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THE USE OF BIOLOGICAL ASSAYS TO EVALUATE BOTANICALS

JERRY L. MCLAUGHLIN, PHD, AND LINGLING L. ROGERS, MS
Department of Medicinal Chemistry and Molecular Pharmacology,
School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana
JON E. ANDERSON, PHD
Estee Lauder, Melville, New York

The Department of Medicinal Chemistry and Molecular Pharmacology's laboratory has adopted four "bench top" bioassays which do not require higher animals to screen and direct the fractionation of botanical extracts in drug discovery efforts. These are: 1. The brine shrimp lethality test (BST) (a general bioassay), 2. The inhibition of crown gall tumors on discs of potato tubers (an antitumor bioassay), 3. The inhibition of frond proliferation in duckweed (a bioassay for herbicides and plant growth stimulants), and 4. The yellow fever mosquito larvae lethality test (a bioassay for pesticides). The materials and procedures for those bioassays will be briefly described. The authors' results in applying these simple methods in the discovery of Annonaceous acetogenins will be presented to illustrate their successful use. The BST is especially suggested as an inexpensive, simple, and rapid means of standardization of bioactivity in heterogeneous botanical products.

Key Words: Brine shrimp; Crown gall tumors; Duckweed; Mosquito larvae; Annonaceous acetogenins; Standardization of botanicals

INTRODUCTION

BIOASSAYS OFFER A SPECIAL advantage in the standardization and quality control of heterogeneous botanical products. Such products can be "heterogeneous" due to the presence of mixtures of bioactive components either from the same or from purposefully mixed botanical sources. Physical analytical methods, such as chromatography, are useless for this purpose as they are usually insensitive to the chemical complexities found in crude botanical extracts. Most often a desired biological response is due to not one but a mixture of bioactive plant components, and the relative proportions of single bioactive compounds can vary from batch to batch while the bioactivity still remains within tolerable limits. Thus, physical or chemical analysis of a single component in such mixtures is not completely satisfactory. The authors have adopted four "bench top" bioassays to aid "drug discovery" work with botanicals. They have used these methods over the past 15 years, and they appear to be adaptable to the purpose of standardization or quality control of bioactive components in such heterogeneous botanicals.

Unfortunately, the goal of many phyto-

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Reprint address: Dr. J. L. McLaughlin, Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907–1333.

chemists has been simply to isolate, characterize, and publish a plethora of novel botanically-derived chemical substances without regard to bioactivities. To achieve applied meaning and significance, today's work in natural product chemistry must incorporate bioassays. Extracts must be screened for biological activity, the "active" extracts selected, fractionations directed with bioassays, and the bioactive compounds identified and then exploited.

This is the salvation of the natural product chemist, and such work must be performed with all useful bioactive botanicals if these products are to be accepted and incorporated into legitimate, long-term, health practices. Three readily available technologies must be combined:

1. Separation techniques (chromatography),
2. Structural elucidation methods (spectrometers and X-ray crystallography), and
3. Simple bioassays.

Today's natural product chemists are very familiar with the first two, but, generally, they ignore the third. It is the hope of the authors of this paper that talents can cease being wasted on the myriad of inactive natural metabolites and can become increasingly devoted to the identification of bioactive botanical products. The health use of heterogeneous standardized botanical products will become generally acceptable in the United States only after their bioactive components have been identified. Standardization of the products by biological assays will then generate reproducible benefits and the resulting consumer confidence.

A rush to very specific, *in vitro*, robotic, mechanism-based bioassays has occurred in recent years. Receptor binding, enzyme inhibition, affinity columns, DNA nicking, tubulin inhibition, and so forth usually mimic the effects of some previously known compound having a specific mechanism of action. Researchers must be cautious, however, with mechanism-based assays. The field of vision of such specific microscopes is very narrow; they must be assured that the scope of their bioassays can be wide enough to include diverse and unknown mechanisms as well as new chemical entities. In addition, in such specific bioassays the same extracts have to be analyzed many times, over and over again, before detecting activities. It would seem more logical to prescreen with general bioassays, throw out the negatives, and then employ specific bioassays on the actives. Unfortunately, this is not the trend today within the major pharmaceutical companies. The molecular pharmacologists have assured themselves of continued employment as each new specific bioassay that they devise must be employed to retest their firm's entire library of substances. Irrespective of whether general or specific bioassays are favored, the use of all bioassays must be promoted, and this promotion is the purpose of this paper. The four bioassays which are described below are easily adapted as "bench top" procedures for use in natural product chemistry. They are inexpensive, rapid, and technologically simple, requiring little technical training.

