Original Article

Antimicrobial activity of formulated clove essential oil spray against biofilm-forming *Malassezia pachydermatis* and *Staphylococcus pseudintermedius* clinical isolates

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Abstract

Yeasts and bacteria that cause infectious skin diseases in animals are becoming increasingly resistant to antimicrobial drugs, especially biofilm-forming microbial strains. Therefore, there is extensive interest in new effective antimicrobial substances. Clove essential oil has broad-spectrum antimicrobial activity and can be developed into various dosage forms. This study investigated the antimicrobial effects of a lipid-based spray containing 5% w/w clove essential oil against biofilm-forming *Malassezia pachydermatis* and *Staphylococcus pseudintermedius* clinical isolates by time-kill test. In addition, the physical stability and antimicrobial activity of the spray was assessed under accelerated storage conditions. The time kill tests revealed that the prepared formulation decreased the number of viable yeast and bacteria by more than 99.9999% (6-log reduction) within 15 mins. After storage at 40^0C and 75% RH for 4 months, the spray showed a slight increase in acidity and a slight darkening in colour. The antimicrobial and bactericidal activity was unaffected but the anti-yeast activity diminished slightly in the 3rd and 4th months under accelerated storage conditions. These results demonstrate the efficacy of clove essential oil lipid-based spray against biofilm-forming *M. pachydermatis* and *S. pseudintermedius*.

Keywords: Clove essential oil, Formulation preparation, Antimicrobial activity, *Malassezia pachydermatis*, *Staphylococcus pseudintermedius*

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Introduction

Infectious dermatitis is the most common disease in dogs, especially canine *Malassezia* dermatitis caused by the yeast *M. pachydermatis* and superficial pyoderma caused by the bacterium *Staphylococcus pseudintermedius* (Hill *et al*., 2006; Hnilica *et al*., 2016; Khurana *et al*., 2016). Nowadays, antimicrobial resistance in these microorganisms is becoming a more serious problem in veterinary medicine, especially among biofilm-forming microbes. Microbes in biofilms can be 10-1,000 times more resistant to antimicrobial drugs than planktonic cells (Mah and O'Toole, 2001; Figueredo *et al*., 2013; Ferran *et al*., 2016). Therefore, there are extensive studies into new effective antimicrobial substances. It has been reported that many Thai traditional medicine plants are highly effective antimicrobial agents (Mekseepralard *et al*, 2010).

The clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) is a plant which is contained in many recipes in the traditional drug list of the Thai National list of essential medicines. It is used as an antimicrobial in the gastrointestinal tract, to relieve colic, as a cough suppressant and a local anaesthetic (Royal Thai Government Gazette, 2013). Dried clove flower buds contain 15-17% w/w essential oil, which is mainly composed of eugenol, caryophyllene and eugenyl acetate (Jirovetz *et al*., 2006; Perry, 2011). Clove essential oil is a broad-spectrum antimicrobial substance. It has anti-biofilm activity against some yeasts and bacteria. In addition, it has antioxidative and anti-inflammatory effects (Perry, 2011; Zhang *et al*., 2017; Satthanakul, 2019). The essential oil from clove can be included in various dosage forms such as emulsion, nanoemulsion, cream and ointment (Nowak *et al*., 2012; Shahavi *et al*., 2019). Since lipid-based dosage forms promote drug penetration of the epidermis, they are ideal for delivery of lipid-soluble topical drugs and are therefore suitable for canine *Malassezia* dermatitis and superficial pyoderma (Quinn *et al*., 2011; Jain *et al*., 2017).

Clove essential oil is more attractive than other essential oils because of its outstanding antimicrobial, anti-inflammatory and anti-oxidation properties. Also, it is easy to find and is low in price. However, there is limited information about the effect of clove formulation on biofilm-forming microbes causing canine dermatitis. Therefore, we evaluated the *in vitro* antimicrobial effects of a lipid-based spray containing 5% w/w clove essential oil against biofilm-forming *M. pachydermatis* and *S. pseudintermedius* clinical isolates and determined the formula's stability under accelerated storage conditions.

