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The Chemical Constituents and the Cytotoxicity, Antioxidant and Antibacterial Activities of the Essential Oil of *Graptophyllum pictum* (L.) Griff.

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Abstract

The essential oil from the leaves of *Graptophyllum pictum* (L.) Griff. was isolated by hydrodistillation and its chemical constituents were investigated by a combination of gas chromatographic (GC-FID) and gas chromatography-mass spectrometric (GC-MS) analysis. Fourteen compounds, comprising 95.0% of the gas chromatographical oil, were identified. The major constituents were phytol (75.7%), *n*-nonacosane (6.5%) and hexahydrofarnesyl acetone (2.6%). The oil showed significant cytotoxicity against KB (epidermoid carcinoma of oral cavity), NCI-H187 (small cell lung carcinoma) and Vero cell lines with IC_{50} values of 27.04, 25.27 and 26.52 µg/ml, respectively. The antioxidant activity of the oil was determined using the ABTS radical cation scavenging assay. The oil had less antioxidant activity than the controls, trolox and ascorbic acid. In a disc diffusion assay the oil exhibited antibacterial activity against *S. aureus* and *E. coli* with MIC values of 11.75 and 35.25 µg/disc, respectively.

Key words: *Graptophyllum pictum* (L.) Griff., Chemical constituents, Antioxidant activity, Cytotoxicity, Antibacterial activity, Essential oil, Phytol.

Introduction

Graptophyllum pictum (L.) Griff., belonging to the family *Acanthaceae*, is a Papua New Guinea native shrub. This plant is commonly known as Bai Ngeon in Thai. It is often cultivated in the tropics as an ornamental plant, but occasional medicinal use has been reported[1,2]. *G. pictum* has been used in Asian folk medicine as a diuretic, an antipyretic and as an antihelmentic. It has also been used to treat the symptoms of constipation, ulcers, earache and syphilis[1,3,4,5]. Previous phytochemical analyses of the plant extract found vomifoliol[6] and flavonoids[7]. The biological activities of the plant extracts have been reported, the ethanolic extract showed anti-inflammatory activity[7], while the aqueous extract of the leaves reduced blood glucose levels[8].

There are no scientific studies on the composition of the essential oil of *G. pictum* and its biological activities. This is the first report on the chemical constituents of the essential oil and its cytotoxicity, antioxidant and antibacterial activities.

Experimental

Plant Material:

The fresh leaves of *G. pictum* (L.) Griff. were collected from the Medicinal Plants Garden, Faculty of Pharmacy, Chiang Mai University (CMU), Chiang Mai, Thailand, in May, 2009. The plant material was identified by Dr J. F. Maxwell from the Department of Biology, CMU. A voucher specimen (N. Jiangseubchatveera 1) was deposited at the herbarium of the Department of Biology at CMU.

Isolation of the essential oil:

Fresh leaves (5.060 kg) were homogenized and hydrodistilled for 6 h, using a modified Clevenger-type apparatus to obtain a pale yellow oil in 0.0002 % yield based on the fresh weight of the sample. Both the fresh leaves and the oil sample had a "woody" odor. The sample was stored at 4°C prior to analysis.

Gas chromatography (GC-FID) and Gas chromatography-Mass spectrometry (GC-MS):

The GC-FID analysis of the essential oil of *G. pictum* was performed on a Shimadzu GC-2010 Plus gas chromatograph. Separation was achieved using H_2 as carrier gas (1.5 ml/min @ 40^{o}C in a constant total flow mode) using a Restek fused silica capillary column (Rxi-5MS: 30 m x 0.25 mm i.d., 0.25 µm film thickness). Injector and detector temperatures were 260°C and 300°C, respectively, with an oven temperature programme starting at 40°C and increasing at 6°C/min to 290°C. Programmed-temperature Kovàts retention indices (RI) were obtained by GC–FID analysis of an aliquot of the essential oil spiked with an *n*-alkane mixture containing each homologue from *n*-C_7 to *n*-C_{30}. GC-MS analysis was performed in the electron impact (EI) mode at 70 eV using a Shimadzu QP5050A GC-MS system. The column and GC-MS chromatographic conditions were as above for the GC-FID analysis with He used as carrier gas.

