ORIGINAL ARTICLE

Cytotoxic and free radical scavenging activities of Zingiberaceous rhizomes

Sariga Zaeoung[1], Anuchit Plubrukarn[2] and Niwat Keawpradub[3]

Abstract

Zaeoung, S., Plubrukarn, A. and Keawpradub, N.
Cytotoxic and free radical scavenging activities of Zingiberaceous rhizomes
Songklanakarin J. Sci. Technol., 2005, 27(4) : 799-812

Methanol extracts, water extracts and volatile oils of the fresh rhizomes of *Alpinia galanga, Boesenbergia pandurata, Curcuma longa, Kaempferia galanga* and *Zingiber officinale* have been assessed for free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and cytotoxic activity against MCF7 (breast adenocarcinoma) and LS174T (colon adenocarcinoma) cell lines. Methanol extract of *C. longa* exhibited the most pronounced radical scavenging activity with an EC_{50} value of 9.7 μg/ml, whereas the water extracts and volatile oils showed weak activity. All volatile oils and the methanol extract of *C. longa* showed strong activity against MCF7 and LS174T with IC_{50} less than 50 μg/ml. The oils of *A. galanga* (AGV), *B. pandurata* (BPV), *C. longa* (CLV), *K. galanga* (KGV) and *Z. officinale* (ZOV) were analyzed by GC/MS. *Trans*-3-acetoxy-1,8-cineole, camphor, ar-turmerone, ethyl cinnamate and geranial (*E*-citral) were detected as main compounds in AGV, BPV, CLV, KGV and ZOV, respectively. The novel compound, *p*-coumaryl-9-methyl ether, was isolated from methanol extract of *A. galanga*. ar-Turmerone, curcumin, demethoxycurcumin and bisdemethoxycurcumin were isolated from the methanol extract of *C. longa* while 6-shogaol, 6-dehydrogingerdione (or 1-dehydrogingerdione) and 6-gingerol were isolated from the methanol

[1]M.Pharm. Student in Pharmaceutical Sciences, [2]Ph.D.(Chemistry), Asst. Prof., [3]Ph.D.(Pharmacognosy), Asst. Prof., Department of Pharmacognosy and Pharmaceutical Botony, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla 90112 Thailand.
Corresponding e-mail: niwat.k@psu.ac.th
Received, 27 August 2004 Accepted, 14 November 2004

extract of *Z. officinale*. Curcumin was the most potent compound for free radical scavenging activity with an EC_{50} value of 2.0 µg/ml. Demethoxycurcumin was found to be the most active compound against LS174T with an IC_{50} value of 0.8 µg/ml and 6-shogaol was the most potent compound against MCF7 with an IC_{50} value of 1.7 µg/ml.

Key words : *p*-coumaryl-9-methyl ether, ar-turmerone, curcuminoids, gingerols, volatile oil, *Zingiberaceae, Alpinia galanga, Boesenbergia pandurata, Curcuma longa, Kaempferia galanga, Zingiber officinale*, cytotoxic, tumour cells, free radical

บทคัดย่อ

สาริกา แซ่อึ้ง อนุชิต พลับรู้การ และ นิวัติ แก้วประดับ

ฤทธิ์ต้านเซลล์มะเร็งและต้านอนุมูลอิสระของเหง้าพืชวงศ์ขิง

ว. สงขลานครินทร์ วทท. 2548 27(4) : 799-812

เมื่อนำสารสกัดเมธานอล สารสกัดน้ำ และน้ำมันหอมระเหยจากเหง้าสดของพืชวงศ์ขิงที่ใช้เป็นเครื่องเทศ 5 ชนิดคือ ข่า (*Alpinia galanga*) กระชาย (*Boesenbergia pandurata*) ขมิ้นชัน (*Curcuma longa*) เปราะหอม (*Kaempferia galanga*) และขิง (*Zingiber officinale*) มาทดสอบฤทธิ์ต้านอนุมูลอิสระด้วยวิธี DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay และฤทธิ์ต้านเซลล์มะเร็งเต้านม MCF7 และมะเร็งลำไส้ LS174T พบว่าสารสกัดเมธานอลจากเหง้าขมิ้นชันมีฤทธิ์ต้านอนุมูลอิสระดีที่สุดโดยมีค่าความเข้มข้นที่กำจัดอนุมูลอิสระได้ 50% (EC_{50}) เป็น 9.7 ไมโครกรัม/มล. ในขณะที่สารสกัดน้ำและน้ำมันหอมระเหยมีฤทธิ์ต้านอนุมูลอิสระต่ำมาก ส่วนการทดสอบฤทธิ์ต้านเซลล์มะเร็ง พบว่าน้ำมันหอมระเหยจากเหง้าพืชทั้ง 5 ชนิด และสารสกัดเมธานอลจากเหง้าขมิ้นชันมีฤทธิ์ดี โดยมีค่าความเข้มข้นที่ยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง 50% (IC_{50}) น้อยกว่า 50 ไมโครกรัม/มล. จากการวิเคราะห์องค์ประกอบทางเคมีด้วย GC/MS พบว่าสารสำคัญที่มีปริมาณมากที่สุดในน้ำมันหอมระเหยจากเหง้าข่า กระชาย ขมิ้นชัน เปราะหอม และขิง คือ *trans*-3-acetoxy-1,8-cineole, camphor, ar-turmerone, ethyl cinnamate และ geranial (*E*-citral) ตามลำดับ และแยกได้สารใหม่ 1 ชนิดคือ *p*-coumaryl-9-methyl ether จากสารสกัดเมธานอลของเหง้าข่า ส่วนสารสกัดเมธานอลของเหง้าขมิ้นชันแยกสารได้ 4 ชนิดคือ ar-turmerone, curcumin, demethoxycurcumin และ bisdemethoxycurcumin และจากสารสกัดเมธานอลของเหง้าขิงแยกสารได้ 3 ชนิดคือ 6-shogaol, 6-dehydrogingerdione (หรือ 1-dehydrogingerdione) และ 6-gingerol จากการทดสอบพบว่า curcumin มีฤทธิ์ต้านอนุมูลอิสระดีที่สุดโดยมีค่า EC_{50} เป็น 2.0 ไมโครกรัม/มล. ส่วน demethoxycurcumin มีฤทธิ์ต้านเซลล์มะเร็งลำไส้ LS174T ดีที่สุดโดยมีค่า IC_{50} เท่ากับ 0.8 ไมโครกรัม/มล. ในขณะที่ 6-shogaol มีฤทธิ์ต้านเซลล์มะเร็งเต้านม MCF7 ดีที่สุดโดยมีค่า IC_{50} เท่ากับ 1.7 ไมโครกรัม/มล.

