CHAPTER III

EXPERIMENTAL

Source of Plant Material

The bark and herbarium specimen of *Paramichelia baillonii* Hu were collected from Doi Suthep-Doi Pui National Park, Chiang Mai Province in July 1985 and authenthicated by comparison with herbarium specimens at Royal Forest Department, Ministry of Agriculture and Co-operatives, Bangkok, Thailand. A voucher specimen of plant material has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

General Techniques

1. Thin-Layer Chromatography (TLC)

Analytical

Technique : one way, ascending

Adsorbent : silica gel G (E.Merck) 30 gm/60 ml of distilled water

Plate size : 10 cm x 20 cm and 20 cm x 20 cm

Layer thickness : 250 μ

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Activation : air dried for 15 minutes and then at 110°C for 1 hour

Solvent systems : A) silica gel G/ethyl acetate : benzene (1:1)

B) silica gel G/chloroform : acetone (8:2)

C) silica gel G/benzene : acetone (8:2)

d) silica gel G/ethyl acetate : acetone (9:1)

Distance : 15 cm

Laboratory temperature

: 24°C-30°C

Detection on chromatographic plate

: spray reagent (299)

2% methanolic solution of resorcin mixed with 2% sulfuric acid solution (1:1)

: colors developed

plates after spraying were warmed in hot air oven at 110°C for 2-4 minutes

2. Column Chromatography

Technique : flash chromatography (300)

Adsorbent : silica gel 0.040-0.063 mm (E.Merck)

Sample loading : the sample is applied by pipette as a 20-25% solution in the eluent to the top of the adsorbent bed

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Solvents : A) ethyl acetate : petroleum ether (7:3)

B) chloroform : petroleum ether (1:1)

C) chloroform

D) chloroform : acetone (8:2)

3. Spectroscopy

3.1 Ultraviolet (UV) Absorption Spectra

Varian DMS 90 spectrophotometer

3.2 Infrared (IR) Absorption Spectra

Perkin-Elmer Model 1330 or 180 spectrophotometer

3.3 Nuclear Magnetic Resonance (NMR) Spectra

^{1}H and ^{13}C nmr spectra were recorded on a Bruker WH 400 spectrometer with TMS (δ=0) as internal standard and with solvents as indicated.

3.4 Mass Spectra

Varian MAT CH 7 or VG Micromass 7070 F spectrometer

4. Physical constants

4.1 Optical rotations

Bendix-NPL automatic polarimeter

4.2 Melting points

Gallenkamp melting point apparatus

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Extraction and Purification

The fresh bark of *Paramichelia baillonii* (Pierre) Hu (3 kg) was blended with 95% ethanol, macerated twice over a period of 3 days (10 and 5 liters) and then filtered. The filtrate was concentrated under reduced pressure to give a residue which was treated with water (5 liters), followed by extraction with chloroform (3 times 2 liters). The combined organic extract was dried (anhydrous Na_2SO_4) and removal of the solvent gave a residue (8.59 g) which was chromatographed on a silica gel column (8 x 15 cm).

The product were eluted with a ethyl acetate : petroleum ether (7:3) solvent system and 25 ml fractions were collected. Fractions 67-126 afforded a crude mixture (0.52 g), which was further purified using the following sequence of solvents and collecting 25 ml fractions: (a) $CHCl_3$: petrol (1:1), 25 fractions, (b) $CHCl_3$, 10 fraction, and $CHCl_3$: acetone (8:2), 15 fractions. Fractions 11-12 gave 85 mg of PB-1, fractions 28-36 gave 136 mg of PB-3 and fractions 49-50 gave 97 mg of a mixture which is presently under investigation.

After increasing the polarity of the solvent in the large 8 x 15 cm column to 100 % ethyl acetate, fractions 152-186 afforded a crude yellow residue (0.89 g) which was further purifued by chromatography with $CHCl_3$ to give a yellow powder (92 mg).

