2015

The chemical constituents and biological activities of the essential oil and the extracts from leaves of Gynura divaricata (L.) DC. growing in Thailand

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Publication Details

Jiangseubchatveera, N., Liawruangrath, B., Liawruangrath, S., Korth, J. & Pyne, S. G. (2015). The chemical constituents and biological activities of the essential oil and the extracts from leaves of Gynura divaricata (L.) DC. growing in Thailand. Journal of Essential Oil Bearing Plants, 18 (3), 543-555.

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Disciplines
Medicine and Health Sciences | Social and Behavioral Sciences

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The Chemical Constituents and Biological Activities of the Essential Oil and the Extracts from leaves of *Gynura divaricata* (L.) DC. Growing in Thailand

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Abstract: The chemical constituents of the essential oil from the leaves of *Gynura divaricata* (L.) DC. were investigated by GC–FID and GC–MS. Seventeen compounds, representing 97.0% of the chromatographical fraction of the oil, were detected. The major constituents, cubenol (65.7%) and spathulenol (6.4%), were isolated using column chromatography and identified by NMR and MS analysis. The antioxidant, cytotoxicity and antimicrobial activities of the essential oil, the hexane, dichloromethane and methanol extracts of *G. divaricata* leaves were investigated. The essential oil and the crude extracts showed antioxidant activity using the ABTS and DPPH methods, respectively. The essential oil exhibited significant cytotoxicity against KB, MCF–7 and NCI–H187 cancer cell lines with the IC_{50} values of 5.79, 47.44 and 17.65 µg/mL, respectively and had a MIC of 50 µg/mL against *Mycobacterium tuberculosis* $H_{37}Ra$. Cubenol had an IC_{50} value of 45.37 µg/mL against the NCI–H187 cancer cell line. All extracts were non–cytotoxic against Vero cells. The essential oil and extracts showed antimicrobial activity using the disc diffusion assay. The methanol extract was the most potent of the three extracts.

Keywords: *Gynura divaricata* (L.) DC., essential oil, extracts, chemical constituents, cytotoxicity, antioxidant activity, antibacterial activity, cubenol.

Introduction

Gynura divaricata (L.) DC. (*Asteraceae*), a traditional medicinal herb, has been used for the treatment of bronchitis, pulmonary tuberculosis, pertussis, toothache, rheumatic arthralgia, uterine bleeding and diabetes in folk medicine[1]. Previous chemical analyses of the crude extract of *G. divaricata* have revealed the presence of alkaloids, cerebrosides, flavonoids, phenolics and polysaccharides[2-6].

The aqueous extracts from the aerial part of *G. divaricata* have decreased the levels of blood glucose in diabetic mice[7], while *in vitro* studies have revealed a hypoglycemic effect via α-amylase and α-glycosidase which are the key enzymes relevant for type 2 diabetes. These extracts also significantly inhibited angiotensin-1 converting enzyme (ACE) that is involved in hypertension pathogenesis[8]. The ethanolic extract of the leaves of *G. divaricata* have significant antioxidant properties[9] and the aqueous extracts of the stem and leaf have been used in preparing antitumor agents[10].

The compositions of the essential oil from the leaves of *G. divaricata* growing in China have been studied. This report showed that the major constituents were τ–cadinene (20.8%) and γ–elemene (10.6%)[11]. During our study, Chen *et al.*[12] reported that the major constituents of this essential oil, from plants growing in two different areas (Nanjing and Nanping) in the east of China, were α–pinene (49.7%) and β–caryophyllene (43.7%) and β–caryophyllene (48.2%) and limonene (21.0%), respectively.

To our knowledge, no previous reports have been made on the biological activity or the isolation of the major components of the essential oil from *G. Divaricata.* Further, different solvent extracts of the leaf have not been investigated for their antioxidant, cytotoxicity, and antimicrobial activities. Therefore, the aim of this study was to identify and isolate the main constituents of this essential oil from the leaves of plants growing in Chiang

Mai, Thailand and determine the antioxidant, cytotoxicity, antimycobacterial and antibacterial activities of the essential oil and the crude extracts.

Experimantal

Plant materials

Leaves of *G. divaricata* (Asteraceae) were collected from a single population in July 2009 from the Medicinal Plants Garden, Faculty of Pharmacy, Chiang Mai University (CMU), Thailand. The plant material was identified by J. F. Maxwell from the Department of Biology at CMU. A voucher specimen (N. Jiangseubchatveera 2) was deposited at the CMU herbarium of the Department of Biology.

