CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of plant extracts

Eighteen Thai medicinal plants were extracted with a less polar organic solvent, ethyl acetate and a polar organic solvent, methanol, consecutively to give eighteen less polar extracts (Table 3.1) and eighteen polar extracts (Table 3.2). *Ocimum sanctum* gave the highest yield for ethyl acetate extract (22.7 %w/w), while *Punica granatum* gave the highest yield (23.0 %w/w) for methanol extract. The extracts were packed in tight containers and kept in desiccator at room temperature.

Table 3.1 Ethyl acetate extracts of Thai medicinal plants

No.	Plant name	Yield (%w/w)	Physical appearance
1	*Ocimum sanctum*	22.7	dark green viscous semisolid
2	*Syzygium aromaticum*	15.8	yellowish brown wax
3	*Azadirachta indica*	6.8	dark green viscous semisolid
4	*Piper betle*	6.3	black viscous semisolid
5	*Ocimum americanum*	5.6	dark green viscous semisolid
6	*Boesenbergia pandurata*	5.1	yellowish brown viscous semisolid
7	*Zingiber officinalis*	4.6	reddish brown viscous semisolid
8	*Senna alata*	4.2	dark green viscous semisolid
9	*Andrographis paniculata*	3.6	greenish solid
10	*Cinnamomum verum*	3.4	brown viscous semisolid
11	*Morus alba*	3.0	dark green viscous semisolid
12	*Centella asiatica*	3.0	dark green viscous semisolid
13	*Alpinia galanga*	3.0	yellow viscous liquid
14	*Rhinacanthus nasutus*	2.7	dark green viscous semisolid
15	*Cymbopogon citratus*	1.8	yellowish brown semisolid
16	*Dioscorea membranacea*	1.3	yellowish brown powder

17	*Punica granatum*	1.2	dark brown viscous semisolid
18	*Plumbago zeylanica*	0.8	dark brown viscous semisolid

Table 3.2 Methanol extracts of Thai medicinal plants

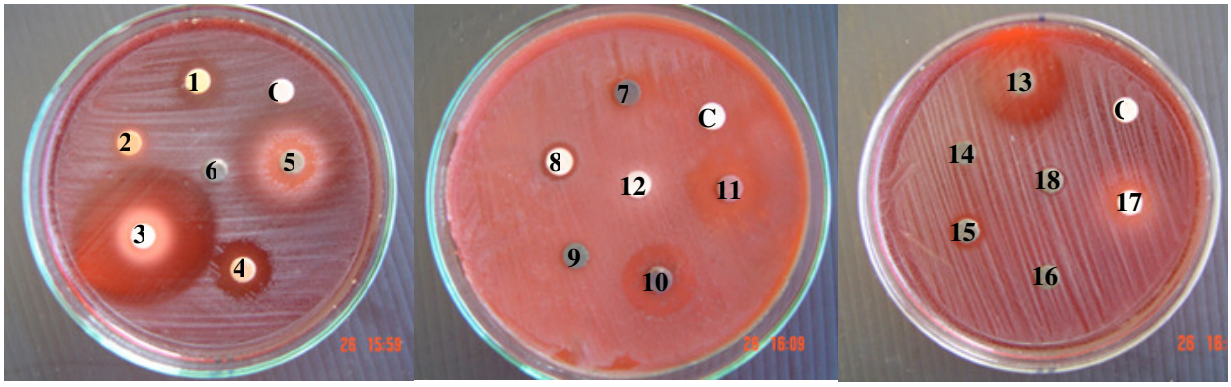
No.	Plant name	Yield (%w/w)	Physical appearance
1	*Punica granatum*	23.0	brown viscous semisolid
2	*Centella asiatica*	17.6	dark green viscous semisolid
3	*Zingiber officinalis*	16.1	reddish brown viscous semisolid
4	*Cymbopogon citratus*	12.0	brown viscous semisolid
5	*Dioscorea membranacea*	10.1	reddish brown viscous semisolid
6	*Piper betle*	9.3	dark green viscous semisolid
7	*Alpinia galanga*	7.8	yellowish brown viscous liquid
8	*Syzygium aromaticum*	7.5	dark brown viscous semisolid
9	*Ocimum sanctum*	6.5	dark green viscous semisolid
10	*Ocimum americanum*	5.7	dark green viscous semisolid
11	*Cinnamomum verum*	5.7	dark brown solid
12	*Senna alata*	5.4	dark brown viscous semisolid
13	*Andrographis paniculata*	4.4	green viscous semisolid
14	*Rhinacanthus nasutus*	4.3	dark brown viscous semisolid
15	*Azadirachta indica*	3.6	dark brown viscous semisolid
16	*Morus alba*	3.6	green viscous semisolid
17	*Boesenbergia pandurata*	2.9	yellowish brown viscous semisolid
18	*Plumbago zeylanica*	2.0	dark brown viscous semisolid

3.2 Evaluation of antibacterial activity against *Propionibacterium acnes*

3.2.1 Antibacterial activity screening

Among thirty-six plant extracts that were investigated for antibacterial activity against *P. acnes*, sixteen plant extracts including the ethyl acetate extracts of *A. galanga*, *P. granatum*, *P. betle*, *P. zeylanica*, *S. aromaticum*, *A. indica*, *C. verum*, *A. paniculata*, *C. citratus*,

R. nasutus and *B. pandurata* (Figure 3.1) and the methanol extracts of *A. galanga*, *P. granatum*, *P. betle*, *P. zeylanica*, *S. aromaticum* (Figure 3.2) were capable of inhibiting the growth of *P. acnes* at the concentration of 5 mg/disk (Table 3.3). In case of *A. indica*, *C. verum*, *A. paniculata*, *C. citratus*, *R. nasutus* and *B. pandurata,* only the ethyl acetate extract exhibited the inhibitory effect, implying that the active components should be less polar compounds. All antibacterial active extracts were subsequently subjected to determination of MIC and MBC values.



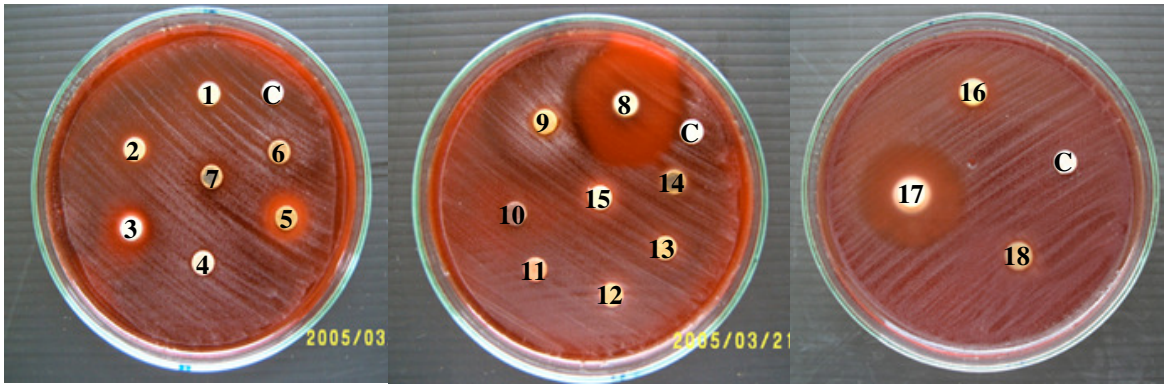
A

B

Figure 3.1 Antibacterial activity screening of the ethyl acetate extracts (A):

1. *Boesenbergia pandurata*; 2. *Zingiber officinalis*; 3. *Alpinia galanga*; 4. *Cymbopogon citrates*; 5. *Piper betle*; 6. *Ocimum americanum*; 7. *Rhinacanthus nasutus*; 8. *Punica granatum*; 9. *Centella asiatica*; 10. *Plumbago zeylanica*; 11. *Cinnamomum verum*; 12. *Dioscorea membranacea*; 13. *Azadirachta indica*; 14.

Senna alata; 15. *Andrographis paniculata*; 16. *Morus alba*; 17. *Syzygium aromaticum*; 18. *Ocimum sanctum*; tetracycline (B) and negative control (DMSO) (C) using disk diffusion method



A

B

Figure 3.2 Antibacterial activity screening of the methanol extracts (A): 1. *Boesenbergia*

pandurata; 2. *Zingiber officinalis*; 3. *Alpinia galanga*; 4. *Cymbopogon citrates*; 5. *Piper betle*; 6. *Ocimum americanum*; 7. *Rhinacanthus nasutus*; 8. *Punica granatum*; 9. *Centella asiatica*; 10. *Plumbago zeylanica*; 11. *Cinnamomum verum*; 12. *Dioscorea membranacea*; 13. *Azadirachta indica*; 14. *Senna alata*; 15. *Andrographis paniculata*; 16. *Morus alba*; 17. *Syzygium aromaticum*; 18. *Ocimum sanctum*; tetracycline (B) and negative control (DMSO) (C) using disk diffusion method

Table 3.3 Inhibition zone of the plant extracts (5 mg/disk) and tetracycline (30 μg/ml)

Medicinal plants/Antibiotic	Inhibition zone (mm)	
	Ethyl acetate extract	Methanol extract
Alpinia galanga	32.2	13.0
Punica granatum	10.3	30.0
Piper betle	11.0	11.0
Plumbago zeylanica	18.5	8.0
Syzygium aromaticum	10.0	25.0
Azadirachta indica	23.9	n.i.
Cinnamomum verum	14.3	n.i.
Andrographis paniculata	8.0	n.i.
Cymbopogon citratus	13.8	n.i.
Rhinacanthus nasutus	10.3	n.i.
Boesenbergia pandurata	11.4	n.i.
Centella asiatica	n.i.	n.i.
Zingiber officinalis	n.i.	n.i.
Ocimum americanum	n.i.	n.i.
Ocimum anctum	n.i.	n.i.
Dioscorea membranacea	n.i.	n.i.
Morus alba	n.i.	n.i.
Senna alata	n.i.	n.i.
Tetracycline hydrochloride	61.0	

n.i. = no inhibition zone

3.2.2 Minimum inhibitory concentration and minimum bactericidal concentration

Determination of MIC and MBC values of the herbal extracts demonstrated that the ethyl acetate extract of *A. galanga* showed the strongest antibacterial activity against *P. acnes*, with MIC and MBC values of 156 and 312 μg/ml, respectively (Table 3.4). In addition, the ethyl acetate extract of *P. betle*, *P. zeylanica* and *A. indica* showed interesting inhibitory activities with MIC values of 312 for *P. betle*, and 625 μg/ml for *P. zeylanica* and *A. indica*. However, MBC

values of *P. betle* and *A. indica* extracts (1250 μg/ml) was higher than that of *P. zeylanica* extract (625 μg/ml). Although the ethyl acetate extract of *A. paniculata* exhibited interesting MIC value of 625 μg/ml, its MBC value was too high (more than 5000 μg/ml). In contrast, the inhibitory activity of all methanol extracts was poor.

