CHAPTER IV
RESULTS

4.1 Growth and appearance of callus cultures

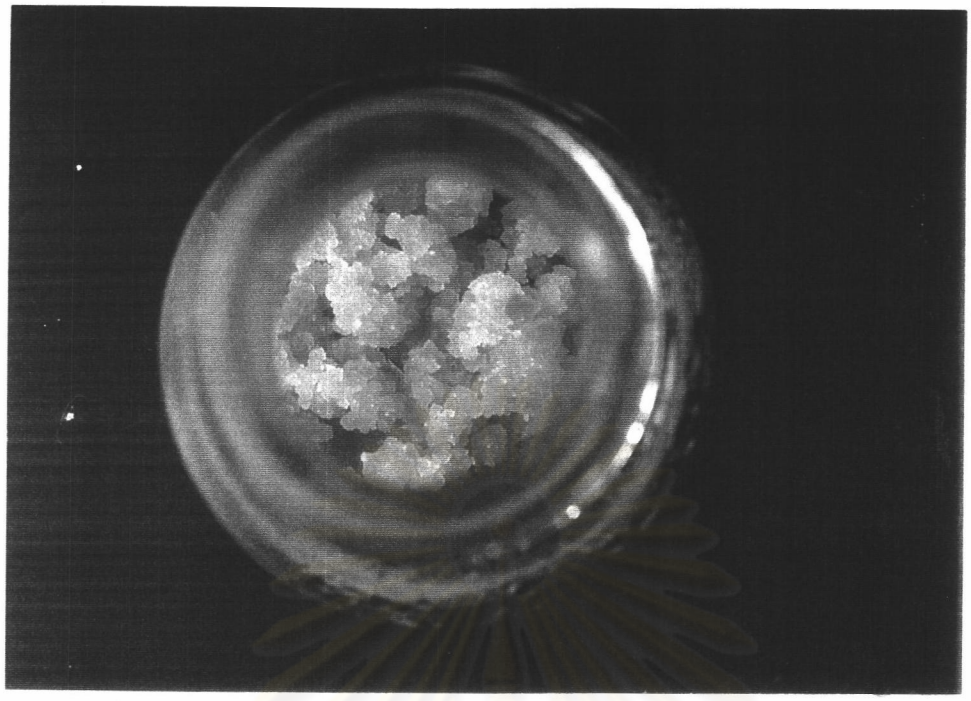
Callus cultures of the investigated plant, *A. dubia* Wall. ex Bess., were initiated by using Murashige & Skoog's (MS) or Gamborg B5 (B5) medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin, 5 ppm *L*-ascorbic acid and 3% sucrose. They were grown at the temperature of 25±2°C under continuous light using cool white fluorescence tubes. Table 11 shows details of growth and appearance of callus cultures and Fig. 7 shows growth and appearance of callus cultures.

Table 11 Growth and appearance of callus cultures of *A. dubia* Wall. ex Bess.

Media	Appearance	Growth*
MS	yellow, friable	4
B5	yellowish-brown, friable	2

* 4 = profuse growth 3 = good growth 2 = moderate growth
1 = slight growth 0 = no growth

From this table, it is shown that callus cultures of *A. dubia* Wall. ex Bess. initiated and incubated on MS or B5 media containing 1 mg/L 2,4-D and 0.1 mg/L kinetin are different in appearance. They were same friable but different in colour. On MS media, they were yellow, but on B5 media they were yellowish-brown. Moreover, the cultures initiated and incubated on MS media grew better than on B5 media. The callus cultures initiated and incubated on MS grew very fast and were subcultured every 2-4 weeks, but another grew very slowly and was subcultured every 4-8 weeks.



on MS medium

on B5 medium

Figure 7 The callus cultures of *A. dubia* Wall. ex Bess.

4.2 Growth and appearance of cell suspension cultures

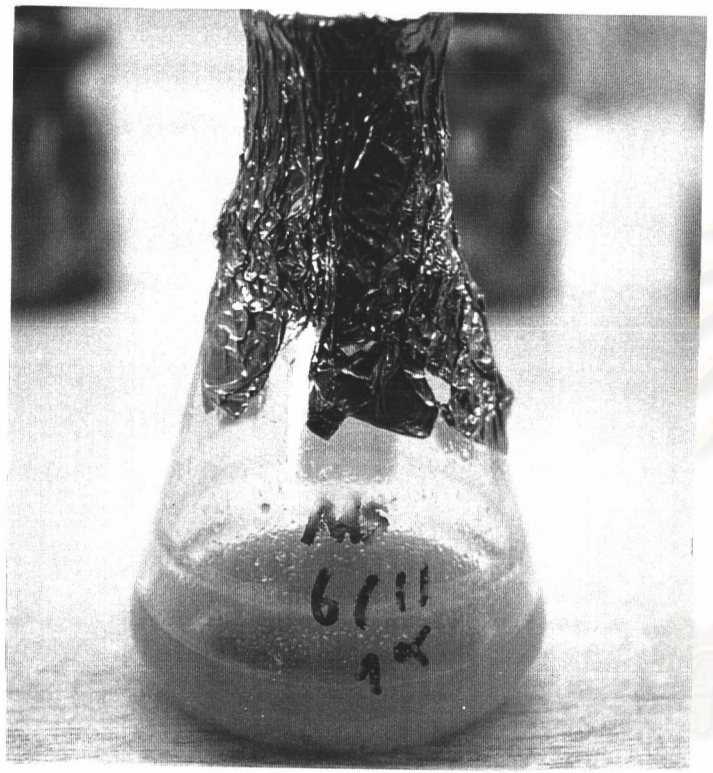
Cell suspension cultures of the investigated plants, *A. dubia* Wall. ex Bess., was carried out by using Murashige & Skoog's (MS) or Gamborg B5 (B5) medium (without agar) supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L kinetin, 5 ppm *L*-ascorbic acid and 3% sucrose. The cell suspension cultures were derived from the fourth generation of callus cultures. Table 12 shows details of growth and appearance of cell suspension cultures and Fig. 8 shows growth and appearance of cell suspension cultures.

Table 12 Growth and appearance of cell suspension cultures of *A. dubia* Wall. ex Bess.

