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Physico-Chemical Properties, Chemical Composition and *In Vitro* Antimicrobial and Free Radical-Scavenging Capacity of Tea Tree Essential Oil in Thailand

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ABSTRACT

The physico-chemical properties, chemical composition and *in vitro* antimicrobial and free radical-scavenging capacity of Tea Tree essential oils (TTOs) from three regions of Thailand were studied. The physico-chemical properties of the TTOs obtained by hydro distillation had specific gravity at 20 °C, refractive index at 20 °C and optical rotation values of 0.900–0.905, 1.475–1.481 and 5.25–6.15 °, respectively. Based on characterization by gas chromatography-mass spectrometry (GC-MS), the chemical composition of the main constituents of the TTOs were terpinen-4-ol (30.42–34.76%), γ-terpinene (25.08–26.23%), α-terpinene (12.31–12.43%) and 1,8-cineole (5.99–9.08%). The TTOs exhibited strong antibacterial activity against *Staphylococcus aureus* (DMST 8840) (MIC 0.064–0.512 mg/mL; MBC 0.128–0.512 mg/mL) and showed interesting antibacterial activity against *Vibrio cholerae* (DMST 15778), *Bacillus cereus* (TISTR 687). The Tea Tree essential oils showed high antifungal activity against *Chaetomium globosum* (TISTR 3093). (AI = 82.76–87.04%) and partially inhibited the growth of *Curvularia lunata* (TISTR 3289), *Aspergillus flavus* (TISTR 3366), *Aspergillus niger* (TISTR 3245) and *Penicillium* sp. (TISTR 3046). The free radical-scavenging capacity of the essential oil was investigated using 2,2-diphenylpicryl-hydrazyl (DPPH) radical scavenging assay, The TTOs showed strong antioxidant activities (IC50 29.34–38.68 mg/mL). The results suggest that TTOs in Thailand had the same quality as in Australia and could be a natural antibacterial and antioxidation agent.

Keywords: tea tree oil, essential oil, antimicrobial activity, free radical-scavenging capacity, chemical composition

INTRODUCTION

Tea Tree *(Melaleuca alternifolia*) is an Australian native plant. It has aromatic foliage. Its valuable oil is extracted from the leaves by steam distillation. Tea Tree Oil (TTO) has been used in Australian traditional medicine, and more recently worldwide, for its wide range of antimicrobial activities. Its chemical composition is strictly related to the quality of the raw plant material; therefore significant differences in the yields and composition of the TTO essential oil can be found. Australian TTO has many therapeutic benefits due to its anti- inflammatory,

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anti-oxidation and anti-cancer properties. It also possesses a broad spectrum of antimicrobial activity (Kim *et al*., 2004; Carson *et al*., 2006). Nowadays, Tea Tree has been cultivated in many regions of Thailand including Chiang Mai, Phetchabun and Prachuap Khiri Khan provinces at the research stations of the Agro-Ecological System Research and Development Institute of Kasetsart University. Thailand. Therefore, the objectives of this study were to determine the physico-chemical properties and chemical composition of Tea Tree oils and to evaluate the *in vitro* antimicrobial and antioxidant activities of the essential oil of Tea Tree in three regions of Thailand.

MATERIALS AND METHODS

Plant material

Tea Tree plants were collected from three sites at the Agro-Ecological System Research and Development Institute research stations at the Ban Tubberg, Phetchabun Research Station, Phetchabun province (TB), the Doi Pui Research Station, Chiang Mai province (DP) and the Sithiporn Kridakorn Research Station, Prachuap Khiri Khan province (SP). All of samples were harvested from the same age of plant (five years old) and at the same time (April 2009) to control the effect of harvesting time and plant age on the properties of the TTO samples.

Extraction of the essential oil

Fresh leaves and twigs of the Tea Tree plants sampled were cut into small pieces and subjected for 5 h hydro distillation using a Clevenger-type apparatus. The essential oil obtained was dried over sulfate sodium anhydrous, filtrated and stored at 4 °C until tested and analyzed.

Analysis of physico-chemical parameters

The specific gravity was measured with a density meter (DA-100M, Mettler, Toledo, Switzerland) at 20 °C. The refractive index was determined using a digital refractometer (RX-5000α, ATAGO, Japan) at 20 °C. Optical rotation was recorded using a polarimeter (POLAX-2L, ATAGO, Japan).

