CHAPTER 3

RESULTS AND DISCUSSION

3.1 Screening of biological activity of crude extracts

The ethanolic extracts and water extracts of leaves of *Bridelia ovata* Decne, rhizomes of *Curcuma zedoaria* (Berg) Roscoe, stems of *Derris scandens* (Roxb.) Benth, rhizomes of *Dioscorea membranacea Pierre*, rhizomes of *Drynaria quercifolia* Linn., stems of *Erythrophleum teysmannii* Craib, barks of *Moringa oleifera* Lamk., flowers of *Nardostachys jatamansi* DC., roots of *Rhinacanthus nasutus* (L.) Kurz., fruits of *Sapindus rarak* DC., rhizomes of *Smilax corbularia* Kunth and seeds of *Strychnos nux-vomica* L were prepared as described in section 2.3. Yields of the extracts are shown in Table 3-1.

Table 3-1 %Yields of the ethanolic and water extracts from the investigated spices.

Plant Species	Part	Extracts	% Yield (w/w)
Bridelia ovata Decne	Leaf	EtOH	12.54
		Water	15.84
Curcuma zedoaria (Berg)Roscoe	Rhizome	EtOH	8.08
		Water	10.39
Derris scandens (Roxb.) Benth	Stem	EtOH	9.14
		Water	12.24
Dioscorea membranacea Pierre	Rhizome	EtOH	2.55
		Water	24.93
Drynaria quercifolia Linn.	Rhizome	EtOH	3.44
		Water	12.97
Erythrophleum teysmannii Craib	Stem	EtOH	2.02
		Water	3.63
Moringa oleifera Lamk.	Bark	EtOH	4.82
		Water	10.28
Nardostachys jatamansi DC	Flower	EtOH	2.73
		Water	6.93
Rhinacanthus nasutus (L.) Kurz.	Root	EtOH	4.88
		Water	16.58
Sapindus rarak DC.	Fruit	EtOH	33.28
		Water	30.15
Smilax corbularia Kunth	Rhizome	EtOH	12.03
		Water	8.25
Strychnos nux-vomica L.	Seed	EtOH	6.35
		Water	9.35

3.1.1 Cytotoxic activity

The cytotoxic activity of the ethanolic and water extracts of the twelve plants was evaluated by the Sulphorhodamine B (SRB) assay. The results of cytotoxic activity of the extracts (screening) are shown in Tables 3-2 and 3-3.

Table 3-2 % survival of cancerous cells (± SEM) (lung adenocarcinoma cell line = COR-L23 and prostate cancer cell line = PC3) treated with extract concentration 50 µg/ml exposure time 72h (n = 3)

Plant Species	extracts	code	Cell line	
			COR-L23	PC3
Bridelia ovata Decne	Et	BO2	4.65±0.09	1.16±0.25
	W	BO1	107±0.17	117.82±1.98
Curcuma zedoaria (Berg) Roscoe	Et	CZ2	0.31±0.07	1.32±0.35
	W	CZ1	122.28±1.2	122.21±1.32
Derris scandens (Roxb.) Benth	Et	DS2	0.04±0.10	42.42±1.04
	W	DS1	133.22±1.7	143.14±2.31
Dioscorea membranacea Pierre	Et	DM2	1.55±0.07	0.73±0.28
	W	DM1	131.23±1.7	137.27±3.6
Drynaria quercifolia Linn.	Et	DQ2	58.18±2.43	83.92±2.58
	W	DQ1	113.76±1.28	119.89±3.22
Erythrophleum teysmannii Craib	Et	ET2	7.39±0.85	98.95±2.30
	W	ET1	112.31±1.77	141.08±2.40
Moringa oleifera Lamk	Et	MO2	105.47±1.15	98.09±3.49
	W	MO1	128.32±1.77	126.66±2.79
Nardostachys jatamansi DC	Et	NJ2	37.27±0.99	24.73±1.33
	W	NJ1	114.12±1.81	133.63±3.47
Rhinacanthus nasutus (L.) Kurz.	Et	RN2	3.44±0.33	2.17±0.61
	W	RN1	124.23±8.27	140.57±2.74
Sapindus rarak DC.	Et	SR2	75.87±5.06	32.93±1.19
	W	SR1	115.0±1.56	124.66±2.01
Smilax corbularia Kunth	Et	SC2	101.93±2.36	124.86±2.12
	W	SC1	98.94±0.82	126.62±2.9
Strychnos nux-vomica L.	Et	SN2	94.34±1.14	103.29±2.48
	W	SN1	117.52±1.45	125.01±2.46

Et= ethanolic extract, W= water extract and n = number of independent experiment which was performed in 6 replicates.

Table 3-3 Cytotoxicity activity (IC_{50} μg/ml±SEM) of plant extracts against two types of cancer cell (COR-L23, PC3) and one type of normal cells (10FS) at exposure time 72 hours (n=3)

Plant Species	Extracts	Cell line		
		10FS	**COR-L23**	**PC3**
Bridelia ovata Decne	EtOH	9.11±0.58	7.11±0.004 **(1.28)**	6.29±0.59 **(1.45)**
	Water	>100	>50	>50
Curcuma zedoaria (Berg) Roscoe	EtOH	55.50±1.32	6.05±0.40 **(9.17)**	17.84±0.36 **(3.11)**
	Water	>100	>50	>50
Derris scandens (Roxb.) Benth	EtOH	32.98±0.07	21.04±0.57 **(1.57)**	43.45±3.60 **(0.76)**
	Water	>100	>50	>50
Dioscorea membranacea Pierre	EtOH	66.05±1.25	4.63±0.20 **(14.27)**	17.55±1.98 **(3.76)**
	Water	>100	>50	>50
Drynaria quercifolia Linn.	EtOH	>100	>50	>50
	Water	>100	>50	>50
Erythrophleum teysmannii Craib	EtOH	>100	37.76±0.01	>50
	Water	>100	>50	>50
Moringa oleifera Lamk.	EtOH	>100	>50	>50
	Water	>100	>50	>50

Table 3-3 (continued)

Plant Species	Extracts	Cell line		
		10FS	**COR-L23**	**PC3**
Nardostachys jatamansi DC	EtOH	>100	45.7±0.70	22.97±1.89
	Water	>100	>50	>50
Rhinacanthus nasutus (L.) Kurz.	EtOH	10.95±2.46	5.05±0.25 **(2.17)**	2.01±0.58 **(5.45)**
	Water	>100	>50	>50
Sapindus rarak DC.	EtOH	>100	37.02±1.21	44.34±1.44
	Water	>100	>50	>50
Smilax corbularia Kunth	EtOH	>100	>50	>50
	Water	>100	>50	>50
Strychnos nux-vomica L.	EtOH	>100	>50	>50
	Water	>100	>50	>50

n = Number of independent experiment, the numbers which were in (...) behind IC_{50} value were the ratio of IC_{50} (µg/ml) normal cells (10FS)/IC_{50} (µg/ml) cancer cells of extracts at exposure time 72 h.

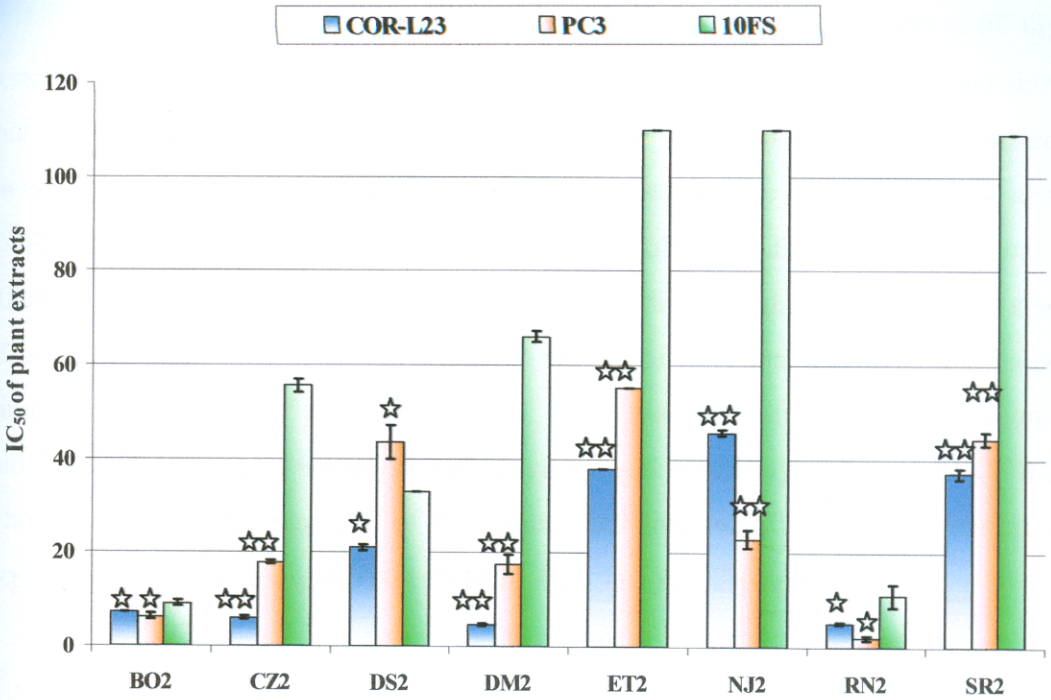


Figure 3-1 IC_{50} (µg/ml) of active crude extracts on cell lines (n=3) exposure time 72h using student t-test from Prism to compare the significant difference between normal cell (10FS) and each cancer cell (COR-L23 and PC3) by ☆ for P<0.05 and ☆☆ for P<0.0001. The ethanolic extract of *Bridelia ovata* (BO2), *Curcuma zedoaria* (CZ2), *Derris scanden* (DS2), *Dioscorea membranacea* (DM2), *Erytropheum teysmannii* (ET2), *Nardostachys jatamansi* (NJ2), *Rhinacanthus nasutus* (RN2), *Sapindus rarax* (SR2)

The results of cytotoxicity evaluation of all plant extracts at 50 μg/ml concentration and exposure time 72 hours are shown in Table 3-2. This data showed that the ethanolic extract of *Bridelia ovata, Curcuma zedoaria, Dioscorea membranacea and Rhinacanthus nasutus* exhibited high cytotoxic activity against COR-L23 and PC3 (in the percent survival range of 0.31-4.65%). Among them, the ethanolic extract of *Dioscorea membranacea* rhizomes was the most cytotoxic against PC3 (% survival = 0.73) and the ethanolic extract of *Curcuma zedoaria* could against lung cancer COR-L23 (%survival = 0.31). The ethanolic extracts of *Derris scandens* and *Erythrophleum teysmannii* showed high cytotoxic activity only against COR-L23 where the percentage survival of the cancer cell line for 50 μg/ml concentration at exposure time 72 hours were 0.04±0.10 and 7.39±0.85, respectively.

Calculations of the IC_{50} values of all plants are shown in Table 3-3. This data showed that the water extract of all plants exhibited no cytotoxic activity against two cancer cell lines and normal cells. The ethanolic extract of four plants showed cytotoxic activity against lung and prostate cancer cells followed by the American National Cancer Institue (NCI) (IC_{50} <20 μg/ml for crude extract) (Boyde,1997). The data showed that IC_{50} of ethanolic extract of *Dioscorea membranacea* showed the highest and *Curcuma zedoaria* rhizome showed the second most effective activity against the lung cancer cell line. They exhibited specific activity against lung cancer cells higher than prostate cancer cells but less active with normal cell. IC_{50} of ethanolic extract of *Dioscorea membranacea* were 4.63, 17.55 and 66.05μg/ml respectively and IC_{50} of ethanolic extract of *Curcuma zedoaria* rhizome were 6.05, 17.84 and 55.50 μg/ml respectively. The ethanolic extract of *Rhinacanthus nasutus* root showed the highest cytotoxic activity against prostate cancer (IC_{50} = 2.01 μg/ml) and the ethanolic extract of *Bridelia ovata* leaves showed the second most effective activity against prostate cancer cells (IC_{50} = 6.29 μg/ml). Although the ethanolic extract of *Rhinacanthus nasutus* root and the ethanolic extract of *Bridelia ovata* leaves exhibited the most activity against prostate cancer but they also showed activity against lung cancer cell lines and normal cells. This was described as nonspecific cytotoxic activity. The comparison between the ratio of normal cell/lung cell found that the ethanolic extracts of *Dioscorea membranacea* and *Curcuma zedoaria* have the highest difference ratio (14.27 and 9.17, respecively) and

value was extremely significant ($p<0.0001$) calculated by student T-test from Prism program. The ratio of normal cell/prostate cancer cells of the ethanolic extract of *Rhinacanthus nasutus* exhibited the highest ratio (5.45). From this result it was concluded that the ethanolic extracts of *Dioscorea membranacea* and *Curcuma zedoaria* showed selective toxicity against lung cancer cells and deserved for looking for the plant extracts or their active ingredients which can kill cancer cells but less harmful to normal cells. So this result related with the objectives of cancer chemotherapy which can kill cancer cells with as little damage as possible to normal cells and should be selectively active (Halliwell and Gatteridge, 1988). Although, the ethanolic extract of *Rhinacanthus nasutus* showed the highest ratio for comparison between normal and prostate cancer cells but it also showed high cytotoxic activity and was not selective of cells. This result related with the previous investigation, which found that many compounds from *R. nasutus* against P388, HL-60, KB, HT-29 and A549 and all compounds killed all cell lines and no had specific cytotoxic activity (Wu *et al.*, 1988; Wu *et al.*, 1998b). Surprisingly, *Bridelia ovata* which showed high cytotoxic activity against all types of cells has no reported on cytotoxic activity. The specific activity against cancer cells will be the first criterion for further investigation. So the ethanolic extracts of *Dioscorea membranacea* and *Curcuma zedoaria* should be selected for further study rather than the ethanolic extract of *Rhinacanthus nasutus.* However, the evaluation of the ingredients in this cancer formula found that nearly 50% of ingredients showed cytotoxic activity followed the previous criteria of NCI on 1990 (IC_{50} of the active plant extracts < 20 μg/ml) (Suffness and Pezzuto, 1991).

So, these data could support using this traditional drug formula to treat cancer patients, but it may depends on a small amount of active ingredients which may still be hidden. From the previous data it was found that Dioscorealide B, isolated from the ethanolic extract of *Dioscorea membranacea* also showed specific cytotoxic activity against lung cancer cell line COR-L23 (Itharat *et al.*, 2003). This compound may be used as a marker for analysis of this preparation against lung cancer cells. However, no report of this compound exhibited cytotoxic activity against prostate cancer and further phytochemical work on the isolation of this compound from *Dioscorea membranacea* is being proposed to test cytotoxic activity against prostate cancer cells. There is one report on data for cytotoxic compounds of

zedoaria against human ovarian cancer cell but it is no report for human lung and prostate cancer (Syu, *et al.*, 1998). These results give support for the use of Thai folk preparations for treating cancer patients (Itharat, 1998) and the result of three active plants (*Dioscorea membranacea, Curcuma zedoaria* and *Rhinacanthus nasutus*) also related with folk medicine texts. In addition, the extract of *Curcuma zedoaria* has been reported to have other activities which relate to cancer such as antimutagenic activity (Lee and Lin, 1988), antitumor activity (Kim *et al.*, 2000), anti-inflammatory activity (Hong *et al.*, 2002a; Hong *et al.*, 2002b; Jang *et al.*, 2001; Lee *et al.*, 2002; Yoshioka *et al.*, 1998) and antihepatotoxic activity (Matsuda *et al.*, 1998; Matsuda *et al.*, 2001; Morikawa *et al.*, 2002). Although the extracts of *Moringa oleifera* and *Smilax corbularia* had no cytotoxic activity in this test, there had been previous reports about cytotoxic acitivity on other cell lines. The extract of *Moringa oleifera* (leaf) showed cytotoxic activity against human promyelocytic leukemia cells (HL-60) (Siddhuraju and Becker, 2003) and leukemia (CEM) (stem) (Costa-Lotufo *et al.*, 2005) including the other cancer related activities i.e. antitumor promoting activity (Guevara *et al.*, 1999) and anti-inflammatory activities (Cáceres *et al.*, 1992). The extract of *Smilax corbularia* showed cytotoxic activity against CA-KB, antitumor activity against tumors in rats (IP) and immunostimulant activity (Pornsiriprasert *et al.*, 1986).

The plant extracts, used in this cancer preparation and had no cytotoxic activity, had been reported with activities which related to cancer i.e. the extract of *Derris scandens* (stem) increased lymphocyte proliferation at concentration of 10, 100 ng/ml, 1, 5 μg/ml (Sriwanthana and Chavalittumrong, 2001) and enhanced the lymphocyte proliferative and function of NK cells of normal individuals induced the IL-2 secretion at 100 ng/ml (Hoult *et al.*, 1997). The aqueous extract of *Derris scandens* (stem) showed reduction in paw edema test (82% for 100 mg/kg; 91% for 500 mg/kg) (Laupattarakasem *et al.*, 2003). *Nardostachys jatamansi* showed hepatoprotective activity at 800 mg/kg bodyweight in mice (Ali *et al.*, 2000). Both brucine and brucine N-oxide from *Strychnos nux-vomica* showed effects 2 h. after drug admistration, 80.3% for brucin N-oxide and 46.1% for brucine at a dose of 200 mg/kg in carrageenan-induced rat paw edema and brucine showed 109% PIP (pain inhibition percentage) with 30 mg/kg at 120 min after administration in hot-plate test. The

maximal inhibition of the nociceptive response was 79.5% for brucine N-oxide at the dose of 200 mg/kg in writhing test (Yin *et al.*, 2003).

3.1.2 Free radical scavenging activity

The antioxidant activity of the ethanolic and water extracts of the twelve plants was evaluated by DPPH radical scavenging assay as described in section 2.5. The results are shown in Table 3-4.

Table 3-4 EC_{50} (μg/ml) of plant extracts on DPPH assay

Plant Species	Ethanolic extracts (Mean±SEM)	Water extracts (Mean±SEM)
Bridelia ovata Decne	23.255±0.6 (n=3)	8.701±0.1 (n=3)
Curcuma zedoaria (Berg) Roscoe	14.031±1.0 (n=3)	47.170±0.2 (n=3)
Derris scandens (Roxb.) Benth	58.312±0.2 (n=3)	13.877±0.2 (n=3)
Dioscorea membranacea Pierre	13.857±1.5 (n=3)	38.614± 1.0 (n=3)
Drynaria quercifolia Linn.	79.265±0.1 (n=3)	23.351±0.2 (n=3)
Erythrophleum teysmannii Craib	2.050±0.2 (n=3)	0.625±0.1 (n=3)
Moringa oleifera Lamk.	>100 (n=2)	44.879±0.5 (n=3)
Nardostachys jatamansi DC	81.176±0.2 (n=3)	22.549±0.1 (n=3)
Rhinacanthus nasutus (L.) Kurz.	79.265±0.1 (n=3)	49.176±0.1 (n=3)
Sapindus rarak DC.	>100 (n=2)	0.625±0.2 (n=3)
Smilax corbularia Kunth	3.091±0.6 (n=3)	3.660±0.5 (n=3)
Strychnos nux-vomica L.	59.634±0.1 (n=3)	17.829±0.2 (n=3)
BHT (positive control)	11.583±0.5 (n=3)	

n = number of independent experiment

The results showing antioxidant activity using the DPPH assay of these twelve plants found that five ethanolic extracts (*Bridelia ovata*, *Curcuma zedoaria*, *Dioscorea membranacea*, *Erythrophleum teysmannii* and *Smilax corbularia*) and

eight water extracts (*Bridelia ovata*, *Derris scandens*, *Drynaria quercifolia*, *Erythrophleum teysmannii*, *Nardostachys jatamansi*, *Sapindus rarak*, *Smilax corbularia* and *Strychnos nux-vomica*) showed high antioxidant activity with EC_{50} in the range of 2.050-23.255 μg/ml and 0.625-23.351 μg/ml, respectively. Interestingly, the ethanolic extracts of *Erythrophleum teysmannii* and *Smilax corbularia*, and the water extracts of *Bridelia ovata*, *Erythrophleum teysmannii*, *Sapindus rarak* and *Smilax corbularia* showed an antioxidative effect higher than BHT which is the standard antioxidant substance especially water extracts of *Erythrophleum teysmannii* and *Sapindus rarak* showed the highest antioxidant activity in this test (EC_{50} = 0.625±0.1 and 0.625±0.2 μg/ml, respectively). Surprisingly, there were no reports on *Erythrophleum teysmannii* and *Sapindus rarak* which showed the highest effectiveness in this test. Although the extracts of *Derris scandens*, *Drynaria quercifolia*, *Erythrophleum teysmannii*, *Nardostachys jatamansi*, *Sapindus rarak*, *Smilax corbularia* and *Strychnos nux-vomica* had no cytotoxic activity against cancer cell lines, they may be useful for protection against free radicals which are more prevalent in cancer patients. These results can support the use of this Thai folk medicine preparation in the treatment of cancer because the tests showed high antioxidant activity for neutralizing free radicals which could cause damage in the initiation and promotion phase of cancer (Thurnham, 1993). In addition, there are several reports which gave support on antioxidant activity of the extracts. For example, the rhizome extract of *Curcuma zedoaria* showed antioxidant activity with DPPH and lipid peroxidation (Mau *et al.*, 2003). The stem extract of *Derris scandens* showed antioxidant activity by DPPH assay (Mahabusarakam *et al.*, 2004) and hypoxanthine/xanthine oxidase system (Laupattarakasem *et al.*, 2003). The rhizome extract of *Dioscorea membranacea* showed antioxidant activity by DPPH and lipid peroxidation of liposome assay (Itarat, 2002). The seed extract of *Strychnos nux-vomica* showed antilipid peroxidative property in the free iron system (Tripathi and Chaurasia, 2000).