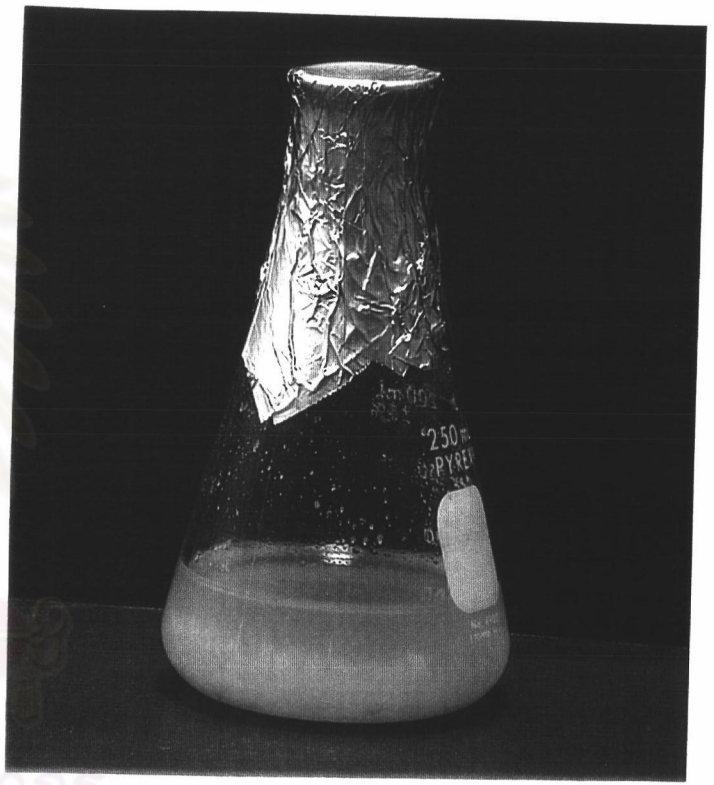
Media	Appearance	Growth*
MS	pale yellow	4
B5	yellowish-brown	2

* 4 = profuse growth 3 = good growth 2 = moderate growth
1 = slight growth 0 = no growth

From this table, it is shown that cell suspension cultures of *A. dubia* Wall. ex Bess. initiated and incubated on MS or B5 media containing 2,4-D and kinetin appeared in different colour. In MS media, they were pale yellows, but in B5 media they were yellowish-brown. Both of them grew well with the fine disperse particles. They were subcultured around every 2 weeks intervals.



in MS medium



in B5 medium

Figure 8 The cell suspension cultures of *A. dubia* Wall. ex. Bess

4.3 Chemical constituents of essential oil from leaves of *Artemisia dubia* Wall. ex Bess. obtained by hydrodistillation method

The yield of essential oil isolated from *Artemisia dubia* Wall. ex. Bess. leaves obtained by hydro distillation of fresh material in Section 3.3 was found to be 0.25 % (v/w) of fresh weight.

The essential oil of *A. dubia* Wall. ex Bess. leaves, , was injected to Gas chromatography-Mass spectrometry (GC-MS) for identification of essential oil chemical constituents. The results of analysis are shown in Fig. 9 and table 13.

These peaks were identified as 6 monoterpenes, 7 oxygenated monoterpenes, 4 sesquiterpenes and 6 oxygenated sesquiterpenes (Table 13). Among these peaks, davanone appeared to be the major constituent (71.56%), followed by chrysanthenone (6.65%) and 9-*epi*-β-caryophyllene (3.80%).

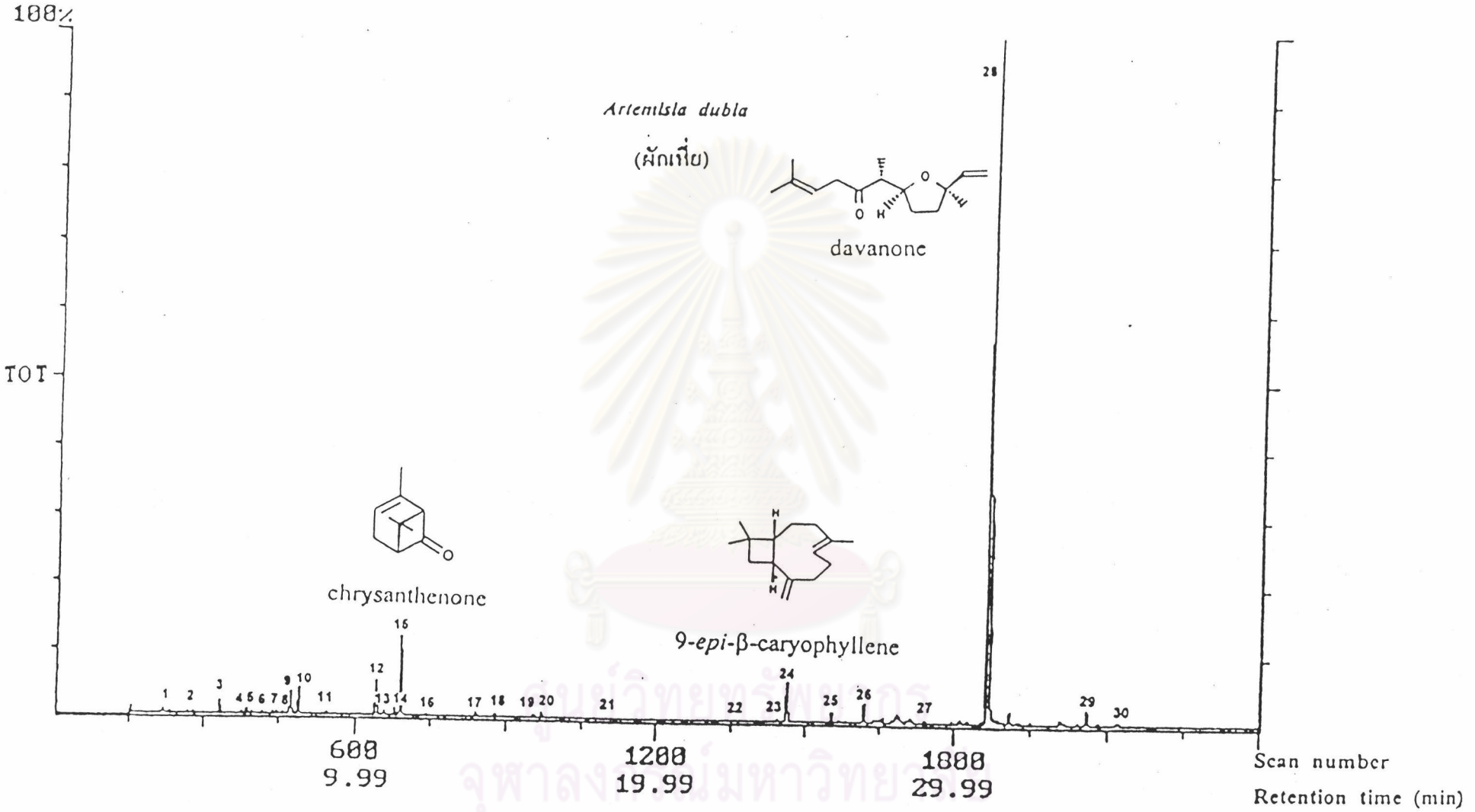


Figure 9 GC chromatogram of the essential oil from *Artemisia dubia* Wall. ex Bess. leaves