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Cancer is perhaps the most progressive and devastating disease posing a threat of mortality to the entire world despite significant advances in medical technology for its diagnosis and treatment. All cells are exposed to oxidative stress, and thus oxidation, and free radicals may be important in carcinogenesis at multiple tumour sites (Sies, 1997). Phytochemical and dietary antioxidants are known to decrease the risk of many chronic diseases such as cancer and cardiovascular disorders. The antioxidant activity may be a result of one of the following: specific scavenging of reactive free radicals, scavenging of oxygen-containing compounds such as hydrogen peroxide, or chelation to metals (Priyadarsini, 1997). The Zingiberaceae is a well-known plant family in Southeast Asia

and many of its species are being used in traditional medicine, which is found to be effective in the treatment of several diseases. The five Zingiberaceous plants, *Alpinia galanga* (Greater galanga), *Boesenbergia pandurata* (Fingerroot), *Curcuma longa* (Turmeric), *Kaempferia galanga* (Proh hom) and *Zingiber officinale* (Ginger), were investigated as they are perennial herbs widely cultivated in Thailand and tropical regions of Asia, and have been commonly used as medicinal plants and spices in Thailand. The rhizomes of these five plants possess diverse biological activities, for instance, antimicrobial (Yamada *et al*., 1992; Hiserodt *et al*., 1998), antiulcer (Al-Yahya *et al*., 1990; Matsuda *et al*., 2003), antiinflammatory (Araujo and Leon, 2001), antioxidant (Selvam *et al*., 1995), cytotoxic and antitumour (Itokawa *et al*., 1987; Murakami *et al*., 1995, 2000; Pal *et al*., 2001), vasorelaxant (Othman *et al*., 2002), antispasmodic (Ammon and Wahl, 1991), antihepatotoxic (Hikino *et al*., 1985) and antidepressant activities (Noro *et al*., 1983; Yu *et al*., 2002). Although there have been many reports concerning chemical constituents and some biological activities of these five species, only a few reports focused on cytotoxic activity against human tumour cells and antioxidative activity against free radicals. Plant materials used in the previous studies were mainly crude extracts except for some curcuminoids from *C. longa* and some gingerols from *Z. officinale* (Hikino *et al*., 1985; Hiserodt *et al*., 1998; Pal *et al*., 2001). Besides, pure compounds responsible for antioxidant activity have not yet been identified from *A. galanga, B. pandurata* and *K. galanga*. Therefore, further study on antioxidant and cytotoxic activities of the extracts and volatile oils, and isolation of the compounds responsible for these two activities would provide additional useful data on biological activities of these five plants.

Materials and Methods

Instrumentation

Optical rotations were measured on a Polax-L polarimeter (sodium D-line 589 nm). Spectronic Genesys 5 UV spectrometer was used for measuring the absorbance in DPPH assay. The Power Wave X plate reader (Bio-TEK Instruments Inc.) was used for measuring absorbance (OD) in cytotoxic assay. IR spectra were recorded on a Jasco IR-810 spectrometer (KBr). UV spectra were obtained from a Hewlett Packard 8452A diode array spectrometer (scanning mode). ^{1}H- and ^{13}C-NMR spectra were recorded at 500/125 MHz on a Varian Inova 500 NMR spectrometer. Chemical shifts (δ) were reported in ppm scale (J in Hz), using either TMS or operating solvent as internal standards. EIMS data were recorded on a Hewlett-Packard HP 5890 Series II Plus GC-HP 5972 Mass Selective Detector (EI mode with mass range of 35-700 amu). FABMS data were recorded by a MAT 95 XL mass spectrometer (solid probe). Analysis of the volatile oils was carried out by GC/MS with a Hewlett-Packard HP5890 Series II Plus GC-HP 5972 Mass Selective Detector. The operating conditions were as follows: inlet temperature 250ºC, initial temperature 70ºC, detector temperature 280ºC and final temperature 280ºC (hold for 5 min). It was performed with column HP-5 (length 30 m, film thickness 0.25 μm and internal diameter 0.25 mm). Carrier gas was ultra high purity helium (UHP He).