In contrast, the bark extract of *Moringa oleifera* showed no antioxidant activity in this result but the leaf extract of *Moringa oleifera* had been reported for antioxidant activity in several systems such as the β-carotene-linoleic acid system, the linoleic acid peroxidation system, scavenging activity on superoxide radicals

(Siddhuraju and Becker, 2003), free radical scavenging activity by DPPH, antioxidant activity on liposome peroxidation induced by Fe^{3+}/H_2O_2/Ascorbic acid and inhibition of the enzymatic lipid peroxidation of microsomal lipids induced by NADPH/ADP/Fe^{3+} (Siddhuraju and Becker, 2003).

It is promising that further work on isolation of pure compounds of the ethanolic extracts of *Curcuma zedoaria* and *Dioscorea membranacea* is of interest because they showed high cytotoxic activity against lung and prostate cancer cell but less activity against normal cells and also showed high antioxidant activity.

3.2 Cytotoxic activity of bioassay-guided fractionation

Results from the preliminary assays for cytotoxic activity in section 2.4 and free radical scavenging activity in section 2.5 of the ethanolic and water extracts of twelve plants are shown in section 3.1. They give evidences of the presence of active constituents in the ethanolic extracts of *C. zedoaria* and *D. membranacea* so a separation of these active extracts was carried out as shown in section 2.6. These six fractions; three fractions from the ethanolic extracts of *C. zedoaria*: FA1, FA2 and FA3, and three fractions from the ethanolic extracts of *D. membranacea*: FB1, FB2 and FB3) were tested for cytotoxic activity using the method described in section 2.4 (data shown in Table 3-5 and Figure 3-2).

Table 3-5 IC_{50} (μg/ml) ± SEM of the fractions from *Curcuma zedoaria* and *Dioscorea membranacea* separated by vacuum liquid chromatography against two cancer cell lines at exposure time 72 hours

Fraction	%Yield	Cell lines	
		COR-L23	PC3
FA1 (Hexane)	2.1	27.073±0.906 (n=2)	29.791±1.173 (n=3)
FA2 ($CHCl_3$)	36.7	25.503±1.365 (n=2)	32.150±0.924 (n=2)
FA3 (MeOH)	47	29.354±0.623 (n=2)	29.094±1.813 (n=2)
FB1 ($CHCl_3$)	10.1	28.047±0.321 (n=3)	28.760±0.569 (n=2)
FB2 (9$CHCl_3$:1MeOH)	0.7	28.798±0.132 (n=2)	9.288±0.655 (n=2)
FB3 (MeOH)	24.1	>100	72.229±0.320 (n=2)

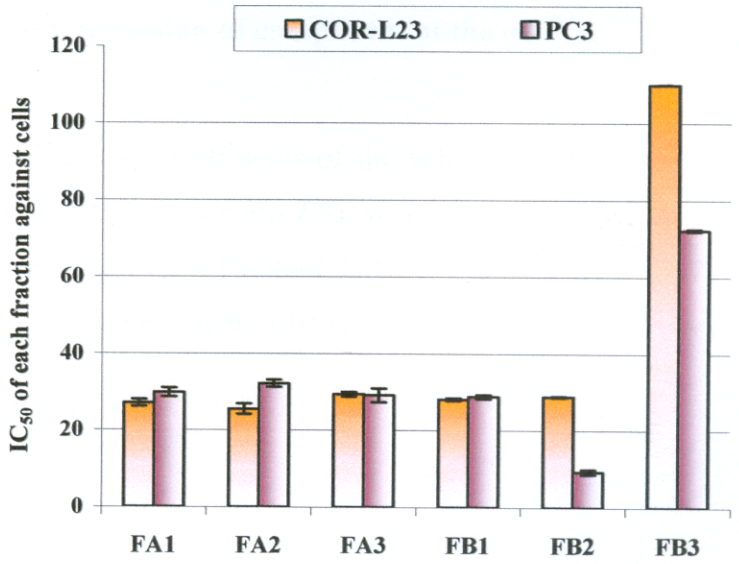


Figure 3-2 Histogram comparing IC_{50} (μg/ml) of each fractions against two cancer cell lines at exposure time 72 h.

FA1-3, FB1 and FB3 showed less cytotoxicity against the human lung cancer cell line (COR-L23) and the human prostate cancer cell line (PC3) than the crude extracts of *C. zedoaria* and *D. membranacea* (Table 3-3 and 3-4). There might be explanation for a reduction of activity after fractionation that it might be due to the breakdown of compounds as a result of the fractionation process, or because of a loss of synergy when some constituents of plant extract were separated (Houghton, 2000). In contrast, FB2 was the only fraction which showed high and specific cytotoxicity against human prostate cancer cell line (PC3) because of a synergy of some constituents as a result of the fractionation. However, following the results of the assay in Tables 3-3 and 3-4, the crude extract of *C. zedoaria* and FB2 were explicitly chosen for separation to find active compounds because of their high and specific cytotoxicity.

3.3 Analysis of chemical composition and structure determination of the isolated compounds

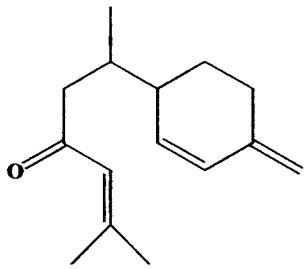
3.3.1 Characterization of component of the oil

Chemical constituents of the yellow oil (2061.4 mg, 29.4%) which is isolated from fractions 1-5 (section 2.8), was analyzed by Gas chromatography/Mass spectrometry with a Hewlett-Packard HP 5890 plus GC-HP 5972 Mass Selective Detector. Chemical constituents of the volatile oil from *C. zedoaria* are shown in Table 3-6.

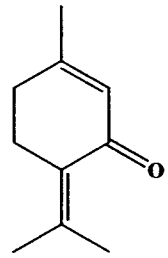
Table 3-6 Chemical constituent of the oil from *Curcuma zedoaria* (GC/MS analysis)

Retention time (min)	% Area	Compound	Molecular weight	Molecular formula
10.54	1.793	Piperitenone	150.10	$C_{10}H_{14}O$
14.62	3.546	Unable to be identified	-	-
15.46	7.369	Unable to be identified	-	-
16.19	34.724	β-Turmerone	218.17	$C_{15}H_{22}O$
16.70	4.296	Unable to be identified	-	-
17.36	2.932	(6S, 1'R)-6-(1', 5'-Dimethylhex-4'-enyl)-3-methylcyclohex-2-enone	220.18	$C_{15}H_{24}O$
18.95	3.521	Unable to be identified	-	-
19.17	4.619	Unable to be identified	-	-
20.25	3.176	Unable to be identified	-	-
20.97	4.154	Unable to be identified	-	-
22.86	1.265	Unable to be identified	-	-

Chemical components of the oil, analyzed by GC/MS technique, were shown in Table 3-6. The oil of *C. zedoaria* (CZV) was composed of at least 11 compounds having β-Turmerone (34.724%) as a major component as found in many previous reports (Mau *et al.*, 2003; Lee *et al.*, 2002; Hong *et al.*, 2001). In addition, piperitenone and (6S,1'R)-6-(1',5'-dimethylhex-4'-enyl)-3-methylcyclo hex-2-enone were in the yellow oil of *C. zedoaria* (CZV). They should be confirmed by GC/MS (high resolution) which not be afforded in this study.



β-turmerone



piperitenone

Figure 3-3 Structures of compounds detected in the volatile oil of *C. zedoaria*.

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File       : C:\HPCHEM\1\DATA\CZF1-5N1.D
Operator   :
Acquired   :  7 Jun 05  15:58     using AcqMethod 6492N2
Instrument :   GC/MS Ins
Sample Name: czf1-5 in hexane
Misc Info  :
Vial Number: 1
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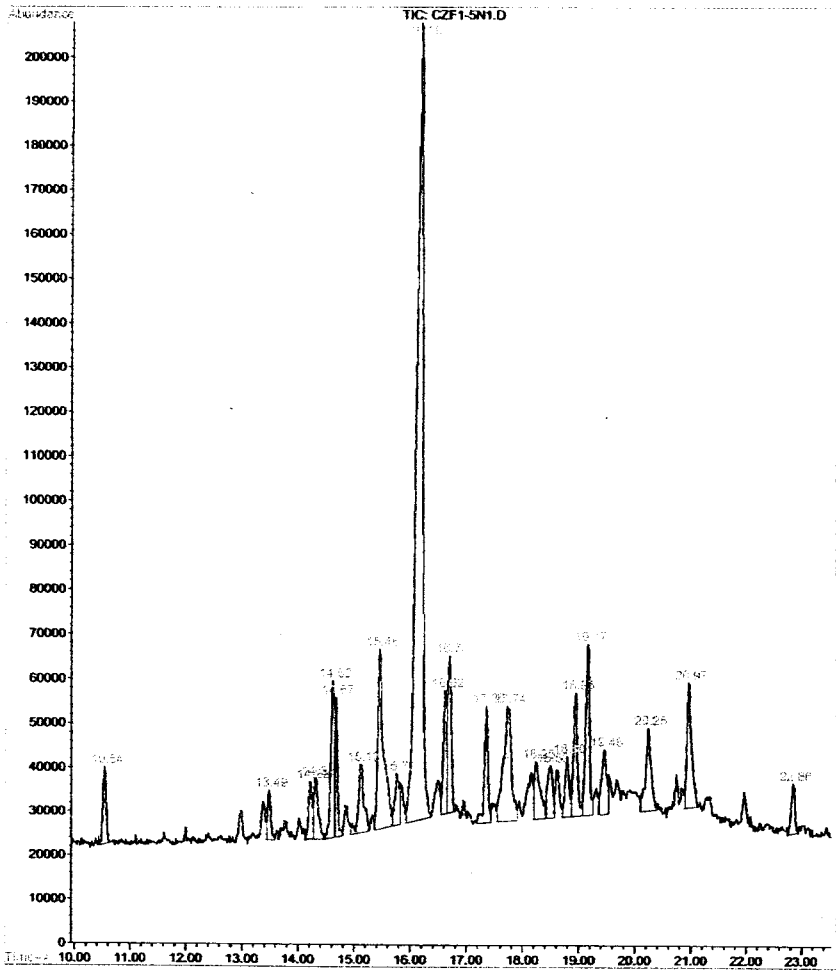


Figure 3-4 GC-MS spectrum of CZV in hexane

3.3.2 Structure elucidation of the isolated compounds

Results from the bioassay-guided fractionation for cytotoxic activity were shown in section 3.2. Thus, a separation of the active extracts was carried out as shown in section 2.8 to give the pure compounds as follows.

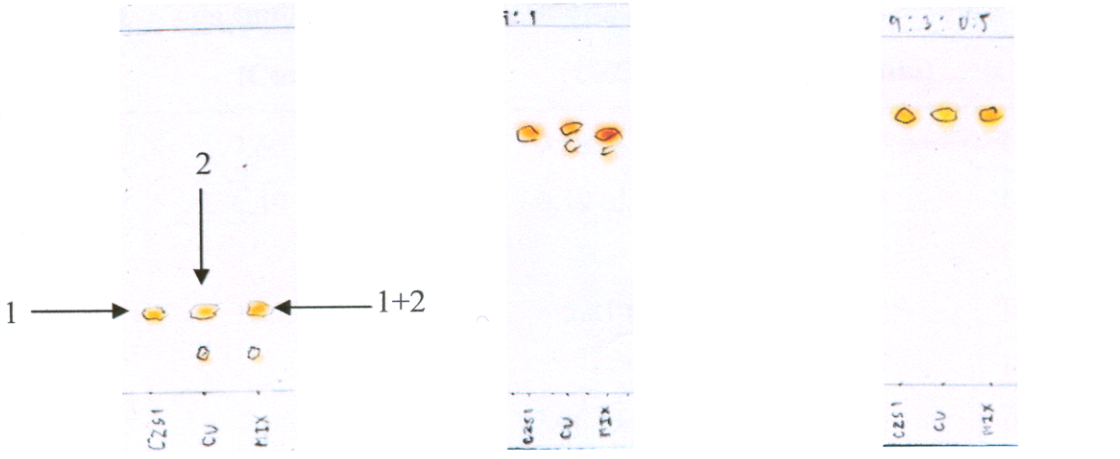
3.3.2.1 CZS1

CZS1 (Curcumin): $C_{21}H_{20}O_6$ (60 mg, 0.8571%); orange crystals; UV ($CHCl_3$) λ_{max} 264, 414 nm; IR ($CHCl_3$) ν_{max} 3400, 3015, 2929, 2852, 1582, 1511, 1434 cm^{-1}; EIMS (low resolution) *m/z* (% relative intensity) 368 (M^+, 41), 350 (42), 272 (14), 217 (17), 191 (32), 190 (41), 177 (100). This compound was compared with authentic sample curcumin (mixture of curcumin, bisdemethoxycurcumin and demethoxtcurcumin) (Sigma) by TLC using 3 solvent systems and gave identical behavior (Figure 3-5).

CZS1 was the major compound of the ethanolic extract of *C. zedoaria*, obtained as orange crystals and showed protonated molecular ion peak in EI mass spectrum at *m/z* 368, corresponding with a molecular formula of $C_{21}H_{20}O_6$ (MW = 368). The IR ($CHCl_3$) spectrum indicated the presence of hydroxyl group at 3400, carbonyl function at 1582 and olefinic carbon stretching at 1511 cm^{-1}. Its UV ($CHCl_3$) spectrum showed absorption maxima at 264 and 414 nm.

^1H and ^{13}C NMR spectra of CZS1 revealed the existence of aromatic, olefinic, methoxyl, hydroxyl and carbonyl functions. Analysis of chemical shifts, integration and spin coupling patterns of these functional groups indicate that CZS1 was a diarylheptanoid. In the ^{13}C NMR (Table 3-7), 11 carbon signals was observed with signal of carbonyl function at δ 183.2. The ^1H NMR showed two methoxy groups at δ 3.96 (6H) which were shown by sharp and strong signals at high field and other important peaks for ^1H NMR and ^{13}C NMR are shown in Table 3-7. The ^1H NMR and ^{13}C NMR spectrum, compared with the previous ^1H NMR and ^{13}C NMR data of curcumin, was the same as the spectrum recorded for curcumin (Zaeoung,

2004). Thus, it was strongly supported that CZS1 to be curcumin.



2 hexane : 8 $CHCl_3$ 9 $CHCl_3$: 1 MeOH 9 $CHCl_3$: 3 MeOH : 0.3 H_2O

Figure 3-5 TLC of CZS1 in 2 hexane : 8 $CHCl_3$, 9 $CHCl_3$: 1 MeOH and 9 $CHCl_3$: 3 MeOH : 0.3 H_2O [1=CZS1, 2=Curcumin(Sigma)]

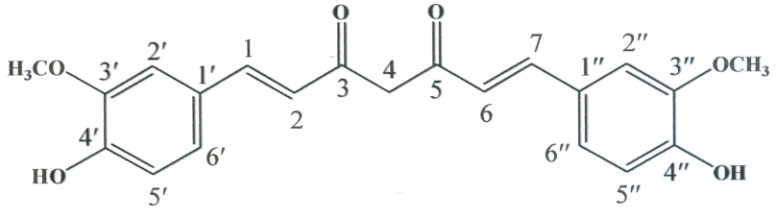


Figure 3-6 CZS1 (Curcumin)

Table 3-7 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of CZS1 and curcumin in $CDCl_3$

atom	δ_H (mult., *J* in Hz) (Curcumin)	δ_H (mult., *J* in Hz) (CZS1)	δ_C (Curcumin)	δ_C (CZS1)
1, 7	7.60 (d, 15.5)	7.60 (d, 16)	140.5	140.5
2, 6	6.49 (d, 15.5)	6.49 (d, 16)	121.8	121.7
3, 5	-	-	183.2	183.2
4	5.81 (s)	5.81 (s)	101.2	101.1
1′, 1″	-	-	127.7	127.6
2′, 2″	7.06 (d, 2.0)	7.06 (d, 2.0)	109.6	109.5
3′, 3″	-	-	146.8	146.7
4′, 4″	-	-	147.8	147.8
5′, 5″	6.92 (d, 8.3)	6.94 (d, 8.0)	114.8	114.8
6′, 6″	7.14 (dd, 8.3, 2.0)	7.13 (dd, 8.0, 2.0)	122.9	122.8
3′-OMe, 3″-OMe	3.96 (s; 6H)	3.96 (s; 6H)	55.9	55.9
4′-OH, 4″-OH	5.88 (br.s; 2H)	5.88 (br; 2H)	-	-

