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Correlations of the components of tea tree oil with its antibacterial effects and skin irritation

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ABSTRACT

Tea tree oil (TTO), the essential oil of *Melaleuca alternifolia* L., is one of the most popular agents used in cosmetics. This study aimed to explore the correlations of components in TTO with its anti-acne activities and skin irritation. TTOs were isolated from the leaves (TTO-L), twigs, and branches of *M. alternifolia* by steam distillation, and the components analyzed by gas chromatography–mass spectrometry. Results showed that components of TTO-L satisfied the International Organization for Standardization (ISO) 4730 guidelines. TTO-L and its components, terpinen-4-ol, terpinolene, α-terpinene, and α-terpineol, had strong inhibitory activities against *Propionibacterium acnes* and *Staphylococcus aureus*. Moreover, six TTO formulas (DF-TTO) were designed according to ISO 4730 guidelines by adjusting the proportions of these four active components. All DF-TTO formulas showed a positive correlation between terpinen-4-ol concentration and anti-*P. acnes* activity. In the skin irritation assay, TTO-L, terpinen-4-ol, and 1,8-cineole did not cause significant skin irritation at 2% per site. In conclusion, terpinen-4-ol is the major active component responsible for TTO's antibacterial efficacy, while minor components in TTO also contributed to its efficacy. Moreover, we suggest that a concentration less than 5% is more suitable and safer for treating acne than higher concentrations.

1. Introduction

Acne is a common skin problem that usually appears during adolescence, and most often manifests in the skin surface of the face, neck, arms, and back. There are several main causes of acne, such as increased sebum production by overactive oil glands, blockage of skin pores by retention hyperkeratosis, normal skin bacterial activities (*Propionibacterium acnes* and *Staphylococcus aureus*), and inflammation. Among these four reasons, the overactivation of *P. acnes* and *S. aureus* plays a

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critical role in acne formation and produces severe inflammatory responses. Therapeutic treatment of bacterium-induced acne is antibiotic application, such as neomycin, tetracycline, and erythromycin. However, excessive use of antibiotics can cause antibiotic resistance that poses a serious threat to public health worldwide [1].

Alternative and complementary medicines have been extensively investigated in recent years. Essential oils, a type of plant secondary metabolites, have been evaluated for their bioactivities and potential therapeutic uses, such as anticancer, antimicrobial, and anti-inflammation [1–3]. The potential synergy of essential oils with antibiotics was previously postulated, with the aim of alleviating the burden of antimicrobial resistance to conventional antimicrobials [4]. Among the different types of essential oils, Australian tea tree oil (TTO), obtained from *Melaleuca alternifolia* L. (Myrtaceae) [5], is one of the best-known essential oils that is frequently used to treat skin, airway, oral, and vaginal infections, or used as an antiseptic and disinfectant [6]. Many reports describing the broad spectrum of TTO's antibacterial and antifungal activities have been published and are summarized in Tables 1 and 2 [7–12].

The main ingredients of TTO are terpene hydrocarbons, such as monoterpenes, sesquiterpenes, and their associated alcohols. Moreover, TTO is reported to contain more than 100 components. According to the International Organization for Standardization (ISO) 4730 guidelines, the concentrations of TTO and terpinen-4-ol should be more than 30%, while that of 1,8-cineole should be less than 15%. In addition, the antibacterial activities of the components have also been reported, such as that of terpinen-4-ol, a well-studied antibacterial component in TTO. The antibacterial activities are summarized in Table 3 [9,13–15]. However, there are many differences among experiments, such as in the methods (agar diffusion or broth dilution), the performance units of the minimum inhibitory concentration (MIC), and in minimum bactericidal concentration (MBC) values (percentage or mg/mL). However, there is no systematic assessment method available to explore the antibacterial effects of each component.

On the other hand, 1,8-cineole, an abundant component in *Eucalyptus polybractea*, is also abundantly found in TTO. There is evidence to show that when koalas are only fed with *E. polybractea*, 1,8-cineole exerts a narcotic effect and results in long periods of sleep [16,17]. However, with increased use of TTO, safety concerns have increased. In Rutherford et al's study, 2320 patients who used a patch-test correlated with TTO and skin irritations over a 5-year period at the Skin and Cancer Foundation were retrospectively reviewed; the results showed that 41 cases had positive reactions to TTO, giving a prevalence of 1.8% [18]. In addition, many studies reported that exposure to a high dose of TTO can cause significant skin and mucous membrane irritation [19].

Table 1 – Reference reviews of the antibacterial activity of TTO [7–9,12].