Table 3.4 The MIC and MBC values of the herbal extracts and tetracycline HCl against *P. acnes*

Extracts/Antibiotic	MIC (μg/ml)	MBC (μg/ml)
Alpinia galanga (e)	156	312
Piper betle (e)	312	1250
Plumbago zeylanica (e)	625	625
Azadirachta indica (e)	625	1250
Andrographis paniculata (e)	625	>5000
Cymbopogon citratus (e)	2500	2500
Rhinacanthus nasutus (e)	>5000	>5000
Boensenbergia pandurata (e)	>5000	>5000
Syzygium aromaticum (e)	>5000	>5000
Punica granatum (e)	>5000	>5000
Cinnamomum verum (e)	>5000	>5000
Piper betle (m)	1250	2500
Punica granatum (m)	1250	2500
Syzygium aromaticum (m)	1250	5000
Plumbago zeylanica (m)	2500	2500
Alpinia galanga (m)	>5000	>5000
Tetracycline hydrochloride	2	8

(e) ethyl acetate extract, *(m)* methanolic extract

3.3 Bioassay-guided isolation of *A. galanga* ethyl actetate extract

Dried powder of *A. galanga* rhizome was extracted with ethyl acetate by maceration method. The yellow oily extract was obtained (4.5 %w/w). The ethyl acetate extract

was subjected to isolation of antibacterial active compound using bioassay-guided purification. Separation of the ethyl acetate extract using a silica gel vacuum chromatography eluted with hexane, chloroform, ethyl acetate and methanol, consecutively gave ten pooled fractions (fractions 1 - 10). Investigation of antibacterial activity against *P. acnes* of each fraction showed that fraction 2, an orange oily liquid exhibited the strongest antibacterial activity with MIC value of 312 μg/ml (Table 3.5).

Table 3.5 Antibacterial activity of the pooled fractions from *A. galanga* ethyl acetate extract isolated by silica gel vacuum column against *P. acnes*

Fraction	Weight (g)	Inhibition zone (mm)	MIC (μg/ml)
1	4.31	14	> 5000
2	13.72	42	312
3	12.38	42	312
4	8.46	40	625
5	1.50	46	1250
6	1.71	20	1250
7	0.85	18	1250
8	0.47	10	1250
9	0.64	n.i	-
10	0.41	n.i	-

n.i. = no inhibition zone

Further purification of the active fraction 2 by Sephadex LH-20 gel filtration column eluted by methanol gave two pooled fractions (fractions I-II). The pooled fraction I showed the inhibitory effect with inhibition zone of 34 mm (Table 3.6). The TLC chromatogram

developed in a mixture of hexane and chloroform (4:6) showed a major spot with Rf value of 0.6. This major spot on TLC should be the antibacterial active compound. Thus, fraction I was subjected to further purification by silica gel column chromatography eluted with the mixture of hexane and chloroform (4:6) to produce five pooled fractions (fraction A-E). A colorless oily liquid (AP1) was obtained from fraction C, which gave the highest inhibitory effect with MIC value of 78 μg/ml (Table 3.7).

Table 3.6 Antibacterial activity of the pooled fractions from *A. galanga* ethyl acetate extract isolated by Sephadex LH-20 column against *P. acnes*

Fraction	Weight	Inhibition zone (mm)
I	12.31	34
II	0.42	n.i.

n.i. = no inhibition zone

Table 3.7 Antibacterial activity of the pooled fractions from *A. galanga* ethyl acetate extract isolated by silica gel column against *P. acnes*

Fraction	Weight (g)	Inhibition zone (mm)	MIC (μg/ml)
A	0.25	14.7	> 5000
B	0.42	23.7	156
C	3.47	32.0	78
D	0.86	27.0	156
E	0.02	12.7	1250

AP1 was subjected to structure determination using spectroscopic techniques, including IR, MS, ^{1}H NMR and ^{13}C NMR.

The IR spectrum (Figure 3.3) displayed strong absorption bands of C=O stretching at 1745 cm^{-1}, C=C stretching of aromatic ring at 1605 cm^{-1}, C-O stretching at 1200 cm^{-1} and C-H stretching around 3000 cm^{-1} both on unsaturated (>3000 cm^{-1}) and on aliphatic (2934, 2979 cm^{-1}) C-bonds. The IR data suggested an aromatic ester.

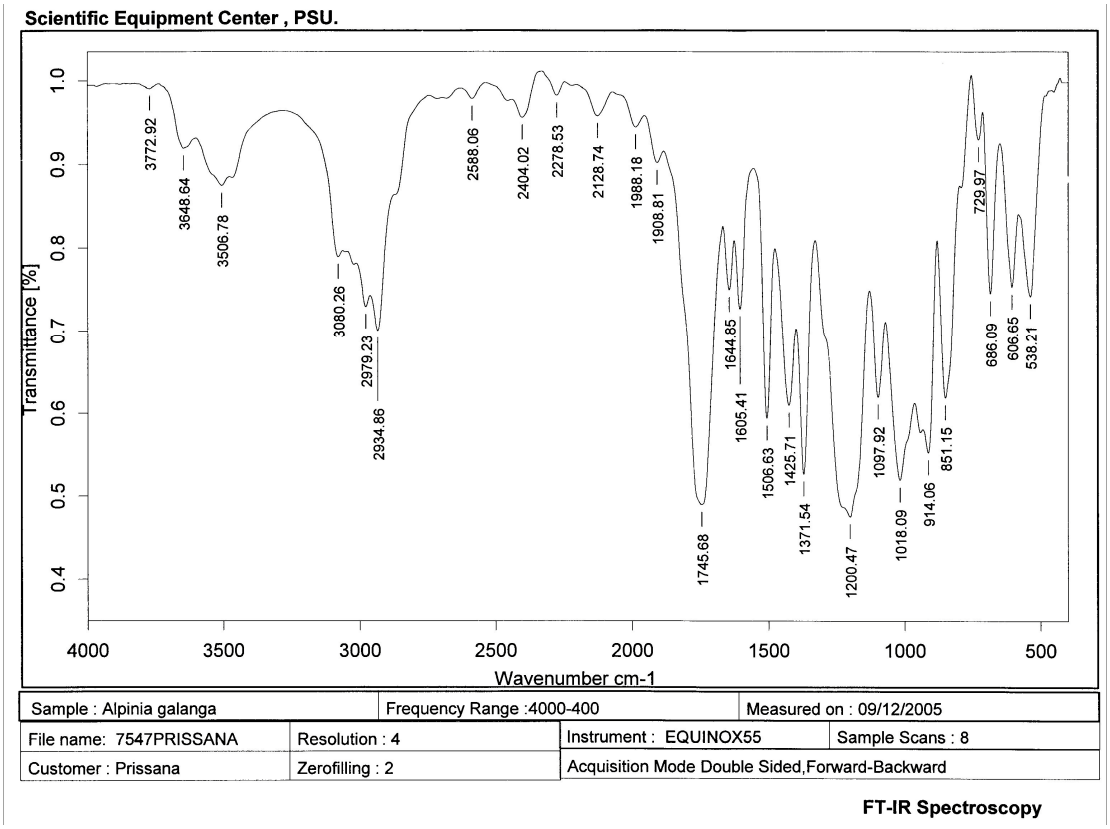


Figure 3.3 IR spectrum of AP1

Its mass spectrum (Figure 3.4) exhibited a molecular ion peak [M^+] at *m/z* 234 corresponding to a molecular formula of $C_{13}H_{14}O_4$. The most abundant mass peaks were *m/z* 192, 150, 132, 131, 121. Characteristic fragments included $C_6H_5^+$ at *m/z* 77 from phenyl derivatives, and loss of mass 42 (192 and 150) due to formation of CH_2=C=O by a McLafferty rearrangement (Yang and Eilerman, 1999).