Note: Curcumin from Zaeoung, 2004

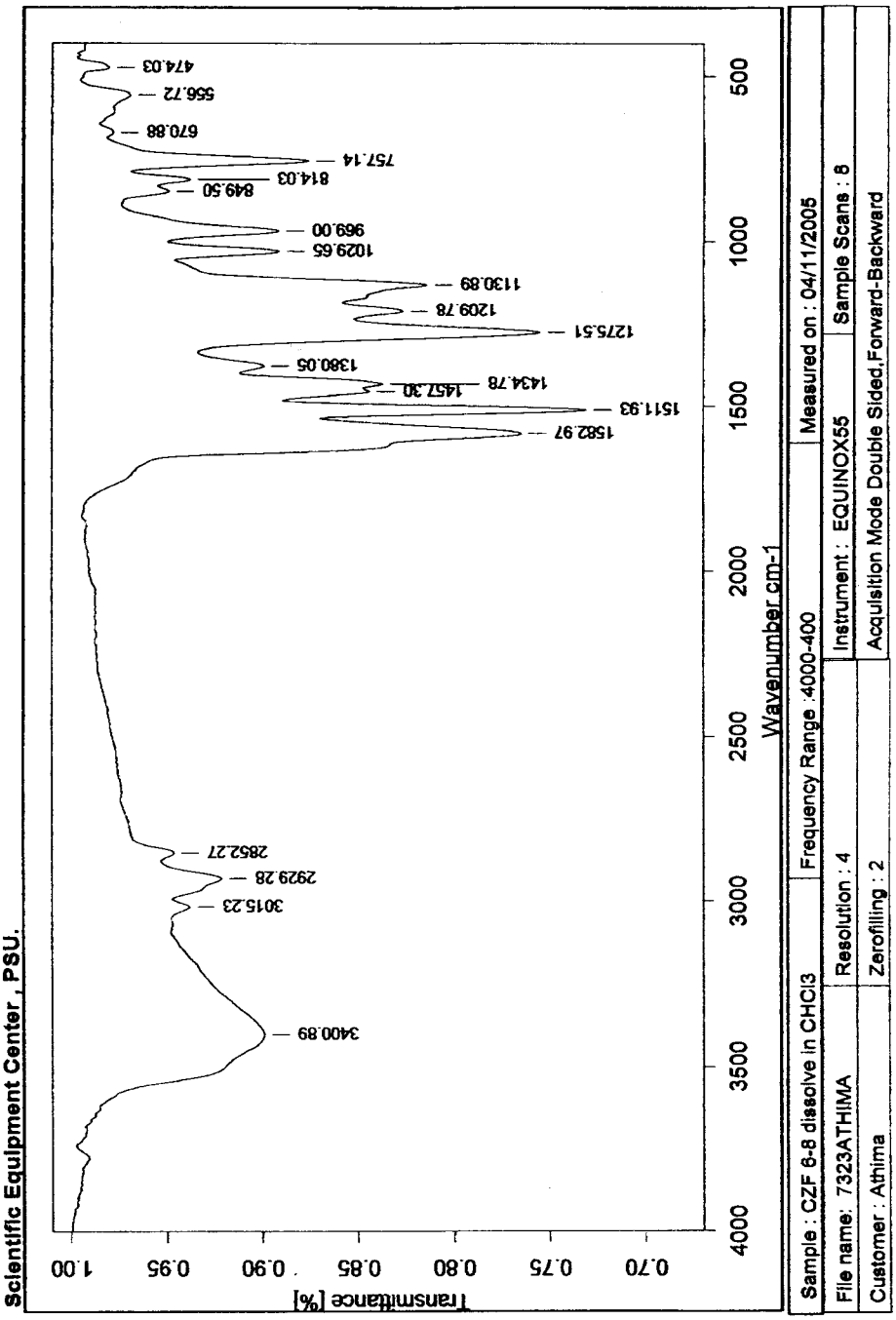


Figure 3-7 IR spectrum of CZS1 in $CHCl_3$

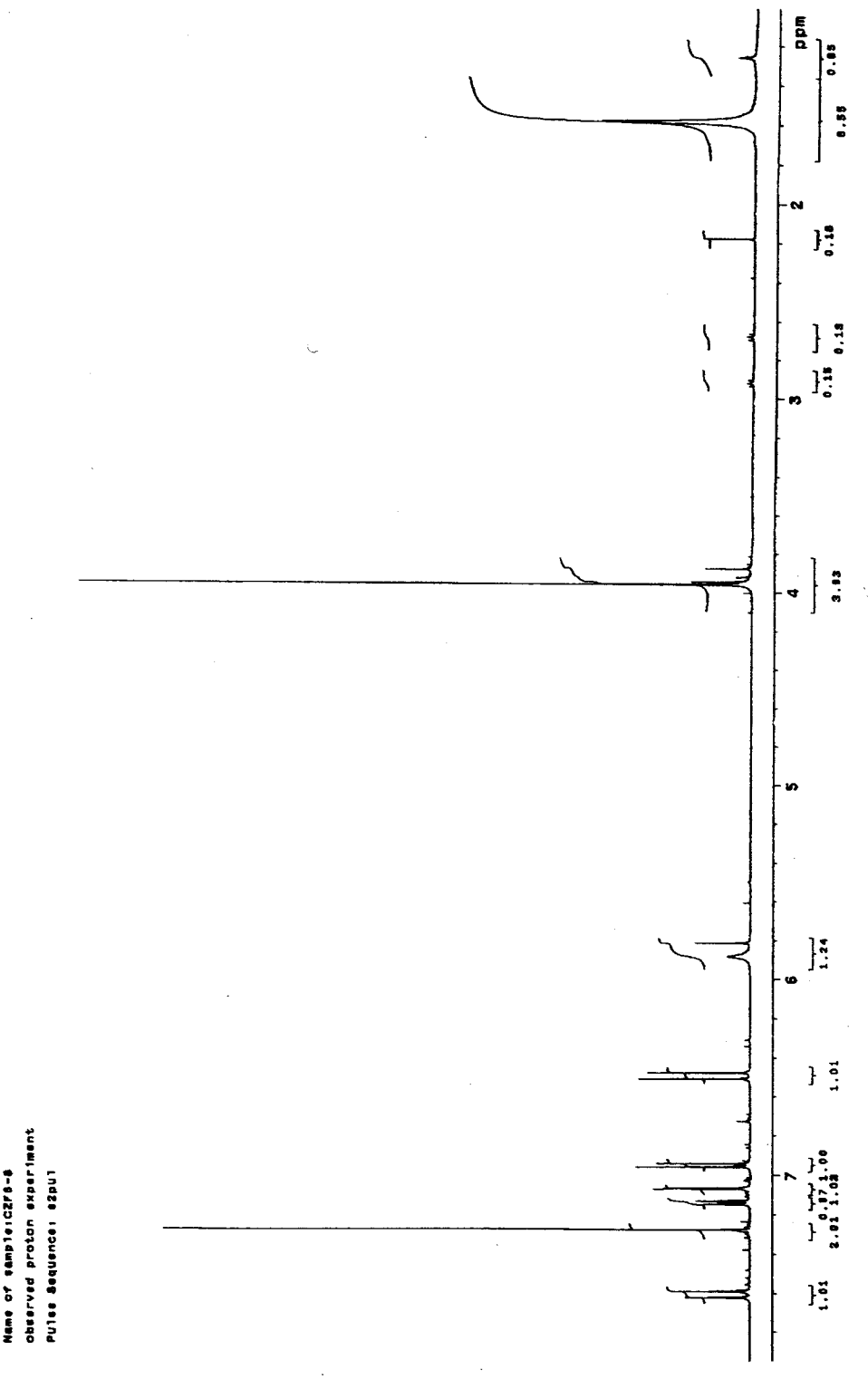


Figure 3-8 ^{1}H NMR spectrum of CZS1 in $CDCl_3$

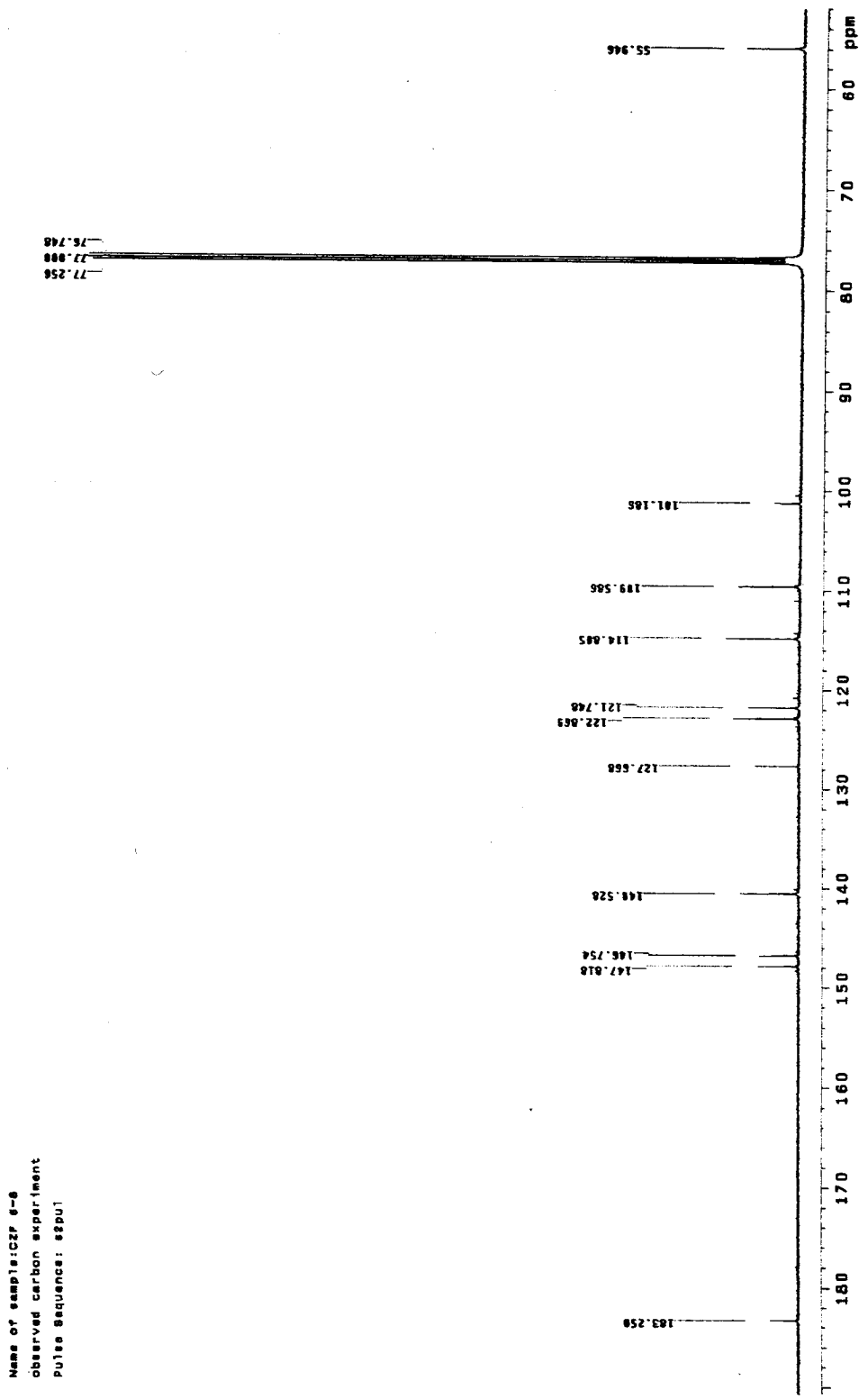


Figure 3-9 ^{13}C NMR spectrum of CZS1 in $CDCl_3$

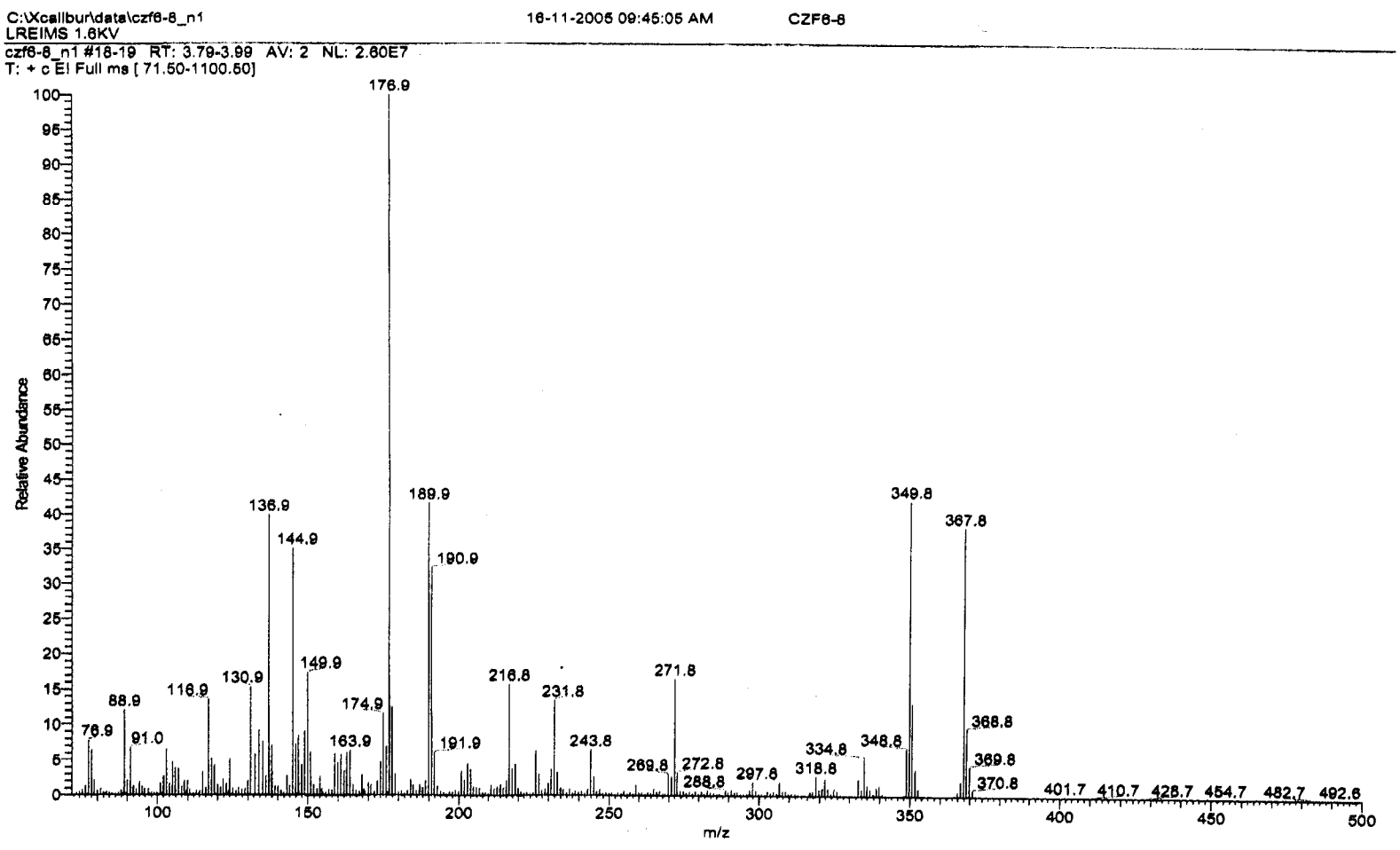
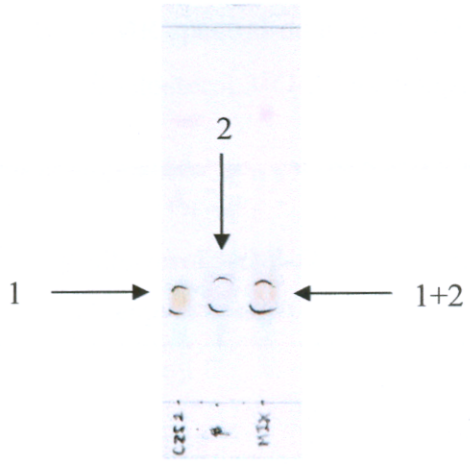


Figure 3-10 EIMS spectrum of CZS1

3.3.2.2 CZS2

CZS2 (β-sitosterol-3-*O*-β-*D*-glucopyranoside): white amorphous solid (2.3 mg, 0.0329%); UV ($CHCl_3$) λ_{max} 248 nm; IR ($CHCl_3$) ν_{max} 3379, 2924, 2858, 1733, 1642, 1456, 1374, 1248, 1160, 1067, 1022 cm^{-1}; EIMS (low resolution) *m/z* (% relative intensity) 396 (M^+: $C_6H_{11}O_5$, 100), 394 (29), 381 (18), 329 (11), 255 (24), 213 (22), 145 (41), 95 (51), 81 (57). This compound was compared with authentic sample from Itharat (Itharat, 2002) using TLC in 3 solvent systems gave identical behavior (Figure 3-11).

The ^1H NMR and ^{13}C NMR of CZS2 was shown in Table 3-8. The ^1H NMR and ^{13}C NMR spectrum, compared with the previous ^1H NMR and ^{13}C NMR data, was the same as that spectrum recorded for β-sitosterol-3-*O*-β-*D*-glucopyranoside (Itharat, 2002). The ^{13}C NMR data was in agreement with this result since the chemical shifts were different for C-3 of the aglycone part which was more down field because it connects with the sugar and C-1, C-2, C-3, C-4, C-5 and C-6 of sugar part was confirmed as glucose (Itharat, 2002). The IR spectra indicated the broad O-H stretching of glucose at 3379 and C-H stretching at 2924 cm^{-1}. The molecular formula from EI mass spectra deduced the molecular ion peak of aglycone-OH of CZS2 as $C_{29}H_{48}$ (*m/z* 396). Thus, it strongly supported that CZS2 is β-sitosterol-3-*O*-β-*D*-glucopyranoside. In pervious study, β-sitosterol-3-*O*-β-*D*-glucopyranoside showed a molecular ion peak in FAB-MS spectrum at *m/z* 576 [which is 599 in MS spectra as M^+ +Na (23)], corresponding with a molecular formula of $C_{35}H_{60}O_6$ (MW = 576) (Itharat, 2002).



9 $CHCl_3$: 1 MeOH

9 $CHCl_3$: 3 MeOH : 0.3 H_2O

Figure 3-11 TLC of CZS2 in 9 $CHCl_3$: 1 MeOH and 9 $CHCl_3$: 3 MeOH : 0.3 H_2O (1=CZS2, 2= β-Sitosterol 3-*O*-β-*D*-glucopyranoside)

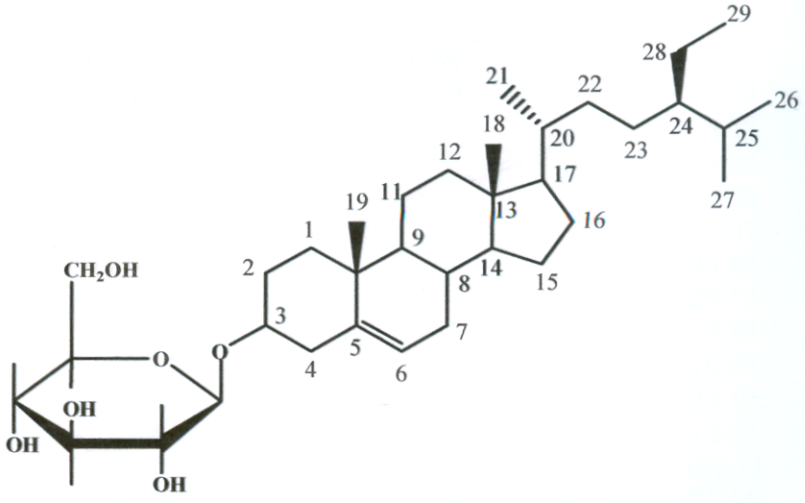


Figure 3-12 CZS2 (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)

Table 3-8 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of CZS2 and β-sitosterol 3-*O*-β-*D*-glucopyranoside in $CDCl_3$+CD_3OD

atom	δ_H (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)	δ_H (CZS2)	δ_C (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)	δ_C (CZS2)
1	-	-	37.28	37.13
2	-	-	31.91	31.76
3	3.58	3.57	78.63	75.49
4	-	-	39.78	39.63
5	-	-	140.26	140.08
6	5.37	5.37	122.30	122.20
7	-	-	31.98	31.83
8	-	-	31.91	31.76
9	-	-	50.20	49.96
10	-	-	36.19	36.05
11	-	-	21.10	20.96
12	-	-	39.78	38.65
13	-	-	42.36	42.28
14	-	-	56.78	56.63
15	-	-	24.33	24.19
16	-	-	28.29	28.15
17	-	-	56.08	55.93
18	0.68	0.68	12.00	11.75
19	1.01	1.00	19.37	19.23
20	-	-	36.19	36.62
21	0.92	0.92	19.00	18.67
22	-	-	33.98	33.83
23	-	-	26.07	25.93
24	-	-	45.88	45.73
25	-	-	29.17	29.62
26	0.81	0.81	19.84	19.72
27	0.83	0.83	19.04	18.91
28	-	-	23.10	22.96
29	0.84	0.85	12.07	11.87
Glu-1	4.42	4.42	101.11	100.94
2	3.45-3.47	3.47-3.49	75.66	73.48
3	3.38-3.41	3.40-3.41	79.27	79.18
4	3.29-3.32	3.28-3.31	70.09	69.82
5	3.75-3.86	3.76-3.87	78.63	76.16
6	3.28	3.26	61.97	61.78

Note: β-Sitosterol 3-*O*-β-*D*-glucopyranoside from Itharat, 2002.