Purification of a portion of this powder (13 mg) was performed by flash chromatography using 2% methanol in benzene giving PB-2 (3.6 mg) followed by PB-4 (8 mg). The large silica gel column was finally eluted with methanol to give a residue (6.64 g) which was not further investigated.

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<u>Characterization and Identification of the Individual Components</u>.

The chemical and physical characteristic properties of the individual components are following described.

PB-1

$[\alpha]_D^{26}$ -62° ($CHCl_3$)

ir ν_{max} (CCl_4) 1775, 1650, 1450, 980 cm^{-1}

^{1}H and ^{13}C nmr, see Table 3 and 4, respectively

eims, *m/z* (rel. int.) 250 (M^{+},2), 232 (3), 203(4), 192(13), 133 (19), 119 (32).

From the above data, PB-1 was identified as a sesquiterpene lactone. It is in complete agreement with published value of (-)-dihydroparthenolide (2,148). Therefore it is concluded that PB-1 is (-) - dihydroparthenolide, the structure of which is shown below.

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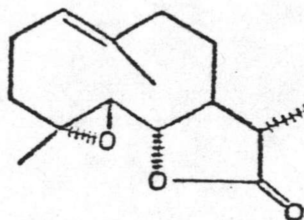

(-)-dihydroparthenolide

PB-2

$[\alpha]^{20}_{D}$ -78^{0} ($CHCl_3$)

ir ν_{max} (CCl_4) 3020, 2920, 1770, 1650, 1281, 1260, 1130, 940 cm^{-1}

^{1}H and ^{13}C nmr, see Table 3 and 4 respectively

eims, *m/z* (rel. int.) 248 (M^+,2), 230 (9), 191 (25), 190 (61), 119(100).

The spectral data of this compound are in complete agreement with the structure of (-)-parthenolide (311-313). Therefore, it is concluded that PB-2 is (-)-parthenolide and the structure of which is shown below .

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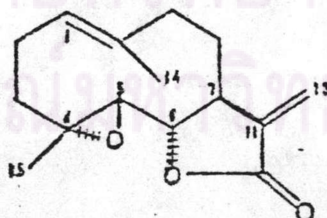

(-)-parthenolide

PB-3

m.p. 100-103° C

$[\alpha]^{20}_{D}$ - 112° ($CHCl_3$)

ir ν_{max} (CCl_4) 3365, 3020, 2920, 1770, 1480, 1215, 1175, 1000, 940 cm^{-1}

^{1}H and ^{13}C nmr, see Table 3 and 4 respectively

eims, *m/z* (rel. int.) 513 (M^{+}, 8), 278 (100), 264 (14)

hrms (composition interpret., calcd. millimass) 513.3077 ($C_{30}H_{43}NO_6$, M^{+}, 513.3090), 278.1752 ($C_{16}H_{24}NO_3$, M - $C_{14}H_{19}O_3$,278.1756), 264.1600 ($C_{16}H_{22}NO_3$, M - $C_{15}H_{21}O_3$, 264.1594).

The results were concluded for the structure of PB-3 as a novel sesquiterpene lactone and the chemical structure was assigned as $C_{30}H_{43}NO_6$ and was named (-)-bis-parthenolidine, the structure of which is shown below.

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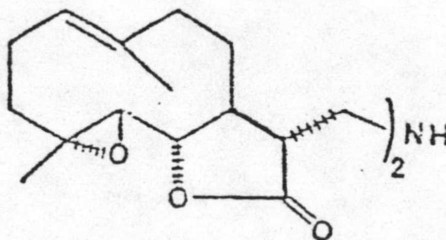


