THE DEVELOPMENT OF ANTI-ACNE PRODUCTS FROM *EUCALYPTUS GLOBULUS* AND *PSIDIUM GUAJAVA* OIL

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ABSTRACT: The aims of this study were to determine the main chemical constituents of volatile oils from leaves of *Eucalyptus globulus* Labill. (eucalyptus) and *Psidium guajava* L. (guava) and to develop stable anti-acne preparations from these medicinal plants. Volatile oils from eucalyptus and guava leaves were extracted by hydrodistillation and then were analyzed of their main chemical components by means of GC/MS. The antimicrobial activity of the oils was determined by agar diffusion and micro-dilution methods. The GC/MS results showed that the main chemical constituents in the volatile oils from eucalyptus and guava leaves were gamma-terpinene and alpha-pinene. These volatile oils showed good antimicrobial activity against *Propionibacterium acnes* (MIC, MBC = 9.38 mg/ml for eucalyptus oil and MIC = 9.38 mg/ml, MBC = 37.50 mg/ml for guava oil). Further, oil-in-water creams incorporating 2% w/w eucalyptus oil and 4% w/w guava oils were prepared. Both of them had satisfied texture and exhibited good stability after stored at -4 °C and 45 °C alternately (freeze-thawing) for 4 cycles. In addition, they had antimicrobial activity against *P. acnes* determined by agar well diffusion method (inhibition zone = 8.0 and 9.0 mm for eucalyptus cream and guava cream, respectively) compared to commercial 5% benzoyl peroxide anti-acne gel.

Keywords: *Propionibacterium acnes*, *Psidium guava*, *Eucalyptus globulus*, anti-acne product, volatile oil

INTRODUCTION: Acne is a skin disease which causes painful social and psychological effects on sufferers. It is a problem for many adults as well as for many teenagers. The primary factors involved in the formation of acne lesions are increased sebum production, sloughing of keratinocytes, bacterial growth and inflammation. *Propionibacterium acnes* (*P. acnes*), an anaerobic pathogen, plays an important role in the pathogenesis of acne by inducing certain inflammatory mediators[1)]. In Thailand, there are many powerful antimicrobial medicinal plants to research and develop for effective antimicrobial products.

Tea tree oil has been used for almost 100 years in Australia but is now available worldwide both as neat oil and as an active component in an array of products. Employed largely for its antimicrobial properties, tea tree oil is incorporated as the active ingredient in many topical formulations used to treat cutaneous infections[2)]. *Eucalyptus globulus* Labill. (eucalyptus) and *Psidium guajava* L. (guava) are the plants in the same family as tea tree (Myrtaceae). These are the plants cultivated in Thailand. The essential oil of eucalyptus and guava show antibacterial activity against wide range of bacteria including *Propionibacterium acnes* (*P. acnes*)[3-4)]. Therefore, the aim of this study is to prepare anti-acne oil-in-water cream incorporated with volatile oil extracted from the fresh leaves of eucalyptus and guava. Furthermore, accelerated stability testing by storing the preparations at 45 °C/ -4 °C for 4 cycles was carried out and antibacterial assay was determined by agar diffusion, microwell dilution and agar well diffusion methods.

MATERIALS AND METHODS:

Plant materials

Fresh leaves of *Eucalyptus globulus* Labill. (eucalyptus) and *Psidium guajava* L. (guava) were collected from botanical garden of Faculty of Pharmacy, Srinakharinwirot University, Nakhon-nayok. The voucher

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specimens (SWU 130507 and SWU 131607) were deposited in the herbarium at Faculty of Pharmacy, Srinakharinwirot University, Nakhon-nayok.

Chemicals

The excipients of the preparations (Table 1) were purchased from Namsiang trading Co., Ltd. Clindamycin (Dalacin C®) from Pfizer international Co., Ltd. and 5% benzoyl peroxide gel (5% Pan-oxyl®) from Stiefel laboratories (Thailand) Ltd. were purchased from local distributors.

Test organisms

*Propionibacterium acnes*DMST14917were purchased from NIH, Thailand.