Chromatography

Analytical TLC was performed on precoated plates of silica gel 60 F_{254} (Merck, 0.2 mm thick). The zones were detected under UV at 254 nm and by spraying with anisaldehyde reagent. Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck, 0.5 mm thick), activated at 105ºC for 30 min before use. Silica gel 60 (Merck, 0.040-0.063 mm) was used for vacuum liquid chromatography (VLC) and column chromatography (CC). Sephadex® LH-20 of Amersham Pharmacia Biotech AB (Sweden) was used for size-exclusion chromatography, and was suspended in methanol overnight before use.

Assay for free radical scavenging activity

The following procedure was modified from those described by Blois (1958) and Yamasaki *et*

al. (1994). Test samples were dissolved in absolute ethanol and diluted for at least 5 concentrations (two-fold dilutions). Each concentration was tested in triplicate. A portion of sample solution (500 µl) was mixed with an equal volume of $6x10^{-5}$ M DPPH (in absolute ethanol) and allowed to stand at room temperature for 20 min. The absorbance (A) was then measured at 520 nm. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage of inhibition, [% inhibition = $(A_{control} - A_{sample})/A_{control}$ x100]. The EC_{50} value (effective concentration of sample required to scavenge DPPH radical by 50%) was obtained by linear regression analysis of dose-response curve plotting between % inhibition and concentrations.

Assay for cytotoxic activity

The human colon adenocarcinoma LS174T and the human breast adenocarcinoma MCF7 cell lines were cultured in Minimum Essential Media (MEM) with Earle's salt, supplemented with 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, 50 IU/ml penicillin G sodium, 50 µg/ml streptomycin sulphate and 0.125 µg/ml amphotericin B. The cells were maintained at 37ºC in a 5% CO_2 atmosphere with 95% humidity. According to growth profiles, the optimal plating density of the cell lines MCF7 and LS174T were determined to be 2000 and 1000 cells/well, respectively, to ensure the exponential growth throughout the experimental period and to ensure a linear relationship between absorbance and cell number when analyzed by SRB assay (Skehan *et al*., 1990). The cytotoxic activity was assessed according to the protocol previously described elsewhere (Keawpradub *et al*., 1997). In brief, the tumour cells were seeded in 96-well plates and incubated to allow for cell attachment (18-24 hr). Cell viability (% survival) after exposure to test samples (serial dilutions) was determined colorimetrically (SRB assay) at 492 nm. Cell survival of the treated wells was measured as the percentage of absorbance compared to the control wells (non-treated cells, taken as 100% survival). The IC_{50} value (effective concentration of sample required to inhibit cell growth by 50%) was calculated from dose-response curves plotting between % inhibition and concentrations.

Plant materials and extraction

Fresh rhizomes of *A. galanga* (L.) Willd., *B. pandurata* (Roxb.) Schltr., *C. longa* L., *K. galanga* L. and *Z. officinale* Rosc. were purchased from a local market in Songkhla in April, 2001. They were identified by Dr.Niwat Keawpradub. The herbarium specimens have been kept at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The cleaned fresh rhizomes were cut into small pieces and divided into two portions. The first portion (1 kg of each plant) was subjected to water distillation for 3 hr. After allowing the system to cool down, the volatile oil of each plant was collected. The yields for *A. galanga, B. pandurata, C. longa, K. galanga* and *Z. officinale* were 0.7 (0.07%), 0.9 (0.09%), 0.6 (0.06%), 0.7 (0.07%) and 0.7 (0.07%) g, respectively. The remaining water in the distillation flask was collected and evaporated to dryness on a water bath (60ºC) to obtain water extract of each plant. The yields for *A. galanga, B. pandurata, C. longa, K. galanga* and *Z. officinale* were 44.2 (4.4%), 24.9 (2.5%), 18.0 (1.8%), 12.7 (1.3%) and 19.2 (1.9%) g, respectively. The second portion of each plant was blended with methanol, soaked for 72 hr, filtered and the marc was then extracted twice with methanol. The filtrates were combined and evaporated to dryness under reduced pressure to yield methanol extract of each plant. The yields for *A. galanga* (2.2 kg of plant material), *B. pandurata* (3.6 kg) *C. longa* (1.6 kg), *K. galanga* (3.1 kg) and *Z. officinale* (4.4 kg) were 113.0 (5.1%), 59.8 (1.7%), 67.2 (4.2%), 61.1 (2.0%) and 115.1 (2.6%) g, respectively.

Isolation of the active extracts

According to the results of preliminary activity assay (Table 1), the active extracts were subjected to chemical investigation as follows:

1. Isolation of methanol extract of *A. galanga* (AGM)

Table 1. Antioxidant and cytotoxic activities of the volatile oils and extracts from *A. galanga, B. pandurata, C. longa, K. galanga* and *Z. officinale*.