Materials and Methods

***Microbial strains and culture conditions*:** The clinical isolates of yeast *M. pachydermatis* and bacteria *S. pseudintermedius*, which had detected the biofilm-forming ability by a Calgary biofilm device (optical density > 0.2 at 595 nm) were obtained from the Veterinary Pharmacology Laboratory. Both organisms were collected from skin lesions of dogs diagnosed with *Malassezia* dermatitis and superficial pyoderma in the animal hospital of the Faculty of Veterinary Medicine, Khon Kaen University, Thailand. The yeast *M. pachydermatis* was the species identified by microscopic appearances and biochemical tests. *M. pachydermatis* cells were oval-shaped and budding yeast cells were observed. It was grown on Dixon agar at a temperature of 32, 37 and 40^{0}C and SDA at 32^{0}C. This yeast also showed positive growth on 2% glucose agar and 1% peptone agar and was positive to the catalase test and all Tweens utilization test (Tween 20, 40, 60 and 80) (Gueho *et al*., 1996). The bacteria *S. pseudintermedius* was the species identified by microscopic appearances, biochemical tests and the PCR-restriction fragment length polymorphism (PCR-RFLP) method using *pta* primers and MboI restriction enzyme. *S. pseudintermedius* were gram-positive round cell bacteria with grape like clusters. Its colonies showed beta haemolysis on blood agar, catalase and coagulase positive, oxidase and hyaluronidase negative and produced anaerobic acid from mannitol, sucrose and trehalose. The DNA fragment of the pre-digest PCR product of *S. pseudintermedius* was 320 bp, digested were 213 and 107 bp (Bannoehr *et al*., 2007; Bannoehr *et al*., 2009).

Before use, the yeast and bacteria were transferred to Sabouraud dextrose broth (SDB, Becton Dickinson, France) and Mueller Hinton broth (MHB, Becton Dickinson, France), respectively, and incubated at 37^{0}C for 24 h. The optical density (OD) at 600 nm of the microbial suspension was measured by Vis-spectrophotometer (Genesys 10 VIS, Thermo Scientific, USA) and adjusted to 10^6 CFU/ml (Usman *et al*., 2013).

***Minimum biofilm eradication concentrations (MBECs) evaluation*:** The MBECs of clove essential oil against *M. pachydermatis* and *S. pseudintermedius* were determined by MBEC assay using calgary biofilm device (CBD) according to the process described by Harrison *et al*., (2010) and Sadekuzzaman *et al*., (2018) with modifications. Briefly, microbial suspension (10^6 CFU/ml) was added to all tested wells of a 96-well flat-bottomed microtiter plate (Thermo Scientific, USA). A CBD lid with 96-pegs (Thermo Scientific, USA) was inserted into the wells of the microtiter plate and they were incubated together for 24 h for bacteria or 48 h for yeast at 37^{0}C. The CBD lid was then transferred to a challenge 96-well flat-bottomed microtiter plate containing the serial 2-fold dilutions of the clove essential oil in MHB (bacteria) or SDB (yeast). The challenge plate and CBD lid were incubated together for 24 h for bacteria or 48 h for yeast at 37^{0}C. The CBD lids with biofilms on the pegs were removed from the clove essential oil challenge plate and submerged in sterile distilled water for 1 min, then submerged into 150 µl/well of recovery medium in a flat-bottomed 96-well microtiter plate. The plate and CBD lid were sonicated for 5 mins, then the CBD lid was removed. The recovery plate was incubated for 24 h at 37^{0}C. The OD_{595} nm of the wells in the recovery plate were measured and adjusted for the OD value in the negative growth control well. The MBEC was defined as the lowest concentration of clove essential oil that inhibited the growth of bacteria in the recovery plate (OD less than 0.1). All tests were performed in triplicate.

***Essential oil spray formulation preparation*:** Clove essential oil prepared by steam distillation was purchased from Thai-China flavours and fragrances industry Co., Ltd., Thailand. Based on a previous preliminary study, the MBECs of clove essential oil against *M. pachydermatis* and *S. pseudintermedius* were 0.0625 and 0.156% w/w, respectively. Thus, in this study, we used 5% w/w clove essential oil in the formulation, which is 80 and 32 times the MBECs. The composition of the clove essential oil spray is shown in Table 1. The clove essential oil was mixed with absolute ethyl alcohol (Merck, Germany) and polyoxyethylene (20) sorbitan monooleate (Tween-80, Ajax Finechem Pty Ltd., Australia). The mixture was mixed by a vortex mixer for 5 mins, then isopropyl myristate (Namsiang Co., Ltd., Thailand) was added up to 100% and mixed by vortex mixer until a homogeneous texture was observed (Asawapattanakul, 2013).