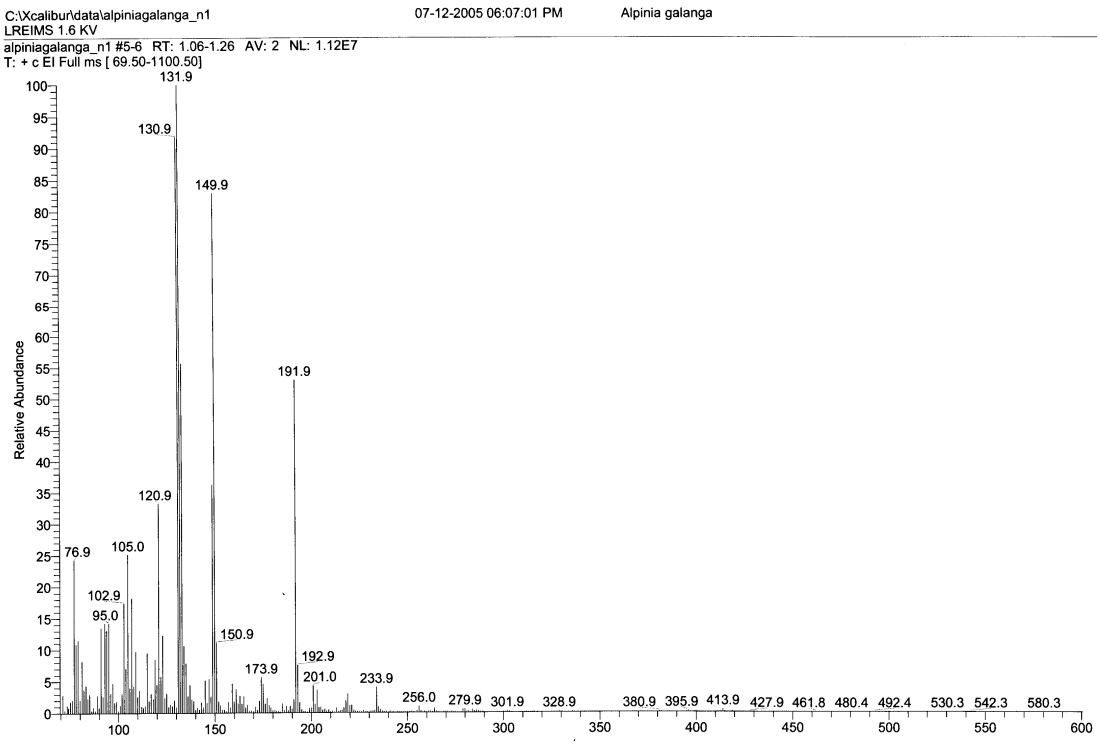


Figure 3.4 Mass spectrum of AP1

Regarding to its NMR properties, the ^{1}H NMR data (Figure 3.5, Table 3.8) showed 14 protons in the molecule. Two symmetrical doublets at δ 7.0 and 7.3 ppm are four protons on a para-substituted benzene ring. The proton resonances at 5.9 (1H, ddd), and 5.2 (1H, ddd) and 6.2 (1H, d) ppm correspond to protons on 2′- and 3′- olefinic carbons, respectively. The signal at 5.2 ppm correspond to protons on 1′ carbon. Two methyl signals of two acetyl groups are clearly due to the presence of two singlet signals at 2.1 and 2.3 ppm. The data was compared

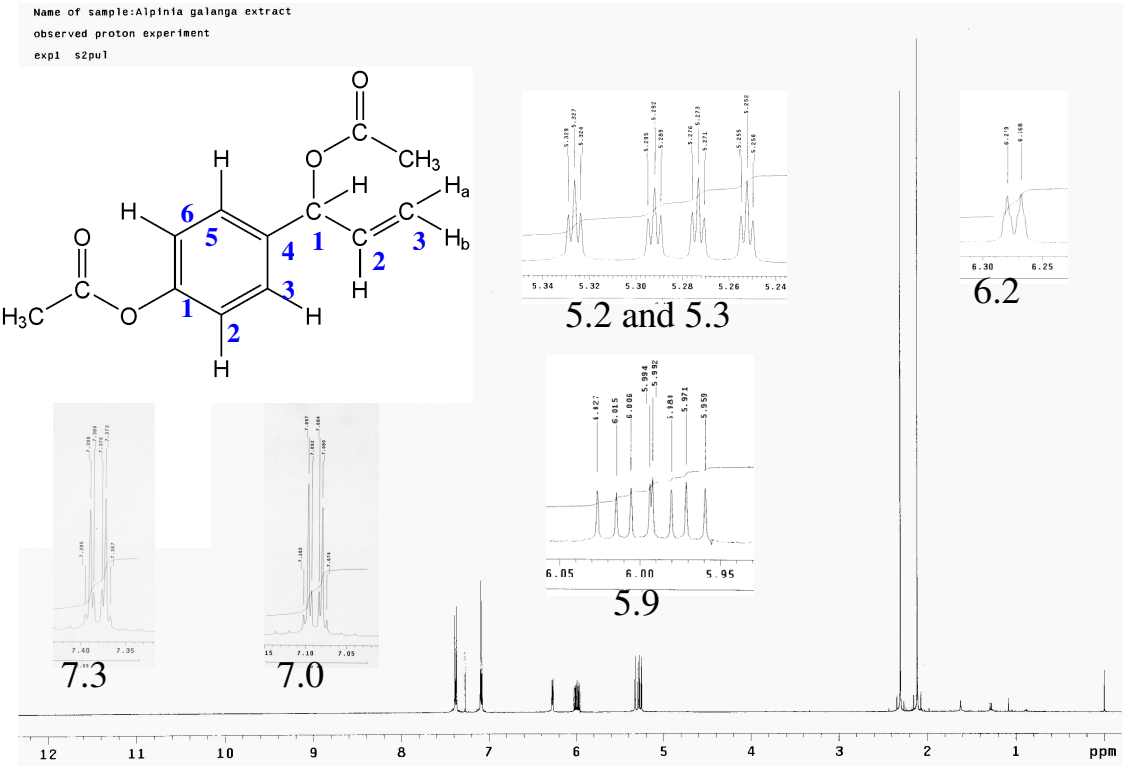
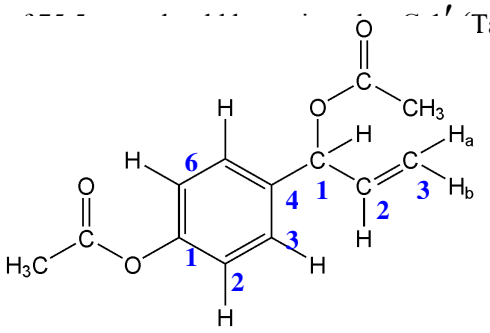


Figure 3.5 ^{1}H NMR spectrum of AP1 (500 MHz, $CDCl_3$)

Table 3.8 H^1-NMR data of 1′-acetoxychavicol acetate (500 MHz, $CDCl_3$)

H	δ (ppm)	Integral	Multiplicity	*J* (Hz)
2	7.0	1H	ddd	2.7, 4.6, 8.5
3	7.3	1H	ddd	2.7, 4.6, 8.5
5	7.3	1H	ddd	2.7, 4.6, 8.5
6	7.0	1H	ddd	2.7, 4.6, 8.5
1′	5.2	1H	ddd	1.2, 1.2, 10.4
2′	5.9	1H	ddd	5.9, 10.4, 17.2
3′a	5.3	1H	ddd	1.2, 1.2, 17.2
3′b	6.2	1H	d	5.9
1′-$COOCH_3$	2.1	3H	s	-
1-$COOCH_3$	2.3	3H	s	-

Figure 3.6 shows the complete proton decoupled ^{13}C NMR spectrum which displayed thirteen carbon signals. Among these, two methyl signals are clear due to the presence of chemical shift value of 21.1 and 21.2 ppm. Two carbon signals at 169.4 and 169.9 ppm are typically from carboxyl groups. Two pairs of carbon signal have chemical shifts at 128.4 and 136.0 ppm, and two quaternary carbon signals at 136.4 and 150.4 ppm confirming a para-substituted benzene ring. The carbon signal with the chemical shift of 121.6 and 117.0 ppm should be assigned as sp^2 carbons at 2′ and 3′, respectively. The carbon signal with the chemical sh[illegible] (Table 3.9).



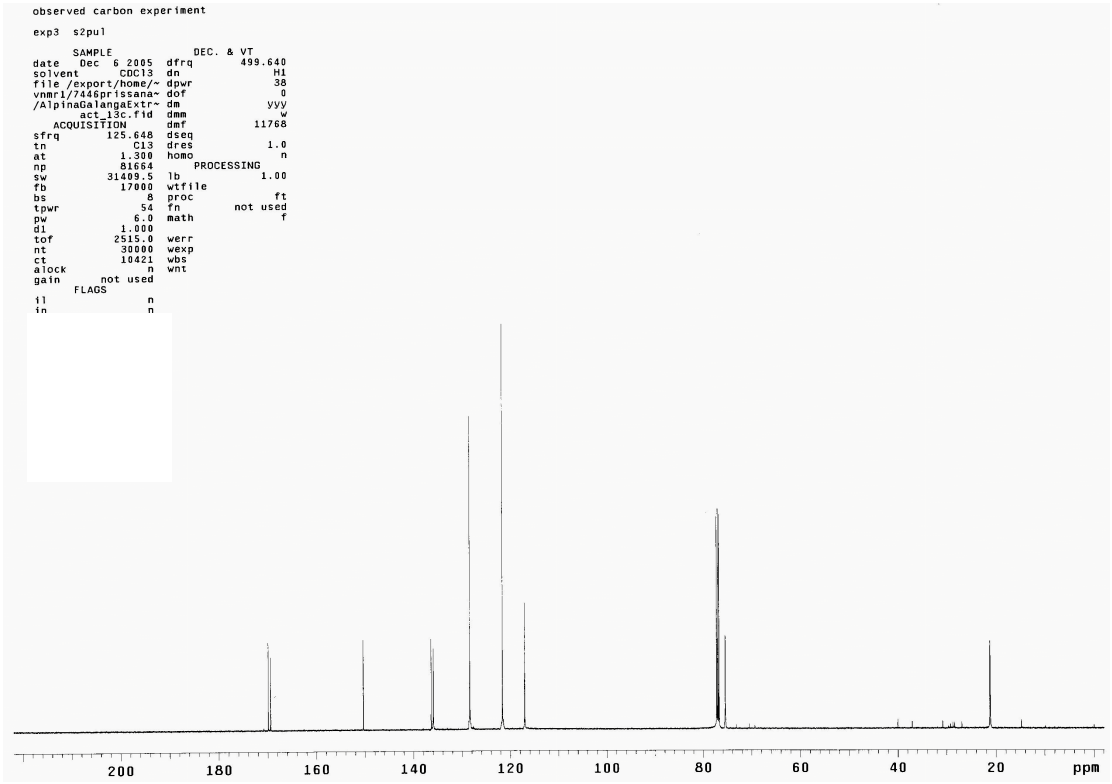


Figure 3.6 ^{13}C NMR spectrum of AP1 (125 MHz, $CDCl_3$)

Table 3.9 ^{13}C-NMR data of 1′-acetoxychavicol acetate (125 MHz, $CDCl_3$)

C	δ (ppm)
CH_3	21.1
CH_3	21.2
1′-CH	75.5

3′-CH_2	117.0
2′-CH	121.6
2-CH	128.4
6-CH	128.4
3-CH	136.0
5-CH	136.0
4-C	136.4
1-C	150.4
C=O	169.4
C=O	169.9

On the basis of these spectroscopic data, AP1 was identified as a phenylpropanoid compound named 1′-acetoxychavicol acetate (Figure 3.7).