Isolation of essential oil and major isolates

Fresh leaves of *G. divaricata* (1.8 kg) were homogenized and hydrodistilled in a modified Clevenger–type apparatus for 6 h. The distillate (142.4 mg) was stored at 4°C until further analysis. The essential oil (72.3 mg) was subjected to column chromatography on silica gel (silica gel 60, 70–230 mesh, Merck, Germany) using a gradient of ethyl acetate in petroleum ether (1:99 to 100:0) as eluent. The constituents of each fraction were examined on silica gel TLC plates with petroleum ether/ethyl acetate (9:1) as the eluent. The TLC plates were stained with ceric ammonium molybdate and heated to allow visualization of the constituents in each fraction. Fractions that showed similar components were combined and the solvent was removed by evaporation and flushing with dry nitrogen gas.

Extraction

The leaves of *G. divaricata* were washed with distilled water and dried in a hot air oven at 40^{o}C for 24 h. Then the dried leaves (472.0 g) were ground and extracted sequentially with 2 litres of hexane, dichloromethane and methanol at room temperature for 3 days. Each extract was filtered and evaporated to dryness under reduced pressure using a rotary evaporator to obtain the crude extracts as follows: the crude hexane extract (9.8 g), the crude

dichloromethane extract (8.9 g) and the crude methanol extract (8.8 g). All the samples were stored at 4^{o}C until further analysis.

GC–FID and GC–MS analysis

The GC–FID analysis of the essential oil was performed on a Shimadzu GC–2010 Plus gas chromatograph (Shimadzu, Kyoto, Japan). Separation was achieved using H_2 carrier gas (1.5 mL/min @ 40^{o}C) in a constant total flow mode using a fused silica capillary column: Rxi–5MS (Nonpolar 5% diphenyl 95% dimethylpolysiloxane phase) 30 m x 0.25 mm i.d., 0.25 μm film thickness (Restek, Bellefonte, PA, USA). 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 Kovats retention indices (RI) for each constituent 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 (Shimadzu). The column and GC–MS chromatographic conditions were as above for the GC–FID analysis with He used as the carrier gas. All determinations were performed in triplicate. Identification of the essential oil constituents was performed by comparison of their mass spectra with the NIST and NISTREP mass spectra libraries of the GC–MS data system and from a comparison of their RI with those in the literature[13-20].

NMR Identification

The two major components of the essential oil were also identified by ^{1}H–NMR spectroscopy. Spectra were recorded on an Innova 500 MHz–NMR spectrometer (Varian, USA) in solutions of $CDCl_3$ with TMS as an internal standard.

Antioxidant activity

The antioxidant activity of the essential oil was investigated using the ABTS radical cation scavenging assay[21], compared with the standards, trolox and ascorbic acid (concentration range 0.5–2.5 mM). For the assay, 20 µL of the essential oil (50 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 using a Jasco 7800 UV–Visible spectrophotometer (USA) at 734 nm. The appropriate solvent blank was run for each assay. Triplicate determinations were performed. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (I %) was calculated as $[(A_{blank} - A_{sample})/A_{blank}] \times 100$ where A_{blank} and A_{sample} are the absorbance of the control reaction (containing all reagents except the test compound) and the the test compound, respectively. The percentage inhibition was calculated and plotted as a concentration of trolox and ascorbic acid for the standard reference data.

The antioxidant activity of the crude extracts of *G. divaricata* was determined by the DPPH method[22]. All crude extracts were prepared in the concentration of 1, 2, 3, 4, 5, 6, 7 and 10 mg/mL using ethanol as a solvent. Then, 180 µL DPPH in ethanol (10 µM) was added to 20 µL samples of different concentrations of the extracts in a 96-well microtitre plate. After 30 min incubation in the dark room at room temperature, the absorbance of each well was measured spectrophotometrically (Spectrophotometer, Multimode detector, Beckman Coulter DTX880, USA) at 540 nm. The DPPH solution was used as a negative control. Trolox and ascorbic acid were used as standard controls in the concentration range 0.01-0.2 mg/mL. All determinations were performed in triplicate. The percentage DPPH scavenging activity was calculated as $(A_c - A_s)/A_c \times 100$ where 'A_c' is the absorbance of the control and 'A_s' is the absorbance of the test sample. The IC_{50} values denote the concentration of the sample which is required to scavenge 50% of DPPH free radicals.