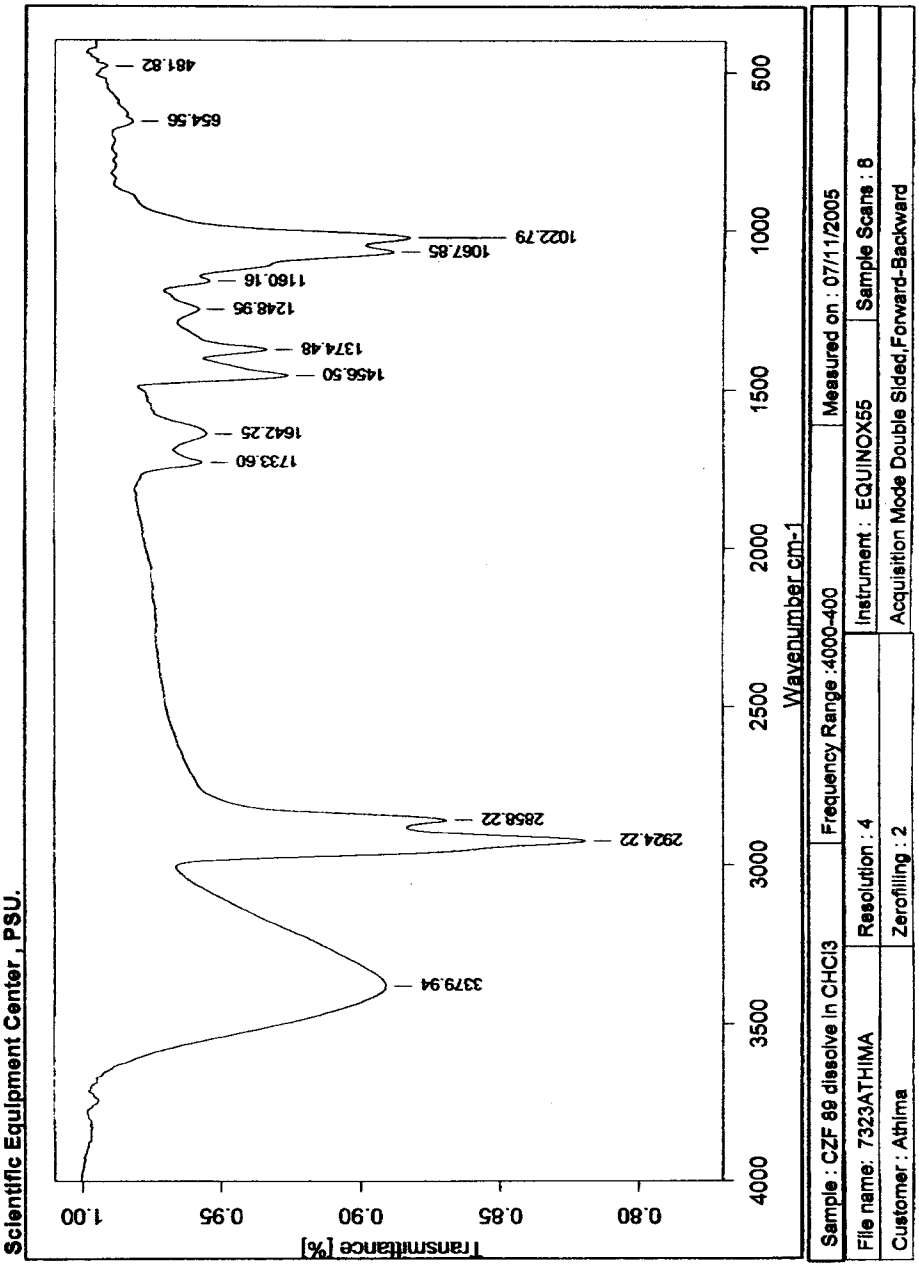


Figure 3-13 IR spectrum of CZS2 in $CHCl_3$

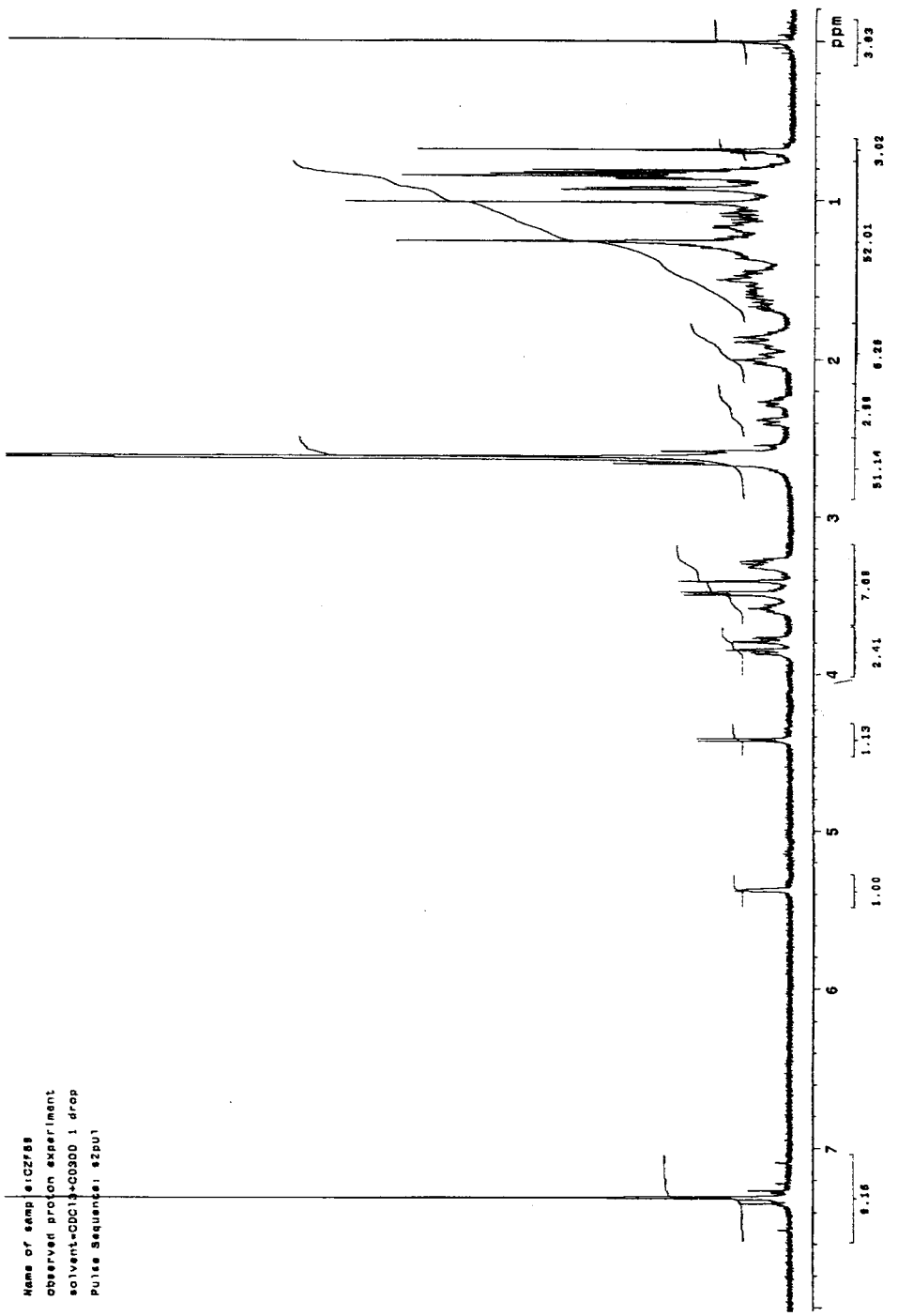


Figure 3-14 ^{1}H NMR spectrum of CZS2 in $CDCl_3$+CD_3OD

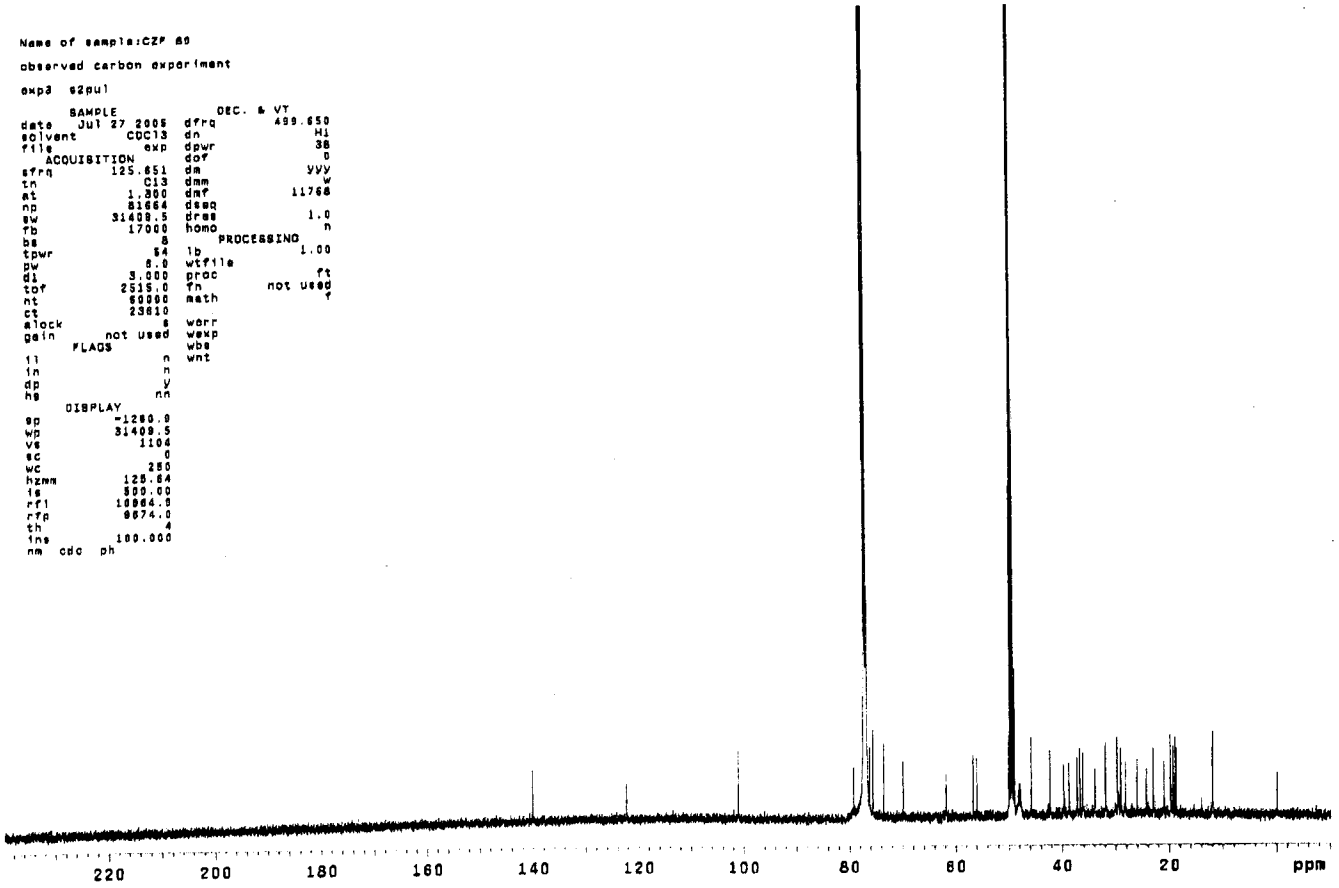


Figure 3-15 ^{13}C NMR spectrum of CZS2 in $CDCl_3$+CD_3OD

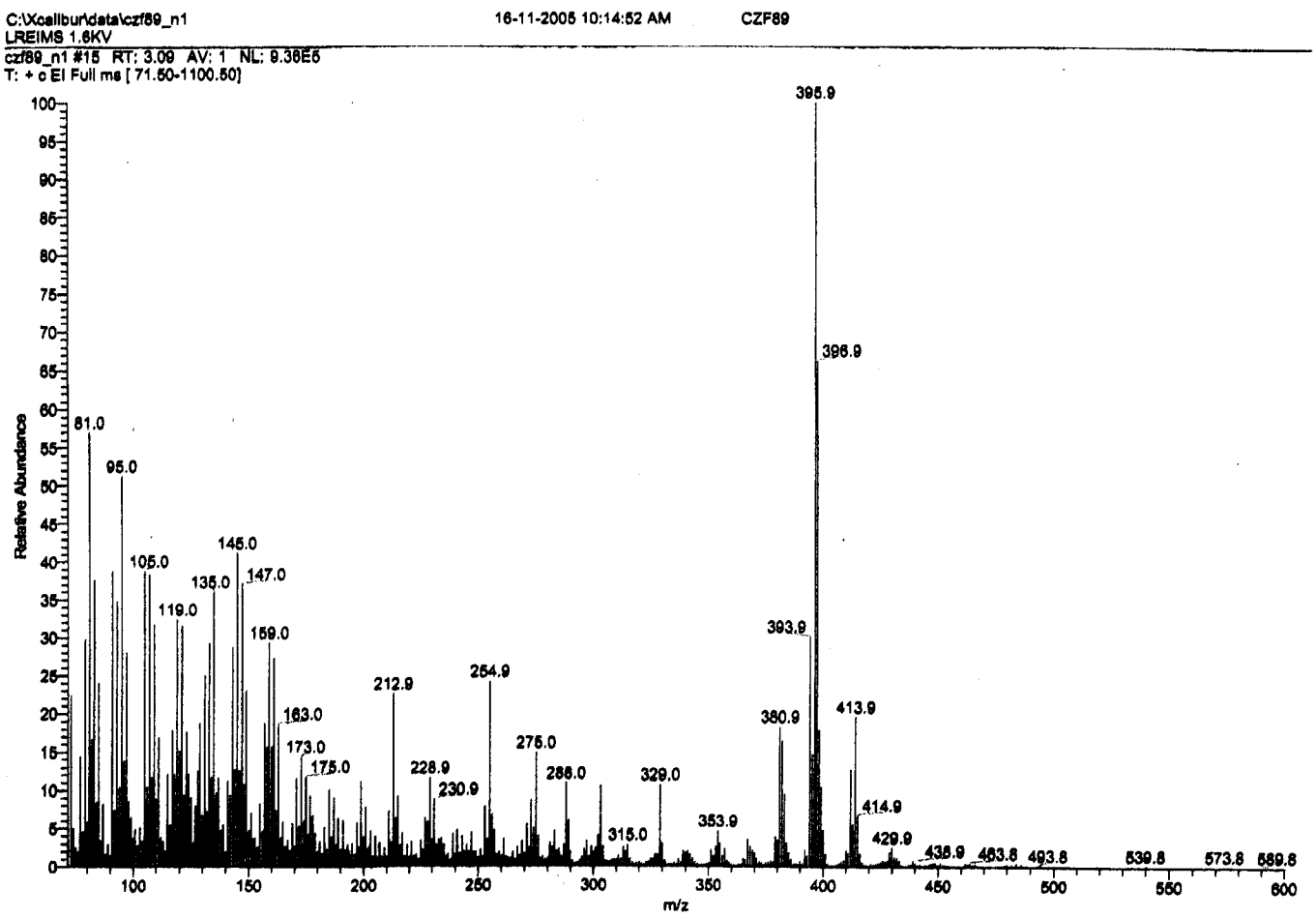
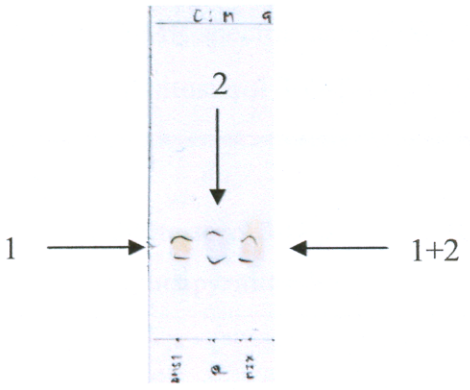


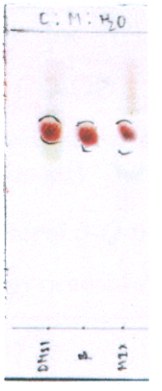
Figure 3-16 EIMS spectrum of CZS2

3.3.2.3 DMS1

DMS1 (β-sitosterol-3-*O*-β-*D*-glucopyranoside): white amorphous solid (22.6 mg, 0.2260%); UV ($CHCl_3$) λ_{max} 246 nm; IR ($CHCl_3$) ν_{max} 3380, 2927, 2860, 1726, 1674, 1629, 1452, 1374, 1246, 1159, 1023 cm^{-1}; EIMS (low resolution) *m/z* (% relative intensity) 396 (M^+: $C_6H_{11}O_5$, 100), 394 (39), 382 (23), 255 (22), 213 (11), 145 (21), 95 (19), 83 (26). This compound was compared with authentic sample from Itharat (Itharat, 2002) using TLC in 3 solvent systems.

The ^1H NMR and ^{13}C NMR of DMS1 is shown in Table 3-9. The ^1H NMR and ^{13}C NMR spectrum, compared with the previous ^1H NMR and ^{13}C NMR data and ^1H NMR and ^{13}C NMR of CZS2, was the same as the spectrum recorded for β-sitosterol-3-*O*-β-*D*-glucopyranoside (Itharat, 2002). The IR spectra indicated the broad O-H stretching of glucose at 3380 and C-H stretching at 2927 cm^{-1}. The molecular formula from EI mass spectra deduced the molecular ion peak of aglycone-OH of DMS1 as $C_{29}H_{48}$ (*m/z* 396). These data support that DMS1 was the same as CZF2. Thus, it is strongly supported that DMS1 is β-sitosterol-3-*O*-β-*D*-glucopyranoside ($C_{35}H_{60}O_6$, MW = 576) (Itharat, 2002).





9 $CHCl_3$: 1 MeOH

9 $CHCl_3$: 3 MeOH : 0.3 H_2O

Figure 3-17 TLC of DMS1 in 9 $CHCl_3$: 1 MeOH and 9 $CHCl_3$: 3 MeOH : 0.3 H_2O (1=DMS1, 2= β-Sitosterol 3-*O*-β-*D*-glucopyranoside)

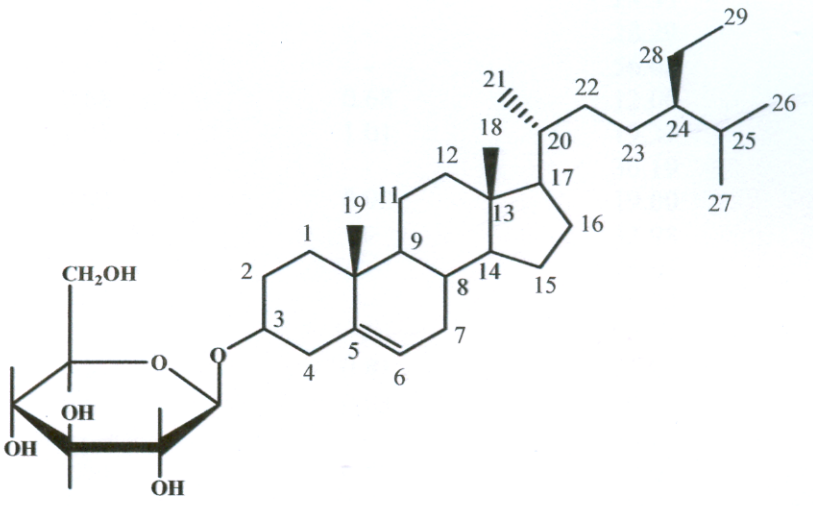


Figure 3-18 DMS1 (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)

Table 3-9 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of DMS1 and β-sitosterol 3-*O*-β-*D*-glucopyranoside in $CDCl_3$+CD_3OD

atom	δ_H (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)	δ_H (DMS1)	δ_C(mult) (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)	δ_C (DMS1)
1	-	-	37.28	37.05
2	-	-	31.91	31.68
3	3.58	3.58	78.63	75.58
4	-	-	39.78	39.47
5	-	-	140.26	140.08
6	5.37	5.37	122.30	122.02
7	-	-	31.98	31.74
8	-	-	31.91	31.68
9	-	-	50.20	49.98
10	-	-	36.19	36.52
11	-	-	21.10	21.03
12	-	-	39.78	38.48
13	-	-	42.36	42.13
14	-	-	56.78	56.55
15	-	-	24.33	24.10
16	-	-	28.29	28.06
17	-	-	56.08	55.85
18	0.68	0.68	12.00	11.75
19	1.01	1.01	19.37	19.59
20	-	-	36.19	35.96
21	0.92	0.92	19.00	18.79
22	-	-	33.98	33.74
23	-	-	26.07	25.84
24	-	-	45.88	45.65
25	-	-	29.17	29.52
26	0.81	0.81	19.84	20.87
27	0.83	0.83	19.04	19.10
28	-	-	23.10	22.86
29	0.84	0.85	12.07	12.03
Glu-1	4.42	4.42	101.11	100.88
2	3.45-3.47	3.44-3.49	75.66	73.30
3	3.38-3.41	3.37-3.41	79.27	79.06
4	3.29-3.32	3.28-3.31	70.09	69.58
5	3.75-3.86	3.76-3.85	78.63	76.15
6	3.28	3.26	61.97	61.36

Note: β-Sitosterol 3-*O*-β-*D*-glucopyranoside from Itharat, 2002.

