CHAPTER IV

RESULTS AND DISCUSSION

Plant extract

The fresh rhizomes of *C. aeruginosa* obtained as a greenish yellow color (Figure 27). The extraction of *C. aeruginosa* rhizome with hexane provided a clear, dark brown and thick liquid (with a yield of 0.6% w/w (dried weight)). Its odour is pleasant and turmeric-liked.

(a) **(b)** **(c)**

Figure 27 The appearance of (a) the fresh rhizomes (b) the dried powder rhizomes and (c) the hexane extract of *C. aeruginosa*

Isolation and structure elucidation

The hexane extract of *C. aeruginosa* was fractionated using chromatography and recrystallization to give compounds **1-6** which were identified as sesquiterpenes (Figure 28): germacrone (**1**), zederone (**2**), dehydrocurdione (**3**), curcumenol (**4**), zedoarondiol (**5**), and isocurcumenol (**6**).

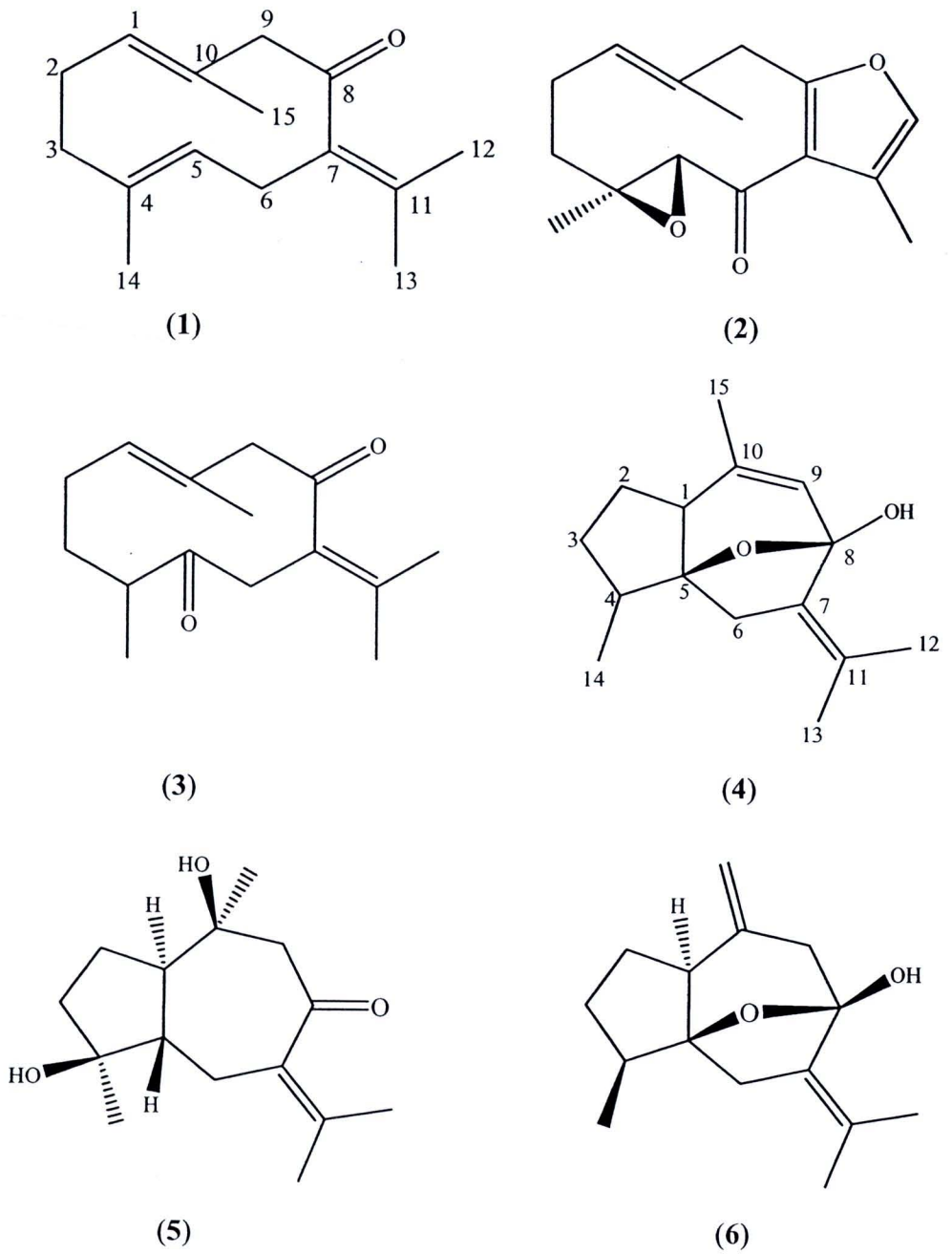


Figure 28 Structures of six sesquiterpenes isolated from *C. aeruginosa* extract

Compound **1** was obtained as colorless needle-shaped crystals; EI-MS (Figure 67): *m/z* 218 $[M]^{+\cdot}$; 200 (6), 176 (18), 136 (37), 107 (85), 91(100), 79 (46), 67 (44). The ^{1}H NMR (in $CDCl_3$) spectrum (Figure 68, 69 and Table 4) showed four 3H-singlets of tertiary methyls at δ 1.74 (H-12), 1.69 (H-13), 1.41 (H-15) and 1.60 (H-14). Two broad doublet signals at δ 4.97 (H-1) and 4.70 (H-5) indicating the vinyl protons in cyclodecadiene ring. The doublet of quartet signal at δ 2.33 belongs to H-2a. The H-2b and methylene proton (H-3) appeared as multiplet at δ 2.05-2.15. Pairs of geminal protons of H-6 and H-9 exhibited the overlapping signal (δ 2.90) and doublet signals (δ 2.82, 3.38). These spectra obtained suggesting a molecular formula of $C_{15}H_{22}O$. Comparing with the data reported, this structure was thus identified as germacrone (**1**) [13,84-86].

Compound **2** was obtained as colorless needle-shaped crystals. The EI-MS (Figure 70) gave a molecular ion peak at *m/z* 246 $[M]^{+\cdot}$; 213(28), 188(38), 175(100), 161(63), 159(47), 119(79), 115(56), 91(98), 77(47). The ^{1}H NMR (in $CDCl_3$) spectrum ((Figure 71 and Table 4) showed one vinyl proton signal at δ 5.47 (dd, H-1). This result supported that its coupling with the adjacent methylene (H-2). One vinyl methyl signal of H-15 was showed at δ 1.59. The methylene proton (H-9) have almost identical chemical shift (δ 3.67 and 3.74). This data show that H-9 was next to the double bond and the carbon adjacent to this methylene was also quaternary. Three-proton resonance signals at δ 1.32 (H-14) arising from a methyl on carbon carrying oxygen. A one-proton signal at δ 3.79 (H-5) originating from a hydrogen on carbon bearing an oxygen and it also showed singlet signal which supported that it no sign of spin coupling with any protons. The low shift at δ 3.79 of H-5 as a proton in an epoxide ring is due to the effect of the adjacent carbonyl group. The ^{13}C NMR spectrum contained 15 carbons (Figure 72 and 73). The assignment of ^{1}H and ^{13}C chemical shift were confirmed by HMQC and HMBC correlation spectra (Figure 74 and 76). The spectral described above suggested that compound **2** was zederone ($C_{15}H_{18}O_3$), which was isolated previously from *Curcuma zedoaria* [87].

Compound **3** was obtained as yellow viscous oil. The positive ESI-MS (Figure 78) gave a pseudo molecular ion peak at *m/z* 235 $[M+H]^+$; 218(39), 190 (31), 135(19). The ^1H NMR (in $CDCl_3$) spectrum (Figure 79, 80 and Table 4) showed quite similar to those of **1**. This compound has a unique germacrone-dione structure. The spectrum showed three 3H-singlets at δ 1.50 (H-12), 1.53 (H-13) and 1.39 (H-15), indicating three methyl groups attached to quaternary carbons. Compared with ^1H NMR of **1**, there were some main points different in the ring. One methyl group showed doublet signal at δ 0.78 (J = 4.0 Hz, H-14), belongs to the proton that attached to methine proton. The geminal proton of H-6 was presented at low field (δ 3.01 and 3.07) due to the effect of carbonyl carbon. The ^1H and ^{13}C assignments were confirmed by ^1H-^1H COSY, HMQC and HMBC (Figure 81-88). Comparing with the reported data, this structure was identified as dehydrocurdione ($C_{15}H_{22}O_2$) [85,86,88].

Compound **4** was obtained as colorless crystals. The negative ESI-MS (Figure 89) gave a pseudo molecular ion peak at *m/z* 233 $[M-H]^-$; 135(17). The ^1H NMR (in $CDCl_3$) spectrum (Figure 90 and Table 5) showed three singlet signals of terminal methyl groups at δ 1.58 (H-12), 1.65 (H-13) and 1.80 (H-15). Another methyl group showed doublet signal at δ 1.02 (J = 6.4 Hz). Two methylene groups (H-2 and H-3) gave multiplet signal at higher field indicating the adjacent methine protons (H-1 and H-4) between δ 1.55-1.95. The geminal methylene protons (H-6) showed as doublets at δ 2.10 and 2.64. Olefinic protons (H-9) appeared as singlet signal at δ 5.75 and the hydroxyl group appeared as a singlet signal at δ 2.88. By using all spectral data obtained and comparing with the literatures, the ^1H NMR and MS are in agreement with curcumenol ($C_{15}H_{22}O_2$), which was isolated previously from the rhizome of *C. aeruginosa* [15].

Compound **5** was obtained as yellow viscous oil. The EI-MS (Figure 91) showed *m/z* 252 $[M]^{+\cdot}$; 234(41), 191(76), 173(28), 121(36), 109(47), 97(34), 81(100), 71(30), 55(24). The ^1H NMR (in $CDCl_3$) and ^{13}C NMR spectra (Figure 95 and Table 5) showed the presence of two tertiary methyl groups (δ 1.17s, 1.19s, and 20.7, 22.3, respectively) on carbon which attached to hydroxyl group (δ 80.1 and 72.8). The presence of these two signals was confirmed by HMBC spectrum (Figure 99-101) with correlation of C-5 (δ 50.2) with H-14 and C-9 (δ 59.9) with H-15. HMBC spectrum

(Figure 104) also indicated that the isopropylidene group conjugated with a carbonyl group (δ 203.2). These data suggested that **5** had a guaiane type skeleton. The compound was then identified as zedoarondiol ($C_{15}H_{24}O_3$). The ^{1}H NMR and ^{13}C NMR assignment were also compared with previous reports [13,85].

Compound **6** was obtained as colorless crystals. The EI-MS (Figure 105) gave *m/z* 234 $[M]^{+\cdot}$; 191(60), 173(20), 121(70), 105(100), 67(40). The ^{1}H NMR (Figure 106, 107 and Table 5) indicated the presence of three methyl groups at δ 1.61 (s, H-12), 1.79 (s, H-13) and 1.00 (d, J =6.4 Hz, H-14). Two pairs of triplets at δ 4.72 and 4.77 were the inequivalent olefinic proton of H-15, while two pairs of doublets at δ 2.51 and 2.66 were the geminal protons of H-9. The singlet peak of hydroxyl group appeared at δ 2.79. Six methylene protons (H-2, H-3 and H-6) showed as multiplets at δ 1.52-2.47. The spectral evidences described above suggested that the compound **6** was isocurcumenol ($C_{15}H_{22}O_2$), which was isolated previously from *Curcuma heyneana* and *C. aeruginosa* [15,86].

The isolated compounds have been reported as composition of volatile oil from the *Curcuma* spp. Various pharmacological activities i.e. anticancer, antitumour, antifungal, antibacterial, anti-inflammatory, hepatoprotection, postcoital contraception, anti-HIV, antioxidation, antinociceptive and platelets-activation reduction effects of volatile oil were reported [7, 9-12].

However, the anti-androgenic activity has not been studied yet. Therefore, we aimed to investigate the anti-androgenic activity of the isolated compounds on both *in vitro* and *in vivo* studies.

Table 4 ^{1}H NMR and ^{13}C NMR data (δ in ppm) of compounds 1-3 recorded at 400 MHz and 100 MHz respectively (in $CDCl_3$).

No.	1	2		3	
	δ_H (J in Hz)	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.
1	4.97 (1H, br d, J = 11.3)	5.47 (1H, dd, J = 3.6, 11.8)	131.0, CH	4.91 (1H, br t)	132.4, CH
2a	2.33 (1H, dq, J = 4.7, 13.3)	2.50 (1H, dq, J = 3.6, 13.3)	24.8, CH_2	1.78-1.93 (3H, m)	25.8, CH_2
2b	2.05-2.15 (3H, m)	2.23 (1H, br d, J = 13.3)			
3a		2.29 (1H, dt, J = 3.5, 13.3)	38.1, CH_2		33.7, CH_2
3b		1.28 (1H, dt, J = 3.5, 13.3)		1.43 (1H, m)	
4			64.0, C	2.19 (1H, m)	45.9, CH
5	4.70 (1H, br d, J = 8.6)	3.79 (1H, s)	66.6, CH		210.3, C
6a	2.90 (1H, overlapping)		192.3, C	3.07 (1H, d, J = 16.0)	43.0, CH_2
6b	2.82 (1H, br d J = 11.1)			3.01 (1H, d, J = 16.0)	
7			122.4, C		129.0, C
8			157.2, C		205.9, C
9a	2.90 (1H, overlapping)	3.74 (1H, d, J = 16.5)	42.0, CH_2	2.97 (1H, d, J = 10.0)	56.4, CH_2
9b	3.38 (1H, d, J = 10.5)	3.67 (1H, d, J = 16.5)		2.82 (1H, d, J = 10.0)	
10			131.2, C		129.6, C
11			123.4, C		136.5, C
12	1.74 (3H, s)	7.07 (1H, s)	138.2, CH	1.50 (3H, s)	20.6, CH_3
13	1.69 (3H, s)	2.09 (3H, s)	10.4, CH_3	1.53 (3H, s)	21.6, CH_3
14	1.41 (3H, s)	1.32 (3H, s)	15.3, CH_3	0.78 (3H, d, J = 4.0)	17.9, CH_3
15	1.60 (3H, s)	1.59 (3H, s)	15.8, CH_3	1.39 (3H, s)	15.8, CH_3

Table 5 ^{1}H NMR and ^{13}C NMR data (δ in ppm) of compounds 4-6 recorded at 400 MHz and 100 MHz respectively (in $CDCl_3$).

No.	4	5		6
	δ_H (J in Hz)	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	1.55-1.95 (6H, m)	1.94 (1H, m)	56.0, CH	2.21 (1H, t, J = 9.5)
2a,b		1.72 (2H, m)	23.0, CH_2	1.52-2.47 (5H, m)
3a		1.66 (1H, m)	39.8, CH_2	
3b		1.77 (1H, m)		
4			80.1, C	
5		1.38 (1H, t)	52.0, CH	
6a	2.10 (1H, d, J = 15.6)	1.95 (1H, m)	28.6, CH_2	1.93 (1H, m)
6b	2.64 (1H, d J = 15.6)	2.80 (1H, d, J = 15.0)		1.99 (1H, d, J = 15.2)
7			134.8, C	
8			203.2, C	
9a	5.75 (1H, s)	2.58 (1H, d, J = 12.6)	59.9, CH_2	2.66 (1H, d, J = 13.6)
9b		2.94 (1H, d, J = 12.6)		2.51 (1H, d, J = 13.6)
10			72.8, C	
11			142.3, C	
12	1.58 (3H, s)	1.82 (3H, s)	22.0, CH_3	1.61 (3H, s)
13	1.65 (3H, s)	1.91 (3H, s)	22.7, CH_3	1.79 (3H, s)
14	1.02 (3H, d, J = 6.4)	1.19 (3H, s)	22.3, CH_3	1.00 (3H, d, J= 6.4)
15a	1.80 (3H, s)	1.17 (3H, s)	20.7, CH_3	4.77 (1H, t, J = 2.1)
15b				4.72 (1H, t, J = 2.1)
OH	2.88 (1H, s)	1.94 (1H, overlapping) and 1.72 (1H, overlapping)		2.79 (1H, s)

The inhibitory activity against conversion of testosterone

Base on the properties of 5α-reductase and tissue distribution of both isozymes (Table 2 and 3), the type 1 isozyme is active at pH 6.0-8.5 while type 2 is active at pH 5.0-5.5. In the assay, rat liver was used as a source of enzyme. The pH dependence of 5α-reductase activity was also studied (Table 6). We found that the enzyme activity was active at pH 7.2 which was corresponded to the pH optima of type 1 isozyme (pH 6.0-8.5) [1].

Table 6 The enzymatic activity in two difference pH conditions

pH	Enzymatic activity*			
	1	2	3	average ± SD
5.3	0.64	0.64	0.79	0.69 ± 0.09
7.2	1.47	1.10	1.37	1.31 ± 0.19

Note: * Enzymatic activity was calculated from r of $control_{0\ min}$ – r of $control_{30\ min}$

In the present study, *C. aeruginosa* has been shown to contain germacrane type sesquiterpenes (**1**, **2**, **3**) and guaiane type sesquiterpenes (**4**, **5**, **6**). The anti-androgenic activity of **1-6**, the *C. aeruginosa* extract (1 mg/mL) and ethinylestradiol (1 mM), as positive control, on the conversion of testosterone were determined using rat liver as a source of enzyme (Table 7).

Among six isolated compounds, **1** expressed the highest inhibitory activity (65.7 ± 4.7%). The concentration that could inhibit 50% of the enzymatic activity (IC_{50}) of **1** was 0.42 ± 0.05 mg/mL (or 1.94 ± 0.23 mM) while that of *C. aeruginosa* was 0.22 ± 0.03 mg/mL. The positive control, ethinylestradiol showed the IC_{50} value of 0.26 ± 0.02 mg/mL or 0.86 ± 0.01 mM which was in agreement to the study of. Matsuda et al. [16,36,37] and Hirata et al. [89] (IC_{50}, 0.81 mM).

Table 7 Inhibition effects of *C. aeruginosa* and its constituents on the conversion of testosterone to DHT

Compounds	Enzymatic inhibition (%)	IC_{50} (mg/mL)
C. aeruginosa extract	72.8 ± 2.6	0.22 ± 0.03
Germacrone (**1**)	65.7 ± 4.7	0.42 ± 0.05
Zederone (**2**)	46.0 ± 12.9	Not determined
Dehydrocurdione (**3**)	45.2 - 48.1	Not determined
Curcumenol (**4**)	17.4 ± 4.5	Not determined
Zedoarondiol (**5**)	16.4 ± 5.1	Not determined
Isocurcumenol (**6**)	4.7 ± 8.5	Not determined
Ethinylestradiol	47.6 ± 5.5	0.26 ± 0.02

Note: Percent enzymatic inhibition of tested samples was measured at the final concentration of 1.0 mg/mL except for **5** which were tested at 0.5 mg/mL (the concentration of **5** was limited by its solubility in DMSO). Ethinylestradiol was used as a positive control and its enzymatic inhibition was measured at 1 mM. The data are expressed as means ± SD. Determinations were done in triplicate except for **3** which was done in duplicate.

From all isolated compounds, the germacrane type sesquiterpenes showed higher inhibitory activity than that of guaiane type sesquiterpenes. The germacrane type sesquiterpenes composed of a cyclodecadiene ring in the structure, while guaiane type sesquiterpenes contained a five-membered ring fused with seven-membered ring. So that, the guaiane type sesquiterpenes were likely to have more rigid conformation comparing to the germacrane type. The structure conformation of molecule might be one of the possible reasons for the difference of enzyme inhibitory activities. In germacrane type, substituted groups and their positions in the molecule changed the %enzymatic inhibition. The most potent inhibitor, compound **1** has two double bonds at position $C_{1\text{-}10}$ and $C_{4\text{-}5}$ in the ten-membered ring. Reduction of double bond at $C_{4\text{-}5}$ can cause the conformation change of the molecule. As seen in compound **3**, the double bond of position $C_{4\text{-}5}$ was reduced and C_5 was oxidized which caused slightly

decrease of % enzymatic inhibition. As for compound **2**, changing the double bond at $C_{4\text{-}5}$ to epoxide ring and α,β-unsaturated ketone to furan ring also reduced the activity. From the data, it was likely that conformation of the ring might play the major role for the inhibitory activity. However, others factors might involve in the activity which are needed to be further investigated.