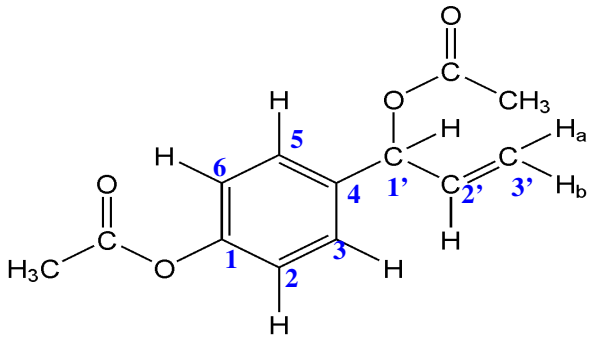
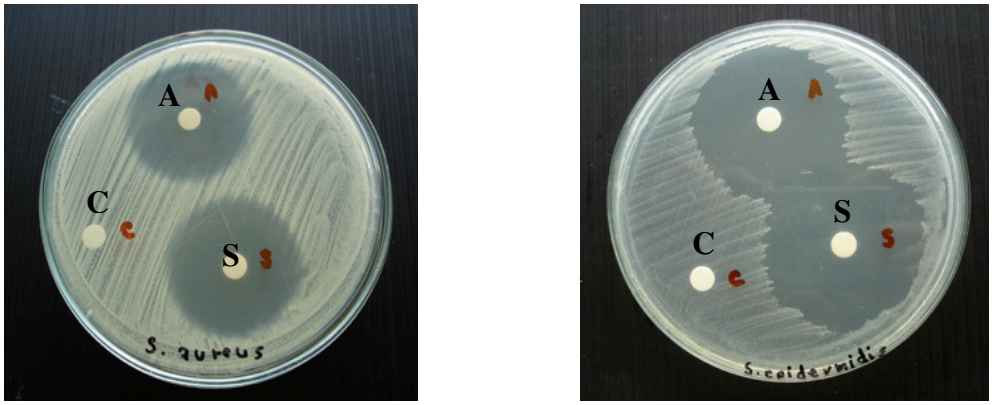


Figure 3.7 Structure of 1′-acetoxychavicol acetate (1′-ACA)

3.4 Antibacterial activity of 1′-ACA

1′-ACA was subjected to evaluation of antibacterial activity against *P. acnes, S. aureus* and *S. epidermidis*. It was found that 1′-ACA possessed antibacterial activity against *P. acnes, S. aureus* and *S. epidermidis* with MIC values of 62, 250 and 250 μg/ml, respectively, and MBC values of 250, 1000 and 1000 μg/ml, respectively (Table 3.10). The result indicated that *P. acnes* is more sensitive to 1′-ACA than *S. aureus* and *S. epidermidis*.



S. aureus ***S. epidermidis***

Figure 3.8 Antibacterial activity screening of *A. galanga* ethyl acetate extract (S) and 1′-ACA (A) against *S. aureus* and *S. epidermidis*; negative control (DMSO) (C)

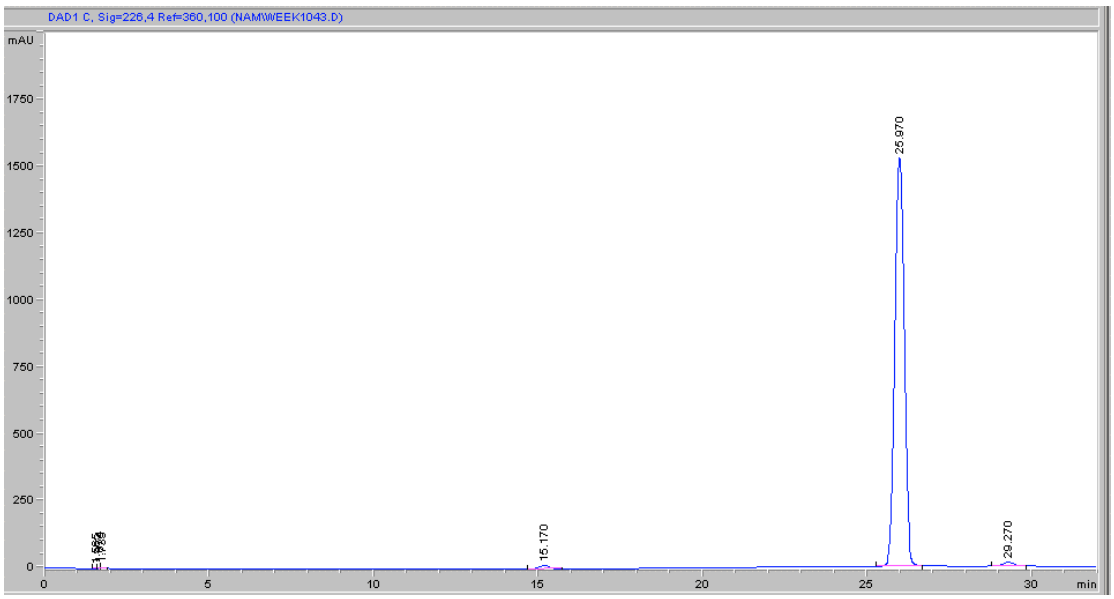
Table 3.10 Antibacterial activity of 1′-ACA, *A. galanga* extract and tetracycline HCl against *P. acnes, S. aureus* and *S. epidermidis*

Substances	*P. acnes*		*S. aureus*		*S. epidermidis*	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
1′-ACA	62	250	250	1000	250	1000
A. galanga extract	156	312	500	>1000	500	>1000
Tetracycline HCl	2	8	0.5	>32	0.5	1

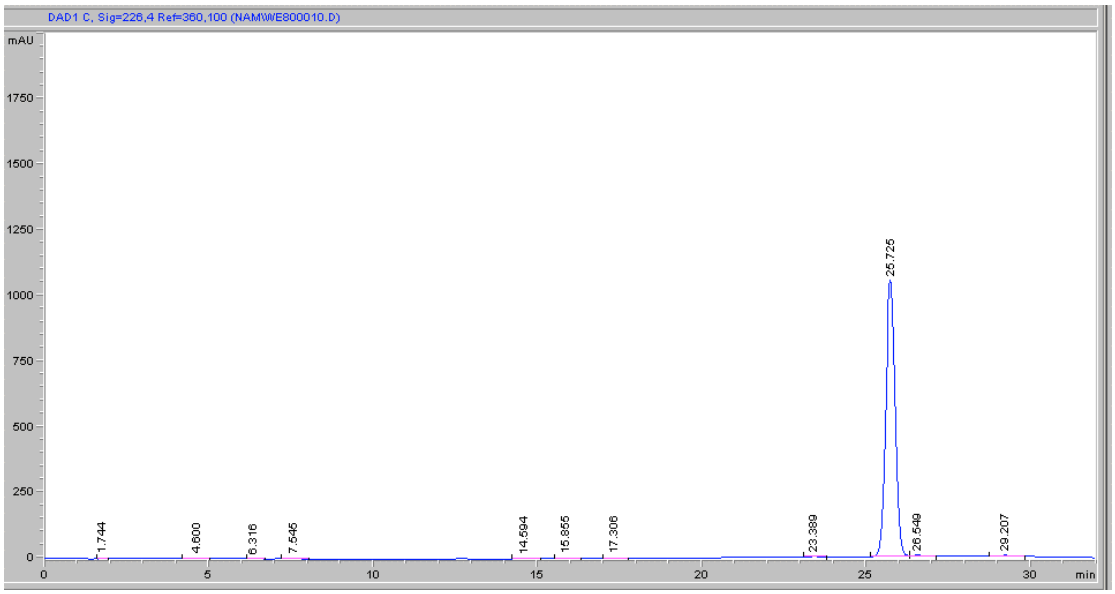
These results suggested that *A. galanga* extract possessed antibacterial activity against normal skin flora including *P. acnes, S. aureus* and *S. epidermidis* through the active constituent, 1′-ACA. Therefore, *A. galanga* extract would be an interesting material for further study on an alternative treatment of acne. It has been reported that 1′-ACA possessed various biological activities, such as antitumor (Itokawa *et al*., 1987; Kondo *et al*., 1993; Moffatt *et al*., 2000; Zheng *et al*., 2002), anti-inflammation (Nakamura *et al*., 1998), antifungal (Janssen and Scherffer, 1985), antioxidative (Kubota *et al*., 2001), and xanthine oxidase inhibitory activity (Noro *et al*., 1988). Thus, 1'ACA could be recommended as an indicative marker for standardization of *A. galanga* extract.

3.5 Quantitative determination of 1′-ACA in *A. galanga* extract by HPLC

An optimal condition for the quantitative analysis of 1′-ACA in *A. galanga* extract was examined using gradient reverse phase HPLC system. A TSK-GEL® ODS-80TS column was eluted with a mixture of water and methanol under gradient conditions as described in the section 2.2.5. Baseline separation of 1′-ACA was achieved within 30 minutes. The retention times of 1′-ACA was 26 minutes (Figure 3.9). The identity of 1′-ACA peak was confirmed by comparison of its absorption spectrum produced by photo-diode array detector with the authentic compound (Figure 3.10). Linearity of the HPLC method was evaluated using standard samples over six calibration concentrations between 0.625 to 20 mg/ml (Figure 3.11). It exhibited good linearity over the evaluated ranges with correlation coefficient of 0.9994.



