

Original Article

EFFECTS OF ALPHA-MANGOSTIN AND CITRONELLA OIL AGAINST ACNE CAUSING BACTERIA

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ABSTRACT

Objective: This study assessed the effects of alpha-mangostin (AM) and citronella oil (CO) working alone or in combination against *Propionibacterium acnes* (*P. acnes*) and *Staphylococcus aureus* (*S. aureus*).

Methods: The screening for antibacterial activity of AM and CO against *P. acnes* and *S. aureus* was carried out using the disk diffusion method. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of these two substances were determined using the broth microdilution method. The fractional inhibitory concentration indices (FICI) of a combination of AM and CO were obtained by checkerboard dilution assay.

Results: The results showed that alpha-mangostin and citronella oil do indeed fight against *P. acnes* and *S. aureus*. The MICs and MBCs of AM against *P. acnes* and *S. aureus* were the same at 6.25 and 50 µg/ml, respectively. Both the MIC and the MBC of CO against *P. acnes* were 27.81 µg/ml. The MIC and the MBC of CO against *S. aureus* were 112.13 and 224.25 µg/ml, respectively. The FICI of a combination of AM and CO against *P. acnes* and *S. aureus* were 2.00, indicating indifferent interaction with no additional inhibitory effect.

Conclusion: AM and CO are very effective against *P. acnes* and *S. aureus*, nevertheless their effect when used together was indifferent from using alone. Further research may find that either or both of these substances combined with yet a different natural agent could provide synergy against *P. acnes* and *S. aureus*.

Keywords: Alpha-mangostin, Citronella oil, Fractional inhibitory concentration index (FICI), *Propionibacterium acnes* (*P. acnes*), *Staphylococcus aureus* (*S. aureus*), Anti-acne

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INTRODUCTION

This study quantitatively analyzed alpha-mangostin (AM), from mangosteens, and citronella oil (CO), from citronella grass, to investigate possible synergistic effects of AM combined with CO against the bacteria that cause acne.

Acne vulgaris is a very common chronic inflammatory disease of the pilosebaceous follicles of the skin which is caused by hyperactive sebaceous glands, hyperkeratosis in the hair infundibulum, *P. acnes* colonization or other bacterial infections. This disease is characterized by both inflammatory and non-inflammatory lesions [1–3]. People of all races and ages get acne, but it is most common in teenagers and young adults. Quality of life is often reduced for patients who suffer from acne vulgaris [4]. Thiboutot *et al.* (2009) reported that acne vulgaris can so disruptive as to sometimes even lead to serious psychiatric issues [5].

P. acnes and *S. aureus*, which are gram-positive bacteria naturally occurring on the skin surface [6], are often what cause the development of acne. Excessive production and secretion of sebum in conjunction with hyperkeratosis in the hair infundibulum result in narrowed and blocked hair follicle pores, causing an increase in the population of *P. acnes*. Subsequently *P. acnes* proliferation, it can induce the production of inflammatory mediators, which are accountable for the inflammation, redness, and pain associated with acne breakouts [7, 8]. In addition, *S. aureus* can cause skin infections and exacerbate pimples and abscesses [9].

Current treatment for acne depends on types and severity of the disease. Normally, patients with moderate to severe infections are treated with topical drugs, which include topical antibiotics and topical retinoids. Unfortunately those drugs often causes adverse side effects, such as skin irritation, dry flaky skin, itching, and erythema [10]. At the same time, there are numerous published