Cell viability tests

1. The cytotoxic effect of the *C. aeruginosa* extract and the isolated compounds on cell viability of human foreskin fibroblast cells (HF)

Since characteristic of androgen action have been described in skin tissue, as well as in cultured human skin fibroblasts, human skin may be considered as a target organ for androgens

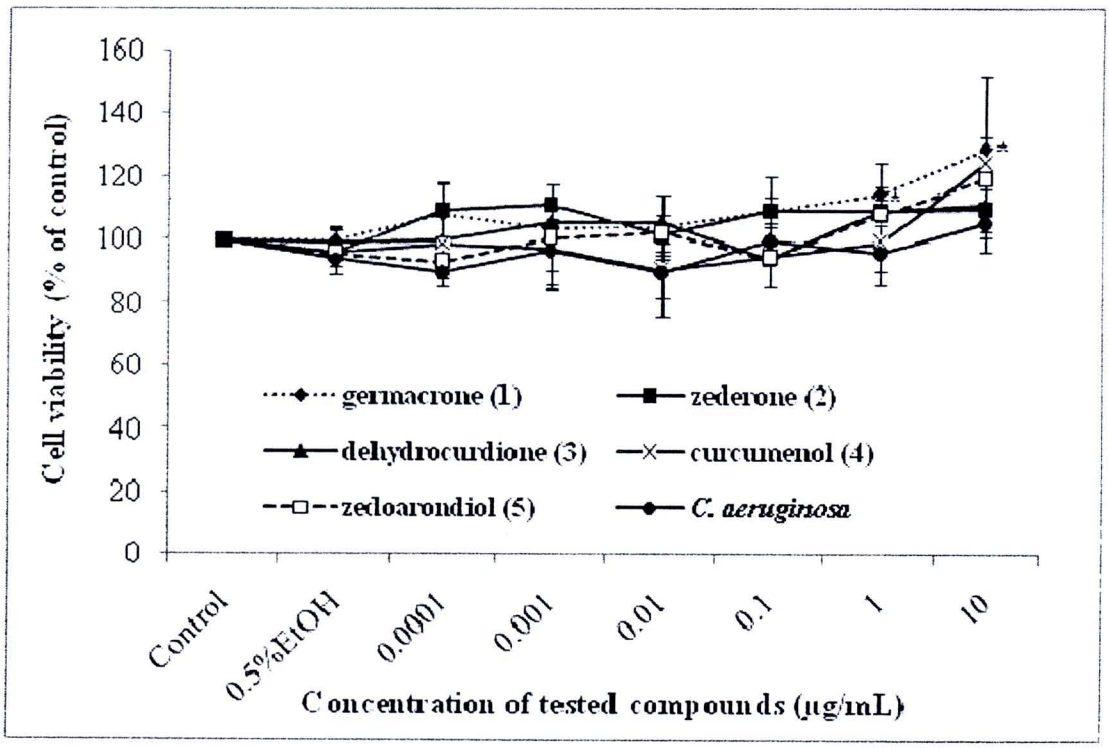


Figure 29 The effect of compounds 1-5 and *C. aeruginosa* extract on the cell viability of HF cells after treatment for 24 hr. (Results represent as mean ± SEM of 3 experiments. *$p < 0.05$ against vehicle (0.5%EtOH) group)

The HF cell was used to assess the cytotoxic effect of tested compounds. Compounds **1-5** together with crude extract of *C. aeruginosa* (0.0001–10 μg/mL) were tested on cell viability of HF cells (Figure 29). Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Most compounds showed no cytotoxic effect on HF cells at the concentration of 10^{-4}-10 μg/mL. Only **1** and **5** tended to significantly increase cell viability at the concentration of 10 and 1 μg/mL, respectively.

2. The cytotoxic effect of the *C. aeruginosa* and isolated compounds on cell viability of human prostate cancer cells (LNCaP)

A cell line derived from androgen dependent human prostate cancer cells (LNCaP cell) was used to: (i) assess the cytotoxicity of compounds and (ii) find a suitable concentration to assess testosterone-induced cell proliferation.

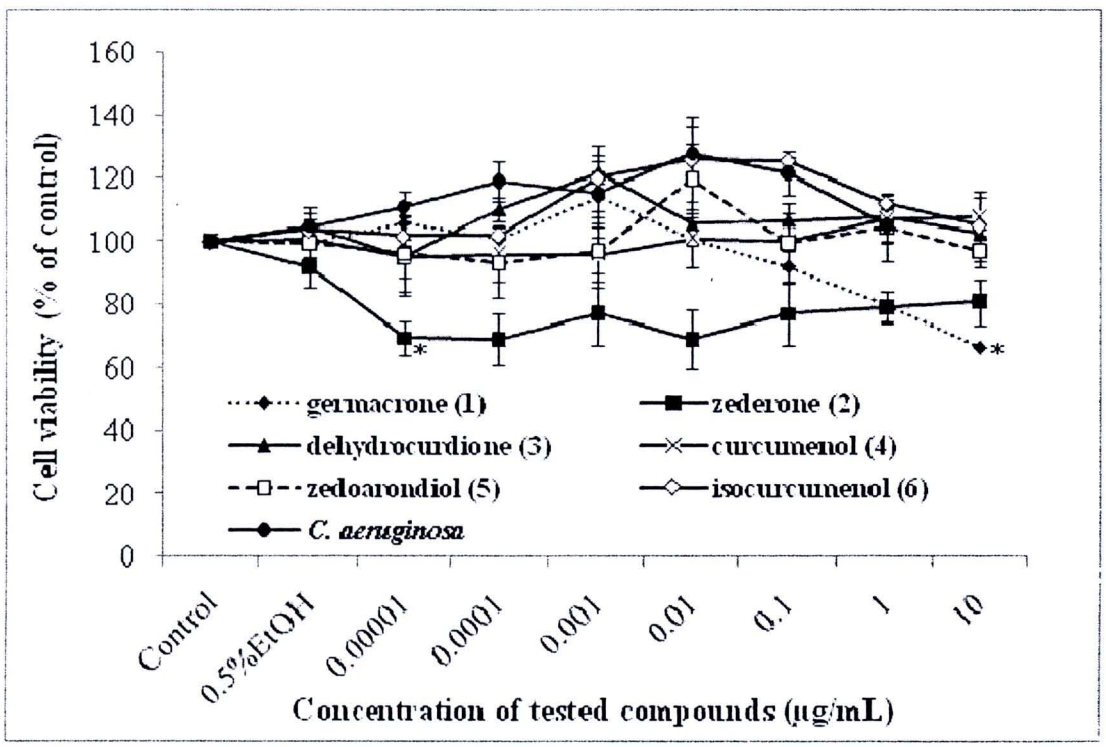


Figure 30 The effect of compounds 1-6 and *C. aeruginosa* extract on the cell viability of LNCaP cells after treatment for 24 hr. (Results represent as mean ± SEM of 3 experiments. *$p < 0.05$ against vehicle (0.5%EtOH) group)

The cells were cultured with compounds **1-6** and the crude extract of *C. aeruginosa* at the concentrations of 0.00001-10 μg/mL (the concentration was limited by their solubility in culture medium). *C. aeruginosa* extract and **3-6** showed no cytotoxic effect on LNCaP cell viability at all tested concentrations. However, **1** at the highest concentration (10 μg/mL or 45 μM) decreased cell viability approximately 30%. The result corresponded to a study by Zhong et al. [90]. They demonstrated that **1** also inhibited the proliferation of breast cancer cell lines (MDA-MB-231 and MCF-7 cells) at the concentration of 50-400 μM. Interestingly, **2** dose-independently reduced LNCaP cell viability by 20-30% at the concentration of 0.00001-10 μg/mL. (Figure 30). Although the concentration of **2** was increased, the cell viability of LNCaP cell was still showed in the range of 70-80%. One of the possible explanations is that **2** might inhibit cell growth rather than kill the cells.

The anti-androgenic effect on testosterone-induced cell proliferation of LNCaP cells

LNCaP cells are widely used in androgen studying related. The cells are source of receptors and enzymes involving in androgen metabolism [91,92]. The most potent inhibitor in the enzymatic assay, **1**, was further investigated for anti-androgenic effects on testosterone-induced growth of LNCaP cells. Compound **1** (0.5 and 5 nM) was incubated with LNCaP cells with or without testosterone for 96 hr. The cell proliferation was quantified using MTT assay.

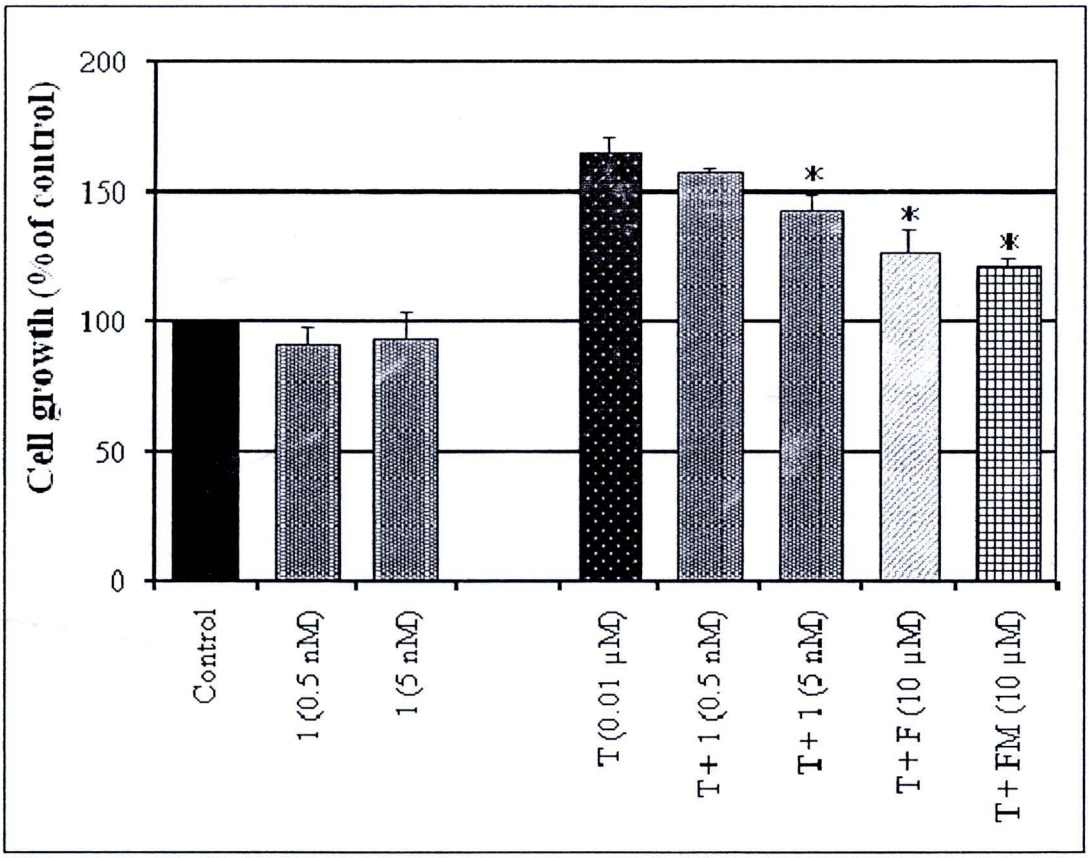


Figure 31 The effect of germacrone (1) in testosterone (T)-induced cell growth of LNCaP cells in comparison with finasteride (F) and flutamide (FM). (Result represent as mean ± SEM of 5 experiments. *$p < 0.05$ in comparison with 0.01 μM testosterone)

Compound **1** had no effect on LNCaP cell proliferation at both concentrations when treated alone (Figure 31). Testosterone obviously stimulated the LNCaP cell proliferation by 60% which corresponded to the study of Liu et al. [92]. Compound **1** (5 nM) inhibited testosterone-induced growth of LNCaP cells while the lower concentration of **1** was ineffective. The similar growth suppression was observed with flutamide (10 μM), an androgen receptor blocker, and 5α-reductase inhibitor, finasteride (10 μM). These results suggested that **1** may exhibit anti-androgenic effect through 5α-reductase inhibition and/or androgen receptor blockage.

Growth suppression of hamster flank glands by germacrone (1)

The hamster flank gland is a well-known model for evaluation of anti-androgen activity of the topically applied compounds [58]. These glands are pigmented macules bilaterally located on a hamster's back and are highly sensitive to androgen stimulation. The androgen-sensitive components of flank glands include dermal melanocytes, sebaceous glands and hair follicles. From our preliminary study, the hexane extract of *C. aeruginosa* (1 mg) showed a similar inhibitory effect to topical finasteride (0.5 mg) on the testosterone-stimulated flank gland growth. In contrast, both could not inhibit DHT-stimulated growth [6].

In the present study, we tested compound **1** using the same model and the results are shown in Tables 8 and 9. After treatment for 4 weeks, the sizes of the flank glands of non-castrated hamsters (intact group) increased by about 13 mm^2 suggesting the role of endogenous androgens. Although these was no control castrated hamster in this experiment, size of this animal was previously reported to remain unchanged [6]. However, when treated with exogenous testosterone or DHT (0.5 μg of each), the flank glands enlarged to 9 and 18 mm^2, respectively and became darker (Table 8).

To investigate the anti-androgenic effect, **1** (3, 30, and 100 μg) and a positive control, finasteride (100 μg) were topically applied on the flank glands together with the exogenous androgen, testosterone or DHT for 4 weeks. Compound **1** at every doses suppressed the testosterone-stimulated growth of the flank gland (79-82 % inhibition) suggesting that **1** was highly potent, ie. effective at 3 μg. While **1** was effective against testosterone, it weakly inhibited DHT-stimulated growth of flank gland (27.7% inhibition at 100 μg). A similar profile was observed on the 5α-reductase inhibitor, finasteride. Thus, these results suggest that **1** inhibited the conversion of testosterone to DHT via 5α-reductase inhibition, with greater effect than that of finasteride at the same concentration. Moreover, the reaction of systemic effect was not observed under our experiment. The flank gland sizes of the untreated sides (vehicle alone) did not change (Table 9).

Table 8 Effects of germacrone (1) and finasteride on androgen-stimulated growth of hamster flank glands

Group'	N	Size of treated flank gland (mm^2± SEM)		Change of flank gland size [a] (mm^2± SEM)	Inhibition (%)
		Week 0	Week 4		
Intact	5	28.9 ± 1.4	41.9 ± 3.7	13.0 ± 2.8	-
Testosterone	8	24.1 ± 2.4	33.1 ± 2.3	9.0 ± 1.8	0
DHT	7	24.1 ± 2.3	42.2 ± 2.9	18.3 ± 3.5*	0
Testosterone + **1** (3 μg)	7	21.2 ± 2.5	22.8 ± 2.3	1.6 ± 2.7*	82.5
Testosterone + **1** (30 μg)	8	22.8 ± 2.2	24.4 ± 1.1	1.6 ± 1.9*	82.4
Testosterone + **1** (100 μg)	7	22.1 ± 2.6	23.9 ± 2.0	1.9 ± 0.8*	79.2
Testosterone + Finasteride (100 μg)	7	22.2 ± 2.4	25.5 ± 2.6	3.3 ± 3.1*	63.4
DHT + **1** (100 μg)	7	21.3 ± 2.7	34.5 ± 2.5	13.2 ± 2.3	27.7
DHT + Finasteride (100 μg)	5	16.2 ± 2.9	33.8 ± 5.4	17.6 ± 6.1	3.7

Note: [a] the changes of flank gland size was calculated by the size of treated flank gland at week 4 subtracted by that at week 0, * $p < 0.05$ represent significant difference when compared with the testosterone group and data are expressed as means ± SEM of 5-8 hamsters.

Table 9 The size of untreated flank gland of castrated hamsters

Group	N	Size of untreated flank gland (mm^2± SEM)	
		Week 0	Week 4
Testosterone	8	25.0 ± 1.6	26.3 ± 1.6
DHT	7	24.4 ± 1.7	25.9 ± 1.8
Testosterone + **1** (3 μg)	7	19.3 ± 7.1	22.4 ± 6.0
Testosterone + **1** (30 μg)	8	22.6 ± 1.9	23.6 ± 1.0
Testosterone + **1** (100 μg)	7	22.5 ± 1.3	22.1 ± 0.7
Testosterone + Finasteride (100 μg)	7	21.3 ± 2.0	22.6 ± 1.1
DHT + **1** (100 μg)	7	23.0 ± 1.6	23.7 ± 0.9
DHT + Finasteride (100 μg)	5	17.0 ± 4.0	17.9 ± 3.7

Note: Data are expressed as means ± SEM of 5-8 hamsters

From our studies, **1** showed similar inhibition effect to finasteride on the testosterone-stimulated growth of flank gland and LNCaP cells. Considering the mechanism of finasteride for inhibition on 5α-reductase, the process was proposed to involve a hydride transfer from NADPH to Δ1-double bond of finasteride. This reduction results in the formation of a lactam enolate which is trapped by the electrophilic pyridinium cation of NADP, yielding a covalent adduct to the cofactor [18,93]. Based on the structure of **1**, it also contains α,β-unsaturated ketone group in the molecule like that of finasteride. Therefore, **1** might bind to 5α-reductase via α,β-unsaturated ketone group.

Androgen receptor binding activity

It is noted that **1** showed more potent inhibitory activity on testosterone-induced growth of LNCaP cells model and *in vivo* model comparing to the effect on the *in vitro* enzymatic assay. Even though the anti-androgenic activity of compound **1** on the growth of hamster flank gland might be related to 5α-reductase inhibition, **1**

may also act through other mechanisms. We, therefore, examined its androgenic receptor binding activity using the fluorescent polarization (FP) and a high affinity fluorescent ligand for androgen receptor. Thus the ability of test compounds to bind to androgen receptors is indicated by the displacement of fluorescent ligand. The binding activity of fluorescent ligand to androgen receptor can be quantified by the change in its speed of the rotation that results in FP values (Figure 32). The receptor-ligand complex will rotate slowly and have high FP value. Increasing concentrations of the competing ligand will displace the fluorescent ligand from the androgen receptor in which free fluorescent ligand will rotate more rapidly and have a low FP value.

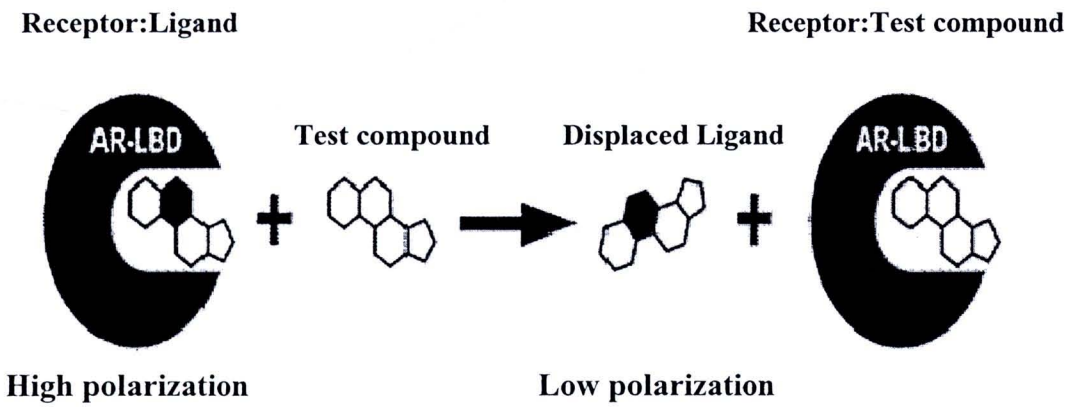


Figure 32 The examination of androgen receptor binding activity [55]

The polarization values were plotted against increasing concentrations of the test compounds. The semilog scale relative of concentration to polarization is shown in Figure 33. The polarization values decreased when the concentrations of DHT and flutamide increased indicated that they both bound to androgen receptors. DHT showed higher affinity than flutamide. The polarization did not change with increasing concentration of compound **1** suggesting that **1** failed to interact with androgen receptors. Thus, the anti-androgenic activity of **1** was clearly not associated with the blockage of androgen receptors.