GC-MS analysis of the essential oil

GC-MS analyses were performed with a Shimadzu QP 5050A gas chromatograph equipped with a DB-5 capillary column (60 m × 0.25 mm; coating thickness 0.25 μm). Analytical conditions were injector and transfer line temperatures of 250 and 250 °C, respectively, programmed from 60 °C (3 min), with an increase of 1°C min^{-1}, to 80 °C and then an increase of 4 °C min^{-1} to 200 °C and hold for 10 min; the carrier gas was helium, at a flow rate of 1.2 mL min^{-1}. Tea Tree essential oils were diluted 1:1000 (v/v) with petroleum ether, and 1.0 μL of the diluted samples was injected automatically in a split ratio of 1:7. The components were identified by comparison of the Kovats index values with those reported in the literature (Adams, 2007) and their identities were confirmed by computer matching of their mass spectral fragmentation patterns with those stored in the mass spectral database at the National Institute of Standards and Technology.

Antimicrobial activity

Microbial strains

Bacterial and fungal strains consisting of *Bacillus cereus* (TISTR 687), *Escherichai coli* (TISTR 780), *Salmonella Typhimurium* (TISTR 292), *Pseudomonas aeruginosa* (TISTR 781), *Aspergillus niger* (TISTR 3245) *Aspergillus flavus* (TISTR 3366) *Penicillium* sp. (TISTR 3046) *Chaetomium globosum* (TISTR 3093) and *Curvularia lunata* (TISTR 3289) were obtained from the culture collection of the Thailand Institute of Scientific and Technological Research (TISTR). *Staphylococcus aureus* (DMST 8840), *Vibrio cholerae* (DMST 15778), *Klebsiella pneumoniae*

(DMST 8216), *Proteus mirabilis* (DMST 8212) and *Shigella flexneri* (DMST 4423) were obtained from the Department of Medical Science (DMSC), Ministry of Health (Bangkok, Thailand).

Antibacterial activity

The disc diffusion method was employed for determination of antibacterial activities (NCCLS, 1999). Erythomycin (15 μg/disc) was used as a positive reference standard to determine the sensitivity of each bacterial species tested. Antibacterial activities of undiluted TTOs (10 μL $disc^{-1}$) were evaluated by measuring the diameter of the clear zone of the tested bacteria expressed in millimeters. The minimum inhibitory concentration (MIC) was determined using a resazurin microtiter assay as described by Nateche *et al*. (2006) with some modifications. Briefly, serial twofold dilutions of oils were performed in Mueller Hinton Broth and prepared in a 96-well microtiter plate over a range of 0.064–131.072 mg mL^{-1}. A suspension of the organism was added to wells at a concentration of 5×10^5 CFU mL^{-1} and the microtiter plates were incubated at 37 °C. After incubation for 24 h, 30 mL resazurin working solution was added to each well; the plates were incubated for 24 h at 37 °C and the results were read visually. The MIC was defined as the lowest oil concentration that prevented a full color change of the resazurin from blue to pink. To determine the minimum bactericidal concentration (MBC), 10 μL of bacterial suspension were removed from each well after overnight growth, (before adding the resazurin working solution) and spread onto Mueller Hinton Agar and incubated at 37 °C. The MBC was defined as the lowest concentration of the essential oil at which inoculated microorganisms were 99.9% killed (Saginur *et al*., 2006). All tests were performed in triplicate.

Antifungal activity

The antifungal activity of undiluted TTOs was undertaken using volatile assay (Alvarez-Castellanos *et al* , 2001). The antifungal activity was determined by an antifungal index. Each experiment was repeated three times. The formula to calculate the antifungal index is shown in Equation 1:

$$\text{Antifungal index (AI,\%)} = (1\text{-}D_a/D_b) \times 100 \quad (1)$$

Where: D_a is the diameter (cm) of the growth zone in the experimental dish and D_b is the diameter of the growth zone in the control dish.

Scavenging activity of DPPH radical

The scavenging activity of each TTO sample against DPPH radicals was assessed according to the method of Karagozler *et al*. (2008) with some modifications. Briefly, TTOs were prepared using ethanol, with each sample of stock solution of 3 mL (10–100mg mL^{-1} in ethanol) mixed with 1 mL of 0.1 mM DPPH ethanol solution. After the solution was incubated for 30 min at 25 °C in the dark, the decrease in the absorbance at 517 nm was measured. Ethanol was used as a blank while throughout the experiment, Butylated hydroxytoluene (BHT) was used as a positive control. The inhibition of DPPH radicals by the samples was calculated according to Equation 2:

$$\text{DPPH radical scavenging activity (\%)} = [(A_0 - A_1) / A_0] \times 100 \quad (2)$$

where: A_0 is the absorbance of the control reaction, and A_1 is the absorbance of the test compound.