Table 13 Chemical constituents of essential oil obtained from *Artemisia dubia* Wall. ex Bess leaves by hydrodistillation

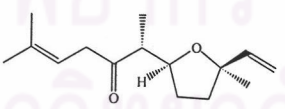
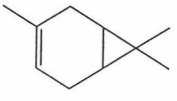
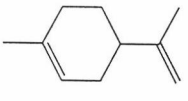
Compound	%Area	Compound	%Area
Monoterpenes		**Oxygenated sesquiterpenes**	
santolina triene	0.15	nordavanone	0.16
sabinene	0.21	*cis*-threo-davanafuran	0.12
α-phellandrene	0.29	artedouglasia oxide	0.24
2-δ-carene	0.26	davanone	71.56
o-cymene	1.94	juniper camphor	1.63
γ-terpinene	0.14	α-bisabolol acetate	0.55
Oxygenated monoterpenes		**Others**	
1,8-cineole	2.07	(*Z*)-3-hexenol	0.43
cis-chrysanthenol	0.46	unidentified 1	1.00
chrysanthenone	6.65	3-octanone	0.21
4-terpineol	0.51	3-octanol	0.27
α-terpineol	0.31	unidentified 2	2.86
cis-chrysanthenyl acetate	0.37	unidentified 3	0.43
bornyl acetate	0.09	unidentified 4	0.22
Sesquiterpenes			
α-copaene	t		
9-*epi*-β-caryophyllene	3.80		
α-humulene	0.98		
germacrenes	1.92		

4.4 GC analysis and GC-MS analysis of dichloromethane extracts from callus and cell suspension cultures

The GC-MS chromatograms of dichloromethane extract from callus and cell suspension cultures of the investigated plant, *A. dubia* Wall. ex Bess. are shown in Fig. 10 and Fig. 12, respectively and the Mass Spectra of the major constituent, namely davanone are also shown in Fig 11 and Fig. 13, respectively, too. Table 14 shows some essential oil constituents of callus and cell suspension cultures, davanone is shown the major constituent, and it is also found another terpenoids e.g. 3-carene, *d*-limonene.

The levels of terpenoids accumulation in cell suspension cultures are much more than in callus cultures, but it is still very low accumulation. So, we will use some biological techniques such as cell immobilisation, precursor feeding and using of adsorbent for improving the levels of essential oil constituents, particularly davanone.

Table 14 Chemical constituents of essential oil obtained from callus and cell suspension cultures *A. dubia* Wall. ex. Bess.

R_t (callus)	R_t (suspension)	Compounds	Structure	MS data
10.21	10.21	davanone		236, 111, 93, 91, 69, 67
8.34	8.32	3-carene		136, 93, 91, 79, 77, 68, 67
8.85	8.85	*d*-limonene		136, 93, 68, 67