Brine Shrimp Lethality: A Rapid General Bioassay for Bioactive Compounds

Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose, and toxicology is simply pharmacology at a higher dose. Thus, *in vivo* lethality in a simple zoologic organism can be used as a convenient monitor for screening and fractionation in the discovery and monitoring of bioactive natural products. The eggs of brine shrimp, *Artemia salina* (Leach), are readily available in pet shops at low cost and remain viable for years in the dry state. Upon being placed in sea water, the eggs hatch within 48 hours to provide large numbers of larvae (nauplii) for experimental use.

Brine shrimp nauplii have been used previously in a number of bioassay systems, but the authors have developed a method whereby natural product extracts, fractions, or pure compounds are tested at initial concentrations of 10, 100, and 1000 ppm (or mcg/ml) in vials containing 5 ml of brine and

10 shrimp in each of three replicates (1,2). Survivors are counted after 24 hours. These data are processed in a simple program for probit analysis on a personal computer to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies. The authors initially found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity ($p = 0.036$ and kappa = 0.56), and now use the brine shrimp test as a prescreen for a panel of six human solid tumor cell lines at the Cell Culture Laboratory of the Purdue Cancer Center. The authors have observed that ED_{50} values for cytotoxicities are generally about one-tenth the LC_{50} values found in the brine shrimp test. Over 300 novel antitumor and pesticidal natural products have now been isolated in the laboratory at Purdue University using this bioassay as the prescreen (2,3,4).

Thus, it is possible to detect and then monitor the fractionation of cytotoxic, as well as 3PS (P388) (*in vivo* murine leukemia) active extracts using the brine shrimp lethality bioassay rather than more tedious and expensive *in vitro* and *in vivo* antitumor assays. The brine shrimp assay has advantages of being rapid (24 hours), inexpensive, and simple (eg, no aseptic techniques are required). It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2–20 mg or less). Furthermore, it does not require animal serum as is needed for cytotoxicities. Animal rights advocates have not yet objected to the use of these invertebrates in experimental work. In the authors' laboratories, each researcher conducts his/her own brine shrimp bioassays on his/her own bench. Self-reliance and rapid results are important advantages in this type of work. Table 1 and Figure 1 summarize the materials and procedures.

Crown Gall Tumors on Potato Discs: An Animal-Sparing Bioassay for Antitumor Compounds

Crown gall is a neoplastic disease of plants induced by specific strains of the Gram negative bacterium, *Agrobacterium tumefaciens* (Smith and Townsend) Conn. The bacteria contain large Ti (tumor-inducing) plasmids which carry genetic information (T-DNA) that transforms normal, wounded, plant cells into autonomous tumor cells. In 1980, Galsky et al. (5,6) at Bradley University demonstrated that the inhibition of crown gall tumors on discs of potato (*Solanum tuberosum* L.) tubers showed an apparent correlation with compounds and plant extracts known to be active in the 3PS (*in vivo*, murine leukemia) antitumor assay. In preliminary studies in 1980–1983 at Purdue, the authors modified the Galsky potato disc method and tested the effectiveness, with appropriate statistical evaluation, of the modified assay as a prescreen for 3PS activity in crude plant extracts. The modified assay was initially performed on a series of natural antitumor compounds, on plant extracts suspected to have 3PS activity, and on ethanol and hexane extracts of seeds of 41 Euphoriaceae species (7); inhibition of 20% or more of the tumors is considered significant.

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.

