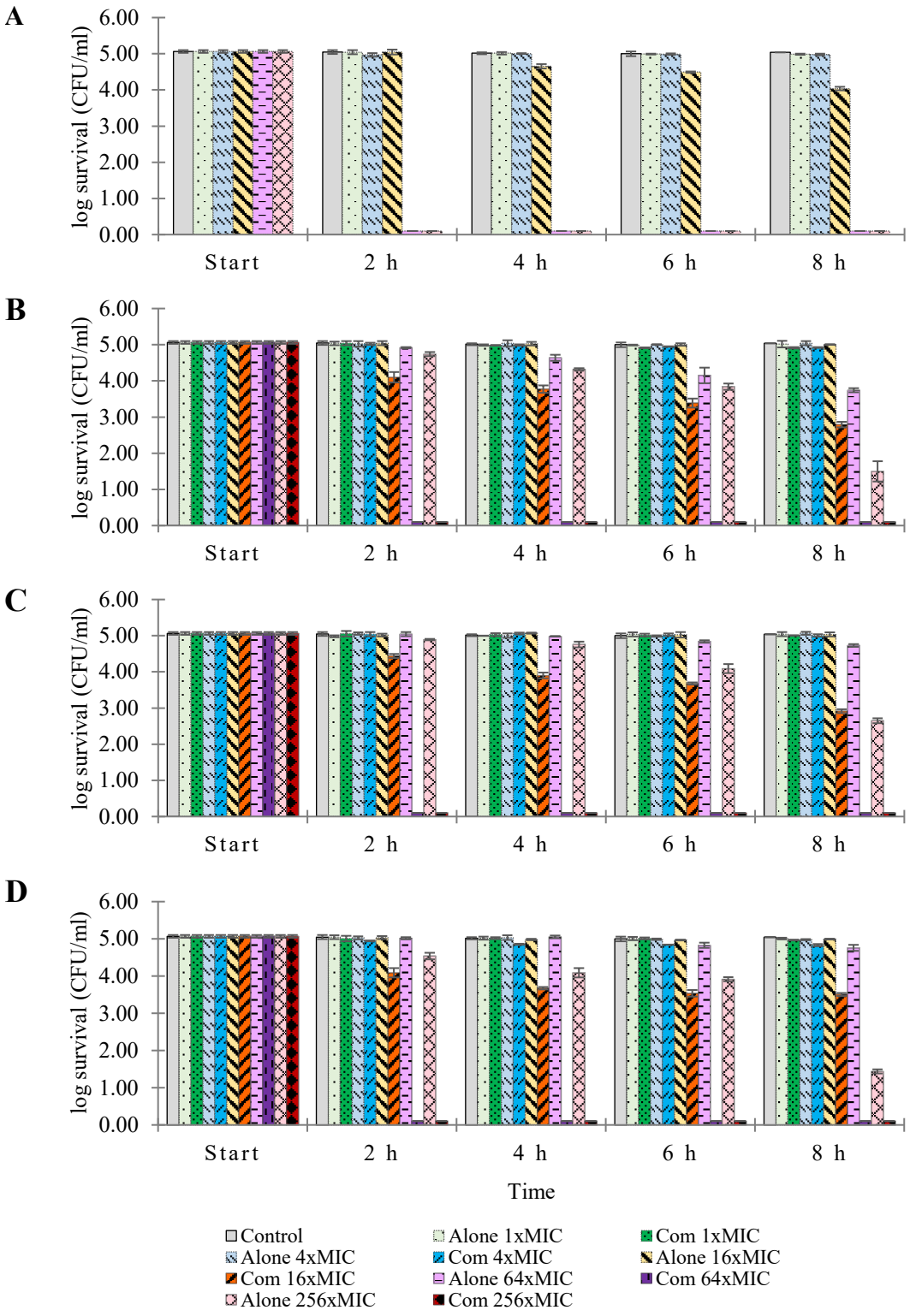

Fig. 1 Time-kill assays of clove essential oil and azole drugs, alone and in combination, against *M. gallinae* ATCC 90749. Clove essential oil alone (A): 1×MIC = 64, 4×MIC = 256, 16×MIC = 1,024, 64×MIC = 4,096, and 256×MIC = 16,384. Clotrimazole (B), ketoconazole (C), and miconazole (D) alone (Alone) and in combination (Com). Alone: 1×MIC = 0.5, 4×MIC = 2, 16×MIC = 8, 64×MIC = 32, and 256×MIC = 128. Com: 1×MIC = 32/0.125, 4×MIC = 128/0.5, 16×MIC = 512/2, 64×MIC = 2,048/8, and 256×MIC = 8,192/32. MIC = minimum inhibitory concentration expressed in μg/ml. Control = phosphate buffer saline. Values represent the mean of triplicate experiments with error bar (SD).

Table 3 Antifungal effect of azole drugs in combination with clove essential oil against *M. gallinae* ATCC 90749.