(A)



(B)

Figure 3.9 HPLC-chromatogram of the authentic 1′-ACA (A) and *A. galanga* extract (B)

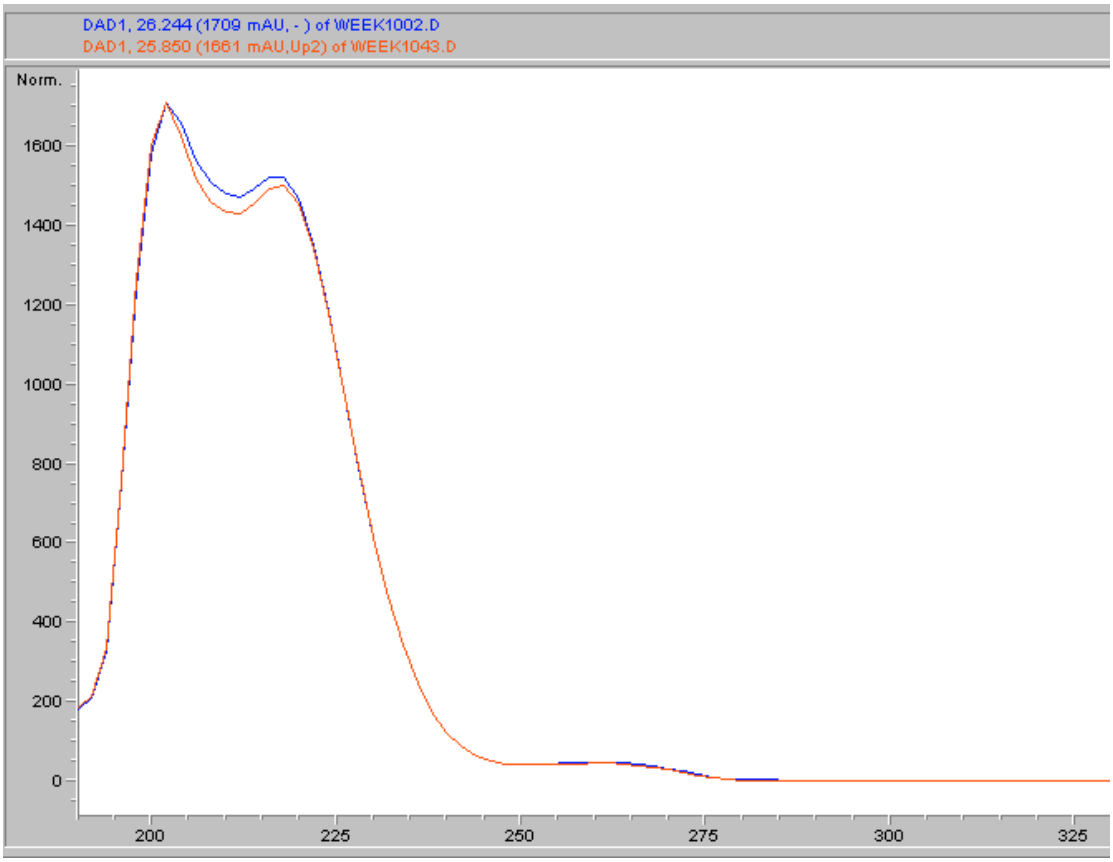


Figure 3.10 Absorption spectra of the peak at retention time 26 minutes ( ) and the authentic 1′-ACA ( )

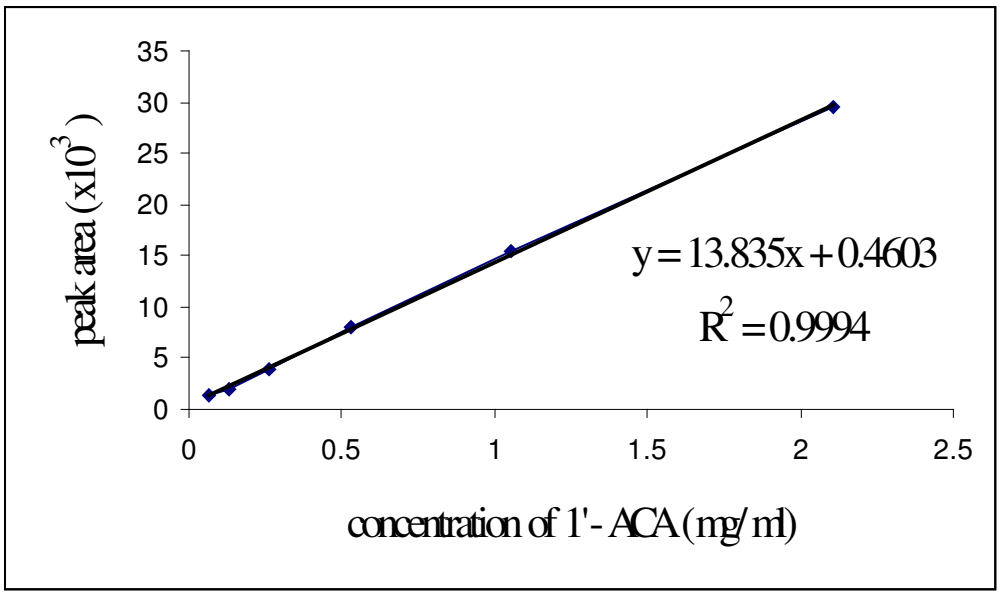


Figure 3.11 Calibration curve of 1′-ACA

3.6 Preparation of *A. galanga* extract for preliminary formulation

According to bioassay guided separation, 1′- ACA was isolated from ethyl acetate extract by hexane elution from silica gel vacuum chromatography. This finding indicates that 1′- ACA is most likely non-polar compound. In order to get the most benefit from this compound, the method for *A. galanga* extract preparation that involved solvent and method of extraction should be revised. According to the polarity 1′- ACA, hexane was selected as the solvent for extraction. The extraction method was changed from maceration to refluxing, due to the time consumed by the maceration method. Therefore, preparation of *A. galanga* extract for preliminary formulation was performed as described in the section 2.2.6. On the basis of HPLC analysis, the obtained yellow oily extract was composed of 76.1 % w/w 1′-ACA.

A. galanga hexane extract was subjected to evaluation of antibacterial activity against *P. acnes*, as described in 2.2.2.2 with the final concentration of 1000 to 1.95 μg/ml. It was found that *A. galanga* hexane extract possessed antibacterial activity against *P. acnes* with MIC value of 250 μg/ml and MBC value of 500 μg/ml,

3.7 Solubility of *A. galanga* extract

There are various cosmetic solvents, co-solvents and some additive compounds that are used in cream base. The solubility test is used to predict the dissolution of *A. galanga* extract in cosmetic solvents that were used in the cream base and the possibility of the homogeneity of *A. galanga* cream. A solubility study showed that *A. galanga* extract was sparingly miscible with ethanol and mineral oil, slightly miscible with propylene glycol, and very slightly miscible with glycerin. It is practically immiscible with water (Table 3.11). *A. galanga* extract is most likely non-polar compound, therefore the suitable solvents for *A. galanga* extract should be a less polarity solvent.

Table 3.11 Solubility of *A. galanga* extract with various cosmetic solvents

Solvent	Volume of solvent in ml/g of solute	Level of solubility
Ethanol	80	Sparingly miscible

Glycerin	1940	Very slightly miscible
Mineral oil	100	Sparingly miscible
Propylene glycol	400	Slightly miscible
Water	>10000	Practically immiscible

3.8 Stability of *A. galanga* extract

Photo and temperature factors affecting the stability of *A. galanga* extract was examined.

3.8.1 Photo-stability

Photo-stability of the extract was examined under fluorescent light as compared with protection from light for a period of 10 weeks. Physical appearance of the extract and the content of 1′-ACA were examined every 2 weeks. The result showed that under light condition, the color of *A. galanga* extract gradually faded, while the viscosity of the extract gradually increased (Table 3.12). In addition, a significant decrease of 1′-ACA content was observed in the sixth week (Table 3.13, Figure 3.12). In contrast, the physical appearance and 1′-ACA content of the extract that kept in the light protecting container were not significantly changed in the period of 10 weeks (Table 3.12, Table 3.13 and Figure 3.12). This finding suggested that *A. galanga* extract should be kept in light protecting container.

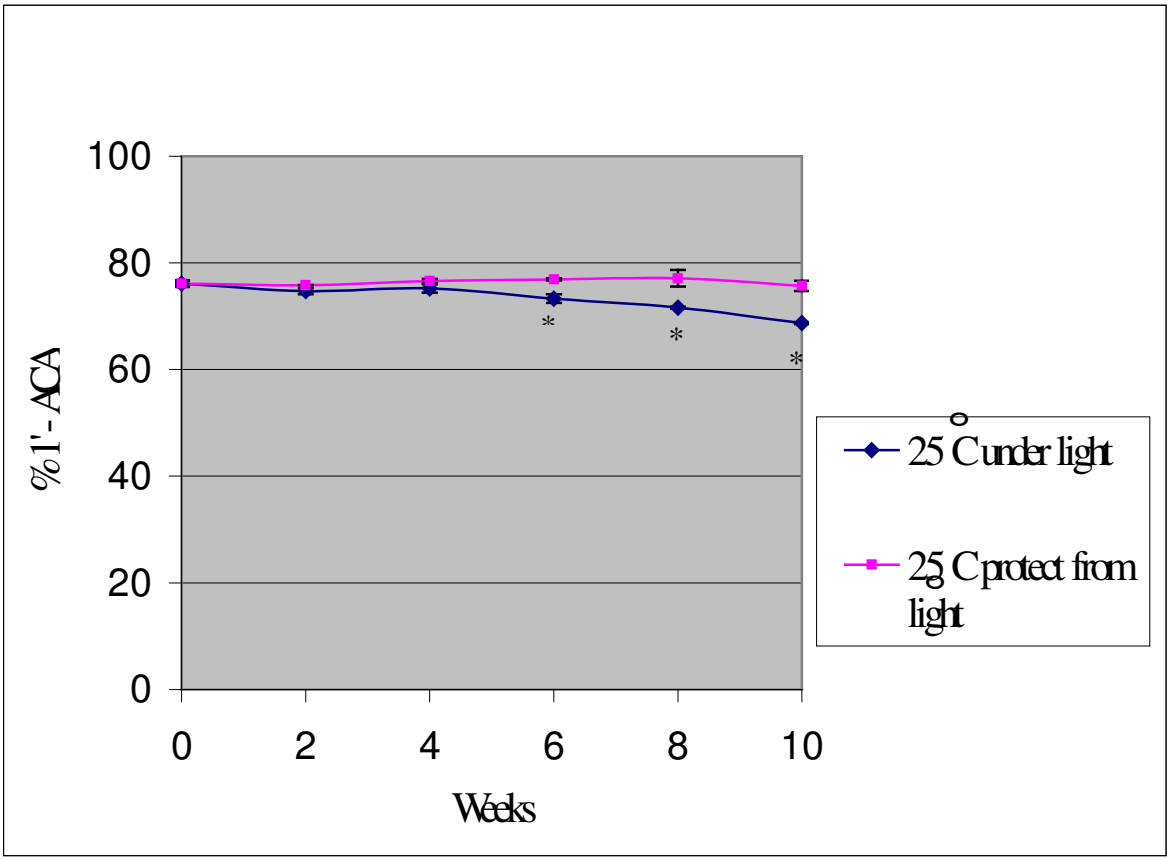
Table 3.12 Physical appearance of *A. galanga* extract under light and with protection from light conditions at 25±2°C