(-)-bisparthenolidine

PB-4

m.p. 278-281° C

ir ν_{max} (CH_2Cl_2) 3040, 2920, 1655, 1590, 1480, 1462, 1438, 1410, 1300, 1220, 1200, 1050, 1010, 965, 890, 865 cm^{-1}

uv λ_{max} (EtOH) 250, 270, 310, 400 (sh), 416 nm

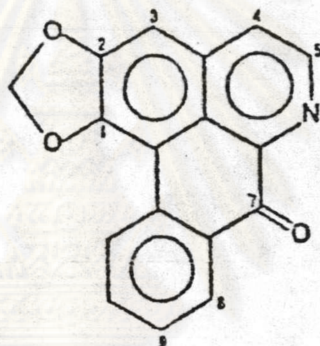
λ_{max} (0.1 N HCl in EtOH) 260, 282, 320, 396,452 nm

eims *m/z* (rel. int.) 275 (M^+, 80), 247 (14), 246 (10).

^1H nmr

Proton	10% DMSO-d_6:$CDCl_3$	$CDCl_3$
3	7.21 (s)	7.17 (s)
4	7.83 (br s)	7.74 (br s)
5	8.90 (br s)	8.80 (br s)
8	8.57 (d, *J*=8.0 Hz)	8.58 (d, 8.1)
9	7.58 (t, *J*=8.0 Hz)	7.57 (t, 8.0)
10	7.77 (t, *J*=8.0 Hz)	7.73 (t, 8.0)
11	8.72 (d, *J*=8.0 Hz)	8.61 (d, 8.1)
OCH_2O	6.41 (s)	6.37 (s)

These data are in agreement with the published values of liriodenine (317-322). Therefore, it is concluded that PB-4 is liriodenine. The structure of which is shown next page.



liriodenine

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Table III ^{1}H NMR Spectra of PB-1, PB-2, PB-3[a]

Hydrogen	PB-1	PB-2	PB-3
1	5.15(dd,2.3,11.9)	5.21(dd,br,4.0, 12.2)	5.27(dd,2.2,9.8)
2 α	2.11(dddd,2.3, 6.0,13.0,13.0)	2.09-2.24(m)[b]	2.26(dd,6.0,12.1)
2 β	2.37(dddd,5.0 11.9,13.0,13.0)	2.46(ddd,13.8, 12.2,12.5)	2.40(m)
3 α	1.21(ddd,6.0, 13.0,13.0)	1.25(m)	1.23(dt,5.9,13.9)
3 β	2.16(m)	2.09-2.24(m)[b]	1.88(dd,5.9,14.6)
5	2.69(d,9.0)	2.79(8.9)	2.74(d,8.8)
6	3.80(dd,8.4,9.0)	3.86(dd,8.9,8.3)	3.86(t,8.8)
7	2.28(m)	2.78(m)	2.40(m)
8 α	2.28(m)	2.09-2.24(m)[b]	2.18(m)
8 β	1.80(m)	1.73(m)	1.70(m)
9 α	1.80(m)	2.09-2.24(m)	2.10-2.18
9 β	2.25(m)	2.38(m)	
11β	2.27(dq,6.8,10.3)	-	2.40(m)
13a	1.25(d,6.8,CH_3)[c]	6.34(d,3.6)	3.15(dd,2.8,13.1)
13b		5.62(d,3.1)	2.92(dd,2.8,13.1)
14	1.68(s)	1.72(s)	1.67(s)
15	1.27(s)[c]	1.31(s)	1.30(s)

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[a] Chemical shifts are in ppm from TMS, coupling constants are in parenthesis in Hertz and the samples were disolved in $CDCl_3$

[b] Specific assignment possible at 400 MHz with 2D - COSY and decoupling experiments; $H_{2\alpha}$, 2.38 (dd, 5.1, 13.1); $H_{3\beta}$, 2.17 (m); $H_{8\alpha}$, 1.72 (m).

[c] In C_6D_6 solvent the C-13 and C-15 methyls were clearly resolved into a doublet at δ 1.05 and a singlet at 0.28, respectively.