Cytotoxic activity

The cytotoxicities of the essential oil and the crude extracts were determined against the 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) cancerous human–cell lines using the Resazurin microplate assay (REMA), using the method described by Brien *et al*[23]. The extracts were first diluted to 50 µg/mL in 0.5% DMSO and then subjected to a doubling series of dilutions. 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 each sample solution and 45 µl cell suspension were added to 384–well plates, incubated at 37ºC in 5% CO_2 in an incubator. After the incubation period (3 days for KB and MCF–7, and 5 days for NCI–H187), 12.5 µL resazurin solution (62.5 µg/mL) was added to each well, and the plates were then incubated at 37ºC for 4 h. The fluorescence was measured using a SpectraMax M5 multi–detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. The percentage inhibition of cell growth (%) was calculated as $[1–(FU_T/FU_C)] \times 100$ where FU_T and FU_C are the mean fluorescent units from treated and untreated conditions, respectively. The IC_{50} values were derived from the dose response curves using the SOFTMax Pro software (Molecular Devices). Triplicate determinations were performed. Ellipticine, doxorubicin and tamoxifen were used as positive controls and 0.5% DMSO was used as a negative control.

The cytotoxicity assay against Vero cells (African green monkey kidney, ATCC CCL–81) was performed using the Green Fluorescent Protein (GFP) detection method[24]. 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 test compounds previously diluted in 0.5% DMSO and then incubating for 4 days in an incubator at 37°C with 5% CO_2. The fluorescence was

measured using a SpectraMax M5 microplate reader in the bottom–reading mode at the excitation and emission wavelengths of 485 and 535 nm. Triplicate determinations were performed. The percentage of cytotoxicity and IC_{50} value of each sample were calculated. Ellipticine was used as a positive control and 0.5% DMSO was used as a negative control.

Antimycobacterial activity

Antimycobacterial activity of the essential oil was determined against *Mycobacterium tuberculosis* H37Ra with the Green Fluorescent Protein (GFP)–based fluorescent detection[25-26]. H_{37}Ra *gfp* was cultivated on 7H10 agar containing 30 µg/mL kanamycin at 37ºC for 4 weeks or until the growth was observed. Starter cultures were prepared by fully looping 2–3 single colony into 7H9 broth supplemented with 0.2% v/v glycerol, 0.1% w/v casitone, 0.05% v/v Tween 80, 10% v/v Middlebrook OADC enrichment solution (BD Biosciences, USA) and 30 µg/mL of kanamycin. The mixture was then incubated at 37ºC in a 200 rpm shaker incubator until the optical density (OD) at 550 nm was between 0.5 and 1. For batch cultivation, the starter cultures were transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37ºC in a 200 rpm shaker incubator until the $OD_{550\ nm}$ was approximately 0.5 to 1. The cells were pelleted, washed and suspended in PBS buffer, and then sonicated 8 times for 15 seconds each. The sonicated samples were then aliquoted and frozen at –80ºC for up to 2 to 3 months prior to use. Titer stocks were determined by the colony forming unit (cfu) assay and the seeding density for the anti–TB assay was optimized by serial dilutions. The dilution that grew at logarithmic phase on day 7 was used as an optimal bacterial seeding density. For assay in 384–well formats, the seeding was approximately $2x10^4$ to $1x10^4$ cfu/mL/well. Each well contained 5µL of test samples serially diluted in 5% dimethyl sulfoxide, followed by 45 µL of cell suspension prepared as described above. Plates were incubated at 37ºC for 7 days. The fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices) in the bottom–reading mode at the excitation and

emission wavelengths of 485 nm and 535 nm. Triplicate determinations were performed. The percentage inhibition of cell growth (%) was calculated as $[1- (FU_T/FU_C)] \times 100$ where FU_T and FU_C are the mean fluorescent units from treated and untreated conditions, respectively. The lowest drug concentration that inhibited cell growth by 90% was reported as the Minimum Inhibitory Concentration (MIC). Rifampicin, streptomycin, isoniacid and ofloxacin were used as positive controls, and 0.5% DMSO was used as a negative control.

Antimicrobial activity

Antibacterial activity of the essential oil

The antibacterial activity of the essential oil was determined against three bacteria (*Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginasa* (ATCC 27853) and *Escherichia coli* (ATCC 25922), which were obtained from the Central Diagnostic Laboratory, Faculty of Medicine, CMU). Tests were performed in triplicate using the disc diffusion assay[27]. Each bacterial suspension, which had been adjusted to 0.5 McFarland, was uniformly spread using a cotton swab onto 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 essential oil of concentrations 100, 70, 50, 30, 10, 5 and 2.5 mg/mL. Plates were incubated for 24 h at 36±0.1°C under appropriate cultivation conditions. 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. Antibacterial activity as minimal inhibitory concentration (MIC) values were determined as the lowest concentration of the essential oil which inhibited the growth of the bacteria.