Bacterial species	% (v/v)	
	MIC	MBC
Gram-positive		
Actinomyces viscosus	0.6	>0.6
Bacillus cereus	0.3	–
Enterococcus faecalis	>8	>8
Staphylococcus aureus	0.5	2
Staphylococcus marcescens	0.25	0.25
MRSA	0.5	8
Gram-negative		
Acinetobacter baumannii	1	1
Fusobacterium nucleatum	0.06	0.06
Klebsiella pneumoniae	0.25	0.25
Pseudomonas aeruginosa	3	3
Mollicutes		
Mycoplasma hominis	0.12	–
Mycoplasma fermentans	0.03	–

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; MRSA = methicillin-resistant *Staphylococcus aureus*.

Table 2 – Reference reviews of the antifungal activity of TTO [10,11].

Fungal species	% (v/v)	
	MIC	MFC
Aspergillus niger	0.02	0.05
Candida species	0.03	0.05
C. albicans	0.25-0.5	–
C. glabrata	0.25-1	–
Madurella mycetomatis	0.25	–

MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration.

Table 3 – Reference reviews of the antibacterial properties of the components of TTO [9,13,15].

Component	Bacterial species	mg/mL (w/v)	
		MIC	MBC
Terpinen-4-ol	*Streptococcus pyogenes* (G+)	0.8	1.6
	Streptococcus gordonii (G+)	0.1	0.2
	MRSA	0.25 (%)	0.5 (%)
	Fusobacterium nucleatum (G–)	0.1	0.4
	Prevotella intermedia (G–)	0.2	0.4
	Porphyromonas gingivalis (G–)	0.1	0.4
1,8-Cineol	*Staphylococcus epidermidis* (G+)	1.6	3.2
	Pseudomonas aeruginosa (G–)	0.25	–
	Salmonella typhimurium (G–)	0.25	–
α-Pinene	*Prevotella intermedia* (G–)	0.8	0.8
	Porphylomonas gingivalis (G–)	0.8	1.6
α-Terpineol	*Streptococcus gordonii* (G+)	0.05	0.1
	Fusobacterium nucleatum (G–)	0.4	0.8
	Prevotella intermedia (G–)	0.2	0.4
	Porphyromonas gingivalis (G–)	0.2	0.8
Sabinene	*Streptococcus pyogenes* (G+)	0.2	0.4
	Streptococcus mutans (G+)	0.8	1.6
	Streptococcus sanguinis (G+)	0.4	0.4
	Streptococcus sobrinus (G+)	0.2	0.2
	Streptococcus criceti (G+)	0.1	0.2

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; G(+) = positive Gram staining; G(–) = negative Gram staining; MRSA = methicillin-resistant *Staphylococcus aureus*.

In this study, TTOs were obtained from *M. alternifolia* and their compositions analyzed by gas chromatography–mass spectrometry (GC–MS). The efficacies of the antibacterial activities of TTO and each component were evaluated, and safety was assayed by acute dermal toxicity and chronic liver toxicity tests using Wistar rats. Newly designed TTO formulas were used, in which the composition percentages of terpinen-4-ol and 1,8-cineole were adjusted so that the correlations between these two TTO components with TTO's antibacterial effects and skin irritation could be determined.

2. Methods

2.1. General

Analytical-grade terpinen-4-ol (Fluka code 86477), γ-terpinene (Fluka code 86476), α-terpinene (Fluka code 86473), 1,8-cineole (Fluka code 29210), terpinolene (Fluka code 86484), ρ-cymene (Fluka code 30039), α-pinene (Aldrich code 14, 752-4), α-terpineol (Aldrich code 43, 262-8), aromadendrene (Fluka code 11067), limonene (Fluka code 89188), sabinene (Fluka code 96573), and globulol (Fluka code 49070) were purchased from Sigma-Aldrich (Milan, Italy). The bacterial culture equipment, including an anaerobic atmosphere by MGC AnaeroPack-Anaero and MGC AnaeroPack-Jar, were from Mitsubishi Gas Chemical Company (Tokyo, Japan). Blood agar plates (BAPs), Bacto™ tryptic soy broth (TSB), and Bacto™ tryptic soy agar (TSA) were from Difco (Detroit, MI, USA).

2.2. Animals

The following animal experiment was conducted according to the Ethical Regulations on Animal Research of Taipei Medical University (approval no. LAC-95-0005). Female Wistar rats (nulliparous and non-pregnant), aged 8–10 weeks and weighing about 200–220 g, were bought from BioLASCO in Taiwan. Rats were maintained at 25 ± 1°C with food and water *ad libitum* and kept on a 12-hour light/dark cycle.

