A preliminary study of the acaricidal activity of clove oil, *Eugenia caryophyllus*

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Summary

Background: **The search for more eco-friendly acaricides has prompted testing of medicinal plants from botanical sources.**

Objective: **To evaluate the eradication of house dust mites (HDM), *Dermatophagoides pteronyssinus*, by direct contact using the essential clove oil (*Eugenia caryophyllus*).**

Methods: **A pilot study was initiated to determine the killing power of clove oil. Synthetic fibers were immersed in 2% clove oil for 30 min, dried in a hot air oven at 60°C for 2 hrs after which 0.5 gm of HDMs were exposed to these coated fibers placed in the Siriraj Chamber (SC). Two additional long-term methods were employed. Ten mites were placed in the SC and 10 µl of clove oil was pipetted or sprayed onto them. These latter two procedures were each carried out for 3 consecutive days at 0, 1, 3 and 6 months. The solutions antimicrobial and antifungal properties were evaluated by exposing common bacteria and fungi to sterile filter disks impregnated with the mixture, and after overnight incubation, the disc diffusion method on nutrient agar was used. Ethyl alcohol served as the placebo.**

Results: **SEMs revealed dead mites on the fibers. The effectiveness of pipetting and spraying was 99% and 81%, respectively, while the placebo mortality was < 5%. The zone of inhibition indicated significant clearance for all the bacteria and fungi indicating greater biocidal activity when compared to the controls.**

Conclusions: **Clove oil is a promising agent for killing dust mites with a potential use in dust-mite laden mattresses. Spraying diminishes in efficiency after 3 months. *(Asian Pac J Allergy Immunol 2014;32:46-52)***

Key words: *anti-mite clove oil, antimicrobial tests, house dust mites, anti-mite agent, Eugenia caryophyllus*

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Introduction

House dust contains the most widespread source of indoor allergens; namely, house dust mites (HDMs). These diminutive creatures affect a sizeable world-wide population and are the predominant source causing allergic pathological states such as asthma and rhinitis. In Thai allergic rhinitis patients, the most important indoor aeroallergen sensitizers were *Dematophagoides farina* (79%) and *D. pteronyssinus* (76%) of 100 allergic rhinitis patients.[1] While the mites themselves are harmless to humans, their feces, in sizes of 10 to 40 microns, contain allergens which, when inhaled, especially over the long term, may cause exacerbation of allergic reactions and eventually asthma. Thus, the avoidance and/or the elimination of HDMs is a recommended initial step for mite allergic patients to follow concomitant with immunological treatments.

Chemical methods to reduce allergen levels and eliminate live mites are practical and readily available. Two of the earlier chemical spray treatments were tannic acid, either alone or in combination with benzyl benzoate. Gutgesell et al.[2] reported a significant reduction in Der p1 exposure following an administrative spray containing both acaricides to allergen-laden mattresses, but no overall improvement in adult patients with atopic

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dermatitis. This contrasts with an earlier study[3] which found that benzyl benzoate was clearly ineffective in reducing the allergen (Der p1) content of mattress covers or carpets.

Currently, attention has shifted away from synthetic acaricides or laboratory-derived chemicals to ecofriendly compounds that are environmentally safe, easily degradable and target-specific. The search for such a compound has been directed extensively towards medicinal plants derived from botanical sources.[4] For example, Raynaud et al.[5] compared a bark extract, dichloromethane, of *Uvaria pauci-ovulata* (Annonaceae) and found that it was 2.14 times more effective than the standard benzyl benzoate against *Dermatophagoides pteronyssinus*. Lee[6] also found that a plant-derived anise seed oil-based compound, *p*-anisaldehyde, was 8.4 and 6.7 times more effective in killing adult mites of the *D. farinae* and *D. pteronyssinus* strains, respectively, than benzyl benzoate. At the very least, these results support the suggestion of Kalpaklioglu et al.[7] that benzyl benzoate may not be as effective when applied only once but may be more so with more frequent applications.