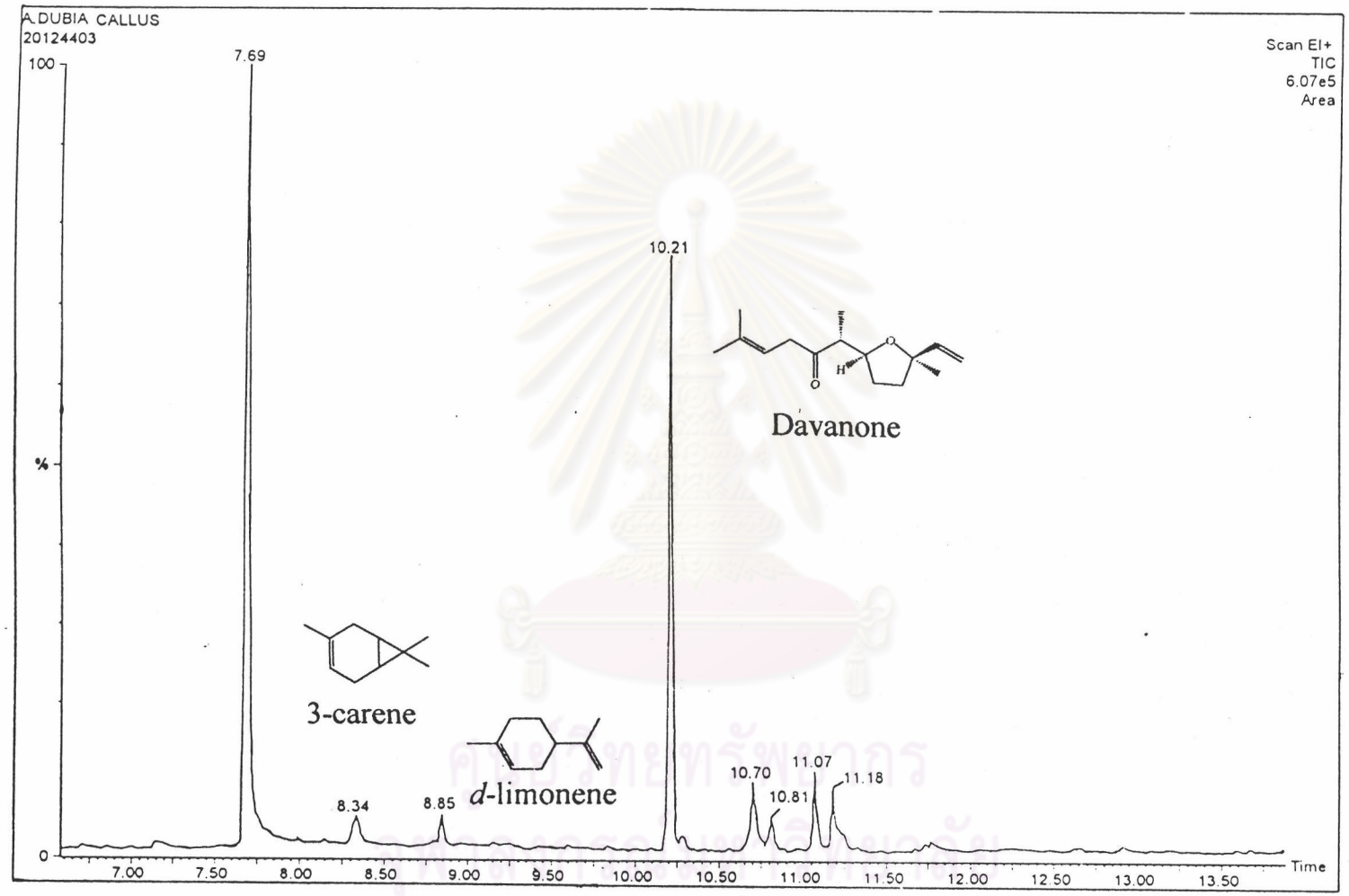


Figure 10 The GC chromatogram of dichloromethane of callus cultures

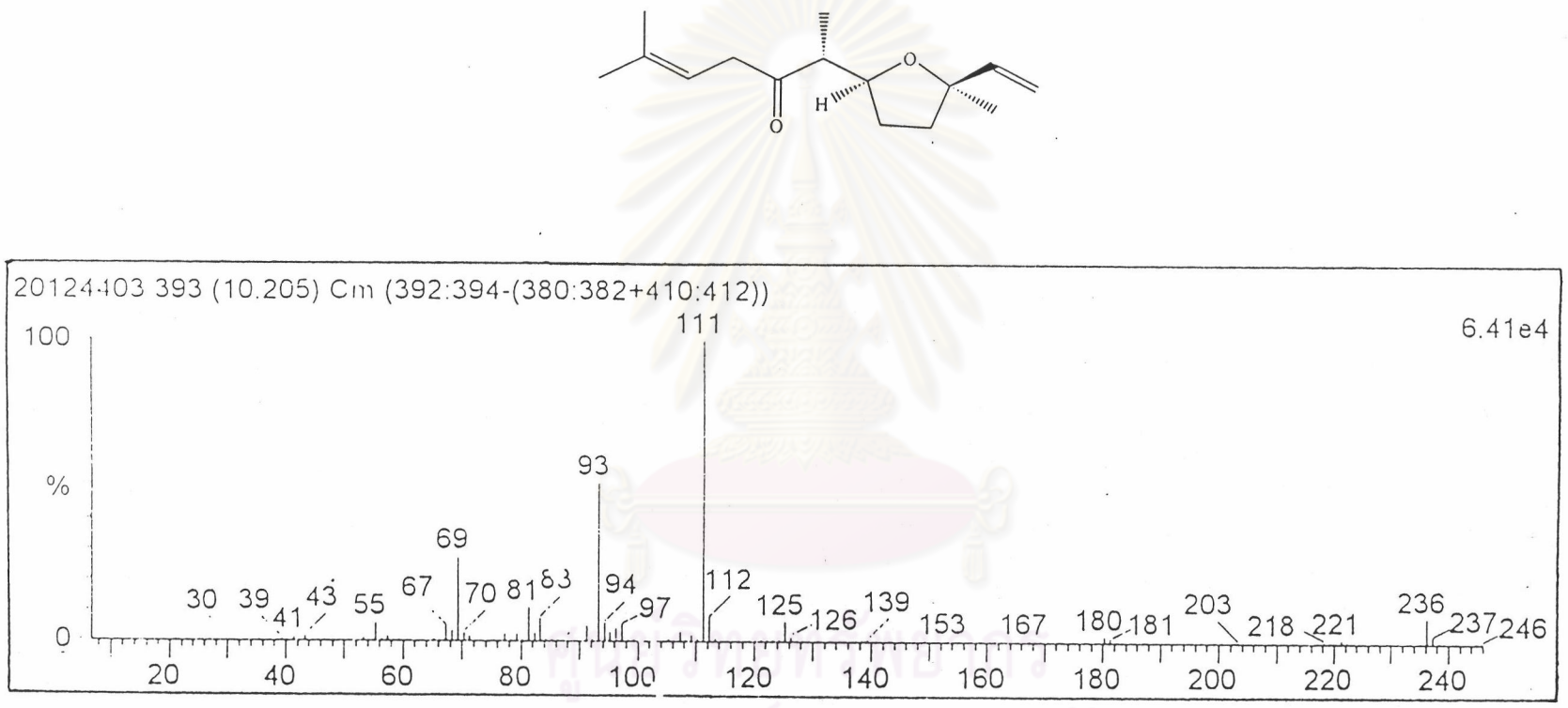


Figure 11 The Mass Spectrum of the major constituent, namely davanone

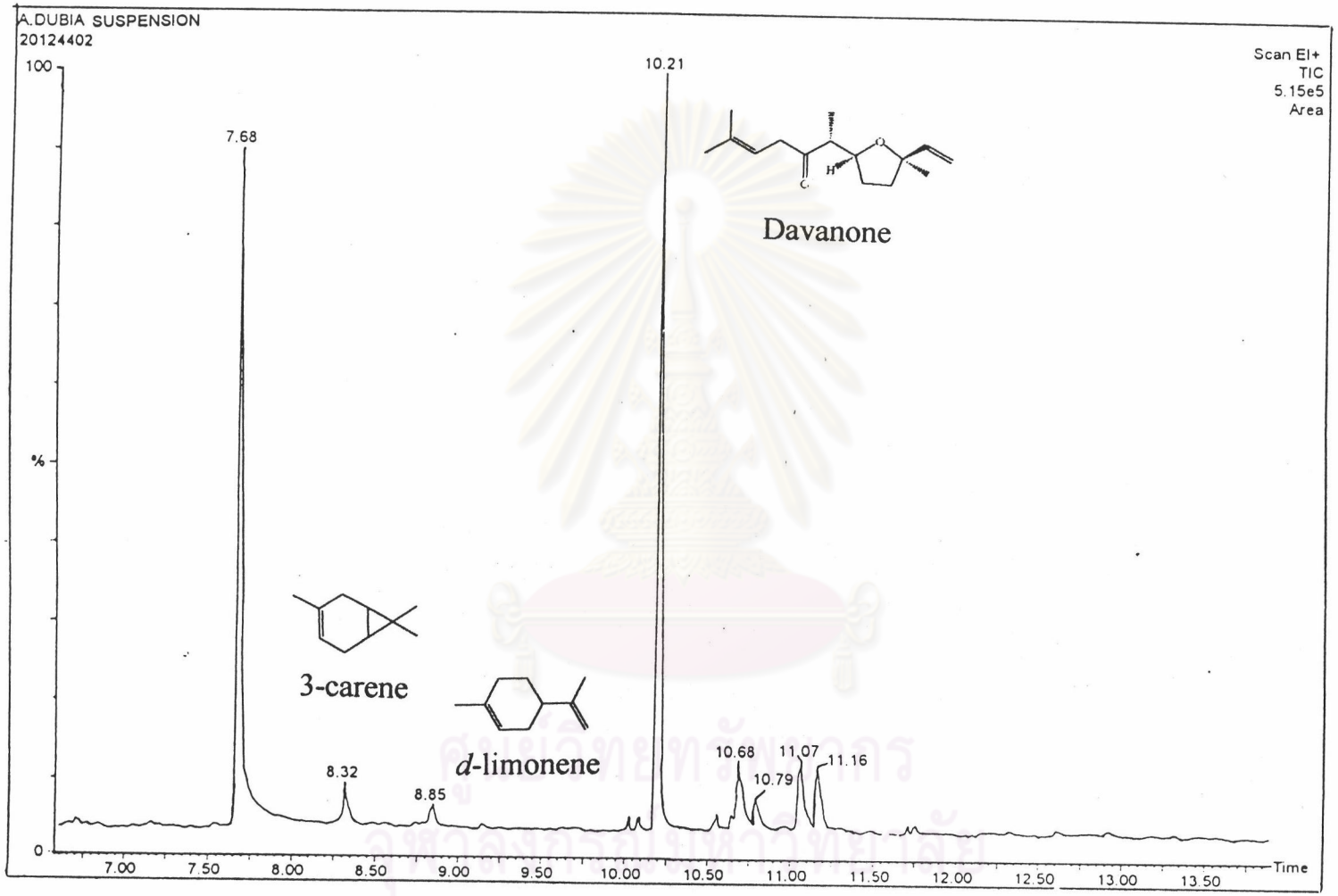


Figure 12 The GC chromatogram of dichloromethane extract of cell suspension cultures