2.3. Preparation of TTO from M. alternifolia

M. alternifolia was collected from the Taiwan Seed Improvement and Propagation Station (TSIPS, Taichung, Taiwan). Voucher specimens (MA-01) were identified and deposited in the TSIPS. TTO was obtained through steam distillation of dried *M. alternifolia* leaves (TTO-L), twigs (TTO-T), and branches (TTO-B) in a Clevenger-type apparatus for 7 hours. Each distillate was partitioned with ether and the ether layer was separated and evaporated to dryness at room temperature to afford different part of TTOs. All TTOs were stored at 4°C until analysis.

2.4. Quality control of TTO by GC–MS

The experimental protocol of the quality control of all TTOs was modified from our previous study [20]. All TTO sample analyses were carried out on a gas chromatographer GC-2010 equipped with a GC–MS (GCMS-QP2010, Shimadzu, Tokyo, Japan). A DB-5MS column (0.25 mm i.d. × 30 m × 0.25 μm film; J&W Scientific, Folsom, CA, USA) was used with helium as the carrier gas at a constant pressure of 73.0 kPa. The injection temperature and ion source temperature were 300°C and 200°C, respectively. The GC oven temperature was programmed to be maintained at 50°C for 3 minutes, raised to 250°C at 20°C/minutes, maintained for 5 minutes, raised to 300°C, and maintained for 3 minutes. The mass range was 30–350 *m/z*. The mass spectra of the GC–MS chromatograms were compared to the database of the NIST/EPA/NIH Mass Spectral Library to identify possible components.

2.5. Growth conditions of P. acnes and S. aureus

Strains of *P. acnes* (BCRC10723) and *S. aureus* (BCRC 10781) were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). *P. acnes* was cultured in BAP, Bacto™ TSB, and Bacto™ TSA in an anaerobic atmosphere using MGC AnaeroPack-Anaero and MGC AnaeroPack-Jar. After that, *P. acnes* was tested by subculturing in BAP at 37°C for 72 hours. *S. aureus* was cultured in Bacto™ TSB and Bacto™ TSA, while the test was performed by subculturing in TSB at 37°C for 24 hours.

2.6. Agar well-diffusion test

The experimental protocol was modified from Cai et al [21]. TTO was tested against *P. acnes* by determining the MIC values by the agar well-diffusion method. Briefly, *P. acnes* was incubated on BAP agar under anaerobic conditions. A freshly grown culture was diluted with Bacto™ TSB, and 10 mL of prepared bacteria [4×10^8 colony-forming units (cfu)/mL] was aseptically added to 90 mL of sterilized media (TSA) at 45°C in a water bath. The seeded agar media were immediately mixed and poured into a Petri dish. Sterilized stainless steel cylinders (8 mm in diameter) were put on a Petri dish to create the sample wells. TTO and different terpene samples were dissolved in jojoba oil and tested in 2-fold serial dilutions. Different kinds of TTO samples, at dosages of 0–80%, were added to the sample wells. In addition, another composition of TTO (devised formula, DF) was designed, in which the percentages of terpinen-4-ol and 1,8-cineole were adjusted to test the antimicrobial activities. Jojoba oil and 10 U of penicillin were used as the respective negative and positive controls. Plates were incubated under anaerobic conditions at 37°C for 24 hours until visible growth of the test microorganisms was evident in the control plates. Inhibition zones in millimeters (including the disc diameter) were measured. The experiment was repeated in triplicate. Moreover, *S. aureus* (5×10^7 cfu/mL) was seeded in agar media and immediately mixed in a Petri dish. The experimental protocol was the same as that described above with some modifications. The antimicrobial activity was expressed as the diameter of the inhibition zones against the test microorganisms as follows:

$$\text{Antimicrobial activity (\%)} = \left(\frac{\text{diameter zone of sample}}{\text{diameter zone of pencillin}}\right) \times 100$$

The MIC and MBC of TTO were determined by agar well-diffusion test. An inoculation loop was used to collect bacteria from clear zone on agar plate. The inoculation loop was enriched in TSB for 24 hours. At the end of the incubation, TSB were put

onto the surface of nutrient agar plates and incubated at 37°C for 24 hours. The MBC was estimated as the least concentration of the TTO where no visible growth was observed. The MIC was estimated as the least concentration of the TTO where visible growth was observed.

2.7. Skin irritation assay of TTO in Wistar rats

The acute primary irritation test was applied by following the classic Draize test [22]. The potential toxicity of TTO was evaluated from a single topical application on Wistar rats. Twenty-four hours before the test, the backs of the rats were shaved free of hair (2.5 × 2.5 cm at each site) and checked for any abnormalities (integrity and allergies) the next day. The foundation color of the skin was measured before the test. An appropriate concentration of a variety of TTOs (50 μL for each sample) was smeared onto the shaved back of each Wistar rat. Each group consisted of five rats. The patches were secured for a 4-hour exposure period and removed with distilled water. After application of the TTOs for 24 and 48 hours, skin irritation was observed, and the extent of evident skin allergic reaction was evaluated for each test animal. Levels of skin irritation were quantified through the Draize score (Table 4).

