

Antibacterial activity of essential oil from *Wedelia trilobata* leaves against *Propionibacterium granulosum*

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ABSTRACT: This study reports on the in vitro antibacterial activity of hydro-distilled essential oil from *Wedelia trilobata* (L.) Hitchc leaves against *Propionibacterium granulosum*. The percentage yield of this essential oil via hydrodistillation was $0.21 \pm 0.01\%$ (v/w). A total of 112 chemical components present in the essential oil from *W. trilobata* leaves representing 62% of the total oil were identified by gas chromatography-mass spectrometry. The main bioactive constituents were monoterpenes, α -pinene (19.5%), α -phellandrene (7.4%), limonene (3.6%), oxazine (3.3%), and β -pinene (3.1%). This essential oil showed the moderate antioxidant capacity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) compared to the trolox equivalent antioxidant capacity and gallic acid equivalent, and possessed anti-*P. granulosum* activity from the inhibition zone from the disc diffusion method. The minimum inhibitory concentration (MIC, 595 ± 206 $\mu\text{g/ml}$) and minimum bacterial concentration (MBC, 1191 ± 413 $\mu\text{g/ml}$) values of this essential oil were higher than the MIC (1.30 ± 0.37 $\mu\text{g/ml}$) and MBC (2.61 ± 0.91 $\mu\text{g/ml}$) values of commercial clindamycin gel and the MIC (52 ± 18 $\mu\text{g/ml}$) and MBC (104 ± 36 $\mu\text{g/ml}$) values of commercial benzoyl peroxide gel. The results provide new information on the hydro-distilled essential oil from *W. trilobata* leaves, which may be important for developing new alternative anti-acne products.

KEYWORDS: antioxidant, anti-acne products, acne vulgaris, clindamycin gel, benzoyl peroxide gel

INTRODUCTION

Wedelia trilobata (L.) Hitchc is an introduced, soil creeping, evergreen perennial herb belonging to the family Asteraceae (formerly Compositae). Its synonym is *Silphium trilobatum*. It is mostly distributed in the tropical and subtropical countries such as Central America, South America, and the Pacific Islands. This invasive species can also be found in Thailand. Over the past decades, it has been historically used as a traditional folk medicinal plant, such as anti-headache, antipyretic¹, and to treat upper respiratory tract infection². In addition, previous studies reported that the methanolic and ethanolic extracts of *W. trilobata* flowers and leaves exhibited antioxidant and antibacterial activities³. In particular, the bioactive compounds from ethanolic extract of *W. trilobata* leaves showed potential wound-healing properties⁴. Crude extracts from other parts of *W. trilobata* also showed antibacte-

rial activity against some strains of Gram-positive bacteria (*Bacillus cereus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*), Gram-negative bacteria (*Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella* group C, *Salmonella paratyphi*, *Shigella sonnei*), and fungus (*Aspergillus flavus*, *Aspergillus niger*, *Aspergillus nidulans*, *Aspergillus flaviceps*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticilloides*)⁵. Several previous studies showed that the essential oil (EO) obtained from the leaves of *W. trilobata* contains a large variety of terpenes⁶ and exhibits antioxidant activity and antibacterial activity against *B. subtilis* and *S. aureus*⁷. No previous study has been reported about the essential oil from *W. trilobata* leaves against *P. granulosum* because *P. granulosum* belongs to the normal human skin flora. However, *P. granulosum* possesses a pathogenic role in acne vulgaris and plays an important synergistic role in the inflammation with *Propi-*

onibacterium acnes which converts sebum oils into free fatty acids that accumulate in the hair follicle, form comedones, and then induce inflammation. Somehow, although topical anti-acne products (clindamycin, erythromycin, azelaic acid, tetracycline, and doxycycline) can reduce both *P. acnes* and *P. granulosum* population and exert anti-inflammatory actions, they lack antioxidant activity⁸ and may cause common side effects such as drug resistance and dark spots on the skin. Alternative natural essential oils can act against *P. granulosum* with a good antioxidant activity and may possess the necessary key activities for effective acne treatment.