The oil concentration providing 50% inhibition (IC50) was calculated from the graph of a plot of the inhibition percentage against the TTO concentration. All tests were performed in triplicate.

RESULTS AND DISCUSSION

The physico-chemical properties of the TTOs obtained from DP SP and TB by hydro distillation showed specific gravity values at 20 °C of 0.905, 0.900 and 0.905, respectively. The refractive indices of the TTOs at 20 °C were 1.476,

1.481 and 1.475, respectively. The values of optical rotation were 6.10, 6.15 and 5.25 °, respectively. The difference between these values depended on the chemical composition of the TTOs (Behera *et al*., 2004). The ISO 4730:2004 recommended physical properties of Australian TTOs are in the range 0.885–0.906, 1.475–1.482 and 5 –15 ° for specific gravity, refractive index and optical rotation, respectively (Southwell *et al*., 2006). The results obtained indicated that the physical properties of the Thai TTOs were in the ranges of the TTOs in Australia.

The chemical composition of each TTO was identified by GC–MS. The qualitative and quantitative analytical results by GC–MS are shown in Table 1. The TTOs from the three regions complied with the ISO 4730:2004 standard analysis with the TTOs from the different regions showing few variations in the contents of major constituents. Terpinen-4-ol (30.42–34.76%) was found as the major component of the TTOs along with γ-terpinene (25.08–26.23%), α-terpinene (12.31–12.43%) and 1,8-cineole (5.99–9.08%), respectively. The TTO from SP showed a high content of terpinen-4-ol, γ-terpinene and α-

Table 1 Main components (%) of Thai TTOs and chromatographic profiles of tea tree oil according to ISO/FDIS 4730:2004.

KI*	Compound	% composition			Chromatographic profile of Tea Tree Oil according to ISO/FDIS 4730:2004**	
		TTO from DP	TTO from SP	TTO from TB	Maximum	Minimum
931	α-thujene	0.12	0.80	0.93	-	-
939	α-pinene	2.25	2.53	2.71	1.00	6.00
974	sabinene	0.16	0.28	0.95	trace	3.50
980	β-pinene	0.71	0.81	0.95	-	-
990	β-myrcene	1.07	1.55	1.22	-	-
1006	α-phellandrene	0.13	0.32	-	-	-
1015	α-terpinene	12.31	12.43	12.33	5.00	13.00
1026	p-cymene	3.58	3.15	2.17	0.50	8.00
1030	Limonene	2.44	2.46	2.35	0.50	1.50
1031	1,8-cineole	9.08	6.67	5.99	trace	15.00
1050	Ocimene	-	0.89	3.70	-	-
1072	γ-terpinene	25.76	26.23	25.08	10	28
1089	Terpinolene	4.11	4.12	4.20	1.50	5.00
1180	Terpinene-4-ol	32.21	34.76	30.42	30.00	48.00
1192	α-terpineol	2.16	2.45	2.56	1.50	8.00
1410	α-gurjunene	0.27	-	-	-	-
-	C15H24	0.28	0.26	-	-	-
1419	β-caryophyllene	1.13	-	3.01	trace	-
1447	Aromadendrene	-	-	1.08	trace	3
-	C15H24	0.39	0.14	-	-	-
-	C15H24	0.15	-	2.61	-	-
1493	Ledene	0.90	-	2.46	trace	3
1524	δ-cadinene	0.79	0.22	2.29	trace	3
1590	Viridiflorol	-	-	0.32	trace	1

* =Kovats index on DB-5 column (Adams, 2007).

** = Normative chromatographic profile of TTO set by ISO/FDIS 4730:2004.

terpinene, while a high content of 1,8-cineole was found in the TTO from DP.

The in vitro antimicrobial activities of the TTOs against all tested bacteria and their activity potentials were qualitatively and quantitatively assessed by the presence or absence of clear zones and MIC and MBC values. The results in Tables 2 and 3 show that the TTOs from various regions had different efficacies to inhibit all of the tested bacteria. All of the TTOs showed good antibacterial activity against *S. aureus*. Their inhibition zone ranged from 15.40 to 21.83 mm, with MIC and MBC values of 0.064–0.512 mg mL^{-1} and 0.128–0.512 mg mL^{-1}, respectively and they showed high activity to control *V. cholerae*, *B. cereus* and *S. Typhimurium*, respectively. The TTOs exhibited weak activity against *P. aeruginosa* and *S. flexneri*. The efficacy of the TTOs is most likely dependent on their chemical compositions as shown in Table 1. TTO from SP

Table 2 Clear zone diameter of 1% of TTOs against test bacterial strains.