Culture methods

The test organisms were stored on Tryptic soy agar (TSA) slopes at 2-4 °C, except for *Propionibacterium acnes*, which was stored on Brain Heart Infusion Agar (BHIA) with 10% sheep blood. The organism used in screening activities with agar diffusion assays and microwell dilution assays were twice pass-aged 16-18 hours cultures grown in Brain Heart Infusion broth (BHIB). The inoculated broths were adjusted to 0.5 McFarland standard turbidity (0.048 M $BaCl_2$ 0.5 ml and added with 0.18 M H_2SO_4 99.5 ml)

The essential oils from the leaves of eucalyptus and guava were determined for antimicrobial activities by agar diffusion and microwell dilution assay. The effectiveness of anti-acne products was determined by agar well diffusion method.

Agar diffusion assay[5-6)]

Antimicrobial tests were carried out by agar diffusion method using 100 µl of suspension containing 10^8 CFU/ml spread on Brain Heart Infusion Agar (BHIA). The discs (9.0 mm in diameter) were impregnated with 30 µl of each solution (in high soluble compounds) and placed on the inoculated agar. The diluted solution impregnated disc was used as negative control. Clindamycin was used as positive controls to determine the sensitivity of tested organism. The inoculated plates were incubated at 37 °C for 18-24 hours and in anaerobic condition for *Propionibacterium acnes.* Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms.

Microwell dilution assay[7-8)]

The minimal inhibition concentration (MIC) values were also studied for the microorganisms which were determined as sensitive to the extracted solutions in agar diffusion assay. The inocula of microorganisms were prepared from 16-18 hours broth cultures and suspensions were adjusted to McFarland standard turbidity number 0.5. The extracted solutions were diluted in serial 2-fold dilutions. The initial concentration was the highest compound of essential oils in tested solution. The 96-well plates were prepared by dispensing into each well 90 µl of broth, 5 µl of 2, 3, 5 Triphenyl-Tetrazolium Chloride (TTC) solution and 5 µl of the inoculum. A 100 µl of highest concentrations were added into the first wells. Then, 100 µl from their serial dilutions was transferred into 4 consecutive wells. The final volume in each well was 200 µl. The diluted solution impregnated discs were used as negative controls. Clindamycin was used as positive controls to determine the sensitivity of tested solutions. The plate was covered with a sterile plate sealer and mixed on plate shaker at 200 rpm for 1 minute. After that, the plates were incubated at appropriate condition for 18-24 hours. The first concentration which not showed the red color was the MIC value. The 5 µl of solution from clear wells were spread on nutrient agar plates. The MBC (Minimum Bactericidal Concentration) was defined as the concentration of the compounds to kill the microorganisms. So that, the sample concentration was not exhibited the growth of microorganisms as the MBC value.

Extraction and Identification of volatile oil

Plant materials were cut into small pieces. They were extracted by hydrodistillation with clevenger apparatus. The volatile oil was collected and stored at 2-4 °C until being used. The chemical analysis was done by gas chromatography mass spectrometry (GC/MS) on a Finnigan Trace GC ultra (Thermo Electron Corpo-ration, USA) with quadrupole mass

spectrometer. The column was ZB-5 fused silica linked methyl silicon capillary column (30 m. x 0.22 mm. i.d.; 0.25 μM); oven temperature programming was 50-250 °C at 7 °C/min; injector and detector temperature were 250 and 280 °C, respectively; sample volume injected was 1 μl; split ratio was 100:1; and the carrier gas was He (2 ml/min).

Compounds were identified by comparing the Kovats gas chromatographic retention indices of the peaks on the HP-5MS column with literature values, computer matching using the Masslynx database, and comparison of the fragmentation patterns of the mass spectra with those reported in the literature[9-10].

Preparation of oil in water anti-acne cream (o/w)

An o/w cream was prepared by addition of the pre-melted lipophilic parts to the aqueous phase components. Emulsification was achieved by low shear homogenization using a lab scale mixer (T25 basic S2, Ika Labortechnik, Staufen, Germany). The preparations were contained 2% and 4% w/w of volatile oils from eucalyptus and guava, respectively. The compositions of the preparations are given in Table 1.

Accelerated stability testing

The effect of freeze thawing on the physical stability of products was examined. The products were kept at -4 °C (48 hours) and 45 °C (48 hours) alternately for 4 cycles, and then the phase separation was observed. In addition, the products were also kept at 45 °C and -4 °C for 30 days.