Surprisingly, the plant screening results of the potato disc assay were very strongly associated with the 3PS results ($p = 0.002$), while cytotoxicity assays, 9PS ($p = 0.114$) and 9KB ($p = 0.140$), were much less reliable as predictors of 3PS activity. The calculated Kappa values for potato disc, 9PS, and 9KB were 0.64, 0.24, and 0.45, respectively. Kappa values greater then 0.75 represent excellent agreement beyond chance; values less then 0.40 represent poor agreement; and values between 0.40 and 0.75 represent fair to good agreement. The combined results showed an even better correlation between the *in vivo* 3PS results and the potato disc method ($p = 2 \times 10^{-6}$).

The authors concluded that, with these modified procedures (Table 2), crown gall

TABLE 1
Materials and Procedures for Brine Shrimp Lethality Bioassay

MATERIALS

1. *Artemia salina* cysts (brine shrimp eggs from store). Most pet shops carry these as a type of fish food.
2. Sea salt (also from fish store).
3. Small tank (hatching chamber) to grow shrimp with dividing dam, cover, and lamp to attract shrimp (The hatching chamber can be conveniently made from a covered plastic soap dish).
4. Syringes: 5 ml, 0.5 ml, 100 mcl, and 10 mcl (smaller syringes are not needed if dilutions are prepared in the alternative dilution procedure as described in Figure 1).
5. Two dram vials (9 per sample + 1 control).

PROCEDURES

1. Prepare sea water according to directions on box (38 g sea salt per liter of water), filter.
2. Put sea water in small tank, add shrimp eggs to one side of the divided tank, and cover this side. The lamp above the other side will attract the hatched shrimp.
3. Allow two days for the shrimp to hatch and mature as nauplii (in warmer climates, hatching may take place sooner, but the authors use 48–72 hr nauplii to be consistent).
4. Prepare vials for testing; for each fraction, test initially at 1000, 100, and 10 mcg/ml; prepare three vials at each concentration for a total of nine vials; weigh 20 mg of sample and add 2 ml of solvent (20 mg/2 ml); from this solution transfer 500, 50, or 5 mcl to vials corresponding to 1000, 100, or 10 mcg/ml, respectively. Evaporate solvent under nitrogen and then put under high vacuum for about 30 min.; volatile solvents will evaporate over night. Alternatively, materials may be dissolved in DMSO (dimethylsulfoxide), and up to 50 mcl may be added per 5 ml of brine before DMSO toxicity will affect the results. An alternative dilution procedure is illustrated in Figure 1; this procedure avoids the need for the expensive 100 mcl and 10 mcl syringes.
5. After two days (when the shrimp larvae are ready), add about 4 ml of sea water to each vial, count 10 shrimp per vial (30 shrimp per dilution), and adjust the volume with sea water to 5 ml/vial. Place the vials, uncovered, under the lamp. Be sure that vials are not overheated by the lamp.
6. Twenty-four hours later count and record the number of survivors.
7. Analyze the data with the Finney computer program for probit analysis to determine LC_{50} values and 95% confidence intervals. A copy of this program for IBM PCs is available from Dr. McLaughlin; a $10.00 donation is requested to cover costs, postage, and handling.
8. Additional dilutions at less than 10 mcg/ml may be needed to determine the LC_{50} values for potent materials; also, intermediate concentrations, eg, at 750, 500, and 250 mcg/ml can be prepared and tested to narrow the confidence intervals. By starting with 2 mg/ml (step 4 above) dilutions at 100, 10, and 1 mcg/ml are easily prepared for more potent materials.

tumors on potato discs could routinely be employed as a comparatively rapid, inexpensive, safe, animal-sparing, and statistically reliable prescreen for *in vivo* 3PS antitumor activity. The methodology is simple, and, although aseptic technique is required, the bioassay can be performed in house with minimal technical training. The authors have since used this assay to detect and isolate several dozen novel, antitumor compounds from various plant species (2,3,4), and it continues to predict, effectively and reliably, *in vivo* animal antitumor activity although the 3PS bioassay has, itself, fallen into disfavor as being too predictive of "soft" versus "solid" antitumor compounds.