Plants	Volatile oils / Extracts	Antioxidant against DPPH (% inhibition ± SD at 100 μg/ml, n=3)	Cytotoxic against LS174T cells (% survival ± SD at 100 μg/ml, n=6)	Cytotoxic against MCF7 cells (% survival ± SD at 100 μg/ml, n=6)
A. galanga	AGV	2.7±1.3	7.5±1.6 (IC_{50} = 47.8±6.0, N=2)	4.8±0.6 (IC_{50} = 30.5±6.8, N=2)
	AGM	92.5±1.3 (EC_{50} = 57.0±3.7, n=6)	45.6±10.9	95.2±11.6
	AGW	18.7±1.7	51.5±19.0	59.8±7.3
B. pandurata	BPV	5.4±1.2	0.6±0.7 (IC_{50} = 12.0±1.6, N=2)	5.2±0.5 (IC_{50} = 31.7±5.4, N=2)
	BPM	47.4±3.5	20.6±7.7	89.9±2.4
	BPW	11.3±1.6	92.6±14.3	79.4±6.0
C. longa	CLV	6.5±1.1	0.9±0.7 (IC_{50} = 20.3±1.4, N=2)	5.2±0.9 (IC_{50} = 20.9±0.0, N=2)
	CLM	91.6±0.2 (EC_{50} = 9.7±0.3, n=6)	0.0±0.0 (IC_{50} = 6.4±1.6, N=2)	1.3±0.4 (IC_{50} = 14.2±2.1, N=2)
	CLW	49.2±0.0	97.2±18.9	66.4±7.1
K. galanga	KGV	2.2±1.8	0.9±0.6 (IC_{50} = 15.9±1.1, N=2)	4.3±0.9 (IC_{50} = 15.4±4.4, N=2)
	KGM	34.0±3.8	88.4±3.8	73.0±2.3
	KGW	33.5±0.6	52.4±13.2	76.6±14.7
Z. officinale	ZOV	4.1±0.8	0.4±0.3 (IC_{50} = 15.9±0.7, N=2)	2.3±0.6 (IC_{50} = 14.2±1.9, N=2)
	ZOM	86.6±0.0 (EC_{50} = 35.6±1.0, n=6)	12.0±6.3 (IC_{50} = 80.0±13.3, N=3)	41.0±7.3 (IC_{50} = 75.0±10.5, N=3)
	ZOW	61.5±0.4	97.2±29.2	94.3±11.7

n = number of samples tested.
N = number of independent experiments (6 replicates in each experiment).

An aliquot of AGM (50 g) was suspended in $CHCl_3$-MeOH (9:1) overnight. It was sonicated for 10 min and filtered. The filtrate was evaporated to afford 4 g of dark brown oily gum, which was subjected to silica gel column chromatography using gradients of solvents $CHCl_3$-MeOH (19:1, 9:1, 4:1) and MeOH to obtain 39 fractions (50 ml each). Fractions 9-12 were combined to obtain green-yellow oil (0.666 g). Further repeated separation by preparative TLC on silica gel plates using $CHCl_3$-MeOH (19:1) as a mobile phase afforded compound **1** as a yellow oil (0.039 g).

2. Isolation of methanol extract of *C. longa* (CLM)

An aliquot of CLM (30 g) was subjected to silica gel column chromatography using gradients of solvents $CHCl_3$-MeOH (19:1, 9:1, 4:1) and MeOH to obtain 75 fractions (50 ml each). Fractions 1-20 gave compound **2** as pale yellow oil (2.137 g). Fractions 24-27 were obtained as dark orange liquid (1.546 g). Upon separation on silica gel column chromatography using gradients of solvents $CHCl_3$-MeOH (19:1, 9:1, 4:1), 39 fractions (50 ml each, 1a-39a) were obtained. Fractions 13a-15a were reseparated by preparative TLC on silica gel plates using $CHCl_3$-MeOH (19:1) as a mobile phase to afford compound **3** as orange crystals (0.161 g) and compound **4** as reddish

orange powder (0.167 g). Fractions 28-53 were separated by repeated preparative TLC on silica gel plates with $CHCl_3$-MeOH (19:1) and acetone-$CHCl_3$-MeOH (10:9.5:0.5) as mobile phases to afford compound **5** as a reddish orange powder (0.002 g).

3. Isolation of methanol extract of *Z. officinale* (ZOM)

An aliquot of ZOM (50 g) was subjected to silica gel vacuum liquid chromatography using $CHCl_3$-MeOH (9:1 and 4:1) and MeOH (1000 ml each) as eluting solvents to obtain 3 fractions, respectively. Fraction 1 ($CHCl_3$-MeOH; 9:1) was separated by preparative TLC on silica gel plates with n-hexane-EtOAc (3:1) as a mobile phase to obtain 5 bands. Band 3 was subjected to Sephadex® LH-20 column chromatography eluting with MeOH to afford 16 fractions (1a-16a). Fractions 8a-10a were further separated by preparative TLC on silica gel plates with *n*-hexane-EtOAc (5:1) as a mobile phase to yield compound **6** as a yellow oil (0.042 g). Fraction 13a, upon standing overnight at room temperature, gave compound **7** as yellow crystals (0.032 g). Band 4 of fraction 1 was separated by Sephadex® LH-20 column eluting with MeOH to obtain 14 fractions (1b-14b). Fractions 7b-8b were separated by preparative TLC on silica gel plates using n-hexane-EtOAc (3:1) as a mobile phase to afford compound **8** as yellow oil (0.078 g).

Physical properties and spectral data of the isolated compounds (1-8)

Compound **1** (*p*-coumaryl-9-methyl ether, $C_{10}H_{12}O_2$): yellow oil; UV (MeOH) λ_{max} nm (log ε): 264 (4.01); IR ν_{max} (KBr) cm^{-1}: 3350, 2920, 1610, 1520; HR-FABMS *m*/*z*: 164.0840 (calc. for $C_{10}H_{12}O_2$: 164.0837); EIMS *m*/*z* (% rel. int.): 165 (11), 164 $[M]^+$ (68), 163 (19), 137 (18), 131 (100); ^1H and ^{13}C NMR (500/125 MHz, $CDCl_3$): Table 3.

Compounds **2-8** were identified by comparison of their spectral data, particularly NMR and mass spectra, with the published data. The physical properties and spectral data of **2-8** obtained from the present work are available upon request.

