CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening of Biological Activity of Crude Extracts and Volatile Oils

The methanol extracts, water extracts and volatile oils of the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* were prepared as described in section 2.3. Yields and characteristics of the extracts and volatile oils were shown in Table 11. Each volatile oil has characteristic aroma with the yield of about 0.1w/w (fresh weight). The water extracts were obtained as gummy residues with mild odour while the methanol extracts were obtained as oily gums with strong odour.

Table 11 Yields and characteristics of the extracts and volatile oils from the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale*

Fresh rhizomes	**Weight (kg)**	**Extracts/volatile oils**	**Physical appearance**	**Weight (g)**	**% Yield (w/w)**
A. galanga	**1**	water extract **(AGW)**	yellowish brown gum	**44.2**	**4.4**
		volatile oil **(AGV)**	yellow oil	**0.7**	**0.1**
	2.2	methanol extract **(AGM)**	black oily gum	**113.0**	**5.1**
B. pandurata	**1**	water extract **(BPW)**	black gum	**24.9**	**2.5**
		volatile oil **(BPV)**	pale yellow oil	**0.9**	**0.1**
	3.6	methanol extract **(BPM)**	dark brown oily gum	**59.8**	**1.7**
C. longa	**1**	water extract **(CLW)**	black gum	**18.0**	**1.8**
		volatile oil **(CLV)**	pale yellow oil	**0.6**	**0.1**
	1.6	methanol extract **(CLM)**	blackish orange oily gum	**67.2**	**4.2**
K. galanga	**1**	water extract **(KGW)**	black gum	**12.7**	**1.3**
		volatile oil **(KGV)**	pale yellow oil	**0.7**	**0.1**
	3.1	methanol extract **(KGM)**	black oily gum	**61.1**	**2.0**
Z. officinale	**1**	water extract **(ZOW)**	blackish brown gum	**19.2**	**1.9**
		volatile oil **(ZOV)**	dark yellow oil	**0.7**	**0.1**
	4.4	methanol extract **(ZOM)**	black oily gum	**115.1**	**2.6**

3.1.1 Free radical scavenging activity

The antioxidative activity of the methanol extracts, water extracts and volatile oils of the five plants was evaluated by DPPH radical scavenging assay as described in section 2.5. The results were shown in Table 12.

Table 12 Percent inhibition of the extracts and volatile oils of the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* tested by DPPH radical scavenging assay (screening) at the final concentration of 100 µg/ml.

Fresh rhizomes	**Extracts/volatile oils**	**% Inhibition±SD**
	water extract **(AGW)**	**18.7±1.7 (n=3)**
A. galanga	volatile oil **(AGV)**	**2.7±1.3 (n=3)**
	methanol extract **(AGM)**	**92.5±1.3 (n=3)**
	water extract **(BPW)**	**11.3±1.6 (n=3)**
B. pandurata	volatile oil **(BPV)**	**5.4±1.2 (n=3)**
	methanol extract **(BPM)**	**47.4±3.5 (n=3)**
	water extract **(CLW)**	**49.2±0.0 (n=3)**
C. longa	volatile oil **(CLV)**	**6.5±1.1 (n=3)**
	methanol extract **(CLM)**	**91.6±0.2 (n=3)**
	water extract **(KGW)**	**33.5±0.6 (n=3)**
K. galanga	volatile oil **(KGV)**	**2.2±1.8 (n=3)**
	methanol extract **(KGM)**	**34.0±3.8 (n=3)**
	water extract **(ZOW)**	**61.5±0.4 (n=3)**
Z. officinale	volatile oil **(ZOV)**	**4.1±0.8 (n=3)**
	methanol extract **(ZOM)**	**86.6±0.0 (n=3)**

n = number of samples tested (triplicate)

The results depicted in Table 12 revealed that the methanol extracts of the fresh rhizomes of *A. galanga, C. longa* and *Z. officinale* possessed strong antioxidative activity against DPPH radical with % inhibition in the range of 86.6-92.5 %. On the contrary, the five volatile oils of the fresh rhizomes showed very weak activity (% inhibition less than 7

%). It is notable that in each rhizome the methanol extract was more active than the corresponding water extract and volatile oil. Subsequently, the methanol extracts of *A. galanga* (AGM), *C. longa* (CLM) and *Z. officinale* (ZOM) were further evaluated for EC_{50} values by DPPH radical scavenging assay as shown in Table 13.

Table 13 EC_{50} values of the methanol extracts of *A. galanga* (AGM), *C. longa* (CLM) and *Z. officinale* (ZOM) against DPPH radical.

Methanol extracts[a]	**EC_{50} (μg/ml) (mean$\pm$SD)**
AGM	**57.0$\pm$3.7 (n=6)**
CLM	**9.7$\pm$0.3 (n=6)**
ZOM	**35.6$\pm$1.0 (n=6)**
BHT (positive standard)	**8.2$\pm$0.2 (n=6)**
Caffeic acid (positive standard)	**0.9$\pm$0.05 (n=6)**

BHT = Butylated hydroxytoluene

[a] See the isolation procedure

n = number of samples tested (6 replicates)

The results of antioxidative activity using the DPPH assay of the methanol extracts of the three fresh rhizomes exhibited that the methanol extract of *C. longa* (CLM) was the most active extract with an EC_{50} value of 9.7 μg/ml.

3.1.2 Cytotoxic activity

The cytotoxic activity of the five rhizomes was evaluated by the Sulphorhodamine B (SRB) assay. The results of cytotoxic activity of the extracts and volatile oils (screening) were shown in Tables 14 and 15.

Table 14 Percent survival of LS174T tested with the extracts and volatile oils of the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* by SRB assay (screening) at final concentration of 100 μg/ml.

Fresh rhizomes	Extracts/volatile oils	% Survival±SD
A. galanga	water extract **(AGW)**	**51.5±19.0 (n=6)**
	volatile oil **(AGV)**	**7.5±1.6 (n=6)**
	methanol extract **(AGM)**	**45.6±10.9 (n=6)**
B. pandurata	water extract **(BPW)**	**92.6±14.3 (n=6)**
	volatile oil **(BPV)**	**0.6±0.7 (n=6)**
	methanol extract **(BPM)**	**20.6±7.7 (n=6)**
C. longa	water extract **(CLW)**	**97.2±18.9 (n=6)**
	volatile oil **(CLV)**	**0.9±0.7 (n=6)**
	methanol extract **(CLM)**	**0.0±0.0 (n=6)**
K. galanga	water extract **(KGW)**	**52.4±13.2 (n=6)**
	volatile oil **(KGV)**	**0.9±0.6 (n=6)**
	methanol extract **(KGM)**	**88.4±3.8 (n=6)**
Z. officinale	water extract **(ZOW)**	**97.2±29.2 (n=6)**
	volatile oil **(ZOV)**	**0.4±0.3 (n=6)**
	methanol extract **(ZOM)**	**12.0±6.3 (n=6)**

n = number of samples tested (6 replicates)

Table 15 Percent survival of MCF7 tested with the extracts and volatile oils of the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* by SRB assay (screening) at final concentration of 100 µg/ml

Fresh rhizomes	Extracts/volatile oils	% Survival±SD
A. galanga	water extract **(AGW)**	**59.8±7.3 (n=6)**
	volatile oil **(AGV)**	**4.8±0.6 (n=6)**
	methanol extract **(AGM)**	**95.2±11.6 (n=6)**
B. pandurata	water extract **(BPW)**	**79.4±6.0 (n=6)**
	volatile oil **(BPV)**	**5.2±0.5 (n=6)**
	methanol extract **(BPM)**	**89.9±2.4 (n=6)**
C. longa	water extract **(CLW)**	**66.4±7.1 (n=6)**
	volatile oil **(CLV)**	**5.2±0.9 (n=6)**
	methanol extract **(CLM)**	**1.3±0.4 (n=6)**
K. galanga	water extract **(KGW)**	**76.6±14.7 (n=6)**
	volatile oil **(KGV)**	**4.3±0.9 (n=6)**
	methanol extract **(KGM)**	**73.0±2.3 (n=6)**
Z. officinale	water extract **(ZOW)**	**94.3±11.7 (n=6)**
	volatile oil **(ZOV)**	**2.3±0.6 (n=6)**
	methanol extract **(ZOM)**	**41.0±7.3 (n=6)**

n = number of samples tested (6 replicates)

In Tables 14 and 15, the five volatile oils were found to be active against the cell lines LS174T and MCF7, which showed percent survival in the range of 0.4-7.5 %. The methanol extract of *C. longa* showed pronounced cytotoxic activity against the two cell lines with percent survival in the range of 0-1.3 % at the final concentration of 100 µg/ml.

On the other hand, the methanol extract of *Z. officinale* exhibited moderate cytotoxic activity with percent survival in the range of 12.0-41.0 %. In contrast, the water extracts of the five plants exhibited slight cytotoxic activity (% survival more than 50 %) on both cell lines at 100 μg/ml. It is interesting to note that the volatile oils and methanol extracts of the five plants showed tendency to be more cytotoxic against LS174T rather than MCF7.

So, the methanol extracts of *C. longa* (CLM), *Z. officinale* (ZOM) and the five volatile oils of fresh rhizomes were evaluated for IC_{50} values by Sulphorhodamine B (SRB) assay as shown in Table 16.