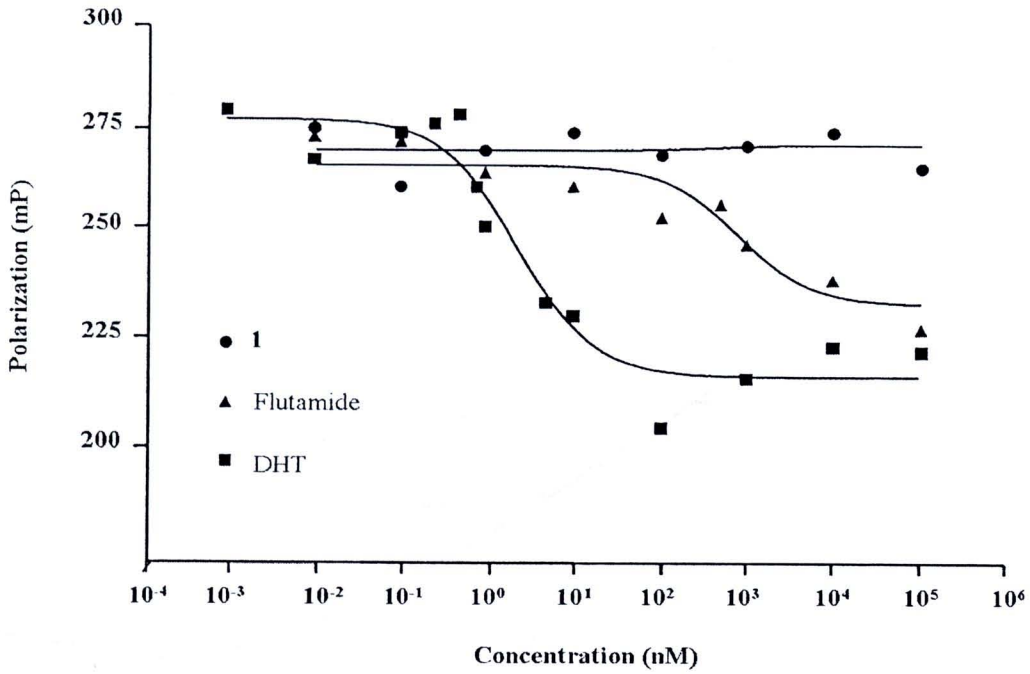


Figure 33 Androgen receptor binding by germacrone (1), flutamide and DHT determined by fluorescent polarization. Experiments were done in triplicate except the test for flutamide which was done in duplicate

From the inhibitory activity profile of **1**, the selectivity of **1** on 5α-reductase isozyme might be explained based on the properties of two isozyme; pH optima and localization in tissue. We found that (i) the pH condition in our *in vitro* enzymatic assay (pH 7.2) was corresponded to the optimal pH range of 5α-reductase type 1 (ii) LNCaP cell which is claimed that expresses only 5α-reductase type 1 was used as *in vitro* model and (iii) the flank gland of hamster which presents predominantly 5α-reductase type 1 was used as an *in vivo* model. Compound **1** showed inhibitory activity against the conversion of testosterone both *in vitro* and *in vivo* studies. These results suggested that **1** might inhibit the 5α-reductase type 1 activity.

Conversion of testosterone to DHT by 5α-reductase is a critical process for androgenic activity. Many androgen target tissues in men, including the prostate and scalp hair follicles are more responsive to DHT than testosterone [94]. In recent years, inhibitors of 5α-reductase have been developed for treatment of androgen-dependent problems in the scalp and prostate [5]. A 5α-reductase inhibitor, finasteride, has been

successfully used for the treatment of benign prostrate hyperplasia and male pattern baldness. From our studies, **1** showed similar inhibition effect to finasteride on the testosterone-stimulated growth of flank gland and LNCaP cells. Therefore, **1** is a promising lead compound for the treatment of the androgen-dependent disorders especially the androgen target tissues responsive to DHT.

Analyses of chemical components of the *C. aeruginosa* extract

The *C. aeruginosa* extract showed significant anti-androgenic activity. It was proved to be an effective ingredient in hair tonic for androgenic alopecia [7]. Thus, the quality control of the extract and the stability studies are very important. In this study, we use 3 chromatographic methods for quality control of the extract i.e. TLC, HPLC and GC.

1. TLC analyses of *C. aeruginosa* extract

TLC is the conventional technique for analysis of the chemical components in the extract. The TLC fingerprint was used for the quality control of plant materials.

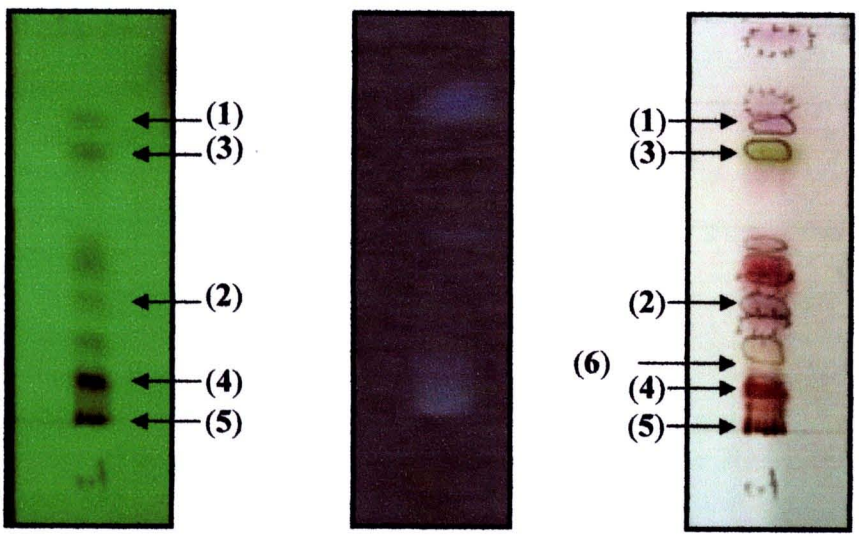


Figure 34 TLC fingerprint of *C. aeruginosa* extract using hexane:$CHCl_3$ (2:8 v/v) as a mobile phase: (a) viewed at 254 nm without coloration (b) viewed at 366 nm without coloration (c) colorized with anisaldehyde reagent

In this study, many TLC mobile phase systems were tested i.e. hexane:$CHCl_3$, hexane: EtOAc, hexane:CH_2Cl_2 to provide the fingerprint of *C. aeruginosa* extract. The mobile phase hexane:$CHCl_3$ (2:8 v/v) seemed to give the most suitable fingerprint. Quenching spots under short UV wavelength (254 nm), and the fluorescence spots under long UV wavelength (366 nm), were detected as shown in Figures 34a and 34b. Most of these spots gave color after sprayed with anisaldehyde reagent (Figure 34c).

2. HPLC analysis of *C. aeruginosa* extract

The series of C-18 column and mobile phase were tested for the separation of chemical components in *C. aeruginosa* extract.

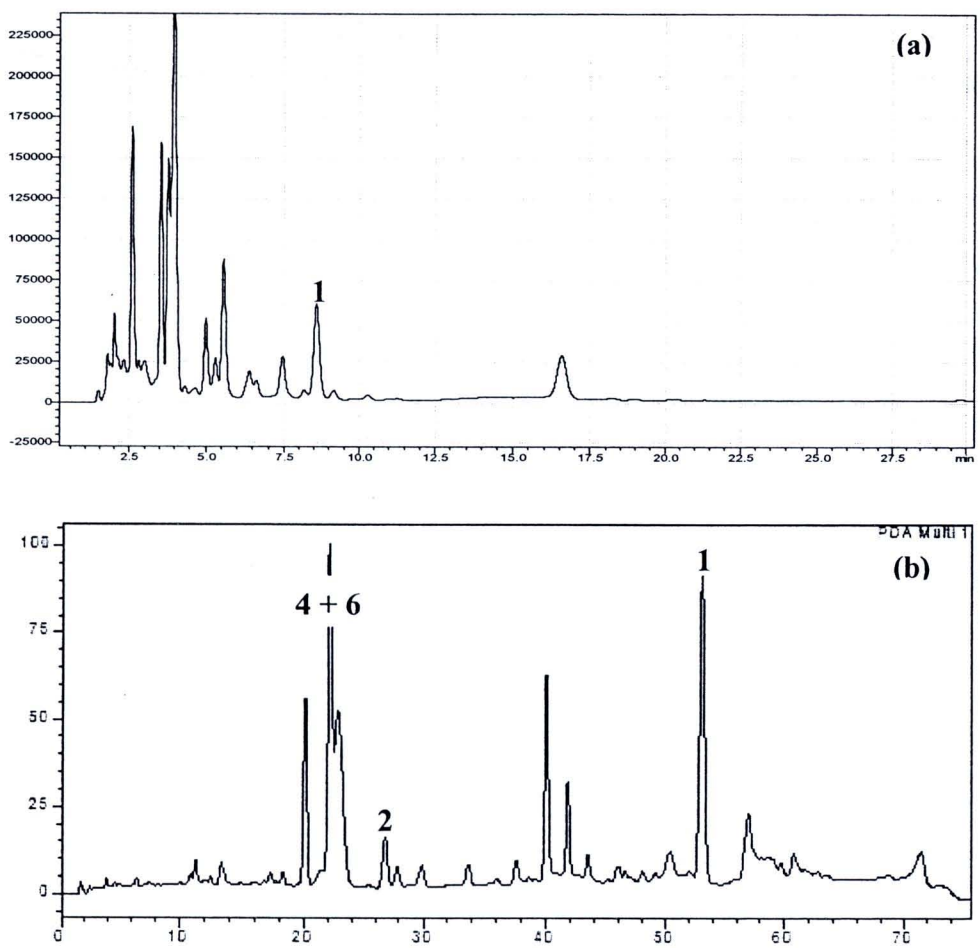


Figure 35 The HPLC fingerprint of 1.0 mg/mL *C. aeruginosa* extract obtained from (a) system 1 viewed at 235 nm and (b) system 2 viewed at 214 nm

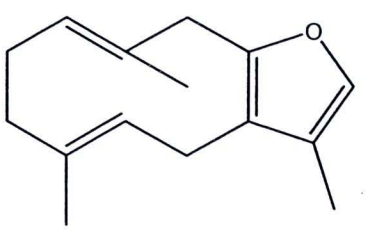
The most suitable chromatographic condition was composed of a Luna C-18 column and a mobile phase, CH_3CN and 0.5 mM phosphate buffer pH 3.0. The injection and elution conditions were optimized as describe in experimental session. Both isocratic and gradient system were developed which are call "HPLC system 1" and "HPLC system 2", respectively.

The HPLC system 1 was optimized for determination of **1** (Figure 35a) in the preliminary stability studies. The suitable mobile phase was CH_3CN and 0.5 mM phosphate buffer pH 3.0 (73:27 v/v). The HPLC system 2 was also optimized. The total run time was 70 min (Figure 35b). The peak of **1**, **2**, **4** and **6** were identified by comparing the retention times of the peaks with that of reference compounds under the same conditions. The result showed that this system could be applied to determination of **1** and **2** in crude extract. Although, the overlapping of peak **4** and **6** was found, the HPLC fingerprint obtained can be useful for control the quality of extract. Moreover, HPLC can be used for the analytes that could not be determined with GC.

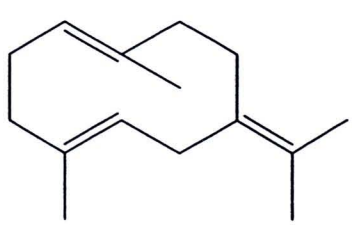
3. GC analysis of *C. aeruginosa* extract

3.1 Optimization of GC conditions

To obtain better resolutions of compounds, GC conditions were optimized. GC is the conventional method for analysis of the essential oil because of its high resolving power and the availability of universal detection using flame ionization detection (FID). The GC-FID systems were developed and validated for determination 6 compounds isolated (compounds **1-6**) and 2 compounds (**7** and **8**) from our colleague, Mr. Jukkarin Srivilai [82]. The structures of **7** and **8** are shown on Figure 36.



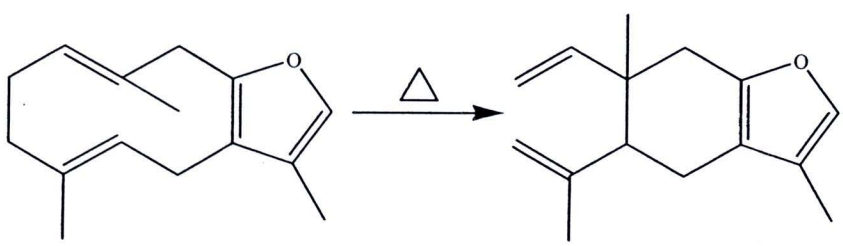
Furanodiene (**7**)



germacrene B (**8**)

Figure 36 Structures of furanodiene (7) and germacrene B (8)

The chromatogram of *C. aeruginosa* extract obtained from GC-FID showed the higher resolution than that from HPLC technique. Two GC systems were then developed. The first system (GC-FID system 1) showed the total run time of 43 min. The injector was set at 150°C and the column temperature program was slowly increased from 50-250°C to avoid thermal degradation. The identification of the components was based on the comparison of their retention time with those of reference compounds under the same conditions. We found the degradation of **7** during analysis in the column. This result was in agreement with the study of Yang et al. [95]. They reported that volatile oil usually contains heat sensitive components which may degrade and give the wrong result during GC analysis. This problem has been encountered with several sesquiterpenes such as germacrene, germacrone, and furanodiene. The skeleton of these compounds rearranges thermally through Cope rearrangement. For example, furanodiene (**7**) changed into curzerene (furanoelemene). The proposed pathway of thermal degradation for furanodiene is shown in Figure 37.



Furanodiene Curzerene

Figure 37 The pathway of thermal degradation for furanodiene [95]

In this study, we also found that compounds **3**, **5** and **8** could not be detected by GC due to the poor resolution and probably heat instability. Fortunately, compound **1** showed no degradation under the optimized condition. Therefore, only four sesquiterpenes (compounds **1**, **2**, **4**, and **6**) were quantified using optimized condition which is called "GC-FID system 1" (Figure 38). Therefore, this system could be applied for determination of chemical components of *C. aeruginosa* extract. The GC fingerprint obtained can be useful for control the quality of *C. aeruginosa* extract (Figure 39).

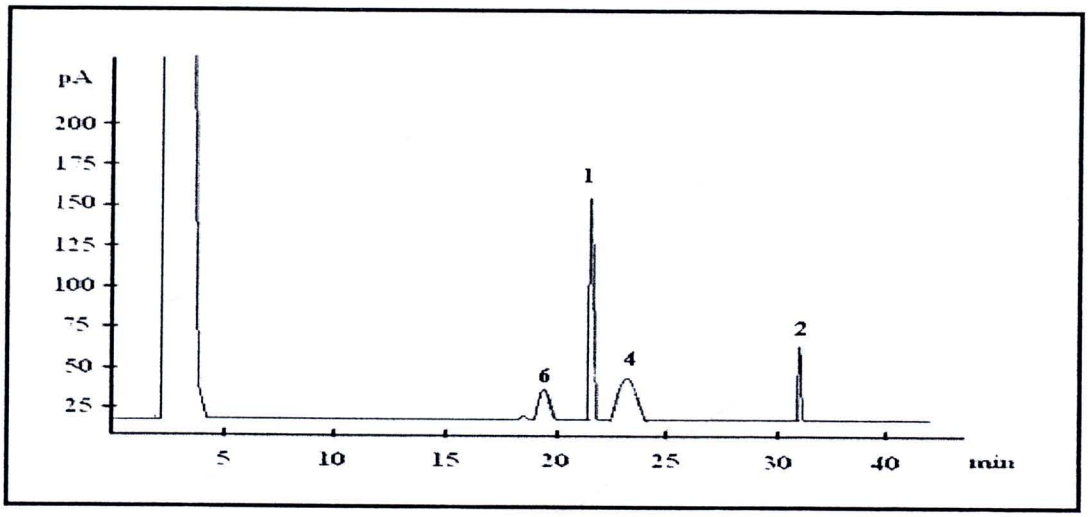


Figure 38 GC chromatograms of 0.5 mg/mL of four reference compounds analyzed using GC-FID system 1.

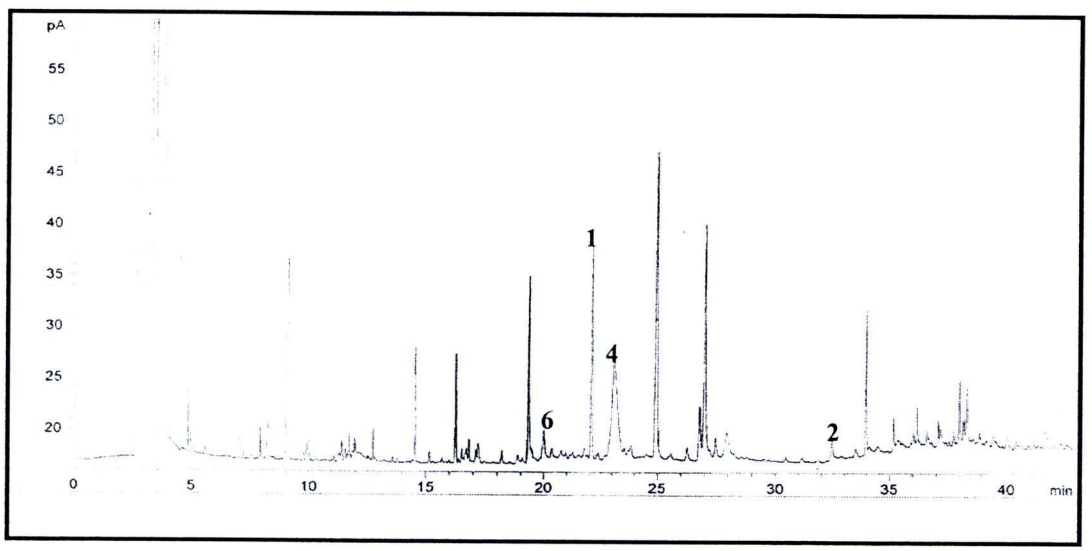


Figure 39 The GC chromatogram of 3.0 mg/mL *C. aeruginosa* extract analyzed using GC-FID system 1

For the stability study of **1**, the separation system with lower retention time of **1** was developed and named as "GC-FID system2". The same GC-FID instrument, column, carrier gas, flow rate, injection mode, injector and detector temperature as "GC-FID system 1" were used. The temperature program was changed as described in experimental session. The peak of **1** was obtained at 13.34 min (Figure 40). The total run time was 16.67 min (GC-FID system 2).

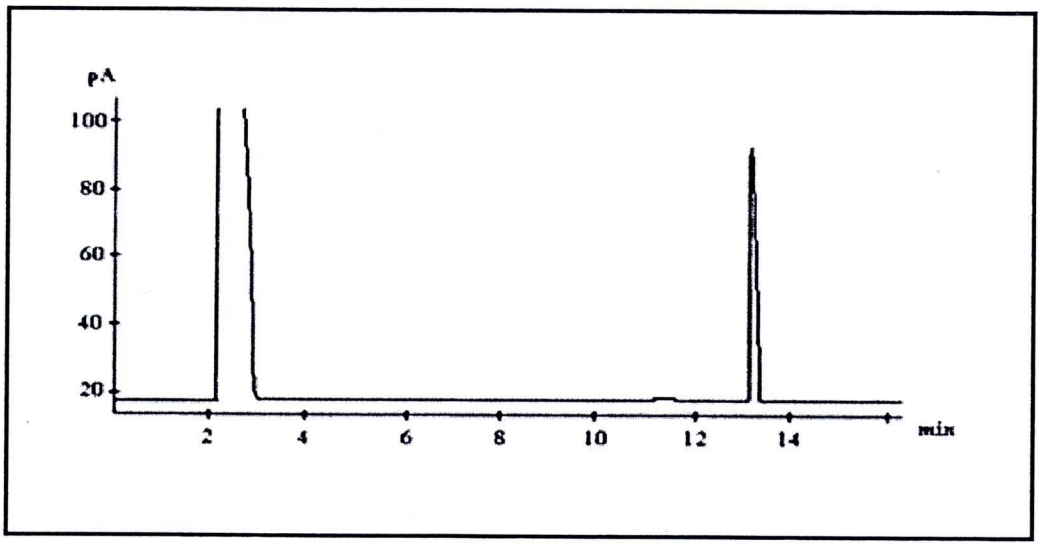


Figure 40 GC chromatogram of 0.5 mg/mL germacrone (1) analyzed using GC-FID system 2

3.2 Method validation for determination the chemical constituents in *C. aeruginosa* extract using GC-FID (system 1)

The proposed GC-FID system 1 was validated by considering the following criteria: linearity of the response, limit of detection, precision and accuracy. Four sesquiterpenes (**1**, **2**, **4**, and **6**) were used as marker compounds. The linearity expressed as correlation coefficient of 0.9946-0.9955 were obtained from the range of the concentration used the calibration curve (18.75-250 μg/mL) as shown in Table 10.