2.8. Chronic liver toxicity of TTO-L in Wistar rats

The experimental protocol for liver toxicity was modified from our previous study [23]. TTO-L (2%, 50 μL) was smeared on the shaved back of three Wistar rats daily for 28 days. On days 0 and 28, rats were lightly anesthetized, blood was obtained through the tail vein, and glutamine-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels were simultaneously measured on a Fuji DRI-CHEM 3500i analyzer (Tokyo, Japan).

2.9. Statistical analysis

Data are presented as the mean and standard deviation (SD). Significance was calculated using Student's *t*-test by SPSS software. Differences were considered significant at $p < 0.05$.

Table 4 – Draize scoring system in albino rats.

Symptom	Irritation reaction
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate-to-severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of site well-defined by definite raising)	2
Moderate edema (site raised 1 mm)	3
Severe edema (site raised >1 mm and extending beyond area of exposure)	4

3. Results

3.1. Composition of TTOs in different parts of M. alternifolia

In the current study, TTO components were extracted from various parts of *M. alternifolia*. *M. alternifolia* was divided into three groups according to branch thickness (Table 5). As shown in Table 5, TTO was predominantly in the leaves rather than the branches. The yields of TTO-L, TTO-T, and TTO-B were 2.02%, 0.59%, and 0.01%, respectively. The percentage compositions of components identified in the TTOs are shown in Table 6. According to ISO 4730 guidelines, the percentage of terpinen-4-ol should be >30.0% and that of 1,8-cineole < 15.0%. Results showed that only TTO-L complied with ISO 4730 specifications. The major components in TTO-L were terpinen-4-ol (47.31%), γ-terpinene (20.59%), and α-terpinene (9.58%); the percentage of 1,8-cineole, another notable component, was 1.71%.

3.2. Antibacterial effects of TTO-L and TTO components

Firstly, TTO-L was dissolved and diluted with jojoba oil before the test. The inhibition ratio of TTO-L is shown in Fig. 1. TTO-L presented high susceptibility and significant and dose-dependent antibacterial activities against *P. acnes*. In *S. aureus*, TTO-L presented slight antibacterial activities. The MIC and MBC of TTO-L were 0.625% and 1.25% against *P. acnes*, and 1.25% and 2.5% against *S. aureus*, respectively.

The antibacterial effects of 12 terpene compounds against the growth of *P. acnes* and *S. aureus* were evaluated at 80% (Table 7). Among the components, terpinen-4-ol, α-terpinene, 1,8-cineole, ρ-cymene, α-pinene, α-terpineol, and limonene exhibited notable antimicrobial activities against *P. acnes*. Terpinen-4-ol and α-terpineol exerted the strongest antimicrobial activities against *P. acnes* with MICs of 2.5%. Both α-terpinene and limonene showed moderate antibacterial activities against *P. acnes* with MICs of 10%. 1,8-Cineole, ρ-cymene, and α-pinene were less effective against *P. acnes* with MICs of 50%. On the other hand, antibacterial properties against *S. aureus* also exhibited a concentration-dependent effect, while α-terpinolene and α-terpinene were the most effective components. The order of the others was α-terpineol, (+)-α-pinene, terpinen-4-ol, γ-terpinene, and aromadendrene.

According to Table 7, the antimicrobial activities of TTO were attributed mainly to terpinen-4-ol, α-terpineol, α-terpinolene, and α-terpinene. Hence, different formulas of TTO were prepared in accordance with the antibacterial data and ISO 4730 guidelines. Compositions of the six DFs are shown in Table 8. The design strategy is described in the following

Table 5 – Diameter and tea tree oil yield of various parts *M. alternifolia*.

	Leaf	Twig	Branch
Diameter (cm)	–	< 0.3	0.3–0.7
Yield (%)	2.02	0.59	0.01

Table 6 – Composition of prepared and commercial TTO.

