CHAPTER V

BIOLOGICAL ACTIVITY

5.1 Biological assay

5.1.1 Cytotoxicity test

Bioassay of cytotoxic activity against P 388 cell culture *in vitro* was performed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. [71-73] In principle, the viable cell number / well is directly proportional to the production of formazan, which following solubilization, can be measured spectrophotometrically.

P 388 cell line was harvested from exponential-phase maintenance cultures (T-75 cm^2 flask), counted by trypan blue exclusion, and dispensed within replicate 96-well culture plates in 100-μl volumes using a repeating pipette. Following a 24-h incubation at 37°C, 5% CO_2, 100% relative humidity, 100 μl of culture medium, culture medium containing sample was dispensed within appropriate wells (control group, N = 6; each sample treatment group, N = 3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N = 2) and medium / tetrazolium reagent blank (N = 6) "background" determinations. Culture plates were then incubated for 4 days prior to the additions of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT / ml PBS was sterile and filtered with 0.45 -μm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1 : 5 (v/v) in prewarmed standard culture medium. MTT working solution (50 μl) was added to each culture well resulting in 50 μg MTT/ 250 μl total medium volume) and cultures were incubated at 37°C for 4 to 24 h depending upon individual cell line requirements. Following incubation cell monolayers and formazan were inspected microscopically: Culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 μl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-

guage needle and replaced with 150 µl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Cell line growth and growth inhibition were expressed in terms of mean (± 1 SD) absorbance units and / or percentage of control absorbance (± 1 SD%) following subtraction of mean "background" absorbance.

Samples were also tested for cytotoxic activity towards 6 cell lines, which contain L929 (fibroblast), S102 (hepatoma), HEP-G2 (heptatoma), SW 620 (colon), chago (lung), Kato-3 (gastric) and BT 474 (breast) following the experimental method of bioassay of cytotoxic activity against 6 cell cultures *in vitro* were performed by MTT colorimetric method in the same manner as for P 388 cell line.

5.1.2 Inhibition of cyclic 3, 5−adenosinemonophosphate phosphodiesterase (cyclic AMP phosphodiesterase)

Inhibition of cyclic AMP phosphodiesterase was measured according to the method described by Chairungsrilerd, N. and co-worker [74]

Cyclic AMP phosphodiesterase activity was determined from the amount of inorganic phosphate liberated from adenosine 5-monophophate by 5-nucleotidase. It was determined by the malachite green method (which is highly sensitive to inorganic phosphate).

A sample (200 µg/ml, 500 µl) was incubated with 400 µl of the enzyme solution (50 mM of $MgCl_2$ 40 µl, 500 mM of Tris-HCl buffer, pH 7.40, 80 µl, 0.30 units/ ml of cAMP phosphodiesterase 50 µl, 13.4 units/ ml of 5-nucleotidase 50 µl, and DI water 180 µl) at 30 ^{O}C for 5 minutes. The reaction was started by adding of cyclic AMP (10 mM, 100 µl). Then 1.0 ml of reagent mixture (malachite green : PVA : ammonium molybdate : DI water = 2 : 1 : 1 : 2) was added and incubated at 30 ^{O}C for 5 minutes. The reaction was stopped with 200 µl of 25 % sodium citrate and left for 30 minutes at room temperature before recording the absorbance at 630 nm. A mixed reagent blank was used as reference. Potassium dihydrogen

phosphate solutions of known concentrations were used as a new solvent compound to determine the amount of phosphorus present in the assay. In the control experiment, dimethyl sulphoxide was added instead of the solution of the sample to minimize the effect of the vehicle solvent. Biological activity of samples was compared with theophylline (reference compound for phosphodiesterase assay). All reagents were prepared freshly and deionized water was used in making these reagents.

Unit of phosphodiesterase activity was expressed in terms of picomoles of cyclic AMP hydrolyzed per minute under the described conditions.

5.2 Result of biological activity test

5.2.1 Cytotoxic activity against P388 cell line

The *in vitro* activity of some compounds from *Croton oblongifolius* Roxb. against P 388 cell line was reported in Table 41.

Table 41 Cytotoxic activity against P 388 cell line of cembranoid compounds from *Croton oblongifolius* Roxb.

Compound	IC_{50} (μg / ml)*
1	> 100
2	41.74
3	6.48
5	42.49

* IC_{50} was the minimum concentration of 50 % inhibitory activity.

The results from Table 39 showed that, compound 3 markedly exhibited cytotoxic activity against P 388 cell line (IC_{50} 6.48 μg/ml).[15]

The *in vitro* activity of some compounds (10 μg/ml) from *Croton oblongifolius* against 6 cell lines, for example, S 102, Hep-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric), BT 474 (breast) cancer was reported in Table 42.

Table 42 Cytotoxic activity against cell line of some compounds from *C. oblongifolius.*

Compound	% Inhibition activity						
	L 929 (fibroblast)	S 102 (hepatoma)	Hep-G2 (hepatoma)	SW 620 (colon)	Chago (lung)	Kato-3 (gastric)	BT474 (breast)
1	18	17	29	4	7	20	3
1a	82	85	64	92	97	90	73
1c	18	5	29	20	38	50	45
2	27	0	29	4	3	10	5
2a	54	3	21	44	83	80	60
2b	45	67	43	32	13	70	9
3	54	25	63	92	88	90	54
4	18	58	29	8	0	30	16
6	36	78	93	97	18	94	89
6a	94	17	93	97	97	93	87
6b	27	83	43	12	41	30	9
6c	0	25	57	0	10	90	69
7	9	47	0	0	7	0	0
7a	82	28	79	36	21	83	71
9	9	38	14	0	0	30	0
11	9	0	21	12	27	30	16

All compounds showed cytotoxic activity against 6 cell lines. Moreover, compound 1a, 3, and 6a, which consisted of methyl ester and an aldehyde group, respectively, showed remarkable cytotoxic activity against all cell lines tested.

5.2.2 Inhibition of cyclic-AMP phosphodiesterase

In order to study and investigate the structure activity relationship. Some compounds from *Croton oblongifolius* Roxb. were tested for cyclic AMP phosphodiesterase inhibitory activity. The results are summarized in Table 43.

Table 43 Inhibitory activity of some compound from *Croton oblongifolius* Roxb. on cyclic AMP phosphodiesterase.

Sample	% Inhibition Activity (100 µg / ml)
Compound 1	44.51
Compound 1a	14.18
Compound 1b	14.80
Compound 1c	9.74
Compound 2	39.41
Compound 2a	9.00
Compound 2b	9.71
Compound 2c	7.46
Compound 5	44.87
Compound 5a	10.00
Compound 5b	11.92
Compound 5c	7.46
Compound 6	18.06
Compound 6a	3.01
Compound 6b	13.55
Compound 6c	5.45
Compound 7	42.47
Compound 7a	18.06
Compound 9	0.00
Compound 9a	0.00

The inhibition activity of cembranoid diterpene compound 1, compound 2, and compound 5 showed high inhibitory activity, whereas their derivatives showed lower inhibitory activity. From these results, it seems that configuration of the carboxylic acid group on these compounds could play a role in the cyclic AMP phosphodiesterase. However, their derivatives exhibited low activity to cyclic AMP phosphodiesterase.