Microorganism	Clear zone (mm)			
	DP	SP	TB	Erythomycin*
Bacillus cereus	13.50±0.32^{b}	15.41±1.59^{a}	12.83±0.92^{b}	14.00±0.89
Escherichai coli	11.83±0.98^{b}	14.42±0.86^{a}	13.00±1.00^{ab}	10.67±0.52
Klebsiella pneumoniae	16.08±0.20^{b}	19.33±0.74^{a}	16.50±0.45^{b}	00.00±0.00
Pseudomonas aeruginosa	8.33±0.52^{a}	8.17±0.26^{a}	8.25±0.61^{a}	00.00±0.00
Proteus mirabilis	12.33±0.82^{a}	11.50±0.84^{a}	11.75±0.76^{a}	00.00±0.00
Salmonella Typhimurium	11.83±1.08^{a}	12.75±1.17^{a}	11.17±0.92^{a}	00.00±0.00
Staphylococcus aureus	21.83±0.93^{a}	18.33±1.03^{b}	15.40±1.02^{c}	39.33±0.52
Shigella flexneri	11.25±1.02^{ab}	12.00±1.51^{a}	10.50±1.44^{b}	00.00±0.00
Vibrio cholerae	17.83±0.98^{a}	18.83±0.93^{a}	16.42±0.80^{b}	22.67±0.26

Mean values followed by different superscripts within a row are significantly different using Duncan's multiple range test ($P < 0.05$).

DP = TTO from Doi Pui Research Station; SP = TTO from Sithiporn Kridakorn Research Station; and TB = TTO from Ban Tubberg, Phetchabun Research Station. * = positive reference standards to determine the sensitivity of each bacterial species tested (15 g $disc^{-1}$).

Table 3 Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of TTOs against test bacterial strains.

Microorganism	MIC (mg mL^{-1})			MBC (mg mL^{-1})		
	DP	SP	TB	DP	SP	TB
Bacillus cereus	2.048	0.256	2.048	2.048	0.256	2.048
Escherichai coli	8.192	4.096	8.192	8.192	4.096	16.384
Klebsiella pneumoniae	8.192	4.096	8.192	8.192	4.096	8.192
Pseudomonas aeruginosa	32.768	8.192	32.768	32.768	16.384	65.536
Proteus mirabilis	4.096	8.192	8.192	4.096	8.192	8.192
Salmonella Typhimurium	2.048	2.048	4.096	2.048	2.048	4.096
Staphylococcus aureus	0.064	0.128	0.512	0.128	0.256	0.512
Shigella flexneri	16.384	8.192	32.768	32.768	16.384	65.536
Vibrio cholerae	0.512	0.128	2.048	0.512	0.128	2.048

DP = TTO from Doi Pui Research Station; SP = TTO from Sithiporn Kridakorn Research Station ; and TB = TTO from Ban Tubberg, Phetchabun Research Station.

with a high amount of terpinen-4-ol represented a high potential to control all bacterial strains. These findings were quite similar with the reports of May *et al*. (2000) and Carson *et al*. (2006). The mechanism of action of TTO against bacteria was made on the basis of its hydrocarbon and oxygenated monoterpene structure such as γ-terpinene 1,8-cineole, terpinen-4-ol, α-terpineol and attendant lipophilicity which were permeated into bacterial cell membranes and disrupted their vital functions resulting in the loss of intracellular material, inability to maintain homeostasis and inhibition of respiration involving the loss of membrane integrity (Carson *et al*., 2006). Moreover, Halcon and Milkus (2004) reported that terpinen-4-ol, the main component of TTO, has been shown to affect the bacterial cell wall, demonstrated by the loss of 260-nm nuclear material, K+, salt tolerance and inhibition of glucose-dependent respiration, and they suggested that TTO compromises the cytoplasmic membrane of *S. aureus*, giving it a bacteriostatic and bactericidal effect.

