CHAPTER TWO

BIOASSAY TECHNIQUES

2.1 The purpose of biological assays

A biological assay (or bioassay) is an experiment for estimating the nature, constitution or potency of a material by means of the reaction that follows its application to living matter. In other words, a biological assay is a measurement of the potency of any stimulus, physical, chemical or biological, by means of the reactions that it produces in living matter. A biological assay most commonly refers to the assessment of the potency of vitamins, hormones, toxicants and drugs of all types by means of responses produced when doses are given to experimental animals. Quantitative assays are similar to methods of physical measurement or of quantitative chemical analysis in that they lead to numerical assessments of some property of the material to be assayed.

The estimation of the potency of a natural product, such as a drug extracted from plant material, in producing a biological effect of a certain type, is often impossible or impracticable by chemical analysis. Even if the chemical constituents of the material has been determined, there may be little knowledge of the magnitude of the effect which the constituents will produce. This difficulty also occurs with many manufactured compounds such as insecticides made to precise chemical specifications but of unknown biological activity.

A typical assay involves a stimulus (for example, a vitamin, a hormone, a fungicide or an insecticide) applied to a subject (for example, an animal, a piece of

animal tissue, a plant or a bacterial culture). The size of the stimulus may be varied, generally up to the investigator. The size of the stimulus, that is the dosage given to the subject can be measured (perhaps a weight, a volume or a concentration). The response of the subject is a measurement of the final value of some characteristic of the subject (body weight, for instance) or of the change in a particular characteristic (increase in body weight, decrease in blood pressure). It may be a simple record of occurrence or non-occurrence of a phenomenon (death for instance). The magnitude or the frequency of the response depends upon the dosage applied. The relationship between dosage and response will be confused by random variations between replicate subjects. Nevertheless, the relation enables the potency of a dosage to be inferred from the responses it induces.

Biological assays are usually comparative, potency being estimated relative to a standard preparation of the stimulus. The standard preparation may be a sample of an internationally agreed standard or of a more readily available working standard whose potency relative to the international standard has been evaluated. The standard preparation may also be a stock maintained as a provisional laboratory standard. The potency of any test sample of the stimulus is assayed by finding the ratio between equivalent doses of it and the standard preparation, equivalence being interpreted as equality of the corresponding mean responses; experimentation with several different doses of one or both preparations is always needed to accomplish this satisfactorily.

2.2 A need to study bioactivities of natural products

The goal of many phytochemists is simply to isolate, characterise and publish novel natural structures. Nature's bioactive compounds are being ignored and are

being thrown down the drain by many phytochemists. Any commercial development of the compounds is difficult since these compounds were only isolated in minute amounts and biological usefulness is of very low priority to the phytochemists. Failures of phytochemists to adopt and use bioassays to direct their work is the main hindrance to the phytochemical discovery of useful bioactive compounds. McLaughlin has developed the application of "bench-top" bioassays to isolate and discover numerous bioactive compounds which have potential as pesticides, antitumour agents, herbicides and plant growth stimulants. These bioassays are: brine shrimp lethality (which is a bioassay indicative of cytotoxicity, various pharmacologic actions and pesticidal effects), the inhibition of crown gall tumours on potato disc (an antitumour bioassay) and monitoring of frond poliferation in Lemna or duckweed (a bioassay for herbicides and plant growth stimulants) (McLaughlin *et al.*, 1991).

Bioactive compounds are much more difficult to isolate, hence such research publications are fewer. However, such papers are more meaningful. Hence it is necessary to incorporate bioassays in natural product chemistry. Extracts need to be screened for biological activity, the active extracts selected, extracts fractionated and fractions bioassayed to identify active fractions. Hence, the bioactive compounds can be isolated and identified. The Third World nations have a wealth of unexplored forests which are fast disappearing due to logging and farming activities. It is hence necessary for phytochemists in this part of the world to learn to use inexpensive, simple bench-top bioassays whereby they can screen and select the bioactive fractions from their plant extracts.

Finally, as McLaughlin suggests scientists need to protect their novel, useful discoveries by patents which precede publications. Patents are needed to guarantee a potential financial return for the investments that are essential for product development.