The present study, therefore, aimed at studying the in vitro antibacterial activity of essential oil from *W. trilobata* leaves against *P. granulosum* gels by disc diffusion technique and two-fold serial dilution method, and compare it to commercial benzoyl peroxide and clindamycin. This study was designed to extract essential oil from *W. trilobata* leaves by hydrodistillation. The biochemical composition of EO was identified by gas chromatography-mass spectrometry (GC-MS). Antioxidant capacity of EO was assessed by the free radical scavenging, DPPH assay. The findings from this study may be valuable to further in-vivo studies for acne product development.

MATERIALS AND METHODS

Solvents and chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), the water-soluble analogue of vitamin E (Trolox), and gallic acid were obtained from Sigma-Aldrich Co., St. Louis (USA). Clindamycin gel (Clindalin gel, Union Drug Laboratories Ltd., Thailand) and 2.5% benzoyl peroxide gel (Benzac, Laboratories Galderma, France) was purchased from the drug store. Brain heart infusion (BHI) broth was purchased from Titan Biotech Ltd. (India), and Tween 80 was purchased from Uniqema Ltd. (Belgium). All solvents were analytical grade and used without further purification.

Collection of plant materials

Fresh specimen of *W. trilobata* leaves was collected from the area in University of Phayao, Phayao, Thailand on May 2015. A voucher specimen of this plant was deposited in the herbarium of the School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand.

Extraction of the essential oil

The fresh leaves of *W. trilobata* were cleaned by distilled water and dried at room temperature. Then 100 g of *W. trilobata* leaves was distilled in water using an all-glass Clevenger-type apparatus ($n = 3$). The obtained essential oil was dried over anhydrous Na_2SO_4 and measured for calculation of percentage yield. The oil was stored in an amber vial at 4 °C until analysis.

GC-MS analysis

The hydro-distilled essential oil from leaves of *W. trilobata* was analysed by GC-MS using Agilent 6890 gas chromatograph equipped with a full-scan Hewlett Packard 5973 mass-selective detector in the electron impact mode (70 eV). Separation was achieved by using Helium as carrier gas (1 ml/min) with a fused silica capillary column (HP5-MSI) of 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. Injector and detector temperatures were 260 °C and 280 °C, respectively; with oven temperature programmes: 3 min isothermal at 70 °C, then raised at 3 °C/min to 188 °C, and 20 °C/min to 280 °C (3 min isothermal). Diluted samples 1/1000 in dichloromethane (v/v) of 1.0 μl were injected manually in the splitless mode. The percentage of peaks area was used for obtaining quantitative data. The programmed-temperature Kováts retention indices (RI) were obtained by GC-MS analysis of an aliquot of the volatile oil spiked with an *n*-alkanes mixture containing homologues from *n*-C₉ to *n*-C₂₀. Identification of the compounds was based on a comparison of their mass spectra database (WILEY&NIST) and spectroscopic data.

Antioxidant capacity analysis

The antioxidant capacity (AOC) of the essential oil of *W. trilobata* leaves was analysed by using DPPH method adapted from Bhatnagar et al⁹. EO with different weights was added to 3 ml of ethanolic DPPH (60 μM) to obtain the final concentrations of 1.5, 3.1, 6.2, and 12.5 mg/ml ($n = 3$). The mixture was shaken vigorously for 20 s at ambient temperature and the decrease in the absorbance at 517 nm was measured in a 1-cm disposable cuvette after 0, 5, 10, 15, 20, 25, and 30 min using double beam UV-Vis recording spectrophotometer (JASCO V-630). The AOC was calculated as $(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}$, where A_{blank} is the absorbance of the control reaction (containing all reagents except EO) and A_{sample} is the absorbance of EO. Inhibition of antioxidant capacity at 50% (IC₅₀) was calculated

and compared with trolox equivalent antioxidant capacity (TEAC) and gallic acid equivalent (GAE) as reference standards.

Bacterial strain identification

P. granulosum (DSM 20700) was originally isolated from disposable specimen of acne lesion from Medicinal Centre, University of Phayao, Phayao, Thailand. This bacterial isolate was identified by the conventional method including microscopy examination, safranin staining protocol, catalase test, and indole test. The identification result was confirmed by 16S ribosomal RNA gene sequencing and compared with nucleotide sequence of the GenBank database reference number 118646.1. The stock culture of *P. granulosum* was preserved in brain heart infusion agar deep tubes kept in anaerobic jar in an oven at 37 °C for 48 h.