Table 1 Composition of 100 g clove essential oil spray formulation.

Ingredients	Amount (g)
Clove essential oil	5
Absolute ethyl alcohol	10
Tween-80	10
Isopropyl myristate	Add to 100 g

***Antimicrobial activity evaluation*:** The antimicrobial activity of prepared clove essential oil spray was determined by a time-kill test according to the method previously described by Prakash and Biary (2012) with modifications. Briefly, 100 µl of microbial suspension was mixed with 900 µl of clove essential oil spray, then mixed by a vortex mixer for 1 min. After incubation for 15 and 30 mins, 3, 6 and 24 h at 37^0C, 100 µl, the mixture was 10-fold diluted with 0.89% sodium chloride solution to stop the antimicrobial reaction of the substances. The whole 100 µl of dilutions 10^{-1} to 10^{-4} was inoculated onto Sabouraud dextrose agar (SDA, Becton Dickinson, France) for yeast or Mueller Hinton agar (MHA, Becton Dickinson, France) for bacteria. After incubation, The colonies of visible growth of tested microorganisms were counted and recorded. Each experiment was performed in triplicate.

***Physical evaluation and stability testing*:** The pH of the clove essential oil spray was measured by pH meter (Lab 850 set, SI Analytics, Germany). The colour and texture were assessed by visual observation and touch. The stability of the prepared formula was tested under accelerated storage conditions at 40^0C and 75% ± 5% RH in the dark. The pH value, color, fractionation and sedimentation change score and time-kill kinetics of the formula were evaluated every month for 4 months (Bajaj *et al*., 2012; World Health Organization Expert Committee on Specifications for Pharmaceutical Preparations, 2014).

***Identification of clove essential oil chemical composition*:** The clove essential oil was identified for chemical constituents by gas chromatography-mass spectrometry (GC-MS) analysis at the start and end of the study periods for evaluating the stability of the chemical composition. During the study period, clove essential oil was stored under accelerated storage conditions similar to the prepared formula as described above for 4 months. The GC-MS analysis was performed according to the method previously described by Aiemsaard *et al*., (2010) and Amelia *et al*., (2017) with modifications for the Agilent CN10402086 gas chromatograph interfaced with the Agilent US35120381 mass spectrometer. The column used was a DB-5ms fused silica capillary column (30 m x 0.25 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 to 120^0C at a rate of 3^0C/min, then from 120 to 270^0C at 5^0C/min. The chemical constituents of clove essential oil were identified by comparing the results of the chromatogram and reference retention times.

***Statistical analysis*:** The normality of the data was assessed by the Shapiro-Wilk test. The stability of the prepared formula was analyzed each month by one-way repeated measure ANOVA for the pH value and by the Wilcoxon signed rank test for colour, fractionation and sedimentation change score. All tests were performed using SPSS software (SPSS Inc., USA; KKU license). Values of $p<0.05$ were considered statistically significant.

Results

The results of MBEC assay revealed that clove essential oil has high anti-biofilms activity against *M. pachydermatis* and *S. pseudintermedius*. The MBEC value for *M. pachydermatis* was 2.5 times lower than *S. pseudintermedius* (0.0625 and 0.156% w/w, respectively). The results from the time-kill assay of 5% w/w clove essential oil spray are shown in Figure 1. The formulated spray showed a marked antimicrobial effect against *M. pachydermatis* and *S. pseudintermedius*, decreasing the number of viable cells by more than 99.9999% (6-log reduction) within 15 mins. After storage at 40^0C and 75% RH for 4 months, the prepared formula was still highly effective against bacteria (retaining its 6-log reduction within 15 mins; Figure 2), however, the anti-yeast activity diminished slightly in the 3rd and 4th months (Figure 3). The anti-yeast activity showed a 3-log (15 mins) and 2-log (30 mins) reduction after 3 months storage, while the activity decreased by 4-log (15 mins) and 2.5-log (30 mins) after 4 months. The formulated spray retained its anti-yeast activity for 4 months for the extended time-kill assay incubation times, maintaining a 6 - log reduction for yeast incubated for 1, 3, 6 and 24 h.