Antifungal agent	MIC (µg/ml)		FIC	FIC index[a]	Outcome
	Alone	Combination			
Clotrimazole	0.50	0.125	0.25	0.75	partial synergy
Clove essential oil	64.00	32.00	0.50		
Ketoconazole	0.50	0.125	0.25	0.75	partial synergy
Clove essential oil	64.00	32.00	0.50		
Miconazole	0.50	0.125	0.25	0.75	partial synergy
Clove essential oil	64.00	32.00	0.50		

[a] FIC index was interpreted as synergy at ⩽0.5, partial synergy (P) at >0.5 – <1.0, additive at 1.0, indifferent at >1.0 – <4.0, and antagonistic at ⩾4.

not fungicidal at 128 µg/ml after 8 h, but all three azole drugs were fungicidal at 8 µg/ml after 2 h when combined with clove essential oil at 2,048 µg/ml. The size of these reductions in the required concentrations of antifungal agents to have a fungicidal effect on *M. gallinae* ATCC 90749 correlated with the partial synergy seen between clove essential oil and the azole drugs in the checkerboard synergy testing assays.

Physical properties and time-kill kinetics of topical solutions

The formulated topical solutions had neutral pH (7.01±0.02 to 7.14±0.06) and were clear colorless liquids free from sedimentation and fractionation (Fig. S1). The time-kill assay of the formulations' antifungal activity against *M. gallinae* ATCC 90749 demonstrated that the solution-based control (F1) showed slightly antifungal activity (1-$\log_{10}$ reduction in the number of viable cells) that might be due to the alcohol solvent and parabens preservative present in the formula. All formulations of the azole drug and clove essential oil combinations showed very strong antifungal activity against *M. gallinae* ATCC 90749. No viable fungal cells were recovered after 2 h (5-$\log_{10}$ reduction; Table 4).

DISCUSSION

The principle of antimicrobial combination is to enhance the antimicrobial activity of combined antimicrobial agents of two or more. Combination can result in additive or synergistic enhancement of antimicrobial activity, or it can result in no effect, or the antimicrobial agents can be antagonistic in combination. Additive and synergistic effects between agents mean that they are effective at lower concentrations, which could reduce the risk of side effects. The results of the current study show that clove essential oil was partially synergistic with azole drugs, reducing MIC values for clotrimazole, ketoconazole, and miconazole against *M. gallinae* by a factor of four. There have been some previous reports on the synergistic effects of clove essential oil with other antimicrobial agents against *Candida* spp. Khan et al [18] reported that clove essential oil reduced MICs of fluconazole and amphotericin B against 20 fluconazole-resistant strains of *Candida albicans* by 2–4 times. Sharifzadeh and Shokri [19] found that eugenol, a key component of clove essential oil, decreased voriconazole MICs against veterinary isolates of *Candida tropicalis* and *Candida krusei* by between 5 and 18 times. Finally, Ahmad et al [20] showed that combining fluconazole with eugenol reduced fluconazole MICs against 100 clinical *Candida* isolates by 4–25 times.

The clove essential oil used in this study contained vastly more eugenol (98.87%) than trans-caryophyllene (1.13%). Therefore, the partially synergistic antifungal activity of the azole drugs and clove essential oil combinations seen in this study were likely to be due to the antifungal activities of the main constituent, eugenol [12, 21]. Azole drugs act on fungi by inhibiting ergosterol synthesis resulting in deformation of cell membranes, which can enhance the entry of eugenol into fungal cells [1]. Eugenol is a phenylpropanoid that shows antimicrobial activity at multiple target sites, inducing damage to mitochondria, cell membranes, and cell walls, leading to increased cell permeability, leakage of intracellular materials and cell lysis [22, 23]. One reason for the partial synergy seen in the checkerboard assay in the current study is that both eugenol and azole drugs target cell membranes. In addition, eugenol has been shown to interact with deoxyribonucleic acid (DNA) and change its structure, reduce the protein and nucleic acid content in cells, and decrease the activity of essential enzymes involved in energy metabolism such as sodium-potassium adenosine triphosphatase (Na^+K^+-ATPase), calcium (Ca^{2+})-ATPase, and magnesium (Mg^{2+})-ATPase [24]. These effects of eugenol on energy metabolism are particularly beneficial in reducing resistance to azole drugs in fungi, which is often attributed to energy-intensive drug efflux mechanisms [25].

Azole drugs are present at a concentration of 1–2% w/w in commercially available topical formulations in Thailand; thus the value was used as the basis for determining the concentration of the azole

Table 4 Log_{10} reductions of viable fungal cells after testing with azole drug/clove essential oil formulations.