Results and Discussion

Chemical analysis of the volatile oils

Upon GC/MS analysis (Table 2), the volatile oil of *A. galanga* (AGV) was found to be composed of at least 13 compounds, 7 of which were identified, having *trans*-3-acetoxy-1,8-cineole (% area 58.5) as a major component. This is similar to the previous data reported by Kubota *et al*. (1998). However, De Pooter *et al*. (1985) reported that 1,8-cineole was a major component in the volatile oil of *A. galanga*. The oil of *B. pandurata* (BPV) contained at least 4 compounds including camphor (% area 81.4), a major constituent. This is in accordance with the results reported by Jantan *et al*. (2001) in which the major constituents were detected as camphor (16.1-32.1%), geraniol (16.2-26.0%) and (*E*)-β-ocimene (19.0-23.7%). The oil of *C. longa* (CLV) was composed of at least 22 compounds, 7 of which were identified, characterized by a high proportion of ar-turmerone (% area 38.0) and β-turmerone (% area 11.4). This is in good agreement with previous reports, in which ar-turmerone was identified as a major component (Gopalan *et al*., 2000; Martins *et al*., 2001). Analysis of the oil of *K. galanga* (KGV) revealed the existence of at least 12 compounds (7 of which were identified), in which the main constituent was identified as ethyl cinnamate (% area 61.8). The volatile oil of *Z. officinale* (ZOV) was found to contain at least 13 compounds, 8 of which were identified, with geranial (% area 33.0) and neral (% area 26.6) being the main compounds. This is in accordance with the results previously reported by Sakamura (1987), in which the volatile oil from the fresh rhizome of *Z. officinale* was characterized by the presence of acyclic oxygenated monoterpenes mainly composed of geranial (*E*-citral) and neral (*Z*-citral).

Chemical structure characterization of the isolated compounds (1-8)

Compound **1** was isolated from the methanol extract of *A. galanga* (AGM). The IR spectrum showed the existence of hydroxyl function at

Table 2. Chemical constituents identified in the volatile oils (GC/MS analysis).

Volatile oil	Compound identified	% Area	Retention time (min)
A. galanga (AGV)	terpinene-4-ol	11.6	3.7
	1-α-terpineol	5.5	3.9
	4-allylphenol	6.9	4.7
	4-allylphenyl acetate	11.4	6.1
	trans-3-acetoxy-1,8-cineole	58.5	6.2
	trans-methyl isoeugenol	< 5	7.0
	α-humulene	< 5	8.1
B. pandurata (BPV)	camphor	81.4	3.2
	geraniol	6.0	4.8
	geranial (*E*-citral)	5.4	4.9
	methyl cinnamate	7.2	6.6
C. longa (CLV)	terpinene-4-ol	2.1	3.7
	1-methyl-4-(2-propanol-2-yl)-cyclohexene	2.1	3.8
	ar-curcumene	2.0	8.5
	β-sesquiphellandrene	1.5	9.2
	ar-turmerone	38.0	11.1
	α-turmerone	7.4	11.2
	β-turmerone	11.4	11.7
K. galanga (KGV)	*l*-borneol	7.6	3.5
	p-cymen-8-ol	1.1	3.6
	terpinene-4-ol	2.0	3.7
	β-fenchyl alcohol	1.2	3.8
	ethyl cinnamate	61.8	8.1
	pentadecane	2.6	9.1
	3-(4-methoxyphenyl)-2-propenoic acid ethyl ester	18.3	12.3
Z. officinale (ZOV)	citronellal	1.9	3.3
	l-borneol	4.3	3.5
	cryptone	3.2	3.6
	1-α-terpineol	2.8	3.9
	neral (*Z*-citral)	26.6	4.5
	geranial (*E*-citral)	33.0	5.0
	2-undecanone	2.0	5.4
	ar-curcumene	3.1	8.5

3350, C-H at 2920 and olefinic/aromatic functions at 1610 and 1520 cm^{-1}. The molecular formula of **1** was $C_{10}H_{12}O_2$ (MW = 164.0840; D.B.E. = 5) as deduced from the HR-FABMS spectrum. The ^{13}C NMR spectrum revealed 10 carbons, 8 of which corresponded with 11 protons as observed from HMQC spectrum. In the ^{1}H NMR spectrum of 1, the signal of a methoxyl group was detected at δ 3.41, and a pair of doublets at δ 6.78 and δ 7.25 both with the same coupling constant (J = 9.0 Hz) indicated a para substituted benzene ring. The upfield shift of proton signal at δ 6.78 (δ_C 115.6) is due to the shielding effect of the oxygenated substitution group at C-4. This leads to the

assignment of two quaternary carbons at δ 129.3 and 155.8 to be C-1 and C-4, respectively. Two signals were observed at δ 6.55 (dt, *J* = 16.0, 1.5 Hz) and δ 6.13 (dt, *J* = 16.0, 6.0 Hz), which are characteristics of trans olefinic protons and subsequently attributed to H-7 and H-8, respectively. Their corresponding carbons (C-7 and C-8) were respectively observed at δ 132.8 and 123.1 in the HMQC spectrum. In addition, H-8 and H-9 showed vicinal coupling with J value of 6.0 Hz, suggesting the presence of a propenoid side chain (C-7, C-8 and C-9). The signal of methylene carbon (C-9) at 73.4 suggested that it is an oxygen-bearing carbon. This was confirmed by analysis of the nuclear Overhauser effect (nOe) difference spectra. Upon irradiation of the signal at δ 3.41 (methoxyl function) there was strong enhancement of the signal at δ 4.10 (H-9) while no enhancement was observed on the aromatic protons, indicating that the methylene function (-CH_2-) should directly connect to the methoxyl function. This leads to the allocation of the methoxyl function at C-9 and the hydroxyl group at C-4. These spectral features suggest that **1** is a derivative of coumaric acid, which has been modified through reduction and methylation in shikimate pathway in the plant. Thus **1** was characterized as a phenylpropanoid and named to be *p*-coumaryl-9-methyl ether. This is the first report of naturally occurring *p*-coumaryl-9-methyl ether with complete ^{1}H and ^{13}C NMR assignments (Table 3).