Table 16 IC_{50} values of the active methanol extracts and volatile oils tested against LS174T and MCF7 (mean±SEM) by Sulphorhodamine B (SRB) assay

Extracts/volatile oils[a]	**IC_{50} against LS174T (μg/ml)[b] (mean±SEM)**	**IC_{50} against MCF7 (μg/ml)[b] (mean±SEM)**
AGV	**47.8±6.0 (N =2)**	**30.5±6.8 (N =2)**
BPV	**12.0±1.6 (N =2)**	**31.7±5.4 (N =2)**
CLV	**20.3±1.4 (N =2)**	**20.9±0.0 (N =2)**
KGV	**15.9±1.1 (N =2)**	**15.4±4.4 (N =2)**
ZOV	**15.9±0.7 (N =2)**	**14.2±1.9 (N =2)**
CLM	**6.4±1.6 (N =2)**	**14.2±2.1 (N =2)**
ZOM	**80.0±13.3 (N =3)**	**75.0+10.5 (N =3)**
Berberine	**0.8±0.0 (N =2)**	**0.6±0.0 (N =2)**
Vinblastine sulphate (nM)	**0.011±0.004 (N =2)**	**0.008±0.004 (N=2)**

[a] See the isolation procedure

[b] Assays for cytotoxic activities were performed in 6 replicates

N = number of independent experiments

The results of cytotoxic activity in Table 16 revealed that the methanol extract of *C. longa* was the most active extract against LS174T and MCF7 with IC_{50} values of 6.4 and 14.2 μg/ml, respectively.

The above results indicated that the five volatile oils were active against LS174T and MCF7 with IC_{50} values in the range of 12.0-47.8 μg/ml but lacked of antioxidative activity. The methanol extracts of the five rhizomes were more active against DPPH radical than their corresponding water extracts. In the antioxidative assay against DPPH radical at final concentration of 100 μg/ml, the methanol extracts of *A. galanga*, *C. longa* and *Z. officinale* exhibited strong antioxidative activity. Their EC_{50} values were less than 100 μg/ml of which the methanol extract of *C. longa* was the most active extract with an EC_{50} value of 9.7 μg/ml. In the cytotoxic activity assay against LS174T and MCF7, the methanol extract of *C. longa* possessed IC_{50} value less than 20 μg/ml (IC_{50} against LS174T was 6.4 μg/ml and that against MCF7 was 14.2 μg/ml). The criteria of cytotoxic activity for the crude extracts, as established by the American National Cancer Institute (NCI), is an IC_{50} value of less than 20 μg/ml in the preliminary assay (Suffness and Pezzuto, 1991).

It is evident that the methanol extracts of *A. galanga*, *C. longa* and *Z. officinale* were of interest for further isolation for pure components (sections 2.3.4, 2.3.5 and 2.3.6) and volatile oils of the five rhizomes, which showed cytotoxic activity, were further analysed to determine chemical constituents by Gas chromatography/Mass spectrometry (section 3.2.1).

3.2 Analysis of Chemical Composition of the Volatile Oils and Structure Determination of the Isolated Compounds

3.2.1 Characterization of component of the volatile oils

The cleaned fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* were prepared for volatile oils (section 2.3.1). Chemical constituents of these volatile oils were analysed by Gas chromatography/Mass spectrometry with a Hewlett-Packard HP 5890 series II plus GC-HP 5972 Mass Selective Detector. Chemical constituents of the volatile oils from *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* were shown in Tables 17, 18, 19, 20 and 21, respectively.

Table 17 Chemical constituents of the volatile oil from *Alpinia galanga* (GC/MS analysis)

Retention time (min)	**% Area**	**Compound**	**Molecular weight**	**Molecular formula**
3.53	Minor compound	Unable to be identified	-	-
3.73	11.59	Terpinene-4-ol	154.14	$C_{10}H_{18}O$
3.88	5.54	1-α-Terpineol	154.14	$C_{10}H_{18}O$
4.74	6.91	4-Allylphenol	134.07	$C_9H_{10}O$
6.06	11.44	4-Allylphenyl acetate	176.08	$C_{11}H_{12}O_2$
6.16	58.47	*trans*-3-Acetoxy-1,8-cineole	212.00	$C_{12}H_{20}O_3$
7.01	Minor compound	*trans*-Methyl isoeugenol	178.10	$C_{11}H_{14}O_2$
7.57	6.05	Unable to be identified	-	-
8.09	Minor compound	α-Humulene	204.19	$C_{15}H_{24}$
8.58	Minor compound	Unable to be identified	-	-
9.05	Minor compound	Unable to be identified	-	-
9.66	Minor compound	Unable to be identified	-	-
11.00	Minor compound	Unable to be identified	-	-

Table 18 Chemical constituents of the volatile oil from *Boesenbergia pandurata* (GC/MS analysis)

Retention time (min)	**% Area**	**Compound**	**Molecular weight**	**Molecular formula**
3.19	81.43	Camphor	152.12	$C_{10}H_{16}O$
4.85	5.99	Geraniol	154.14	$C_{10}H_{18}O$
4.95	5.37	Geranial (*E*-Citral)	152.12	$C_{10}H_{16}O$
6.57	7.21	Methyl cinnamate	162.07	$C_{10}H_{10}O_2$

Table 19 Chemical constituents of the volatile oil from *Curcuma longa* (GC/MS analysis)

Retention time (min)	**% Area**	**Compound**	**Molecular weight**	**Molecular formula**
3.70	2.07	4-Terpineol	154.14	$C_{10}H_{18}O$
3.85	2.11	1-Methyl-4-(2-propanol-2-yl)-cyclohexene	154.14	$C_{10}H_{18}O$
4.48	5.20	Unable to be identified	-	-
5.15	1.00	Unable to be identified	-	-
5.57	0.92	Unable to be identified	-	-
5.65	1.24	Unable to be identified	-	-
8.52	1.98	ar-Curcumene	202.17	$C_{15}H_{22}$
9.21	1.48	β-Sesquiphellandrene	204.19	$C_{15}H_{24}$
9.88	2.66	Unable to be identified	-	-
10.17	1.06	Unable to be identified	-	-
10.77	1.95	Unable to be identified	-	-
11.13	38.00	ar-Turmerone	216.17	$C_{15}H_{20}O$
11.23	7.41	α-Turmerone	218.17	$C_{15}H_{22}O$
11.69	11.43	β-Turmerone	218.17	$C_{15}H_{22}O$
12.54	1.43	Unable to be identified	-	-
12.96	1.43	Unable to be identified	-	-
13.65	4.79	Unable to be identified	-	-
13.79	4.36	Unable to be identified	-	-
13.85	2.12	Unable to be identified	-	-
14.03	1.53	Unable to be identified	-	-
14.73	5.24	Unable to be identified	-	-
15.38	0.62	Unable to be identified	-	-

Table 20 Chemical constituents of the volatile oil from *Kaempferia galanga* (GC/MS analysis)

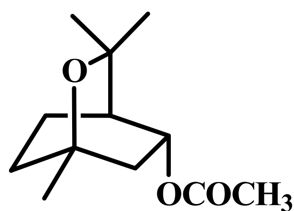
Retention time (min)	**% Area**	**Compound**	**Molecular weight**	**Molecular formula**
3.29	0.66	Unable to be identified	-	-
3.43	2.31	Unable to be identified	-	-
3.55	7.58	*l*-Borneol	154.14	$C_{10}H_{18}O$
3.67	1.11	*p*-Cymen-8-ol	150.10	$C_{10}H_{14}O$
3.72	1.95	Terpinene-4-ol	154.14	$C_{10}H_{18}O$
3.87	1.23	β-Fenchyl alcohol	154.14	$C_{10}H_{18}O$
3.97	0.69	Unable to be identified	-	-
4.20	0.87	Unable to be identified	-	-
4.55	0.86	Unable to be identified	-	-
8.09	61.81	Ethyl cinnamate	176.08	$C_{11}H_{12}O_2$
9.16	2.60	Pentadecane	212.25	$C_{15}H_{32}$
12.33	18.33	3-(4-Methoxyphenyl)-2-propenoic acid ethyl ester	206.09	$C_{12}H_{14}O_3$

Table 21 Chemical constituents of the volatile oil from *Zingiber officinale* (GC/MS analysis)

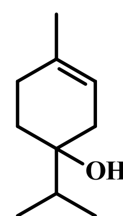
Retention time (min)	**% Area**	**Compound**	**Molecular weight**	**Molecular formula**
3.35	1.87	Citronellal	154.14	$C_{10}H_{18}O$
3.53	4.26	*l*-Borneol	154.14	$C_{10}H_{18}O$
3.62	3.18	Cryptone	138.10	$C_9H_{14}O$
3.87	2.82	1-α-Terpineol	154.14	$C_{10}H_{18}O$
4.54	26.59	Neral (*Z*-Citral)	152.12	$C_{10}H_{16}O$
4.97	33.02	Geranial (*E*-Citral)	152.12	$C_{10}H_{16}O$
5.44	1.99	2-Undecanone	170.17	$C_{11}H_{22}O$
5.83	5.17	Unable to be identified	-	-
6.39	6.87	Unable to be identified	-	-
6.73	8.32	Unable to be identified	-	-
8.08	1.52	Unable to be identified	-	-
8.54	3.07	ar-Curcumene	202.17	$C_{15}H_{22}$
10.56	1.34	Unable to be identified	-	-

Chemical compositions of the volatile oils, analyzed by GC/MS technique, were shown in Tables 17-21. Volatile oil of *A. galanga* (AGV) was composed of at least 13 compounds having *trans*-3-acetoxy-1,8-cineole (% area 58.47) and terpinene-4-ol (% area 11.59) as major components. This was partly similar to the previous data reported by De Pooter, *et al*. (1985) of which α-pinene, 1,8-cineole, bornyl acetate, geranyl acetate, α-bergamotene, *trans*-β-farnesene and β-bisabolene were identified as major compounds of the volatile oil of *A. galanga*. The oil of *B. pandurata* (BPV) contained at least 4 compounds including camphor (% area 81.43) and methyl cinnamate (% area 7.21) which were the main constituents. This was in accordance with the result reported by Jantan, *et al*. (2001) of which the major constituents of the rhizome oil of *B. pandurata* were detected as camphor (16.1-32.1 %), geraniol (16.2-26.0 %), (*E*)-β-ocimene (19.0-23.7 %), 1,8-cineole (7.5-13.9 %), camphene (5.4-6.0 %) and methyl cinnamate (2.2-5.8 %). The oil of *C. longa* (CLV) was composed of at least 22 compounds, characterized by high proportion of ar-turmerone (% area 38.00) and β-turmerone (% area 11.43). Analysis of the oil of *K. galanga* (KGV) revealed the existence of at least 12 compounds, in which the main constituents were identified as ethyl cinnamate (% area 61.81) and 3-(4-methoxyphenyl)-2-propenoic acid ethyl ester (% area 18.33). The volatile oil of *Z. officinale* (ZOV) were composed of at least 13 compounds with geranial (% area 33.02) and neral (% area 26.59) being main compounds. It is notable that volatile oils of the five spices were mainly composed of monoterpenes, sesquiterpenes and phenylpropanoids. The major component in the volatile oil from *C. longa* rhizomes was identified as ar-turmerone which was in good agreement with the previous reports (Gopalan, *et al*., 2000; Negi, *et al*., 1999; Martins, *et al*., 2001). The volatile oil from the fresh rhizome of *Z. officinale* was characterized by presence of acyclic oxygenated monoterpenes mainly composed of geranial (*E*-citral) and neral (*Z*-citral), which was in accordance with the result reported by Sakamura (1987). These major and minor compounds in the volatile oils could be responsible for the observed cytotoxic activity of the volatile oils. For example, growth of V-79 cells (lung fibroblasts of Chinese hamster) was completely inhibited on treatment with camphor at 0.3 % for 24-48 hr (Toshihiko, 1987). A