Table 10 Linear regression data of four marker compounds (n=4)

Compounds	Regression equation[a]	Correlation coefficient (r^2)
germacrone (**1**)	y = 1.1882x – 8.1168	0.9955
zederone (**2**)	y = 0.3967x – 1.2160	0.9947
curcumenol (**4**)	y = 1.0416x – 3.0374	0.9946
isocurcumenol (**6**)	y = 0.3881x – 2.7033	0.9953

Note: [a] x is the concentration of compound in μg/mL, y is the peak area obtained from GC-FID

Table 11 Intra-day and inter-day precision of four marker compounds by GC-FID (system 1, n=3)

Concentration (μg/mL)	Compounds	RSD (%)	
		Intra-day	Inter-day
25	**1**	7.42	3.00
	2	5.01	9.86
	4	4.69	9.16
	6	5.89	6.29
100	**1**	7.46	4.96
	2	9.47	7.68
	4	7.04	4.15
	6	6.55	5.28
200	**1**	5.61	1.15
	2	6.22	4.16
	4	2.57	4.88
	6	8.53	3.43

The LOD and LOQ of compounds **1**, **2**, **4** and **6** were obtained as 0.1 ng and 18.75 ng, respectively. The inter-day and intra-day precisions of marker compounds in different concentration are given in Table 11. The result showed acceptable precision, expressed by less than 10%RSD. Recovery test was used to evaluate the accuracy of this method. Accurate amounts of individual 4 compounds were added to approximate 2.0 mg/mL of *C. aeruginosa* extract. The results are shown in Table 12. The performance of the method was tested by applying it to determination of marker compounds content in crude extract from the stability study and the quality control.

Table 12 Recovery study of four reference compounds in the GC-FID method (system 1, n=3)

Serial no.	Compounds	Amount present in 2.0 mg/mL extract (μg/mL)	Amount added (μg/mL)	Amount found [a], (μg/mL)	Recovery (%) [a]
1	**1**	77.35	10	76.58 ± 1.34	87.67 ± 1.53
	2	35.69	10	47.94 ± 2.34	104.92 ± 5.13
	4	131.46	10	144.52 ± 7.69	102.16 ± 5.44
	6	69.69	10	75.80 ± 1.71	95.11 ± 2.15
2	**1**	77.35	50	112.55 ± 5.50	88.38 ± 4.32
	2	35.69	50	88.38 ± 8.38	103.14 ± 9.78
	4	131.46	50	181.07 ± 21.38	99.78 ± 11.78
	6	69.69	50	119.25 ± 15.18	99.63 ± 12.68
3	**1**	77.35	100	163.12 ± 3.08	91.97 ± 1.74
	2	35.69	100	135.84 ± 2.73	100.11 ± 2.01
	4	131.46	100	232.71 ± 7.85	100.54 ± 3.39
	6	69.69	100	151.60 ± 6.32	89.34 ± 3.73

Note: [a] expressed as mean ± SD (n=3)

3.3 Method validation for determination of germacrone (1) isolated from *C. aeruginosa* extract using GC-FID (system 2)

The system 2 for determination of **1** was validated. A linear relationship was obtained with the concentration range of 15.625-250 μg/mL. The calibration curve constructed was evaluated by its correlation coefficient. The calibration equation from quadruplet (n=4) experiments was $y = 0.3401x - 0.2960$ ($r^2 = 0.9958$). The LOD and LOQ values for analytes were found to be 0.1 and 15.625 ng, respectively. The intra-day and inter-day precision of **1** are shown in Table 13.

Table 13 Intra-day and inter-day precision of 1 by GC-FID (system 2, n=3)

Concentration (μg/mL)	RSD (%)	
	Intra-day	Inter-day
25	9.12	5.34
100	5.68	7.30
200	0.34	2.22

Table 14 Recovery study of 1 in the proposed GC-FID (system 2, n=3)

Amount added (μg/mL)	Amount present in sample (μg/mL)	Amount found [a], (μg/mL)	Recovery [a] (%)
5	27.40	30.05 ± 1.04	92.76 ± 3.21
50	27.40	73.90 ± 3.79	95.48 ± 4.89
150	27.40	168.29 ± 11.37	94.86 ± 6.41

Note: [a] expressed as mean ± SD (n=3)

The recovery at 3 different levels of **1** was found to be 92.76–94.86% (Table 14), which indicates good accuracy of the method. This system was applied to determination of **1** in the sample of stability studies.

Qualitative and quantitative analyses of *C. aeruginosa* from different sources

For the use of *C. aeruginosa* extract as an ingredient in cosmetics or drugs, the extract should be standardized. The TLC and GC analyses were conducted to obtain the information of the variation of chemicals components of *C. aeruginosa* extract from the different sources and different extraction solvent.

The samples used in this experiment were:

CA = hexane extract of *C. aeruginosa* used in our study from Khaokhor district, Phetchabun province.

HFF = hexane extract of fresh rhizomes of *C. aeruginosa* collected from the Faculty of Pharmaceutical Sciences

HWF = hexane extract of fresh rhizome of *C. aeruginosa* purchased from the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple

HFD = hexane extract of dried powder rhizome of *C. aeruginosa* collected from the Faculty of Pharmaceutical Sciences

HWD = hexane extract of dried powder rhizome of *C. aeruginosa* purchased from the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple

HSD = hexane extract of dried powder rhizome of *C. aeruginosa* purchased from Sukhothai province

DFF = CH_2Cl_2 extract of fresh rhizome of *C. aeruginosa* collected from the Faculty of Pharmaceutical Sciences

DWF = CH_2Cl_2 extract of fresh rhizome of *C. aeruginosa* purchased from the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple

DFD = CH_2Cl_2 extract of dried powder rhizome of *C. aeruginosa* collected from the Faculty of Pharmaceutical Sciences

DWD = CH_2Cl_2 extract of dried powder rhizome of *C. aeruginosa* purchased from the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple

DSD = CH_2Cl_2 extract of dried powder rhizome of *C. aeruginosa* purchased from Sukhothai province.

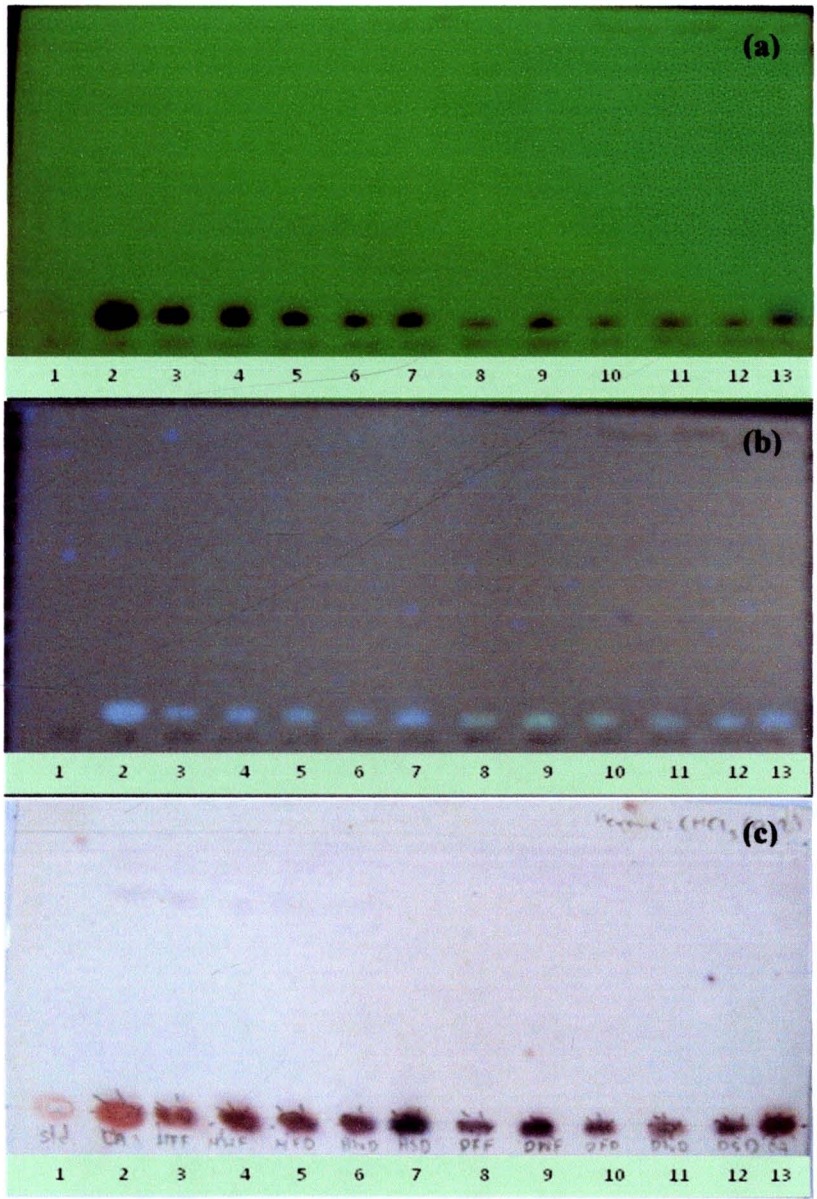


Figure 41 TLC fingerprints of the *C. aeruginosa* extract from different sources using hexane:$CHCl_3$ (9:1 v/v) as a mobile phase: (a) viewed at 254 nm without coloration (b) viewed at 366 nm without coloration (c) colorized with anisaldehyde reagent. (1 = mixture marker compounds (1, 2, 4 and 9), 2 = CA, 3 = HFF, 4 = HWF, 5 = HFD, 6 = HWD, 7 = HSD, 8 = DFF, 9 = DWF, 10 = DFD, 11 = DWD, 12 = DSD, and 13 = CA)

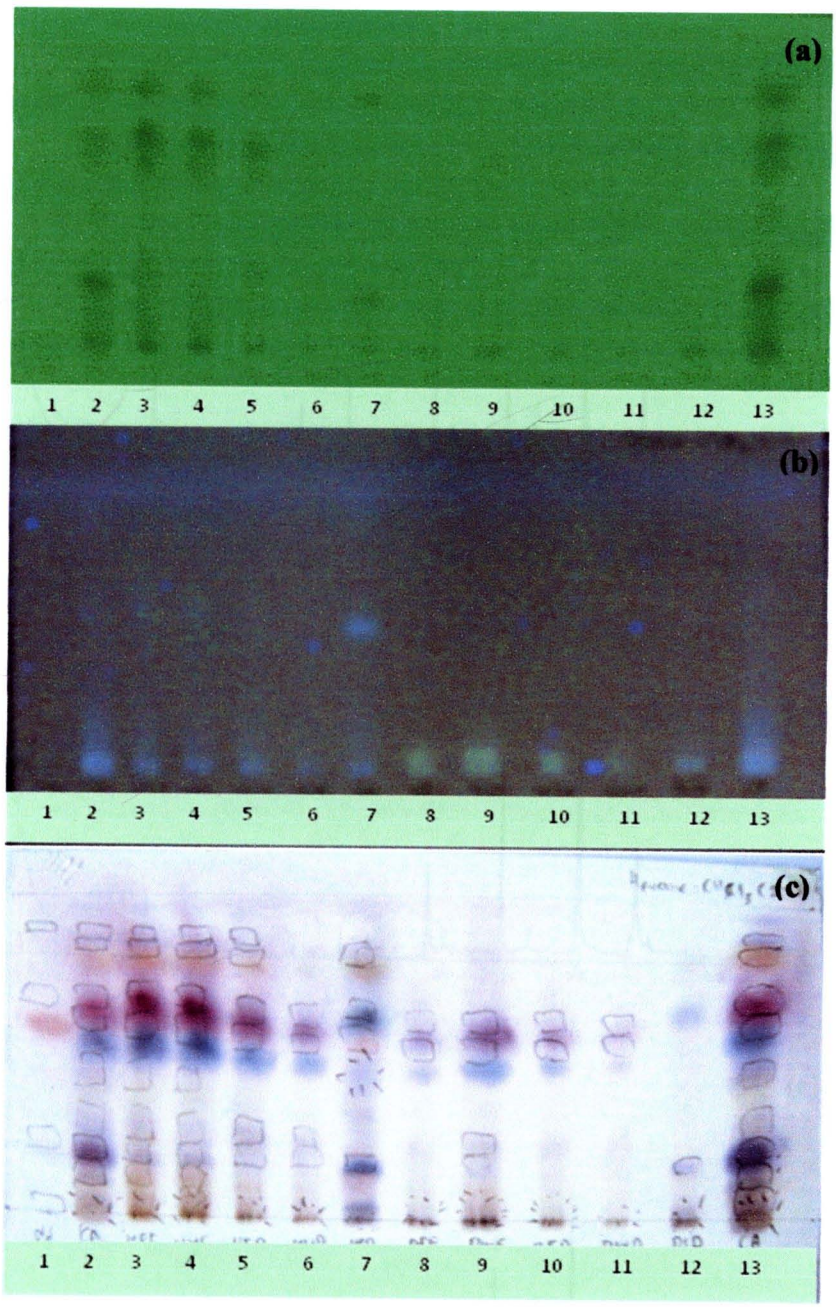


Figure 42 TLC fingerprints of the *C. aeruginosa* extract from different sources using hexane:$CHCl_3$ (2:8 v/v) as a mobile phase: (a) viewed at 254 nm without coloration (b) viewed at 366 nm without coloration (c) colorized with anisaldehyde reagent. (1 = mixture marker compounds (1, 2, 4 and 9), 2 = CA, 3 = HFF, 4 = HWF, 5 = HFD, 6 = HWD, 7 = HSD, 8 = DFF, 9 = DWF, 10 = DFD, 11 = DWD, 12 = DSD, and 13 = CA)

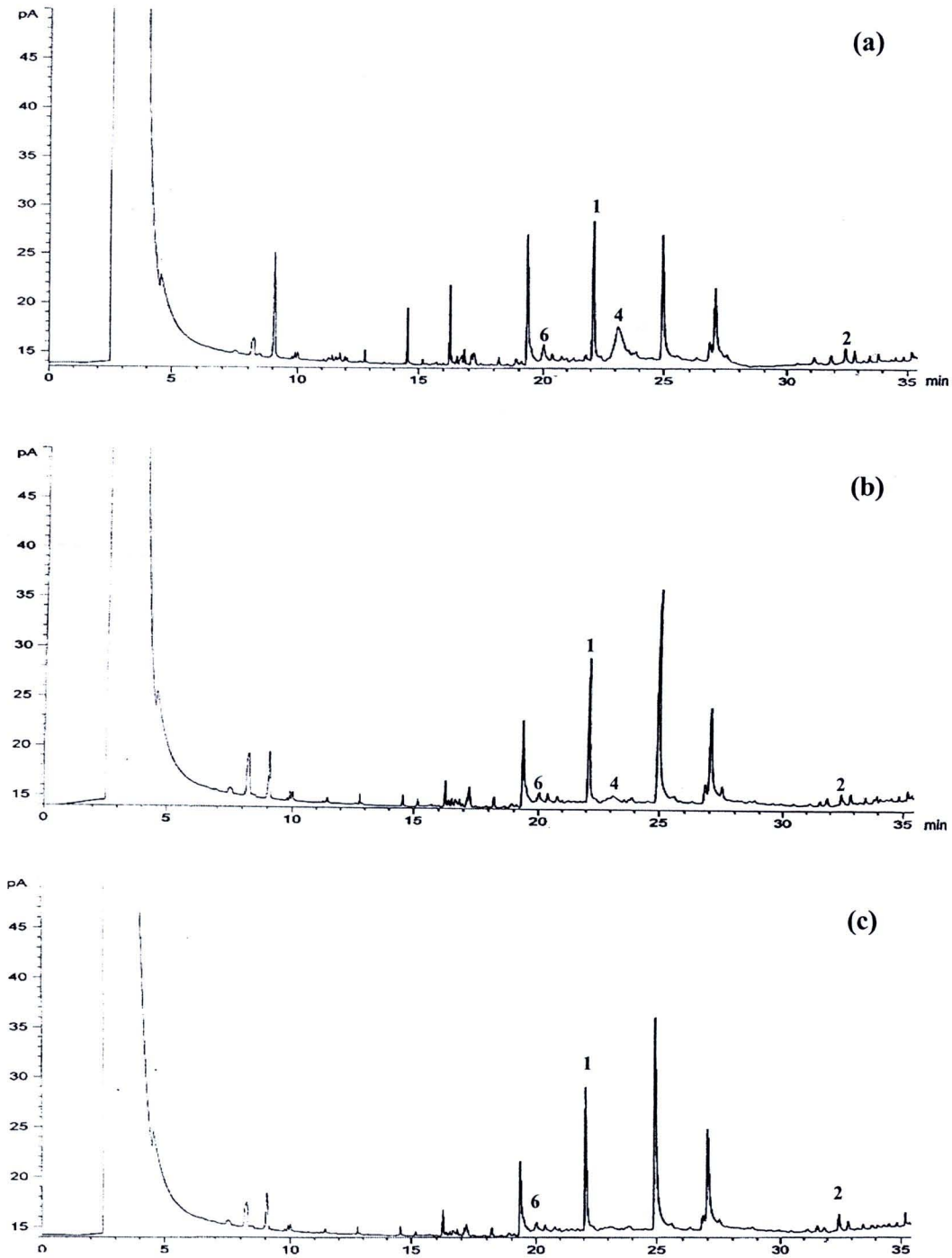


Figure 43 GC chromatograms of 3.0 mg/mL (a) *C. aeruginosa* extract in our study, (b) HFF and (c) HWF obtained from GC-FID analysis (system 1)

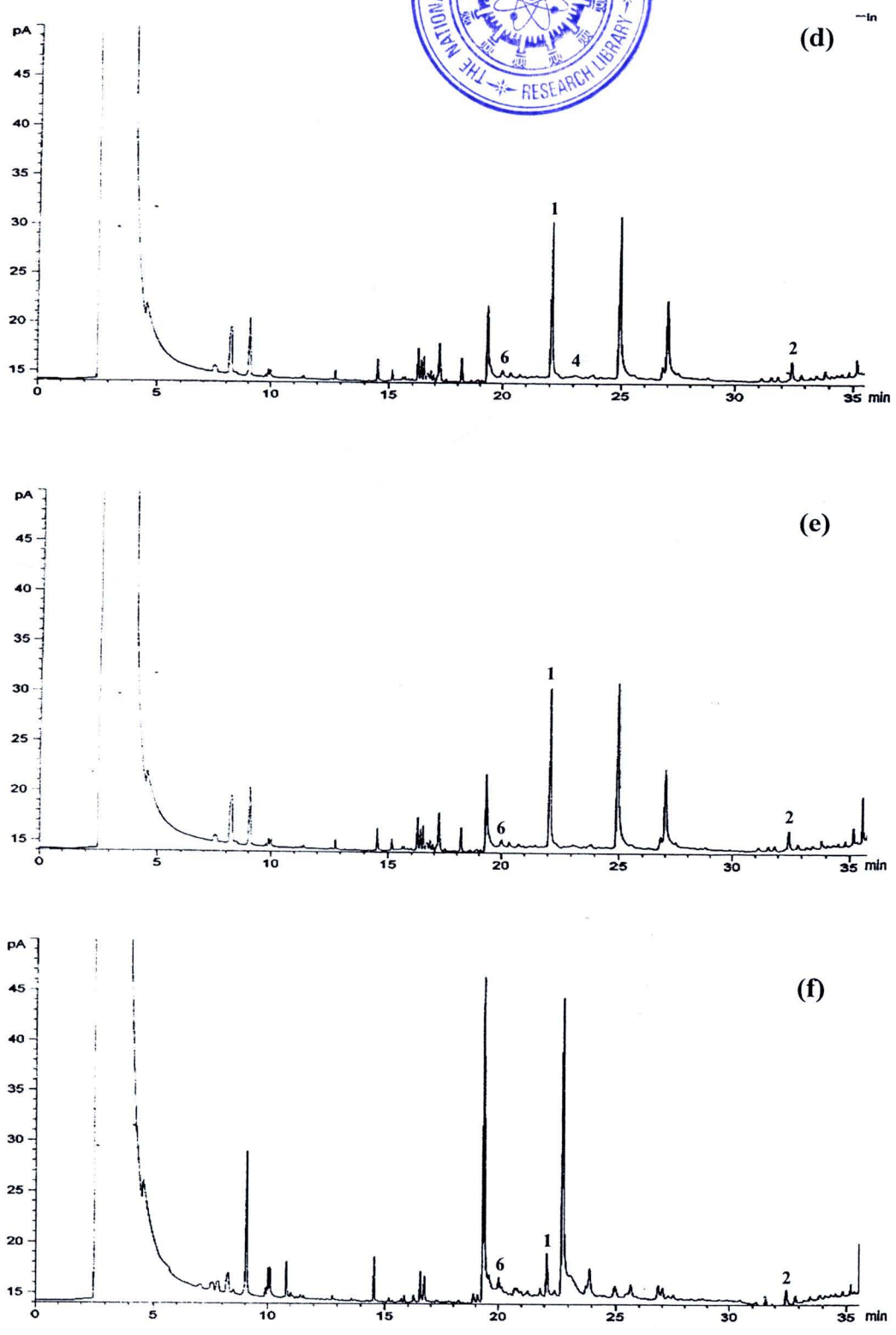


Figure 44 GC chromatograms of 3.0 mg/mL (d) HFD, (e) HWD and (f) HSD obtained from GC-FID analysis (system 1)