The essential oil constituents were identified by comparison of their mass spectra with the literature data (NIST and NISTREP) and by a comparison of their programmed-temperature Kovàts retention indices (RI) with those in the literature[9,10,11,12].

Cytotoxic activity:

The cytotoxicities of the essential oil were determined against KB (epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF-7 (breast adenocarcinoma, ATCC HTB-22) and NCI-H187 (small cell lung carcinoma, ATCC CRL-5804) cancer cell lines. This assay was performed using the Resazurin microplate assay (REMA) described by O'Brien *et al.*[13]. Cells at a logarithmic growth phase were harvested and diluted to $7x10^4$ cells/ml for KB and $9x10^4$ cells/ml for MCF-7 and NCI-H187, in fresh medium. Successively, 5 µl of test sample diluted in 5% DMSO, and 45 µl of cell suspension were added to 384-well plates, incubated at 37ºC in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF-7, and 5 days for NCI-H187), 12.5 µl of 62.5 µg/ml resazurin solution was added to each well, and the plates were then incubated at 37ºC for 4 hours. Fluorescence signals were measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Ellipticine and doxorubicin were used as positive controls and 0.5% DMSO was used as a negative control. The maximum final test concentration was 50 µg/ml. Triplicate determinations were performed. The percent inhibition of cell growth was calculated as $[1–(FU_T/FU_C)]$ x100, where FU_T and FU_C were the mean fluorescent unit from treated and untreated cells, respectively. The inhibition of cell growth by 50% (IC_{50}) values were derived from dose-response curves by the SOFTMax Pro software (Molecular Devices, USA).

The cytotoxicity against a Vero cell line (the African green monkey kidney, ATCC CCL-81) was performed using the Green Fluorescent Protein (GFP) detection method[14]. The assay was carried out by adding 45 µl of cell suspension at $3.3x10^4$ cells/ml to each well of 384-well plates containing 5 µl of the essential oil previously diluted in 0.5% DMSO, and then incubating for 4 days at 37°C in an incubator with 5% CO_2. Fluorescence signals were measured using a SpectraMax M5 microplate reader in the bottom-reading mode with

excitation and emission wavelengths of 485 and 535 nm. Triplicate determinations were performed. Ellipticine and 0.5% DMSO were used as a positive and a negative control, respectively. The maximum test concentration was 50 µg/ml.

Antioxidant activity:

The antioxidant activity of the essential oil was investigated using the ABTS radical cation scavenging assay, which was carried out according to the procedure described by Re *et al.*[15] with comparisons made with the standards, trolox and ascorbic acid (concentration range 0.5-2.5 mM). For the assay, 20 µl of the essential oil (10.6 mg/ml) was mixed with 2.0 ml of diluted ABTS solution (A_{734nm} = 0.700 ± 0.020) and incubated at room temperature for 5 min. The absorbance was determined at 734 nm. The appropriate solvent blank was run in each assay. All determinations were repeated three times, and in triplicate. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (I%) was calculated as $[(A_{blank} - A_{sample})/A_{blank}]$ x100, where A_{blank} was the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} was the absorbance of the test compound. The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a concentration of antioxidants and of trolox and ascorbic acid for the standard reference data.

Antibacterial activity:

The minimal inhibitory concentration (MIC) of the essential oil against three bacteria (*Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginasa* ATCC 27853 and *Escherichia coli* ATCC 25922) was determined using the disc diffusion assay[16]. Each bacterial suspension which has been adjusted to 0.5 McFarland was uniformly spread using a cotton swab on a nutrient agar Petri dish. Five sterile paper discs (5 mm filter paper disc,

Whatmann no. 1) were placed on the surface of each agar plate and were impregnated with 10 µl of the diluted concentration of 9.40, 7.05, 4.70, 3.525, 2.35, 1.7625 and 1.175 mg/ml for the essential oil. Plates were incubated for 24h at 36±0.1°C under appropriate cultivation conditions. Antibacterial activity as MIC was determined as the lowest concentration of the essential oil which inhibits the growth of bacteria. A disc impregnated with ethanol served as a negative control and discs with vancomycin 30 µg and amikacin 30 µg (Oxoid, UK) served as positive controls. Tests were performed in duplicate.