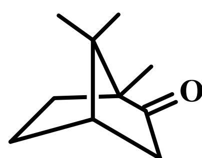
sesquiterpene β-sesquiphellandrene, one of the minor compounds detected in CLV, was reported to be cytotoxic against mouse lymphocytic leukaemia cells L1210 (Ahn and Lee, 1989). Citral, one of the major compounds of ZOV, was found to be cytotoxic against P388 mouse leukaemia cells (Dubey, Takeya and Itokawa, 1997). 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). These studies gave 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 are accounted for one of the cytotoxic constituents against tumour cells of the five rhizomes, which is of interest for medicinal purposes.



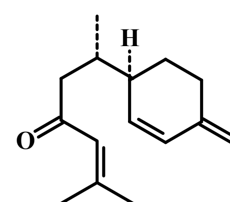
***trans*-3-Acetoxy-1,8-cineole**



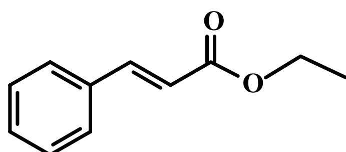
Terpinene-4-ol



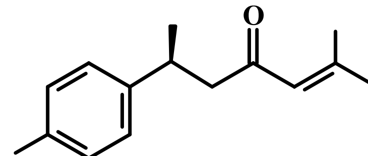
Camphor



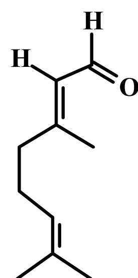
β-Turmerone



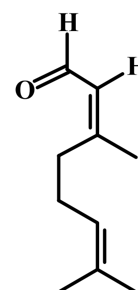
Ethyl cinnamate



ar-Turmerone



Geranial (*E*-Citral)



Neral (*Z*-Citral)

Figure 6 Structures of some major compounds detected in the volatile oils of *A. galanga*, *B. pandurata*, *C. longa, K. galanga* and *Z. officinale*.

3.2.2 Structure elucidation of the isolated compounds

Results from the preliminary assays for free radical scavenging activity in section 2.5 and cytotoxic activity in section 2.6 of the methanol extracts, water extracts and volatile oils of the fresh rhizomes of *A. galanga*, *B. pandurata*, *C. longa*, *K. galanga* and *Z. officinale* were shown in section 3.1. They gave evidences of the presence of active constituents in the volatile oils of the five fresh rhizomes and the methanol extracts of *A. galanga*, *C. longa*, and *Z. officinale*. Thus, a separation of the active extracts was carried out as shown in section 2.3 to give the pure compounds as follows:

AGM1 from the methanol extract of the fresh rhizome of *A. galanga*
CLM01 from the methanol extract of the fresh rhizome of *C. longa*
CLM02 from the methanol extract of the fresh rhizome of *C. longa*
CLM03 from the methanol extract of the fresh rhizome of *C. longa*
CLM06 from the methanol extract of the fresh rhizome of *C. longa*
ZOM0 from the methanol extract of the fresh rhizome of *Z. officinale*
ZOM1 from the methanol extract of the fresh rhizome of *Z. officinale*
ZOM3 from the methanol extract of the fresh rhizome of *Z. officinale*

3.2.2.1 AGM1

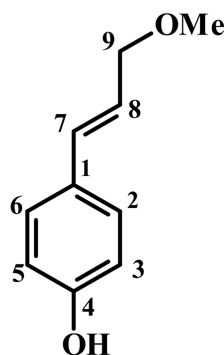
AGM1 was obtained as yellow oil. Its UV spectrum showed maximum absorption at 264 nm. The IR spectrum (KBr disc) showed the presence of hydroxyl function at 3350, C-H stretching at 2920 and olefinic carbon stretching at 1610 and 1520 cm^{-1}.

The molecular formula of AGM1 was proposed to be $C_{10}H_{12}O_2$ (MW = 164, D.B.E. = 5) as deduced from EIMS spectrum. The ^{13}C NMR (125 MHz, $CDCl_3$) revealed 10 carbons, 8 of which corresponded with 11 protons as observed from HMQC spectrum. This corresponded well with the observed molecular mass from HR-FABMS at *m/z* 164.0840. In the ^1H NMR spectrum of AGM1 (500 MHz; $CDCl_3$),

the 3-H singlet signal of a methoxyl group was detected at δ 3.41 and 2 aromatic spin systems (integrated two protons each) were observed. They are in *ortho* position of benzene ring (δ 6.78, d, J = 9.0 Hz, H-3, H-5 and δ 7.25, d, J = 9.0 Hz, H-2, H-6). This suggested that the benzene ring had substituted groups in *para* position. The upfield shift of proton signal at δ 6.78 (δ_C 115.6) was due to the shielding effect of the hydroxyl group at C-4. The existence of OH function was confirmed with the IR absorption band at 3350 cm^{-1}. The two quaternary carbons at δ 129.3 and 155.8 were attributed to C-1 and C-4 respectively, based on hydroxyl substitution at C-4. Two proton signals were observed at δ 6.55 (br.d, J = 16.0 Hz) and δ 6.13 (dt, J = 16.0, 6.0 Hz), which were characteristic of two *trans* olefinic protons and subsequently assigned for 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.

Chemical shifts of the remaining H-bonded carbons were allocated by HMQC experiment as shown in Table 22. The position of methoxyl group was allocated by analysis of nuclear Overhauser effect (nOe) difference spectra. Upon irradiation of the signal at δ 3.41 (methoxyl group) resulted in strong enhancement to signal at δ 4.10 (H-9), indicating that these methylene protons ($-CH_2-$) were adjacent to the methoxyl group. In addition, H-8 and H-9 showed vicinal (3-bond) coupling with coupling constant of 6.0 Hz, suggesting the presence of a propenoid side chain (C-7, C-8 and C-9).

These spectral features suggested that AGM1 was a derivative of coumaric acid, which has been modified through reduction and methylation in shikimate pathway in plant. Thus, AGM1 was characterized as a phenylpropanoid and named to be *p*-coumaryl-9-methyl ether. Some phenylpropanoids, e.g. *p*-coumaryl alcohol, *p*-coumaric acid and cinnamic acid were isolated from the rhizome of *A. galanga* (Matsuda *et al*., 2003) but this compound has not previously been reported from *A. galanga*. This is the first report of naturally occurring *p*-coumaryl-9-methyl ether with complete ^{1}H and ^{13}C NMR assignments.



***p*-Coumaryl-9-methyl ether (AGM1)**

Table 22 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of AGM 1[a]

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

Note; [a]In $CDCl_3$

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

3.2.2.2 CLM01

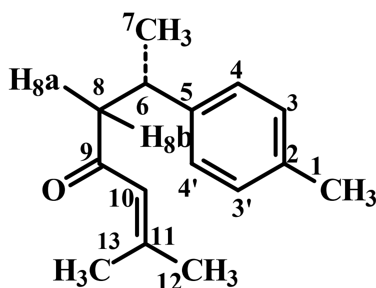
Phytochemical investigation of the methanol extract of *C. longa* led to the isolation of pure compound CLM01 as pale yellow oil. The EI mass spectrum of CLM01 showed a protonated molecular ion peak at *m/z* 217 consistent with the molecular formula $C_{15}H_{20}O$ (MW = 216, D.B.E. = 6). The IR spectrum (KBr disc) of CLM01 showed C-H stretching at 3000, carbonyl stretching at 1700 and double bond stretching at 1600 cm^{-1}. CLM01 showed UV absorption at 238 nm and specific optical rotation of +64.3° (c 0.7, $CHCl_3$).

The ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HMQC and HMBC spectra of CLM01 showed the existence of one benzene ring, one olefinic function, four methyl groups, one methylene group and four quaternary carbons (including a carbonyl function). Chemical shifts of all protonated carbons were assigned by HMQC experiment. The quaternary carbon at δ 199.9 was attributed to a carbonyl function, which showed two-bond cross peaks with protons at δ 2.60 (H-8a), δ 2.70 (H-8b) and δ 6.02 (H-10) in the HMBC spectrum. This resulted in the assignments of carbon signals at δ 52.6 and 124.0 to be C-8 and C-10, respectively, according to the HMQC correlation with their corresponding proton signals. Having allocated the chemical shift of olefinic carbon (C-10) at δ 124.0, the two methyl functions at δ 1.85 (3H, d, 1.3 Hz) and δ 2.10 (3H, d, 1.3 Hz), which showed 3-bond correlation with C-10 in the HMBC spectrum, were assigned to be 12-CH_3 and 13-CH_3, respectively. The downfield shift of 13-CH_3, comparing with 12-CH_3, was due to deshielding effect of the carbonyl function. The quaternary carbon at δ 155.2 was attributed to C-11 due to the correlation with the two methyl functions at δ 1.85 and δ 2.10 in the HMBC spectrum. The methine carbon at δ 35.2 (δ_H 3.28) was attributed to C-6 according to two-bond correlation with H-8a and H-8b in the HMBC spectrum. The methyl function at δ 1.23 (3H, d, 7.1 Hz), which showed cross peaks with C-6 (δ 35.2) and C-8 (δ 52.6) in the HMBC spectrum, was therefore assigned to be 7-CH_3. Furthermore, H-6 (δ 3.28) also showed cross peaks with quaternary carbon at δ 143.6 and protonated carbon at δ 126.6 leading to

assignments of these two signals as C-5 and C-4/C-4′ (equivalent carbons), respectively.