Week	Physical appearance of the extract	
	Light	Protect from light
0	Yellow oily liquid	Yellow oily liquid
2	Yellowish oily liquid	Yellow oily liquid
4	Colorless oily liquid	Yellow oily liquid
6	Colorless viscous oily liquid	Yellow oily liquid
8	Colorless viscous oily liquid	Yellow oily liquid
10	Colorless viscous oily liquid	Yellow oily liquid

Table 3.13 Content of 1′-ACA in *A. galanga* extract under light and with protection from light conditions at 25±2°C

Week	Mean content of 1′-ACA (% w/w ± S.D.)	
	Light	Protect from light
0	76.1 ± 0.62	76.1 ± 0.62
2	74.7 ± 0.57	75.8 ± 0.03
4	75.2 ± 0.79	76.6 ± 0.39
6	73.3 ± 0.82*	76.9 ± 0.22
8	71.6 ± 0.19*	77.1 ± 1.56
10	68.7 ± 0.18*	75.7 ± 0.96

* Significance at $P < 0.05$ when compared with the content at initial time



* Significance at $P < 0.05$ when compared with the content at initial time

Figure 3.12 Content of 1′-ACA in *A. galanga* extract under light and with protection from light conditions at 25±2°C

3.8.2 Thermal-stability

Thermal stability of galangal extract was examined under light protecting condition and temperatures of 25°C, 30°C and 45°C, for 10 weeks-period. Physical appearance of the extract and the content of 1′-ACA were examined every 2 weeks. The result showed that all tested temperatures did not affect the physical appearance of the extract and 1′-ACA content when the extracts were kept in light protecting container within 10 weeks (Table 3.14 and Figure 3.13). It implies that *A. galanga* extract is stable under room temperature but unstable under light condition.

Table 3.14 Content of 1′-ACA in *A. galanga* extract under light protecting container at 25° C, 30°C and 45°C

Week	Mean content of 1′-ACA (% w/w ± S.D.)		
	25 ± 2 °C	30 ± 2 °C	45 ± 2 °C
0	76.1 ± 0.62	76.1 ± 0.62	76.1 ± 0.62
2	75.8 ± 0.03	75.2 ± 0.09	76.4 ± 1.13
4	76.6 ± 0.39	77.2 ± 0.37	76.8 ± 0.77
6	76.9 ± 0.22	76.7 ± 0.63	77.1 ± 1.19
8	77.1 ± 1.56	77.6 ± 0.94	77.2 ± 1.43
10	75.7 ± 0.96	77.0 ± 0.45	77.3 ± 0.54

***** Significance at $P < 0.05$ when compared with the content at initial time

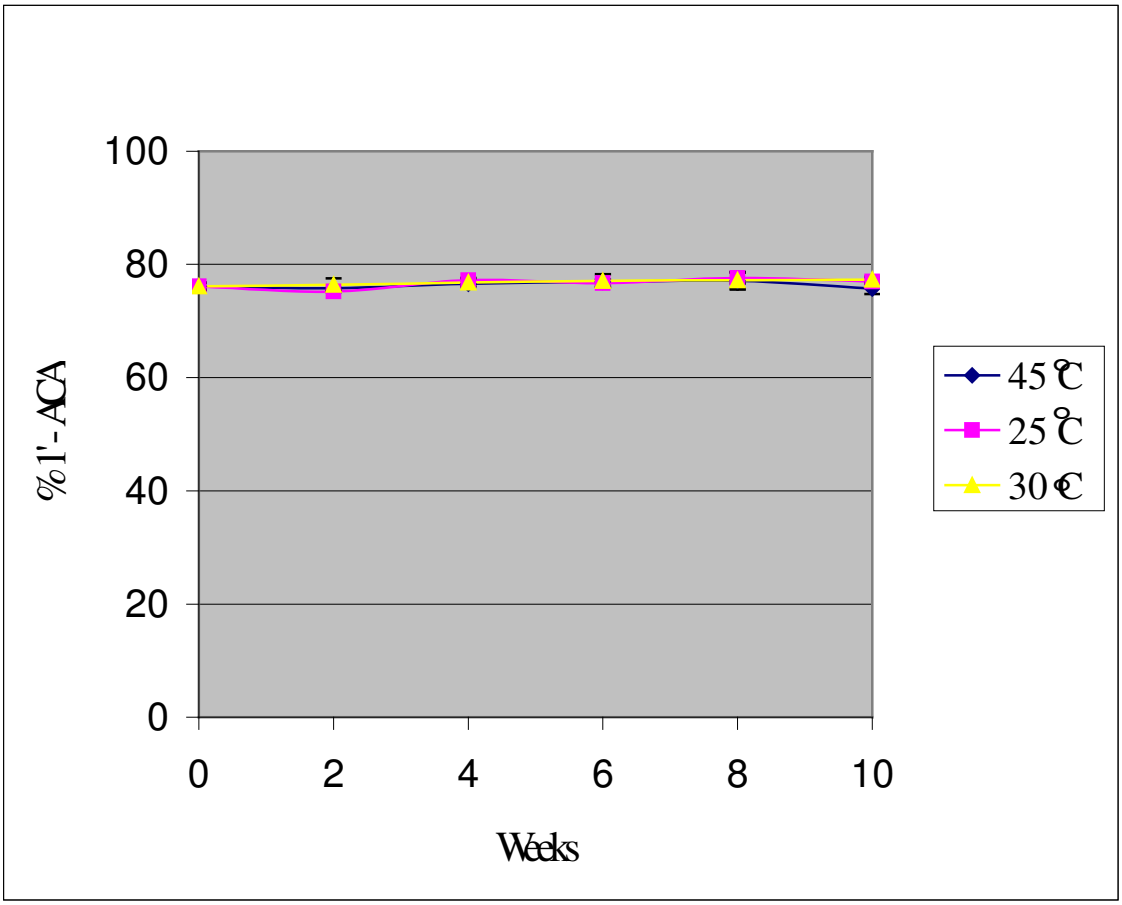


Figure 3.13 Content of 1′-ACA in *A. galanga* extract under light protecting container at 25° C, 30°C and 45°C

3.9 Preliminary formulation study of *A. galanga* extract cream

Five cream bases were prepared and tested for their stability by heating-cooling cycle method as described in section 2.2.8. An observation of the physical appearance, including color, smoothness, phase separation, viscosity and pH, before and after heating-cooling cycles test led to the selection of a suitable cream base (Table 3.15, Figure 3.14). The cream base Rx 2 exhibited a high basic property (pH 8.8), which is not suitable for a human skin. Thus, this cream base was excluded. The cream bases Rx 1, Rx 4 and Rx 5 showed good physical appearance and the pH of the cream bases was closed to 5.5, with is suitable for human skin. In addition, their pH and viscosity did not significantly change after the test. In contrast, a lot of bubbles were

produced in the cream Rx 3 after heating-cooling cycle. Therefore, the cream bases Rx 1, Rx 4 and Rx 5 were selected for further formulation studies.

Table 3.15 Physical properties of cream bases before and after heating and cooling cycle

Physical properties	Before heating-cooling cycle					After heating-cooling cycle				
	Rx 1	Rx 2	Rx 3	Rx 4	Rx 5	Rx 1	Rx 2	Rx 3	Rx 4	Rx 5
Color	W	W	W	W	OW	W	W	W	W	OW
Smoothness	S	SB	S	S	S	S	nd	SB	S	S
Phase separation	No	No	No	No	No	No	nd	No	No	No
Viscosity (cps) Mean ± S.D.	37000± 2160.2	nd	66000± 1414.2	196333± 1247.2	63333± 2357.0	48333± 2357.0	nd	68333± 2357.0	202333± 1699.7	92333± 2084.8
PH	5.56	8.80	5.88	6.64	5.68	5.76	nd	5.73	6.61	5.76

W = white, OW = off-white, S = smooth, SB = smooth cream with bubble, nd = not determine