Results and discussion

Fresh leaves of *G. pictum* were subjected to hydrodistillation using a modified Clevenger-type apparatus to yield 0.0002 % (w/w) of a pale yellow oil. GC-FID and GC-MS analysis of the oil identified fourteen compounds, collectively accounting for 95.0% of the gas chromatographical components. The major constituents were phytol (75.7%), *n*-nonacosane (6.5%) and hexahydrofarnesyl acetone (2.6%). The components were identified by comparison of their mass spectra from NIST and NISTREP libraries and by their retention indices (RI) relative to *n*-alkane indices on a Rxi-5MS capillary column with the data report in the literature[9,10,11,12]. The components, their retention indices and percentage composition, in order of their elution, are summarized in Table 1 with peak areas expressed as a percentage of the total chromatographable components of the essential oil assuming equal relative FID response for each component. Five components, collectively accounting for 4.8% of the total chromatographical oil, could not be identified due to their relatively low abundance and/or lack of reference spectra.

The most abundant compounds in the oil were oxygenated acyclic diterpenes 76.8%, followed by *n*-alkanes 13.8%, a carbonylic compound 2.6%, and a small amount of fatty acid

esters 1.8% (Table 1). The major component, phytol, is an oxygenated acyclic diterpene which can be produced from cleavage the phytyl side chain of chlorophyll and has been subjected to catalytic cracking to produce gasoline in a renewable route to motor fuels[17]. It can also be used as a precursor for the production of the synthetic forms of vitamin E[18] and vitamin K1[19]. Phytol exhibited anti-mycobacterium tuberculosis activity against *Mycobacterium tuberculosis* H_{37}Rv strain at 100 μg/ml[20]. In fragrance manufacturing, phytol and isophytol are used as ingredients in cosmetics, fine perfumes, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents[21,22].

The cytotoxicity of the essential oil against three cancerous human-cell lines, KB (epidermoid carcinoma of oral cavity), MCF-7 (breast adenocarcinoma) and NCI-H187 (small cell lung carcinoma) were determined using the Resazurin microplate assay (REMA). The results are summarized in Table 2. The essential oil inhibited the growth of the KB and NCI-H187 cell lines with IC_{50} values of 27.04 and 25.27 μg/ml, respectively. It did not however, inhibit growth of the MCF-7 cell line. The essential oil also exhibited cytotoxicity against the Vero cell line with an IC_{50} value of 26.52 μg/ml using the Green Fluorescent Protein (GFP) detection method (Table 2)**.** In contrast, a previous study showed that phytol had cytotoxicity against KB (IC_{50} 11.95 μg/ml) and MCF-7 (IC_{50} 39.60 μg/ml) cell lines, but was non-cytotoxic to NCI-H187 and Vero cell lines[23]. These contrasting results suggest that the other components of the essential oil, rather than phytol, are responsible for the cytotoxicity against NCI-H187 and Vero cells. While the inactivity of the oil against MCF-7 suggests that the other components of the oil inhibit or mask the cyctotoxicity of phytol. Phytol has also been reported to have cytotoxicity against HT-29 human colon cancer cells, MG-63 osteosarcoma and AZ-521 gastric cancer cells[24] while hentriacontane, a minor (1.7%) component of the oil

and the active component of Natto extracts, has been shown to have possible anti-tumor promoter activity[25].

The antioxidant activity of the essential oil was investigated using the ABTS radical cation scavenging assay by comparison with the standards, trolox and ascorbic acid. The essential oil showed trolox and ascorbic acid equivalent antioxidant capacities of 0.16 and 0.17 mM/mg, respectively, which indicated that the essential oil had less antioxidant activity than the standards controls.

The antibacterial activity of the essential oil against three bacterial strains was assessed by determination of MIC values using a disc diffusion assay. The results are shown in Table 3. The essential oil had antibacterial activities against both Gram positive *S. aureus*, with the most potent activity at 11.75 µg/disc, and Gram negative *E. coli* with an activity of 35.25 µg/disc. The oil was not active against Gram negative *P. aeruginosa*. Notably, essential oils rich in nonacosane and hexahydrofarnesyl acetone, which comprise 9.1% of the oil of this study, have shown broad spectrum antimicrobial activities[26,27].