Antimicrobial activity of the crude extracts

The antimicrobial activity of the crude extracts was determined using the agar diffusion assay[28]. The samples were tested against the three aforementioned bacterial strains

and Aspergillus flavus, *Candida albican* and *Trichophyton mentagrophyte*. All microorganisms were obtained from the Central Diagnostic Laboratory, CMU. The microorganisms were maintained in agar conservation at room temperature. The cell suspensions were adjusted to 0.5 McFarland standard turbidity (10^{7-8} CFU/mL) for bacteria and 1.0 McFarland standard turbidity (10^{15} CFU/mL) for fungi. Every cell suspension was spread over plates containing Nutrient Agar (NA) for bacteria and Potato Dextrose Agar (PDA) for fungi. In each of these plates, 4 wells (9 mm) were cut out using sterilized cork borer. The extracts were diluted with ethanol and screened for antibacterial activity using concentrations of 20 mg/mL. One hundred microtiter sample solution was carefully transferred to the wells and the same volume of ethanol was used as a negative control. The samples were incubated at 37 ºC for 24 h for bacterial pathogens and 3 days for fungal pathogens. After incubation, the diameter of the inhibition zone was measured. A positive control was also assayed to check the sensitivity of the tested organisms using gentamicin (75 μg/mL) and ketoconazole (250 μg/mL). Experiments were performed in triplicate.

Statistic analysis

All values are expressed as the mean ± S.D. and all the experiments were performed in triplicate. Linear regression analysis R^2 was performed on the standard and test extracts. The IC_{50} values were calculated by linear regression analysis.

Results and discussion

Fresh leaves of *G. divaricata* were hydrodistilled to afford a yellow oil in 0.0078 % (w/w). The essential oil was analysed by GC–FID and GC–MS. A GC chromatogram is presented in Figure 1. Thirteen components were identified, representing 97.0% of the chromatographical fraction of the oil (Table 1). Oxygenated sesquiterpenes (73.2%) were the

main group of the constituents. The major compounds were cubenol (65.7%) and spathulenol (6.4%). These results were different from the previous studies. The first study showed that τ–cadinene (20.8%) and γ–elemene (10.6%) were the major components[11], while a more recent study, from plants collected in two different areas of China, revealed the major components of the essential oil were α–pinene (49.7%) and β–caryophyllene (43.7%) and β–caryophyllene (48.2%) and limonene (21.0%)[12].

<Figure 1 near here>

Cubenol was not identified as a component in this earlier study. This variation in the two major essential oil components might be because of the different plant locations (Thailand versus China), habitats and plant collection times. However, the minor components β-pinene, copaene, α–humulene, β–elemene, spathulenol, phytol and *n*–hexadecanoic acid were found both in this study and the earlier ones. The two major components were isolated by column chromatography to give cubenol (9 mg, 12.4%) and spathulenol (1.6 mg, 2.21%) as colourless oils. Their ^{1}H NMR spectroscopic data was identical to those reported in the literature[29-30].

<Table1 near here>

The antioxidant activity of the essential oil was investigated using the ABTS assay. The percentage inhibitions were plotted against the trolox and ascorbic acid concentrations to obtain the linear calibration curves which gave the linear regression equations for trolox and ascorbic acid (Figure 2). These equations were used to calculate the equivalent capacities of the essential oil. The results showed that the essential oil had trolox and ascorbic acid equivalent capacities of 5.10 ± 0.08 and 3.77 ± 0.06 mg/g extract, respectively.

<Figure 2 near here>

The extraction yields and the antioxidant activity of the different crude extracts are presented in Table 2. The hexane extract gave the highest percentage yield (2.98%), whereas

the dichloromethane and methanol extracts gave yields of 1.90 and 1.87%, respectively. The antioxidant activities of the extracts were evaluated using the DPPH assay. In the DPPH assay, the ability of the examined crude extracts to act as donor of hydrogen atoms or electrons in transformation of the purple-coloured DPPH radical into its yellow-coloured reduced form DPPH-H was investigated. A plot of the percentage inhibition of DPPH against the concentration of the sample solutions was prepared and the IC_{50} values of each crude extract was determined from the calibration curve.

