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A Comparative Study of the Essential Oil from Flowers and Fruits of *Lepisanthes rubiginosa* (Roxb.) Leenh.

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Abstract

The essential oils extracted by hydrodistillation from the flowers and fruits of *Lepisanthes rubiginosa* (Roxb.) Leenh. were analyzed by capillary GC and GC-MS. The major components of the flower essential oil were nerolidol (34.8 %), palmitic acid (13.2 %) and farnesol (10.0 %). For the fruit essential oil, palmitic acid (66.1 %), myristic and (10.0 %) and linoleic acid (5.5 %) appeared to be the major constituents. Evaluation for *in vitro* anticancer activity of the essential oils was done against three human cancer cell lines (KB-Oral cavity cancer, MCF7 Breast cancer, NCI-H187 Small cell lung cancer). Only the flower essential oil exhibited anticancer activity against NCI-H187. Small cell lung cancer with the IC_{50} of 43.90 μgmL^{-1}. The flower essential oil also possessed antioxidant activity (ABTS method) with the % inhibition of 25.4 %, whereas the fruit essential oil did not show anticancer activity and possessed low antioxidant activity with the % inhibition of 6.4 %. The flower and fruit essential oils exhibited strong antimicrobial activity against *Trichophyton mentagophyte* and showed moderately activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans.*

Keywords: *Lepisanthes rubiginosa* (Roxb.) Leenh., essential oil, chemical constituents, anticancer activity, antimicrobial activity.

Introduction

Natural product provides a great molecular diversity and biological functionality, and so are indispensable for novel drug discovery. Traditional tropical herbs contain many useful compounds which are used for the treatment of diseases. Many reports confirmed the potentials of medicinal herbs in the prevention of some infection diseases (Ung et al. 2007).

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Cancer is the third leading cause of death worldwide, only preceded by cardiovascular disease, infectious and parasitic disease (Mathers et al. 2001). Interestingly, cancer has been the leading cause of death in Thailand for several years, with an increase in the death rate every year. In Thailand, many people use traditional medicine as an alternative treatment for cancer (Subchareon 1998). Folk doctors of Southern Thailand used many medicinal plants in cancer drug formulae (Itharat et al. 1998).

Lepisanthes Rubiginosa (Roxb.) Leenh. is one of Sapindaceae family, flowering shrub native to tropical southeastern Asia. The fruit ripens from red to blackish purple, it is sweet and can be eaten raw.

The young leaves and fruits are edible. The root (boiled) is used to treat coughing. The roots and leaves are used to treat fever. It has been externally used as antipruritic; apply locally to forehead for fever, headache and decoction. The family Sapindaceae is known for its variety of saponins particularly hederagenin glycosides (Delaude 1993). Chemical investigation of the methanolic fraction of *Lepisanthes rubiginosa* bark has led to the isolation and characterization of a new tetrasaccharide derivative of farnesol named rubiginoside along with known triterpenoid saponins (Saburi et al. 1999).

There is no previous report describes the chemical constituents and biological activities of the essential oils from *L.rubiginosa*.

Material and Method

Plant Material

Fresh flowers and fruits of *L. rubiginosa* were collected from Kamphaengphet province, Thailand in February and April 2008, repectively. A voucher specimen (Herbarium No.: J. Chuangbunyat-1) was deposited at the Herbarium of Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

GC and GC-MS Analysis of the Essential Oils

Fresh flowers and fruits (500 g each) of *L. rubiginosa* were homogenized and hydrodistilled separately for 8 h, using a modified Clevenger-type apparatus yielded the percentage of essential oils (calculated based on a dry weight basis) 0.05 % (flower) and 0.03 % (fruit) as light brown oils. The essential oils were analysed by GC and GC-MS. GC analyses were carried out on a Hewlett-Packard 19091S-933E gas chromatograph equipped with a flame ionization detector (FID) and DB-1 capillary fused silica column (30 m, 0.25 mm I.D.; 0.25 μm film thickness). The oven temperature was held at 80 °C for 4 min then programmed at 8 °C min^{-1} to 260 °C, held for 15 min. Other operating conditions were as follows: carrier gas, He, inlet pressure 9.32 psi, with a linear velocity of 20 cms^{-1}; injector temperature, 250 °C; detector temperature, 280 °C; split ratio, 1:20. GC–MS analyses were performed on a HP-6850 GC system coupled with a 5973 network mass selective detector and equipped with a DB1-MS capillary fused silica column (30 m, 0.25 mm I.D.; 0.25 μm film thickness) temperature programmed as above. The carrier gas was He at a flow rate of 1.0 $mLmin^{-1}$ and the split mode had a ratio of 1:20. The injection port was set at 250 °C. The effluent from the capillary column went directly into the mass spectrometer and operated in the electron impact (EI) mode with an ionization voltage of 70 eV. The ion source temperature was 250 °C, and the GC-MS transfer line was set to 280 °C. The identification of the oil components was accomplished by comparison of their GC retention indices as well as their mass spectra with corresponding data of authentic compounds or published spectra.