studies on natural extracts and essential oils that are derived from plants and used instead of antibiotic drugs for treatment of acne. The current study investigated and combined two such natural substances, AM and CO, with the hypothesis that this mixture might produce a synergistic effect that enhances their activity, while at the same time causing fewer adverse side effects than conventional treatments. AM is a major constituent of the pericarp of mangosteen fruit, and it shows promising antibacterial activity against *P. acnes* and *S. aureus* [11, 12]. AM has also been found to possess anti-inflammatory and antioxidant properties [13]. Reassuringly, a study by Larson *et al.* (2010) investigated the toxicity of AM against young rats and found that it did not demonstrate any adverse effects, even at a high doses of 80 mg/kg [14]. Moreover, a study by Sakagami *et al.* (2005) found a synergistic effect when AM isolated from the stem bark of *Garcinia mangostana* was combined with vancomycin hydrochloride against methicillin resistant *Staphylococcus aureus* (MRSA) [15]. There are also preliminary published studies on the biological activities of essential oils obtained from the leaves and stems of *Cymbopogon nardus* (Citronella grass). This grass is widely used as a fragrant herb in Asian cooking, and also as a medicinal herb due to its inhibitory effects against a variety of bacteria [16, 17]. Citronella grass is also easy to grow, widely available, and inexpensive to purchase. The U. S. Environmental Protection Agency (1997) reported that citronella oils have a relatively low acute skin toxicity (LD₅₀>2000 mg/kg) [18]. Furthermore, a study by Afsar *et al.* (2017) investigated antibacterial efficacy of creams and gels containing the extracts from *Cassia fistula*, *Ficus religiosa*, *Milletiapiinnata* and *Wendlandiathyrsoida* in a combination with citronella oil at various concentrations against *P. acnes* and *S. epidermidis* and found that all the formulations displayed antibacterial activity against the selected strains [19]. Although there have been previous studies of AM or CO used individually or used in combination with other antibacterial substances, the current

study is believed to be the first to look for possible synergistic benefits from using these two substances together.

MATERIALS AND METHODS

Test substances and chemical reagents

Alpha-mangostin was obtained from Chroma Dex Inc., Santa Ana, CA. Citronella oil was purchased from Thai-China Flavours and Fragrances Industry Co., Ltd., Bangkok, Thailand. Geraniol was purchased from Sigma-Aldrich, Inc., Steinheim, Germany. The solvents and other chemicals were analytical grade.

Quantitative analysis of AM and geraniol

The purity of AM was tested using high performance liquid chromatography (HPLC) analysis as per Pothitirat *et al.*'s method, somewhat modified [20]. The AM was dissolved at six different concentrations ranging from 0.25 to 50 µg/ml. These six solutions were separated using a Vertiseq C-18 column (5 µm, 4.6 x 250 mm) and a guard column. Acetonitrile and 0.2% o-phosphoric acid in water were mixed at a ratio of 9:1 (v/v), and this solution was used as the mobile phase. The mobile phase flow rate was set at 1 ml/min with a UV detection wavelength of 320 nm. In order to determine the calibration curve, the resulting HPLC chromatogram data was plotted in a graph showing peak area versus concentration.

The geraniol content of the CO was determined using gas chromatography-flame ionization detection (GC-FID) analysis as per Jumpeaeng *et al.*'s method, somewhat modified [19]. The geraniol was dissolved at six different concentrations ranging from 62.5 to 2,000 µg/ml. These six solutions were separated using an HP-5 column (0.25 mm, 30m x 0.25 mm), using split injection mode 7:1 at 240 °C. Helium was the carrier gas at a flow rate of 2 ml/min. The oven temperature was initially at 50 °C for 2 min, then gradually increased to 150 °C, with the heat increase at a rate of 5 °C/min, and finally increased to 240 °C at a rate of 10 °C/min. The temperature was held at 240 °C for 2 min. The amount of geraniol in the CO was calculated using a calibration curve.

Test organisms

The antibacterial activity of AM and citronella oil were evaluated using the two bacteria types that are considered to be the main cause of acne. *P. acnes* DMST 14916 and *S. aureus* ATCC 25923 were obtained from the Department of Medical Sciences, Naresuan University, Thailand.

Inoculum preparation

The inocula were prepared using the direct colony suspension method as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines [21]. *P. acnes* was cultivated on brain heart infusion agar (BHI) for 72 h under anaerobic conditions at 37±2 °C, and *S. aureus* was cultivated on nutrient agar (NA) for 24 h under aerobic conditions, also at 37±2 °C. The inoculum of each bacteria strain was prepared by the direct colony suspension as recommended by the CLSI.