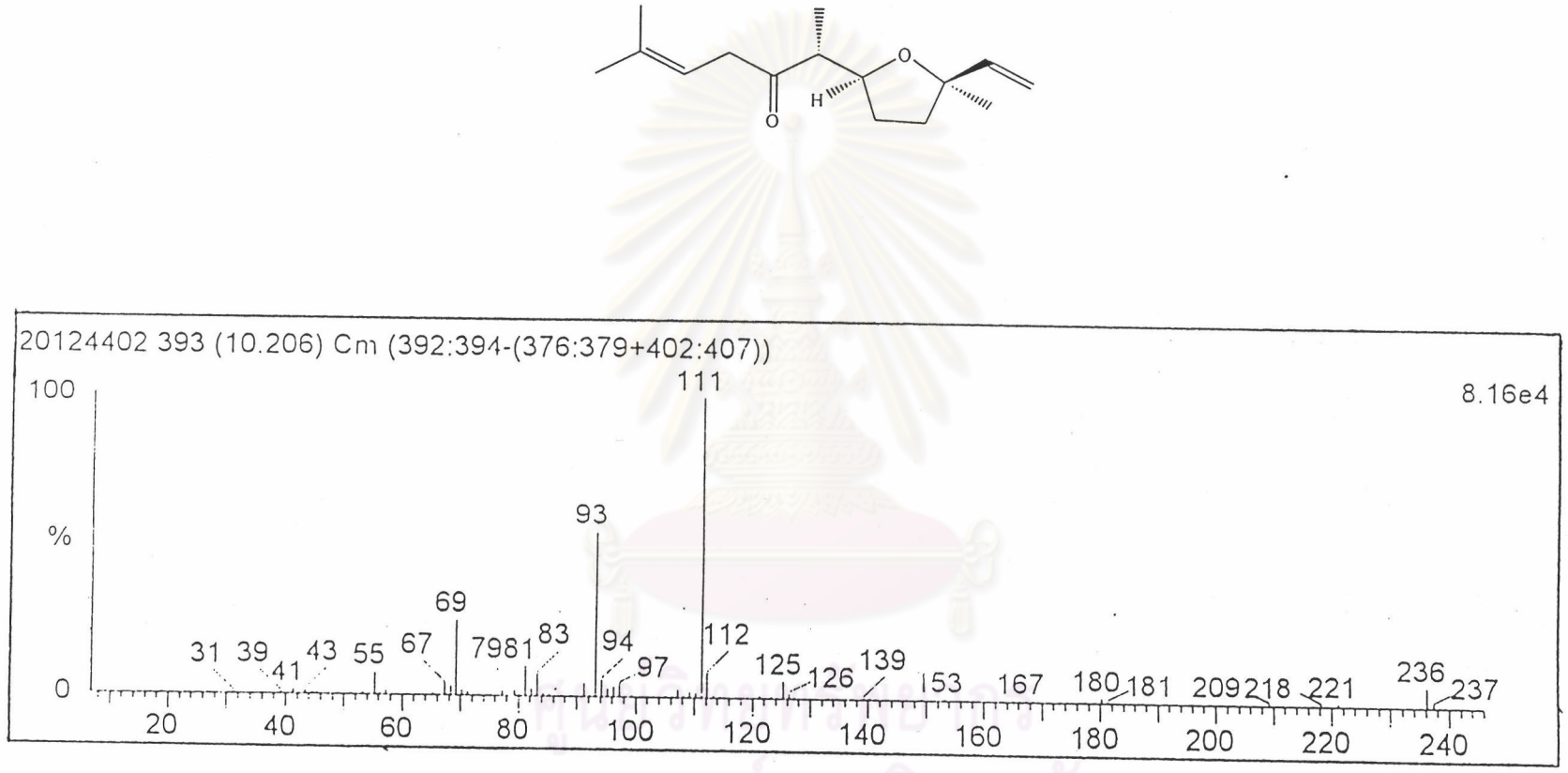


Figure 13 The Mass Spectrum of the major constituent, namely davanone

4.5 GC analysis and GC-MS analysis of dichloromethane extract of cell suspension cultures after using the biotechnological techniques

Many researchers reported low yield of essential oil obtained from plant cell cultures, so biotechnological techniques had been used for improving the level of essential oil e.g. cell immobilisation, two-phase systems, elicitation, precursor feeding or permeabilisation (Buitelaar and Tramper, 1992, Hunter, 1993, Banthope, 1994 and Lockwood, 2000). In this experiment, cell immobilisation, feeding of precursor and using of adsorbent were used together for improving level of essential oil, especially the major constituent, namely davanone.

Nylon meshes (1x1x1 cm^3), pore size 10 ppi was used for immobilising cell, various concentrations of geranyl acetate (5, 10, 50 and 100 ppm) fed into the liquid media were used as a precursor and Porapak Q was used for adsorbing the essential oil.

Fig. 14 shows comparison of GC chromatogram of dichloromethane extracts of intact plant (a), callus (b), cell suspension cultures (c) and immobilised cell after using biotechnological techniques (d-g).

Fig. 15 shows standard curve using for calibration of davanone quantity.

Table 15, shows quantity of davanone obtained from dichloromethane extracts of intact plant, callus cultures, cell suspension cultures and immobilised cells after using biotechnological techniques calculated by area under peak of davanone.

Fig. 16 shows davanone contents (μg/g FW) obtained from dichloromethane extracts of intact plant (a), callus cultures (b) and suspension cultures (c). It was shown that, davanone content obtained from intact plant (70.82 μg/g FW) is much more than in callus cultures (13.90 μg/g FW) and cell suspension cultures (15.09 μg/g FW), respectively.