The essential oils offer additional anti-synthetic compounds drawing attention. Eucalyptus oil (2%) when applied as an emulsion in low concentrations of a laboratory detergent was reported to kill live mites in 30° C wash water after 30 or 60 minutes, when compared with liquid detergent alone.[8] Additional essential oils which have been shown to have a miticidal effect upon direct contact (in order of their mortality [24 h-LC_{50}]) were clove, matrecary, chenopodium, fennel and caraway.[9] Moreover, preparations from traditional Chinese medicines, such as *Cinnamon cassia*, *Eugenia caryophyllata, Asarum sieboldii* and *Pogostemon cablin* have also provided "green" biodegradable petroleum ether or ethyl acetate extracts that were better than or comparable to benzyl benzoate in killing HDMs.[10]

It was the purpose of this study to present preliminary data of the effectiveness of a solution of clove oil in killing live adult mites through pipetting, spaying and also as a dry agent on bedding fibers where mites and their developmental stages typically reside. The bactericidal and anti-fungal properties of clove oil were also evaluated. It was hypothesized that the herbal solution would efficiently eradicate HDMs and their developmental levels, and exhibit significant antimicrobial activity.

Methods

Preparation of the Herbal solution

The herbal solution used in this study was 2% clove oil preparing by dissolving clove oil in 95% ethanol, yielding a final concentration of 2% (v/v). The herbal solution was stored in closed containers at ambient temperature (23° C) until use. Ethyl alcohol served as the inactive placebo for the control group.

House dust mites

The species of *Dermatophagoides pteronyssinus* used in these experiments were cultured in the Siriraj Dust Mite Center, Department of Parasitology, Mahidol University, Bangkok, Thailand. Adult mites were isolated from the culture medium and sequestered in a standard Petri dish until ready for use at the time of testing.

Siriraj Chamber

The Siriraj chamber restricts house dust mites to a specified area in order to evaluate the efficacy of the test solution against dust mites. It was designed to prevent the mites from escaping during the experiment. The chamber is a small acrylic box containing a 1 cm diameter aperture in the middle for ventilation. Mites were constrained by an acrylic ring, covered with the chamber lid, which is then locked to prevent mite escape. After direct contact was made by the test solution, live and dead mites were counted under a stereomicroscope.

Pipetting

A sample of 10 house dust mites were placed on a 2 cm^2 filter paper in the Siriraj chamber. Then 10 µL of the tested solution was pipetted onto the mites' bodies. The chamber was then kept at room temperature (25 ± 2° C and 75% relative humidity) and examined every 24 hrs (5 – 10 mins observation time) under a stereomicroscope. Thus, 3 samples of 10 mites each in 3 separate chambers were observed for mortality on 3 consecutive days at 0, 1, 3, and 6 months, respectively. Since 95% ethyl alcohol was used as the diluent for the test solution, it served as the inactive placebo in the control groups. The number of live mites and dead mites were enumerated according to the following definitions: a live mite was one that exhibited movement especially when prodded by a thin filament attached to a small diameter wooden probe, whereas a dead mite would appear shriveled, immobile and dark brown in color.

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Spraying

This procedure was identical to the pipetting method (3 samples of 10 mites observed after 24 hrs for 3 consecutive days at 0, 1, 3, and 6 mos.), except that the experimental or control solution was applied by spraying. This delivery method delivered a volume of ~10 µl for 2 sec onto the trapped mites.

Coating fibers

Synthetic fibers were immersed in 2% clove oil for 30 min and then dried in a hot air oven at 60°C for 2 hours. The coated fibers were separately kept in plastic bags on an open work bench at the aforementioned temperature and RH until ready for use. Testing consisted of exposing 0.5 gm of mite culture to a cluster of coated fibers placed in the Siriraj Chamber.

Scanning electron micrographs were taken 24 hrs post-seeding of eggs and adult mites amid the matrix of clove-oil saturated fibers. This initial non-quantitative pilot study was designed to test the efficacy of the clove oil as an acaricide as well as determining the potential usefulness of coated fibers in mattresses or bedding for the alleviation of allergy symptoms in hypersensitive patients.

Antimicrobial tests

These procedures are designed to detect the presence of microbes and to prevent their growth and potential pathogenic activity. It is imperative for human safety that the herbal solution be nontoxic; i.e. be bacteriologically-free from nosogenic microorganisms, as well as exhibiting biocidal activity.

Tested organisms

Bacteria: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were used. They were cultured over night on blood agar at 37°C before testing.

Fungi: *Candida albicans* ATCC 90028 and *Aspergillus fumigatus* (clinical isolate) were used. *Candida* was cultured on Sabouraud dextrose agar at 37°C for 24 hours. *Aspergillus fumigatus* was cultured on Sabouraud dextrose agar for 4 days at room temperature.