<Table 2 near here>

The methanol extract possessed the highest antioxidant activity with an IC_{50} of 2.87 ± 0.02 mg/mL. The hexane and dichloromethane extracts also showed antioxidant activity with IC_{50} values of 5.28 ± 0.01 and 4.28 ± 0.01 mg/mL, respectively. The ethanolic extract of *G. divaricata* leaves was reported to have significant antioxidant properties[9] and our studies reported here support these earlier observations. This activity may be due to the presence of phenolic and flavonoid compounds which have significant correlations with the total antioxidant activity and free radical-scavenging capacities[31]. While phytol, a minor component of this essential oil, has been reported to possess antioxidant activity (by measuring the cupric ion reducing antioxidant capacity)[32]. This plant thus has potential for future development as a source of natural antioxidants to prevent or treat diseases involving oxidative stress.

The essential oil exhibited cytotoxicity against the KB, MCF–7 and NCI–H187 cell lines with the IC_{50} values of 5.79, 47.44 and 17.65 µg/mL, respectively. Cubenol showed cytotoxicity only against NCI–H187 cells with an IC_{50} value of 45.37 µg/mL. Spathulenol and all crude extracts were inactive against all cancer cell lines (Table 3). The essential oil and all crude extracts were non–cytotoxic against Vero cells. The assay of the essential oil against *Mycobacterium tuberculosis* H37Ra indicated a MIC value of 50 µg/mL. However,

cubenol and spathulenol were inactive in this assay (Table 3). A previous study reported that spathulenol had weak cytotoxicity (IC_{50} values > 20 μg/mL) against eight different human carcinogenic cell lines, including the KB cell line[33]. This compound was inactive against the three cell lines tested (KB, MCF–7 and NCI–H187 cell lines). These results indicated that some of the other more minor components were responsible for the higher cytotoxic activities of the essential oil. Indeed, previous reports have shown that of the minor components of the essential oil, β–elemene inhibited the growth of human non-small cell lung cancer H460 and A549 cells (IC_{50} 40−60 μg/mL)[34] and α–humulene was active against several solid tumor cell lines, including MCF–7 (GI_{50} 55 −73 μM)[35]. Phytol has been reported to have cytotoxicity against HT-29 human colon cancer cells, MG-63 osteosarcoma and AZ-521 gastric cancer cells[36] and also exhibited antituberculosis activity with a MIC value of 12.5 mg/ml against *M. tuberculosis* H_{37}Rv in *vitro*[37]. *n*-Hexadecanoic acid (or palmitic acid) has shown cytotoxicity against human leukemic cells, MOLT-4, and also showed *in-vivo* antitumor activity in mice[38]. Moreover, *n*-hexadecanoic acid had antituberculosis against H_{37}Rv with a MIC value of 25 μg/mL using the Microplate Alamar Blue Assay[39]. Therefore, the cytotoxicity and antimycobacterial activity of the essential oil may be due to the activity of these components or the synergistic effects of these compounds.

<Table 2 near here>

The antimicrobial activity of the essential oil and the crude extracts were assessed using a disc diffusion assay. The MIC values and the inhibition zones of the essential oil are summarised in Table 4 while the inhibition zones of the crude extracts are shown in Table 5. The lowest concentration of the essential oil that had antibacterial activity against *S. aureus*, *P. aeruginosa* and *E. coli* showed inhibition zones of 6.00 ± 0.26, 6.00 ± 0.44 and 6.00 ± 0.53 mm, respectively. These results have been used to determine the MIC values of 100, 50 and 500 μg/disc, respectively.

<Table 4 near here>

The methanol extract exhibited antibacterial activity against *S. aureus* and *P. aeruginosa* with inhibition zones of 11.00 ± 0.05 and 15.00 ± 0.26 mm, respectively. The hexane extract only showed antibacterial activity against only *P. aeruginosa* with an inhibition zone of 12.00 ± 0.20 mm, while the dichloromethane extract had no antibacterial activity against all tested bacterial strains. The dichloromethane and methanol extracts exhibited antifungal activities against *A. flavus*, *C. albican* and *T. mentagrophyte* with inhibition zones of 10.00 ± 0.20, 12.00 ± 0.26 and 13.00 ± 0.26 mm (for the dichloromethane extract) and 10.00 ± 0.42, 13.00 ± 0.26 and 14.00 ± 0.40 mm (for the methanol extract), respectively. The hexane extract showed antifungal activity against *A. flavus* and *C. albican* with the inhibition zones of 10.00 ± 0.44 and 10.00 ± 0.55 mm, respectively. Spathulenol has been reported to possess modest antimicrobial activity (MIC 136 μg/mL) against *S. aureus* and *Proteus mirabilis*[30]. *n*-Hexadecanoic acid and phytol have been reported to possess antibacterial activity against *S. Aureus*[40-41]. Cubenol, isolated from the essential oil of *P. uviferum*, was one of the most active antibacterials of the sesquiterpene components[42]. The results from this investigation indicated that the essential oil and the crude extracts of *G. divaricata* leaves have antimicrobial activities which may be useful for future medical applications.