Screening for antibacterial activity of AM and CO

AM and CO were tested against *P. acnes* and *S. aureus* using the agar disk diffusion method of A. Klančnik *et al.* (2010) [22] with some modifications. First, test disks were prepared in advance by placing 20 µl of test solution (a broth medium solution containing 100 mg/ml of either AM or CO, along with 0.5% Tween 80 as co-solvent) onto the center of a sterile filter-paper disk (6 mm in diameter). The standard commercially prepared disks of clindamycin (2 µg/disk) were used as a positive control.

Next, for each of the two inocula, the bacterial suspension was swabbed over the surface BHI agar for *P. acnes* and Müller-Hinton agar (MHA) for *S. aureus*. The plates were allowed to dry for 5 min. The prepared test disks were then placed on the agar surfaces, and the plates were incubated under the previously described culture conditions for the respective bacteria. Finally, the inhibition zone diameters of each plate were measured in millimeters. Each test was repeated in triplicate.

Determination of MIC and MBC of AM and CO

The MICs of AM and CO alone were determined by using 2,3,5-triphenyltetrazolium chloride (TTC) as a chromogenic marker, based on the method of Athikomkulchai *et al.* (2008) with some modifications [23]. Two sets of eight test solutions were prepared containing AM at various concentrations ranging from 1.56 to 200 µg/ml, along with 1% DMSO and 0.5% Tween 80. The first set was in BHI media broth for testing on the *P. acnes*. The second set was in Müller-Hinton broth (MHB) for testing on the *S. aureus*. Similarly, two sets of eight CO test solutions ranging from 3.50 to 448.5 µg/ml were also prepared in the same way. These AM solutions and CO solutions were prepared by serial two-fold dilution with their respective broths in 96-well plates. Two growth controls were set up, one containing *P. acnes* in its medium and the other containing *S. aureus* in its medium. Clindamycin was used as a positive control, while negative controls were the media contained 1% DMSO and 0.5% Tween 80.

After incubation, 20 µl of TTC solution (20 mg/ml) was added to all the plates, which were then all incubated at 37±2 °C for 3 h. If the TTC in the plates encounters bacteria cells that are still viable, those viable cells convert the colorless TTC to red formazan, with the color intensity proportional to the amount of viable cells, i.e. the more viable cells there are, the darker the red color. The color changes were evaluated visually. The lowest concentrations of AM and CO that showed no visible growth of bacteria or no red color were recorded as their respective MIC values. The MBCs of AM and CO were then determined by subculturing from the well broth used for the MIC test onto agar plates and incubated under the respective conditions used earlier to make each of the two inocula. The concentration of AM or CO required to kill all the bacteria were recorded as their respective MBC values.

Determination of synergistic activity from a combination of AM and CO

Synergistic activity of the combination of AM and CO was determined by the checkerboard dilution assay using the method of Thathaisong and Sangnual (2011) with some modifications [24]. Fresh new stock solutions of AM and CO were prepared. The respective MICs of AM and CO were used together to define a fractional inhibitory concentration (FIC) of 1. Then dilutions of each agent's FIC were prepared in a series of 0xMIC, 0.25xMIC, 0.50xMIC, 0.75xMIC and 1xMIC in a 96 well-plate. Then, the bacterial inoculum was added and all the plates were then incubated under the respective conditions used. The FIC index (FICI) was determined according to the following equation (1):

$$FICI = FIC_A + FIC_B = \frac{[A]}{MIC_A} + \frac{[B]}{MIC_B} \dots\dots\dots (1)$$

Where [A] = MIC of AM combined with CO

[B] = MIC of CO combined with AM

MIC_A = MIC of AM alone

MIC_B = MIC of CO alone

A FICI ≤ 0.5 indicates synergy, 0.5 ≤ FICI ≤ 1 indicates addition, 1 ≤ FICI ≤ 4 indicates indifference, and FICI > 4 indicates antagonism.