Frond Inhibition of *Lemna* (duckweed): A Bioassay for Plant Growth Stimulants and Inhibitors

Lemna minor L. (duckweed) is a miniature aquatic monocot. *Lemna* plants consist of a central oval frond or mother frond with two attached daughter fronds and a filamentious root. Under normal conditions, the plants re-

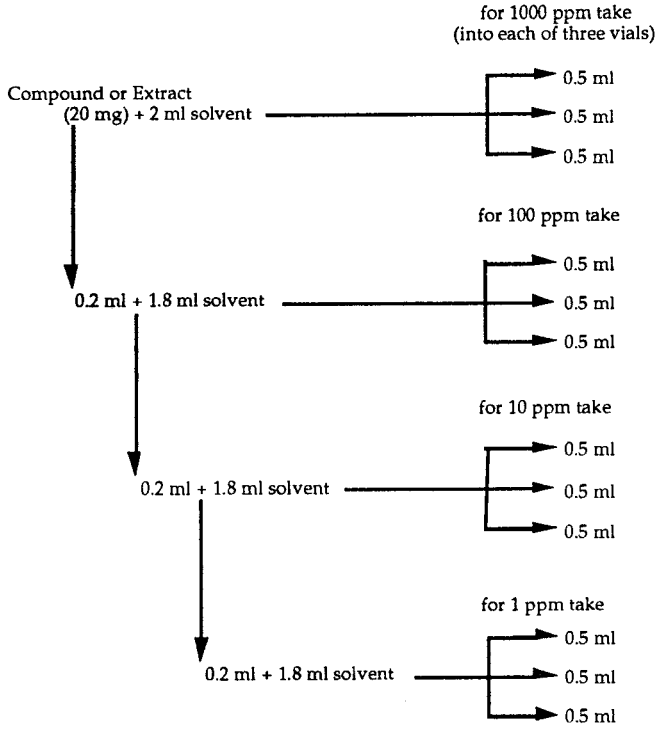

FIGURE 1. Flow chart for alternate dilution procedure for brine shrimp bioassay.

produce exponentially with buddings of daughter fronds from pouches on the sides of the mother fronds. A previous report by Einhellig et al. (8) provided general guidelines for developing a *Lemna* bioassay to screen a large number of plant extracts and chemical substances for their effects on plant growth.

Single *Lemna* plants, consisting of three fronds (1 mother and 2 daughter fronds), are placed into two 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, to avoid moisture loss, and placed in a plant growth chamber at 27–29°C with 24 hours of fluorescent and incandescent light. After seven days the number of fronds are counted, and FI_{50} values (concentrations necessary to inhibit 50% of frond proliferation) or FP_{50} values (concentrations causing 50% increase in proliferation of fronds) are determined using the Finney program on an IBM personal computer; 95% confidence intervals are also determined to provide statistical relevance.

The search for biodegradable herbicides may be extended to include natural compounds (allelochemicals), and this is a simple screen for such activity. In addition, the authors find that some natural substances stimulate frond proliferation, and the assay may be useful to detect new plant growth stimulants. Usnic acid was shown to be a potent herbicide ($FI_{50} = 0.91$ ppm). Hippuric acid was shown to be a potent ($FP_{50} = 125$ ppm) plant growth stimulant; this unexpected activity of this common urinary constituent had never before been documented. The commercial need for such natural, biodegradable, herbicides and plant growth stimulants may someday be filled with natural products detected by this simple and convenient *Lemna*

TABLE 2
The Potato Disc Bioassay Modified for Plant Extracts and Compounds

MATERIALS

A. Preparation of *Agrobacterium tumefaciens* (two days before assay): Prepare growth medium by adding 0.5 g sucrose, 0.8 g nutrient broth (Difco), and 0.1 g yeast extract (Difco) to 100 ml of water in a 250 ml flask. Plug the flask with cotton, cover with aluminum foil, and sterilize in an autoclave for 12 minutes. Allow the medium to cool and add 1 loop of *Agrobacterium tumefaciens*, from a storage culture on an agar slant, using sterile technique. Place the flask on a shaker for 48 hours below 30°C.