2.3 Benchtop bioassays commonly used - a review

A number of simple rapid, reliable and inexpensive antitumour prescreens have been presented (see for example White, 1982; Otani *et al.*, 1984) to the NCI and they have considered several alternatives (see Table 2.1) (Suffness and Douros, 1982).

Table 2. 1: Some novel antitumour prescreens

Assay	Biological activities selected
Astrocytoma	Mitotic inhibition
Phage induction	DNA damage
Aminopeptidase B	Cell surface changes
Candida	Cell membrane activities, antifungals
Xanthomonas	Glycosylation inhibitors
Agrobacterium	Plasmid transfer tumour inhibition
Protein kinase	Inhibition of cell growth proteins
Topoisomerase	DNA conformation
Strand scission	DNA strand cleavage

Source: McLaughlin, (1991).

Of these prescreens, the one which offers any advantage of detecting a broad range of both known and novel antitumour effects is the inhibition of crown gall tumours in plants.

The following describes three simple, bench top bioassay techniques developed and adopted by McLaughlin *et al.*, (1991). The first is the brine shrimp

lethality test which detects a broad range of biological activities for a diversity of chemical structures. The second bioassay is the inhibition of the development of crown gall tumours on discs of potato tubers (potato disc assay). The third bioassay determines the effect of test materials on the growth of fronds of *Lemna minor* (duckweed). This test is to detect herbicides as well as plant growth stimulants.

2.3.1 Brine shrimp lethality test

The eggs of brine shrimp, *Artemia salina* (Leach) are available in pet shops or shrimp hatcheries. When placed in sea water, the eggs hatch within 48 hours to provide large numbers of larvae called nauplii for bioassay use. Natural product extracts, fractions or pure compounds are tested at initial concentrations of 10, 100 and 1000 ppm (µg/ml) in vials containing 5 ml of brine and ten shrimps in each of three replicates. Mortality is recorded after 24 hours. These data are analysed using the probit analysis method described by Finney to determine the estimated LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies (Meyer *et al.*, 1982).

2.3.2 The Potato disc bioassay (inhibition of crown gall tumours)

Crown gall is a neoplastic disease of plants induced by specific strains of the Gram negative bacterium *Agrobacterium tumefaciens* (Lippincott and Lippincott, 1975). These bacteria contain tumour-inducing plasmids which carry genetic information (T-DNA) that transforms normal, wounded plant cells into tumour cells (Zaener *et al.*, 1974; Watson *et al.*, 1975). Galsky demonstrated that inhibition of crown gall tumour initiation on potato discs showed good correlation with compounds and plant extracts known to be active in the 3PS (*in vivo*, mouse leukaemia) antitumour assay (Galsky *et al.*, 1980). This inhibition of the growth of the tumours,

in addition to the inhibition of tumour initiation correlates well with 3PS activity (Galsky *et al.*, 1981). Active samples do not affect bacterial viability when added to the bacterial growth medium; thus the antitumour effect with crown gall is independent of antibiosis.

A modified inhibition of crown gall bioassay was performed in McLaughlin's laboratory on a series of active natural compounds, plant extracts and ethanol or hexane seed extracts of 41 Euphorbiaceae species (Ferrigni *et al.*, 1982). Statistical relationships were assessed by (1) four-fold tables; (2) the Fisher-Irwin test for significance of association and (3) calculation of Kappa values to indicate the degree of data agreement. The modified procedures are summarised below (Ferrigni *et al*, 1982).

A. Preparation of *Agrobacterium tumefaciens* (two days before assays).

Prepare the growth medium by adding 0.5 g sucrose, 0.8 g nutrient broth (Difco) and 0.1 g yeast extract (Difco) to 100 ml water in a 250 ml flask. Plug the flask with cotton, cover with aluminium foil and sterilise in autoclave for 12 minutes. Allow the medium to cool and add 1 loop of *Agrobacterium tumefaciens* using sterile technique. Place the flask on a shaker for 48 hours below 30 ^{o}C. (The strain of *A. tumefaciens* used must carry a tumour inducing plasmid).