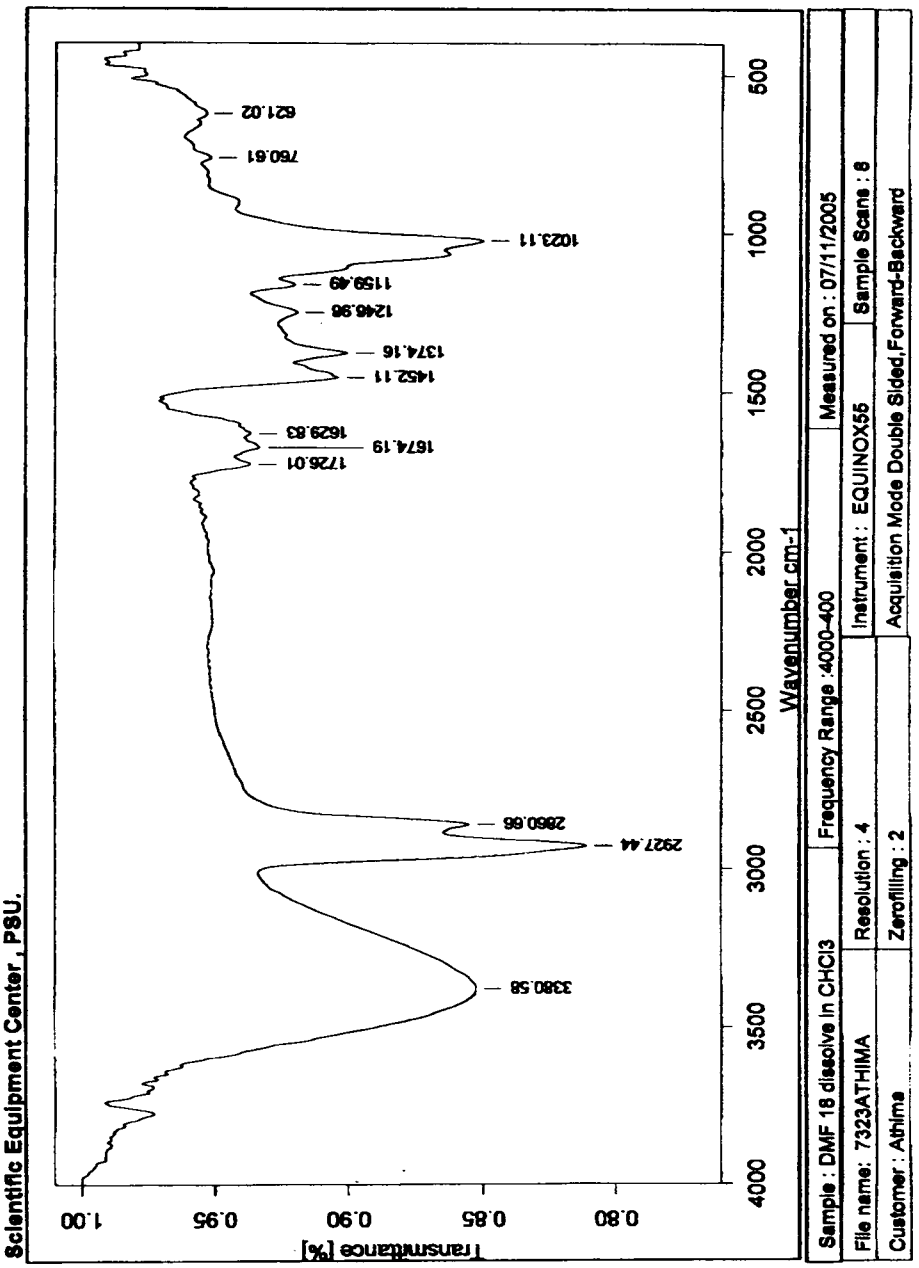


Figure 3-19 IR spectrum of DMS1 in $CHCl_3$

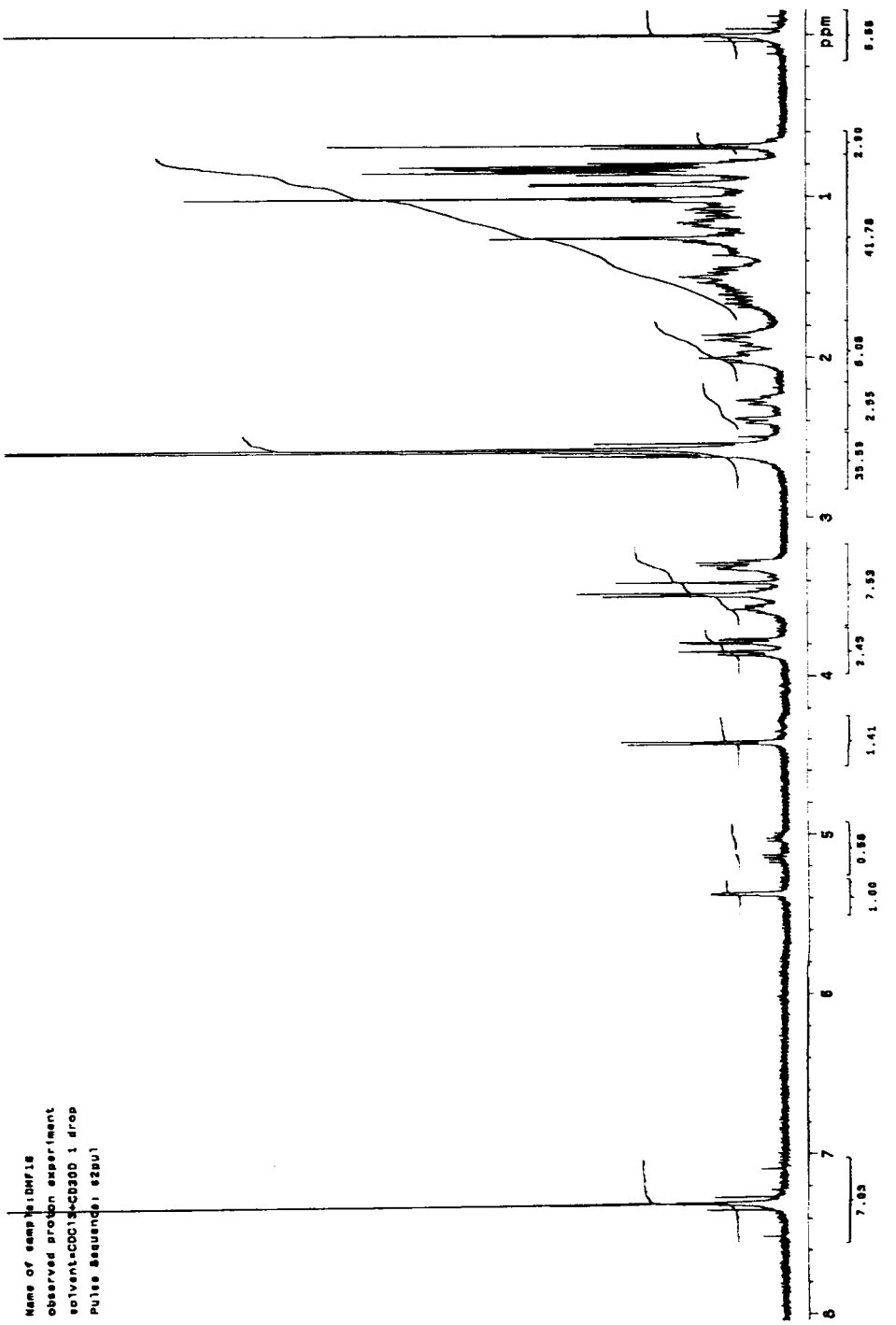


Figure 3-20 ^{1}H NMR spectrum of DMS1 in $CDCl_3$+CD_3OD

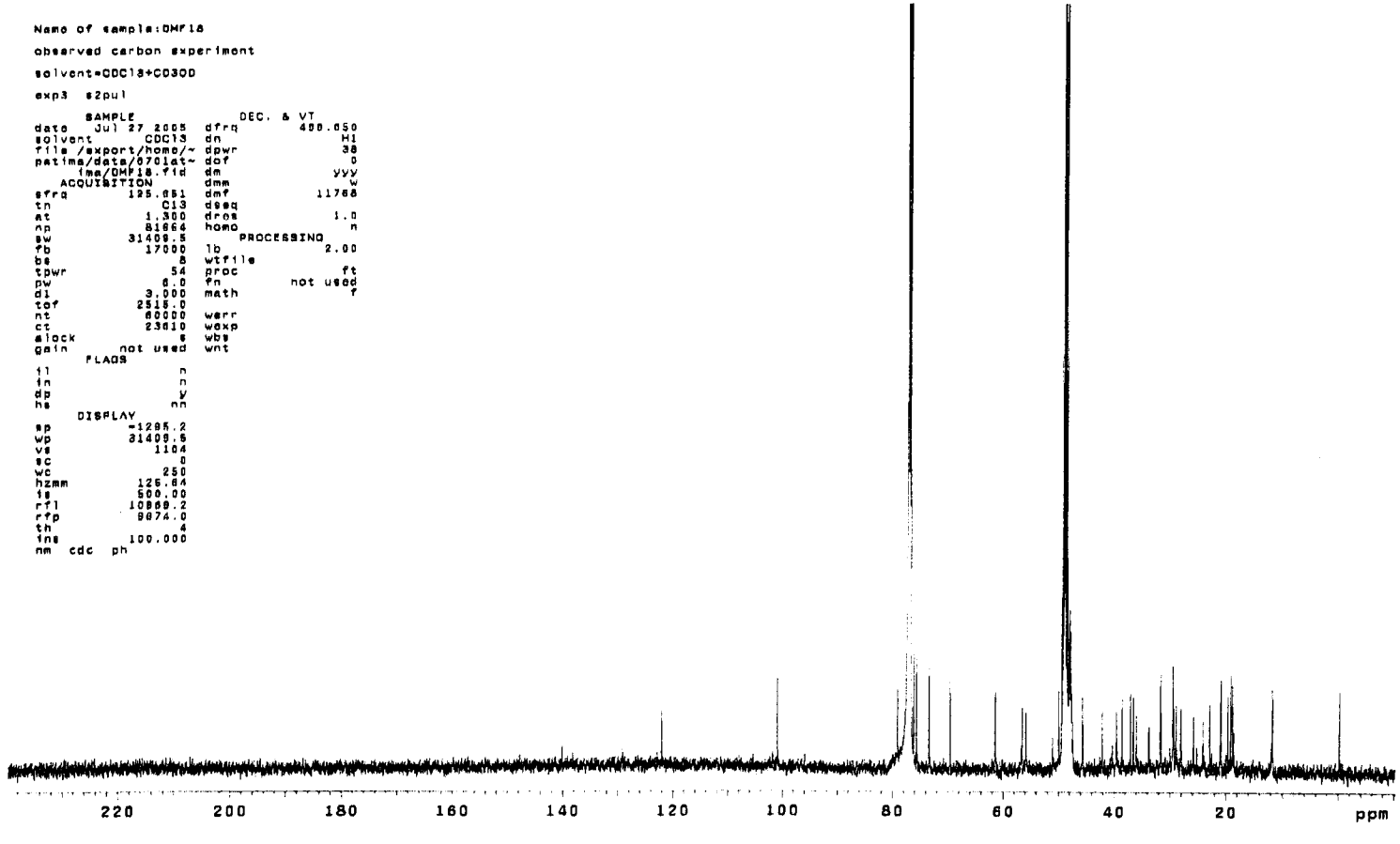


Figure 3-21 ^{13}C NMR spectrum of DMS1 in $CDCl_3$+CD_3OD

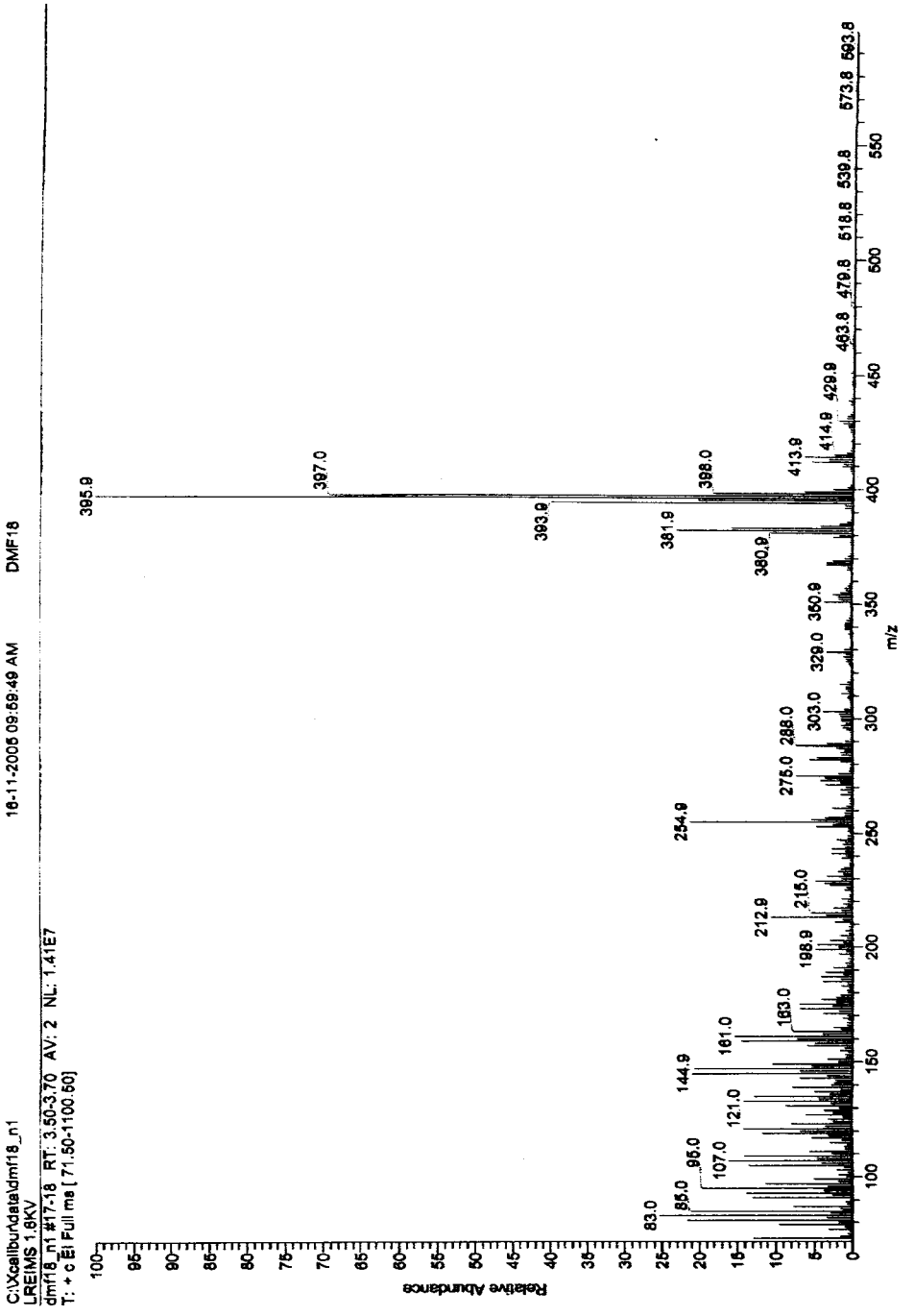
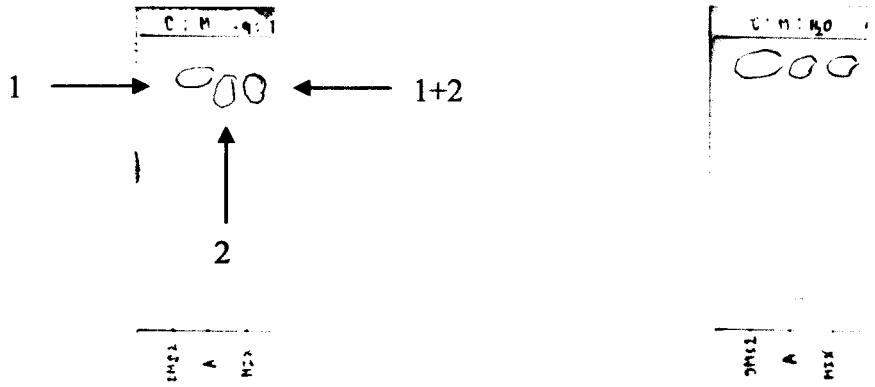


Figure 3-22 EIMS spectrum of DMS1

3.3.2.4 DMS2

DMS2 (Dioscorealide A): pale yellow crystal (17.7 mg, 2.514 %); UV ($CHCl_3$) λ_{max} 272, 310, 352 nm; IR ($CHCl_3$) ν_{max} 2943, 1765, 1683, 1624 cm^{-1}; EIMS (low resolution) *m/z* (% relative intensity) 314 (M^+; $C_{17}H_{14}O_6$, 51), 283 (100), 227 (13). This compound was compared with authentic samples from Itharat (Itharat, 2002) using TLC in 3 solvent systems.

The molecular formula of DMS2 was $C_{17}H_{14}O_6$ as deduced from EI mass spectra (*m/z* 314; M^+). The proposed molecular formula gave rise to the unsaturation degree of 11, which deduced from the IR ($CHCl_3$) spectrum as 1 ester carbonyl function at 1765 and C-H stretching at 2943 cm^{-1}, and the ^{13}C NMR (Table 3-10) as 1 ester carbonyl (δ 166.8), six olefinic double bonds (ranging from δ 160.9-103.5) and four ring systems. In the ^1H NMR spectrum (Table 3-10), two aromatic spin systems were observed. The first one was two *ortho* protons of a tetrasubstituted benzene ring (δ 7.75, d, J=8.5 Hz, H-2; and 7.69, d, J= 8.5 Hz, H-3), and the other was two *meta* protons of another tetrasubstituted benzene (δ 7.05, d, J=2.2 Hz, H-4; and 6.96, d, J= 2.2 Hz, H-6). The three methoxy groups showed signal at δ 3.96, 3.56 and 4.42. These ^1H NMR and ^{13}C NMR spectrum, compared by the previous ^1H NMR and ^{13}C NMR data of dioscorealide A showed identical spectra and also was the same as the spectrum recorded for dioscorealide A (Itharat *et al.*, 2003). Thus, it was strongly supported that DMS2 to be dioscorealide A.



9 $CHCl_3$: 1 MeOH 9 $CHCl_3$: 3 MeOH : 0.3 H_2O

Figure 3-23 TLC of DMS2 in 9 $CHCl_3$: 1 MeOH and 9 $CHCl_3$: 3 MeOH : 0.3 H_2O (1=DMS2, 2= Dioscorealide A)

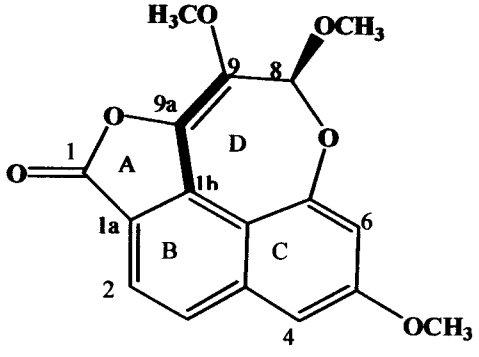


Figure 3-24 DMS2 (Dioscorealide A)

Table 3-10 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of DMS2 and dioscorealide A in $CDCl_3$

atom	δ_H (mult., *J* in Hz) (Dioscorealide A)	δ_H (mult., *J* in Hz) (DMS2)	δ_C (Dioscorealide A)	δ_C (DMS2)
1	-	-	166.8	166.8
1a	-	-	115.6	115.6
1b	-	-	136.7	136.7
2	7.72 (d, 8.5)	7.75 (d, 8.5)	121.4	121.4
3	7.66 (d, 8.5)	7.69 (d, 8.5)	127.9	127.9
3a	-	-	138.6	138.6
3b	-	-	116.2	116.1
4	7.03 (d, 2.0)	7.05 (d, 2.2)	103.6	103.5
5	-	-	161.0	160.9
6	6.94 (d, 2.0)	6.96 (d, 2.2)	110.5	110.5
6a	-	-	151.0	150.9
8	5.57 (s)	5.59 (s)	102.7	102.7
9	-	-	140.3	140.2
9a	-	-	130.4	130.3
5-OMe	3.96 (s; 3H)	3.96 (s; 3H)	55.7	55.6
8-OMe	3.56 (s; 3H)	3.58 (s; 3H)	56.8	56.8
9-OMe	4.24 (s; 3H)	4.26 (s; 3H)	60.9	60.9

Note: Dioscorealide A from Itharat *et al*, 2003.