Biological Activity

Comparative evaluations of the biological activities of the flower and fruit essential oils of L. rubiginosa were also investigated.

Cytotoxic Assay

The anticancer activities of the essential oils from flowers and fruits of *L. rubiginosa* were performed by using KB (Oral Cavity cancer), MCF7 (Human breast adenocarcinoma) and NCI-H187 (Human small cell lung carcinoma) and determined by Resazurin Microplate Assay (REMA) following a modified method of the use of a fluorescent dye for mammalian cell cytotoxicity according to Brien et al. (2000). Ellipticine and doxorubicin were used as positive controls. DMSO and sterile distilled water were used as negative controls. Cells at a logarithmic growth phase were harvested and diluted to 10^5 cellsml^{-1} in fresh medium and gently mixed. Test compounds were diluted in culture medium in a ratio of 1:2 giving 8 concentrations. Five microliters of the test sample and 45 μL of cells were put into 96 well microtiter plates with a total volume of 50 μLwell^{-1}. Plates were incubated at 37 °C, 5 % CO_2, for 72 h for KB and MCF7 and 5 days for NCI-H187. After the incubation periods, 12.5 μL of resazurin solution was added to each well and the plates were incubated at 37 °C for 4 h. The plates were then processed for optical density absorbance analysis using a microplate reader at dual wavelengths of 530 and 590 nm.

The cytotoxicity against primate cell line (Vero) of the oils was assayed by using Green fluorescent protein (GFP) detection described by Hunt et al. (1999). In brief, the GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N-1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 gL^{-1} sodium bicarbonate and 0.8 mgmL^{-1} geneticin, at 37 °C in a humidified incubator with 5 % CO_2.

The assay was carried out by adding 45 μL of cell suspension at 3.3×10^4 cellsmL^{-1} to each well of 384-well plates containing 5 μL of test compounds previously diluted in 0.5 % DMSO, and then incubating for 4 days in 37 °C incubator with 5 % CO_2. Fluorescence signals were measured by using SpectralMax M5 microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 was subtracted with background fluorescence at day 0. The percentage of cytotoxicity was calculated by the following equation below, where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cell, respectively.

$$\% \text{ Inhibition} = [\, 1 - FU_T/FU_C]^* 100$$

Antimicrobial Activity

The antimicrobial activities of the flower and fruit essential oils of *L. rubiginosa* were determined by agar diffusion method. For antibacterial activity, two Gram-negative (*Escherichia coli, Pseudomonas aeruginosa*) and one Gram-positive (*Staphylococcus aureus*) bacteria were employed. The tested microorganisms were grown in nutrient broth at 37 °C for 24 h. The culture suspensions were adjusted by comparing against 5.0 McFarland Petri dishes with 20 mL of nutrient agar were prepared, previously inoculated with 200 μL of the culture suspension. Five milligrams per milliter of the essential oils were transferred to each well (9.0 mm in diameter). The inoculated plates were incubated for 24 h. After incubation, the diameter of inhibition zone was measured and reported in the scale of millimeter. The diameter of zone of the inhibition zone produced by the essential oil was then compared with the standard antibiotic, gentamycin 75 μgmL^{-1}. The antifungal activity of the essential oils was also tested by agar diffusion method against the three pathogenic fungi: *Candida albican, Aspergillus flavus* and *Trichophyton mentagrophyte*, using the procedure as described above. Ketoconazole was used as positive control.

Antioxidant Activity

The antioxidant activity of the flower and fruit essential oils was performed by the ABTS method (Re et al. 1999). The essential oil 5 mgmL^{-1} in ethanol was mixed with 1 mL of ABTS solution and the absorbance was determined at 734 nm after 10 min of incubation at room temperature. The antioxidant activity was expressed as Trolox equivalent.

Results and Discussion

Analysis of the Essential Oils

The essential oils obtained from the flowers and fruits of *L. rubiginosa* were analysed by means of GC and GC-MS. Identification of oil constituents were analysed by means of GC and GC-MS. Identification of the compounds was based on retention indices and computer matching with the NIST and Wiley 7n.1 libraries as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature (Table 1 and Table 2). Retention indices were determined using retention times of n-alkanes that have been injected to the same instrument and under the same chromatographic conditions. Table 1 and Table 2 list the identified compounds in the order of their elution on the DB-1 capillary column used for GC-FID analysis.