B. Procedure for bioassay

1. Prepare 1.5% agar by adding 100 ml water per sample to 1.5 g bacto-agar (Difco) for each sample including control.

2. Sterilise by autoclave for 15 minutes items that are needed for the experiment like aluminium foil, tweezers, cutter, cork borer (1.8 cm, size

13), tray, 1 tube rack with 1 screw cap tube per sample including control containing 2 ml water per tube.

3. Wash potatoes (red-skinned) with water and soak in chlorox.

4. Clean Laminar flow hood with ethanol.

5. Pour 20 ml sterilised agar solution per petri dish and let it cool.

6. Dissolve 4 mg sample in 1 ml DMSO.

7. Use 1 ml of DMSO as blank standard.

8. Preparation of inoculum.

   - add 1.5 ml water, 2.0 ml bacteria and 0.5 ml sample to tube
   - prepare standard by replacing sample with 0.5 ml DMSO.
   - use sterile technique (frame and cap)

9. Take potato out of bleach and bore out cylinders into a tray.

10. Cut cylinders into discs and place 5 discs per petri dish.

11. Prepare at least 3 petri dishes per sample and control.

12. Add one drop (0.05 ml) inoculum per disc.

13. Wrap each dish with parafilm strips.

14. Keep in dark at 27 ^{o}C and count the tumours after 12 to 21 days.

15. Calculate percent inhibition of crown gall tumours.

$$\% \text{ inhibition} = 100 - \frac{\text{Ave \# tumours per disc of sample}}{\text{Ave \# tumours per disc of control}} \times 100$$

With these modified procedures, crown gall tumours on potato discs could routinely be employed as comparatively rapid, inexpensive, safe and statistically reliable prescreens for 3PS antitumour activity.

2.3.3 *Lemna minor* (duckweed): A bioassay for inhibitors and promotors of plant growth (McLaughlin *et al.*, 1991)

Lemna minor (duckweed) is a miniature aquatic monocot. Single *Lemna* plants, consisting of three fronds (1 mother and 2 daughter fronds) are placed into 2 dram vials containing 2 ml of a special medium (E. medium). By evaporation of volatile solvents, appropriate dilutions of test substances have been previously evaporated in the vials to deliver initial concentrations of 500, 50 and 5 ppm in the medium. The vials are placed in translucent, glass-covered dishes and placed in a plant growth chamber at 27-29 ^{o}C for 24 hours of fluorescent and incandescent light. After seven days, the number of fronds are counted, and FI_{50} values (conc. necessary to inhibit 50% of frond poliferation) or FP_{50} values (conc. causing 50% increase in proliferation of fronds) are determined using a Finney program on an IBM personal computer, 95% confidence intervals are also determined to provide statistical relevance.

Using the *Lemna* bioassay, McLaughlin and co-workers (1991) observed that natural antitumour compounds can inhibit *Lemna* growth. This bioassay technique can be a simple screening method in the search for biodegradable herbicides. This method is probably useful in the detection of new plant growth stimulants. However, the *Lemna* bioassay method is not good for predicting antitumour activity.

2.3.4 The susceptibility or resistance of mosquito larvae to insecticides tests (WHO/VBC/81.807)

This susceptibility test is a simple bench top bioassay against mosquito larvae namely the *Aedes aegypti* (Dengue fever mosquito larvae), the *Culex quinquefasciatus*, and the *Anopheles maculatus*. This test involves suspending the larvae in aqueous solutions containing various concentrations of the test solutions and determining the mortality after 24 hours.

A. Establishing the base-line

Batches of mosquito larvae are exposed to different concentrations of the plant extract/compound and the mortality at each level is determined. A preliminary test is usually made on a wide range of concentrations using the standard exposure of 24 hours. This will indicate the general level of susceptibility. Further tests are then made with at least 4 concentrations, some of which will give partial mortality. Four replicate tests at each concentration should then be made and from the results a log-probit regression line constructed. The line should be straight if the population is homogeneous and if so, the concentration expected to produce 99.9% mortality can be extrapolated from it.

B. Procedure for carrying out bioassays

Larvae (about 300 individuals) should be in their third or early fourth instar. Any larvae having abnormalities, for example, a fuzzy appearance due to the presence of parasites on the body surface should be discarded. Lots of 20-25 larvae are distributed in each of 120 ml small beakers, each containing 25 ml of water. Their transfer is effected by means of a dropper.