The correlation of H-4/H-4′ (δ 7.09) with protonated carbon signal at δ 129.0 and quaternary carbon at δ 135.5 in the HMBC spectrum resulted in assignments of these two signals to be C-3/C-3′ (equivalent carbons) and C-2, respectively. The spin coupling between H-3/H-3′ and H-4/H-4′ as doublet with coupling constant (*J* value) of 8.5 Hz (*ortho* coupling) and the presence of two quaternary carbons at δ 135.5 (C-2) and δ 143.6 (C-5) were indicative of a 1,4-disubstitued benzene ring. The remaining methyl function at δ 2.30 (3H, s), which showed correlation with carbon signals at δ 135.5 (C-2) and δ 129.0 (C-3/C-3′) in the HMBC spectrum, was attributed to 1-CH_3.

These spectral features of CLM01 were in good agreement with those of the sesquiterpene ar-turmerone previously isolated from the rhizome of *Curcuma 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 on the other way round (C-11 should be more downfield than C-5) according to the data obtained in this present work. The stereochemistry at C-6 was assumed to be *S*-configuration based on the similar profiles of optical rotation (+64.3°; literatures: +82.7° and +68°) and proton NMR characteristics of H-6 and 7-CH_3 of CLM01 to those of the published data (Honwad and Rao, 1964; Li *et al*., 2003). The unambiguous ^{1}H and ^{13}C NMR assignments of CLM01 obtained from the present work were shown in Table 23.



ar-Turmerone (CLM01)

Table 23 NMR spectral data (500 MHz for ^1H and 125 MHz for ^{13}C) of CLM01[a]

Position	δ_H (mult., *J* in Hz)[b]	δ_C	HMBC $^{13}C \rightarrow {}^1H$
1	2.30 (s, 3H)	20.9	-
2	-	135.5	H-1, H-3, H-3′, H-4, H-4′
3, 3′	7.11 (d, 8.5, 2H)	129.0	H-1, H-4, H-4′
4, 4′	7.09 (d, 8.5, 2H)	126.6	H-3, H-3′, H-6
5	-	143.6	H-3, H-3′, H-4, H-4′, H-6, H-7, H-8a, H-8b
6	3.28 (br.dq, 8.4, 7.1)	35.2	H-7, H-8a, H-8b
7	1.23 (d, 7.1, 3H)	21.9	H-6, H-8a, H-8b
8	2.60 (dd, 15.8, 8.4, H-8a)	52.6	H-6, H-7
	2.70 (dd, 15.8, 6.2, H-8b)	52.6	H-6, H-7
9	-	199.9	H-8a, H-8b, H-10
10	6.02 (septet, 1.3)	124.0	H-12, H-13
11	-	155.2	H-12, H-13
12	1.85 (d, 1.3, 3H)	27.6	H-10, H-13
13	2.10 (d, 1.3, 3H)	20.7	H-10, H-12

Note; [a]In $CDCl_3$

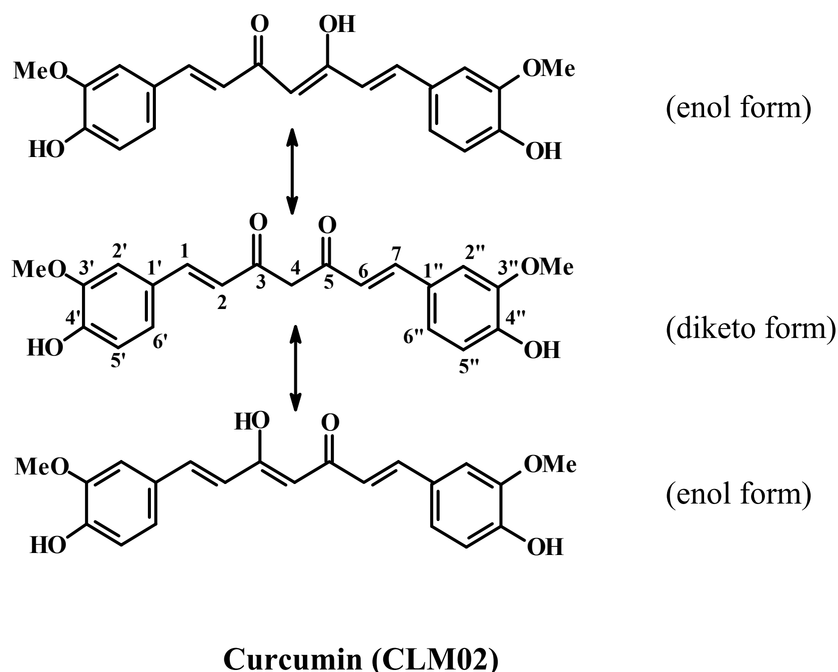
[b]If not indicated, the integration of each proton signal is equal to 1 proton.

3.2.2.3 CLM02

CLM02 was the major compound of the methanol extract of *C. longa*, obtained as orange crystals and showed protonated molecular ion peak in FAB mass spectrum at *m/z* 369, corresponding with a molecular formula of $C_{21}H_{20}O_6$ (MW = 368, D.B.E. = 12). The IR (KBr disc) spectrum indicated the presence of hydroxy group at 3500, carbonyl function at 1630 and olefinic carbon stretching at 1510 cm^{-1}. Its UV (MeOH) spectrum showed absorption maxima at 236 and 424 nm.

^{1}H and ^{13}C NMR spectra of CLM02 revealed the existence of aromatic, olefinic, methoxyl, hydroxyl and carbonyl functions. Analysis of chemical shifts, integration and spin coupling patterns of these functional groups gave evidence that CLM02 was a diarylheptanoid. The signal of carbonyl function at δ 183.2, which showed correlation with *trans*-olefinic protons at δ 7.60 (2H, d, 15.5 Hz) and δ 6.49 (2H, d, 15.5 Hz) in the HMBC spectrum, was attributed to C-3/C-5 (equivalent carbons). The substantial downfield shift of signal at δ 7.60, comparing with the signal at δ 6.49, was typical for the β-olefinic proton of the α,β-unsaturated ketone. Hence the signals at δ 7.60 and δ 6.49 were assignable for H-1/H-7 and H-2/H-6, respectively. The corresponding C-1/C-7 and C-2/C-6 were assigned by HMQC experiment at δ 140.5 and 121.8, respectively. The cross peaks of C-1/C-7 with proton signals at δ 7.06 (H-2′ and H-2″) and δ 7.14 (H-6′ and H-6″) in the HMBC spectrum yielded additional support for the presence of 1,6-heptadiene-3,5-dione moiety with two equivalent phenyl groups attached to C-1 and C-7 of the molecule. It was notable that the signal of H-4 was assignable as an enol form at δ 5.81 (δ_C 101.2). The spin coupling patterns of H-2′/H-2″, H-5′/H-5″ and H-6′/H-6″, which were doublet (2.0 Hz), doublet (8.3 Hz) and doublet of doublets (8.3 and 2.0 Hz), respectively, indicated the presence of 1,3,4-trisubstituted benzene ring. The 3-bond cross peaks of quaternary carbon signal at δ 146.8 with proton signals at δ 6.92 (H-5′/H-5″) and δ 3.96 (3′-OCH_3/3″-OCH_3) in the HMBC spectrum enabled us to assign this signal as C-3′/C-3″. The quaternary carbon signal at δ 147.8 was attributed to C-4′/C-4″ due to the 3-bond cross peaks with proton signals at δ 7.06 (H-2′/H-2″) and δ 7.14 (H-6′/H-6″) in the HMBC spectrum.

These spectral features of CLM02 was in accordance with those of published data of curcumin or 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (Kosuge, Ishida and Yamazaki, 1985; Uehara *et al*., 1987; Masuda *et al*., 1992). Curcumin has been identified as the major constituent in Turmeric (*Curcuma longa*) and also has been isolated from some other *Curcuma* species, e.g. *C. xanthorrhiza* and *C. aromatica* (Kosuge, Ishida and Yamazaki, 1985; Ishida *et al*., 2002). The complete ^{1}H and ^{13}C NMR assignments of curcumin (CLM02) obtained from the present work were shown in Table 24.



Curcumin (CLM02)

Table 24 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of CLM02[a]

Position	δ_H (mult., J in Hz)[b]	δ_C	HMBC $^{13}C \rightarrow {}^1H$
1,7	7.60 (d, 15.5, 2H)	140.5	H-6′, H-6″, H-2′, H-2″
2, 6	6.49 (d, 15.5, 2H)	121.8	H-4
3, 5	-	183.2	H-1, H-2, H-4, H-6, H-7
4	5.81 (s)[c]	101.2	H-2, H-6
1′, 1″	-	127.7	H-2, H-6, H-5′, H-5″
2′, 2″	7.06 (d, 2.0, 2H)	109.6	H-1, H-7, H-6′, H-6″
3′, 3″	-	146.8	H-5′, H-5″, OMe
4′, 4″	-	147.8	H-2′, H-2″, H-6′, H-6″
5′, 5″	6.92 (d, 8.3, 2H)	114.8	H-6′, H-6″
6′, 6″	7.14 (dd, 8.3, 2.0, 2H)	122.9	H-1, H-7, H-2′, H-2″
3′-OMe, 3″-OMe	3.96 (s, 6H)	55.9	-
4′-OH, 4″-OH	5.88 (br.s, 2H)	-	-

Note; [a]In $CDCl_3$

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

[c]Detected as an enol form.