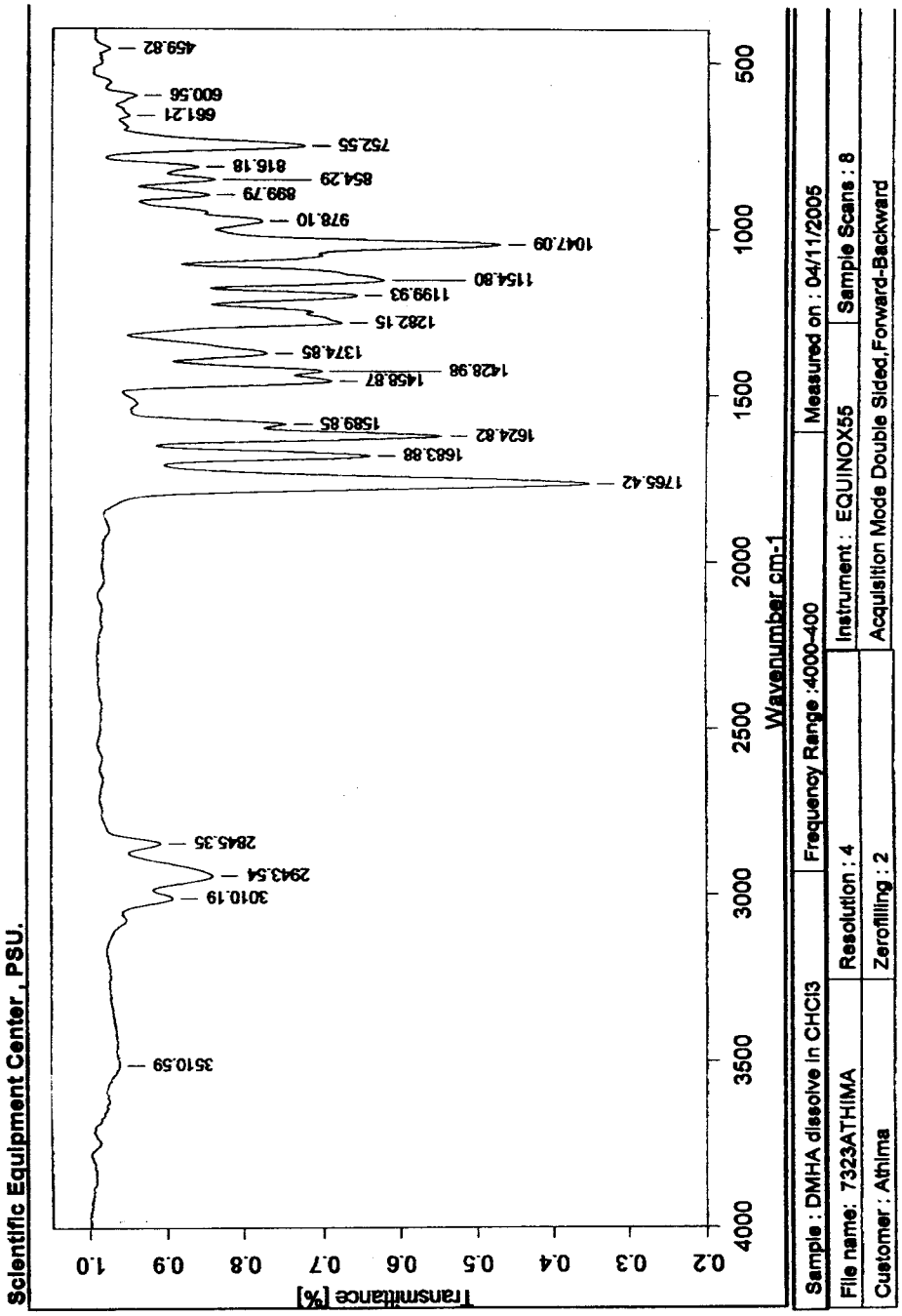


Figure 3-25 IR spectrum of DMS2 in $CHCl_3$

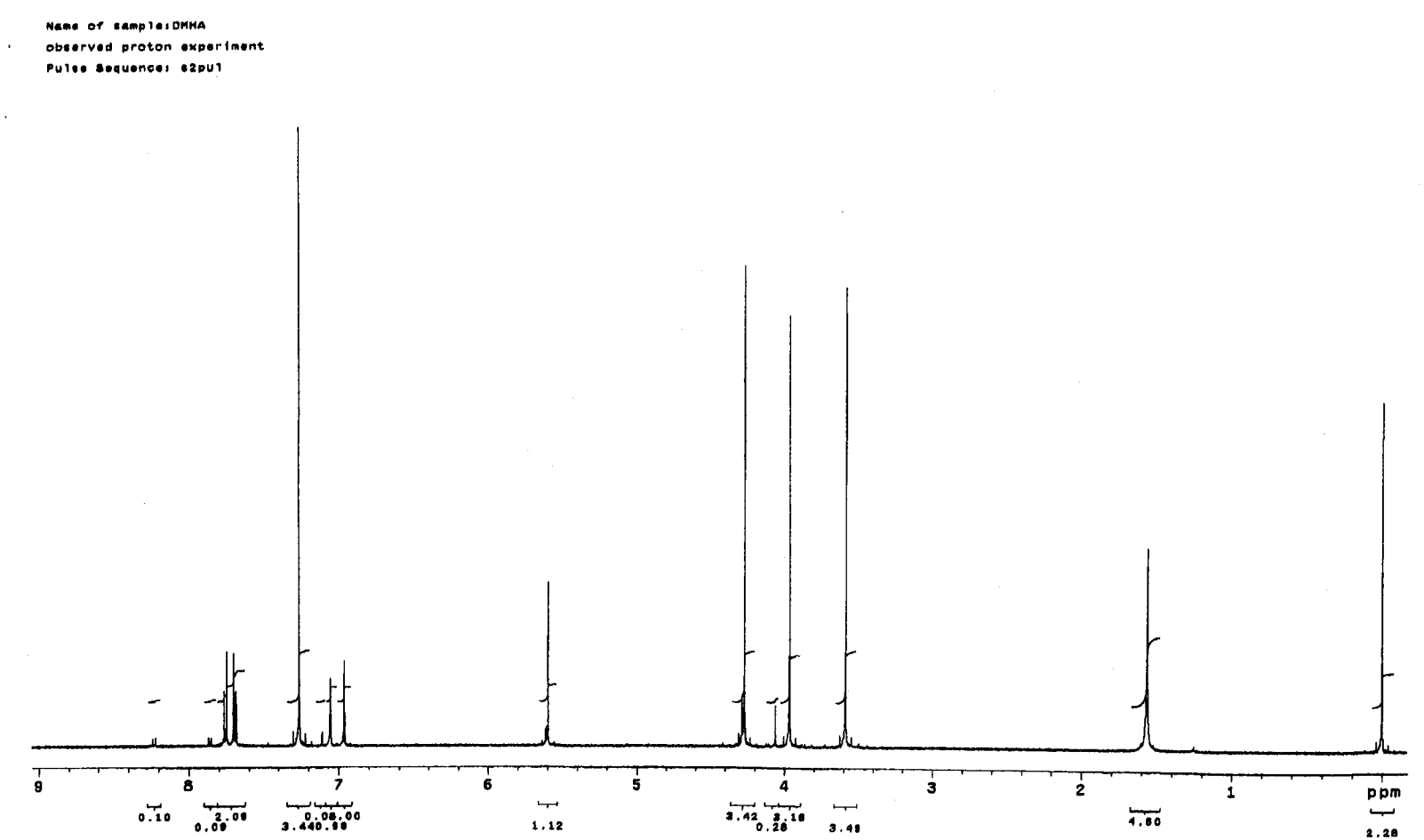


Figure 3-26 ^{1}H NMR spectrum of DMS2 in $CDCl_3$

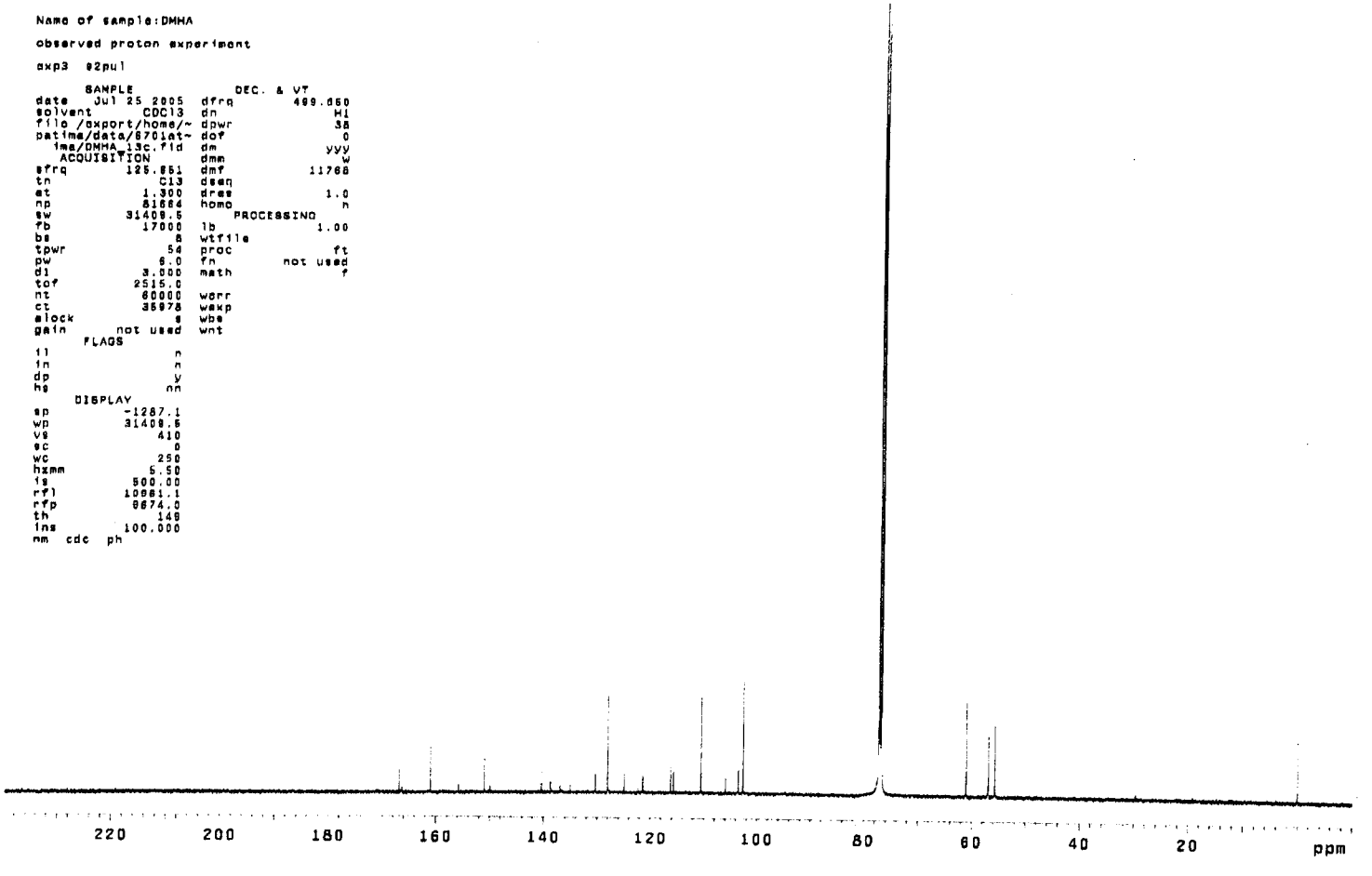


Figure 3-27 ^{13}C NMR spectrum of DMS2 in $CDCl_3$

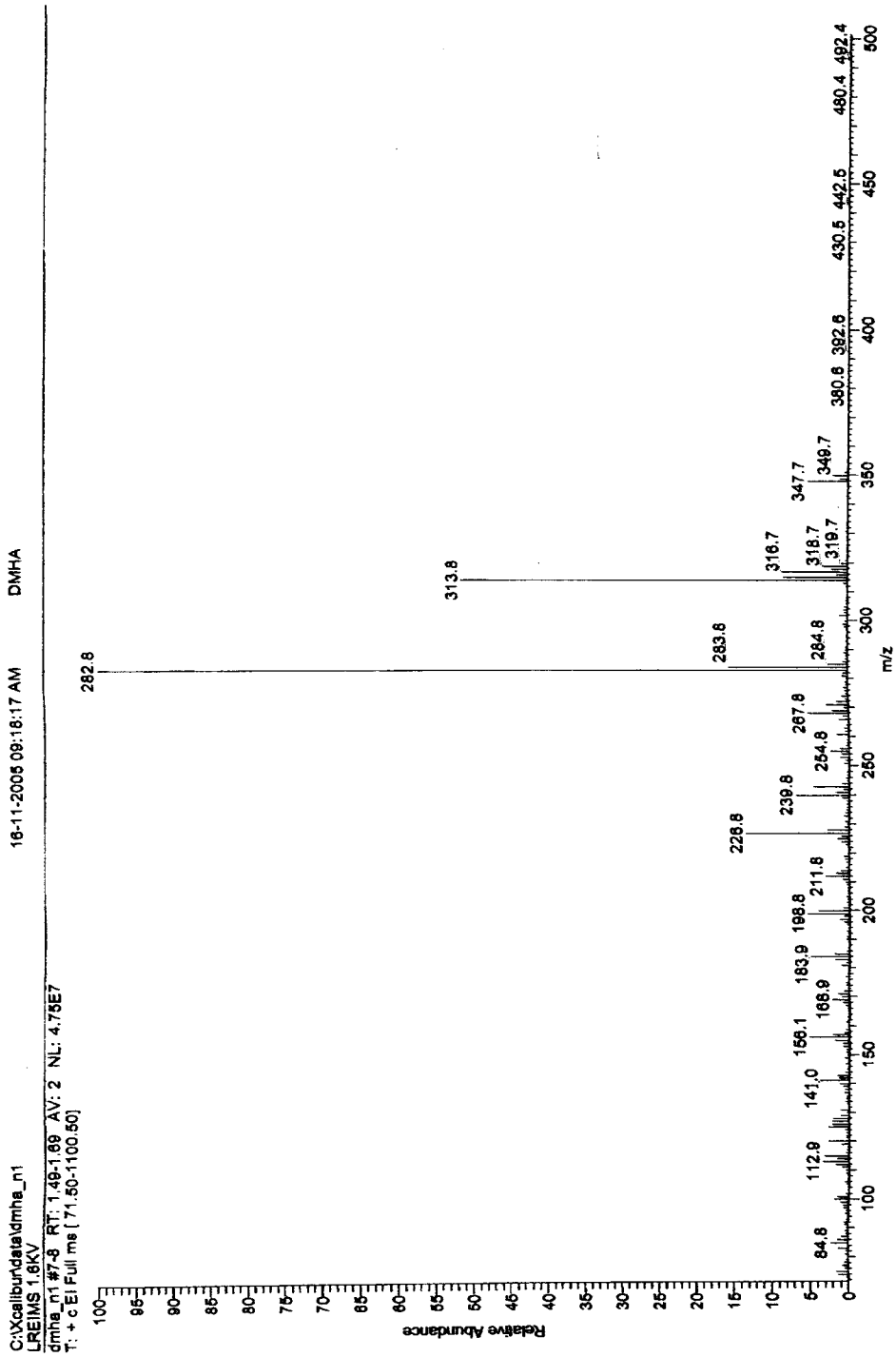
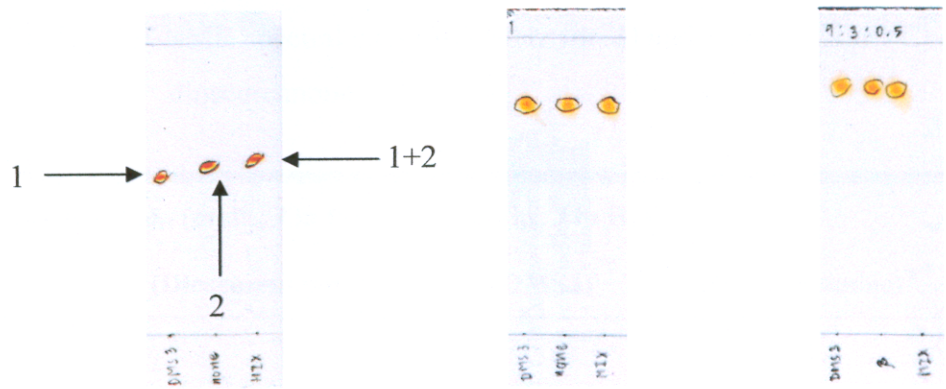


Figure 3-28 EIMS spectrum of DMS2

3.3.2.4 DMS3

DMS3 (Dioscoreanone): red crystal (6.5 mg, 13.829 %); UV ($CHCl_3$) λ_{max} 248, 292, 304 nm; IR ($CHCl_3$) ν_{max} 3450, 2921, 2853, 1737, 1667, 1477 cm^{-1}; EIMS (low resolution) *m/z* (% relative intensity) 284 (M^+; $C_{17}H_{14}O_6$, 51), 283 (100), 227 (13). This compound was compared with an authentic sample from Itharat (Itharat, 2002) using TLC in 3 solvent systems gave identical behavior.

The molecular mass of DMS3 (*m/z* 284; M^+) was proposed to have a molecular formula of $C_{17}H_{14}O_6$. Its degree of unsaturation was 11, which deduced from the IR ($CHCl_3$) spectrum as quinone function groups at 1665 cm^{-1}, corresponding to carbonyl carbon observed at δ 188.9 and 181.1 in ^{13}C NMR (Table 3-11) and six olefinic-aromatic double bonds (twelve olefinic ^{13}C signals from δ 158.5-106). In the ^1H NMR spectrum (Table 3-11), only two aromatic doublets, three aromatic singlets, and two methoxy signals were observed. Among these, the two doublets at δ 8.08 (J=8.3 Hz, H-10) and 7.93 (J= 8.5 Hz, H-9) were assigned as two *ortho* protons on a tetrasubstituted benzene ring system. Three aromatic singlets showed signals at δ 6.09, 9.16 and 6.19, and two methoxy signals were observed at δ 3.91 and 4.13. These ^1H NMR and ^{13}C NMR spectrum, compared with the previous ^1H NMR and ^{13}C NMR data of dioscoreanone showed identical spectra and also were the same as the spectrum recorded for dioscoreanone (Itharat *et al.*, 2003) except the only signal of H-8. The H-8 signal of the previous ^1H NMR data appeared at δ 6.19, differed from the H-8 signal of DMS3; due to the effect solvent of the previous study ($CDCl_3$+CD_3OD) could have effect on the chemical shift of H-8. Thus, it was strongly supported that DMS3 to be dioscoreanone.



2 hexane : 8 $CHCl_3$ 9 $CHCl_3$: 1 MeOH 9 $CHCl_3$: 3 MeOH : 0.3 H_2O

Figure 3-29 TLC of DMS3 in 2 hexane : 8 $CHCl_3$, 9 $CHCl_3$: 1 MeOH and 9 $CHCl_3$: 3 MeOH : 0.3 H_2O (1=DMS3, 2=dioscoreanone)

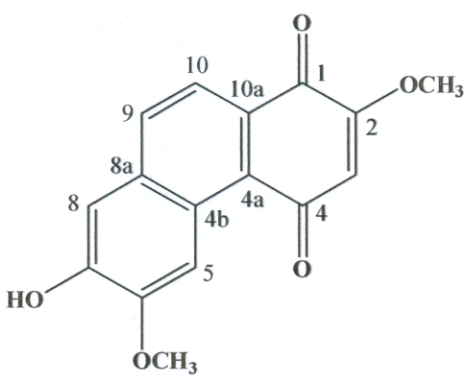


Figure 3-30 DMS3 (Dioscoreanone)

Table 3-11 NMR spectral data (500 MHz for ^{1}H and 125 MHz for ^{13}C) of DMS3 and dioscoreanone

atom	δ_H (mult., *J* in Hz) (Dioscoreanone)[a]	δ_H (mult., *J* in Hz)[b] (DMS3)	δ_C (Dioscoreanone)[a]	δ_C (DMS3)[b]
1	-	-	181.4	181.1
2	-	-	158.6	158.5
3	6.11 (s)	6.09 (s)	111.3	111.2
4	-	-	189.3	188.9
4a	-	-	125.7	125.6
4b	-	-	126.3	125.9
5	9.13 (s)	9.16 (s)	106.4	106.1
6	-	-	151.6	150.5
7	-	-	149.0	147.9
8	7.24 (s)	6.19 (s)	110.1	109.7
8a	-	-	135.1	134.8
9	7.92 (d, 8.5)	7.93 (d, 8.3)	132.3	132.4
10	8.06 (d, 8.5)	8.08 (d, 8.3)	121.0	121.1
10a	-	-	129.4	129.6
2-OMe	3.93 (s; 3H)	3.91 (s; 3H)	56.3	56.2
6-OMe	4.12 (s; 3H)	4.13 (s; 3H)	56.1	56.2