Sensitivity of *P. granulosum* by disc diffusion

The disc diffusion method was used to determine the inhibition zone of *P. granulosum* from EO of *W. trilobata* leaves¹⁰. Approximately 10⁶ CFU/ml of *P. granulosum* was seeded onto 20 ml of BHI agar. Then 10 µl of essential oil was aseptically impregnated onto 6 mm paper disc in one application and dried at room temperature for 30 min. An empty disc was used as a negative control. Discs containing commercial 2.5% benzoyl peroxide gel as lipophilic standard compound and 1% clindamycin gel as lyophobic standard compound were used as positive controls. The discs were then placed on the surface of the seeded agar. Then the plates were kept in anaerobic jar and incubated at 37 °C for 48 h. The sensitivity of *P. granulosum* was defined by measuring the diameter of the growth inhibition zone. Each sample was performed in triplicate.

Confirmation of essential oil from *W. trilobata* leaves against *P. granulosum*

Anti-*P. granulosum* activity of EO was confirmed by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a dilution broth method. The MIC and MBC values of EO were compared to commercial 2.5% benzoyl peroxide gel and 1% clindamycin gel. Tween 80 was used to stabilize the EO. All tests were investigated in BHI containing 1% glucose. A two-fold dilution of EO and commercial 1% clindamycin gel was prepared in test tubes at concentrations that ranged from 0.39–1000 µg/ml, whereas commercial 2.5% benzoyl peroxide gel concentrations

ranged from 7.9–2000 µg/ml. The culture of *P. granulosum* was prepared by incubating in BHI broth with 1% glucose for 72 h under anaerobic condition. The bacterial culture was then added to each tube to make a final concentration of approximately 10⁶ CFU/ml. In each test, *P. granulosum* in BHI broth and BHI broth alone were used as a positive and negative growth control, respectively. All test tubes were then incubated in an anaerobic jar at 37 °C for 72 h before determination of the MIC and MBC. The cell pellet at the bottom was used to indicate the bacterial growth. The MIC value was defined as the lowest concentration of essential oil at which the bacteria do not express visible growth. To determine the MBC value, 10 µl of broth was transferred from each test tube and spotted onto BHI agar. The number of surviving *P. granulosum* was determined after incubated at 37 °C under anaerobic conditions for 72 h. The lowest concentration where no initial inoculum survived was defined as MBC value. Each experiment was performed in triplicate.

Statistic analysis

All determinations were carried out in triplicate and the results are given as means ± standard deviation. The statistical analysis was performed by using one-way ANOVA for 3 samples comparison at a significance level of 0.05.

RESULTS AND DISCUSSION

Extraction of the essential oil and GC-MS analysis

The hydrodistillation of 100 g fresh *W. trilobata* leaves yielded 0.21 ± 0.01% (v/w). This hydro-distilled EO is a clear light-yellow oil with a distinctive odour. The hydro-distilled EO was analysed by GC-MS, and the bioactive constituents were identified based on their retention time values compared to those reported in the mass spectra database (WILEY&NIST) and spectroscopic data representing the Kováts RI. The GC-MS analysis of this hydro-distilled EO of *W. trilobata* indicated that the main bioactive constituents were monoterpenes, such as α-pinene (19.46 ± 0.04%), α-phellandrene (7.39 ± 0.04%), limonene (3.63 ± 0.05%), oxazine (3.34 ± 0.03%), and β-pinene (3.12 ± 0.03%) (Table 1). The percentage of most of the individual constituents present in essential oil of *W. trilobata* leaves was not significantly changed during the hydrodistillation ($p < 0.05$). The current results indicate that the essential oil of *W. trilobata* leaves contains compo-