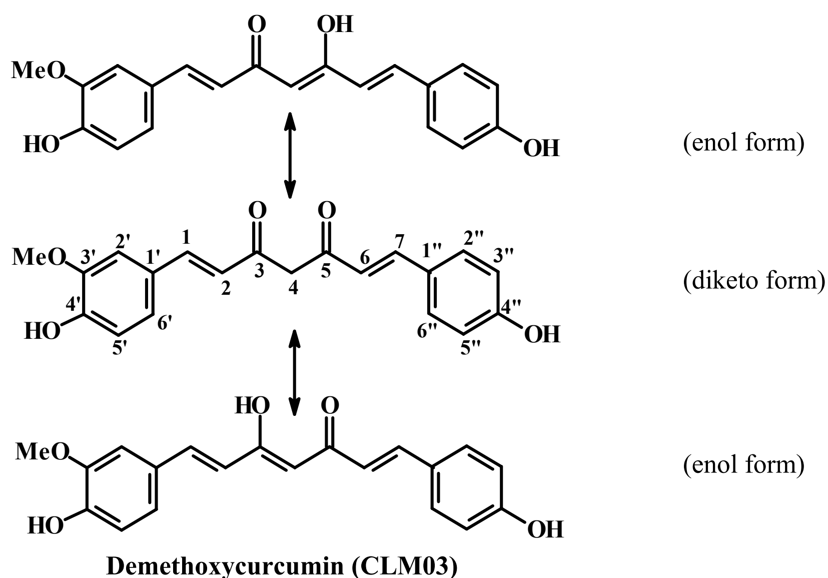
3.2.2.4 CLM03

CLM03 was isolated from the methanol extract of *C. longa* as reddish orange powder. The FAB mass spectrum (low resolution) spectrum of CLM03 showed protonated molecular ion peak at *m/z* 339, consistent with the molecular formula $C_{20}H_{18}O_5$ (MW = 338, D.B.E. = 12). The IR (KBr disc) spectrum indicated the presence of hydroxy group at 3450, carbonyl function at 1650 and olefinic carbon stretching at 1500 cm^{-1}. Its UV (MeOH) spectrum showed absorption maxima at 240 and 420 nm.

^1H and ^{13}C NMR spectra of CLM03 showed the existence of functional groups almost similar to those of CLM02 (curcumin), suggesting that CLM03 should be a diarylheptanoid. This was strongly supported by the presence of two carbonyl signals at δ 183.3 and δ 183.4 and four signals of *trans*-olefinic protons at δ 7.60 (1H, d, 15.8 Hz), δ 6.50 (1H, d, 15.8 Hz), δ 6.49 (1H, d, 15.8 Hz) and δ 7.62 (1H, d, 15.8 Hz), which indicated the characteristics of 1,6-heptadiene-3,5-dione skeleton. Furthermore, signals of two aromatic systems, which showed different substitution patterns, were observed. The first system was composed of three signals at δ 7.06 (1H, d, 1.8 Hz), δ 6.95 (1H, d, 8.4 Hz) and δ 7.13 (1H, dd, 8.4 and 1.8 Hz) suggesting the presence of 1,3,4-trisubstituted benzene ring according to the spin couplings. These three signals were attributed to H-2′, H-5′ and H-6′, respectively, based on the 3-bond correlation of the olefinic carbon signal at δ 140.6 (C-1) with signals at δ 7.06 (H-2′) and δ 7.13 (H-6′) in the HMBC spectrum (Table 25). The signal of methoxyl group at δ 3.96 (3H, s) was assigned as 3′-OCH_3 due to the cross peaks with C-3′ (δ 146.8) in the HMBC spectrum. Having allocated the position of C-1, the signal of carbonyl function at δ 183.3 was assignable to C-3 due to the cross peaks between C-3 and proton signals at δ 7.60 (H-1) and δ 6.50 (H-2). The second aromatic system was composed of two proton signals at δ 7.45 (2H, d, 8.7 Hz) and δ 6.87 (2H, d, 8.7 Hz), indicating the existence of 1,4-disubstituted benzene ring due to the *ortho* couplings. These two signals were assigned to be H-2″/H-6″ and H-3″/H-5″, respectively, according to the correlation with carbon signals at δ 128.0 (C-1″) and δ 157.5 (C-4″), and the

correlation between H-2″/H-6″ and the olefinic carbon at δ 140.0 (C-7) in the HMBC spectrum. The noticeable upfield shift of H-3″/H-5″, comparing with that of H-2″/H-6″, was due to the shielding effect of the hydroxyl substitution at C-4″. The carbonyl signal at δ 183.4 was attributed to C-5 based on the HMBC correlation with proton signals at δ 7.62 (H-7), δ 6.49 (H-6) and δ 5.80 (H-4) in the HMBC spectrum. All protonated carbons were assigned by HMQC experiment including C-4 (δ 101.3), which was assignable to an enol form.

The obtained spectral information indicated that the structure of CLM03 related to that of curcumin (CLM02) with an absence of one methoxyl group. This was also supported by the difference of molecular weight of the two compounds, of which CLM03 was 30 amu less than that of curcumin. Hence, CLM03 was concluded to be demethoxycurcumin or 1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl)-1,6-heptadiene-3,5-dione. Demethoxycurcumin was previously isolated from the rhizome of *C. longa* and some other *Curcuma* species (Kosuge, Ishida and Yamazaki, 1985; Masuda *et al*., 1992). However, the chemical shifts of carbons and protons of demethoxycurcumin have only been partially assigned in the previous publications. The unambiguous ^{1}H and ^{13}C NMR assignments of demethoxycurcumin (CLM03) was achieved in the present work through 2D-NMR experiments as shown in Table 25.



Demethoxycurcumin (CLM03)

Table 25 NMR spectral data (500 MHz for ^1H and 125 MHz for ^{13}C) of CLM03[a]

Position	δ_H (mult., J in Hz)[b]	δ_C	HMBC $^{13}C \longrightarrow {}^1H$
1	7.60 (d, 15.8)	140.6	H-2′, H-6′
2	6.50 (d, 15.8)	121.8	H-4
3	-	183.3	H-1, H-2, H-4
4	5.80 (s)[c]	101.3	H-2, H-6
5	-	183.4	H-4, H-6, H-7
6	6.49 (d, 15.8)	121.7	H-4
7	7.62 (d, 15.8)	140.0	H-2″, H-6″
1′	-	127.4	H-5′
2′	7.06 (d, 1.8)	109.6	H-1, H-6′
3′	-	146.8	H-5′, 3′-OMe
4′	-	147.8	H-2′, H-6′
5′	6.95 (d, 8.4)	114.8	H-6′
6′	7.13 (dd, 8.4, 1.8)	122.9	H-1, H-2′
1″	-	128.0	H-6, H-3″, H-5″
2″, 6″	7.45 (d, 8.7, 2H)	130.0	H-3″,H-5″, H-7
3″, 5″	6.87 (d, 8.7, 2H)	115.9	H-2″, H-6″
4″	-	157.5	H-2″, H-3″, H-5″,H-6″
3′-OMe	3.96 (s, 3H)	56.0	-
4′-OH	5.88 (br.s)	-	-
4″-OH	not detected	-	-

Note; [a]In $CDCl_3$,

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

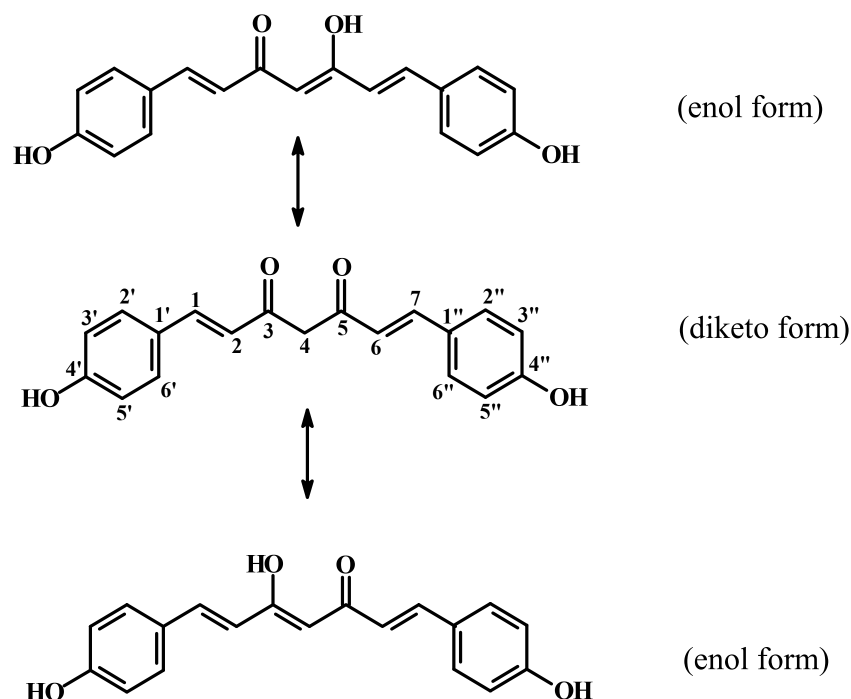
[c]Detected as an enol form.

3.2.2.5 CLM06

CLM06 was isolated from the methanol extract of *C. longa*. The FAB mass spectrum (low resolution) of CLM06 showed protonated molecular ion peak at *m/z* 309 (M+H) consistent with the molecular formula $C_{19}H_{16}O_4$ (MW = 308, D.B.E. = 12). The IR spectrum indicated the presence of hydroxy group and carbonyl function at 3500-3200 (broad) and 1600 cm^{-1}, respectively. Its UV spectrum showed absorption with λ_{max} at 246 and 414 nm.