The major components of the flower essential oil (Table1) were identified as nerolidol (34.8), palmitic acid (13.2 %) and farnesol (10.0 %). The minor components were triacontane (7.2 %), tetradecanoic acid (4.6 %), nonanoic acid (1.7 %) and pentacosane (1.3 %). The major components of the fruit essential oil (Table 2) were palmitic acid (66.1 %), tetradecanoic acid (10.0 %) and linoleic acid (5.5 %). The minor components were nonacosane (2.0 %), nerolidol (1.6 %), heptacosane (1.3 %), (E,E)-farnesol (0.3 %), pentacosane (0.3 %) and methyl linoleate (0.2 %).

The flower essential oil of *L. rubiginosa* consisted of two important sesquiterpenes, nerolidol and farnesol. Nerolidol is an antiulcer (Klopell et al. 2007) and also possesses antileishmanial activity. It is also currently under testing as a skin penetration enhancer for transdermal delivery of therapeutic drugs (Arruda et al. 2005). Farnesol is an acyclic sesquiterpene alcohol. It has been suggested to function as a chemopreventative and anti-tumor agent (Joo and Jetten 2009). It is used in perfumery to emphasize the odours of sweet floral perfumes.

Table 1. Chemical constituents of the flower essential oil of *L. rubiginosa*

Compounds	RT	RI	Area (%)	Methods of Identification	References
Nonanoic acid	15.3	1256	1.7	RI, MS	Peng 2000
Nerolidol	21.6	1528	34.8	RI, MS	Velasco Negueruela et al. 2002
Farnesol	24.5	1624	10	RI, MS	Hadian et al. 2006
Tetradecanoic acid (Myristic acid)	25.2	1741	4.6	RI, MS	Hadian et al. 2006
Palmitic acid	28.6	1949	13.2	RI, MS	Hadian et al. 2006
Pentacosane	35.4	2501	1.3	RI, MS	Hadian et al. 2006
Triacontane	38	3000	7.2	RI, MS	Carlson et al. 2001
Total identified (%)			72.8		

RT = Retention time; RI = Retention index on DB-1 capillary column (relative to n-alkane); Methods of identification: MS, comparison of the mass spectrum with MS libraries; RI of literature

Table 2. Chemical constituents of the fruit essential oil of *L. rubiginosa*

Compounds	RT	RI	Area (%)	Methods of Identification	References
Nerolidol	13.5	1528	1.6	RI, MS	Velasco Negueruela et al. 2002
(E,E)-Farnesol	15.3	1657	0.3	RI, MS	Hadian et al. 2006
Tetradecanoic acid (Myristic acid)	15.9	1723	10	RI, MS	Ghazghazi et al. 2010
Palmitic acid	18.1	1949	66.1	RI, MS	Hadian et al. 2006
Methyl linoleate	19.1	2063	0.2	RI, MS	Hadjikhoondi et al. 2006
Linoleic acid	19.7	2125	5.5	RI, MS	Ghazghazi et al. 2010
Pentacosane	22.6	2497	0.3	RI, MS	Hadjikhoondi et al. 2006
Heptacosane	24.7	2698	1.3	RI, MS	Hadjikhoondi et al. 2006
Nonacosane	27.8	2898	2	RI, MS	Hadjikhoondi et al. 2006
Total identified (%)			87.1		

RT = Retention time; RI = Retention index on DB-1 capillary column (relative to n-alkane); Methods of identification: MS, comparison of the mass spectrum with MS libraries; RI of literature

The fruit essential oil consisted of saturated fatty acids, palmitic acid and tetradecanoic acid. Palmitic acid (hexadecanoic acid) is a saturated fatty acid. The most widely known use of palmitic acid is that it is an essential ingredient in soap making. Palmitic acid derivatives are used in different psychotic medicines especially in the treatment of schizophrenia (palimperdone palmitate is an anti-psychotic medication). The fruit oil also consisted of unsaturated fatty acid (linoleic acid) and methyl linoleate. Linoleic acid is a polyunsaturated fatty acid used in the biosynthesis of arachidonic acid and thus some prostaglandins. It is found in the lipids of cell membranes. It is abundant in many vegetable oils, comprising over half (by weight) of poppy seed, safflower, sunflower and corn oils (US Department of Agriculture, Agricultural Research Service, 2007). Linoleic acid is an essential fatty acid that must be consumed for proper health. A lack of linoleic acid and other n-6 fatty acids in the diet causes dry hair, hair loss (Cunnane and Anderson 1997), and poor wound healing (Ruthig and Meckling-Gill 1999). There are small amount of nerolidol (1.6 %) and (E,E)-farnesol (0.3 %) present in the fruit essential oil.