Component	CAS no.	Molecular weight (g/mol)	Retention time (min)	ISO 4730 range (%)	Prepared TTO (%)	
					TTO-L	TTO-T
Terpinen-4-ol	562-74-3	154.25	8.575	>30	47.31	39.09
γ-Terpinene	99-85-4	136.1	7.292	10–28	20.59	6.71
α-Terpinene	99-86-5	136.1	6.724	5–13	9.58	1.01
1,8-Cineole	470-82-6	154.25	7.075	<15	1.71	ND
α-Terpinolene	586-62-9	136.1	6.936	1.5–5	2.96	1.45
ρ-Cymene	99-87-6	134.22	6.967	0.5–12	1.51	ND
(+)-α-Pinene	80-56-8	136.1	5.842	1–6	1.96	1.70
α-Terpineol	98-55-5	154.25	8.692	1.5–8	3.02	ND
Aromadendrene	489-39-4	204.35	10.867	Trace–7	<0.1	3.34
(+)-Limonene	5989-27-5	136.24	7.017	Trace–8	0.47	ND
Sabinene	3387-41-5	136.24	6.350	0.5–4	1.55	0.44
Globulol	489-41-8	222.37	11.810	Trace–3.5	0.26	1.38

TTO-L = tea tree oil from leaves; TTO-T = tea tree oil from twigs; ND = not detected.

statement. First, the effective components of α-terpinene, terpinolene, α-terpineol, and α-pinene were adjusted to the maximum allowed ratios. Then, moderate antimicrobial ingredients such as γ-terpinene, ρ-cymene, and limonene were added to half of the amount. Components with small or negligible concentrations, which presented no antibacterial effect, were reduced to zero. Finally, because the ISO 4730 guidelines do not limit the maximum percentage of terpinen-4-ol, the strongest antibacterial component, three different percentages of terpinen-4-ol (30%, 40%, and 50%) were designed. On the other hand, the percentage of 1,8-cineole used was 3%.

As shown in Fig. 2, all DFs displayed significant and dose-dependent antibacterial effects against *P. acnes*. Among them, DF5 and DF6 presented the strongest antibacterial activities with both MICs of 1.25%. Referring to the design rule, the antibacterial activities of DF5 and DF6 could probably be attributed to the high content of terpinen-4-ol (50%). However, all DFs displayed lower antibacterial effects against *S. aureus*, which were dose-dependent (10–40%). DF1 and DF2 showed stronger antibacterial activities against *S. aureus* than the others, because of the higher percentages of α-terpinolene and α-terpinene, the most effective components against *S. aureus*.

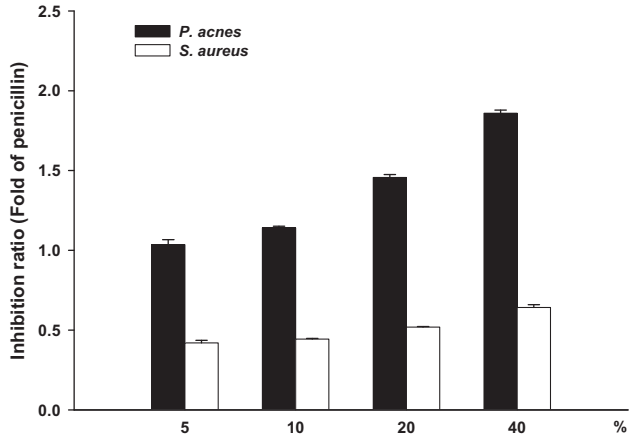

Fig. 1 – Inhibition ratio of TTO-L against *P. acnes* and *S. aureus*. Each test was performed in triplicate.

3.3. *Toxicity effects of TTO-L*

The toxicity effects of TTO-L were evaluated by a skin irritation assay and liver function evaluation. TTO-L and two major components, terpinen-4-ol and 1,8-cineole, were used to evaluate skin toxicity by a single topical application. Firstly, no significant erythema and edema were found at 0 hours. As shown in Fig. 3A, well-defined erythema (level 2) and very slight edema (level 1) were found in the TTO-L-treated group at 10% per site for 24 hours. Even when the application time increased to 48 hours, the erythema and edema levels did not change. When the concentration was reduced to 5% per site, the irritation was diminished. No skin irritation was observed when the concentration was decreased to 2.5%. On the other

Table 7 – Antibacterial activity of terpenes from TTO-L against *P. acnes* and *S. aureus*.[a]

Terpene	*P. acnes*		*S. aureus*	
	Diameter of inhibition zone (multiples of penicillin)	MIC (%)	Diameter of inhibition zone (multiples of penicillin)	MIC (%)
Terpinen-4-ol	0.73 ± 0.01	2.5	0.50 ± 0.03	25
γ-Terpinene	0.00 ± 0.00	–	0.39 ± 0.02	50
α-Terpinene	0.45 ± 0.02	10	1.00 ± 0.02	6.25
1,8-Cineole	0.35 ± 0.00	80	0.00 ± 0.00	–
Terpinolene	0.00 ± 0.00	–	1.03 ± 0.03	6.25
ρ-Cymene	0.38 ± 0.01	60	0.00 ± 0.00	–
α-Pinene	0.35 ± 0.01	60	0.52 ± 0.06	30
α-Terpineol	0.69 ± 0.01	2.5	0.73 ± 0.06	10
Aromadendrene	0.00 ± 0.00	–	0.27 ± 0.02	50
Limonene	0.51 ± 0.01	40	0.00 ± 0.00	–
Sabinene	0.00 ± 0.00	–	0.00 ± 0.00	–
Globulol	0.00 ± 0.00	–	0.00 ± 0.00	–
Jojoba oil [b]	0.00 ± 0.00	–	0.00 ± 0.00	–
Penicillin [c]	1.00 ± 0.00		1.00 ± 0.01	