^1H and ^{13}C NMR spectra of CLM06 exhibited the 1,6-heptadiene-3,5-dione skeleton, according to the appearance of *trans*-olefinic proton signals at δ 7.54 (H-1/H-7) and δ 6.68 (H-2/H-6) together with the carbonyl signal at δ 183.4 (C-3/C-5). The two signals at δ 7.55 (4H, d, 8.6 Hz) and δ 6.81 (4H, d, 8.6 Hz) were assignable to protons of two 1,4-disubstituted benzene rings. The proton signal at δ 6.00 (1H, s) and its corresponding carbon signal at δ 101.1 were attributed to H-4 and C-4, respectively (detected as enol form). These spectral features suggested that CLM06 had a highly symmetrical structure, regarding to the molecular weight of 308 amu (60 amu less than that of curcumin). The absence of methoxyl signal in NMR spectra of CLM06 gave additional evidence to propose the assumption that CLM06 should be a bisdemethoxy derivative of curcumin. The ion peak at *m/z* 147 (base peak) in the mass spectrum of CLM06, which was assignable to a $[HO\text{-}C_6H_4\text{-}CH\text{=}CH\text{-}C\text{=}O]^+$ fragment, gave strong support to the assignment. Furthermore, the quaternary carbon signal at δ 160.0 (C-4′/C-4″) suggested the hydroxyl substitution on C-4′ and C-4″.

Comparison of ^1H and ^{13}C NMR assignments of CLM06 with the published data (Kosuge, Ishida and Yamazaki, 1985; Masuda *et al*., 1992) resulted in determination of CLM06 as bisdemethoxycurcumin or 1,7-bis (4-hydroxyphenyl)-1,6-heptadiene-3,5-dione, which was previously isolated from the rhizome of *C. longa* and *C. xanthorrhiza* (Kosuge, Ishida and Yamazaki, 1985; Uehara *et al*., 1987).



Bisdemethoxycurcumin (CLM06)

Table 26 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of CLM06[a]

Atom	δ_H (mult., J in Hz)[b]	δ_C
1, 7	7.54 (d, 15.4, 2H)	140.5
2, 6	6.68 (d, 15.4, 2H)	121.0
3, 5	-	183.4
4	6.00 (s)[c]	101.1
1′, 1″	-	126.0
2′, 2″, 6′, 6″	7.55 (d, 8.6, 4H)	130.5
3′, 3″, 5′, 5″	6.81 (d, 8.6, 4H)	116.1
4′, 4″	-	160.0
4′-OH, 4″-OH	not detected	-

Note; [a]In DMSO-d_6

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

[c]Detected as an enol form.

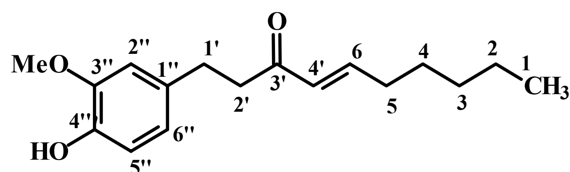
3.2.2.6 ZOM0

Phytochemical investigation of the methanol extract of *Z. officinale* led to the isolation of pure compound ZOM0 as yellow oil. Its FAB mass spectrum (low resolution) established a molecular formula of $C_{17}H_{24}O_3$ (MW = 276, D.B.E. = 6) according to the protonated ion peak at *m/z* 277 (M+H). Its UV (MeOH) spectrum showed absorption with λ_{max} at 226 nm. The IR (KBr disc) spectrum indicated the presence of hydroxy group, C-H, carbonyl function and olefinic carbon stretching at 3400, 2960, 1700 and 1500 cm^{-1}, respectively.

The ^{1}H, ^{13}C NMR and HMQC spectra of ZOM0 exhibited a carbonyl function, a methoxyl group, a methyl group, five olefinic methines and six methylene functions. The chemical shifts and spin couplings of proton signals at δ 6.71 (1H, d, 2.0 Hz), δ 6.82 (1H, d, 8.0 Hz) and δ 6.68 (1H, dd, 8.0 and 2.0 Hz) suggested the existence of 1,3,4-trisubstituted benzene ring. These aromatic protons and their corresponding carbons were attributed to H-2″ (δ_C 111.1), H-5″ (δ_C 114.3) and H-6″ (δ_C 120.8), respectively, base on the correlation observed in the ^{1}H-^{1}H COSY and HMQC spectra. The HMBC correlation between methoxyl signal at δ 3.87 (3H, s) and quaternary carbon signal at δ 146.3 helped allocating the two signals as 3″-OCH_3 and C-3″, respectively. The HMBC cross peaks of C-3″ and proton signals at δ 6.71 (H-2″) and δ 6.82 (H-5″) confirmed the assignment of signal at δ 146.3 as C-3″. As a consequence, the hydroxyl substitution was allocated to be at C-4″ (δ 143.8), the quaternary carbon which showed HMBC cross peaks with H-2″, H-5″ and H-6″. The signal of quaternary carbon at δ 133.2, which correlated with methylene signal at δ 2.85 (H-1′) and signals of aromatic protons at δ 6.71 (H-2″) and δ 6.82 (H-5″) in the HMBC spectrum, was attributed to C-1″. The assignment of carbon signal at δ 29.8 (δ_H 2.85) as C-1′ was deduced from the HMBC cross peaks with H-2′, H-2″ and H-6″. The assignments of methyl signal at δ 0.88 (1-CH_3) and four methylene signals at δ 1.29 (H-2), δ 1.31 (H-3), δ 1.44 (H-4) and δ 2.20 (H-5) were deduced from ^{1}H-^{1}H COSY, HMQC and HMBC spectra. The chemical shifts and spin couplings of these five signals suggested the existence of –CH_2-CH_2-CH_2-CH_2-CH_3 side chain. The

downfield shift of methylene signal at δ 2.20 (2H, tdd, 7.2, 7.0 and 1.4 Hz), which was attributed to H-5, suggested the proximity to the olefinic function. This was confirmed by the cross peaks of H-5 with two *trans*-olefinic protons at δ 6.83 (1H, dt, 16.0 and 7.0 Hz) and δ 6.09 (1H, dt, 16.0 and 1.4 Hz) in the ^{1}H-^{1}H COSY spectrum. The signal at δ 6.83 (δ_C 147.9) was attributed to H-6 due to the HMBC correlation of C-6 with H-5 (δ 2.20) and H-4 (δ 1.44). The other signal at δ 6.09 was, therefore, assigned as H-4′. The assignments of all protonated carbons were deduced from the HMQC and ^{1}H-^{1}H COSY spectra. The HMBC cross peaks of carbonyl signal at δ 199.8 (C-3′) with proton signals at δ 6.83 (H-6), δ 6.09 (H-4′) and δ 2.84 (H-2′) allowed the aliphatic moiety and the aromatic moiety to be connected.

Comparison the spectral data of ZOM0 with the published assignments led to the determination of ZOM0 as 6-shogaol [or 1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one] one of the pungent principles previously isolated from the rhizome of *Z. officinale* (Chen, Rosen and Ho, 1986b). The unambiguous ^{1}H and ^{13}C NMR assignments of 6-shogaol (ZOM0) obtained from the present work were shown in Table 27.



6-Shogaol (ZOM0)

Table 27 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of ZOM0[a]

Atom	δ_H (mult., *J* in Hz)[b]	δ_C	HMBC $^{13}C \rightarrow {}^{1}H$
1	0.88 (t, 7.0, 3H)	13.9	H-3
2	1.29 (m, 2H)	22.4	H-1, H-4
3	1.31 (m, 2H)	31.3	H-1, H-5
4	1.44 (quintet, 7.2, 2H)	27.7	H-5, H-6
5	2.20 (tdd, 7.2, 7.0, 1.4, 2H)	32.4	H-4, H-6, H-4′
6	6.83 (dt, 16.0, 7.0)	147.9	H-4, H-5
1′	2.85 (m, 2H)	29.8	H-2′, H-2″, H-6″
2′	2.84 (m, 2H)	41.9	H-1′
3′	-	199.8	H-6, H-2′, H-4′
4′	6.09 (dt, 16.0, 1.4)	130.3	H-5
1″	-	133.2	H-1′, H-2″, H-5″
2″	6.71 (d, 2.0)	111.1	H-1′
3″	-	146.3	H-2″, H-5″, OMe
4″	-	143.8	H-2″, H-5″, H-6″
5″	6.82 (d, 8.0)	114.3	H-6″
6″	6.68 (dd, 8.0, 2.0)	120.8	H-1′, H-2″, H-5″
OMe	3.87 (s, 3H)	55.8	-

Note; [a]In $CDCl_3$

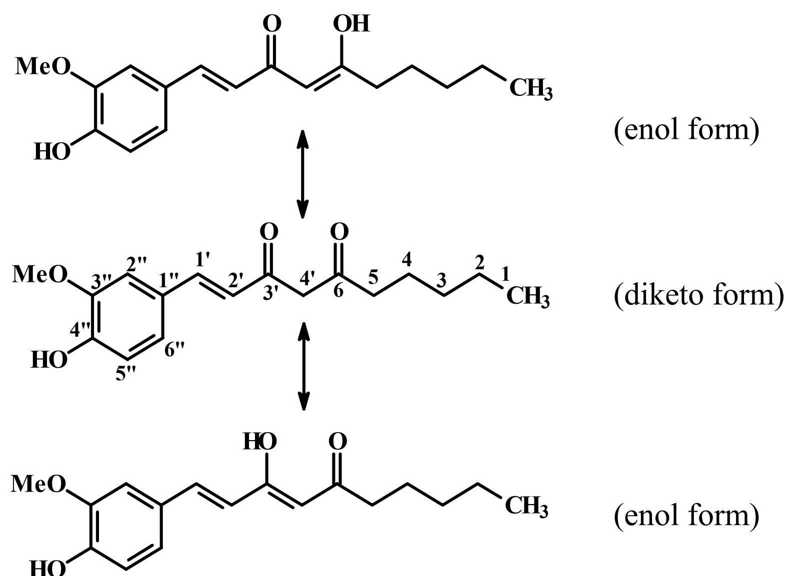
[b]If not indicated, the integration of each proton signal is equal to 1 proton.