Fig. 17 shows davanone contents (μg/g FW) obtained from dichloromethane extracts of immobilised cells after feeding various concentrations of geranyl acetate, all of the experiments were used Porapak Q adsorbing tube fixed at the outlet to collected volatile oil compounds. It was shown that, after increasing of geranyl acetate fed into the immobilised cells, the davanone contents were obtained increased. Immobilised cells which are fed 5 ppm (d), 10 ppm (e), 50 ppm (f) and 100 ppm (g) geranyl acetate, obtained davanone content 18.02 μg/g FW, 0.26 μg/g FW, 56.73 μg/g FW and 52.17 μg/g FW, respectively. According to, high concentration of geranyl acetate, one of the monoterpenes, may damage cells and caused cells died, immobilized cells which are fed 100 ppm geranyl acetate, was obtained lower yield of davanone than which are fed 50 ppm geranyl acetate.

Fig. 18 shows davanone contents (μg/g FW) obtained from dichloromethane extracts of intact plant (a), callus cultures (b), suspension cultures (c), immobilised cells fed 5 ppm geranyl acetate (d), immobilised cells fed 10 ppm geranyl acetate (e), immobilised cells fed 50 ppm geranyl acetate (f) and immobilised cells fed 100 ppm geranyl acetate (g). It was shown that davanone content were obtained increased after using biotechnological techniques, such as cell immobilisation, feeding of precursor and biotransformation, and use of adsorbent,

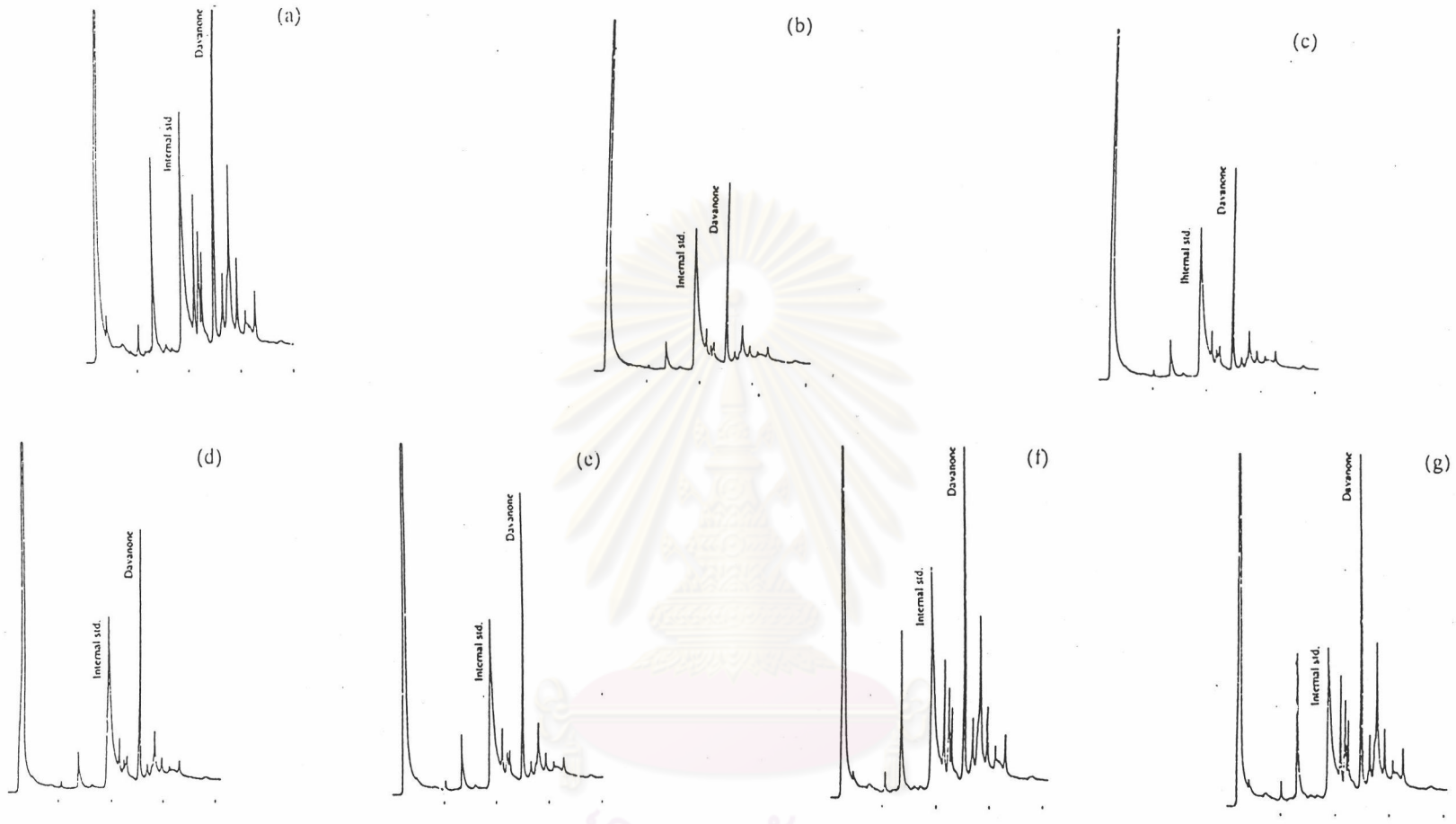


Figure 14 The GC chromatogram of dichloromethane extract

(a) = Intact plant , (b) = Callus cultures , (c) = Cell suspension cultures , (d) = Immobilised cells + 5 ppm geranyl acetate, (e) = Immobilised cells + 10 ppm geranyl acetate, (f) = Immobilised cells + 50 ppm geranyl acetate and (g) = Immobilised cells + 100 ppm geranyl acetate