a = in $CDCl_3$+CD_3OD

b = in $CDCl_3$

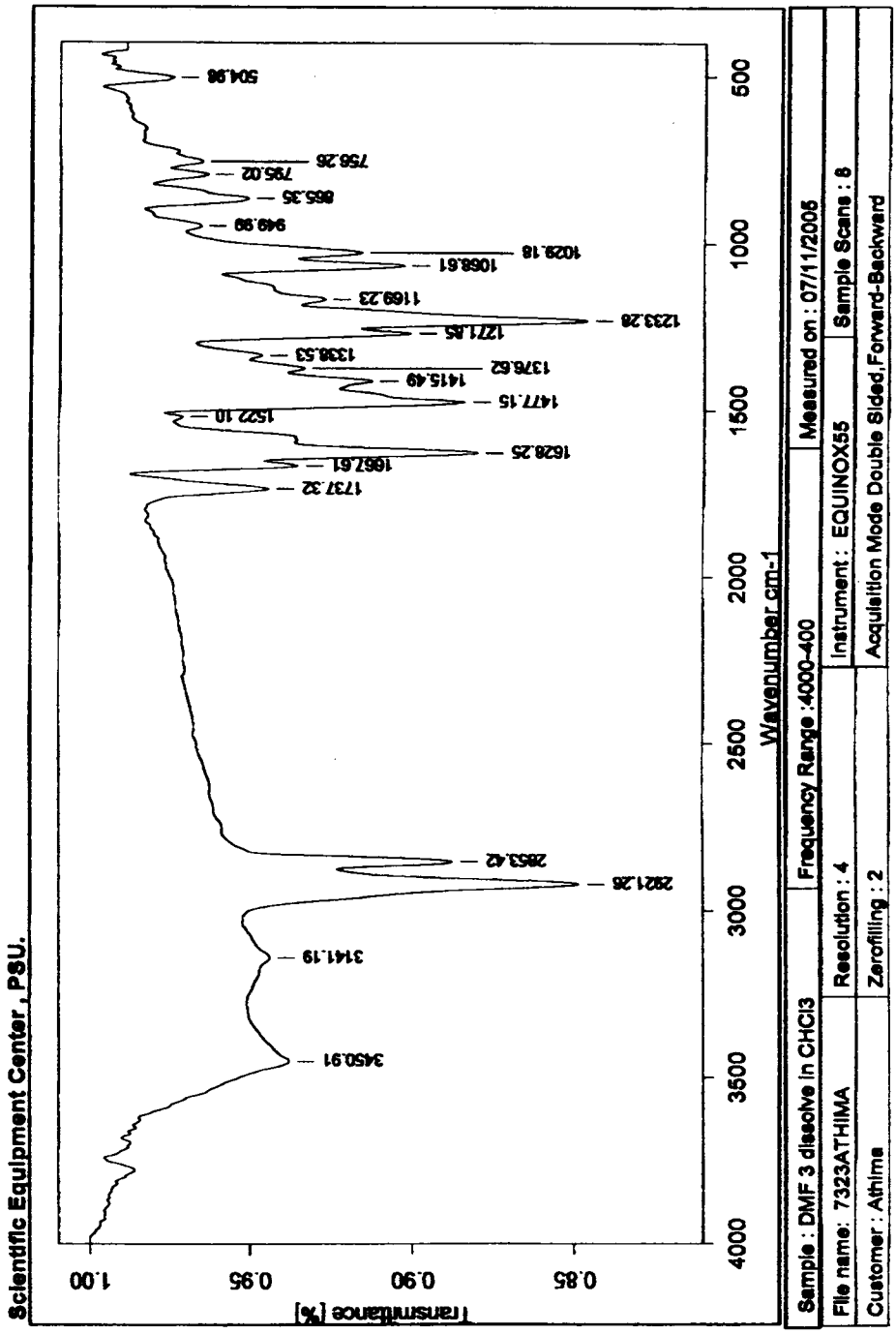


Figure 3-31 IR spectrum of DMS3 in $CHCl_3$

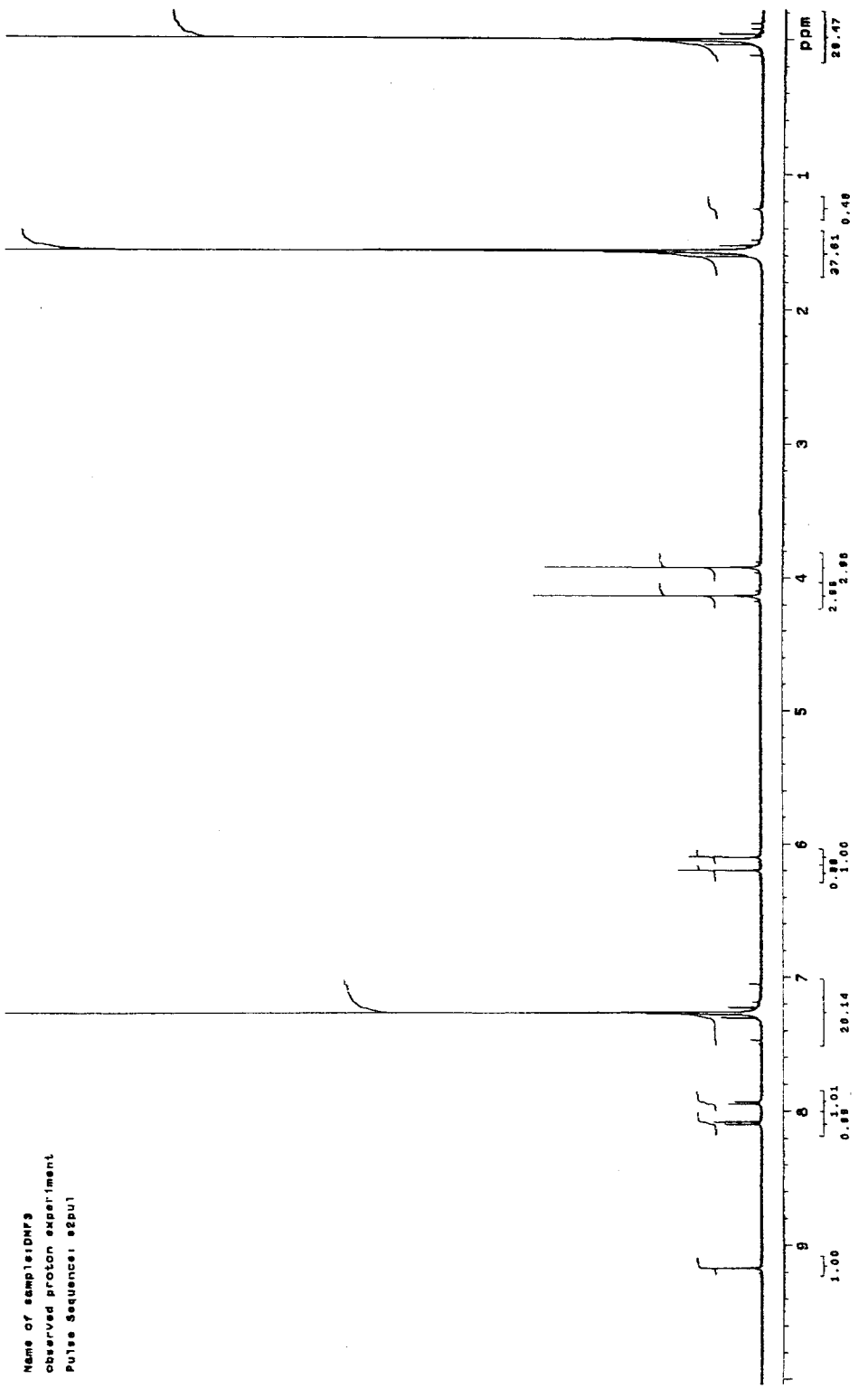


Figure 3-32 ^{1}H NMR spectrum of DMS3 in $CDCl_3$

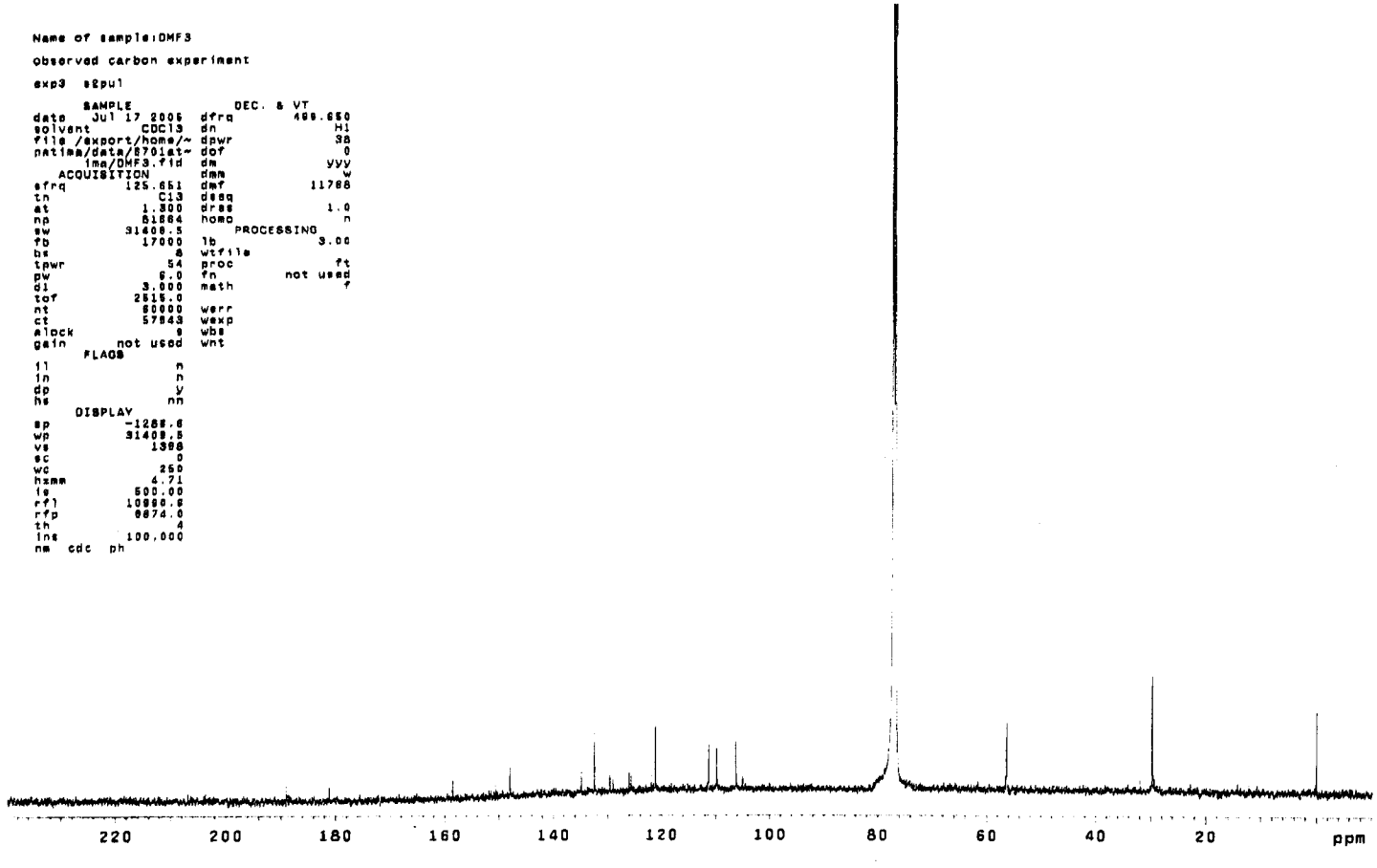


Figure 3-33 ^{13}C NMR spectrum of DMS3 in $CDCl_3$

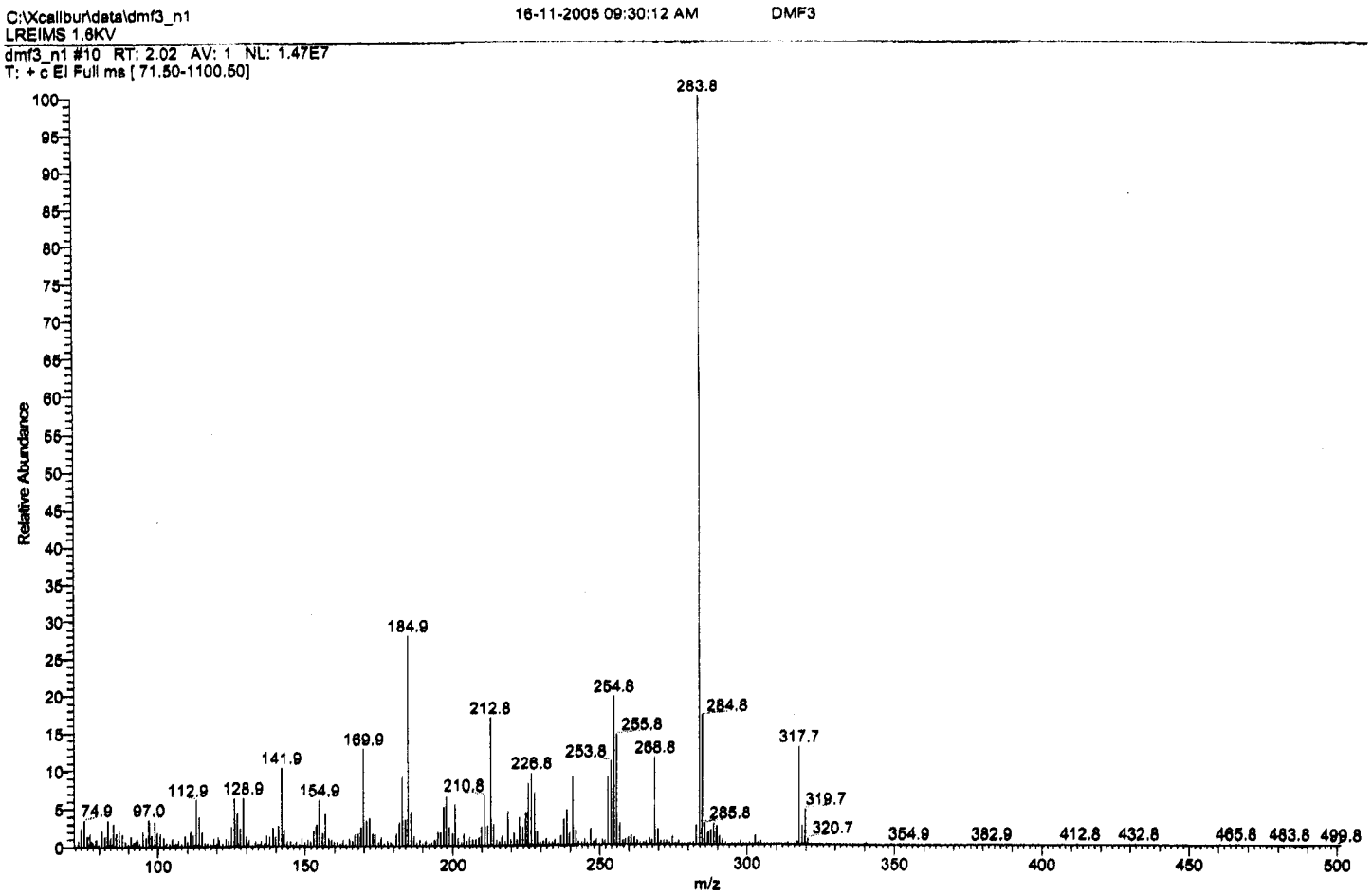
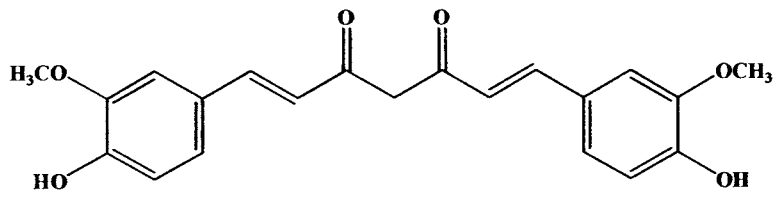


Figure 3-34 EIMS spectrum of DMS3

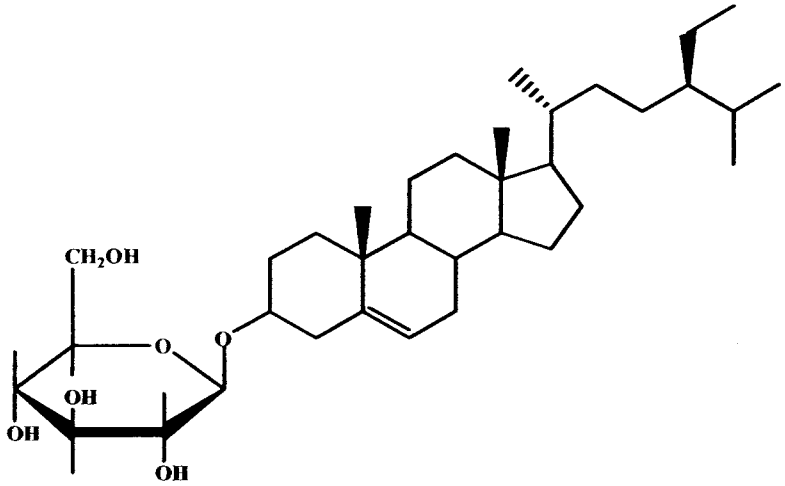
3.4 Discussion on phytochemical investigation

The ethanolic extracts of *Curcuma zedoaria* and *Dioscorea membranacea* were separated by column chromatography and used a solvent gradient of hexane, chloroform and methanol. A mixture of volatile and four compounds were isolated in this research. All pure compounds were detected by use of the general spraying reagent anisaldehyde in sulphuric acid and giving different colours after heating. CZS1 (yellow colour in daylight) showed a yellow brown colour, CZS2 and DMS1 showed a violet colour, DMS2 showed a green colour but DMS3 remained yellow as in daylight. Only three compounds (CZS1, DMS2 and DMS3) could be detected by UV 254 nm.

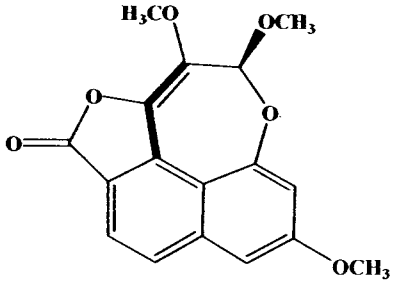
The four compounds from *Curcuma zedoaria* and *Dioscorea membranacea* could be divided into four chemical groups. They were diarylheptanoid (CZS1 or curcumin), naphthofuranoxepin (DMS2 or dioscorealide A), naphthoquinone (DMS3 or dioscoreanone) and sterols (CZS2 and DMS1 or β-sitosterol-3-*O*-β-*D*-glucopyranoside). The structures are shown in Figure 3-35. The investigation on chemical constituents of *Curcuma zedoaria* found that curcumin which is the main compound and the volatile oil, mixed with many compounds, are normally found in *curcuma* species such as *Curcuma longa*. Dioscoreanone, isolated from *Dioscorea membranacea*, was found in small amount and this compound caused the yellow colour in the rhizome when broken. The structure of dioscorealide A, a novel cytotoxic compound, was first reported in *Dioscorea membranacea* by Itharat (2003). β-Sitosterol-3-*O*-β-*D*-glucopyranoside from the ethanolic extracts are common sterols which were found in higher plants and have been found in many species.



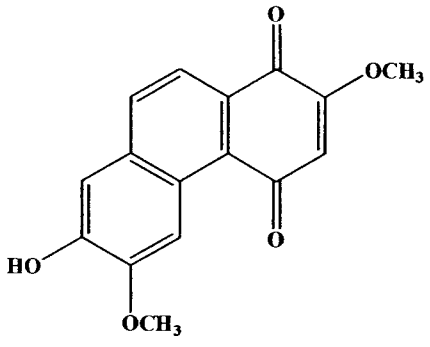
CZS1 or Curcumin



CZS2 and DMS1 or β-sitosterol glucopyranoside



DMS2 (Dioscorealide A)

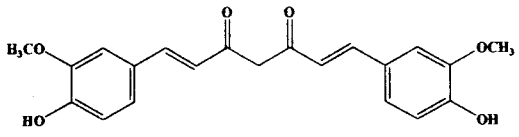


DMS3 (Dioscoreanone)

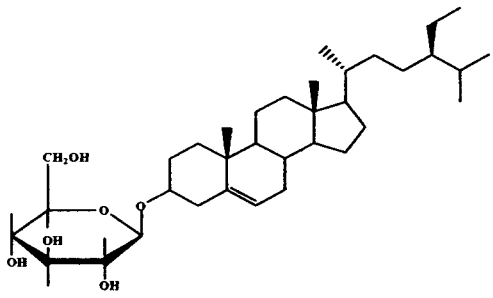
Figure 3-35 The chemical structure of four compounds isolated from the ethanolic extracts of *Curcuma zedoaria* and *Dioscorea membranacea*

3.5 Activities of the isolated compounds

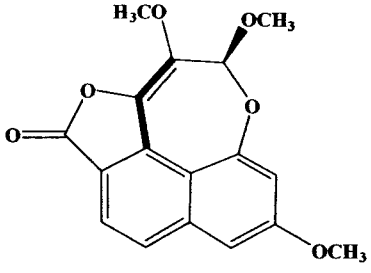
The CZV (oil), CZS1, CZS2, DMS1, DMS2 and DMS3, isolated in this study, and dioscorealide B, stigmasterol, β-sitosterol, diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside, diosgenin 3-*O*-β-*D*-glucopyranosyl (1→3)-β-*D*-glucopyranoside, taken from Arunporn Itharat, were assessed for cytotoxic activity against cancer cell lines (COR-L23 and PC3) and normal cell lines (10FS). The chemical structures are shown in Figure 3-36.



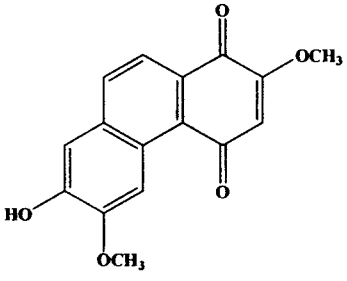
CZS1 (Curcumin)



CZS2 and DMS1
(β-Sitosterol 3-*O*-β-*D*-glucopyranoside)

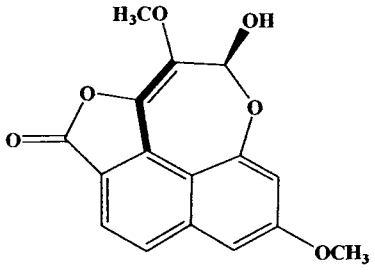


DMS2 (Dioscorealide A)

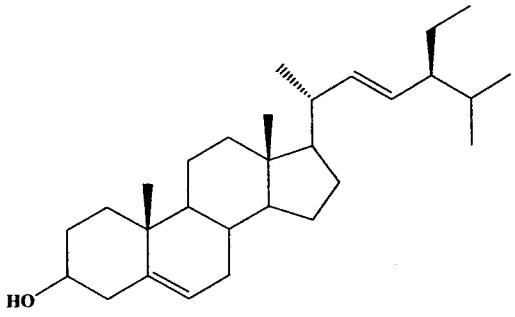


DMS3 (Dioscoreanone)

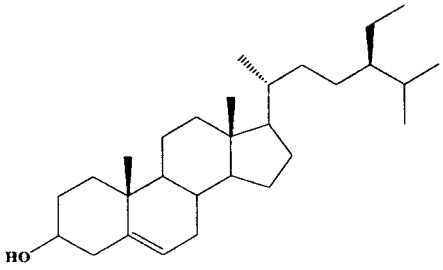
Figure 3-36 Structure of the isolated compounds from rhizomes of *Curcuma zedoaria* and *Dioscorea membranacea*.