In conclusion, the compositions of the essential oil from the leaves of *G. divaricata* growing in Chiang Mai, Thailand have been analysed by GC and GC-MS. The essential oil contained seventeen compounds of which thirteen components were identified. The major components were cubenol (65.7%) and spathulenol (6.4%). These major components are different to those found in the essential oil from previous studies on this plant growing in China. The major constituents were isolated using column chromatography and identified by NMR and MS analysis. The essential oil exhibited significant cytotoxicity against three

cancer cell lines, and showed antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra. Cubenol had cytotoxicity against NCI–H187 cells. The essential oil, pure compounds and all crude extracts were non–cytotoxic. The essential oil and the crude extracts, especially the methanol extract, had potentially useful antioxidant and antimicrobial activities. This suggested the essential oil and the methanol extract of *G. divaricata* could be used as an alternative therapeutic to, or in combination with, synthetic anticancer, anti-TB or antibiotic drugs and may be used as a health supplement. However, further investigation using bioassay directed methods, are in progress to determine more bioactive compounds in the leaves of this medicinal plant which may be useful for new drug discovery.

Acknowledgements

The authors thank the Thailand Research Fund for a Royal Golden Jubilee Grant PHD/0144/2550 (to N. J.), the Graduate School, Chiang Mai University and the University of Wollongong (UOW) for support. We thank Dr. Minyan Tang (UOW) and Prof. Yang Ye (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) for translating reference 11.

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Table 1. Chemical constituents of the essential oil from the fresh leaves of *G. divaricata* (L.) DC.

	Compounds[a]	RI_{lit}	RI_{exp}	%	Method of Identification
1	β–Pinene	981	982	0.7	RI[15], MS
2	Linalool	1100	1100	0.9	RI[14], MS
3	Copaene	1391	1387	1.7	RI[17], MS
4	β–Elemene	1393	1400	1.8	RI[15], MS
5	Aromadendrene	1437	1434	2.8	RI[13], MS
6	α–Humulene	1467	1470	3.0	RI[14], MS
7	δ–Cadinene	1536	1535	1.8	RI[18], MS
8	Spathulenol	1591	1593	6.3	RI[16], MS, ^{1}H NMR[29]
9	Unidentified	–	1600	4.2	–
10	Viridiflorol	1612	1609	1.2	RI[18], MS
11	Unidentified	–	1627	1.9	–
12	Cubenol	1645	1648	65.7	RI[14], MS, ^{1}H NMR[28]
13	Unidentified	–	1683	0.8	–
14	α–Calacorene[b]	–	1693	0.8	MS
15	Unidentified	–	1737	0.7	–
16	*n*-Hexadecanoic acid	1957	1963	1.4	RI[13], MS
17	Phytol	2132	2156	1.3	RI[13], MS
	Monoterpene hydrocarbon			0.7	
	Oxygenated monoterpene			0.9	
	Sesquiterpene hydrocarbons			11.9	
	Oxygenated sesquiterpenes			73.2	
	Fatty acid			1.4	
	Oxygenated acyclic diterpene			1.3	
	Unidentified (4 in total)			7.6	
	Total			97.0	

All determinations were performed in triplicate; RI_{lit}, RI_{exp}, reference and experimental retention index (RI_{exp} determined with reference to a homologous series of normal alkanes on Rxi–5MS capillary column); [a]Compounds listed in order of elution from a Rxi–5MS capillary column; [b]Tentative identification, $RI_{lit.}$have been reported as 1859[14], 1744[19] and 1540[20].

Table 2. Extraction yield, antioxidant activity of the crude extracts from *G. divaricata* (L.) DC.