B. Items to prepare for experiment:
1. strips of parafilm
2. Four (4) mg of sample in a 2 dram (10 ml) vial
3. 1.5 g bacto-agar (Difco) for each sample including control
4. Cover with aluminum foil: tweezers, cutter, 1.8 mm cork borer (size 13), and tray
5. One (1) tube rack with 1 screw cap culture tube per sample including control
6. For each sample, 1 screw cap culture tube containing 2 ml distilled water
7. Red-skinned potatoes (*Solanum tuberosum*) and bleach (Chlorox™)
8. Ethanol for disinfection
9. Three (3) Petri dishes per sample and three (3) for the control

PROCEDURES

1. Prepare 1.5% agar by adding 100 ml water per sample to step B.3
2. Sterilize by autoclave all things in steps B. 3–6 for 15 minutes
3. Wash potatoes with water and soak in bleach
4. Clean laminar flow hood with ethanol
5. Pour 20 ml sterilized agar solution per Petri dish and let cool
6. Dissolve 4 mg sample in 1 ml DMSO
7. Use 1 ml of DMSO as blank standard
8. Preparation of inocculum
   a. add 1.5 ml water, 2.0 ml of the 48 hr bacterial culture, and 0.5 ml sample in DMSO to tube
   b. prepare control by replacing sample with 0.5 ml DMSO
   c. use sterile technique (flame and cap).
9. Take potato out of bleach, cut away ends, and bore out cylinders onto the sterile tray
10. Cut cylinders into discs and place 5 discs per Petri dish by gently pushing the discs into the agar using aseptic technique
11. Prepare at least 3 Petri dishes per sample and control
12. Add one drop (0.05 ml) of the prepared innoculum per disc
13. Seal the edge of each Petri dish with parafilm strips to prevent moisture loss during the incubation period; keep the dish level at all times to keep the innoculum on the tops of the discs
14. Keep in the dark at 27°C and count the tumors after 12 to 21 days
15. Calculate the percent inhibition of crown gall tumors as follows:

$$\% \text{ inhibition} = 100 - \frac{\text{Average number tumors of sample}}{\text{Average number tumors of control}} \times 100$$

bioassay. The materials and procedures are listed in Table 3.

Yellow Fever Mosquito (YFM) Test: A Bioassay for Pesticides

The yellow fever mosquito larvae microtiter plate assay (YFM) (9) is a simple "bench top" bioassay that can be used to determine the pesticidal activities of botanical extracts, fractions, and isolated compounds. The eggs of the yellow fever mosquitoes, *Aedes aegypti* (Linnaeus), are obtained from Dr. Steven Sackett, of the New Orleans Mosquito Control Board, and are stored at room temperature in a sealed container. Another small

TABLE 3
The *Lemna* Bioassay Modified for Plant Extracts and Compounds (8)

MATERIALS

1. *Lemna minor* prepared by aseptic technique described below, if contaminated
2. E Medium (about 80 ml per compound)
3. Syringes: 10 µl, 100 µl, 1 ml, 2 ml
4. 2 dram vials (40 per compound)
5. Large glass container to hold vials; translucent plate glass to form a lid; stopcock grease to form a seal to avoid moisture loss
6. Growth chamber with temperature range of 27 to 29°C and 24 hours of fluorescent and incandescent lights

PROCEDURES

1. Prepare inorganic medium E (see below); add KOH pellets to pH 5.5–6.0
2. Prepare vials for testing: 10 vials per dose (500, 50, 5 ppm, control)
   a. Weigh 15 mg of compound and dissolve in 15 ml solvent
   b. Add 1000, 100, and 10 µl solution to vials for 500, 50 and 5 ppm. Allow solvent to evaporate overnight
   c. Add 2 ml of E Medium and then a single plant containing a rosette of three fronds to each vial
3. Place vials in glass dish filled with about 2 cm water, seal container with stopcock grease and glass plate
4. Place dish with vials in growth chamber for seven days
5. Count and record number of fronds per vial on days 3 and 7
6. Analyze data as percent of control with ED_{50} computer-program (available from Dr. McLaughlin) to determine FI_{50} and FP_{50} values and 95% confidence intervals
   500 ppm = 15 mg compound/15 ml solvent × 1000 ml solution/2 ml E Medium
   50 ppm = 15 mg compound/15 ml solvent × 100 ml solution/2 ml E Medium
   5 ppm = 15 mg compound/15 ml solvent × 10 ml solution/2 ml E Medium