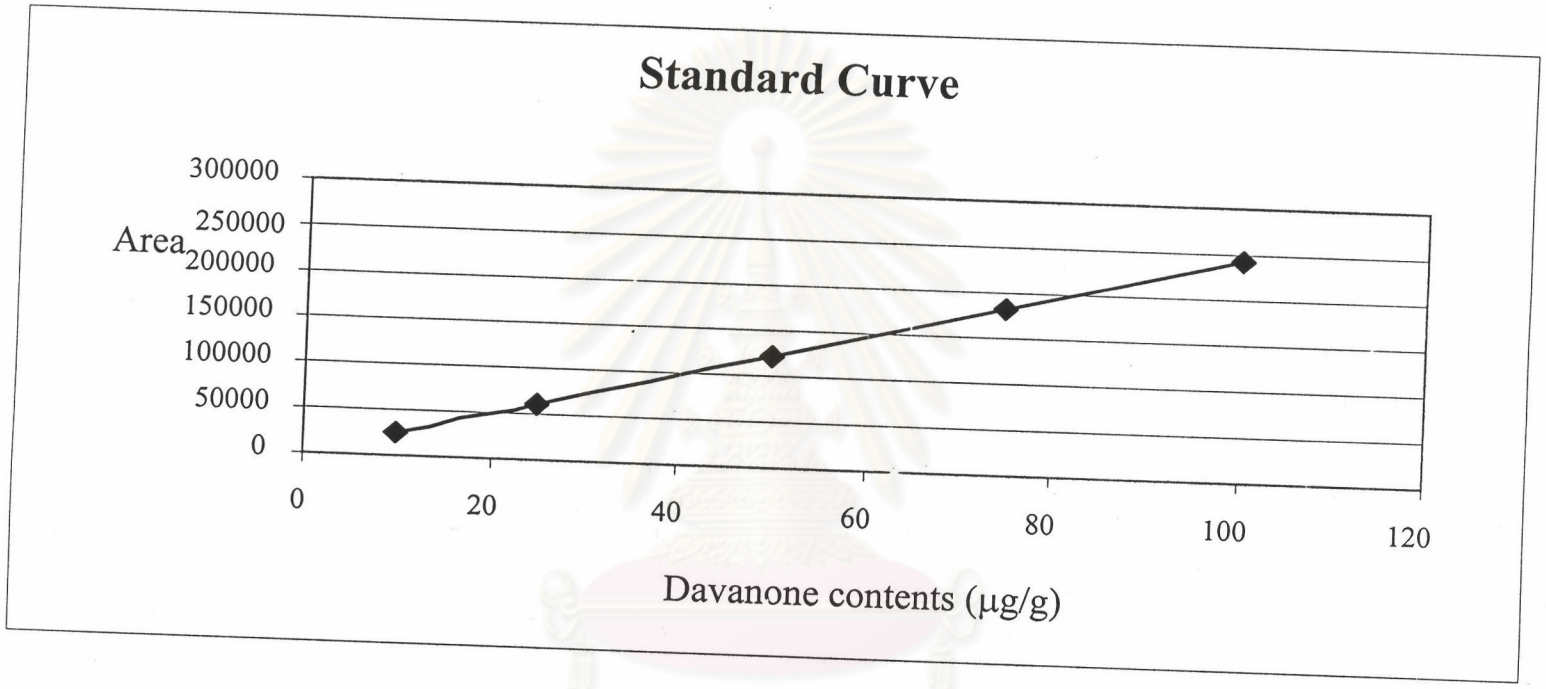


Figure 15 A standard curve of davanone contents

Table 15 Davanone contents obtained from dichloromethane extracts

Dichloromethane extracts	**Area under peak**	**Davanone (μg/g FW)**
Intact plant (a)	172627	70.82
Callus cultures (b)	33045	13.90
Cell suspension cultures (c)	36498	15.09
Immobilized cells + 5 ppm geranyl acetate (d)	44764	18.02
Immobilized cells + 10 ppm geranyl acetate (e)	49782	20.26
Immobilized cells + 50 ppm geranyl acetate (f)	141890	56.73
Immobilized cells + 100 ppm geranyl acetate (g)	128770	52.17

(SD < 5%, n = 3)

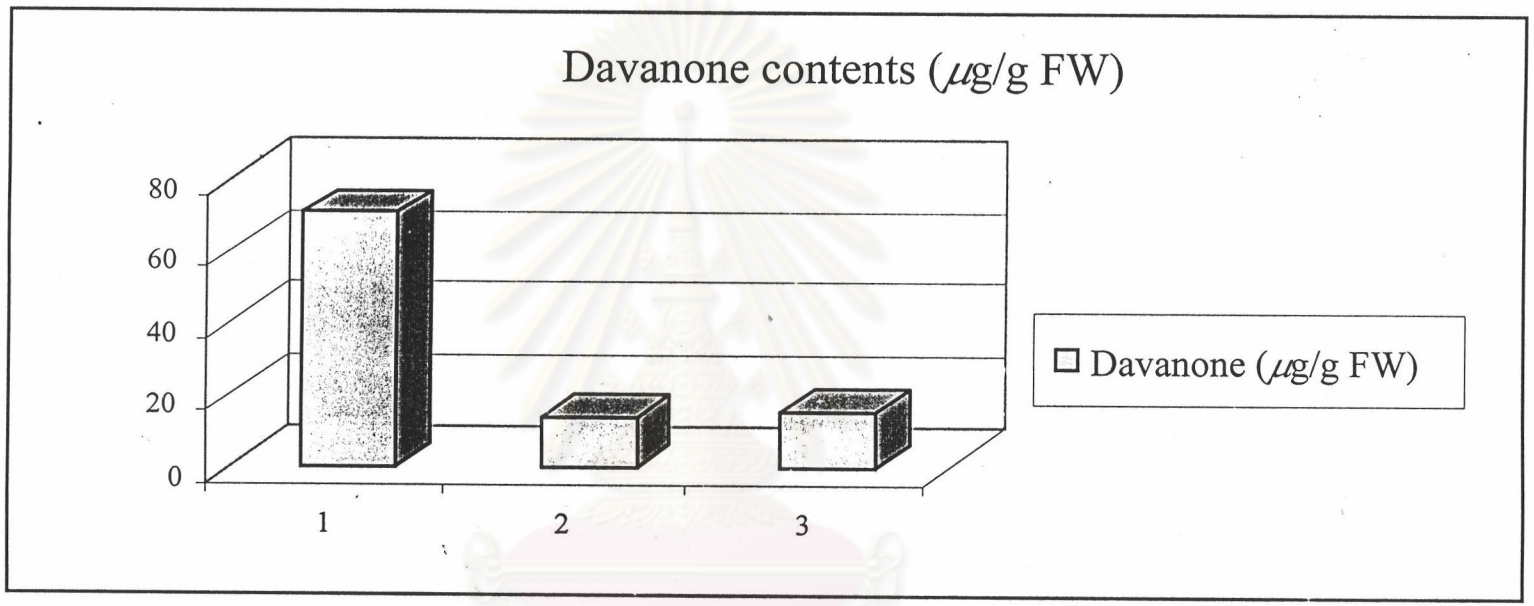


Figure 16 Davanone contents (μg/g FW) obtained from dichloromethane extracts, 1 = Intact plant (a), 2 = Callus cultures (b) and 3 = Cell suspension cultures (c)