Statistical analysis

All tests were performed in triplicate, and the resulting data is presented here as mean±standard deviation (SD) of the three experiments.

RESULTS

Quantitative analysis of AM and geraniol

The purity of the AM powder used in this study was tested by HPLC analysis at 320 nm. The calibration curve of AM in concentration in the range of 0.25-50 µg/ml showed a good linear relationship with a correlation coefficient (r²) of 0.9999. HPLC chromatograms of AM showed a retention time at 8.2 min. The purity of AM was found to be 95%. Next the geraniol content of the CO used in this study was determined by GC-FID analysis. The calibration curve of geraniol within the concentration range of 62.5-2,000 µg/ml showed a good linear relationship with an r² of 0.9998. GC-FID chromatograms of CO showed

a retention time at 2.9 min. The geraniol content of the CO was found to be 25%.

Screening antibacterial activities of AM and CO

The antibacterial activity of AM and CO against *P. acnes* and *S. aureus* were determined by the disk diffusion method. Clindamycin was used as a positive control. The results are shown

in table 1, where they are displayed in the average zone of inhibition (mm) including the disk diameter (6 mm). The zones of inhibition of Clindamycin against *P. acnes* and *S. aureus* were 50.0±0.50 and 20.5±0.50 mm, respectively, and the zones of inhibition of AM against *P. acnes* and *S. aureus* were 10.5±1.25 and 8.5±1.00 mm, respectively. Meanwhile, CO exhibited larger zone sizes of 18.3±1.00 mm and 15.9±1.25 mm.

Table 1: Antibacterial activity of AM and CO against *P. acnes* and *S. aureus*

Substance tested	Average zone of inhibition (mm)	
	<i>P. acnes</i>	<i>S. aureus</i>
AM (2 mg/disk)	10.5±1.25	8.5±1.00
CO (2 mg/disk)	18.3±1.00	15.9±1.25
Clindamycin* (2 µg/disk)	50.0±0.50	26.5±0.50

*positive reference standard, mean±SD; n=3

Determination of MIC and MBC values of AM and CO

The MIC and MBC of AM, CO and clindamycin against *P. acnes* and *S. aureus* are shown in table 2. When the positive control clindamycin was tested against *P. acnes*, both the MIC and MBC values of clindamycin were 0.16 µg/ml. In comparison, both the MIC and MBC values of AM against *P. acnes* were 6.25 µg/ml. CO did not perform as well, with MIC and MBC values of 27.81 µg/ml.

Against *S. aureus*, both the MIC and MBC values of clindamycin were 0.63 µg/ml. AM again performed better than CO, with AM's MIC and

MBC values at 50 µg/ml, while CO had an MIC value of 112.13 µg/ml and an MBC value of 224.25 µg/ml.

Determination of synergistic activity of a combination of AM and CO

The synergistic effects of a combination of AM and CO against *P. acnes* and *S. aureus* were tested using the checkerboard method, and the FICI results of this combination are reported in table 3. The FICI values against both bacteria were 2, indicating a test interpretation of "Indifferent with no additional inhibitory effect".

Table 2: The MIC and MBC values of AM and CO against *P. acnes* and *S. aureus*

Substance tested	<i>P. acnes</i>		<i>S. aureus</i>	
	MIC	MBC	MIC	MBC
AM (µg/ml)	6.25	6.25	50	50
CO (µg/ml)	27.81	27.81	112.13	224.25
Clindamycin* (µg/ml)	0.16	0.16	0.63	0.63

*positive reference standard, n=3

Table 3: MICs, FICs and FICIs of a combination of AM and CO against *P. acnes* or *S. aureus*