Table 1 The compositions of the 2% eucalyptus and 4% guava oil creams

Excipients	% w/w	
	2% Eucalyptus	4% Guava
Stearic acid	4.0	4.0
Spermaceti	4.0	4.0
Glycerylmonostearate	6.0	6.0
White petrolatum	1.0	1.0
Silicone oil	9.0	7.0
Carbopol 940	0.3	0.3
Glycerine	2.0	2.0
PEG 1500	1.0	1.0
Citric acid	0.5	0.5
Sodium citrate	0.05	0.05
Sodium metabisulfate	0.1	0.1
Methyl paraben	0.1	0.1
Propyl paraben	0.01	0.01
Triethanolamine	1.5	1.5
Water	68.5	68.5

Agar well diffusion method[11]

The agar well diffusion method was the method to test the anti-acne products. This method was the same principle as the agar diffusion assay. It was changed only the application of the sample. The agar well diffusion method was used the reservoir of the anti-acne cream instead of the paper disc. And so on, the antimicrobial activity was determined by the diameter of inhibition zone against the tested organisms compared with the reference standard.

RESULTS ANS DISCUSSION: The extraction of volatile oil from the fresh leaves of eucalyptus and guava yields 0.4% and 0.2% w/w, respectively. Both are clear-yellow oil with characteristic odor.

The results of the screening activity of eucalyptus and guava volatile oil showed antibacterial activity against *P. acnes* by agar diffusion method. The mean diameter of the inhibition zones from eucalyptus and guava volatile oil in the concentration 62.5 mg/ml were in the range of 10.0 to 11.0 mm. The broth dilution results of eucalyptus and guava volatile oil, MIC and MBC values against *P. acnes* were determined. MIC values of eucalyptus and guava volatile oil were 9.375 mg/ml whereas MBC values of eucalyptus and guava volatile oil were 9.375 and 37.5 mg/ml, respectively. The MIC and MBC of positive control, clindamycin, were 0.5 mg/ml.

The GC/MS results in Table 2 and 3 showed that the major compounds in the volatile oil from the leaves of eucalyptus and guava were gamma-terpinene and alpha-pinene, respectively (Figure 1).

Gamma-terpinene Alpha-pinene

Figure 1 Chemical structure of gamma-terpinene and alpha-pinene

Gamma-terpinene is a monoterpene that plays an important role for antibacterial activity. It might cross the cell membranes, thus penetrating into the interior of the cell and interacting with intracellular sites critical for antibacterial activity[12].

Alpha-pinene is found in the oils of many species of many coniferous trees, especially the pine. It shows antimicrobial activity against wide range of bacteria and fungal[13].

Both eucalyptus and guava oil creams showed good texture and have proper pH to be used topically. After stored under freeze thaw condition, phase separation was not observed.

Table 2 Chemical constituents of *E. globulus* volatile oil from hydrodistillation

Compounds	%Area	Retention Time
Monoterpenes		
α -Pinene	8.57	5.67
α -Phellandrene	2.78	7.67
α -Terpinene	0.41	8.09
p- Cymene	28.75	8.36
D-Sylvestrene	2.00	8.50
γ -Terpinene	44.60	9.56
Terpinolene	0.82	10.68
Oxygenated monoterpenes		
1,8-Cineol	4.48	8.59
4-Terpineol	5.42	14.24
α -Terpineol	0.55	14.80

The antimicrobial activities against *P. acnes* of eucalyptus and guava oils containing creams were determined by agar well diffusion method (Table 4). The results demonstrated the good activities of 2% eucalyptus and 4% guava oil creams (the mean diameter of the inhibition zones were in the range of 8.0 to 9.0 mm.) compared to the commercial 5% benzoyl peroxide anti-acne gel (the mean diameter of the inhibition zone was 10.0 mm.). Their efficacy was decreased after stored under accelerated conditions (-4 °C, 45 °C, freeze thawing).

This study indicated that gamma-terpinene and alpha-pinene were the monoterpenes that having antimicrobial activity against *P. acnes*. They are the major chemical components of volatile oils from eucalyptus and guava. These results suggested that the preparations incorporating the volatile oils of eucalyptus and guava could be used as anti-acne products, however further clinical research will be necessary.