a The results are presented as the average of three independent experiments.
b jojoba oil was the negative control.
c penicillin was the positive control. MIC = minimum inhibitory concentration; – = no antibacterial activity.

Table 8 – Composition of six devised TTO formulas.

Component	Percentage of components (%)					
	DF1	DF2	DF3	DF4	DF5	DF6
Terpinen-4-ol	30.0	30.0	40.0	40.0	50.0	50.0
γ-Terpinene	19.0	19.0	19.0	19.0	19.0	19.0
α-Terpinene	13.0	13.0	13.0	13.0	13.0	13.0
1,8-Cineole	3.0	0.0	3.0	0.0	3.0	0.0
Terpinolene	5.0	5.0	5.0	5.0	5.0	5.0
ρ-Cymene	2.5	2.5	2.5	2.5	2.5	2.5
α-Pinene	6.0	6.0	6.0	6.0	6.0	6.0
α-Terpineol	8.0	8.0	8.0	8.0	8.0	8.0
Limonene	1.5	1.5	1.5	1.5	1.5	1.5

hand, with different concentrations of terpinen-4-ol, no evidence of erythema, edema, or any other skin reactions were observed at 24 hours and 48 hours (Fig. 3B). However, slight irritation from 1,8-cineole was found at 0.75% and 1.5% per site for 24 hours (Fig. 3C).

According to the above results, TTO-L did not display significant dermal toxicity when the concentration was decreased to 2.5%. In this study, 2% TTO-L was applied daily to Wistar rats and liver function was monitored through serum GOT and GPT levels on days 0, 14, and 28. As shown in Fig. 4, no significant change in GOT or GPT levels were found on days 14 and 28, indicating that 2% TTO-L did not have liver toxicity.

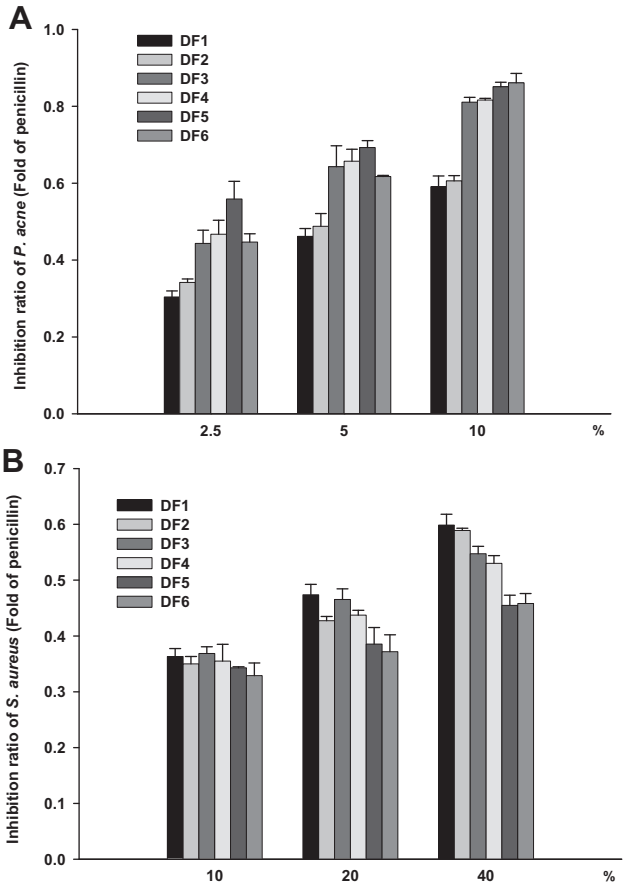

Fig. 2 – Antibacterial effects of devised formulas of TTO-L against different bacteria. Each test was performed in triplicate. (A) *P. acnes*. (B) *S. aureus*.