The prepared formula was a clear, slightly pale yellow liquid, free of fractionation and sedimentation (Figure 4). It had low viscosity, good dispersion and a neutral pH (7.07±0.01, Table 2). Following storage under accelerated conditions, the prepared spray showed a slight darkening in colour and an increase in acidity at the 2nd, 3rd and 4th months ($p<0.05$). The pH

values did not decrease further after the 2nd month (range 6.67±0.15 to 6.76±0.04) The formula showed no fractionation and sedimentation in all tested months.

The results of the chemical composition of clove essential oil are shown in Table 3. The 100 and 97.21% of total clove essential oil composition were identified at the start of the study and after 4 months of accelerated condition stored, respectively. Eugenol and *trans*-caryophyllene were the main constituents of tested clove essential oil. Eugenol had the highest concentration (98.87%), followed by *trans*-caryophyllene with a concentration of 1.13%. After the accelerated stability test, the eugenol had 10.83% reduction from the start of the study (88.16%), while the *trans*-caryophyllene had increased 8 times (9.05%).

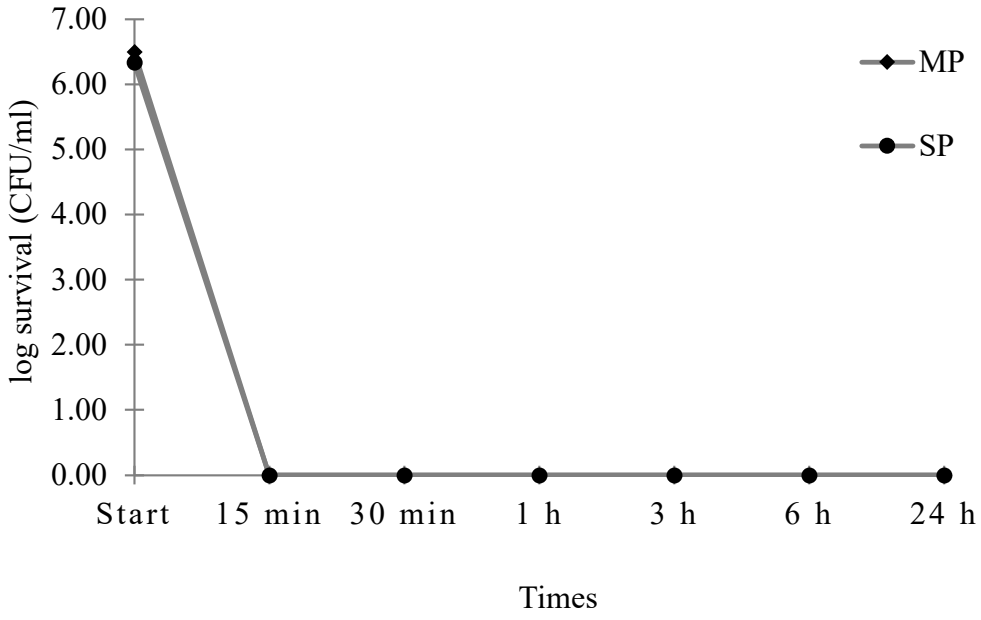

Figure 1 Time-kill assay of clove essential oil spray against *M. pachydermatis* (MP) (n=6) and *S. pseudintermedius* (SP) (n=6). Values represent the means of triplicates with error bar (SD).