Time (h)	Log_{10} reduction (CFU/ml)							
	Control	F1	F2	F3	F4	F5	F6	F7
2	0.01 ± 0.05	1.04 ± 0.05	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00
4	0.04 ± 0.03	1.18 ± 0.03	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00
6	0.05 ± 0.06	1.25 ± 0.06	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00
8	0.01 ± 0.00	1.60 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00	5.05 ± 0.00

Values represent the mean ± SD of triplicate experiments.
Control = phosphate buffer saline, F1 = solution base, F2 = 0.25% clotrimazole and 0.75% clove essential oil, F3 = 0.5% clotrimazole and 1.5% clove essential oil, F4 = 0.25% ketoconazole and 0.75% clove essential oil, F5 = 0.5% ketoconazole and 1.5% clove essential oil, F6 = 0.25% miconazole and 0.75% clove essential oil, F7 = 0.5% miconazole and 1.5% clove essential oil. All amounts in % are w/w.

drugs and clove essential oil to be used in formulations F2–F7. Since the checkerboard assay revealed that the combination of clove essential oil with azole drugs reduced the azole effective concentration by 4 times, the concentration of clotrimazole, ketoconazole and miconazole in the formulations was set at one-quarter of 1 and 2% w/w, with the remainder of the active ingredient fraction made up with clove essential oil. Therefore, the developed formulations were 0.25% w/w azole drug with 0.75% w/w clove essential oil and 0.5% w/w azole drug with 1.5% w/w clove essential oil. The formulations were prepared as solutions that could be applied directly to the skin by spraying. Spray application has an advantage over topical creams and ointments as it can be applied from a small distance meaning users do not have to touch the medication directly. In addition, solutions can spread through the skin more evenly and quickly than semisolid formulations [2]. This study provided the first report on the preparation of clove essential oil in combination with azole drugs for *M. gallinae* infection treatment.

In the preparation of topical solutions for the current study, ethyl alcohol was used as the solvent and polyethylene glycol 40 hydrogenated castor oil (PEG-40 hydrogenated castor oil) as the emulsifier. This allowed the hydrophobic azole drugs and clove essential oil to dissolve into the water that made up the remaining third of the solution base. PEG-40 hydrogenated castor oil is biocompatible and acts as skin moisturizer to reduce skin irritation from other substances in the formula. Additionally, PEG-40 hydrogenated castor oil helps the drug to adhere to skin and prolongs contact time, which promotes the action of time-dependent antimicrobial agents [26].

CONCLUSION

Clove essential oil in combination with clotrimazole, ketoconazole, or miconazole showed a partial synergistic effect against *M. gallinae* ATCC 90749 (FICI = 0.75), reducing the concentrations of clove essential oil and azole drug required to inhibit fungal growth by 2- and 4-times, respectively. Topical solutions containing 0.25% w/w of clotrimazole, ketoconazole, or miconazole with 0.75% w/w clove essential oil were highly fungicidal, reducing the number of viable *M. gallinae* ATCC 90749 by 99.999% within 2 h. Further studies should investigate the stability of formulations and examine their *in vivo* efficacy in animal models.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.026.

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Appendix A. Supplementary data

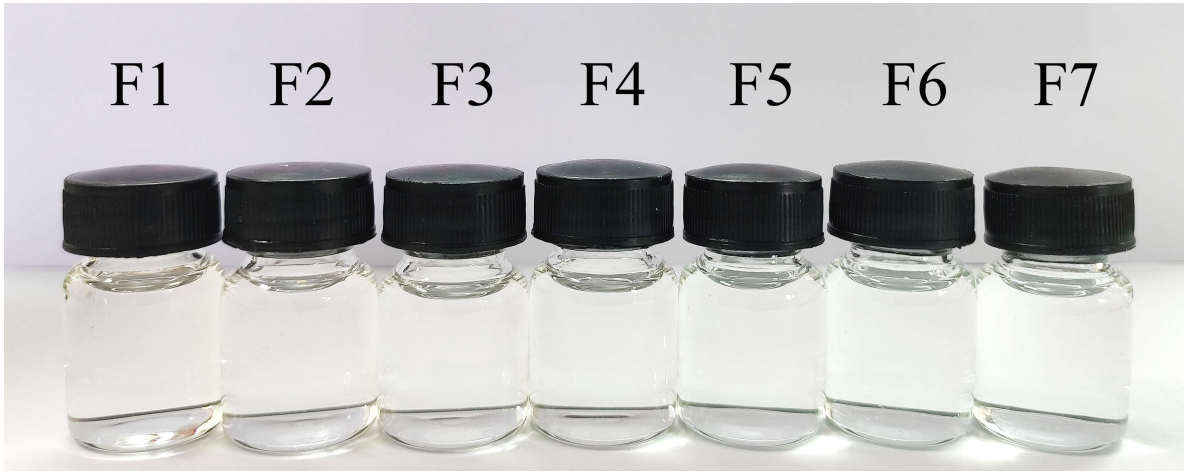

Fig. S1 Appearance of azole drug/clove essential oil formulations. F1 = solution base, F2 = 0.25% clotrimazole and 0.75% clove essential oil, F3 = 0.5% clotrimazole and 1.5% clove essential oil, F4 = 0.25% ketoconazole and 0.75% clove essential oil, F5 = 0.5% ketoconazole and 1.5% clove essential oil, F6 = 0.25% miconazole and 0.75% clove essential oil, F7 = 0.5% miconazole and 1.5% clove essential oil. All amounts in % are w/w.