4. Discussion

Use of TTO, an important complementary and alternative medicine, has widely increased in recent years. In the 18th century, the aboriginals in northern Australia (New South Wales) used TTO to treat coughs and minor skin diseases [7]. From the early 1990s, bioactivities of TTO were increasingly published in the scientific literature, with most attention focused on its antimicrobial activity. In our study, TTO presented dose-dependent inhibitory effects against the growth of *P. acnes* and *S. aureus*, while the inhibitory effects against *P. acnes* were stronger than those against *S. aureus* (Table 7). These findings are similar to those shown in Wilkinson and Cavanagh's [24] and Carson et al's [25] papers that TTO presented better antibacterial activity toward anaerobic bacteria than aerobic bacteria. However, the main constituents of TTO that are active against the growth of *P. acnes* and *S. aureus* were not well studied.

In 1925, Penfold used phenol as a disinfectant standard to compare the antibacterial activity of the components of TTO and presented them as the Rideal-Walker (RW) coefficient. The RW coefficient was defined as a ratio of the antibacterial activity of a sample compared to the antibacterial activity of phenol. RW coefficients of TTO and TTO components were as follows: TTO (RW:11), cineole (RW:3.5), cymene (RW:8), linalool (RW:13), terpinen-4-ol (RW:13.3), and terpineol (RW:16) [26]. Based on the above reference, terpinen-4-ol and α-terpineol are the main active ingredients in TTO. However, no research paper has reported the antibacterial activities of each component of TTO. This is the first study to evaluate the antibacterial effects of the components of TTO according to ISO 4730 guidelines. As shown in Table 7, we also found that terpinen-4-ol and α-terpineol presented the strongest antibacterial activities against *P. acnes*. On the other hand, terpinolene, α-terpinene, and α-pinene, minor components in TTO-L, also contributed strong antibacterial activities against *S. aureus*. In treating acne, anti-inflammation also plays a critical role [26,27]. Hart and colleagues found that terpinen-4-ol, the richest component in TTO, was also a potential anti-inflammatory component that suppressed the production of tumor necrosis factor-α, interleukin (IL)-1β, IL-8, IL-10 and prostaglandin E_2 by lipopolysaccharide (LPS)-activated human peripheral blood monocytes for 40 hours [5]. Therefore, when TTO is used to treat acne, it not only inhibits microbial growth but also suppresses the inflammatory response.

ISO 4730 specifies the characteristics of 15 kinds of TTO constituents to facilitate assessment of its quality. However, whether ISO 4730 can facilitate assessment of antibacterial activities is still unknown. As shown in Fig. 1, four commercial TTOs were in compliance with ISO 4730 standards, and all of these commercial TTOs displayed antibacterial activities. However, there were still some differences in the antibacterial activities among the compounds. Hence, we suggest that ISO 4730 is a good standard to evaluate the compositions of TTO but not its bioactivity. Based on the above findings, six devised TTO formulas were prepared by adjusting the proportions of the main components of TTO, and these were used to evaluate the antibacterial activities. Results showed that DF5 and DF6, which contained the highest terpinen-4-ol levels, exhibited