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Table 3 Chemical constituents of *P. guajava* volatile oil from hydrodistillation

Compounds	%Area	Retention Time
Monoterpenes		
α -Pinene	23.89	5.65
Sylvestrene	7.85	8.44
Oxygenated monoterpenes		
1,8-Cineol	6.32	8.54
Sesquiterpenes		
E-Caryophyllene	14.30	24.18
Aromadendrene	3.01	24.96
α –Humulene	1.51	25.54
β- Bisabolene	2.19	27.74
Oxygenated sesquiterpenes		
E-Nerodiol	3.33	29.85
Caryophyllene oxide	17.25	30.59
Caryophylla-4(12), 8(13)-dien-5-alpha-ol	2.50	32.58

Table 4 The inhibition zone of the preparations under various conditions

Preparations	Inhibition zone (mm)
2% Eucalyptus	
Freshly prepared	8.0
Stored under freeze thaw condition	6.0
Stored at 45°C (30 days)	6.0
Stored at -4°C (30 days)	7.0
4% Guava	
Freshly prepared	9.0
Stored under freeze thaw condition	7.0
Stored at 45°C (30 days)	7.0
Stored at -4°C (30 days)	8.0
Cream base	-
5% benzoyl peroxide gel	10.0

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การพัฒนาตำรับผลิตภัณฑ์ต้านสิวจากน้ำมันหอมระเหยของยูคาลิปตัสและฝรั่ง

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บทคัดย่อ: วัตถุประสงค์ของงานวิจัยนี้คือการพัฒนาตำรับผลิตภัณฑ์รักษาสิวและวิเคราะห์องค์ประกอบทางเคมีของน้ำมันหอมระเหยจากใบยูคาลิปตัส (*Eucalyptus globulus* Labill.) และใบฝรั่ง (*Psidium guajava* L.) ซึ่งได้จากการกลั่นด้วยน้ำ (hydrodistillation) จากนั้นนำไปวิเคราะห์หาองค์ประกอบทางเคมีด้วย GC/MS พบว่าองค์ประกอบหลักของน้ำมันหอมระเหยจากใบยูคาลิปตัสและใบฝรั่ง คือ gamma-terpinene และ alpha-pinene ตามลำดับ สำหรับการพิจารณาฤทธิ์ต้านสิวของน้ำมันหอมระเหยนั้น พิจารณาจากฤทธิ์ในการยับยั้งเชื้อ *Propionibacterium acnes* (*P. acnes*) ด้วยวิธี agar diffusion และวิธี microwell dilution จากผลการทดลองพบว่า น้ำมันหอมระเหยจากใบยูคาลิปตัส (MIC, MBC = 9.38 mg/ml) และน้ำมันหอมระเหยจากใบฝรั่ง (MIC = 9.38 mg/ml, MBC = 37.50 mg/ml) มีผลในการยับยั้งเชื้อ *P. acnes* ได้ดี หลังจากนั้นนำน้ำมันหอมระเหยที่ได้มาพัฒนาตำรับเป็นครีมแบบน้ำมันในน้ำ ครีมที่ได้มีลักษณะที่ดี มีความคงสภาพหลังจากเก็บที่ -4°C หรือ 45°C สลับกัน 4 รอบ ทำการประเมินประสิทธิภาพของครีมในการต้านเชื้อ *P. acnes* โดยวิธี agar well diffusion ตำรับครีมที่ผสมน้ำมันหอมระเหยจากใบยูคาลิปตัสร้อยละ 4 w/w มี inhibition zone เท่ากับ 8.0 มิลลิเมตรและตำรับครีมที่ผสมน้ำมันหอมระเหยจากใบฝรั่งร้อยละ 2 w/w มี inhibition zone เท่ากับ 9.0 มิลลิเมตร ในการยับยั้งเชื้อ *P. acnes* เทียบกับเจลรักษาสิว 5% benzoyl peroxide

คำสำคัญ: *Propionibacterium acnes* ยูคาลิปตัส ฝรั่ง น้ำมันหอมระเหย ผลิตภัณฑ์รักษาสิว

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