Table IV ^{13}C NMR Spectra of PB-1, PB-2 and PB-3[a]

carbon	PB-1	PB-2[c]	PB-3
1	125.1(-)	125.3	125.3(-)
2	24.0(+)[b]	24.2[b]	24.2(+)[b]
3	36.6(+)[b]	36.2[b]	36.5(+)[b]
4	61.4(+)	61.5	61.6(+)
5	66.3(-)	66.4	66.1(-)
6	82.1(-)	82.5	82.3(-)
7	51.9(-)	47.7	49.0(-)
8	29.7(+)[b]	41.2[b]	30.2(+)[b]
9	41.1(+)	30.2[b]	40.9(+)
10	134.4(+)	134.7	134.3(+)
11	42.4(-)	139.5	45.5(-)
12	179.6(+)	169.3	176.7(+)
13	13.2(-)	121.0	46.2(+)
14	17.1(-)	17.3	17.2(-)
15	16.8(-)	17.0	16.8(-)

[a] Chemical shifts are in ppm from TMS, solvent was $CDCl_3$, (+) and (-) are signed from the attached proton test (APT).

[b] Assignments may be interchanged.

[c] Data taken from reference (310).

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<u>Cytotoxic Activity Test</u>

Cell Culture Screen, KB

KB (Eagle) cell culture, a cell line derived from a human carcinoma of the floor of mouth has been used by the National Cancer Institute (NCI) of U.S.A. as an antitumor assay for screening plants extracts since 1960. KB is the *in vitro* system of choice which is more sensitive to most antitumor agents than *in vivo* assays. It is also less expensive and requires less test material and time. KB (Eagle) cell culture has played a powerful role in discovery of antitumor agents from higher plants (301).

According to the known compounds, which obtained, had been investigated their cytotoxic activity. Therefore, this experiment investigates for crude extract and bisparthenolidine only.

1. Materials

KB Cell Line

The origin of KB cell line was derived from human epidermoid carcinoma of the floor of mouth (302,303).

The cell culture was provided by Virus Research Institute, Department of Medical Science, Ministry of Public Health, Thailand. It was propagated in 10% FBS in EMEM and cultivated in incubator (37°C) and subcultured twice a week.

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The KB was cultivated in culture flasks (Costar®) with surface area of 25 Sq.cm and capacity of 40 ml.

Medium

EMEM (Eagle's Minimum Essential Medium, GIBCO Laboratories, Grand Island, U.S.A.) was sterilized by Millipore filtration (Millipore Corporation, Bedford, Massachusetts; porosity = 0.22 micron) prior to addition of 100 unit /ml of Penicillin G Sodium (Dumex, Bangkok) and 100 mcg/ml of Streptomycin Sulfate (Dumex, Bangkok) and supplemented with 10% FBS (FBS, Gibco Laboratory, Grand Island, U.S.A.) freshly prepared.

Reagents

Acacia

Acacia is used as suspending agent for water non soluble test samples.

Alkaline Copper Solution

The preparation method has been described by Oyama V.I. and Eagle H (304).(see appendix I)

Bovine Serum Albumin

Albumin from bovine serum, lyophilized powder (Sigma Chemical Company, U.S.A.) was prepared at the concentration of 50 mcg/ml and kept frozen.

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Folin-Ciocalteau Reagent

(see appendix VI)

Hank's Balance Salt Solution (HBSS)

(see appendix II)

Phosphate Buffer Saline (PBS)

(see appendix III)

Positive Control Sample

5-Fluorouracil was used as positive control.

0.4% Trypan Blue Dye

This aqueous solution was made up by Phillips' method (see appendix IV).

0.25% Trypsin in Hepes-Buffered Saline (HBS)

This solution was prepared following to the method of Shipman C. et al (see appendix V).

2. **Methods**

Preparation of Cell Suspension

KB culture was cultivated in 10% FBS in EMEM. Stock, which cell content was appropriated, cells were refed 24 hours before testing.