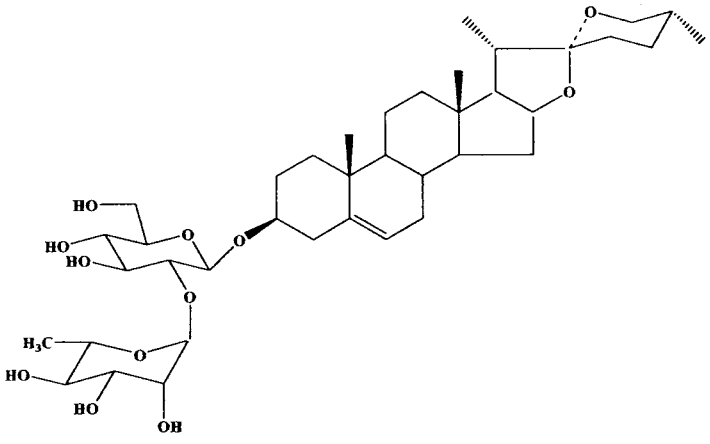
Dioscorealide B



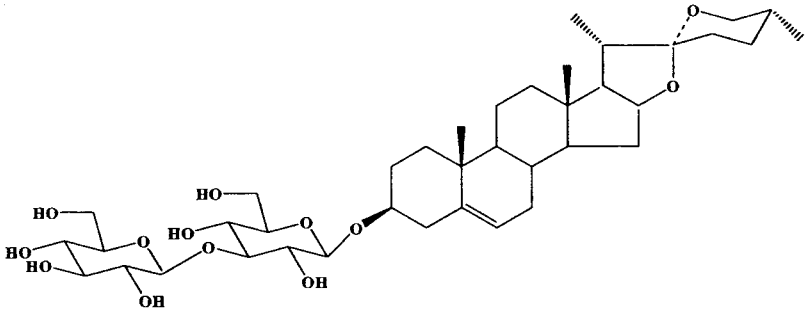
Stigmasterol



β-sitosterol



Diosgenin 3-*O*-α-*L*-rhamnopyranosyl (1→ 2)-β-*D*-glucopyranoside



Diosgenin 3-*O*-β-*D*-glucopyranosyl (1→ 3)-β-*D*-glucopyranoside

Figure 3-36 (continued)

3.5.1 Cytotoxic activity

Results of pure compounds which were isolated from the ethanolic extract of *C. zedoaria* and *D. membranacea* and oil isolated from the ethanolic extract of *C. zedoaria* exhibited against lung cancer cell line (COR-L23), prostate cancer cell lines (PC3) and normal cell line (fibloblast, 10FS) at exposure time 72 hours are shown on Table 3-13 and Figure 3-32. Vinblastine sulphate was used as the standard drug in the cytotoxicity assay. The results indicate that the volatile oil showed less cytotoxicity against PC3 followed by the American National Cancer Institute (NCI) (IC_{50} < 20 μg/ml for the extract) and less toxicity against 10FS. From the previous study the volatile oil from the rhizome of *C. zedoaria* had not reported cytotoxic activity against prostate cancer cell lines. Following the criteria for cytotoxic activity of pure compounds established by the American National Cancer Institute (NCI) should show the IC_{50} less than 4 μg/ml. Among these compounds, curcumin showed slight cytotoxic activity against PC3 with IC_{50} 18.29 μM or 6.73 μg/ml, whereas dioscoreanone (IC_{50} = 8.137 μM or 2.3 μg/ml) and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside (IC_{50} = 5.88 μM or 4.24 μg/ml) exhibited high potency.

The effect of the compound on COR-L23, dioscoreanone showed the highest activity (IC_{50} = 2.45 μM or 0.7 μg/ml) and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside showed the next highest activity (IC_{50} = 5.61 μM or 4.05 μg/ml). Although both compounds exhibited high cytotoxic activity against COR-L23, they also showed activity against PC3. It showed that there is nonspecific cytotoxic activity. The comparison between ratio of normal cell/lung cancer cell found that dioscoreanone and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside have the highest difference ratio (8.73 and 6.77, respectively) and P-value was extremely significant (P<0.001) calculated by student T-test from Prism program (Figure 3-32). The ratio of normal cell/prostate cancer cell of curcumin, dioscoreanone and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside exhibited different ratio (4.71, 2.36 and 6.47, respectively). From these results it was concluded that diosgenin 3-*O*-α-*L*-

rhamnopyranosyl(1→2)-β-*D*-glucopyranoside was selective toxicity against PC3 but less effect against normal cells. From the comparison of ratio of IC_{50} (μM) normal cell/ IC_{50} (μM) cancer cells of dioscoreanone it was found that it showed the highest ratio between lung cancer cells and normal cells. It indicated that dioscoreanone exhibits the best potency against lung cancer cell but less effect on the normal cell.

The cytotoxic activity of curcumin has been reported as showing cytotoxic activity against ovarian cancer OVCAR-3 cells with IC_{50} 4.4 μg/ml (Syu *et al.*, 1998), urinary bladder cancer cells (Sindhwani *et al.*, 2000) and human colon cancer cells (Goel *et al.*, 2001) etc. The cytotoxicity of dioscoreanone and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside observed in the present work is in accordance with the previous study in which these compounds showed cytotoxicity against lung cancer cell line (COR-L23) with IC_{50} 2.89 μg/ml and human beast cancer cell line (MCF-7) with IC_{50} 3.76 μg/ml (Itharat, 2002) but there is no report about prostate cancer cell lines. In conclusion, these active compounds could be studied further for mechanisms against cancer.

Table 3-12 IC_{50} value of compounds tested against lung and prostate cancer cell lines (COR-L23 and PC3), and normal cell lines (fibloblast, 10FS) (mean±SEM) exposure time 72 hours (n=3).

Compounds	IC_{50} (μM)		
	COR-L23	**PC3**	**10FS**
CZV (Oil, μg/ml)	29.196±0.795	17.616±1.303	>100
CZS1 (Curcumin)	22.016±0.111 (3.91)	18.288±0.450 (4.71)	86.177±3.185
CZS2, DMS1 (β-Sitosterol 3-*O*-β-*D*-glucopyranoside)	>100	>50	>100
DMS2 (Dioscorealide A)	>50	>50	>100
DMS3 (Dioscoreanone)	2.450±0.475 (8.73)	8.137±0.167 (2.63)	21.408±1.368
Dioscorealide B	NT	>50	NT
Stigmasterol	>50	>50	>100
β-sitosterol	>100	>50	>100
Diosgenin 3-*O*-β-*D*-glucopyranosyl(1→3)-β-*D*-glucopyranoside	40.140±0.898	17.694±1.267	NT
Diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside	5.612±0.375 (6.77)	5.878±0.047 (6.47)	38.048±0.815
Vinblastine sulphate (nM)	0.566±0.017	0.675±0.026	NA

n = Number of independent experiments, the numbers which were in (…) behind IC_{50} value were the ratio of IC_{50} (μM) normal cells (10FS)/IC_{50} (μM) cancer cells of extracts at exposure time 72 h., NT = not sufficient for test., NA = not assay.

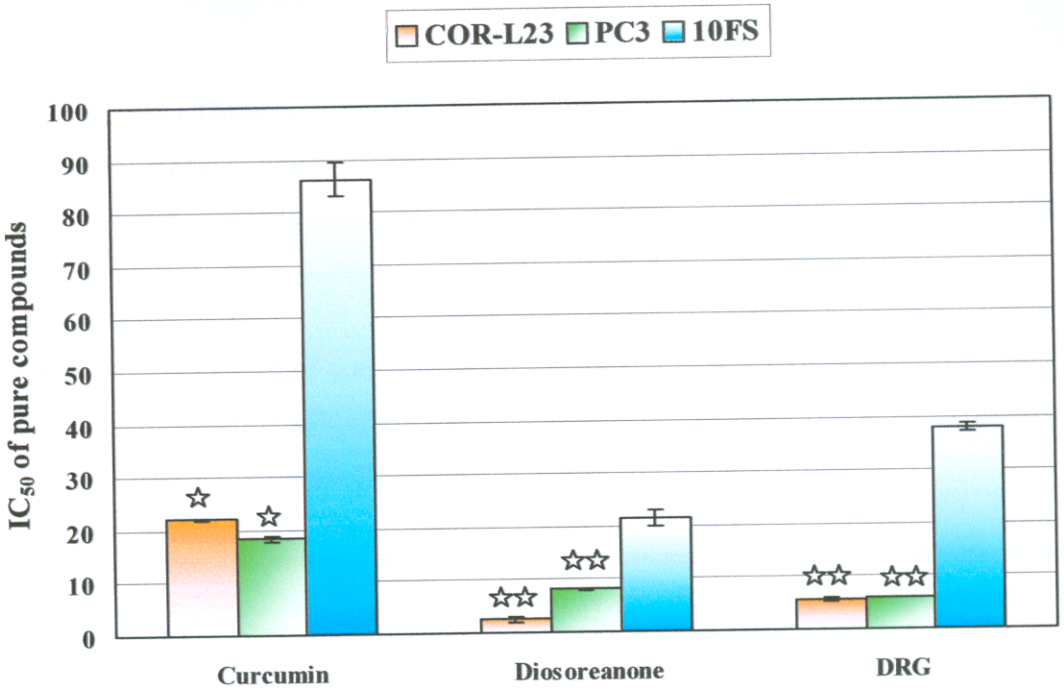


Figure 3-37 IC_{50} values (μM) and SEM of cytotoxic compounds isolated from the ethanolic extracts of *C. zedoaria* and *D. membranacea* against lung and prostate cancer cell lines (COR-L23 and PC3) and normal cell lines (10FS) at exposure time 72h using student t-test from Prism to compare the significant difference between normal cell (10FS) and each cancer cell (COR-L23and PC3) by ☆ for $P<0.01$ and ☆☆ for $P<0.001$.

(DGR= diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside)

3.5.2 Apoptosis assay

Three compounds: CZS1 (curcumin), DMS3 (dioscoreanone), and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside were assessed for apoptosis in large lung carcinoma cell lines (COR-L23) and human prostate cancer cell lines (PC3). The results are depicted in Table 3-14, Figure 3-34 and Figure 3-35.

Table 3-13 Effects of compounds on the induction of apoptosis in lung cancer cell lines (COR-L23) and prostate cancer cell lines (PC3) at exposure time 48 hours (n=2)

Cell line	Compound	% apoptosis±SEM
COR-L23	Control	1.480±0.350
	0.1% DMSO	1.605±0.565
	5 μM Dioscoreanone	2.855±0.005
	10 μM Dioscoreanone	7.555±1.145
	5 μM Diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside	1.165±0.185
	10 μM Diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside	3.455±0.575
	5 μg/ml Curcumin	4.635±0.035
	10 μg/ml Curcumin	21.400±0.820
PC3	Control	0.820±0.040
	0.1% DMSO	1.200±0.350
	5 μM Dioscoreanone	3.375±0.015
	10 μM Dioscoreanone	3.705±0.015
	5 μM Diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside	3.430±0.050
	10 μM Diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside	7.285±0.095
	5 μg/ml Curcumin	2.500±0.710
	10 μg/ml Curcumin	4.260±0.260

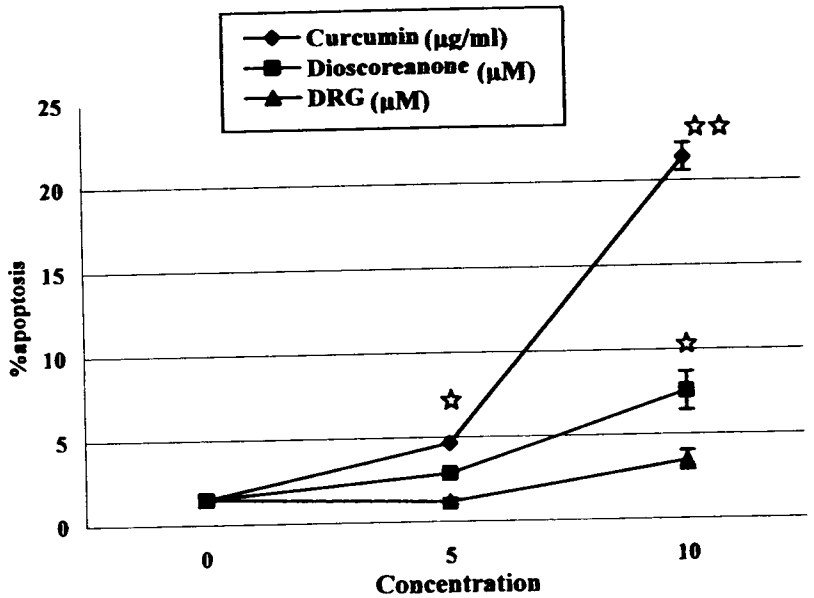


Figure 3-38 Compound-induced apoptosis in COR-L23 cell after 48 h incubation. Student t-test from Prism was used to compare the significant difference between control and treatment by ☆ for $P<0.05$ and ☆☆ for $P<0.01$.

(DGR= diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside)

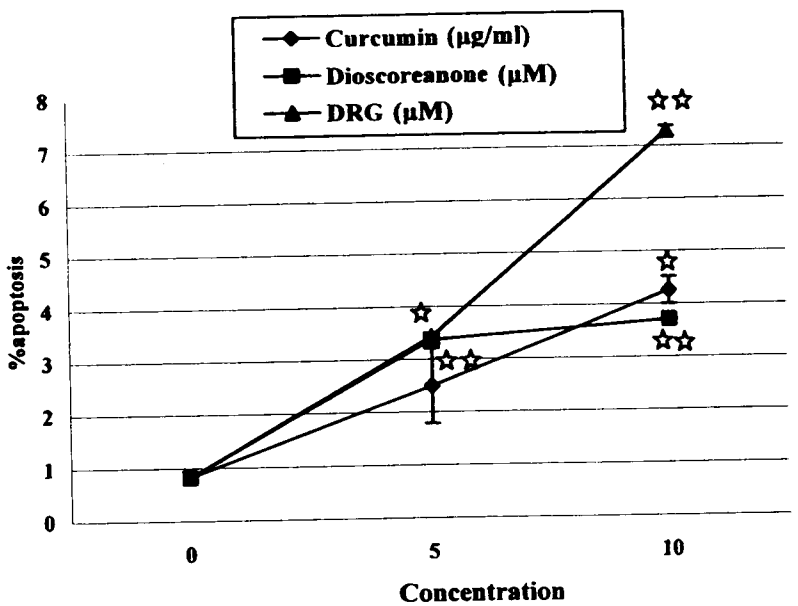


Figure 3-39 Compound-induced apoptosis in PC3 cell after 48 h incubation. Student t-test from Prism was used to compare the significant difference between control and treatment by ☆for $P<0.05$ and ☆☆ for $P<0.01$.

(DGR= diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside)

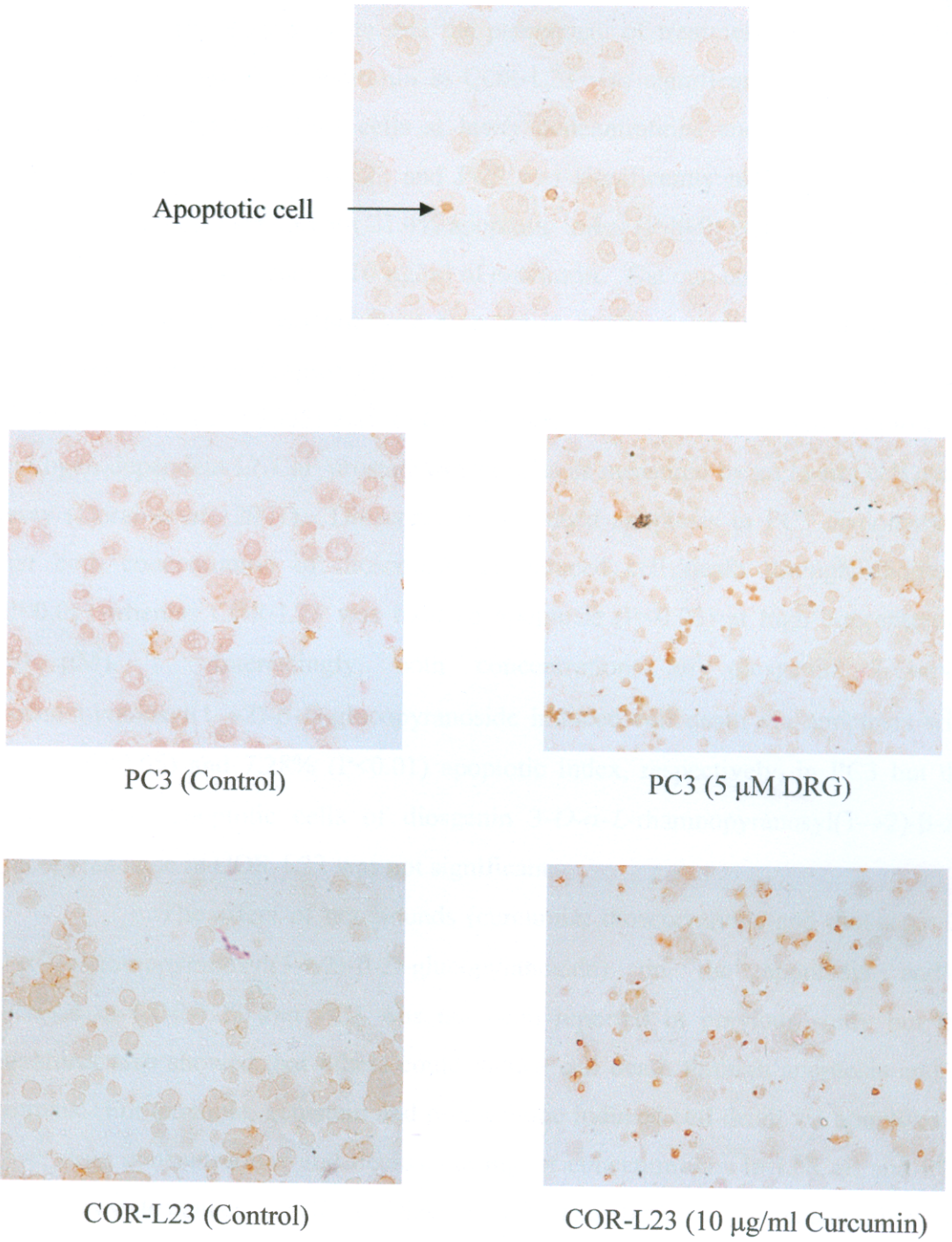


Figure 3-40 Apoptotic cells were demonstrated in PC3 and COR-L23 cells by colorimetic TUNEL assay after treatment with curcumin and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside (DRG) for 48 hours and staining. (Note that the apoptotic cells showed brown-staining nuclei)

The data indicate that the percentage of treatment-induced apoptotic cells (apoptosis index) of curcumin in COR-L23 was significantly higher ($P<0.05$) than apoptotic index in PC3 cells at lower concentrations and the percentage of apoptotic cells in both COR-L23 and PC3 was significantly higher than apoptosis index of the control group with 21.4% apoptotic index ($P<0.01$) and 4.26% apoptotic index ($P<0.05$) respectively at 10 μg/ml of curcumin. The curcumin-induced apotosis in COR-L23 and PC3 was not been reported in previous studies but curcumin has been shown to induce apoptosis (23.7%) in the human squamous cell lung carcinoma H520 cell (Sen *et al.*, 2005) and it also causes a significantly increased apoptosis of androgen-dependent LNCaP prostate cancer cells as measured by an in situ cell death assay (Dorai *et al.*, 2001). Dioscoreanone-induced apoptosis in PC3 demonstrated that both concentration of dioscoreanone induced cell death through apoptosis ($P<0.01$) whereas COR-L23 was induced apoptosis ($P<0.05$) at high concentration (10 μM). Interestingly, both concentrations of diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside induced cell death via apoptosis with 3.43% ($P<0.05$) and 7.28% ($P<0.01$) apoptotic index, respectively, in PC3 but the percentage of apoptotic cells of diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside in COR-L23 was not significant.

The effect of compounds (curcumin, dioscoreanone and diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside) on the apoptosis and/or necrosis in COR-L23 and PC3 was not been reported in previous study but the literatures also showed that others could induce cell death through apoptosis and/or necrosis. For example, genistein and β-lapachone induced cell death via apoptosis at lower drug concentrations and necrosis at higher concentrations in LNCaP and PC3 human prostate cancer cell (Kumi-Diaka, 2000). The alkylating agents can kill tumor cells in mice deficient in p53 and BCL-2 genes by necrosis rather than by inducing apoptosis by blocking their energy supply (Miller, 2004). Saikosaponin-d, a triterpene saponin from *Bupleurum falcatum* L., induced apoptosis as well as necrosis at concentration of 10^{-5}-10^{-4} M in human CEM lymphocytes (Hsu *et al.*, 2000). Zinc at concentrations of 150 μM or higher induced necrosis as well as apoptosis in thyroid cancer cell lines and necrosis was increased in a dose-dependent rate (Litaka *et al.*, 2001). The effects of vitamin C and vitamin K3 on a human bladder carcinoma cell

line (T24) following 1 hour and 2 hours after vitamin treatment showed both apoptotic and necrotic morphologic characteristic induced by oxidative stress (Gilloteaux *et al.*, 1998). Therefore, these data indicate that diosgenin 3-*O*-α-*L*-rhamnopyranosyl(1→2)-β-*D*-glucopyranoside may induce cell death via the necrosis or another pathway. These data support that the study of the apoptosis and necrosis mechanisms of these compounds should be continued.