3.2.2.7 ZOM1

ZOM1 was obtained as yellow crystals. Its FAB mass spectrum showed the protonated ion peak at *m/z* 291 corresponding with the molecular formula of $C_{17}H_{22}O_4$ (MW = 290, D.B.E. = 7). From the ^{13}C NMR spectrum, seventeen carbon signals were found including two carbonyl functions at δ 200.2 (C-6) and δ 178.0 (C-3′). The correlation between C-3′ and *trans*-olefinic proton signals at δ 7.53 (1H, d, 16.0 Hz) and δ 6.34 (1H, d, 16.0 Hz) in the HMBC spectrum suggested the existence of the α,β-unsaturated ketone moiety, resulting in assignments of the two signals as H-1′ and H-2′, respectively. Their corresponding carbons were observed in the HMQC spectrum at δ 139.8 (C-1′) and 120.6 (C-2′). The chemical shifts and spin coupling patterns of three proton signals at δ 7.02 (1H, d, 1.8 Hz), δ 6.92 (1H, d, 8.3 Hz) and δ 7.09 (1H, dd, 8.3 and 1.8 Hz) indicated the presence of the 1,3,4-trisubstituted benzene ring. The 3-bond cross peaks between C-1′ (δ 139.8) and signals at δ 7.02 and δ 7.09 in the HMBC spectrum gave evidence for conectivity and assignments of these two aromatic protons as H-2″ and H-6″, respectively. As a consequence, the signal at δ 6.92 was attributed to H-5″. The methoxyl signal at δ 3.93 (3H, s), which showed cross peak with quaternary carbon signal at δ 147.6 in the HMBC spectrum, was assigned to 3″-OCH_3. The allocation of C-3″ at δ 147.6 was supported by the cross peaks with proton signals at δ 7.02 (H-2″) and δ 6.92 (H-5″). The quaternary carbon at δ 146.7, which correlated with signals of H-5″ in the HMBC spectrum, was attributed to C-4″. The hydroxyl signal at δ 5.84, which was the characteristic for the hydroxyl function of the 4-hydroxy-3-methoxyphenyl moiety as that of curcumin (CLM02), was assigned to be 4″-OH. This was further supported by its HMBC cross peaks with carbon signals at δ 147.6 (C-3″) and δ 114.8 (C-5″). The presence of hydroxyl group was also supported by the absorption peak at 3350 cm^{-1} in the IR spectrum. The methyl signal at δ 0.88 (3H, t, 6.8 Hz) and four signals of methylene groups at δ 1.31 (H-2), 1.32 (H-3), 1.65 (H-4) and 2.37 (H-5) showed typical spin coupling patterns of $–CH_2$-CH_2-CH_2-CH_2-CH_3 chain, as deduced from ^1H-^1H COSY, HMQC and HMBC spectra. Assignment of the aliphatic side chain was also supported by the HMBC cross peak between C-6 (δ 200.2) and methylene signal at δ 2.37 (H-5). The proton signal at δ 5.62 (δ_C 100.1),

which correlated with carbon signals at δ 200.2 (C-6), δ 178.0 (C-3′) and δ 120.6 (C-2′), was attributed to H-4′ (detected as an enol form).

The spectral data mentioned above were in good agreement with those of 6-dehydrogingerdione, which was previously isolated from *Z. officinale* (Kiuchi, Shibuya and Sankawa, 1982). ZOM1 was, therefore, concluded to be 6-dehydrogingerdione, which has also been known as 1-dehydrogingerdione (Charles, Garg and Kumar, 2000). The unambiguous ^{1}H and ^{13}C NMR assignments of ZOM1 obtained from the present work were shown in Table 28.



6-Dehydrogingerdione (ZOM1) (also known as 1-dehydrogingerdione)

Table 28 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of ZOM1[a]

Atom	δ_H (mult., J in Hz)[b]	δ_C	HMBC ^{13}C ⟶ ^{1}H
1	0.88 (t, 6.8, 3H)	13.9	-
2	1.31 (m, 2H)	22.4	H-1, H-3
3	1.32 (m, 2H)	31.5	H-1, H-2, H-4, H-5
4	1.65 (quintet, 7.5, 2H)	25.3	H-5
5	2.37 (br.t, 7.5, 2H)	40.1	H-4
6	-	200.2	H-5, H-4′
1′	7.53 (d, 16.0)	139.8	H-2″, H-6″
2′	6.34 (d, 16.0)	120.6	H-4′
3′	-	178.0	H-1′, H-2′, H-4′
4′	5.62 (s)[c]	100.1	H-5, H-2′
1″	-	127.7	H-2′, H-5″
2″	7.02 (d, 1.8)	109.4	H-1′, H-6″
3″	-	147.6	H-2″, H-5″, OMe, 4″-OH
4″	-	146.7	H-5″
5″	6.92 (d, 8.3)	114.8	H-6″, 4″-OH
6″	7.09 (dd, 8.3, 1.8)	122.6	H-1′, H-2″, H-5″
OMe	3.93 (s, 3H)	55.9	-
4″-OH	5.84 (br.s)	-	-

Note; [a]In $CDCl_3$

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

[c]Detected as an enol form.

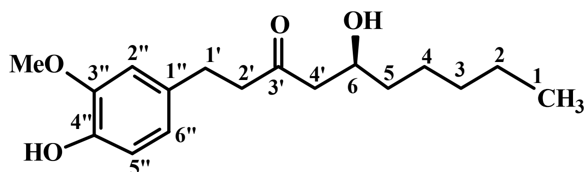
3.2.2.8 ZOM3

Phytochemical investigation of the methanol extract of *Z. officinale* led to the isolation of pure compound ZOM3 as yellow oil. Its FAB mass spectrum (low resolution) established a molecular formula of $C_{17}H_{26}O_4$ (MW = 294, D.B.E. = 5) according to the protonated ion peak at *m/z* 295 (M+H). Its UV (MeOH) spectrum showed absorption with λ_{max} at 282 nm and specific optical rotation of +29.3° (c 0.478, $CHCl_3$). The IR (KBr disc) spectrum indicated the presence of hydroxy group, C-H and carbonyl function at 3450, 2960 and 1700 cm^{-1}, respectively.

The majority of ^1H and ^{13}C NMR profiles of ZOM3 were in accordance with those of ZOM0 (6-shogaol) and ZOM1 (6-dehydrogingerdione). The exceptions were the absence of *trans*-olefinic function and the presence of oxygen-bearing methine carbon at δ 67.6 (δ_H 4.03) in the HMQC spectrum of ZOM3. The chemical shifts and spin couplings of proton signals at δ 6.69 (1H, d, 1.8 Hz), δ 6.83 (1H, d, 7.7 Hz) and δ 6.66 (1H, dd, 7.7 and 1.8 Hz) revealed the existence of 1,3,4-trisubstituted benzene ring, which were assigned as H-2″, H-5″ and H-6″, respectively. Their corresponding carbons, as deduced from the HMQC spectrum, were allocated at δ 110.9 (C-2″), 114.4 (C-5″) and 120.7 (C-6″). Allocation of the methoxyl function at C-3″ was confirmed by the HMBC correlation between the signal of quaternary carbon at δ 146.4 (C-3″) and the methoxyl signal at δ 3.87. The assignment of signal at δ 146.4 as C-3″ was supported by the HMBC cross peaks with H-2″ and H-5″. The hydroxyl signal at δ 5.59 was assigned as 4″-OH due to its HMBC cross peaks with carbon signals at δ 146.4 (C-3″), δ 143.9 (C-4″) and δ 114.4 (C-5″). The assignment of C-4″ was confirmed by the cross peaks with H-2″, H-5″ and H-6″ in the HMBC spectrum. The signal of quaternary carbon at δ 132.6, which correlated with two methylene functions at δ 2.84 (1′-CH_2) and δ 2.73 (2′-CH_2), H-2″ and H-5″ in the HMBC spectrum, was attributed to C-1″. The assignment of signal at δ 29.2 (δ_H 2.84) as C-1′ was confirmed by the HMBC correlation with H-2″ and H-6″. The assignments of 2′-CH_2 and 4′-CH_2 was deduced from the HMBC correlation with the carbonyl signal at δ 211.5 (C-3′) and the cross peaks between H-1′ and H-2′ in the ^1H-^1H COSY spectrum. The

methylene carbon at δ 49.3 was attributed to C-4′ due to the HMQC correlation with two proton signals at δ 2.57, 2.49 and HMBC correlation with H-2′. The downfield proton signal at δ 4.03 (1H, br.m) was assigned to be H-6 according to the HMQC cross peak with carbon signal at δ 67.6 (C-6), and the couplings with H-4′ and H-5 (δ 1.43) in the ^{1}H-^{1}H COSY spectrum. The NMR features of H-6 and C-6 suggested the presence of hydroxyl function as secondary alcohol at C-6, which was allocated at δ 3.00 (6-OH). The assignments of methyl signal at δ 0.88 (H-1) and four methylene signals at δ 1.28 (H-2), 1.29 (H-3), 1.30 (H-4) and 1.43 (H-5) were deduced from the ^{1}H-^{1}H COSY, HMQC and HMBC spectra.

The mentioned spectral features of ZOM3 were consistent with those of 6-gingerol (Shoji, *et al*., 1982; Yamada, Kikuzaki and Nakatani, 1992). The relative configuration at C-6 of ZOM3 (6-gingerol) was assumed to be (*S*)-configuration based on the similarity of specific optical rotation and NMR profiles of H-6 (δ 4.03) and H-4′ (δ 2.57 and δ 2.49) of ZOM3 to those of published (*S*)-6-gingerol previously isolated from the rhizome of *Z. officinale* (Shoji, *et al*., 1982; Yamada, Kikuzaki and Nakatani, 1992). It is of interest to note that although the chemical shifts of protons and carbons of 6-gingerol were reported, but they have not yet been completely assigned. The present work contributed unambiguous ^{1}H and ^{13}C NMR assignments of ZOM3 (6-gingerol) as shown in Table 29.