The antifungal activities of the TTOs through the vapor phase against five fungi are shown in Table 4. The results showed wide variation in the antifungal activities of the TTOs which showed high efficacy against *C. globosum* followed by *C. lunata*, *A.flavus* , *A. niger and Penicillium* sp., respectively. The TTOs from the three regions exhibited significant activity against all tested fungi except for *Chaetomium globosum*. The TTOs from DP gave high antifungal activity against all tested fungi. The efficacy of the TTOs to control the growth of fungi was supported by the report of Carson *et al*. (2006) who suggested a mechanism of antifungal action whereby the TTO causes changes or damage to the functioning of the fungal membranes effected by terpinen-4-ol 1,8-cineole as the main compound in the TTOs. Hammer *et al*. (2003) reported that the components of TTO such as terpinen-4-ol, α-terpineol, linalool, α-pinene and β-pinene exhibited strong antifungal effects against yeast and filamentous fungi, and 1,8-cineole showed moderate control over the growth of fungi (Hammer *et al*., 2003; Mondello *et al*., 2006).

The antioxidative capacity of the TTOs was determined by a free radical scavenging test using DPPH solution and by the decrease in absorbance at 517 nm, due to reduction by the antioxidant (AH) or reaction with a radical species. Substances that perform this reaction can be considered as antioxidants or scavengers (Brand-Williams *et al*., 1995) and as shown in Figure 1, all of the TTOs studied showed appreciable free radical scavenging activity. Among the samples tested in this study, the SP sample had the strongest radical scavenging activity (IC50 29.34 mg mL^{-1}) followed by DP and TB with IC50 values of 34.03 and 38.68 mg mL^{-1}, respectively. The efficacy of TTOs to inhibit free radicals depends on their

Table 4 Antifungal activity of TTOs against fungi.

Microorganism	Antifungal index (%)		
	DP	SP	TB
Aspergillus flavus	24.56±0.44^{a}	15.29±1.15^{c}	18.04±1.51^{b}
Aspergillus niger	23.67±1.53^{a}	17.05±1.06^{b}	14.25±1.36^{c}
Chaetomium globosum	84.57±0.81^{a}	82.76±1.56^{a}	87.04±1.00^{a}
Curvularia lunata	59.88±0.41^{a}	53.24±1.07^{b}	49.38±1.53^{c}
Penicillium sp.	19.05±1.38^{a}	18.52±1.99^{a}	3.17±1.59^{b}

Mean values followed by different superscripts within a row are significantly different using Duncan's multiple range test ($P < 0.05$).

DP = TTO from Doi Pui Research Station; SP = TT0 from Sithiporn Kridakorn Research Station; and TB = TTO from Ban Tubberg, Phetchabun Research Station.

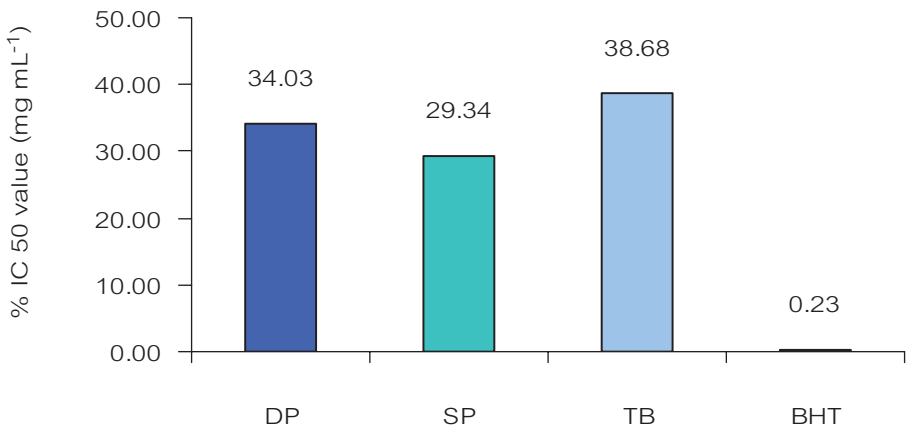

Figure 1 DPPH radical scavenging activity of TTOs on DPPH free radicals as compared to standard BHT. Values are the average of triplicate experiments. DP = TTO from Doi Pui Research Station; SP = TT0 from Sithiporn Kridakorn Research Station; and TB = TTO from Ban Tubberg, Phetchabun Research Station.

chemical composition. Kim *et al*. (2004) revealed that the major antioxidant activity in TTO was attributed to the three terpenic compounds—namely, α-terpinene, terpinolene and γ-terpinene, rather than terpinen-4-ol, a major component of the TTO that showed weak antioxidative activity. TTO from SP had a high content of α-terpinene and γ-terpinene and had strong radical scavenging activity.

CONCLUSION

TTOs in various regions of Thailand showed different physico-chemical properties and antimicrobial and antioxidant activity due to differences in their chemical composition. Moreover, Thai TTOs complied with the international standard (ISO 4730:2004) and were similar in content to those reported for Australian TTOs.

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