Conclusions

In conclusion, we report the first analysis of the chemical constituents of the essential oil from the fresh leaves of *G. pictum* (L.) Griff. and its cytotoxicity, antioxidant and antibacterial activities. The essential oil was analyzed by GC-FID and GC-MS and fourteen compounds were identified. The main compounds were phytol, *n*-nonacosane and hexahydrofarnesyl acetone. The essential oil exhibited significant cytotoxicity against KB-, NCI-H187 and Vero cell lines. The essential oil was also possessed antioxidant activity. The antibacterial activity against *S. aureus* and *E. coli* suggests that the essential oil may be used for the treatment of bacterial infections or in combination with synthetic antibiotic drugs.

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Table 1. Chemical constituents of the essential oil of *G. pictum* (L.) Griff.

Peak	Compounds	RA[a]	RI[b] (Exp.)	RI[c]	Identification[d]
1	Unidentified[e]	0.8	1638		
2	Hexahydrofarnesyl acetone	2.6	1847	1845	RI, MS
3	Unidentified	1.2	1881		
4	Isophytol	1.1	1950	1946	RI, MS
5	Isopropyl Palmitate	0.4	2026	2026.9	RI, MS
6	Unidentified	0.9	2084		
7	Linolenic acid, methyl ester	1.4	2103	2098	RI, MS
8	Phytol	75.7	2116	2011	RI, MS
9	*n*-Tricosane	0.5	2300	2300	RI, MS
10	*n*-Tetracosane	0.7	2400	2400	RI, MS
11	*n*-Pentacosane	0.9	2500	2500	RI, MS
12	Unidentified	0.8	2554		
13	*n*-Hexacosane	0.7	2600	2600	RI, MS
14	*n*-Heptacosane	1.6	2700	2700	RI, MS
15	*n*-Octacosane	0.8	2800	2800	RI, MS
16	Unidentified	1.1	2835		
17	*n*-Nonacosane	6.5	2900	2900	RI, MS
18	*n*-Triacontane	0.4	3000	3000	RI, MS
19	*n*-Hentriacontane	1.7	3100	3100	RI, MS
	Oil components (total)	99.8			
	Carbonylic compound	2.6			
	Oxygenated acyclics	76.8			
	Fatty acid esters	1.8			
	n-alkanes	13.8			
	Unidentified	4.8			

[a] RA, relative area (peak area relative to total peak area)

[b] RI (Exp), programmed temperature retention indices as determined on a Rxi-5MS column using a homologous series of *n*-alkanes (C_7-C_{30}) as internal standard and H_2 as carrier gas

[c] RI (Lit.) values from literature data

[d] MS, From a comparison of the mass spectrum with MS libraries and RI of literature

[e] EI MS *m/z* 204 (40%), 179 (31%), 161 (54%), 119 (100%)

Table 2. Cytotoxicity of the essential oil of *G. pictum* (L.) Griff.

Sample	IC_{50}[a] (μg/ml)			
	KB cell line	MCF-7 cell line	NCI-H187	Vero cell line
G. pictum oil	27.04	inactive	25.27	26.52
Phytol[b]	11.95	39.60	inactive	non-cytotoxic
Ellipticine[c]	0.261	-	0.420	1.621
Doxorubicin[c]	0.099	0.860	0.040	-

[a]Concentration that killed 50% of cells

[b] IC_{50} values from Keawsa-ard, *et al*.[23].

[c]Anticancer drug used as positive control

Table 3. Antibacterial activity of the essential oil of *G. pictum* (L.) Griff.

	Gram positive bacteria	Gram negative bacteria	
	MIC (µg/disc)		
	S. aureus	*P. aeruginosa*	*E. coli*
G. pictum oil	11.75 µg (5.5 mm)	NA	35.25 µg (5.5 mm)
Positive control	Vancomycin 30 µg (16 mm)	Amikacin 30 µg (17 mm)	Amikacin 30 µg (17 mm)
Negative control (ethanol)	NA	NA	NA

NA= No activity