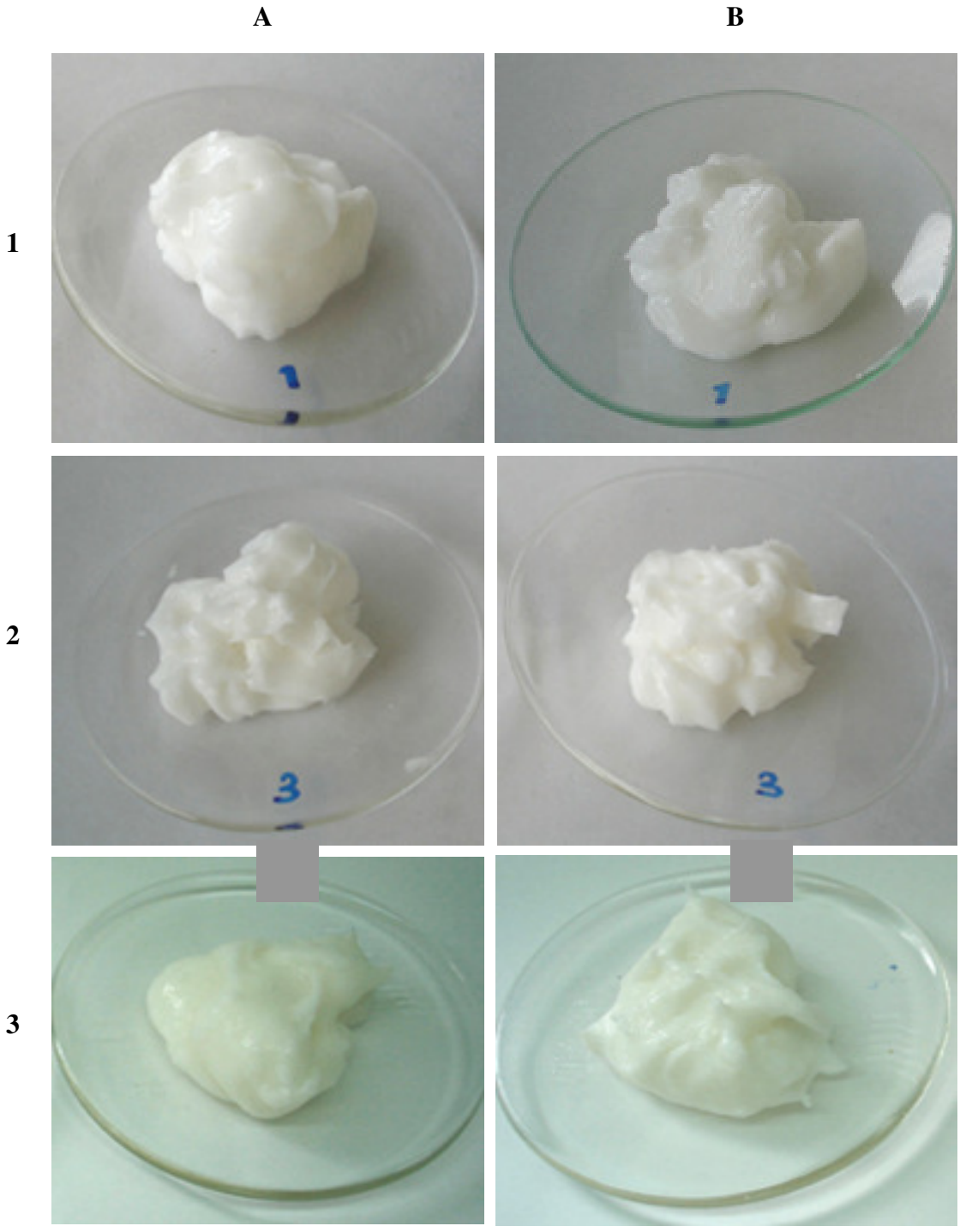

A
B
1
2
3
1
1
3
3

Figure 3.14 Physical appearance of cream bases before (A) and after (B) heating-cooling cycle test: Rx1 (1), Rx 4 (2), Rx 5 (3)

In order to formulate an anti *P. acne* cream, *A. galanga* creams were prepared from hexane extract of *A. galanga* at the concentration of 1% and 2% w/w, which equivalent to 1′-ACA concentration of 0.76% w/w and 1.52% w/w, respectively. The hexane extract was added into three selected cream bases, Rx 1, Rx 4 and Rx 5 to produce six anti *P. acne* preparations (Rx 1.1, Rx 1.2, Rx 4.1, Rx 4.2, Rx 5.1 and Rx 5.2). The physical appearances (color, smoothness, and phase separation) before and after 8 cycles of heating cooling test and at room temperature after 30 days of storage, pH and viscosity of the preparations were shown in Table 3.16 and Table 3.17. All six preparations had good appearance with a little odor at initial time. The colors of preparations varied from off-white to pale yellow. The tone of color depended on the concentration of the extract in the preparation. However, the colors of all preparations may be acceptable as skin creams. After a heating and cooling cycle test and storaged at room temperature for 30 days, it was found that the colors of all preparations were little changed. Phase separation was not observed in all preparations. However, pH and viscosity changes were observed in all preparations, especially Rx 1.1 and 1.2. In addition, oil bubbles were observed only in Rx 1.1 and Rx 1.2. Thus, Rx 1.1 and 1.2 were excluded. The pictures of 1% and 2% *A. galanga* creams before and after heating and cooling cycle were shown in Figure 3.15. These results showed that Rx 4.1, Rx 4.2, Rx 5.1 and Rx 5.2 exhibited good physical properties after heating and cooling cycle and at room temperature after 30 days.


A
B
1
2
3
4
5
6

Figure 3.15 *A. galanga* preparations, Rx 1.1 (1), Rx 1.2 (2), Rx 4.1 (3), Rx 4.2 (4), Rx 5.1 (5), Rx 5.2 (6) before (A) and after (B) heating and cooling cycle

Table 3.16 Physical properties of *A. galanga* cream containing 1% and 2% *A. galanga* extract before and after heating and cooling cycle

Physical properties	*A. galanga* preparation											
	Rx 1.1		Rx 1.2		Rx 4.1		Rx 4.2		Rx 5.1		Rx 5.2	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Color	O	OB	O	OB	Y	Y	Y	Y	O	O	O	O
Phase separation	No	No	No	No	No	No	No	No	No	No	No	No
PH	4.22	3.96	4.17	3.64	5.94	5.13	5.89	4.87	4.64	4.17	4.62	3.90
Viscosity (cps)	31866± 611.0	21733± 461.9	28933± 1006.6	20400± 400	71866± 230.9	65333± 1154	51866± 611.0	40666± 611.0	42000± 400	40000± 800	44266± 461.9	40000± 800

O= off-white OB= off-white with bubble Y= pale yellow

Table 3.17 Physical properties of *A. galanga* cream containing 1% and 2% *A. galanga* extract before and after 30 days of storage at room temperature

Physical properties	*A. galanga* preparation											
	Rx 1.1		Rx 1.2		Rx 4.1		Rx 4.2		Rx 5.1		Rx 5.2	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Color	O	OB	O	OB	Y	Y	Y	Y	O	O	O	O
Phase separation	No	No	No	No	No	No	No	No	No	No	No	No
PH	4.22	4.03	4.17	3.85	5.94	5.17	5.89	4.89	4.64	4.20	4.62	3.96
Viscosity (cps)	31866± 611.0	21266± 692.8	28933± 1006.6	20266± 461.9	71866± 230.9	62666± 611.0	51866± 611.0	42400± 400	42000± 400	40533± 461.9	44266± 461.9	41733± 230.9

O= off-white OB= off-white with bubble Y= pale yellow

Regarding of chemical stability of the preparations, quantitative determination of 1′-ACA content in the preparation before and after heating-cooling cycle and at room temperature after 30 days was examined. It was found that at the initial time all preparations were composed of 1′-ACA content between 92.9-101.9 % labeled amount (Table 3.18). Unfortunately, after heating-cooling cycle and at room temperature after 30 days the content of 1′-ACA in all preparation was markedly attenuated. Two major peaks of degradation products of 1′-ACA were found in the HPLC-chromatograms of all preparations after heating-cooling cycle. These degraded compounds were observed at the retention times of 12.4 and 13.3 minutes (Figure 3.16). The absorption spectrum of the peak at 13.3 minutes was similar to that of 1′-ACA (Figure 3.17). It has been reported that 1′-ACA was not stable in aqueous solution and undergoes hydrolysis reaction and rearrangement when refluxed with water. These reactions produced 1′-hydroxychavicol acetate and *p*-acetoxy-trans-cinnamic alcohol as hydrolysis products (Figure 3.18) (Yang and Eilerman, 1999). Therefore, hydrolysis of 1′-ACA may occur in oil in water cream bases and the degraded products may be 1′-hydroxychavicol acetate and *p*-acetoxy-trans-cinnamic alcohol.

Table 3.18 Content of 1′-ACA in *A. galanga* creams at initial, after 8 cycles of heating-cooling test and after 30 days of storage at room temperature

A. galanga preparation	Concentration of the extract (%w/w)	% Labeled amount		
		Initial	After heating-cooling cycle test	30 days of storage at room temperature
Rx 4.1	1	101.9 ± 5.93	1.4 ± 0.34	5.26±0.24
Rx 4.2	2	98.9 ± 2.58	1.2 ± 0.02	5.18±1.81
Rx 5.1	1	92.9 ± 2.73	1.0 ± 0.37	1.43±0.16
Rx 5.2	2	96.0 ± 2.24	0.1 ± 0.02	1.33±0.12