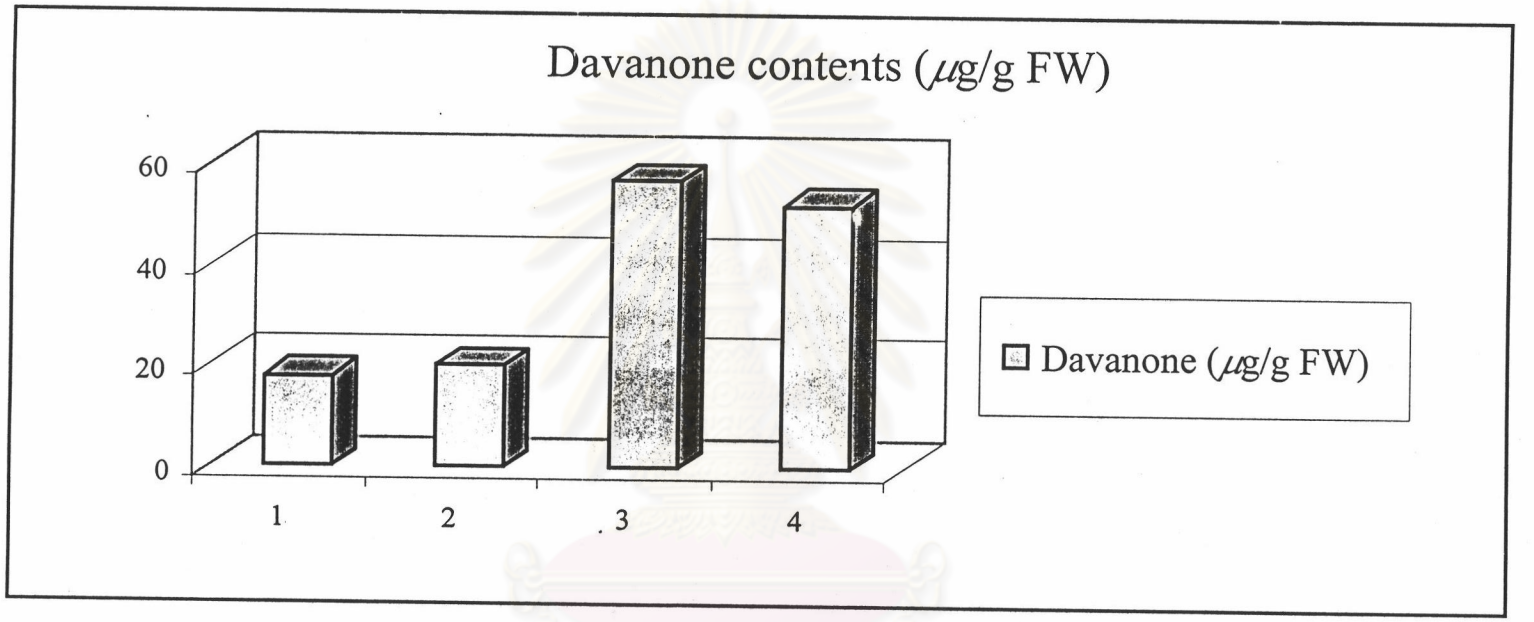


Figure 17 Davanone contents (μg/g FW) obtained from dichloromethane extracts, 1 = Immobilized cells + 5 ppm geranyl acetate (d), 2 = Immobilized cells + 10 ppm geranyl acetate (e), 3 = Immobilized cells + 50 ppm geranyl acetate (f) and 4 = Immobilized cells + 100 ppm geranyl acetate (g)

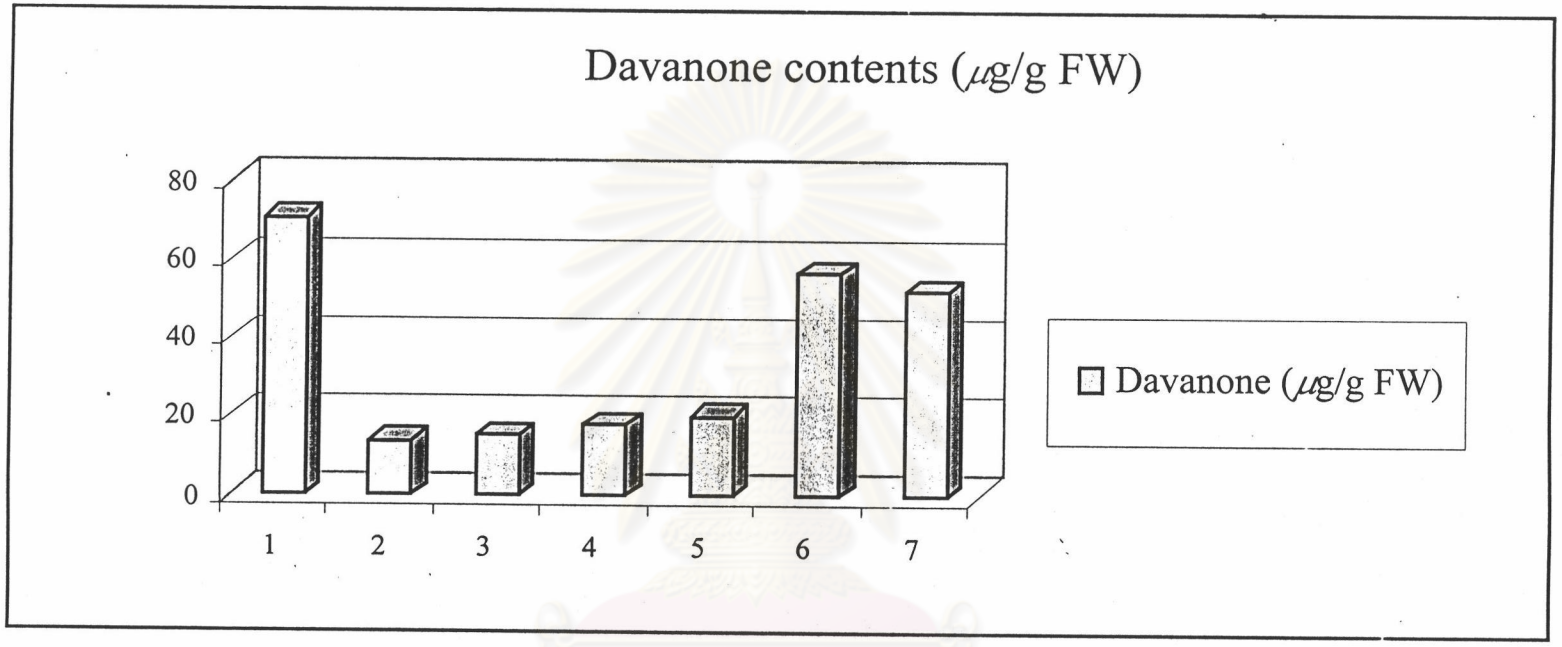


Figure 18 Davanone contents (μg/g FW) obtained from dichloromethane extracts, 1 = Intact plant (a), 2 = Callus cultures (b), 3 = Cell suspension cultures (c), 4 = Immobilised cells + 5 ppm geranyl acetate (d), 5 = Immobilised cells + 10 ppm geranyl acetate (e), 6 = Immobilised cells + 50 ppm geranyl acetate (f) and 7 = Immobilised cells + 100 ppm geranyl acetate (g)