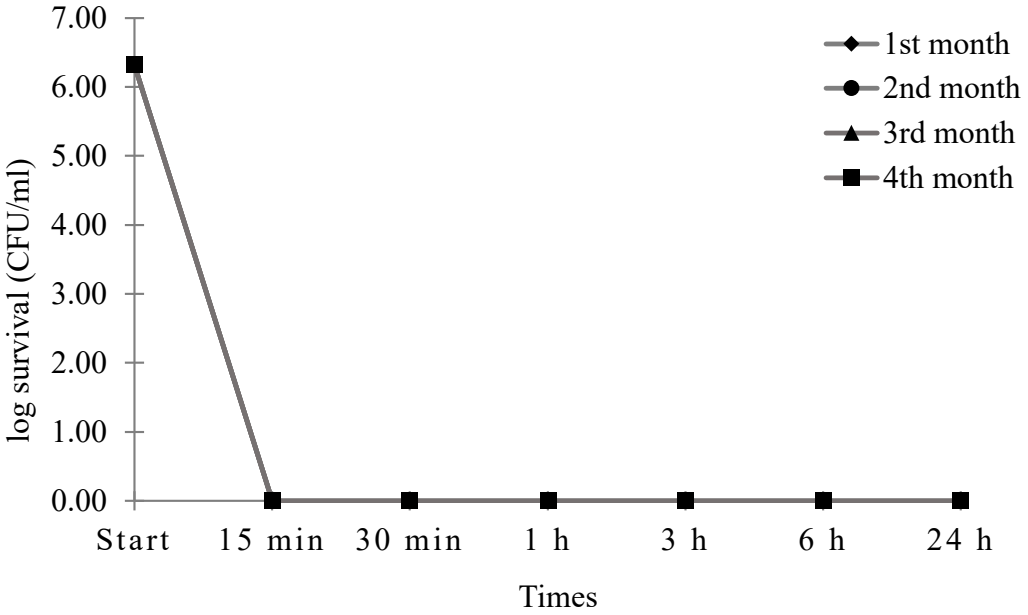

Figure 2 Stability of clove essential oil spray time-kill activity against *S. pseudintermedius* (n=6). Values represent the means of triplicates with error bar (SD).

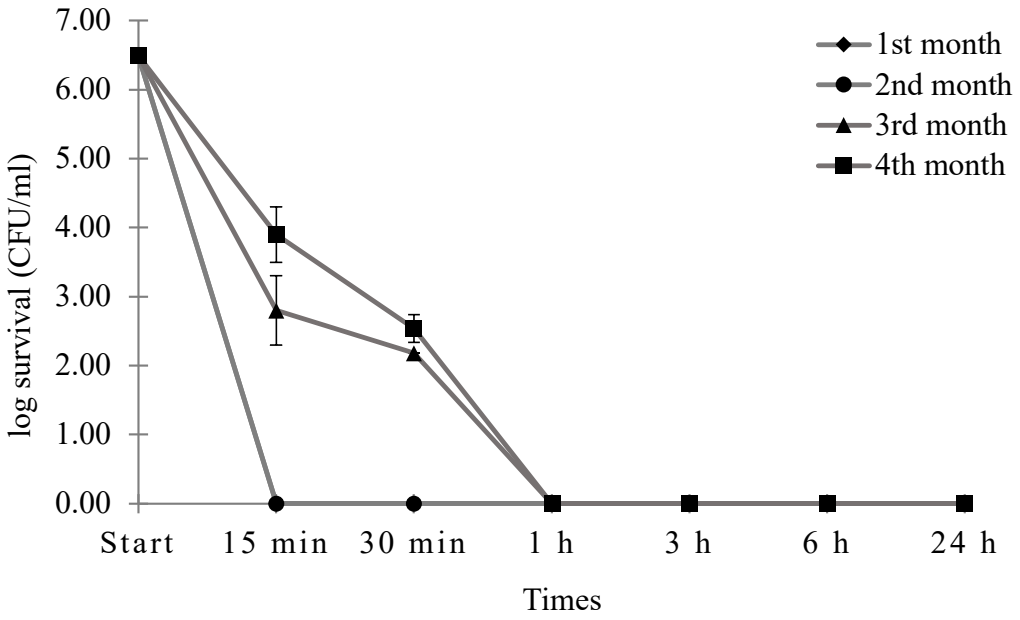

Figure 3 Stability of clove essential oil spray time-kill activity against *M. pachydermatis* (n=6). Values represent the means of triplicates with error bars (SD).

Figure 4 The physical appearance of clove essential oil spray.

Table 2 The pH value, colour, fractionation and sedimentation change score of clove essential oil spray (measured at 25^{0}C).