Antimicrobial disc diffusion tests

The antimicrobial test was carried out by the disc diffusion method using a 0.5 McFarland standard inoculum spread on Mueller-Hinton agar surface for bacteria or Sabouraud dextrose agar for fungi and allowed to dry. Twenty µl of 2% clove oil and its solvent (ethyl alcohol) were impregnated into sterile 6 mm diameter filter discs. The prepared discs were aseptically placed onto the surface of the implanted bacterial or fungi (inoculated) plate and the plates were incubated at 37° C overnight for bacterial testing or at room temperature for 72 hours for fungi. Inhibition was assessed by measuring the zone of clearing (mm) from the edge of the disc to the growing culture. Negative controls were prepared using an inactive solvent. Each experiment was repeated twice. If the saturated water containing the 2% Clove oil has a biocidal action, then an area of clearing where bacteria or fungi are incapable of growing will surrounds the impregnated disk. No zone of inhibition should surround the inactive solvent.

Data Analysis

For the pipetting and spraying conditions, the percentage mortality data were combined for all

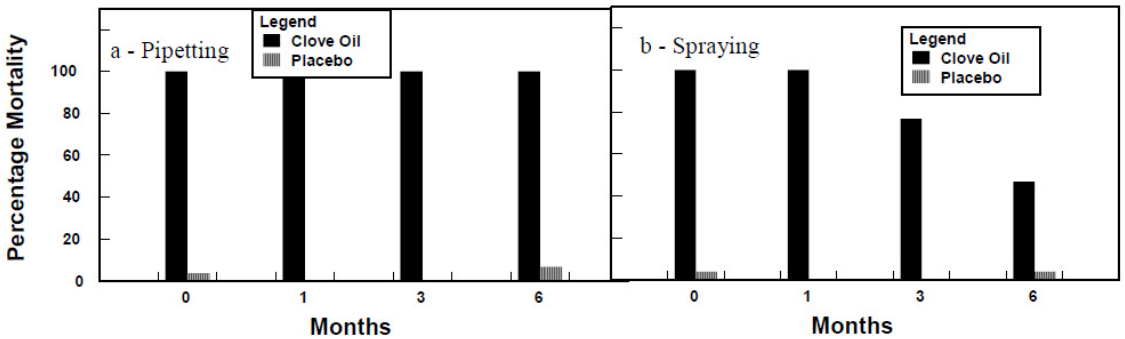

Figure 1. Panels a and b show the effects of pipetting and spraying, respectively, on the percentage mortality of each of 3 samples at 0, 1, 3 and 6 months after 24 hours exposure of either the herbal compound or the placebo.

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3 samples and where appropriate chi-square tests were used to compare the number of dead mites in the treatment groups across all time intervals. If the p value was < 0.05, the differences were considered significant. Since there was almost a complete dichotomy between the effects of the herbal solution and the placebo, inferential statistics were unnecessary. The same situation occurred for the antimicrobial tests which were presented in tabular form. For the qualitative pilot study, standard scanning electron micrographs depicted the effects of the herbal acaricide on the mite culture.

Results

Anti-mite testing

Figure 1, panel a, presents the mortality results for pipetting of clove oil over the 6 month time course of this study. The 3 samples for each time period were averaged and the total percentage was used as the major dependent variable. When applied by pipetting, the clove oil killed 119/120 mites giving a 99.1% mortality ratio, while the ethyl alcohol resulted in a 2.5% (3/120) ratio with no mites killed at 1 and 3 months. Thus, the herbal solution was effective across all time periods.

Panel b presents the mortality percentages for clove oil applied by spraying. All mites were killed within 24 hrs after the initial application of the herbal solution at 0 and 1 month, but at 3 and 6 months, the percentage of mites killed was 76.7% and 46.7%, respectively. The differences in the mortality rates of the herbal solution between 0 or 1 months and 3 and 6 months were significant (p = 0.025, $p \leq .0005$) respectively. However, the difference in mortality rates between 3 and 6 months was insignificant (p = 0.06). For the control treatment no mites were killed at 0 and 3 months while 1 was killed at 1and 6 months.

Figure 2(A) presents an SEM of the normal shape and smooth shell of a Dp egg, while (B) shows the rough deformed skin of an egg shell after treatment.

Figure 3(A) presents a micrograph of the hatching of a normal egg, while in (B) the deformity of an emerging larva is seen post-treatment.

Figure 4 presents a matrix of treated fibers with one dead mite (upper view) and the deformity of an emerging egg (lower view) following treatment.