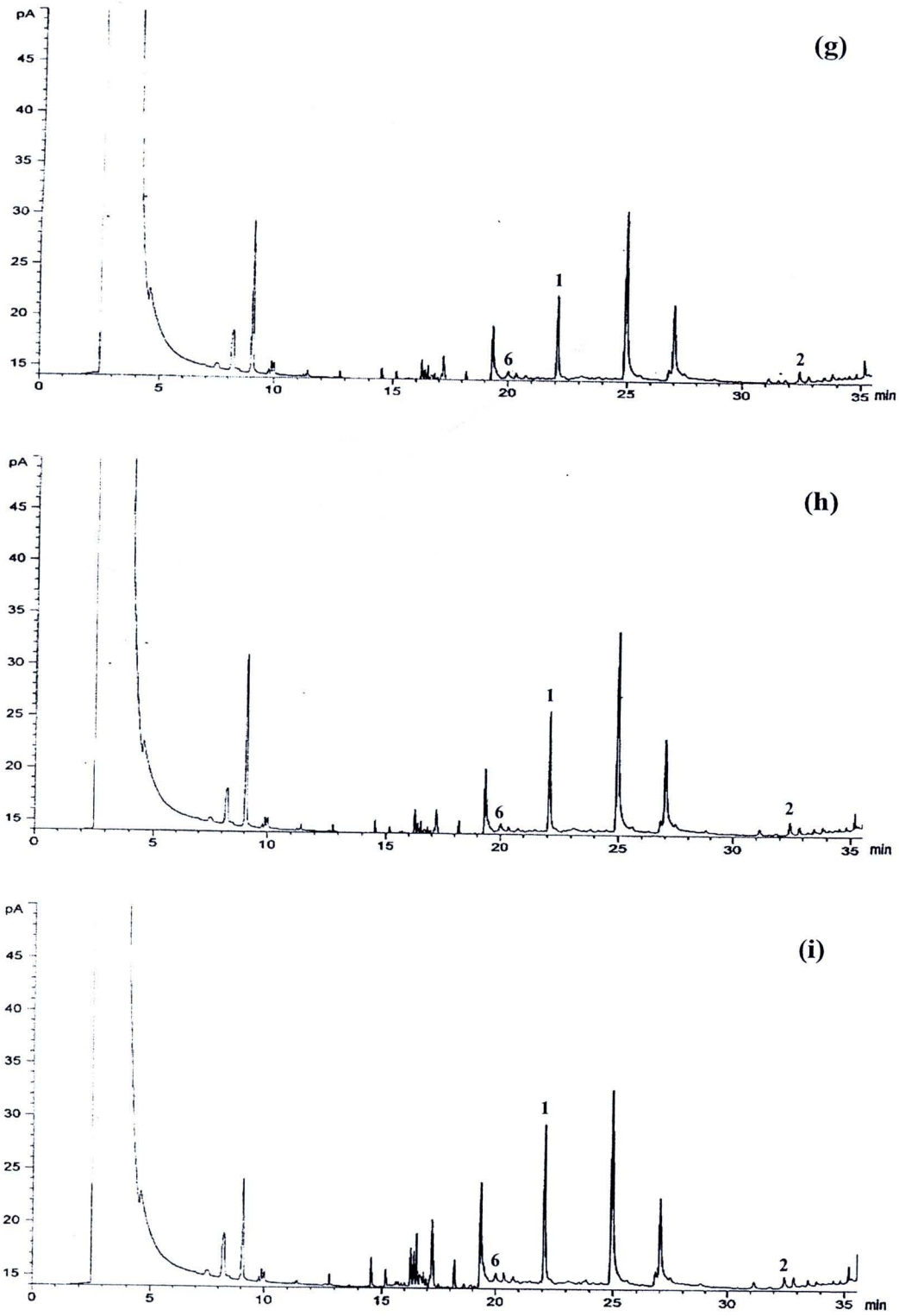


Figure 45 GC chromatograms of 3.0 mg/mL (g) DFF, (h) DWF and (i) DFD obtained from GC-FID analysis (system 1)

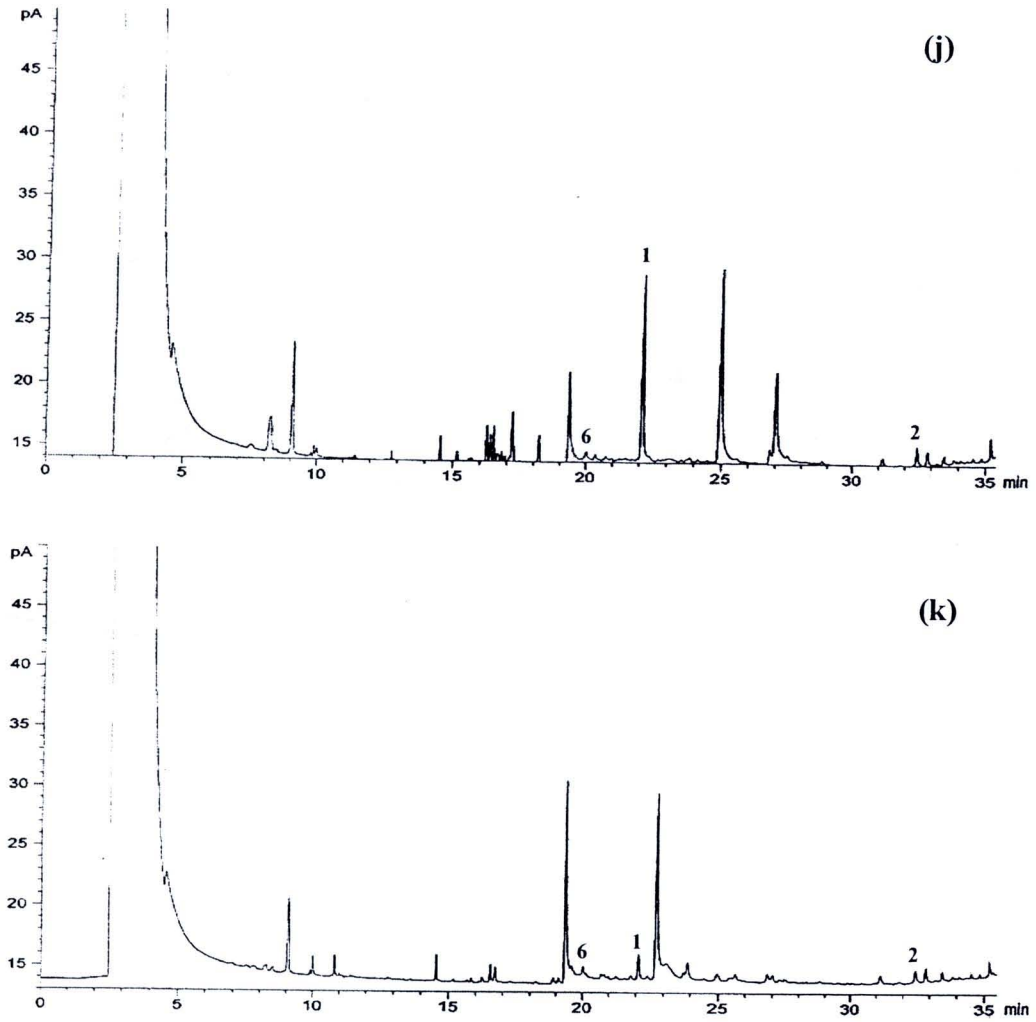


Figure 46 GC chromatograms of 3.0 mg/mL (j) DWD and (k) DSD obtained from GC-FID analysis (system 1)

Both fresh rhizomes and dried rhizomes were extracted in hexane and in CH_2Cl_2. The fingerprints and identification of different components present in *C. aeruginosa* were done using TLC (Figures 41 and 42) and GC-FID (Figures 43-46). The chemical profiles obtained from TLC and GC-FID for the crude extract of *C. aeruginosa* in different sources were almost the same except the crude extract from Sukhothai that showed different fingerprint from the other sources. To quantify the contents of marker compounds, 3 mg/mL of crude extracts were injected to GC-FID system 1. The results of GC fingerprints are shown on Figures 43-46.

Table 15 Contents of 4 marker compounds in *C. aeruginosa* extract from different sources in Thailand and different solvent extraction

Samples	Fresh/ dried rhizomes	Solvent	Yield of crude extract [a]	Marker compound contents in crude extract (%w/w)				
				germacrone	zederone	curcumenol	isocurcumenol	Total
HFF	Fresh	Hexane	0.37	2.13	0.62	0.62	0.88	4.50
HWF	Fresh	Hexane	0.44	2.13	1.10	-[b]	1.06	4.29
CA	Dried	Hexane	0.60	2.11	0.98	2.82	1.66	7.56
HFD	Dried	Hexane	3.15	2.12	1.30	0.38	0.82	4.62
HWD	Dried	Hexane	2.59	2.31	0.97	-[b]	0.67	3.95
HSD	Dried	Hexane	0.52	0.78	0.83	-[b]	0.95	2.56
DFF	Fresh	CH_2Cl_2	1.15	1.32	0.61	-[b]	0.68	2.61
DWF	Fresh	CH_2Cl_2	1.50	1.74	0.76	-[b]	0.65	3.14
DFD	Dried	CH_2Cl_2	5.39	2.18	0.58	-[b]	0.97	3.73
DWD	Dried	CH_2Cl_2	5.67	2.22	0.71	-[b]	0.71	3.64
DSD	Dried	CH_2Cl_2	3.57	0.51	0.66	-[b]	0.56	1.73

Note: [a] % dried weight or fresh weight, [b] undetected

From the GC fingerprints, germacrone (**1**) was found as the minor constituents in *C. aeruginosa* while the major compound was unidentified peaks. The amounts of some sesquiterpenes (compounds **1**, **2**, **4** and **6**) in *C. aeruginosa* from different sources and different extraction solvents were analyzed from above mentioned study. Table 15 shows the summary results. The results showed the variation of the chemical constituents in *C. aeruginosa* from different sources and locations.

Table 16 Contents (mg/g) of 4 marker compounds in dried rhizomes of *C. aeruginosa* from different sources in Thailand. (The data expresses as mean ± SD (n=3))

Samples[a]	germacrone	zederone	curcumenol	isocurcumenol	Total
CA1	0.31 ± 0.04	0.18 ± 0.03	-[b]	0.23 ± 0.02	0.71 ± 0.08
CA2	0.39 ± 0.02	0.16 ± 0.03	-[b]	0.22 ± 0.01	0.77 ± 0.06
CA3	0.22 ± 0.01	0.30 ± 0.08	-[b]	0.31 ± 0.07	0.83 ± 0.14

Note: [a] CA1-CA3 were *C. aeruginosa* from Faculty of Pharmaceutical Sciences, the market at Pra Sri Rattana Mahathat Vora Maha Vihar temple and Sukhothai province.

[b] undetected

We found that the hexane extracts of both fresh and dried rhizomes contained the higher yield of total 4 marker compounds contents than that of CH_2Cl_2 extracts. When we focus on the anti-androgenic compound, germacrone (**1**), the result showed that the contents of **1** in all of the hexane extracts and CH_2Cl_2 extracts were ranged from 0.78 to 2.31 %w/w and 0.51 to 2.22 %w/w, respectively. The level of **1** in the dried powder rhizomes from three different sources were 0.31 ± 0.04, 0.39 ± 0.02 and 0.22 ± 0.01 mg/g of dried powder (Table 16). Of 3 different sources, CA1 and CA2 showed similar level of **1** while CA3 contained lower amount of **1**. The results of relative amount could be used for evaluating of different samples or batches of *C. aeruginosa* for the further study on anti-androgenic activity.

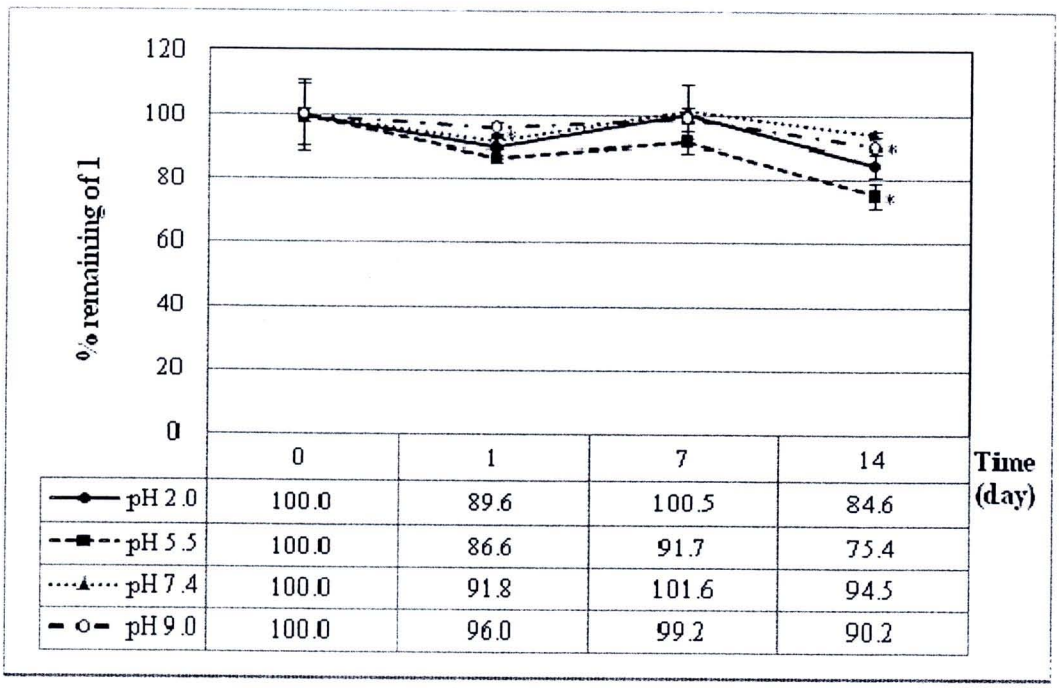
Stability studies of *C. aeruginosa* extract and its anti-androgenic components

The effect of pH, temperature and light on stability of marker compounds of *C. aeruginosa* was studied. The relative concentrations of marker compound, **1**, in crude extract or **1** which was studied as a pure compound were plotted against time intervals.

1. Effect of pH

In the preliminary study, the effect of pH on the stability of **1** was tested and analyzed using HPLC method with isocratic system (system 1). The remaining amount of **1** was observed both in crude extract and as a pure compound. The samples were dissolved in PEG-40 hydrogenated castor oil and diluted with buffer solution to obtain the pH needed.

The results of stability studies at different pH (at 25 ± 2°C) indicated that the remaining amounts of **1** in the extract and as a pure form were still higher than 75% at all of pH conditions when the samples were kept for 14 days (Figure 47 and 48). We selected pH 5.5 which is the general pH for cosmetic products for further study on the effect of temperature and light.



	0	1	7	14
pH 2.0	100.0	89.6	100.5	84.6
pH 5.5	100.0	86.6	91.7	75.4
pH 7.4	100.0	91.8	101.6	94.5
pH 9.0	100.0	96.0	99.2	90.2

Time (day)

Figure 47 The effect of various pH condition on the stability of 1 in *C. aeruginosa* extract analyzed by HPLC (*$p < 0.05$ against day 0, n=3)

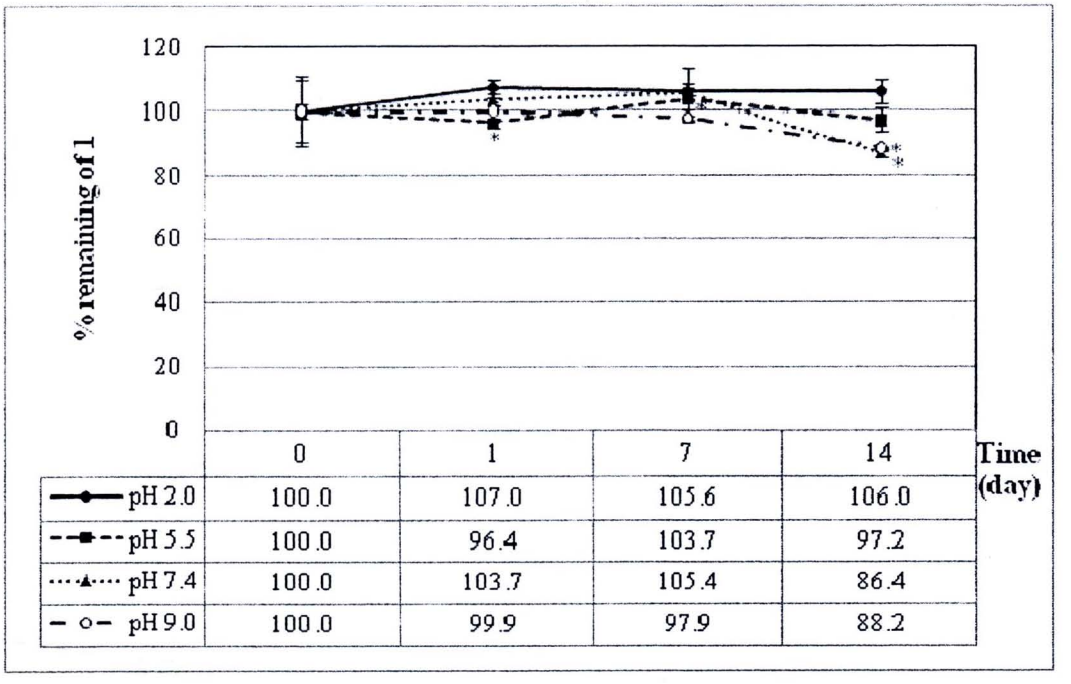
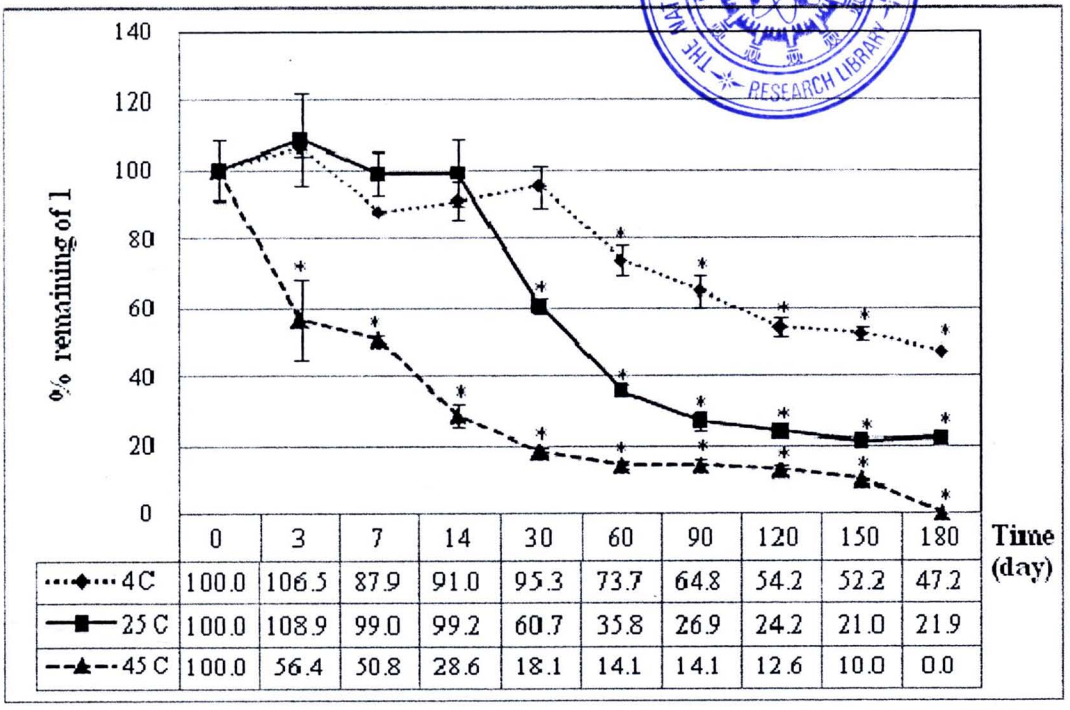