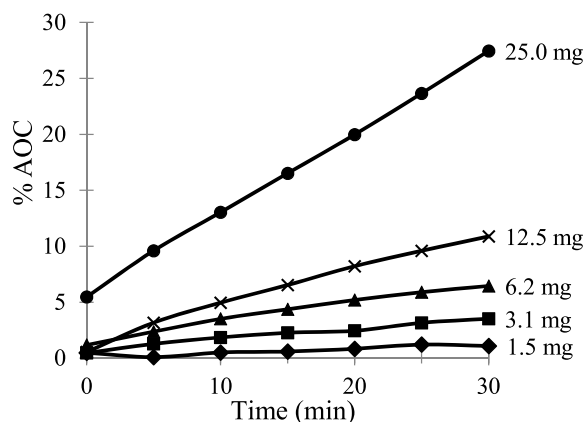
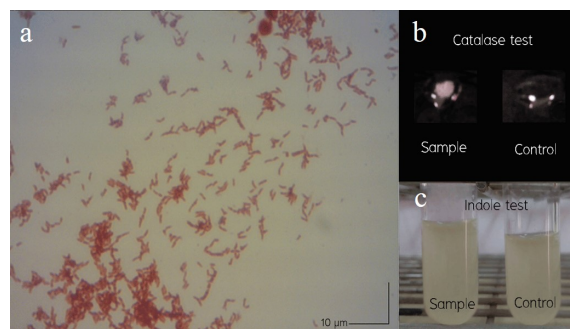
Table 1 Chemical constituents of hydro-distilled essential oil from *W. trilobata* leaves by GC-MS.

Chemical compound	Area (%)	Retention time (m)	Kováts RI
α -pinene	19.46 \pm 0.04	4.15 \pm 0.01	982 \pm 1
α -phellandrene	7.39 \pm 0.04	5.81 \pm 0.01	1003 \pm 1
limonene	3.63 \pm 0.05	6.39 \pm 0.01	1029 \pm 1
oxazine	3.34 \pm 0.03	32.2 \pm 0.01	1153 \pm 1
β -pinene	3.12 \pm 0.03	5.07 \pm 0.01	964 \pm 1
γ -terpinene	0.03 \pm 0.01	7.41 \pm 0.01	1060 \pm 1
α -terpinolene	0.03 \pm 0.01	8.40 \pm 0.02	1089 \pm 1
4-terpineol	0.05 \pm 0.02	11.89 \pm 0.02	1177 \pm 2
aromadendrene	4.02 \pm 0.01	23.00 \pm 0.02	1490 \pm 2
caryophyllene	0.32 \pm 0.01	20.54 \pm 0.01	1419 \pm 1
caryophyllene oxide	0.92 \pm 0.01	27.99 \pm 0.01	1583 \pm 1

nents relatively similar to those of other *Wedelia* species that showed monoterpenes, such as α -pinene, α -phellandrene, limonene, and caryophyllene, as the major compounds^{6,11-14}. The percentage difference of chemical components in essential oils may be due to different material resources, seasonal variation, and extraction methods.

Antioxidant capacity analysis

The scavenging of free radicals is one of the well-known mechanisms by which antioxidants inhibit lipid oxidation¹⁴, and the scavenging activity on DPPH radicals has been widely used to determine the free radical scavenging activity of active compounds as antioxidant. The DPPH radical scavenging activity can be reduced by the hydrogen donating ability¹⁵. Hence the measurement of the comparative interceptive ability of antioxidant EO from *W. trilobata* leaves by determining its ability to scavenge DPPH radical has been proposed. As a dose-dependent manner, the increasing concentrations of hydro-distilled EO from *W. trilobata* leaves ($IC_{50} = 23.31 \pm 0.10$ mg/ml) was a trend towards increased antioxidant capacity with moderate antioxidant activity for a period of 30 min as shown in Fig. 1 compared to TEAC ($IC_{50} = 9.37 \pm 0.08$ μ g/ml) and GAE ($IC_{50} = 5.73 \pm 0.04$ μ g/ml). This current result indicates that the essential oil of *W. trilobata* with moderate antioxidant activity contains α -pinene as a major bioactive compound relatively similar to a previous study that also reported moderate antioxidant activities for the essential oil of *Wedelia* species and its main components (limonene and α -pinene) compared to α -tocopherol and limonene¹².