6-Gingerol (ZOM3)

Table 29 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of ZOM3[a]

Atom	δ_H (mult., *J* in Hz)[b]	δ_C	HMBC $^{13}C \rightarrow {}^{1}H$
1	0.88 (t, 7.3, 3H)	14.0	H-2
2	1.28 (m, 2H)	22.6	H-1
3	1.29 (m, 2H)	25.1	H-4
4	1.30 (m, 2H)	31.7	H-5
5	1.43 (m, 2H)	36.4	H-4′
6	4.03 (br.m)	67.6	H-4′
1′	2.84 (ddd, 7.6, 7.6, 1.4, 2H)	29.2	H-2′, H-2″, H-6″
2′	2.73 (ddd, 7.6, 7.6, 1.4, 2H)	45.4	H-1′, H-4′
3′	-	211.5	H-1′, H-2′, H-4′
4′	2.57 (dd, 17.6, 3.0)	49.3	H-2′
	2.49 (dd, 17.6, 8.9)		
1″	-	132.6	H-1′, H-2′, H-2″, H-5″
2″	6.69 (d, 1.8)	110.9	H-1′, H-6″
3″	-	146.4	H-2″, H-5″, OMe, 4″-OH
4″	-	143.9	H-2″, H-5″, H-6″, 4″-OH
5″	6.83 (d, 7.7)	114.4	H-6″, 4″-OH
6″	6.66 (dd, 7.7, 1.8)	120.7	H-1′, H-2″, H-5″
OMe	3.87 (s, 3H)	55.8	-
4″-OH	5.59 (br.s)	-	-
6-OH	3.00 (br.s)	-	-

Note; [a]In $CDCl_3$

[b]If not indicated, the integration of each proton signal is equal to 1 proton.

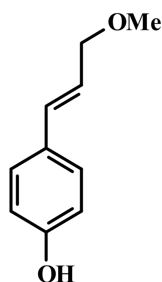
3.3 Activity of the Isolated Compounds

The eight isolated compounds (AGM1, CLM01, CLM02, CLM03, CLM06, ZOM0, ZOM1 and ZOM3) 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 7 and the results are depicted in Table 30. BHT (butylated hydroxytoluene) 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. The strong radical scavenging activity was exhibited by CLM02 (curcumin), CLM03 (demethoxycurcumin), ZOM0 (6-shogaol), ZOM1 (6-dehydrogingerdione) and ZOM3 (6-gingerol) with EC_{50} values ranging from 2.0-4.7 µg/ml. This was about 2-4 times more active than that of BHT, the well-known synthetic antioxidant. By contrast, CLM01 (ar-turmerone) showed very slight antioxidant effect ($EC_{50} > 100$ µg/ml). AGM1 (*p*-coumaryl-9-methyl ether) and CLM06 (bisdemethoxycurcumin) were found to possess moderate activity with EC_{50} values of 73.9 and 40.9 µg/ml, respectively. Rao (1996) reported that antioxidative activity of the curcuminoids from turmeric is in the following order: curcumin > demethoxycurcumin > bisdemethoxycurcumin, which is consistent with the results obtained from the present work. The strong antioxidant activity of CLM02 (curcumin) could be due to its capability to stabilize the two aroxyl radicals, which occurred after donating two hydrogen atoms of the hydroxyl functions to DPPH radicals, through aromatic system, conjugated double bonds and carbonyl functions. This, however, the two phenolic moieties of CLM06 are more exchangeable than that of CLM02, suggesting some degree of reversibility of reaction between CLM06 and DPPH radicals, hence being less active. Furthermore, it has been reported that the antioxidant mechanism of curcumin 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 (CLM02, CLM03 and CLM06) are regarded as main antioxidative constituents of *C. longa* rhizome. Radical scavenging activity of the three gingerol

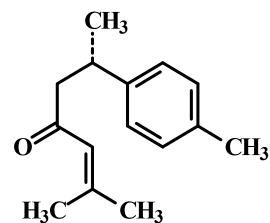
derivatives (ZOM0, ZOM1 and ZOM3) could be attributed to the hydroxyl group of the 4-hydroxy-3-methoxyphenyl moiety, which is almost comparable to that of CLM03 (demethoxycurcumin). The present results gave additional evidence to support the assumption that gingerols are responsible for antioxidant activity of *Z. officinale* rhizome (Sekiwa, Kubota and Kobayashi, 2000; Chung, *et al*., 2001). The extract of *A. galanga* rhizome has been known to possess antioxidative effect (Cheah and Gan, 2000) but the active constituents has not yet been identified. The radical scavenging activity against DPPH of AGM1 with EC_{50} value of 73.9 μg/ml, although less active than its corresponding methanol extract (EC_{50} = 57.0 μg/ml), provided new evidence of active compound responsible for antioxidative activity of *A. galanga* rhizome. It is likely that some other antioxidative compounds remain to be identified in the rhizome of *A. galanga*. Natural antioxidants have been known to be capable of reducing toxic from oxygen species or free radicals thus inhibiting formation of carcinogens from precursor substances such as lipid peroxidation. They may also improve the resistance of tissue to oxidative damage and enhance immune system. These suggested that natural antioxidants could provide preventive effect against cancer (Gordon, 1996). The antioxidative activity of the active compounds obtained from the present work give strong evidence of chemopreventive potential of the rhizomes of *C. longa*, *Z. officinale* and *A. galanga*.

Pronounced cytotoxic activity against the two tumour cell lines were observed for CLM03 (demethoxycurcumin) and ZOM0 (6-shogaol) 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). CLM02 (curcumin) and AGM1 (*p*-coumaryl-9-methyl ether) were slightly less active than CLM03 and ZOM0 with IC_{50} values on LS174T and MCF7 in the range of 5-10 μg/ml. On the other hand, ZOM1, ZOM3 and CLM01 were considerably less active than CLM03 and ZOM0 with IC_{50} values against both cell lines in the range of 11-32 μg/ml. The strong cytotoxic activity of CLM03 (demethoxycurcumin) observed in the present work

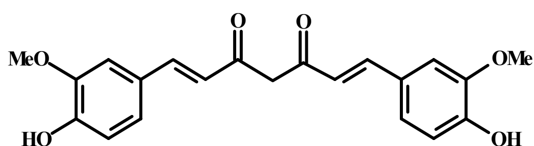
is in accordance with the previous study of Simon, *et al*. (1998) of which demethoxycurcumin showed stronger cytotoxic activity than curcumin and bisdemethoxycurcumin. Furthermore, the activity of CLM02 (curcumin) against MCF7 cells with IC_{50} value of 8.3 μg/ml was in the similar range with the previous report as 10 μg/ml (Kuo, Huang and Lin, 1996). According to the chemical structures, it could be proposed that the hydroxylated benzene ring and/or the α,β-unsaturated ketone moieties are essential for cytotoxic activity of the eight 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 be active through inhibition of telomerase activity in human breast cancer cells (Ramachandran, *et al*., 2002). Huang *et al*. (1997) pointed out that this effect of curcumin may be linked to its strong inhibitory action on DNA and RNA synthesis that has been previously shown on cultured Hela cells. 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 CLM03 (demethoxycurcumin) and ZOM0 (6-shogaol). However, the anticancer drug vinblastine sulphate was found to be far more active than all of 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 AGM1 (*p*-coumaryl-9-methyl ether) as a new cytotoxic compound in the present work provided 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 therapeutic potential against tumour cells of the rhizomes of *A. galanga*, *C. longa* and *Z. officinale.*



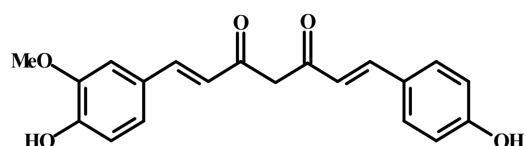
AGM1 (*p*-Coumaryl-9-methyl ether)



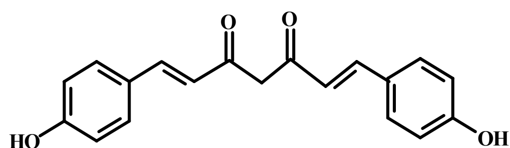
CLM01 (ar-Turmerone)



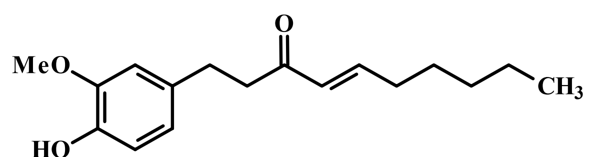
CLM02 (Curcumin)



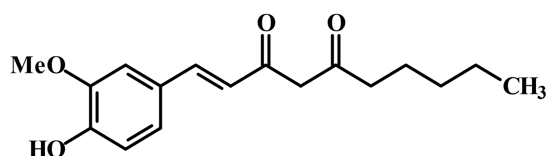
CLM03 (Demethoxycurcumin)



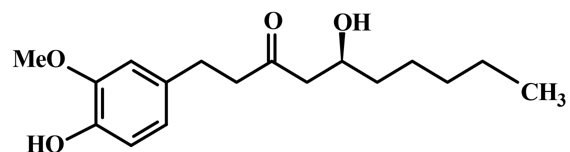
CLM06 (Bisdemethoxycurcumin)



ZOM0 (6-Shogaol)



ZOM1 (6-Dehydrogingerdione or 1-Dehydrogingerdione)



ZOM3 (6-Gingerol)

Figure 7 Structure of the isolated compounds from the rhizome of *Alpinia galanga*, *Curcuma longa* and *Zingiber officinale*.

Table 30 EC_{50} values against DPPH radical and IC_{50} values against LS174T and MCF7 cells of the isolated compounds from *A. galanga*, *C. longa* and *Z. officinale*.

Compounds	EC_{50} (μg/ml) (DPPH) (mean±SD)	IC_{50} against LS174T (μg/ml) (mean±SEM)	IC_{50} against MCF7 (μg/ml) (mean±SEM)
AGM1 (*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)
CLM01 (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)
CLM02 (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)
CLM03 (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)
CLM06 (Bisdemethoxycurcumin)	40.9±4.8 (n=6) (132.4±16.2 μM)	*	*
ZOM0 (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)
ZOM1 (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)
ZOM3 (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)
Butylated hydroxytoluene (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±0.00 nM (N=2)

* CLM06 was obtained only 2 mg, not sufficient for cytotoxic test.

n = number of samples tested

N = number of independent experiments (6 replicates in each experiment)