Antimicrobial testing

Table 1 presents the antimicrobial testing for the disc diffusion procedures. The three strains of bacteria yielded an average zone of inhibition of 10.2 mm while the 2 strains of fungi exhibited a greater average clearance of 19.25 mm. For the solvent, all 5 tests showed no clearing whatsoever.

A.

B.

Figure 2. presents micrographs of the smooth shell of a normal egg (A) and the rough, deformed shell after treatment (B).

Discussion

The development of 2% clove oil, an essential oil, constitutes a promising anti-mite agent for the eradication of adult dust mites and their eggs while simultaneously displaying anti-fungal and bactericidal activity. The essential oils, having distinct volatile aromas derived from plants, have been used throughout antiquity for medicinal purposes ranging from dermatological treatments to remedies for cancer. However, such claims in recent years have become vaguer to comply with regulations imposed by host countries.

The primary constituents of essential clove oil are the phenylpropanoids. These are a diverse family of organic compounds synthesized by plants from the amino acid phenylalanine, of which eugenol (*Eugenia caryophyllata*) is a main component. Chaieb et al.[11] have enumerated a plethora of biological activities related to the use of

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A.

B.

Figure 3. shows hatching of a normal larva (A) while (B) depicts the malformation of an emerging larva after acaricidal treatment.

E. caryophyllata including antimicrobial, antioxidant, antifungal, antiviral, anti-inflammatory, cytotoxic, biopesticide and anesthetic properties.

The acaricidal activity of clove (*Eugenia caryophyllata)* bud oil derived eugenol and 3 of its congeners were evaluated by direct contact application and by a fumigation test for their induction of mortality in 2 species of HDMs and contrasted with benzyl benzoate and N,N-diethyl-m-toluamide (DEET).[12] It was found that on the basis of LD 50 values for the direct contact test, the most to least toxic for *Dermatophagoides pteronyssinus*, were methyleugenol, isoeugenol, eugenol, acetylyeugenol, benzyl benzoate and DEET. For *Dermatophagoides farinae*, the same order held for the first 3 phenylpropenes but benzyl benzoate was more toxic than acetyleugenol with DEET showing the least activity. In the fumigation test using both mite species all 4 eugenol congeners in the closed container procedure were more effective than the open container procedure. These data indicate that the lipophilicity of the clove bud oil compounds plays a critical role in dust mite mortality.

Li et al.[13] replicated the above mentioned fumigation procedures. When a vapour phase toxic

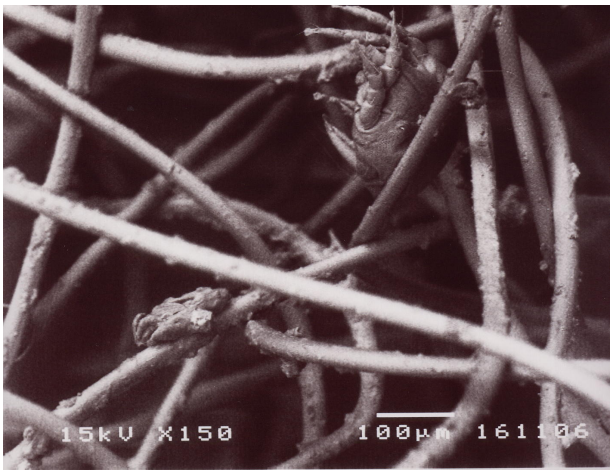

Figure 4. presents an array of clove oil treated fibers with an adult dead mite in the upper right view and a deformed emerging larva in the bottom left view.

preparation had been extracted (Soxhlet Extractor) from clove buds (12.2µg/cm^2), the volatile oil was applied against *Dermatophagoides farinae* in a fumigant test. The LD_{50} values after 24 hrs indicated potent fumigant activity, thereby supporting the acaricidal activity of clove oil. Saad et al.[9] exposed dust mites to five concentrations of 14 essential oils. The 50% lethal concentration after 24 hrs indicated that clove oil was the most effective while eucalyptus and cinnamon were ranked 8th and 11th respectively, in mortality. In a study measuring the herbicidal effects of essential oils, it was found that cinnamon and clove were the most phytotoxic causing electrolyte leakage resulting in cell death, and that eugenol was determined to be the major component of cinnamon oil (84%,v/v), as it was for clove oil.[14]

In the present study, pipetting, in which the mites' body was completely saturated, was the most effective method for eradicating mites. Its acaricidal

Table 1. Disc diffusion results

Organisms	zone of inhibition in diameter (mm)	
	2% clove oil	solvent
Escherichia coli	10	0
Staphylococcus aureus	10	0
Pseudomonas aeruginosa	10.5	0
Candida albicans	19.5	0
Aspergillus fumigatus	19	0

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activity was observed within 24 hours, and most importantly, its shelf life was > 6 months. Further, we have unpublished data suggesting an even lower (15 min) latency of action. Spraying was an equally effective killing method for only months 0 and 1. Thereafter, its potency diminished, but not to the level of the placebo. This may be due either to a deteriorating shelf life or an incomplete saturation of the mites' bodies.