For the GC and GC-MS analysis (Table 1 and Table 2), seven compounds in the flower essential oil were identified, corresponding to 72.8 % of the total oil that consisted mainly of sesquiterpenes (44.8 %), fatty acids (19.5 %) and hydrocarbons (8.5 %).

For the fruit essential oil, nine compounds were identified corresponding to 87.1 % of the total oil that consisted mainly of fatty acids (81.4 %), methyl linoleate (0.2 %), sesquiterpenes (1.9 %) and hydrocarbons (3.6 %).

Cytotoxic Assay

The anticancer activity of the essential oils was performed using the Resazurin Microplate Assay. Results are presented in Table 3. The flower essential oil exhibited anticancer activity against NCI-H187-small cell lung human cancer with the IC_{50} of 43.90 μgmL^{-1}. But the fruit essential oil did not show anticancer activity, because it possessed only small amount of farnesol (0.3 %) compared to that present in the flower essential oil (farnesol 10.0 %). Both essential oils were non-cytotoxic to Vero cells.

Table 3. Cytotoxic activity of the flower and fruit essential oils of *L. rubiginosa*

Sample	IC50a (μg mL^{-1})			
	Vero cells	KB	MCF-7	NCI-H187
Flower essential oil	Non-cytotoxic	Inactive	Inactive	43.9
Fruit essential oil	Non-cytotoxic	Inactive	Inactive	Inactive
Ellipticineb	1.67	0.39	-	1.18
Doxorubicine c	-	0.15	2.84	0.06

[a]Concentration that killed 50 % of cell lines; [b,c]Anticancer drugs used as positive control

Antimicrobial Activity

The *in vitro* antimicrobial activities of the flower and fruit essential oils of *L. rubiginosa* were evaluated by agar diffusion method. The flower essential oil inhibited antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa* with the inhibition zones of 10 mm, 10 mm and 12 mm, respectively. It also showed antifungal activity against *C. albican* and *T. mentagophyte* with the inhibition zones of 12 mm and 15 mm, respectively.

The fruit essential oil exhibited antibacterial activity against *S. aureus* and *P. aeruginosa* with the inhibition zones of 13 mm and 12 mm, respectively, but did not inhibit the growth of *E. coli*. This essential oil also showed antifungal activity against *C. albican* and *T. mentagophyte* with the inhibition zones of 10 mm and 15 mm, respectively. The flower and fruit essential oils strongly inhibited the hyphal growth of *Trichophyton mentagrophyte*. The fruit essential oil was highly effective against *S. aureus* strain. Results are presented in Table 4.

Table 4. Antimicrobial activity of *L. rubiginosa* essential oils

Test sample	Concentration	Zone of inhibition (mm)					
		Bacteria strains				Fungi strains	
		E.coli	S.aereus	P.aeruginosa	A.flavus	C.albican	T.mentographyte
Flower essential oil	5 $mgmL^{-1}$	10	10	12	-	12	15
Fruit essential oil	5 $mgmL^{-1}$	-	13	12	-	10	15
Gentamicin	75 μgmL^{-1}	27	27	35	-	-	-
Ketoconazole	250 μgmL^{-1}	-	-	-	25	37	16

Antioxidant Activity

The antioxidant activity of the flower and fruit essential oils was carried out using ABTS method. The flower essential oil possessed antioxidant activity with the % inhibition of 25.4%, whereas the fruit essential oil possessed antioxidant activity with the % inhibition of 6.4%.

Conclusions

The chemical constituents of the flower and fruit essential oils of *L. rubiginosa* were analysed by GC and GC-MS. The flower essential oil consisted of sesquiterpenes; nerolidol (34.8 %) and farnesol (10.0 %), whereas the fruit essential oil consisted of fatty acids; palmitic acid (66.1 %) and myristic acid (10.0 %). The flower essential oil exhibited anticancer activity

against NCI-H187 with the IC_{50} of 43.90 μg mL^{-1} and also possessed antioxidant activity with the % inhibition of 25.4 %. The flower and fruit essential oils exhibited strong antimicrobial activity against *T. mentagrophyte* and also showed moderately activity against *E. coli, S. aureus*, and *C. albican.*

This study suggests that the essential oil of *L. rubiginosa* have profound antimicrobial, antioxidant and cytotoxic effect. The obtained results could form a good basis for further investigation in the potential discovery of new natural bioactive compounds.

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