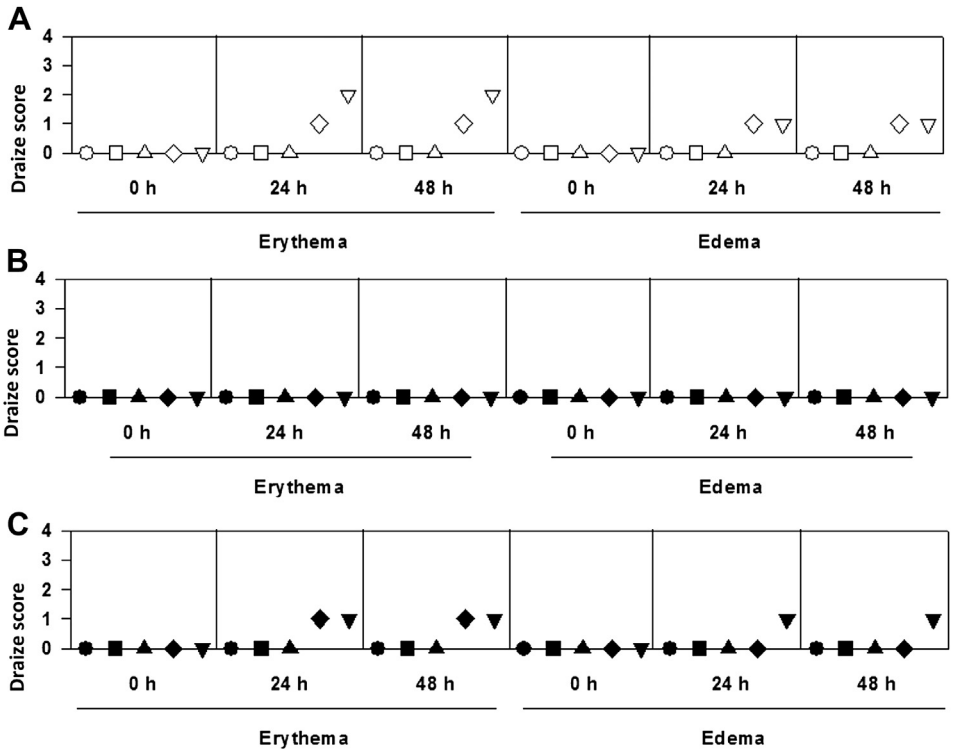

Fig. 3 – Skin erythema and edema levels after applying various samples of different concentrations on Wistar rats. (A) TTO-L: ○ = 0.625%; □ = 1.25%; △ = 2.5%; ◆ = 5%; ▽ = 10%. (B) Terpinen-4-ol. (C) 1,8-Cineole. ● = 0.094%; ■ = 0.188%; ▲ = 0.375%; ◆ = 0.750%; ▼ = 1.500%.

the strongest antibacterial activities. On the other hand, DF1 and DF2 showed stronger antibacterial activities than the others against *S. aureus* because of the higher percentages of α-terpinolene and α-terpinene, the most effective components against *S. aureus* (Fig. 2). However, compared to the prepared TTO-L, none of the antibacterial activities of any DF was as good as that of natural TTO-L.

With increasing reports of the therapeutic properties of TTO, several toxicity review papers of TTO were published. TTO produces many local adverse reactions, such as contact allergy, irritation, and dermatitis in humans. However, most of the literature suggests that levels of allergy and skin irritation can be reduced by diluting TTO [18,19,28]. In this study, several diluted concentrations were used to test the acute dermal toxicity of TTO. The results showed that skin irritation was significantly reduced when TTO concentration was less than 2.5%. The components in TTO that caused the skin irritation were thus further explored. Terpinen-4-ol, the major component in TTO, and 1,8-cineole, a limited component in TTO, were tested to determine if they were the major reasons for skin irritation. First, TTO caused significant skin irritation at 5%, while the maximum percentage of terpinen-4-ol in TTO was 30%. Hence, the skin irritation effect of terpinen-4-ol was evaluated at a concentration of 1.5% and was found not to cause skin irritation (Fig. 3). No document describes possible skin toxicity caused by terpinen-4-ol. On the other hand, the main toxic component in TTO, 1,8-cineole, was also assessed using the same concentration. As shown in Fig. 3C, 1,8-cineole at a concentration of 1.5% raised mild skin irritation, but the irritation was much alleviated when the concentration was less than 0.375%. However, the percentage of 1,8-cineole is regulated to below 18% in ISO 4730, while 0.375% 1,8-cineole is much lower than this regulated percentage. When TTO is applied to the face to treat acne, safety is an important issue. In this study, 2% TTO-L, a non-irritating dose, was applied to the rats' shaved backs daily to evaluate the liver toxicity of TTO-L. As shown in Fig. 4, 2% TTO-L did not significantly elevate GOT or GPT levels when it was applied daily, indicating that TTO-L did not produce liver toxicity.

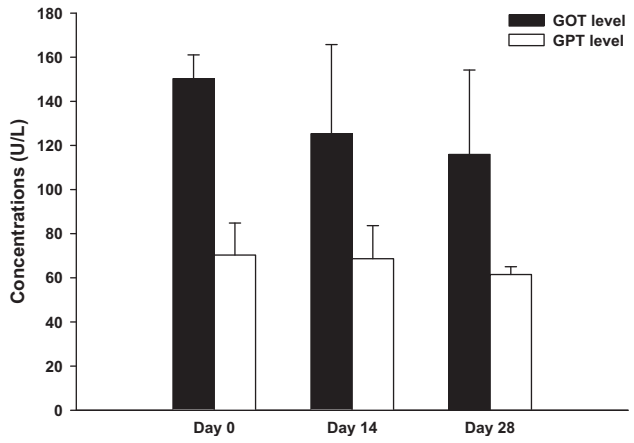

Fig. 4 – Serum GOT and GPT levels of Wistar rats after applying TTO-L for 28 days. The concentration of TTO-L was 2%, and each test was performed in triplicate.

Taken together, the antibacterial efficacy and safety of TTO for treating acne were investigated in this study. In terms of its antibacterial efficacy, TTO-L significantly decreased acne formation via inhibiting the growth of acne-related bacteria, *P. acnes* and *S. aureus*, and reducing acne-caused inflammation. In addition, terpinen-4-ol, the major component in TTO-L, was also the major active component in its antibacterial efficacy, while minor components in TTO contributed to its efficacy as well. A common suggestion is that TTO be used at less than 10%. In conclusion, TTO and its components exhibited good antibacterial efficacy, but in terms of safety, we suggest that less than 5% is more suitable and safer for treating acne.

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