**Fig. 1** The % AOC of different concentrations from hydro-distilled essential oil from *W. trilobata* leaves.**Fig. 2** (a) Gram stain and morphology of *P. granulorum* under light microscopy by 100 \times 10-fold magnification, (b) positive result in catalase test, and (c) negative result in indole test.

Bacterial strain identification

The morphology and biochemical tests of isolated bacterial from disposable specimens were examined as preliminary identification. The results showed that the isolates of disposable specimen is Gram-positive bacteria with a rod-shaped bacterium because they counter-stained pink safranin as observed under light microscopy (Fig. 2a). The specimen (sample) also showed a positive result in catalase test after hydrogen peroxide was dropped into the specimen (Fig. 2b), whereas the indole test of both sample and control showed no cherry-red ring (Fig. 2c). From these results, the specimen could be *P. granulorum* or *P. avidum*. To distinguish between *P. granulorum* and *P. avidum*, the 16S ribosomal RNA gene was sequenced, and the result showed 99% homology with *P. granulorum* reference strain DSM 20700, and no gaps of the nucleotide sequence between the isolated specimen and the reference

Score 1354 bits (733)	Expect 0.0	Identities 735/736 (99%)	Gaps 0/736 (0%)	Strand Plus/Plus
Query 1		AACACGTTGAGTAACTCCGCCCAACTTTGGGATAACGCTAGGAACCTGGTCTAATCTG		60
Sbjct 93		AAACGCTTGAGTAACTCCGCCCAACTTTGGGATAACGCTAGGAACCTGGTCTAATCTG		152
Query 61		GATATGCTCCTCTCGCATGTGGGGGTTGGAAAGCTCCGGCGGTTGTGGATGGACTGG		120
Sbjct 153		GATATGCTCCTCTCGCATGTGGGGGTTGGAAAGCTCCGGCGGTTGTGGATGGACTGG		212
Query 121		CGGCCTATCAGTTGTTGGTGGGTAAGTGGCTACCAAGCGCGCGACGGGTAGCCGGCT		180
Sbjct 213		CGGCCTATCAGTTGTTGGTGGGTAAGTGGCTACCAAGCGCGCGACGGGTAGCCGGCT		272
Query 181		GAGAAGGTGACCGCCACATTTGGGACTGAGATACGGCCAGACTCTTACGGGAGCGACGA		240
Sbjct 273		GAGAAGGTGACCGCCACATTTGGGACTGAGATACGGCCAGACTCTTACGGGAGCGACGA		332
Query 241		GTGGGAAATTTGCACAAATGGCGCAAGCTGATGCGAGCAACCGCGGTGGGATGAGC		300
Sbjct 333		GTGGGAAATTTGCACAAATGGCGCAAGCTGATGCGAGCAACCGCGGTGGGATGAGC		392
Query 301		GCCTCGGGTTTAAACCGCTTTTCCAGCGGACGAAGCTTTTGTGACGCTACTGACGA		360
Sbjct 393		GCCTCGGGTTTAAACCGCTTTTCCAGCGGACGAAGCTTTTGTGACGCTACTGACGA		452
Query 361		AGAGCACCGGCTAACTACTGTCGCGAGCGCGCGGTGATAGGTTGGAGGCTTGTCT		420
Sbjct 453		AGAGCACCGGCTAACTACTGTCGCGAGCGCGCGGTGATAGGTTGGAGGCTTGTCT		512
Query 421		CGGATTTATTTGGGCTAAAGGCGCTGAGGCGGTTGATCGCTCGAAGTGGAACTTGA		480
Sbjct 513		CGGATTTATTTGGGCTAAAGGCGCTGAGGCGGTTGATCGCTCGAAGTGGAACTTGA		572
Query 481		TGCTTAACTTTGAGGCTGCTTTCGATACGGTTCACCTGAGGAAAGTGGGGAGAATGA		540
Sbjct 573		TGCTTAACTTTGAGGCTGCTTTCGATACGGTTCACCTGAGGAAAGTGGGGAGAATGA		632
Query 541		ATTCCTGTGGAGCGTGGAAATGCGCAGATATCAGGAGAACACAGTGGCGAAGCGGT		600
Sbjct 633		ATTCCTGTGGAGCGTGGAAATGCGCAGATATCAGGAGAACACAGTGGCGAAGCGGT		692
Query 601		TCTCTGGATTTTCTGACCGTGAAGGCGAAGCGTGGGAGCGAACAGGCTTAGATAC		660
Sbjct 693		TCTCTGGATTTTCTGACCGTGAAGGCGAAGCGTGGGAGCGAACAGGCTTAGATAC		752
Query 661		CCTGGTAGTCCACGCTGTAACCGGTGGGATAGGTGTGGGCTCATTCCAGGATTTCTG		720
Sbjct 753		CCTGGTAGTCCACGCTGTAACCGGTGGGATAGGTGTGGGCTCATTCCAGGATTTCTG		812
Query 721		TCCGCTAGCTAACGCA 736		
Sbjct 813		TCCGCTAGCTAACGCA 828		