E MEDIUM

	mg/L
KH_2PO_4	680
KNO_3	1515
$Ca(NO_3)_24H_2O$	1180
$MgSO_47H_2O$	492
H_3BO_3	286
$MnCl_24H_2O$	3.62
$FeCl_36H_2O$	5.40
$ZnSO_47H_2O$	0.22
$CuSO_45H_2O$	0.22
$Na_2MoO_42H_2O$	0.12
EDTA	11.2

open jar with saturated zinc sulfate solution can be put in the container to maintain a relative humidity between 80–90%.

After being hatched in warm water overnight, the larvae are allowed to develop in bovine liver powder solution for four days. The surviving larvae are then transferred to MES (2[N-morpholino] ethanesulfonic acid) buffer solution and used for the tests. Natural product extracts, fractions, or pure compounds are tested in five, 1 : 10, dilutions starting at 5000 µg/ml. After four days of incubation with test material, the surviving larvae are counted, and the data can be processed in the simple probit analysis program on a personal computer to estimate LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.

The amounts of samples used in this assay are fairly small, 5–25 mg (corresponding to testing concentrations of 1000 µg/ml to 5000 µg/ml). Rotenone can be used as a positive control for the assay. Pure substances with LC_{50} values ≤1.0 µg/ml are worthy of com-

mercial development. Table 4 summarizes the materials and procedures.

VALIDATION AND THE ANNONACEOUS ACETOGENINS

The first three simple "bench top" bioassays, as well as cytotoxicities in three human solid tumor cell lines, were compared for accuracies to detect known-3PS *in vivo* (P-388) active antitumor agents supplied blind by Dr. Matthew Suffness of the United States National Cancer Institute. The potato disc assay was the best predictor and showed excellent correlation to *in vivo* activities ($p = 0.008$). The brine shrimp bioassay ($p = 0.033$) proved to be superior or equally as accurate as the three *in vitro* human solid tumor cell lines ($p = 0.033–0.0334$); it gave no false positives and only one false negative; as expected, the *Lemna* assay showed the poorest correlation ($p = 0.708$) with 3PS; however, it detected plant growth stimulation as well as inhibition effects among the several test substances (10). The authors now suggest the brine shrimp and potato disc bioassays as convenient in-house prescreens to the more expensive mechanism-based, cytotoxicity, or *in vivo* antitumor assays. In addition, the brine shrimp test is an effective screening method for pesticides, as exemplified by the authors' discovery of the Annonaceous acetogenins as a new class of natural pesticides (11,12). These compounds are also powerful as *in vivo* active antitumor agents with bullatacin (2) (Figure 2) showing 300 times the potency of taxol against L1210 murine leukemia (13).

Since their discovery in 1982, the Annonaceous acetogenins (named for the plant family, Annonaceae) have become one of the most rapidly growing classes of bioactive natural products. Chemically, they are C-32 or C-34 long chain fatty acids which have been combined with a 2-propanol unit at C-2 to form a γ-lactone. Biogenetically, double bonds along the fatty acid chain seem to epoxidize and cyclize to form one, two, or three tetrahydrofuran (THF) rings, often with flanking hydroxyls; other functional groups include hydroxyls, acetoxyls, carbonyls, and double bonds. Recently, the authors have found acetogenins bearing tetrahydropyran (THP) rings (14). Biologically, these compounds are among the most potent of the known inhibitors of complex I (NADH : ubiquinone oxidoreductase) in mitochondrial electron transport systems and of the plasma membrane NADH : oxidase that is characteristic of cancerous cells; these actions seem to induce apoptosis (programmed cell death), perhaps as a consequence of ATP deprivation. Applications as pesticides and antitumor agents hold excellent potential, especially in the thwarting of resistance mechanisms which require an ATP-dependent efflux (15,16,17). The authors' laboratory has published four comprehensive reviews on the Annonaceous acetogenins (18–21), and this work has been guided by the above bioassays to yield over 180 of these potent compounds. Structure-activity relationship studies, using these, including the YFM test and other bioassays, permit prediction of the optimum structures for antitumor and pesticidal effects (22,23, 24). Asimicin (1) and bullatacin (2) (Figure 2) are two of the most potent compounds in this series and have been patented (25,26).