Samples	Extraction yield (%)	DPPH method* IC_{50} (mg/mL)
Hexane extract	2.08	5.28 ± 0.01
Dichloromethane extract	1.90	4.28 ± 0.02
Methanol extract	1.87	2.87 ± 0.02
Standard trolox		0.14 ± 0.01
Standard ascorbic acid		0.05 ± 0.00

*Results summarized here are: mean ± S.D. values (n=3)

Table 3. Cytotoxicity and antimycobacterial activity of the essential oil and the crude extracts from *G. divaricata* (L.) DC.

Sample	IC_{50}[a] (μg/mL)				MIC[b] (μg/mL)
	KB	MCF–7	NCI–H187	Vero cells	H_{37} Ra strain
Essential oil	5.79 ± 0.04	47.44 ± 0.19	17.65 ± 0.13	NA	50.00
Cubenol	NA	NA	45.37 ± 2.94	–	NA
Spathulenol	NA	NA	NA	–	NA
Hexane extract	NA	NA	NA	NA	–
Dichloromethane Extract	NA	NA	NA	NA	–
Methanol extract	NA	NA	NA	NA	–
Ellipticine[c]	0.44 ±0.09	–	0.86 ± 0.40	1.62 ± 0.17	–
Doxorubicin[c]	0.61 ± 0.19	9.16 ± 0.39	0.10 ± 0.02	–	–
Tamoxifen[c]	–	4.94 ± 0.84	–	–	–
Rifampicin[d]	–	–	–	–	0.003–0.012
Streptomycin[d]	–	–	–	–	0.156–0.313
Isoniazid[d]	–	–	–	–	0.023–0.046
Ofloxacin[d]	–	–	–	–	0.391–0.781

[a]Results summarized here are: mean ± S.D. values (n=3), NA = No activity, (–) = Not tested; [b]Results are ranges from three experiments; [c]Anticancer drugs used as positive controls.; [d]Antimycobacterial drugs used as positive controls.

Table 4. Antibacterial activity of the essential oil from *G. divaricata* (L.) DC. leaves

	Diameter of inhibition zone[a] (mm)			MIC (μg/disc)		
Samples	*S. aureus*	*P. aeruginosa*	*E. coli*	*S. aureus*	*P. aeruginosa*	*E. coli*
Essential oil	6.00 ± 0.26	6.00 ± 0.44	6.00 ± 0.53	100.00	50.00	500.00
Vancomycin[b]	16.00 ± 0.26	–	–	30.00	–	–
Amikacin[b]	–	17.00 ± 0.50	17.00 ± 0.20	–	30.00	30.00
Ethanol[c]	NA	NA	NA			

[a] The diameter of inhibition zone of the lowest concentration that inhibited the growth of the bacteria; results summarized here are: mean ± S.D. values (n=3); NA= No activity; [b]Standard antibiotics used as positive controls; [c]negative control

Table 5. Antibacterial activity of the crude extracts from *G. divaricata* (L.) DC. leaves

Samples	Diameter of inhibition zone[a] (mm)					
	Bacteria			Fungi		
	S. aureus	*P. aeruginosa*	*E. coli*	*A. flavus*	*C. albican*	*T. mentagrophyte*
Hexane	NA	12.00 ± 0.20	NA	10.00 ± 0.50	10.00 ± 0.50	NA
DCM[b]	NA	NA	NA	10.00 ± 0.20	12.00 ± 0.26	13.00 ± 0.26
Methanol	11.00 ± 0.50	15.00 ± 0.26	NA	10.00 ± 0.42	13.00 ± 0.26	14.00 ±0.40
Gentamicin[c]	35.00 ± 0.30	27.00 ± 0.50	27.00 ± 0.10	–	–	–
Ketoconazole[c]	–	–	–	25.00 ± 0.50	37.00 ± 0.40	16.00 ± 0.20
Ethanol[d]	NA	NA	NA	NA	NA	NA

[a] Results summarized here are: mean ± S.D. values (n=3); NA= No activity; (–) = Not tested;

[b] DCM = Dichloromethane; [c] Standard antibiotics used as positive controls (Gentamicin 75µg/mL and Ketoconazole 250 µg/mL); [d] negative control