Figure 48 The effect of various pH condition on the stability of 1 (pure compound) analyzed by HPLC (*$p < 0.05$ against day 0, n=3)

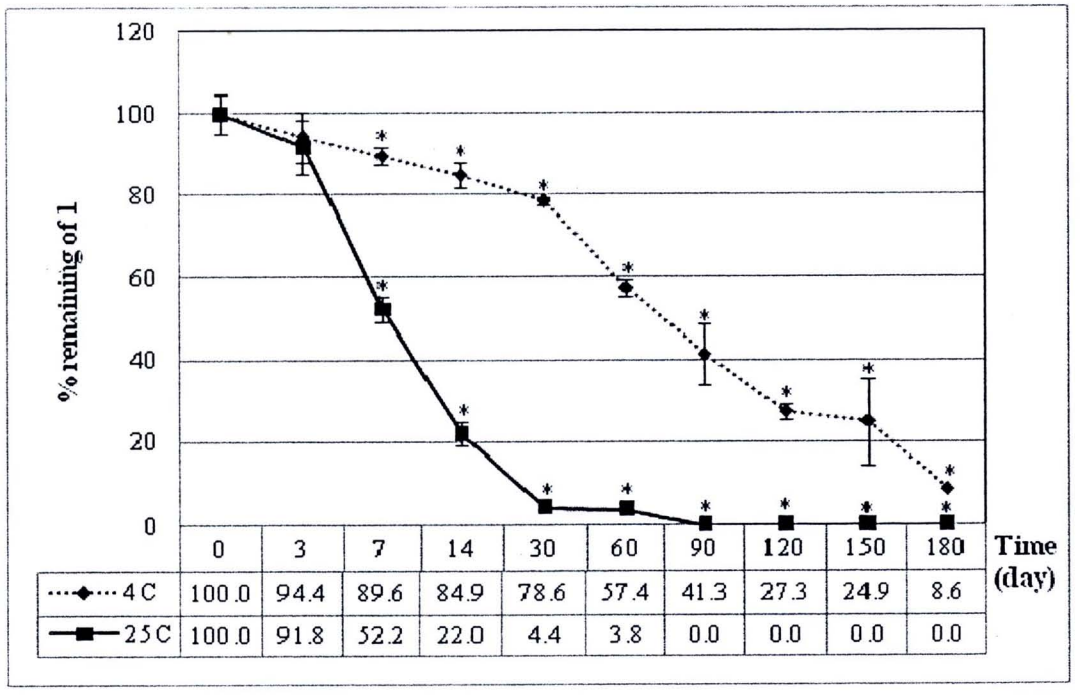
2. Effect of temperature

The samples were kept at 4, 25 and 45°C for different duration of time. The content of **1** in the samples was determined using GC-FID (Figure 49). The results showed that the %remaining of **1** in solid form of crude extract slightly decreased at 4 and 25°C in the first month and dropped to lower than 50 and 20%, respectively in 6 months. At 45°C, however, the remaining of **1** quickly decreased to lower than 20% in the first month and approached to undetectable level at 6 months of the study. The content of **1** which was studied as solid form of pure compound decreased more quickly than that of **1** in crude extract (Figure 50). At 45°C, **1** rapidly decreased to undetectable level within 3 days. Our results revealed that **1** was sensitive to temperature change. Since the melting point of this compound is rather than low (55-56°C), it might be thermally changed to other chemical structures or it was vaporized.



	0	3	7	14	30	60	90	120	150	180
4 C	100.0	106.5	87.9	91.0	95.3	73.7	64.8	54.2	52.2	47.2
25 C	100.0	108.9	99.0	99.2	60.7	35.8	26.9	24.2	21.0	21.9
45 C	100.0	56.4	50.8	28.6	18.1	14.1	14.1	12.6	10.0	0.0

Figure 49 The effect of temperature on the stability of 1 in solid form of *C. aeruginosa* extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



	0	3	7	14	30	60	90	120	150	180
4 C	100.0	94.4	89.6	84.9	78.6	57.4	41.3	27.3	24.9	8.6
25 C	100.0	91.8	52.2	22.0	4.4	3.8	0.0	0.0	0.0	0.0

Figure 50 The effect of temperature on the stability of 1 (solid form of pure compound) analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

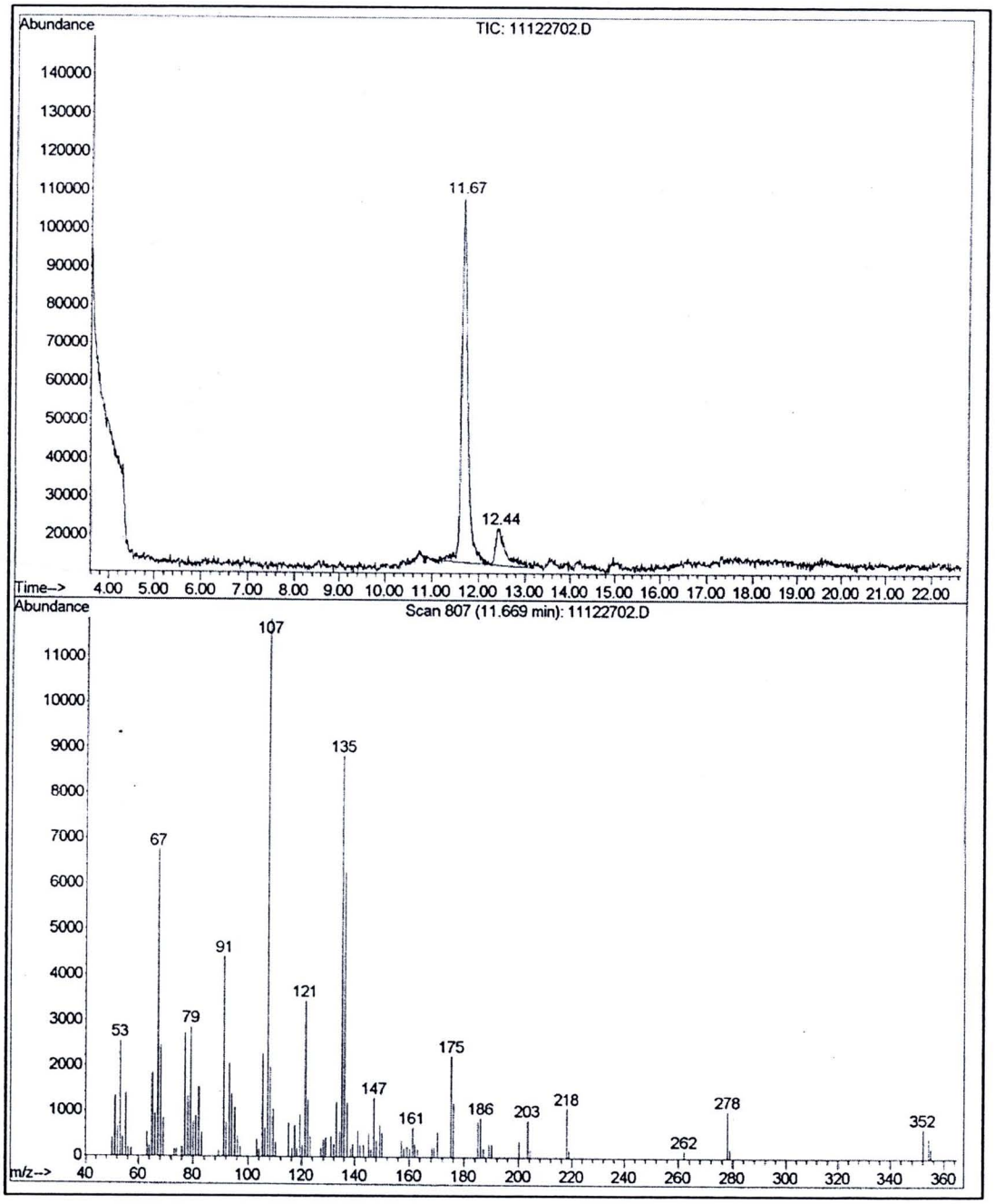
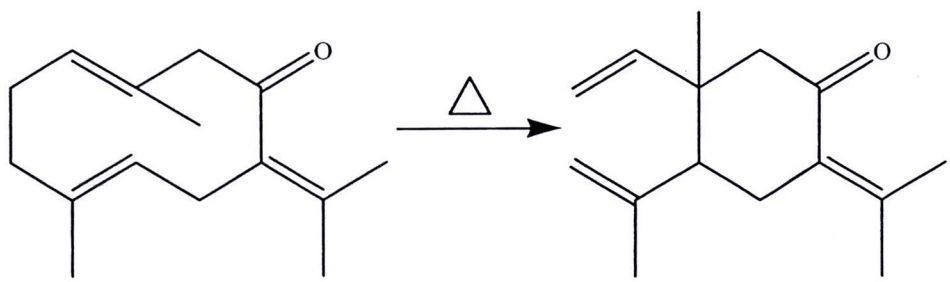


Figure 51 EI-MS spectrum of unknown compounds in solid form sample (pure form of 1) after kept at 25°C for 6 months.

Interestingly, **1** was kept for 4-6 months at 4 and 25 °C, the peaks of unknown compounds were observed in the chromatogram. This degradation peaks were also observed on the samples at 45°C after kept for 6 months. To identify the possible degradation products, the sample of **1** after stored at 25 °C for 6 months was

analyzed using GC-MS. From the EI-MS spectrum (Figure 51), one of the degradation products (at retention time 11.67 min) showed the *m/z* = 218 $[M]^+$; 203(6), 186(8), 175(20), 161(5), 147(11), 135(75), 121(30), 107(100), 91(38), 79(24), 67(58), 53(22) . This MS spectrum was compared with the study of Zhou, X. et al. [96], and the possible structure was thus identified as elemenone. Reichardt, P.B. et al. and Yang, F.Q. et al. proposed that **1** can changed into β-elemenone ($C_{15}H_{22}O$) (Figure 52) [95,97]. The corresponded result was also found from the study of our colleagues. When **1** was refluxed in DMSO at 250°C for 2 hr, elemenone was obtained [82].



Germacrone (**1**) β-elemenone

Figure 52 The pathway of thermal degradation for germacrone [95,97]

When analyzing the sample of **1** after kept for 3 days at 45°C, there was no peak detected by GC. In this case, TLC analyses were useful to compare the fingerprint of degradation products. The TLC result showed the unknown degradation products while these products could not be detected by GC. The results supported that **1** was a heat sensitive compound.

Oxygen might be one of the factors causing degradation of **1**. To prove this, **1** was kept in nitrogen at 45°C. Interestingly, the degradation of **1** was not observed after kept for 3 days. The result supported that oxygen might play role on the degradation process of **1**. However, **1** is still sensitive to heat because the appearance was changed from solid form to oil after kept for 7 days. The peaks of unknown compounds were observed in the GC chromatogram which was similar to that of **1** in oxygen atmosphere after kept for 6 months at 25 °C. The TLC result clearly showed the degradation products of **1** (Figure 53). Therefore, keeping the *C. aeruginosa* extract and pure compound at low temperature could retard the degradation of **1**.

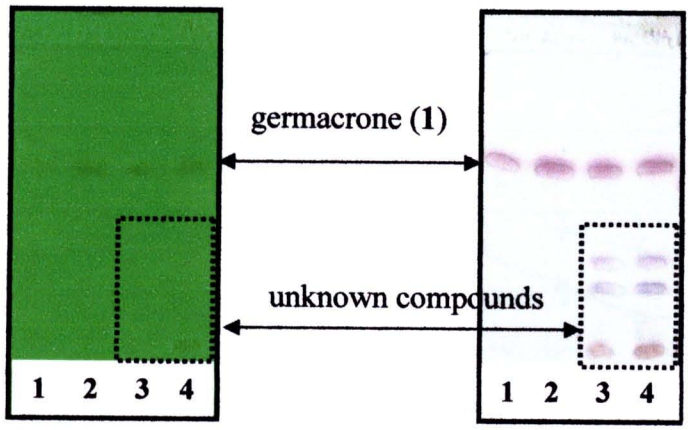
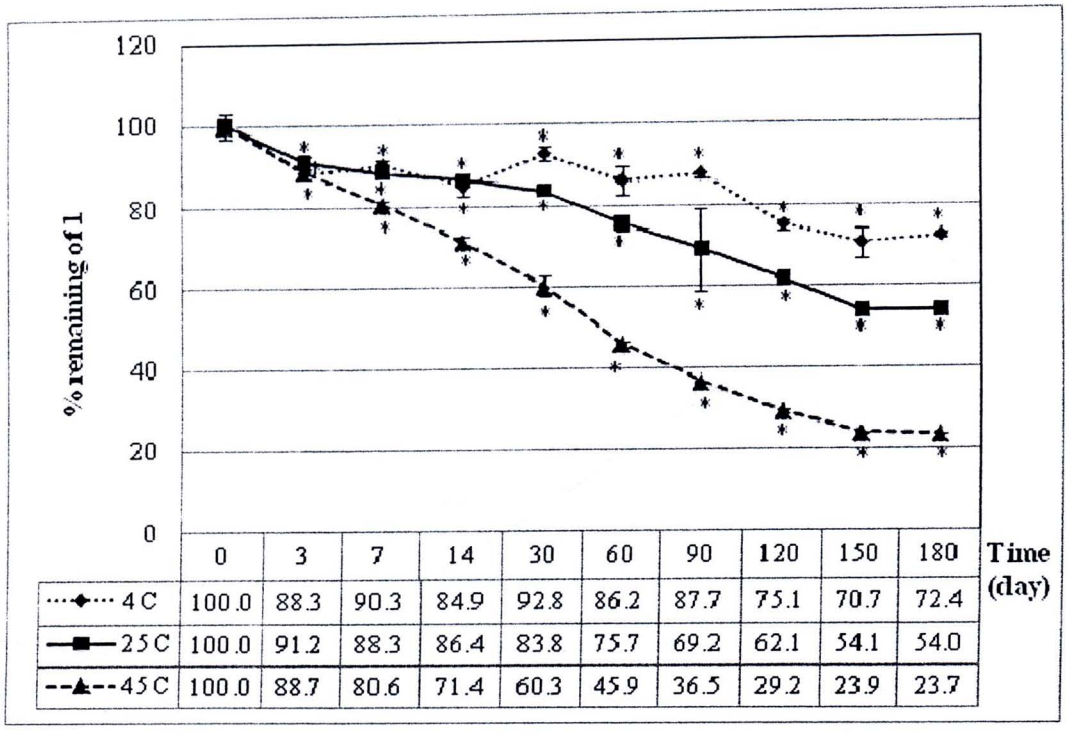


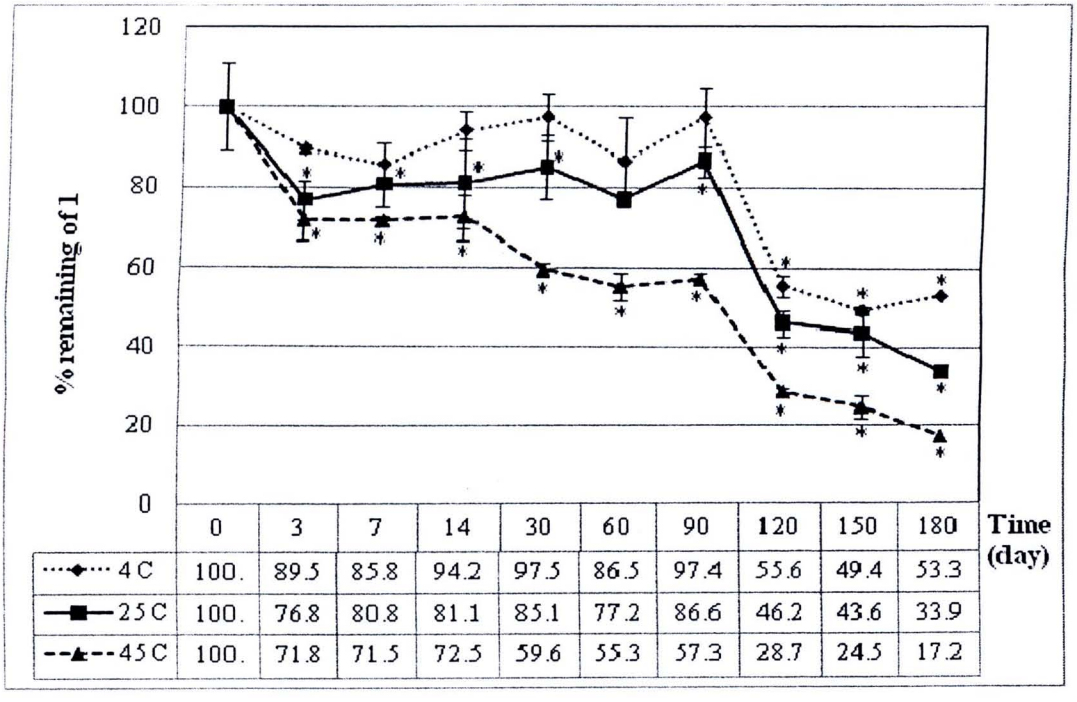
Figure 53 TLC fingerprints of sample of 1 in solid pure form after kept for various time intervals using hexane:EtOAc (95:5 v/v) as a mobile phase: (a) viewed at 254 nm without coloration and (b) colorized with anisaldehyde reagent. (1 = 0 day, 2 = 3 days, 3 = 7 days and 4 = 14 days)

Figures 54 and 55 showed the effect of temperature on the stability of **1** in solution form of crude extract and pure compound. The rapid degradation profile of **1** was expected since the degradation of most chemicals is generally faster in solution than in solid form due to higher molecular mobility in solution. Surprisingly, the solution form showed lower rate of degradation than solid form. The remaining of **1** in crude extract solution was unchanged at 4°C for 2 months, while it slightly decreased and remained higher than 50 and 20% at 25 and 45°C after kept for 6 months (Figure 54). The similar effect was also observed on the stability profile of **1** as pure compound solution (Figure 55). Although the solution form with PEG-40 was diluted in buffer solution pH 5.5, the remaining amounts of **1** were still higher than that of solid form.



	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.0	88.3	90.3	84.9	92.8	86.2	87.7	75.1	70.7	72.4	
25 C	100.0	91.2	88.3	86.4	83.8	75.7	69.2	62.1	54.1	54.0	
45 C	100.0	88.7	80.6	71.4	60.3	45.9	36.5	29.2	23.9	23.7	

Figure 54 The effect of temperature on the stability of 1 in solution form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.	89.5	85.8	94.2	97.5	86.5	97.4	55.6	49.4	53.3	
25 C	100.	76.8	80.8	81.1	85.1	77.2	86.6	46.2	43.6	33.9	
45 C	100.	71.8	71.5	72.5	59.6	55.3	57.3	28.7	24.5	17.2	

Figure 55 The effect of temperature on the stability of 1 (solution form of pure compound) analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

As *C. aeruginosa* extract and **1** are poorly soluble in water, PEG-40 hydrogenated castor oil was added as a surfactant before adding of buffer solution (15:85 %v/v) to improve the water solubility. Polyethylene glycol (PEG) is a hydrophilic nonionic polymer used in many biochemical and industrial applications. This chemical can be found in cosmetics, food, and pharmaceutical products [98,99]. One important property of surfactants is the formation of colloidal-sized clusters in solutions, known as micelles which able to increase the solubility of sparingly soluble substances in water [100]. The above results may be explained by one of the possible mechanisms, the dispersion of active component into aqueous buffer environment through micelles process and its volatility property was also decreased. The crude extract or pure compound which is hydrophobic substance might be incorporated into the hydrophobic core of micelles by hydrophobic interaction and prevent the effect of pH from the outer surface of micelles.