Into each of 12 glass vessels, approximately 7.5-10.0 cm in diameter (jars, bowls or 500 ml beakers) place 225 ml of water. The vessels should be such that the depth of water is between 2.5 and 7.5 cm. Distilled water, rain water or tap water may be used. Even water obtained from a well or stream which is free from chlorine or organic contaminants may be used. It should be noted that distilled water obtained commercially may contain traces of poisonous heavy metals and this will give high mortalities in the controls. The average temperature of the water should be approximately 25 ^{o}C and it must not be below 20 ^{o}C or above 30 ^{o}C.

Prepare the test concentrations by pipetting 1 ml of the appropriate standard insecticide solution just above the surface of the water in each of the glass vessels and stirring vigorously for 30 seconds with a glass rod. In preparing a series of concentrations, the most dilute should be prepared first. There should be two replicates. The two controls should be prepared by the addition of 1 ml of the alcohol to the water in each container. To obtain intermediate concentrations, pipette 0.5 ml of any standard solution instead of 1 ml.

Within 15-30 minutes of the preparation of the test concentrations, add the mosquito larvae to them by tipping the contents of the small beakers into the vessels.

After a period of 24 hours, make mortality counts. In recording the percentage mortalities for each concentration the moribund and dead larvae in both replicates should be combined. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region. Moribund larvae are

those incapable of rising to the surface (within a reasonable period of time) or of showing the characteristic diving reaction when the water is disturbed.

Discard the larvae that have pupated during the test. If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded. Tests with a control mortality of 20% or more are unsatisfactory and should be repeated.

When four replicates have been performed with the same population of mosquito larvae, adequate data should be available for constructing a base-line susceptibility. The results should be recorded.

C. Results

To construct the dosage-mortality regression line the results obtained should be plotted on the logarithmic-probability paper provided. The regression line may be fitted by eye and the concentrations expected to kill various percentages can be read from it. The concentration to kill 50% is known as LC_{50}; that for 95% kill as LC_{95} etc. The curve can be extended to estimate the $LC_{99.9}$ (though it must be realized that this is very approximate).

If the control mortality is between 5% and 20%, the percentage mortalities should be corrected by Abbott's formula:

$$\frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

D. Estimation of the median effective dose

The median effective dose is the dose that will produce a response (other than death) in half the population. The median effective dose is referred to as the ED_{50}, the median lethal dose as the LD_{50}; ED_{90} is the dose which causes 90% to respond.

Below is an example of the outcome of an experiment on different doses of an insecticide applied under standardised conditions to samples of an insect species.

Table 2. 2: A test of the toxicity of rotenone to *Macrosiphoniella sanborni*

Dose of rotenone (mg/l)	No. of insects (n)	No. affected (r)	% kill (p)
10.2	50	44	88
7.7	49	42	86
5.1	46	24	52
3.8	48	16	33
2.6	50	6	12
0	49	0	0

Source: Finney, (1971).

The table shows one such set of results in which the percentage of insects dead or seriously affected in each dose group has been entered alongside the raw data. An estimate of the ED_{50}, the dose that an average would produce 50% response is calculated.

When the experimental data on the relation between dose and mortality have been obtained, either a graphical or an arithmetical process can be used to estimate the parameters. Both employ the probit transformation. The graphical approach is rapid and sufficiently good for many purposes. The percentage response observed for each dose should first be calculated and converted to probits by means of Table 2.4 which transforms percentages to probits. The probits are then plotted against the dose

metameter, and a straight line is drawn to fit the points as satisfactorily as possible. In drawing the line and judging its agreement with the data, only the vertical deviations of the points must be considered; the line must be so placed that the differences between the probit values which are plotted and the probits given by the line of each are as small as possible. Very extreme probit, say outside the range 2.5 to 7.5, carry little weight and may be disregarded unless many more subjects were used than in the batches giving intermediate probit values. For many carefully conducted experiments, however, the empirical probits lie so close to a straight line that the provisional graphical line is good enough. The log ED_{50} is estimated from the line as m, the dose at which Y = 5. The slope of the line b, is an estimate of $1/\sigma$ and is obtained as the increase in Y for a unit increase in X.

$$Y = 5 + 1/\sigma(X - \mu) \qquad (1)$$

These two estimates are then substituted for the parameters in equation (1) to give the estimated relation between dose and response. To test whether the line is an adequate representation of the data, a χ^2 (chi-square) test may be used. A value of χ^2 within the limits of random variation indicates satisfactory agreement between theory (the line) and observation (the data). A significantly large χ^2 may arise either because individual test subejcts do not react independently or because the straight line does not adequately describe the relation between dose and probit. Below is an example of fitting a probit regression by eye to the results of an insecticidal test.