Time	pH	Colour change score	Fractionation and sedimentation change score
0 month	7.07±0.01^a	0^a	0^a
1st month	7.10±0.05^a	0^a	0^a
2nd month	6.67±0.15^b	1^b	0^a
3rd month	6.70±0.02^b	1^b	0^a
4th month	6.76±0.04^b	2^c	0^a

Superscript letters within a column indicate statistically significant differences (p<0.05). The pH values represent the means ± SD of triplicate experiments. The colour, fractionation and sedimentation change score are ratio scale data and the values represent the modes of triplicate experiments. The colour change scores are provided as follows: 0 = the colour is unchanged or remains close to the initial colour, 1 = the colour is slightly darker than the initial colour, 2 = the colour is moderately darker than the initial colour, and 3 = the colour is much darker than the initial colour. The fractionation and sedimentation change scores are provided as follows: 0 = the fractionation and sedimentation is unchanged, 1 = less than 25% separation or precipitation, 2 = 25-50% separation or precipitation, and 3 = more than 50% separation or precipitation.

Table 3 Chemical compositions of clove essential oil.

Components	Molecular formulas	Retention times (min)	Area (%)	
			At the start of study	After 4 months of accelerated condition stored
Eugenol	$C_{10}H_{12}O_2$	23.37	98.87	88.16
Trans-caryophyllene	$C_{15}H_{24}$	26.35	1.13	9.05

Discussion

Figures 1-3 show that the anti-microbial activity of the formulated clove essential oil spray was related to incubation time, in accordance with previous studies (Chamdit and Siripermpool, 2012; Zhang *et al*., 2017). The main components of clove essential oil are monoterpenes and sesquiterpenes such as eugenol and *trans*-caryophyllene. This result is consistent with previous studies that have reported eugenol and caryophyllene being found in 47.64-88.55% and 1.39-13%, respectively. Of these, Eugenol is the principal component of clove essential oil and has been shown to act as an antimicrobial agent against both biofilms and planktonic cells (Chaieb *et al*., 2007; Perry, 2011; Satthanakul *et al*., 2019). Previous studies demonstrated that clove essential oil's minimum inhibitory concentrations (MICs) against *M. pachydermatis* and Staphylococcal bacteria were in the range of 0.06-2 µl/ml and 1.25-10 µl/ml, respectively (Asawapattanakul *et al*., 2013; Rani and Gopinath, 2017). Eugenol is believed to affect the cell membrane of microorganisms, disrupting the structure and resulting in cell lysis and death (Guimaraes *et al*., 2019). In addition, Eugenol has been reported to eradicate biofilms by inhibiting adhesion of cells to material surfaces and decreasing expression of biofilm-related genes such as the intercellular adhesion gene (*icaD*), *Staphylococcus* enterotoxin A gene (*seA*) and the Staphylococcal accessory regulator A gene (*sarA*) (Yadav *et al*., 2015; Rajkowska *et al*., 2019).

The clove essential oil formula prepared in this study is a lipid-based preparation that used isopropyl myristate as the lipid base. Tween-80 and absolute ethyl alcohol were used as the surfactant and co-surfactant, respectively. This formula has the potential to promote the penetration of lipid-soluble clove essential oil through the epidermis enhancing its antimicrobial activity (Rashmin *et al*., 2009). Therefore, the clove essential oil formula is possible for use as an alternative treatment or combination with antibiotics to control skin infection caused by *M. pachydermatis* and *S. pseudintermedius* in animals, which reduces the use of antibiotics. However, the experiments in this report are only *in vitro* studies and are also limited to local microbial strains. For more information on the effectiveness of this clove essential oil formula, tests on other microbial strains and *in vivo* study are required.

The results of the stability test indicated that the formula retained all antimicrobial activity for 4 months. However, the anti-yeast activity decreased slightly, which could be due to the partial decomposition of eugenol. Although it is a test for the stability of the composition of raw clove essential oil, not the formula, the results are that the formula may also show a decrease in eugenol which is in agreement with the previous study of Shahtalebi *et al*., (2016). It is not likely that the observed increase in acidity of the stored formula would result in a decrease in the antimicrobial efficacy since the clove essential oil has been shown to have good antimicrobial activity, both neutral and acidic pH (Knight *et al*., 2007).

In conclusion, the clove essential oil lipid-based spray formulation showed good potential for development as a topical drug against *M. pachydermatis* and *S. pseudintermedius*. The prepared formula had good stability under accelerated storage conditions, retaining its physical and antimicrobial properties for 4 months. The *in vivo* antifungal and antibacterial efficacy and any potential skin irritation or sensitization properties of the topical formulation should be further studied.

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