Compounds **2-5** were isolated from the methanol extract of *C. longa* (CLM). The EIMS, ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC spectra of **2** were consistent with those of the sesquiterpene ar-turmerone previously isolated from the rhizome of *C. longa* (Ferreira *et al.,* 1992). However, the published chemical shift assignments of C-5 (δ 154.6) and C-11 (δ 143.5) in the previous work should be reassigned the other way round (C-11 should be more downfield than C-5) according to the data obtained in the present work. Analysis of the FABMS, ^{1}H and ^{13}C NMR spectra of compounds **3-5** gave evidence that they should be diarylheptanoids. Compounds **3**, one of the major constituents of CLM, was identified as curcumin or 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Kosuge *et al*., 1985; Uehara *et al*., 1987; Masuda *et al*., 1992). Compounds **4** and **5** were identified as demethoxycurcumin or 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione, and bisdemethoxycurcumin or 1,7-bis (4-hydroxyphenyl)-1,6-heptadiene-3,5-dione, respectively (Kosuge *et al*., 1985; Masuda *et al*., 1992). Compounds **6-8** were isolated from the methanol extract of *Z. officinale* (ZOM). Analysis of the FABMS, ^{1}H and ^{13}C NMR spectra of **6-8** indicated that they should be gingerols, the major components of *Z. officinale* rhizome. Compounds **6, 7** and **8** were characterized to be 6-shogaol or 1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one (Chen *et al*., 1986),

Table 3. ^{1}H and ^{13}C NMR assignments of compound 1 (*p*-coumaryl-9-methyl ether) [δ, ppm, 500/125 MHz, in $CDCl_3$]

Position	δ_H (ppm, Multiplicity, *J* in Hz)	δ_C (ppm)
1	-	129.3
2, 6	7.25, 2H, d, 9.0 Hz	127.9
3, 5	6.78, 2H, d, 9.0 Hz	115.6
4	-	155.8
7	6.55, 1H, dt, 16.0, 1.5 Hz	132.8
8	6.13, 1H, dt, 16.0, 6.0 Hz	123.1
9	4.10, 2H, dd, 6.0, 1.5 Hz	73.4
OCH_3	3.41, 3H, s	57.8

6-dehydrogingerdione or 1-dehydrogingerdione, (Kiuchi *et al*., 1982; Charles *et al*., 2000), and 6-gingerol (Shoji *et al*., 1982; Yamada *et al*., 1992), respectively.

Activity of the extracts and volatile oils

The results depicted in Table 1 reveal that the methanol extracts of the fresh rhizomes of *A. galanga* (AGM), *C. longa* (CLM) and *Z. officinale* (ZOM) possess strong antioxidative activity against the DPPH radical with % inhibition in the range of 86.6-92.5%. CLM was the most active extract with an EC_{50} value of 9.7 μg/ml. On the contrary, the five volatile oils showed very weak activity (percent inhibition less than 7%). In general, the methanol extract was more active against the DPPH radical than the corresponding water extract and volatile oil of each rhizome. CLM showed pronounced cytotoxic activity against the two cell lines with percent survival in the range of 0-1.3% (at 100 μg/ml). In contrast, the water extracts of the five plants exhibited slight cytotoxic activity. CLM was found to be the most active extract against the tumour cells LS174T and MCF7 with IC_{50} values of 6.4 and 14.2 μg/ml, respectively. All volatile oils (at 100 μg/ml) were capable of inhibiting proliferation of the two cell lines with percent survival in the range of 0.4-7.5% (IC_{50} ranging from 12.0-47.8 μg/ml). Remarkably, the volatile oils and methanol extracts showed tendencies to be more cytotoxic against LS174T rather than MCF7. It is notable that volatile oils of the five rhizomes were mainly composed of monoterpenes, sesquiterpenes and phenylpropanoids (Table 2). These compounds could be responsible for the observed cytotoxic activity of the volatile oils. For example, the growth of V-79 cells (lung fibroblasts of Chinese hamster) was completely inhibited on treatment with camphor (detected in BPV) at 0.3% for 24-48 hr (Toshihiko, 1987). Geraniol (at 400 μM), one of the minor compounds of BPV, inhibited the growth of Caco-2 (human colon cancer cells) by 70% (Carnesecchi *et al*., 2001). This should be an explanation for the strong cytotoxic activity of BPV against LS174T cells with an IC_{50} value of 12.0 μg/ml (Table 1). The sesquiterpene β-sesquiphellandrene, one of the minor compounds detected in CLV, was reported to be cytotoxic against mouse lymphocytic leukaemia cells L1210 (Ahn and Lee, 1989). Citral, one of the major compounds of ZOV, was found to be cytotoxic against P388 mouse leukaemia cells (Dubey *et al*., 1997). These previous studies give support to the pronounced cytotoxic activity against tumour cells of the five volatile oils observed in the present work. It is obvious that the volatile oils account for one of the cytotoxic constituents against tumour cells of the five rhizomes, which is of interest for medicinal purposes.