E. Fitting a probit regression line

The results in Table 2.2 is referred. The number affected is the total of insects apparently dead, moribund or so badly affected as to be unable to walk more than a few steps.

Table 2.3 summarises the dose metameter, percentage kill and empirical probit values for the experiment.

Table 2. 3: Empirical probits and approximate expected probits for data of Table 2.2

Log dose (x)	No. of insects (n)	% kill (p)	Empirical probit	Expected probit (Y)
1.01	50	88	6.18	6.30
0.89	49	86	6.08	5.83
0.71	46	52	5.05	5.10
0.58	48	33	4.56	4.58
0.41	50	12	3.82	3.90

Source: Finney, (1971).

Over the range of concentrations tested, the Sigmoid nature of the relation between percentage kill and log concentration is not very apparent. The percentages are plotted against the dose in Figure 2.1 together with the normal Sigmoid curve fitted to them by the present analysis. Between 25% and 75% this curve is practically indistinguishable from a straight line; a line drawn to fit the five points would give x = 0.68 as approximately corresponding to 50% kill, in good agreement with the value obtained later for the log LD_{50}.

The probits of p read from Table 2.4, have been entered in Table 2.3. When plotted against dose they lie nearly on a straight line (see Figure 2.2). From this line,

probits corresponding to many different values of x have been read, converted back to percentages by using Table 2.4 inversely and plotted against x in Figure 2.1 to give a Sigmoid curve. This plot facilitates prediction of either the mortality to be expected at any given dose or the dose which will kill, on average, a stated percentage of insects. In practice, the sigmoid seldom need to be constructed, as all predictions can be made directly from the probit diagram. For example, in Figure 2.2 a probit value of 5.0 corresponds on the x-scale to a dose m = 0.687; this is the estimate of log LD_{50} whence the LD_{50} is estimated as a concentration of 4.86 mg/l. Similarly, since the probit of 90% is 6.28, the log LD_{90} can be read from Fig. 2.2 as 1.0003, hence the LD_{90} is estimated as 10.1 mg/l.

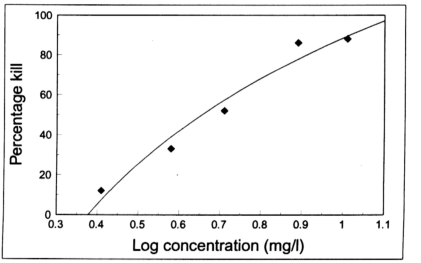


Figure 2. 1: Relation between percentage kill of *Macrosiphoniella sanborni* and doses of rotenone, showing normal sigmoid curve represented by equation Y = 2.25 + 4.01x (Source: Finney, 1971: 30)

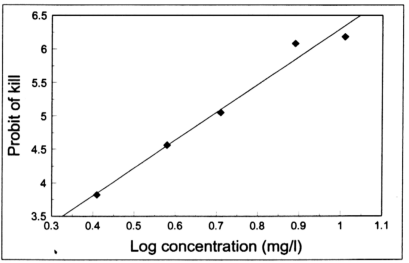


Figure 2. 2: Relation between probit of kill of *Macrosiphoniella sanborni* and dose of rotenone, showing probit regression line from equation Y = 2.25 + 4.01x (Source: Finney, 1971: 31)

Substitution of the actual values of x from the experiment gives the expected probits, the final column of Table 2.3. These are used in Table 2.5 to give the corresponding expected percentages, P; a probit of 6.30 in Table 2.4 falls between 90% and 91%, more exactly (90 + 2/6)%. Multiplication of each proportion by n, the number of insects tested, gives the expectation or expected number of responding insects; this is the average number that would respond in a batch of n insects. The numbers nP may then be compared with the observed r as a guide to the agreement of the experiment with theory. In the above example, only the second concentration tested shows appreciable discrepancy with three more insects being affected than the theory predicts. This is actually within the limits of random variation.