The results derived from inspecting the SEMs suggest that coating of synthetic fibers with dried clove oil may also be an effective procedure for eradicating mites and their eggs. Additionally, we have seen dead adult mites manifesting phenotypical color changes in the exoskeleton from translucent to brown, a deformity of the legs, an upright stance similar to that which Kim et al.[12] described as a "death symptom of the forelegs extended forward together, leading to death without a knockdown" ,and immobility after prodding. A recent study reported exposing *D. pteronyssinus* to cotton fabrics containing eugenol- loaded chitosan nanoparticles. The effect of the encapsulated nanoparticles on dust mite mortality depended on the percentage loading and exposure time. High eugenol loading (8.24% coated fabric) with direct contact and a 48 hr exposure time resulted in greater percentage of mites killed than 24 hrs exposure (50% vs. 28% mortality). The authors speculated that the lower percentage of dust mite mortality may have been due to a delayed release of the entrapped eugenol when compared to direct contact.[15]

This initial qualitative pilot study was successful in establishing clove oil as a potent herbal acaricide. While the constraints of this procedure made it impossible to accurately determine the number of mites and/or their eggs destroyed (some mites may have dropped down deep into the matrix of the fibers, and therefore, were not captured by the micrograph), the potential usefulness of coating fibers used in bedding should be explored over a substantially longer period of time than 24 hrs.

Regarding antimicrobial testing, a greater zone of inhibition/clearance was observed for the fungi than the bacteria, although for both clove oil was clearly superior to the solvent alone. These results suggest that fungi are more susceptible to the herbal mixture than bacteria. In evaluating biocidal activity, the action on both types of organism was clearly superior to the solvent only. In 1973, van Bronswijk[16] analyzed the gut contents of over 600 specimens of *D. pteronyssinus* from bedroom floors and mattresses and reported finding evidence of fungal mycelia/hyphae, unspecified bacteria, pollen and spores of microorganisms. Colloff[17] also reports similar findings (e.g. macerated skin scales, fungal hyphae and spores, yeasts and bacteria) in the alimentary canal of *D. pteronyssinus* mites. The eradication of mites and a reduction of their food source might be accomplished by applying the herbal solution to mattresses. Of 4 common Thai mattresses, mite allergen permeability was higher in kapock and synthetic mattresses than coconut and polyurethane mattresses.[18] It may be speculated that if the clove oil solution was applied to any of the above mentioned Thai mattresses as well as to tightly woven synthetic fibers comprising a mattress encasement (which has been demonstrated to prevent mite penetration[19] and has a 98.5% allergen impenetrability),[20] enhanced protection from mites and their allergens might accrue, thereby reducing the risk of developing allergic symptomatolgy.

Before this acaricide can be commercially marketed as an anti-mite herbal solution for home use, it must undergo rigorous testing. Many of the studies described above, including the present undertaking have killed mites in the laboratory but their effectiveness in the home has not been evaluated. All acaricides must (a) be proven non-toxic and odor free; (b) not destroy or markedly damage the substrate to which it is applied; (c) be easy to apply and penetrate deeply within the bedding and soft furnishing; and (d) not require multiple applications that is costly and time-consuming.[21]

Within the limitations of this study, it may be concluded that: (a) the anti-mite herbal solution is effective against all stages of live mites including mature and immature mites, larvae and egg stages when direct contact is made; (b) pipetting has a longer duration of action than spraying, the shelf life of which diminishes after 3 months; (c) exposing mites to clove oil embedded on synthetic fibers is effective in killing mites and their developmental stages; (d) the herbal solution has definitive antimicrobial properties which are effective against certain strains of bacteria and fungi and might also reduce a potential food source for mites in mattresses. Thus, 2% clove oil can and should be used as an anti-mite agent both in wet and dry form. It is this latter property which has great potential/relevance for treating fabrics used in bedding and soft furniture for the control of HDMs in allergy-prone patients.

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