To prove this hypothesis, **1** was prepared in MeOH and kept at 45°C for 14 days. The remaining amounts of **1** were analyzed and compared to that of solution form with PEG-40. Surprisingly, the level of **1** only slightly decreased and remained higher than 70% at both systems when the samples were kept for 14 days (Figure 56). Therefore, the retarding of degradation of **1** in solution form might be involved with other processes rather than micelle formation. Since, oxygen could be one of the factors causing degradation of **1**, being dissolved in solution form might protect **1** from oxygen. Our results revealed that the solution form could enhance the stability of **1** either in the extract or as pure compound.

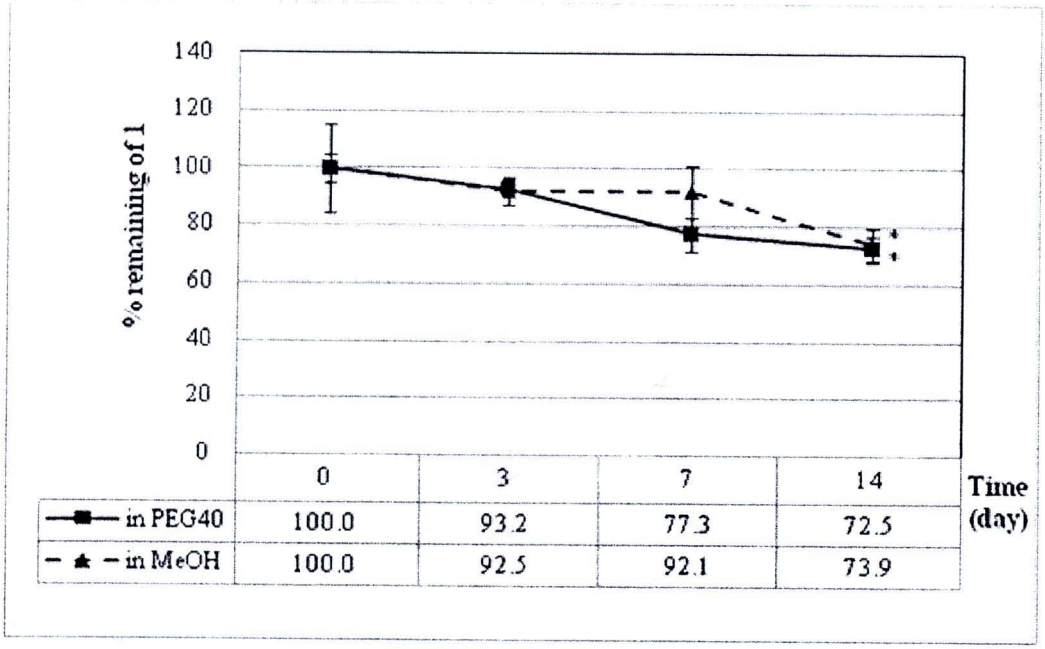
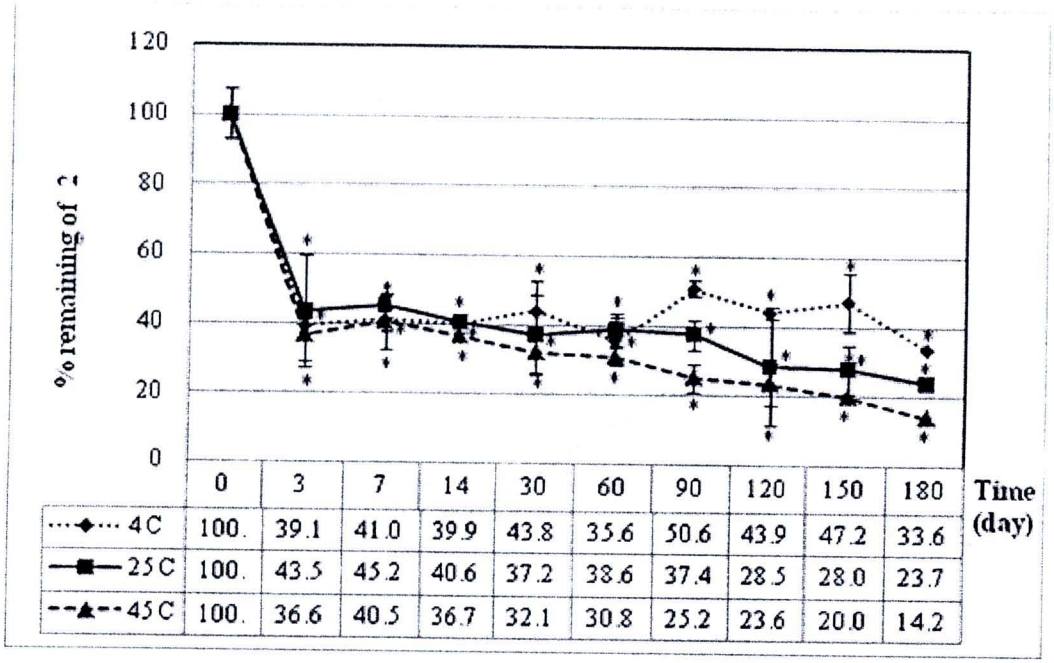


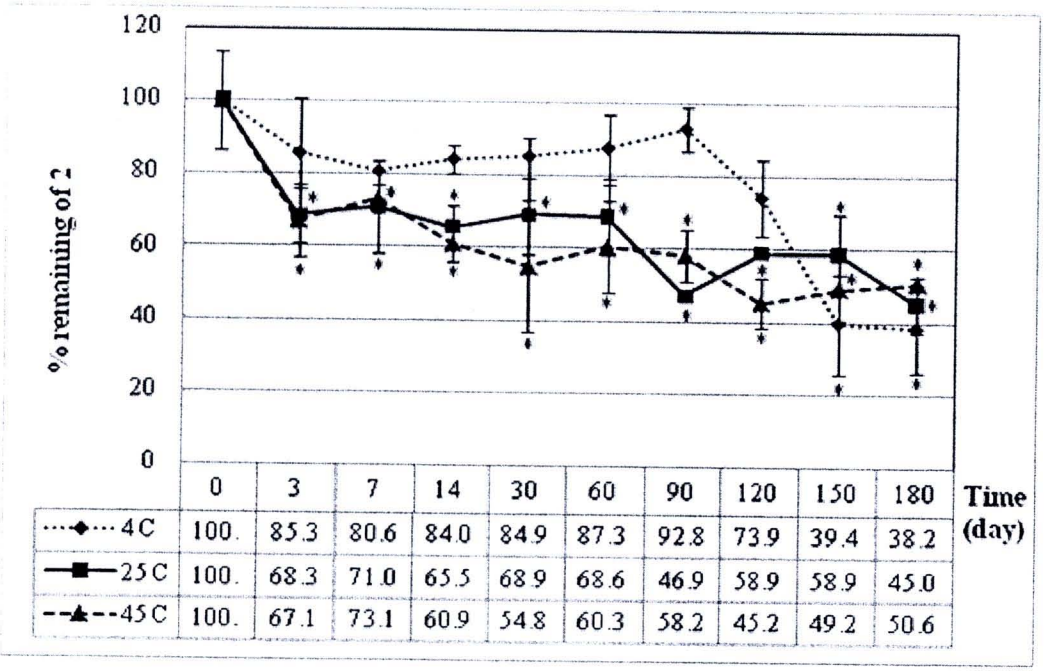
Figure 56 The stability of 1 (as pure compound) in solution form with PEG-40 and MeOH after kept at 45°C analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

To obtain more information about the stability of other chemical constituents of *C. aeruginosa* extract, the %remaining of compounds **2**, **4** and **6** in both solid and solution form were also determined using GC-FID system 1. The effect of temperature on the stability of **2**, **4** and **6** are shown on Figure 57-62. Sometimes the interfering peaks can hamper the analysis on the GC chromatogram. For example, the peak overlapping was observed when analyzing the content of **4** in solid form after kept for 1 month. However, the results showed that the content of these compounds (compounds **2**, **4** and **6**) which were studied as solid form decreased more quickly than that of solution form. Our results revealed that these isolated sesquiterpenes were sensitive to temperature change. Therefore, keeping the *C. aeruginosa* extract at low temperature could retard the degradation of these constituents.



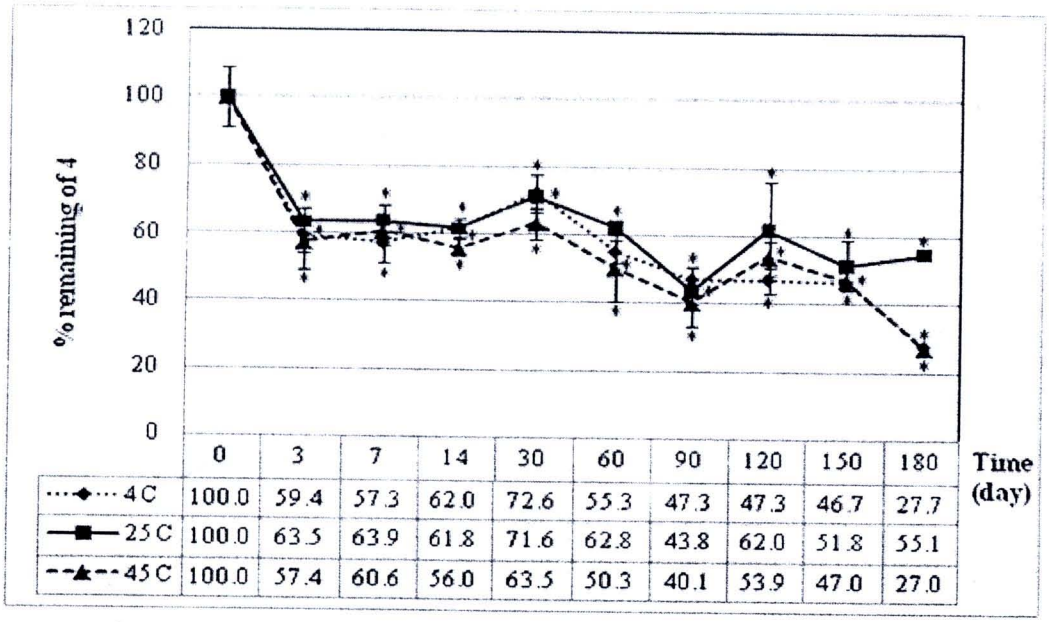
	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.	39.1	41.0	39.9	43.8	35.6	50.6	43.9	47.2	33.6	
25 C	100.	43.5	45.2	40.6	37.2	38.6	37.4	28.5	28.0	23.7	
45 C	100.	36.6	40.5	36.7	32.1	30.8	25.2	23.6	20.0	14.2	

Figure 57 The effect of temperature on the stability of zederone (2) in solid form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



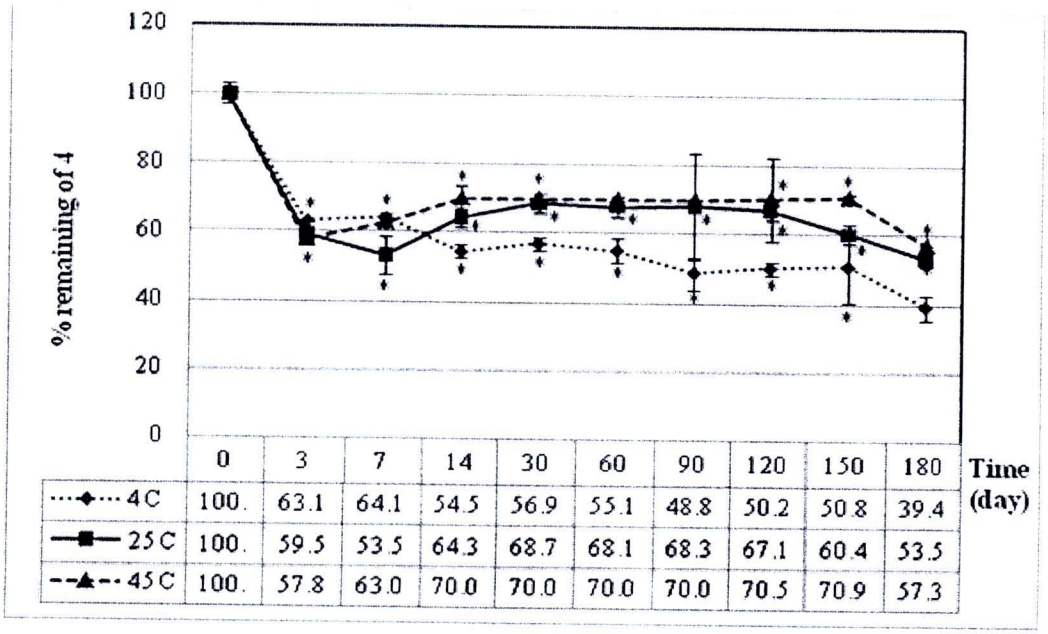
	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.	85.3	80.6	84.0	84.9	87.3	92.8	73.9	39.4	38.2	
25 C	100.	68.3	71.0	65.5	68.9	68.6	46.9	58.9	58.9	45.0	
45 C	100.	67.1	73.1	60.9	54.8	60.3	58.2	45.2	49.2	50.6	

Figure 58 The effect of temperature on the stability of 2 in solution form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.0	59.4	57.3	62.0	72.6	55.3	47.3	47.3	46.7	27.7	
25 C	100.0	63.5	63.9	61.8	71.6	62.8	43.8	62.0	51.8	55.1	
45 C	100.0	57.4	60.6	56.0	63.5	50.3	40.1	53.9	47.0	27.0	

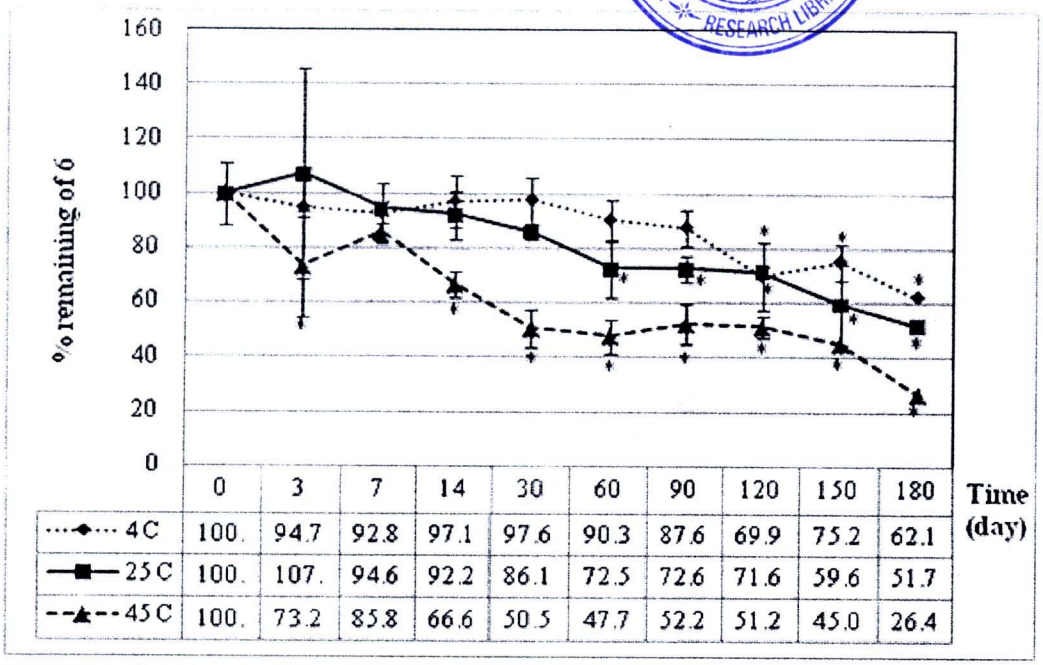
Figure 59 The effect of temperature on the stability of curcumenol (4) in solid form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



	0	3	7	14	30	60	90	120	150	180	Time (day)
4 C	100.	63.1	64.1	54.5	56.9	55.1	48.8	50.2	50.8	39.4	
25 C	100.	59.5	53.5	64.3	68.7	68.1	68.3	67.1	60.4	53.5	
45 C	100.	57.8	63.0	70.0	70.0	70.0	70.0	70.5	70.9	57.3	

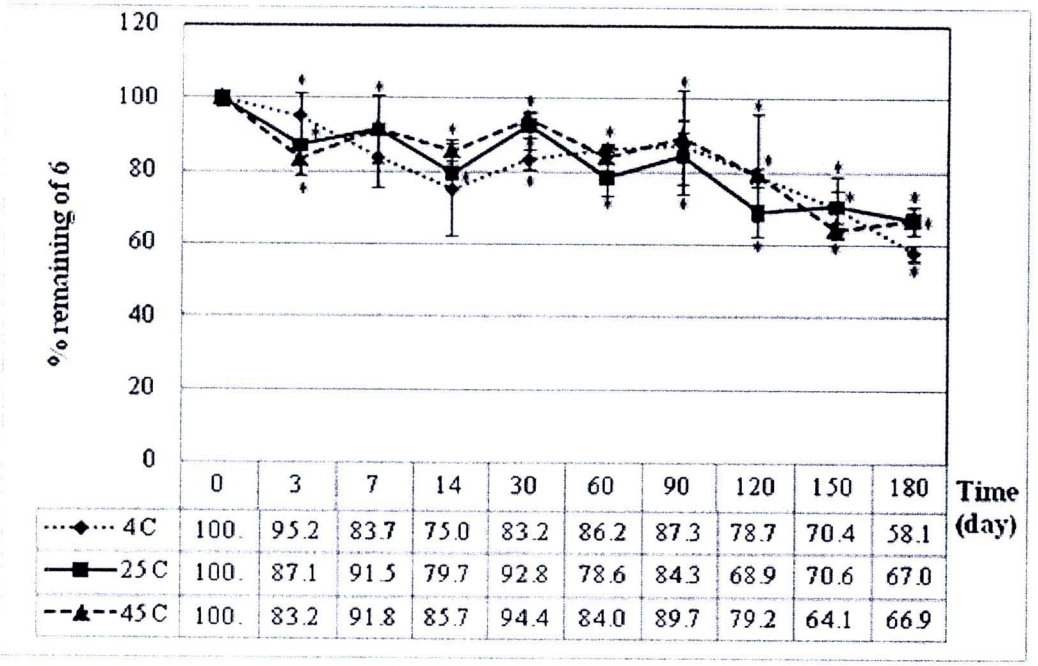
Figure 60 The effect of temperature on the stability of 4 in solution form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

Note: At 45°C, the remaining of **4** was not determined after kept for 1-3 months.



	0	3	7	14	30	60	90	120	150	180
4C	100.	94.7	92.8	97.1	97.6	90.3	87.6	69.9	75.2	62.1
25C	100.	107.	94.6	92.2	86.1	72.5	72.6	71.6	59.6	51.7
45C	100.	73.2	85.8	66.6	50.5	47.7	52.2	51.2	45.0	26.4

Figure 61 The effect of temperature on the stability of isocurcumenol (6) in solid form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)



	0	3	7	14	30	60	90	120	150	180
4C	100.	95.2	83.7	75.0	83.2	86.2	87.3	78.7	70.4	58.1
25C	100.	87.1	91.5	79.7	92.8	78.6	84.3	68.9	70.6	67.0
45C	100.	83.2	91.8	85.7	94.4	84.0	89.7	79.2	64.1	66.9

Figure 62 The effect of temperature on the stability of 6 in solution form of crude extract analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

3. Effect of light

At 25°C, when *C. aeruginosa* extract in solid form were exposed to light, the remaining of **1** in slightly decreased to 60% within 7 days while the level of that kept in dark environment did not change (Figure 63a). This result suggests that **1** is sensitive to light. However, the degradation was not clearly observed in *C. aeruginosa* extract in solution form either the samples were exposed to light or not. To see the results, the longer observation time is needed (Figure 63b). It is noted that when the same experiment was conducted in **1** as a pure compound instead of in the extract, no differences of degradation rate of **1** were observed either with or without light environment (Figures 64a and 64b).