The old medium of the stock culture was decanted before washing with 5 ml of HBSS then trypsinized with 3 ml of 0.25% trypsin in HEPES-buffered solution. Single cell

solution was obtained after adding 15 ml of 10% FBS in EMEM and pipetting. The cell suspension at the concentration of 4 x 10^4 cells/ml was obtained by viable count and diluting (157).

Preparation of Positive Control Sample

Fluorouracil Injection U.S.P. 250 mg in 5 ml (David Bull, Batch No. 2670) was diluted with sterile 1 % acacia in normal saline to the concentration of 4 x 2.5 mcg/ml.

Preparation of Test Solution

According to the samples are water insoluble. The test solutions were prepared immediately before testing by

1. dispersing in sterile 20% acacia in normal saline
2. diluting the suspensions with normal saline (pH=7) to
   - 10 mcg/ml of crude extract in 1% acacia
   - 4 mcg/ml of bisparthenolidine in 1% acacia
3. sterilizing by autoclave
4. diluting again with sterile technique (in laminar flow hood, aseptic room) for 3 doses of each test solution
   - 10 mcg/ml of crude extract in 1% acacia
   - 5 mcg/ml of crude extract in 1% acacia
   - 2.5 mcg/ml of crude extract in 1% acacia
   - 4 mcg/ml of bisparthenolidine in 1% acacia

- 2 mcg/ml of bisparthenolidine in 1% acacia
- 1 mcg/ml of bisparthenolidine in 1% acacia

Measurement of Cell Growth in Cell Culture

Measurement of cell growth was done by the method of Oyama V.I. and Eagle H.(156) which is a modification of the colorimetric method of Lowry, *et al.*(306), using a phenol reagent (Folin-Ciocalteau) for the development of color. (see appendix VI).

KB Cell *in vitro* Assay

The assay was scheduled as following ;

Day 0:- 4×10^4 cells/ml cell suspension was prepared as described in "Preparation of Cell Suspension"

- 1 ml of each samples was transfered into each cultured flasks. Two cultured flasks were added for each sample as duplicate. The samples were the following ;
  - 1 ml of NSS as negative control
  - 1 ml of 4x2.5 mcg/ml of 5-FU as positive control
  - 1 ml of 10.0 mcg/ml of crude extract
  - 1 ml of 5.0 mcg/ml of crude extract
  - 1 ml of 2.5 mcg/ml of crude extract
  - 1 ml of 4 mcg/ml of bisparthenolidine
  - 1 ml of 2 mcg/ml of bisparthenolidine
  - 1 ml of 1 mcg/ml of bisparthenolidine
- 3 ml of cell suspension were added into prepared cultured flasks (with 1 ml sample), simultaneously..

- All culture flasks with samples were incubated at 37°C until day 4.

- 2 samples of 3 ml cell suspension which were added nothing were centrifuged at 1974 x 3 g for 5 minutes, there after removing the media, the cells were washed with NSS and kept frozen until day 4. These 2 flasks were negative control at day 0 (baseline protein).

Day 4:- Cells in flasks were harvested after PBS washed and cell protein analysis were conducted according to the method of Oyama and Eagle. (see appendix VI)

The assay was repeated two times.

3. Result

Measurement of cell growth was determined through protein determinations according to the method of Oyama and Eagle. The KB screening demonstrated that ED_{50} of crude extract of *Paramichelia baillonii* is 1.95 mcg/ml and ED_{50} of Bisparthenolidine is 0.73 mcg/ml. (Calculation of two assays by Linear Regression Relationship)

O. Masaru *et al* (1978) found that ED_{50} of Parthenolide, Liriodenine and Dihydroparthenolide are 0.45 mcg/ml, 3.8 mcg/ml and inactive respectively (2).

Criteria of cytotoxic activity according to the NCI of U.S.A. is the followings (307).

synthetics : $ED_{50} \leq$ 4 mcg/ml

plant and animal extracts : $ED_{50} \leq$ 20 mcg/ml

Table V Cell protein analysis, showing the electrophotometric absorption at 660 nm and percent growth rate .