Fig. 3 Partial nucleotide sequence of 16S ribosomal RNA of the isolated specimen of *P. granulosum*.

were found in the rage from 93–828 (Fig. 3). This isolated specimen could be considered as *P. granulosum* strain that can possess the pathogenic role in acne vulgaris and plays an important synergistic role in the inflammation with *P. acnes*. Although the EO extracted from the leaves of *W. trilobata* showed antioxidant activity and exhibited antibacterial activity against both Gram-positive and -negative bacteria³⁻⁵, no anti-*P. granulosum* activity was reported. Hence sensitivity of *P. granulosum* by disc diffusion method was studied in the next step.

Sensitivity of *P. granulosum* to the essential oil from *W. trilobata*

The sensitivity of hydro-distilled EO from *W. trilobata* leaves against *P. granulosum* was qualitatively and quantitatively assessed by the presence of inhibition zones, MIC and MBC values (Table 2). Concerning disc diffusion method, clindamycin gel possessed the highest susceptibility against *P. gra-*

Table 2 Sensitivity of *P. granulosum* to hydro-distilled essential oil from *W. trilobata* leaves by disc diffusion and two-fold serial dilution methods.

Subject	Inhibition zone (mm)	MIC (µg/ml)	MBC (µg/ml)
empty disc	0	-	-
clindamycin gel	53.5 ± 3.1	1.3 ± 0.4	2.6 ± 0.9
benzoyl peroxide gel	18.3 ± 2.1	52 ± 18	104 ± 36
essential oil	12.3 ± 1.7	595 ± 206	1191 ± 413

nulosum, followed by benzoyl peroxide and hydro-distilled EO, whereas the empty disc did not affect the growth of *P. granulosum* as for the negative control. The results of MIC and MBC values showed that hydro-distilled EO (MIC 595 ± 206 and MBC 1191 ± 413 µg/ml) was higher than clindamycin (MIC 1.3 ± 0.4 and MBC 2.6 ± 0.9 µg/ml) and benzoyl peroxide gels (MIC 52 ± 18 and MBC 104 ± 36 µg/ml). This means the essential oil of *W. trilobata* leaves has lower anti-*P. granulosum* than clindamycin and benzoyl peroxide gels. Although clindamycin and benzoyl peroxide have a good efficacy against *P. granulosum*, they may induce side effects and oxidation reaction after application. To overcome the disadvantages of clindamycin and benzoyl peroxide, the hydro-distilled EO from *W. trilobata* leaves may decrease the side effects and oxidation reaction of clindamycin and benzoyl peroxide and give valuable information for the development of a new alternative anti-acne product with mild antioxidant activity. In addition, the synergistic effects of the diverse major and minor chemical components of essential oils from *W. trilobata* leaves should be further taken into consideration to account for the oil biological activity.

CONCLUSIONS

The hydro-distilled EO of *W. trilobata* leaves possesses antibacterial activity against *P. granulosum* with moderate antioxidant capacity compared to commercial clindamycin and benzoyl peroxide gels. This may be due to the synergistic effects of α-pinene and other bioactive compounds in the oil. This present study suggests a possible alternative option from this oil for the development of new effective anti-acne products in the near future. However, the toxicological properties should be further studied in an in vivo model to support the information of this study.

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