The effects of light on the stability of **2, 4** and **6** in *C. aeruginosa* extract were also studied. Light did not seem to effect the stability of compounds **2**, **4** and **6** in solid *C. aeruginosa* extract during 7 days of study. However, the degradation rate of **4** and **6** in *C. aeruginosa* extract solution increased when exposed to light (Figures 65-67). This information can be useful for the storage of *C. aeruginosa* extract. For long-term keeping, it should be kept in dark for retarding the degradation of chemical constituents.

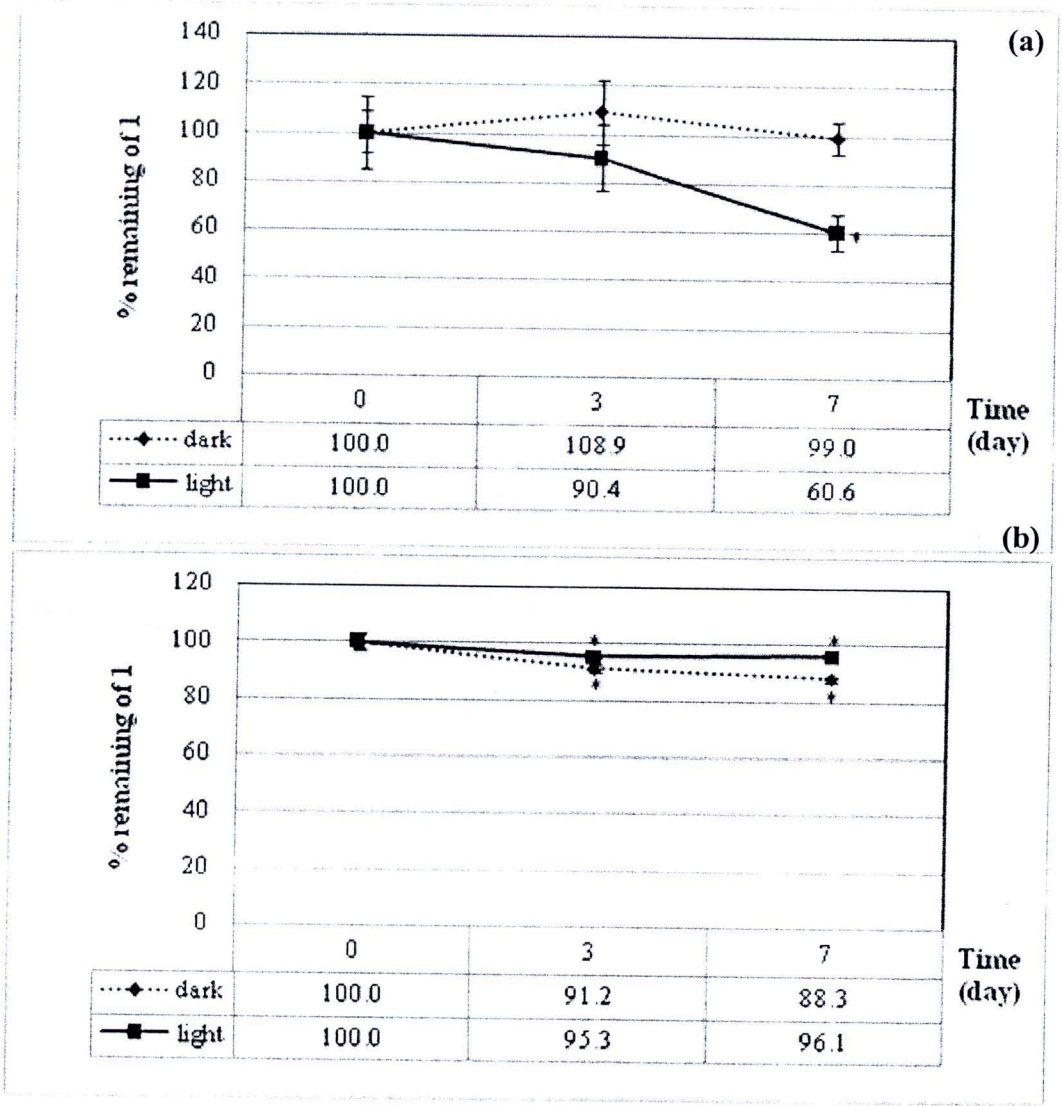


Figure 63 The effect of light on the stability of 1 in *C. aeruginosa* extract in (a) solid form and (b) solution form stored at 25°C. The remaining of 1 were analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

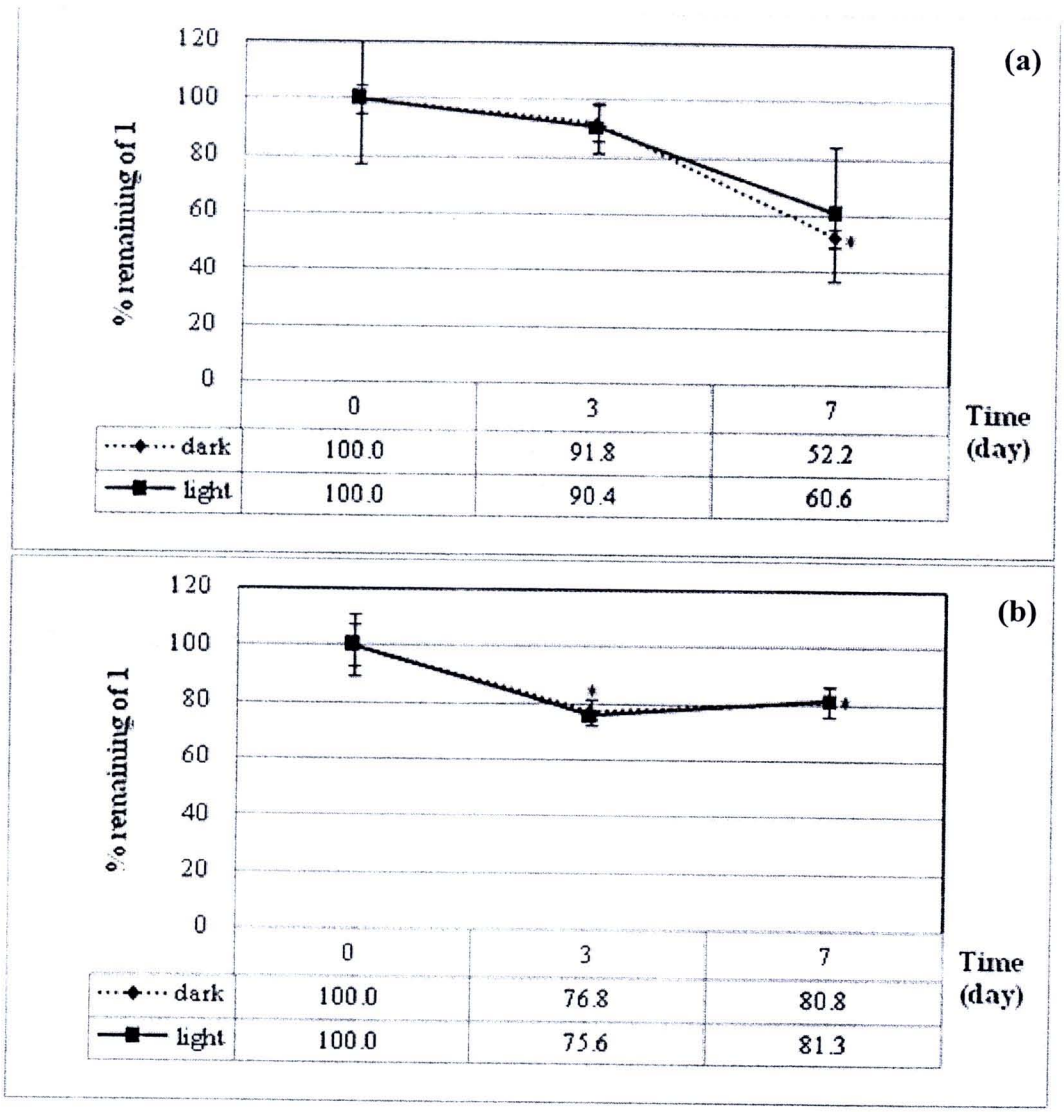


Figure 64 The effect of light on the stability of 1 (pure compound) in (a) solid form and (b) solution form stored at 25°C. The remaining of 1 were analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

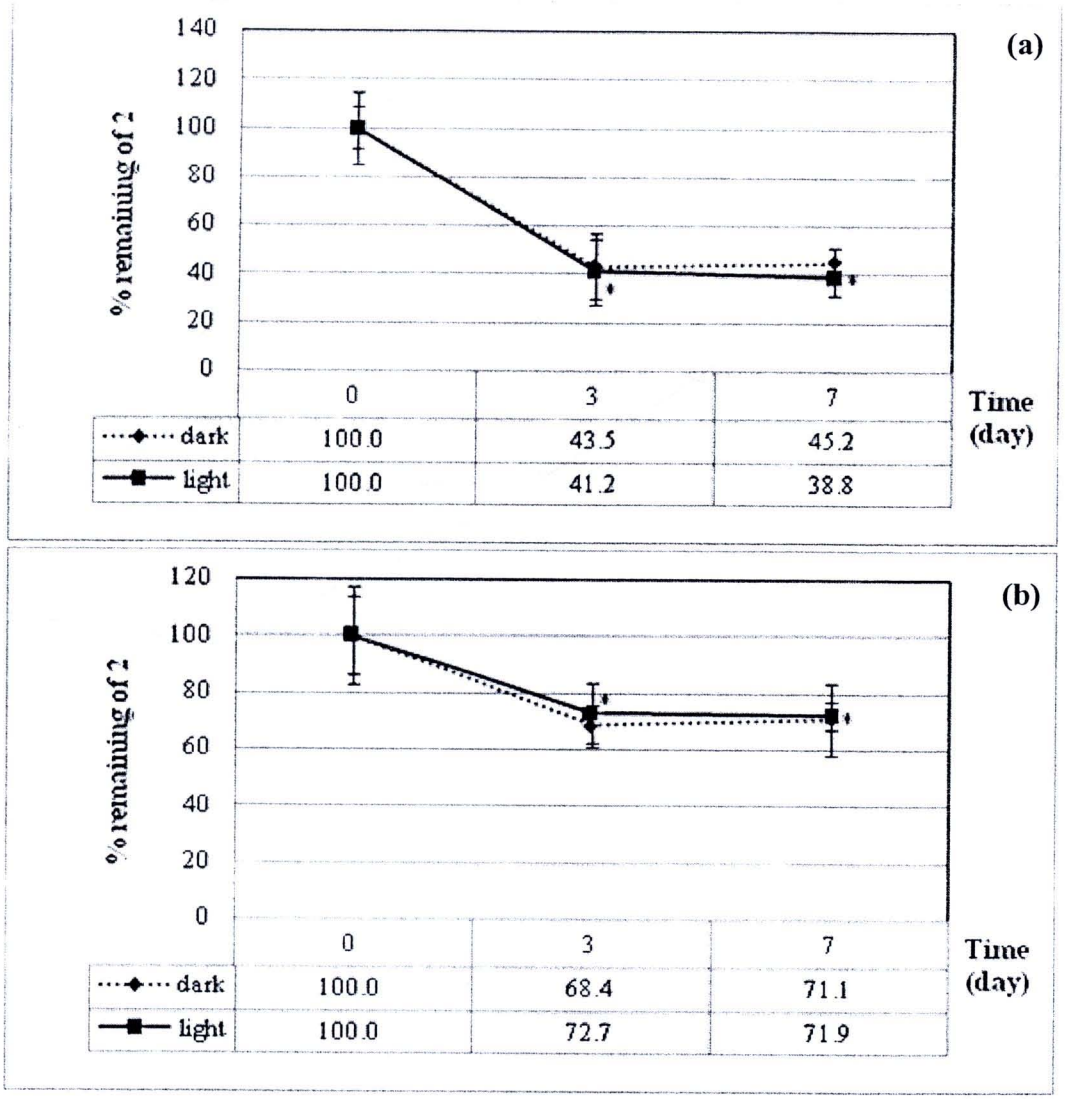


Figure 65 The effect of light on the stability of 2 in *C. aeruginosa* extract in (a) solid form and (b) solution form stored at 25°C. The remaining of 2 were analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

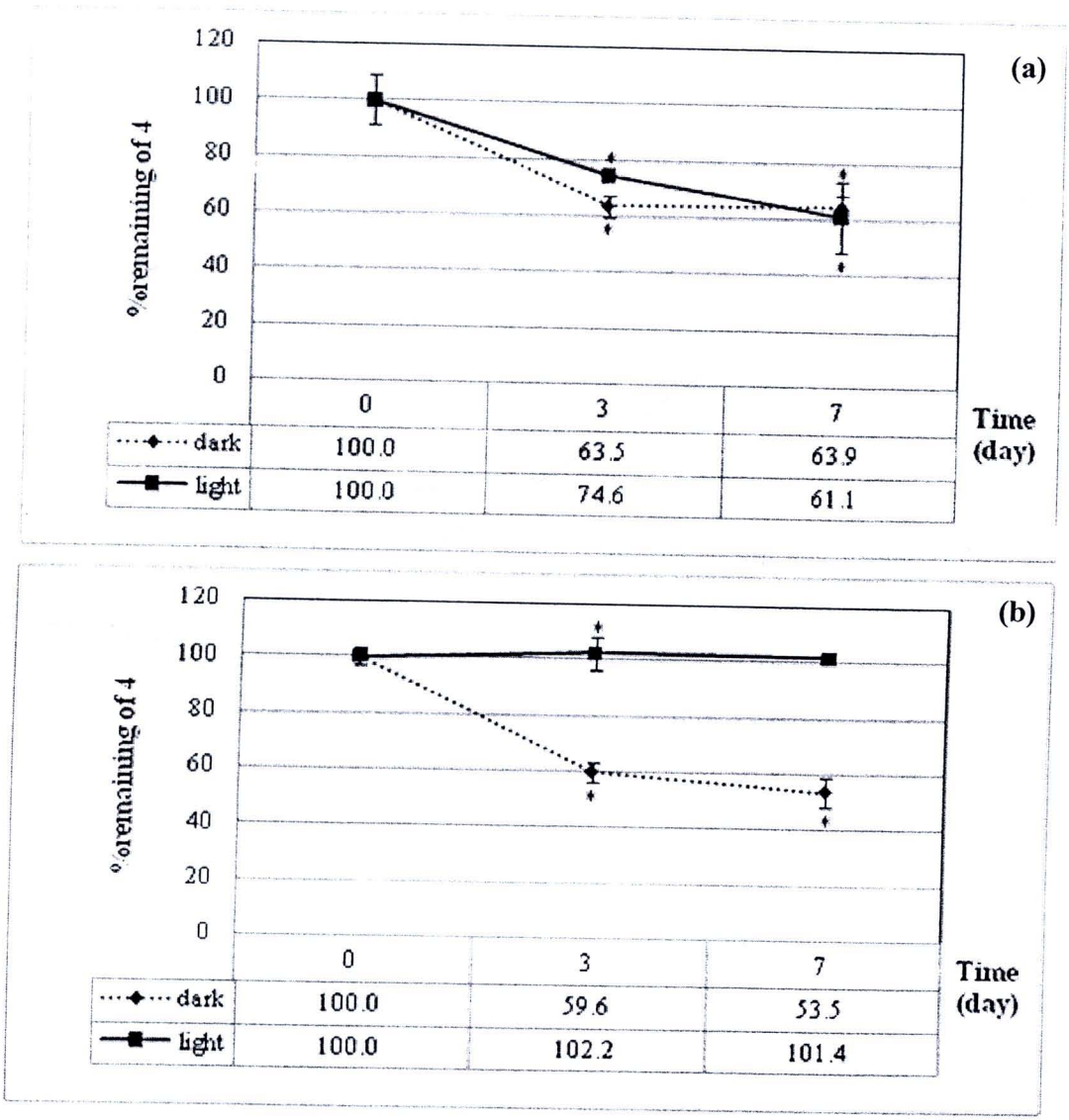


Figure 66 The effect of light on the stability of 4 in *C. aeruginosa* extract in (a) solid form and (b) solution form stored at 25°C. The remaining of 4 were analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)

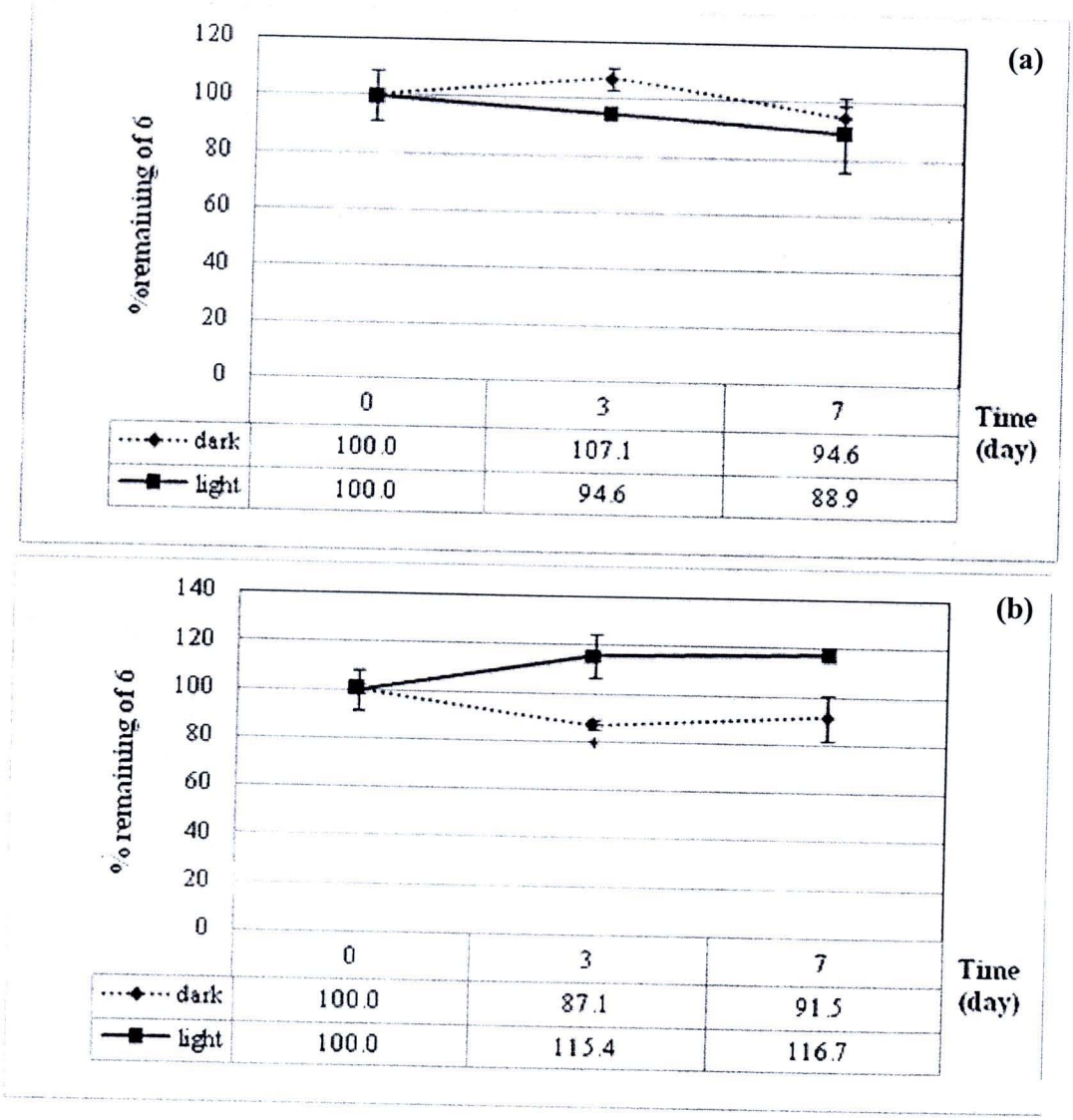


Figure 67 The effect of light on the stability of 6 in *C. aeruginosa* extract in (a) solid form and (b) solution form stored at 25°C. The remaining of 6 were analyzed by GC-FID (*$p < 0.05$ against day 0, n=3)