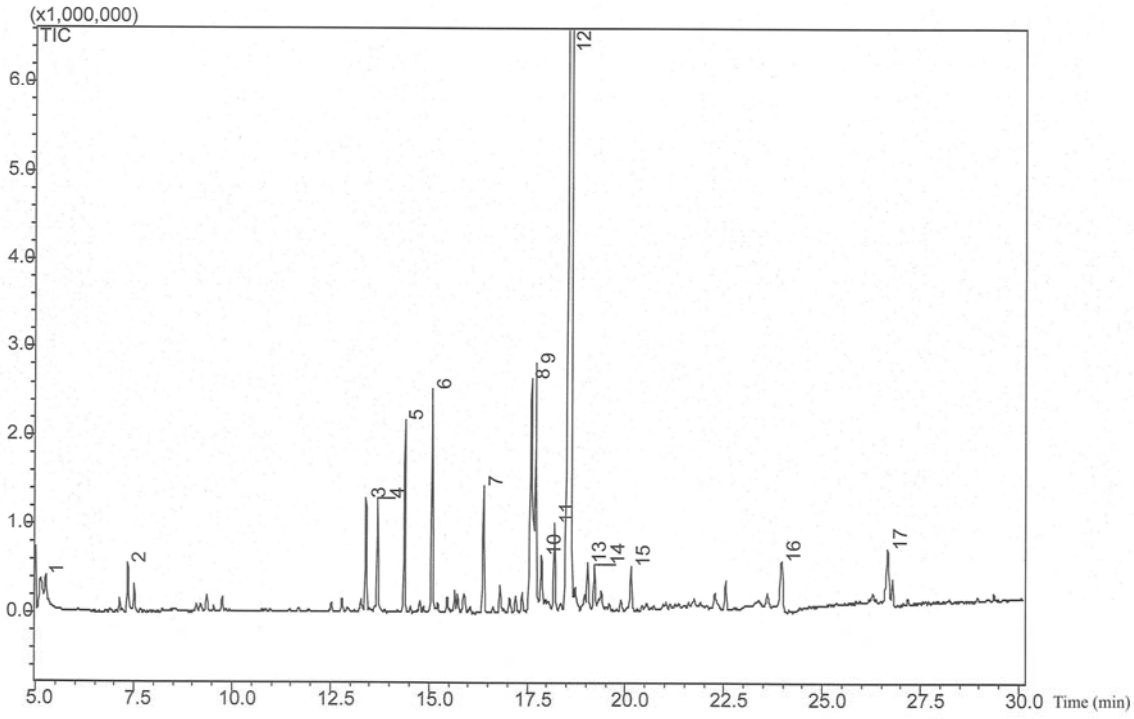

Figure 1. GC chromatogram of the leaves essential oil of *G. divaricata* (L.) DC.

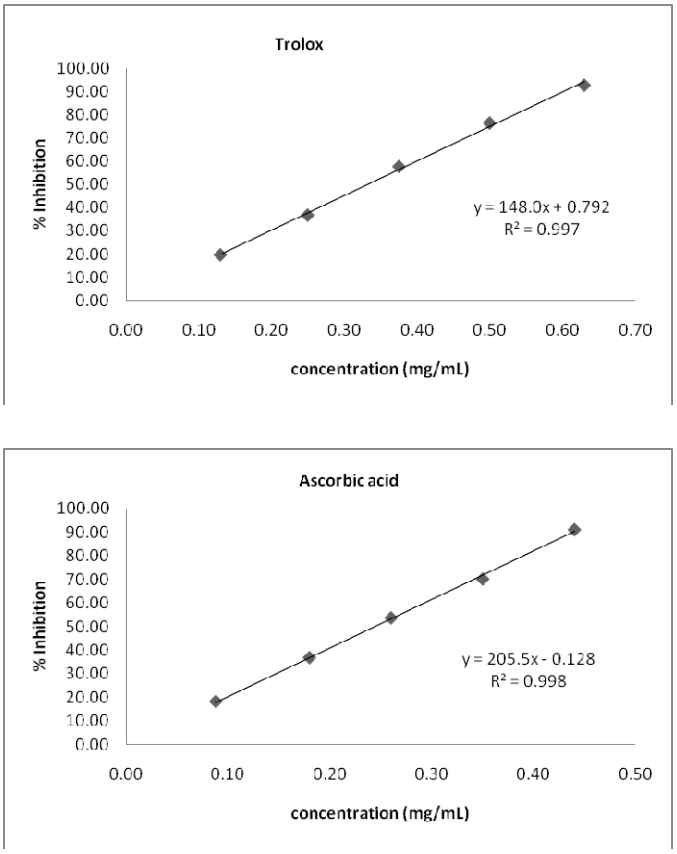

Figure 2. Calibration curves of standard trolox and ascorbic acid solutions by ABTS method