Table 2. 4: Transformation of percentages to probits

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.00	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Source: Finney, (1971: 25).

A test of significance of the discrepancies may be obtained by squaring each dividing the square by (1-P) and again dividing by the tabulated value nP. The sum of these quantities is, to a sufficiently close approximation if the line in Figure 2.2 has been well drawn, a χ^2.

Table 2. 5: Comparison of observed and expected mortality in eye estimation for rotenone - *Macrosiphoniella sanborni* test

x	Y	P	No. of insects (n)	No. affected		Discrepancy (r-nP)	$(r - nP)^2$ / nP(1 - P)
				observed (r)	expected (nP)		
1.01	6.30	90.3	50	44	45.2	-1.2	0.33
0.89	5.83	79.7	49	42	39.1	+2.9	1.06
0.71	5.10	54.0	46	24	24.8	-0.8	0.06
0.58	4.58	33.7	48	16	16.2	-0.2	0.00
0.41	3.90	13.6	50	6	6.8	-0.8	0.11
							$\chi^2_{(3)} = 1.56$

Source: Finney, (1971).

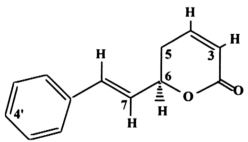
2.4 Bioactivities of the styrylpyrones from the genus *Goniothalamus*

Goniothalamin the first strylpyrone to be isolated from the genus *Goniothalamus* (Jewers *et al.*, 1972) and was shown to be the same as the compound isolated from *Crytocarya caloneura* (Hlubucek *et al.*, 1961) was claimed to be bioactive but no biological testing were carried out. Later, Talapatra reported four new styrylpyrones, goniodiol diacetate, goniodiol-7-monoacetate, goniodiol and goniotriol but there was no mention on biological activity (Talapatra *et al.*, 1985).

A little later, McLaughlin and co-workers reported the weakly 3PS active goniothalenol from *Goniothalamus giganteus*. The bioactivity was monitored using the brine shrimp lethality test. Goniothalamin was isolated alongside goniothalenol and was also active against the brine shrimp (El-Zayat *et al.*, 1985). Goniothalenol in the NCI human tumour cell panel, showed good cytotoxicity but little selectivity. It was active *in vivo* against 3PS adriamycin resistant leukemia. From the same plant was also isolated the third weakly active styrylpyrone goniotriol. This compound was previously isolated by Talapatra from the *Goniothalamus sesquipedalis* but was not completely characterised. This compound showed some activity in the brine shrimp lethality test and the potato disc assay but mild activity to human tumour cell (Alkofahi *et al.*, 1989). Sam and co-workers reported the embryotoxic compound goniothalamin epoxide (LD_{50}, i.p., 36 mg/kg and 16.2% abnormality). Again goniothalamin was mentioned and shown to be embroytoxic with a LD_{50} on mice, i.p., 76 mg/kg and 32.5% abnormality at a dose of 7 mg/25 g (Sam *et al.*, 1987). The McLaughlin group reported three additional styrylpyrone derivatives of moderate activity from *Goniothalamus giganteus*. They were all active against the brine shrimp

and cytotoxic to human tumour cells. These three compounds were 8-acetylgoniotriol, goniofufurone and goniopypyrone (Fang *et al.*, 1990). 7-epi-Goniofufurone and 5-deoxygoniopypyrone two new styryl lactones were also isolated from *Goniothalamus giganteus*; however they were not significantly bioactive to human tumour cells. Alongside these two mildly bioactive compounds were also reported the known goniodiol which showed significant and selective cytotoxicity against human lung tumour cells (A-549) (Fang *et al.*, 1991). Goniodiol-7-monoacetate was reported again in 1991 but this time from *Goniothalamus amuyon* by another group of scientists (Wu *et al.*, 1991) and shown to possess potent (ED_{50} values < 0.1 μg/ml) cytotoxicities against KB, P-388, RPM1 and TE671 tumour cells. The same group, a year later, reported the compound goniodiol-8-monoacetate which showed cytotoxicity against KB, P-388, A-549, HT-29 and HL-60 with ED_{50} values of 4.85, 1.68, 4.79, 3.99 and 1.85 μg ml^{-1}, respectively (Wu, 1992).