Absorption at 660 nm		Protein control				Day 0			Day 4			% Growth	Cell* Day 4 / Day 0
Exp.No.	Blank	1	2	3	average	1	2	Average	1	2	average		
1	0	0.098	0.093	0.096	0.096								
		Negative control (NSS)				0.012	0.034	0.023	0.176	0.217	0.196	100.00	8.5
		Positive control (5-FU 2.500 mcg/ml)							0.066	0.061	0.064	32.65	
		Test solution:											
		Crude extract;											
		2.500 mcg/ml							0.061	0.061	0.061	31.12	
		1.250 mcg/ml							0.136	0.137	0.136	69.39	
		0.625 mcg/ml							0.166	0.168	0.167	85.20	

* Control Growth Rate

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Table V Cell protein analysis, showing the electrophotometric absorption at 660 nm and percent growth rate. (continued)

Absorption at 660 nm		Protein control				Day 0			Day 4			% Growth	Cell* Day 4 / Day 0
Exp.No.	Blank	1	2	3	average	1	2	Average	1	2	average		
2	0	0.106	0.102	0.098	0.102								
		Negative control (NSS)				0.046	0.048	0.047	0.163	0.148	0.156	100.00	3.3
		Positive control							0.063	0.049	0.056	36.13	
		(5-FU 2.500 mcg/ml)											
		Test solutions:											
		Bisparthenolidine;											
		1.000 mcg/ml							0.045	0.048	0.046	29.68	
		0.500 mcg/ml							0.091	0.103	0.097	62.58	
		0.250 mcg/ml							0.104	0.096	1.000	64.52	

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* Control Growth Rate

Table V Cell protein analysis, showing the electrophotometric absorption at 660 nm and percent growth rate. (continued)

Absorption at 660 nm		Protein control				Day 0			Day 4			% Growth	Cell* Day 4 / Day 0
Exp.No.	Blank	1	2	3	average	1	2	Average	1	2	average		
3	0	0.112	0.122	0.112	0.115								
		Negative control (NSS)				0.036	0.034	0.035	0.182	0.216	0.199	100.00	5.7
		Positive control (5-FU 2.500 mcg/ml)							0.064	0.068	0.066	33.17	
		Test solution:											
		Crude extract;											
		2.500 mcg/ml							0.082	0.084	0.083	41.71	
		1.250 mcg/ml							0.134	0.136	0.135	67.84	
		0.625 mcg/ml							0.151	0.148	0.150	75.37	

* Control Growth Rate

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Table VI Cytotoxic activity of isolates of *P. baillonii*

compound	9 KB (mcg/ml)
Crude extract	1.95
Bisparthenolidine	0.73
Parthenolide	0.45#
Dihydroparthenolide	inactive#
Liriodenine	3.80#

\# = taken from reference (2)