Activity of the isolated compounds

The eight isolated compounds (**1-8**) were assessed for free radical scavenging activity against DPPH radical and cytotoxic activity against the tumour cell lines LS174T and MCF7. The chemical structures are shown in Figure 1 and the results are depicted in Table 4. BHT and caffeic acid were used as positive standards for antioxidative assay while vinblastine sulphate and the cytotoxic alkaloid berberine were used as standard drugs in the cytotoxicity assay. Strong radical scavenging activity was exhibited by **3, 4, 6, 7** and **8** with EC_{50} values ranging from 2.0-4.7 μg/ml. This was about 2-4 times more active than BHT, and about 2-8 times less active than caffeic acid. Compounds **1** and **5** were found to possess moderate activity with EC_{50} of 73.9 and 40.9 μg/ml, respectively. The results obtained from the present work are in good agreement with those reported by Rao (1996), in which the antioxidative activity of the curcuminoids from turmeric is in the following order: curcumin > demethoxycurcumin > bis-demethoxycurcumin. The strong antioxidant activity of **3** could be due to its capability to stabilize the two aroxyl radicals, which occurred after donating hydrogen atoms of the hydroxyl functions to DPPH radicals, through the aromatic system and the conjugated double bonds. However, the two phenolic moieties of **5** are more exchangeable than those of **3**, suggesting some degree of reversibility of reaction between **5** and DPPH radicals, hence

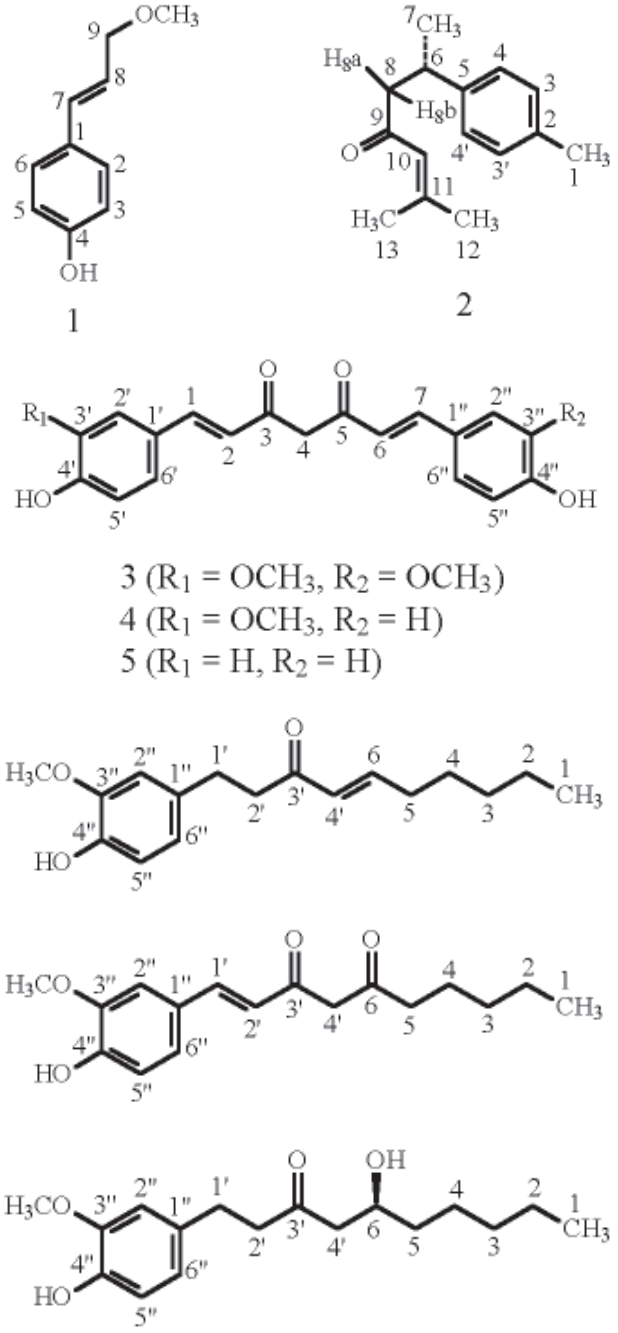

Figure 1. Chemical structures of the isolated compounds (1-8).

being less active. Furthermore, it has been reported that the antioxidant mechanism of **3** in polyunsaturated lipid (ethyl linoleate) was proposed to be an oxidative coupling reaction at the phenolic moiety of the curcumin with the peroxyl radical (Masuda *et al*., 2001). In general, these three curcuminoids (**3-5**) are regarded as main antioxidative constituents of *C. longa* rhizome. Radical scavenging activity of the three gingerol derivatives (**6-8**) could be attributed to the hydroxyl

Table 4. EC_{50} values against DPPH radical and IC_{50} values against LS174T and MCF7 cell lines of the isolated compounds from *A. galanga, C. longa* and *Z. officinale*.