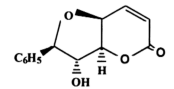
Figure 2. 3: Styrylpyrones and their biological activities (McLaughlin *et al.*, 1991).



(+)-Goniothalamin

Goniothalamin:

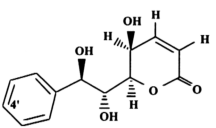
BS LC_{50} 10 ppm;
3PS T/C 124% at 80 mg kg^{-1};
9KB ED_{50} 3 x 10^{-1} μg ml^{-1}



(+)-Goniothalenol

(+)-Goniothalenol:

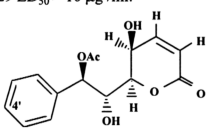
BS LC_{50} 234 ppm;
Potato disc 50-70% tumour inhibition;
9KB ED_{50} 2 μg ml^{-1};
3PS T/C 122%, 118% at 25 mg/kg;
3PS adriamycin resistance T/C 132% at 25 mg/kg;
Inhibits growth and germination of velvet leaf seeds (USDA);
NCI tumour panel av. molar log IC_{50} 5.5, delta 0.6, range 1.6;
Attenuates gastric ulceration.



Goniotriol

Goniotriol:

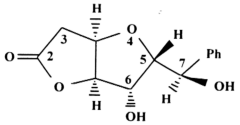
BS LC_{50} 269 ppm;
Potato disc 38% tumour inhibition;
A-549 ED_{50} < 10 μg/ml;
MCF-7 ED_{50} 5.9 μg /ml;
HT-29 ED_{50} > 10 μg /ml.



8-Acetylgoniotriol

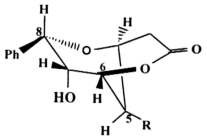
8-Acetylgoniotriol:

BS LC_{50} 99 ppm;
Potato disc 56% tumour inhibition;
A-549 ED_{50} 9.26 μg/ml;
MCF-7 ED_{50} 10 μg/ml;
HT-29 ED_{50} 4.70 μg/ml.



Goniofufurone

Goniofufurone:
BS LC_{50} 123 ppm;
Potato disc 14% tumour inhibition;
A-549 ED_{50} 4.76 µg/ml;
MCF-7 ED_{50} > 10 µg/ml;
HT-29 ED_{50} > 10 µg/ml.



(+)-Goniopypyrone, R = OH;
(+)-5-Deoxygoniopypyrone, R = H

Goniopypyrone, R = OH:
BS LC_{50} 12 ppm;
Potato disc 67% tumour inhibition;
A-549 ED_{50} 6.6 x 10^{-1} µg/ml;
MCF-7 ED_{50} 6.72 x 10^{-1} µg/ml;
HT-29 ED_{50} 6.78 x 10^{-1} µg/ml.

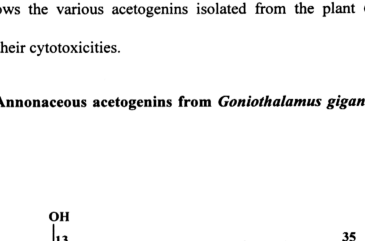
5-Deoxygoniopypyrone, R = H:
BS LC_{50} > 400 µg/ml;
A-549, MCF-7 and
HT-29 ED_{50} all > 10 µg/ml.

2.5 Bioactivities of acetogenins from the genus *Goniothalamus*

The bark extracts of the *Goniothalamus giganteus* was very toxic to mice during the P388 *in vivo* antileukemic screen, as well as brine shrimp. Definitely, the brine shrimp toxicity could not be explained by the moderately active styrylpyrones.