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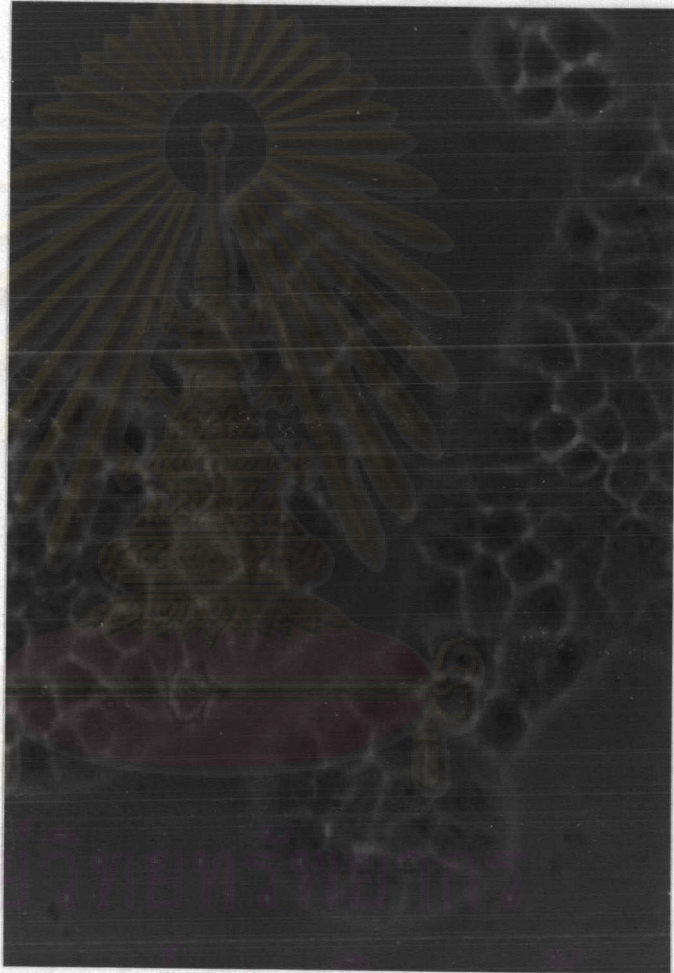

Figure 3.1 Microscopic appearance (100X) of 4-day culture of KB cell after normal saline addition. (negative control)

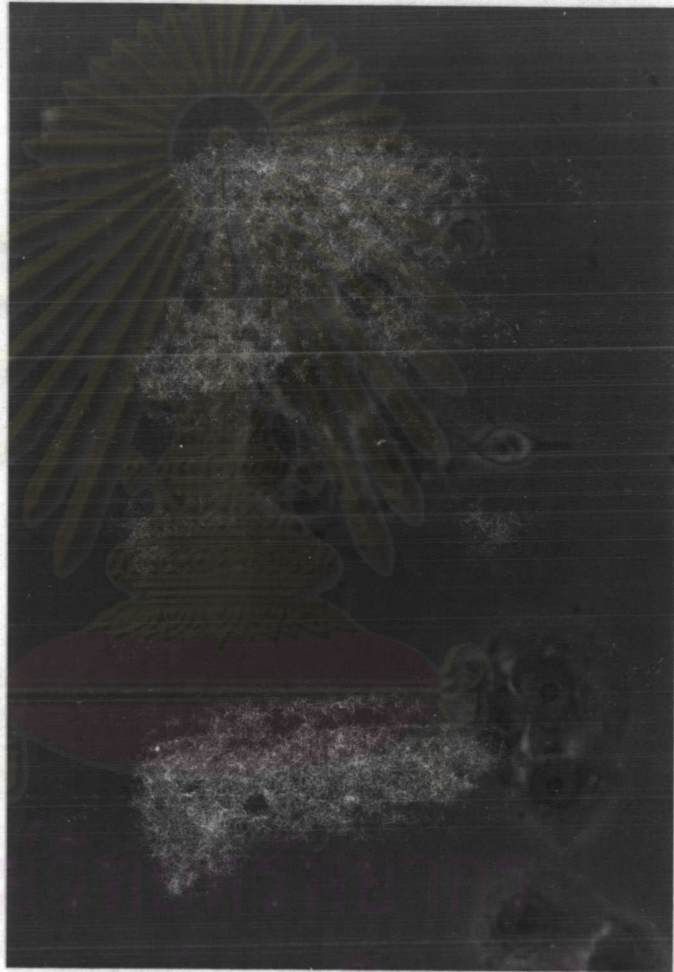

Figure 3.2 Microscopic appearance (100X) of 4-day culture of KB cell after 5-FU addition. (concentration 2.5 mcg/ml)(positive control)

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Figure 3.3 Microscopic appearance (100X) of a 4-day culture of KB cell after bisparthenolidine addition. (concentration=1.0 mcg/ml)