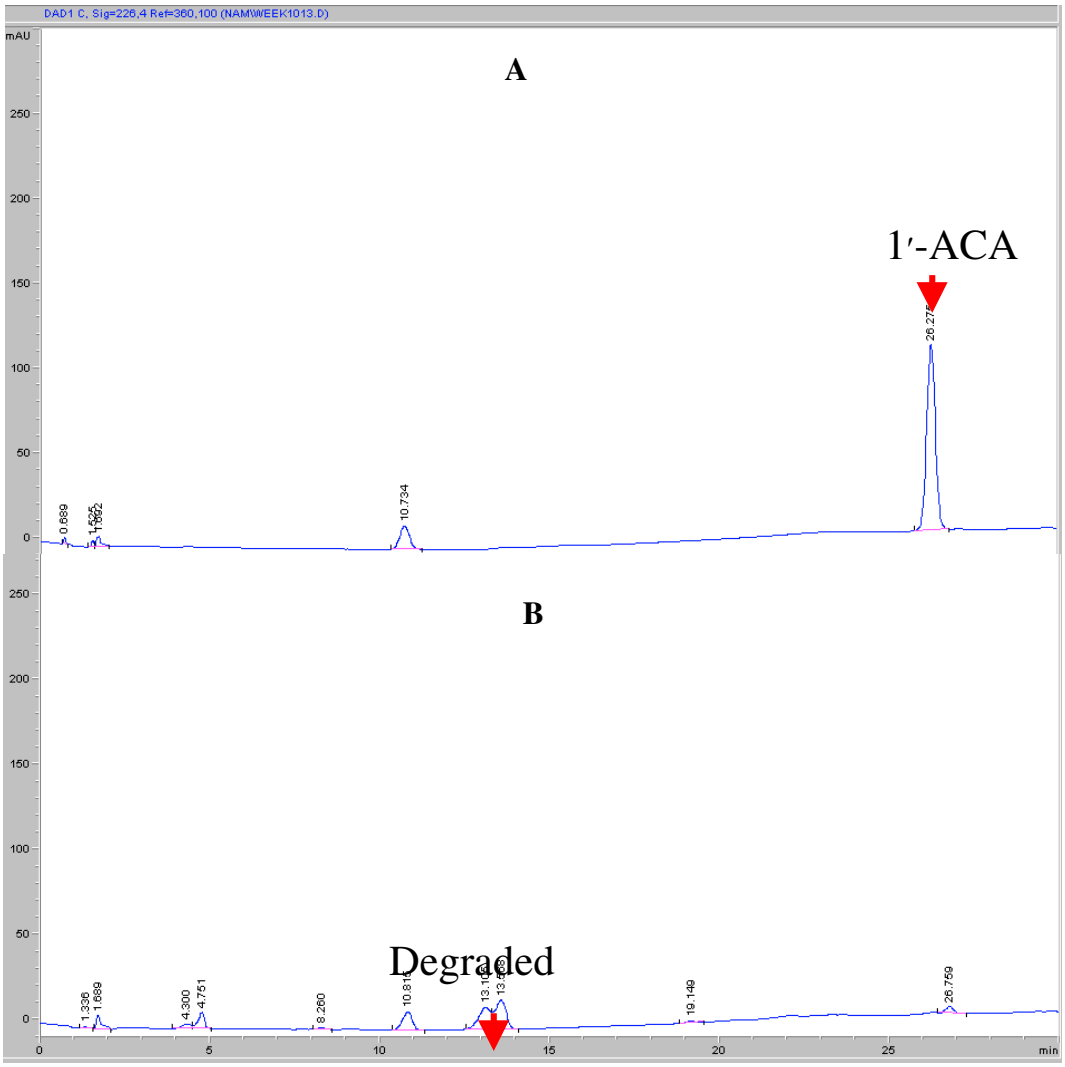


Figure 3.16 HPLC-chromatogram of the preparation extracts at initial time (A) and after heating-cooling cycle test (B)

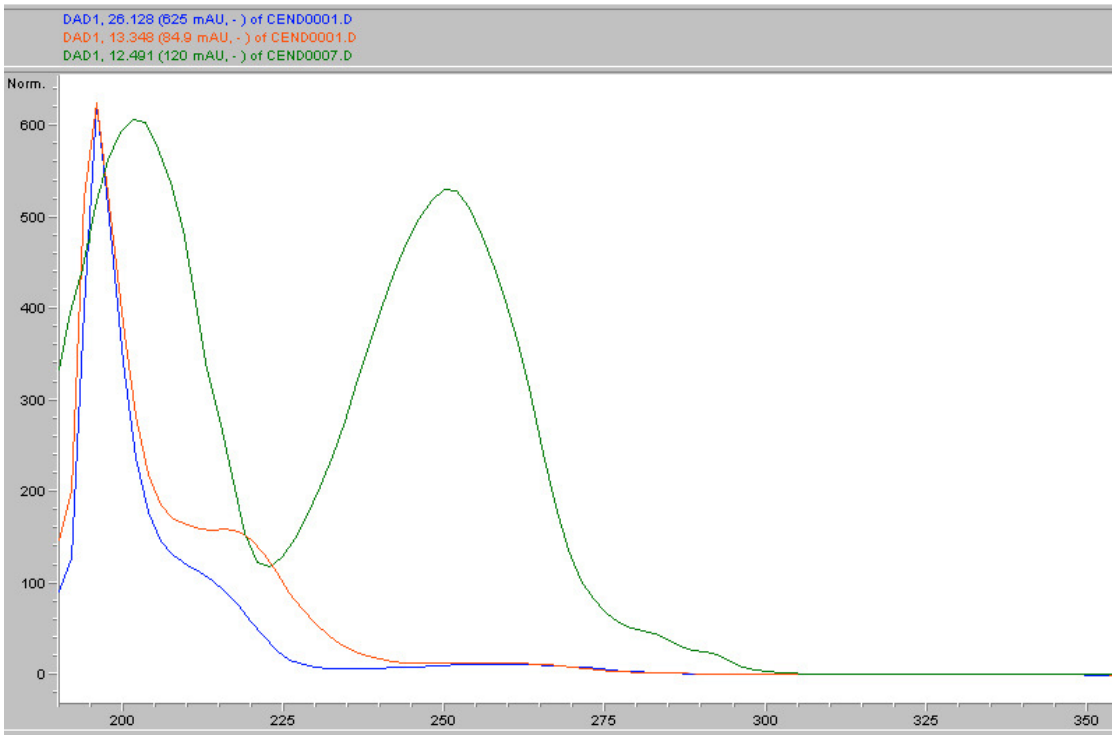


Figure 3.17 UV absorption spectra of 1′-ACA ( ) and degradation products at retention time 13.3 ( ) and 12.4 minutes ( )

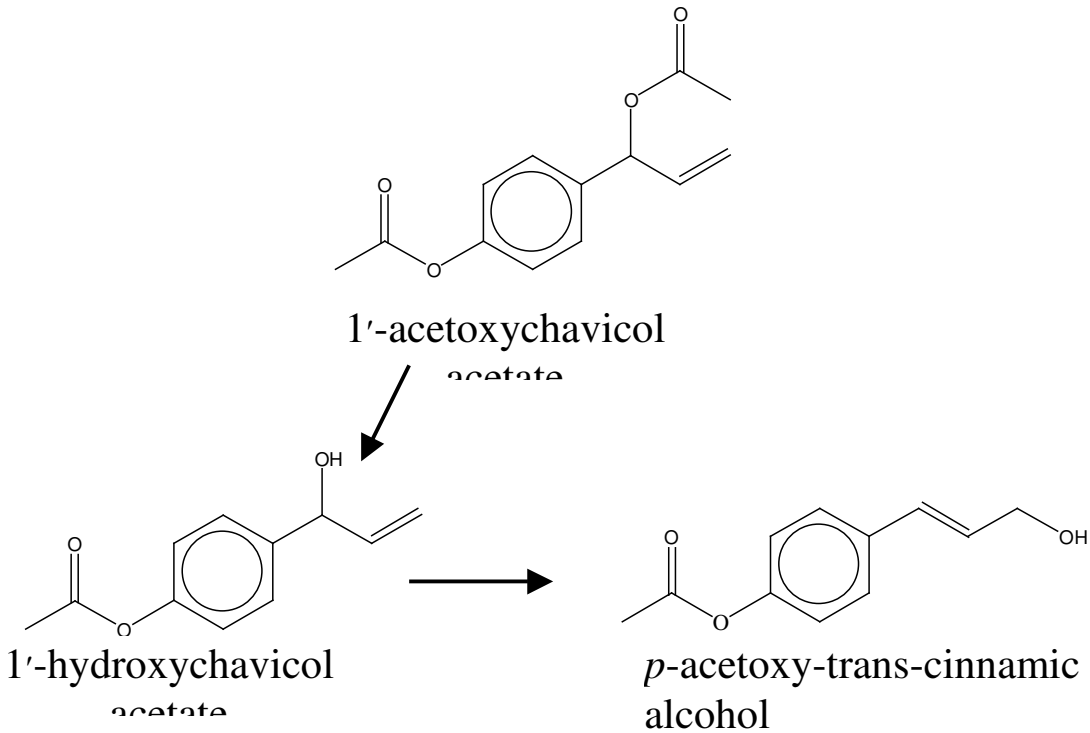


Figure 3.18 Hydrolysis of 1′-acetoxychavicol acetate

In contrast, investigation of antibacterial activity of 1% and 2% w/w *A. galanga* creams showed that the inhibition zones of the preparations Rx 4.1, Rx5.1, Rx 5.2 were not different when compared between before and after heating-cooling cycle test and Rx 4.1 and 5.2 were not different when compared between before and at room temperature after 30 days of storage, however, that of Rx 4.2 was higher (Table 3.19). This implies that the degraded products still possessed antibacterial activity against *P. acnes*. In addition, the results also showed that the concentration of *A. galanga* extract at 2%w/w exhibited higher antibacterial activity than that of 1% w/w.

The result also indicated that the cream base Rx4 exhibited antibacterial activity with and inhibition zone of 12.1± 0.31 mm. This may be due to polysorbate 20, which is a surfactant.

Table 3.19 Antibacterial activity of *A. galanga* creams against *P. acnes* investigated by disk diffusion method

Formulation	Concentration of the extract (% w/w)	Inhibition zone (mm)		
		Before heating-cooling cycle	After heating-cooling cycle	After room temperature 30 days
Cream base Rx 4	0	12.1 ± 0.31	12.8 ± 0.30	12.6±0.80
Rx 4.1	1	27.7 ± 0.66	29.1 ± 1.11	31.2± 2.2
Rx 4.2	2	33.1 ± 0.72	38.2 ± 0.75*	42.8±3.93*
Cream base Rx 5	0	0	0	0
Rx 5.1	1	28.2 ± 1.00	21.0 ± 2.63	20.5±2.33*
Rx 5.2	2	33.7 ± 0.30	27.1 ± 4.67	30.2±2.21
Clindalin gel 1%		53.4 ± 0.74		

***** Significance at $P < 0.05$ when compared with the content at initial time

Preliminary formulation study of anti-acne cream using *A. galanga* extract found that although, the *A. galanga* cream exhibited inhibitory effect after heating and cooling cycle test and at room temperature after 30 days of storage, 1′-ACA may not be stable in the oil in water cream bases. Stability of *A. galanga* cream in this research was not success. Therefore, it requires further studies for searching a suitable cream base or suitable dosage form. In addition, skin irritation should be a further study.