The presence of the very bioactive annonaceous acetogenins were the contributing factor to the high cytotoxicities of the bark extracts. Initially, two C-35, 4-hydroxylated mono-THF, linear acetogenins goniothalamicin and annonacin were isolated. Later, a series of other annonaceous acetogenins were isolated from the same plant. Goniothalamicin and annonacin show selective cytotoxic activities in the NCI human tumour panel. Gigantetronenin and gigantrionenin, both having a mono-THF ring with a double bond along the hydrocarbon chain show selective and potent cytotoxicities to human tumour cells in culture as well as toxicity to brine shrimp. Figure 2.4 shows the various acetogenins isolated from the plant *Goniothalamus giganteus* and their cytotoxicities.

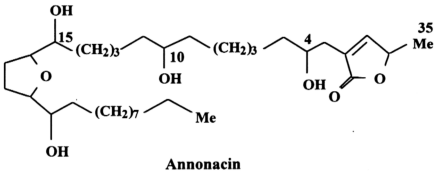
Figure 2. 4: Annonaceous acetogenins from *Goniothalamus giganteus* and their cytotoxicities.



Goniothalamicin

Goniothalamicin:

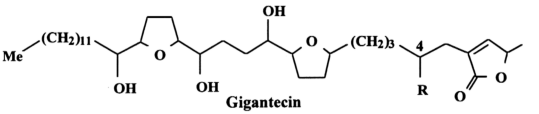
BS LC_{50} 37 ppm;
Potato disc 68% tumour inhibitor;
3PS inactive up to 2.5 mg/kg, toxic at higher doses;
9ASK inactive;
9KB $ED_{50} < 10^{-2}$ µg/ml;
9PS $ED_{50} < 10^{-1}$ µg/ml;
Selective in the NCl human panels av. molar log IC_{50} -0.9, delta 1.7, range 3.5;
Insecticidal and antimalarial.



Annonacin

Annonacin:

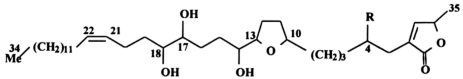
BS LC_{50} 3.3 ppm;
Potato disc 72% tumour inhibition;
3PS T/C 124% at 0.9 mg/kg;
9ASK 15.30% reversal at 100 µg/ml;
Insecticidal and antimalarial;
NCI human tumour panel av. IC_{50} - 0.75, delta 2, range 3.7.



Gigantecin

Gigantecin:

BS LC_{50} 222 ppm;
Potato disc 83% tumour inhibition;
9KB $ED_{50} < 10^{-5}$ µg/ml;
9PS $ED_{50} < 10^{-2}$ µg/ml;
A-549 ED_{50} 2.19×10^{-7} µg/ml;
MCF-7 ED_{50} 4.11×10^{-9} µg/ml;
HT-29 2.68×10^{-4} µg/ml;
9ASK 31-50% reversal at 10 µg/ml.



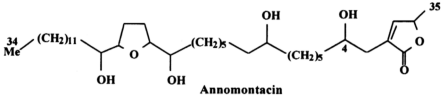
Gigantetronenin, R = OH;
Gigantrionenin, R = H

Gigantetronenin, R = OH:

BS LC_{50} 9.4 ppm;
A-549 ED_{50} 4.71 x 10^{-3} µg/ml;
MCF-7 ED_{50} 6.03 x 10^{-1} µg/ml;
HT-29 ED_{50} 5.37 x 10^{-2} µg/ml.

Gigantrionenin, R = H:

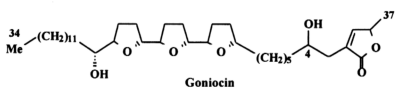
BS LC_{50} 13.9 ppm;
A-549 ED_{50} 3.94 x 10^{-3} µg/ml;
MCF-7 ED_{50} 8.06 µg/ml;
HT-29 ED_{50} 2.92 x 10^{-3} µg/ml.



Annomontacin

Annomontacin:

BS LC_{50} 12.5 ppm;
A-549 ED_{50} 7.72 x 10^{-3} µg/ml;
MCF-7 ED_{50} 1.65 x 10^{-1} µg/ml;
HT-29 ED_{50} 2.58x 10^{-3} µg/ml.



Goniocin

Goniocin:

BS LC_{50} 57 ppm;
A-549 ED_{50} 9.42 x 10^{-1} µg/ml;
MCF-7 ED_{50} 4.85 µg/ml;
HT-29 human solid tumour cells 1.61 x 10^{-2} µg/ml.