Bacterial type	Substance tested	MIC(µg/ml)		FIC	FICI	Interpretation
		Alone	Combination			
<i>P. acnes</i>	AM	6.25	6.25	1	2	Indifferent with no additional inhibitory effect
	CO	27.81	27.81	1		
<i>S. aureus</i>	AM	50	50	1	2	Indifferent with no additional inhibitory effect
	CO	112.13	112.13	1		

n=3

DISCUSSION

In this study, various antibacterial properties of alpha-mangostin and citronella oil were tested, specifically their MIC, MBC and FICI values, in order to look for a synergistic effect when AM and CO were combined. In the disc diffusion test for screening antibacterial activity, AM showed a smaller inhibition zone against *P. acnes* and *S. aureus* than CO. Mostly the inhibition zone is related to the MIC value, in that usually the larger the inhibition zone, the lower the MIC value. However, that was not the case when comparing AM and CO. Although AM showed lower MIC and MBC values against these bacteria types than CO, but it manifested smaller inhibition zone than CO. One possible explanation of this uncommon result might be that the size of inhibition zone also depends on its ability to diffuse through the agar [25], which can be expressed by the partition coefficient ($\log P_{o/w}$) value. A lower $\log P_{o/w}$ value means the compound is more hydrophilic and more likely to diffuse through agar media. The $\log P_{o/w}$ of AM is 4.64 [26]. Unfortunately the $\log P_{o/w}$ of CO has not been previously reported and is not readily available. Nevertheless, geraniol, known for CO's active constituents,

possesses a $\log P_{o/w}$ value of 2.5 [27]. As a consequent, CO may possess the greater diffusion ability than AM. Thus, the small zone of inhibition observed may not necessarily preclude better antibacterial activity from that substance.

Looking at the FICI test results, the FICI of the combination of AM and CO against *P. acnes* and *S. aureus* was 2.00, indicating an interpretation of "indifferent with no additional inhibitory effect", which means this combination did not create any synergy beyond the individual antibacterial abilities of the individual substances.

Several studies have reported that the antibacterial mechanisms of AM and CO against *P. acnes* and *S. aureus* are actually the same mechanism, namely membrane disruption. A study by Koh *et al.* (2013) reported that AM rapidly disrupts the cytoplasmic membrane of gram positive-bacteria, thereby causing leakage of intracellular content. Koh's study hypothesizes that AM's main antibacterial activity comes from its isoprenyl groups, which are driven by hydrophobic interaction. In order to reduce free energy, the phenyl ring of the isoprenyl group embeds itself into the

hydrophobic region of the bacteria's membrane. This results in the membrane damage, thereby increasing penetration of AM [12]. Similarly, Khunkitti *et al.* (2010) reported that the main components of CO are monoterpene alcohols, including citronellal, geraniol, and citronellol. These compounds variously dehydrate and/or denature membrane proteins of gram positive-bacteria, causing damage to the cytoplasmic membranes and cell walls. Then the cells lose their rigid structures, leading to morphological changes, intercellular leakage, and cell lysis. In this way, the compounds inhibit or kill the bacteria cells. Moreover, Khunkitti reported that CO's activity against bacteria was greater than that of any of its major components in isolation, due to synergistic effects of multiple components [16].

It is unfortunate that no synergistic effect was found when AM and CO were combined to counter *P. acnes* and *S. aureus*. Even though, it could be used together as there is no antagonist interaction observed. In this verity, another possible avenue for future research might be to combine either AM or CO alone with one of the pharmaceutical acne treatments, and investigate if that sort of combination will yield a synergistic effect. Such a combination might exhibit considering synergy, since the mechanism of AM or CO and the mechanism of the pharmaceutical drug will probably be different.

CONCLUSION

The two natural substances alpha-mangostin and citronella oil are good candidates for the development of anti-acne products. However, using them in combination may not have substantial benefit as it was indifferent from using alone, so at least for now they might better considered independent antibacterial agents and used as such in natural products for the treatment of acne.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The two bacteria types (i.e. *P. acnes* DMST 14916 and *S. aureus* ATCC 25923) were used in this research, and it has been permitted by Technical Biosafety Committee of Naresuan University, Phitsanulok, Thailand (No. NUIBC MI 59-08-35).

AUTHORS CONTRIBUTIONS

All listed authors have contributed equally to the design and perform of the research, to the analysis of the results and to the writing of the manuscript.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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