Compounds	EC_{50} against DPPH (μg/ml, mean±SD)	IC_{50} against LS174T (μg/ml, mean±SEM)	IC_{50} against MCF7 (μg/ml, mean±SEM)
1 (p-coumaryl-9-methyl ether)	73.9±2.1 (n=6) (450.3±13.1 μM)	7.5±0.7 (N=2) (45.8±4.3 μM)	7.4±0.3 (N=2) (44.8±2.1 μM)
2 (ar-turmerone)	> 100 (n=6) (> 463.0 μM)	19.3±0.6 (N=2) (89.6±3.0 μM)	14.6±2.1 (N=2) (67.8±13.4 μM)
3 (curcumin)	2.0±0.2 (n=6) (5.4±0.6 μM)	5.9±0.6 (N=2) (16.2±1.8 μM)	8.3±1.4 (N=2) (22.7±5.2 μM)
4 (demethoxycurcumin)	2.8±0.1 (n=6) (8.4±0.4 μM)	0.8±0.0 (N=2) (2.3±0.0 μM)	2.8±0.3 (N=2) (8.5±1.1 μM)
5 (bisdemethoxycurcumin)	40.9±4.8 (n=6) (132.4±16.2 μM)	*	*
6 (6-shogaol)	4.0±0.1 (n=6) (14.5±0.5 μM)	1.2±0.1 (N=2) (4.2±0.2 μM)	1.7±0.1 (N=2) (6.0±0.8 μM)
7 (6-dehydrogingerdione) (or 1-dehydrogingerdione)	4.7±0.1 (n=6) (16.2±0.3 μM)	11.3±3.2 (N=2) (39.2±11.0 μM)	13.9±0.9 (N=2) (47.8±3.2 μM)
8 (6-gingerol)	4.4±0.1 (n=6) (14.8±0.4 μM)	30.6±7.5 (N=2) (104.1±25.5 μM)	31.6±0.3 (N=2) (107.5±1.4 μM)
BHT (positive standard)	8.2±0.2 (n=6) (37.3±0.9 μM)	N/A	N/A
Caffeic acid (positive standard)	0.9±0.1 (n=6) (5.2±0.3 μM)	N/A	N/A
Berberine (positive standard)	N/A	0.8±0.0 (N=2) (2.4±0.0 μM)	0.6±0.0 (N=2) (1.8±0.0 μM)
Vinblastine sulphate (positive standard)	N/A	0.011±0.004 nM (N=2)	0.008±0.001 nM (N=2)

n = number of samples tested.
N = number of independent experiments (6 replicates in each experiment).
*** = Only 2 mg of 5 was obtained, which was not sufficient for the cytotoxic test.**
BHT = butylated hydroxytoluene
N/A = test not done.

group of the 4-hydroxy-3-methoxyphenyl moiety, which is almost comparable to that of **4**. The present results give additional evidence to support the assumption that gingerols are responsible for antioxidant activity of *Z. officinale* rhizome (Sekiwa *et al*., 2000; Chung *et al*., 2001). The extract of *A. galanga* rhizome has been known to possess an antioxidative effect (Cheah and Gan, 2000) but the active constituents have not yet been identified. The radical scavenging activity against DPPH of **1** with an EC_{50} value of 73.9 μg/ml, although less active than its corresponding methanol extract (EC_{50} = 57.0 μg/ml), provides new evidence of active compound responsible for antioxidant activity of *A. galanga* rhizome.

Pronounced cytotoxic activity against the tumour cell lines were observed for **4** and **6** with IC_{50} values in the range of 0.8-2.8 μg/ml. These two compounds were considered to be significantly cytotoxic according to the criteria for cytotoxic activity of pure compounds established by the American National Cancer Institute (IC_{50} < 4 μg/ml) (Suffness and Pezzuto, 1991). Compounds **3** and **1** were slightly less active than **4** and **6** with IC_{50} values in the range of 5-10 μg/ml. The strong cytotoxic activity of **4** observed in the present

work is in accordance with the previous study of Simon *et al*. (1998) in which demethoxycurcumin showed stronger cytotoxic activity than curcumin and bisdemethoxycurcumin. According to the chemical structures, it is likely that the hydroxylated benzene ring and/or the α,β-unsaturated ketone moieties would be essential for cytotoxic activity of the isolated compounds. However, further study is needed to determine the exact essential structure and mechanism of action of these cytotoxic compounds. So far, curcumin was reported to possess strong inhibitory action on DNA and RNA synthesis of cultured Hela cells (Huang *et al*., 1997) and to be active through inhibition of telomerase activity in human breast cancer cells (Ramachandran *et al*., 2002). It is also of interest to note that, for each compound investigated in the present work, there was no significant cell-type selectivity. Under the same test conditions, the positive standard berberine showed cytotoxic activity with IC_{50} values in the same range as those of **4** and **6**. However, the anticancer drug vinblastine sulphate was found to be far more active than the isolated compounds. The eight isolated compounds were mainly responsible for the cytotoxic activity against LS174T and MCF7 tumour cell lines observed in their corresponding methanol extracts. The finding of **1** as a new cytotoxic compound in the present work provides additional evidence of the presence of antitumour principles in the rhizome of *A. galanga* of which 1′-acetoxychavicol acetate and 1′-acetoxyeugenol acetate were previously identified (Itokawa *et al*., 1987). The obtained results confirm the therapeutic potential against proliferation of tumour cells of the rhizomes of *A. galanga, C. longa* and *Z. officinale*. The present work suggest that the rhizomes of the five species commonly used as spices and medicinal plants in Thailand are potential sources of antioxidants (by acting as free radical scavengers) and/or cytotoxic agents against tumour cells. Further study in an animal model is strongly recommended in order to evaluate whether these plants are promising for clinical trials.

Acknowledgements

This work obtained financial support from the Shell Centennial Education Fund (The Shell Company of Thailand Limited) for students in Master's degree and Ph.D. programmes; Graduate School, Prince of Songkla University; Graduate Studies Related Research Fund (GSRRF) 2002, Prince of Songkla University. We thank The Scientific Equipment Center, Prince of Songkla University for NMR, MS and GC-MS measurements.

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