Two Annonaceous species, *Asimina triloba* Dunal ("paw paw") and *Annona muricata* L. ("sour sop," "guanabana"), are, respectively, abundant as fruit trees in temperate eastern North America and in the tropics worldwide. Both of these species have now yielded a variety of new acetogenins, and their crude extracts exhibit potent pesticidal effects. These crude extracts can be employed as safe, effective, economical, and environmentally friendly pesticides with an emphasis on the home garden, ornamental, greenhouse, and produce markets, pending regulatory approval.

The complexities of these acetogenin mixtures, undoubtedly, serve as a benefit in the protection of the trees against a host of microbes, pests, and herbivores. It is known that such a series of structurally related secondary plant products can even display a level of toxicity to pests that is lacking in the individual compounds (27). The recent work of Feng and Isman (28) showed that, after 40 generations, green peach aphids, which were repeat-

TABLE 4
Materials and Procedures for the Yellow Fever Mosquito (YFM) Test

MATERIALS

1. *Aedes aegypti* (yellow fever mosquito) eggs (from Dr. Steven Sackett of the New Orleans Mosquito Control Board, New Orleans, LA 70126)
2. MES (2-[N-morpholino] ethanesulfonic acid) (from Sigma)
3. Bovine liver powder (from ICN Biochemicals)
4. 96 U and F microwell plates and lids (from VWR Scientific)
5. Syringes: 10 μl, 50 μl, 100 μl
6. Pipette
   a. regular dispo-pipette
   b. a motorized microliter pipette with liquid ends (Rainin Instrument Company)
   c. a Vaccu-Pette/96 pipette (Vanguard International)
7. Vials: 4 ml glass vials with septums (five for each sample tested)
8. A glass vessel, a jar, or a 500 ml beaker for storing developed mosquito larvae

PROCEDURES

1. Put YFM eggs in a small vial with 5–10 ml of warm water, let stand for 2–3 hours
2. Transfer the above contents into a jar or a beaker containing bovine liver powder solution at a concentration of ∼4 mg/ml
3. Allow to develop for four days
4. Harvest the live larvae with a pipette, transfer them into a jar or beaker which contains 5 mM MES pH 6.5 solution
5. Use a Vaccu-Pette/96 to fill 96-well microwell plate with 240 μl 5 mM MES solution per well
6. Prepare solutions for testing: to start with a concentration of testing material of 5000 μl/ml, 25 mg of the test material is weighed and dissolved in 100 μl of methanol in a sealed vial; this testing solution (250 μl/ml) is then injected into eight wells on the microliter plate with 5 μl each. The final concentration of the testing material in these eight wells will be 5000 μg/ml. Another 5 μl of this solution is injected into another vial and diluted with 45 μl of methanol to make a solution with a concentration of testing material of 500 μg/ml of methanol; further diluted solutions can be made in the same way. Each 96 well plate can hold two testing materials as shown below. The contents of the control wells are the same as the other wells except that 5 μl of pure methanol should be used instead of 5 μl of testing material in methanol

Compound 1					Controls				Compound 2		
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000
5000	500	50	5	0.5	C	C	0.5	5	50	500	5000

7. Use the motorized microliter pipette to add larvae in the wells, one larvae per well in as close to 10 ml as possible
8. Cover the plate and incubate in the dark at room temperature for four days. (Keep the environment humid to avoid drying the plate)
9. To score for dead larvae, tap the plates sharply with a pencil to induce movement
10. Analyze the mortality data with the Finney computer program to determine LC_{50} values and 95% confidence intervals. A copy of this program for IBM PC is available from Dr. McLaughlin

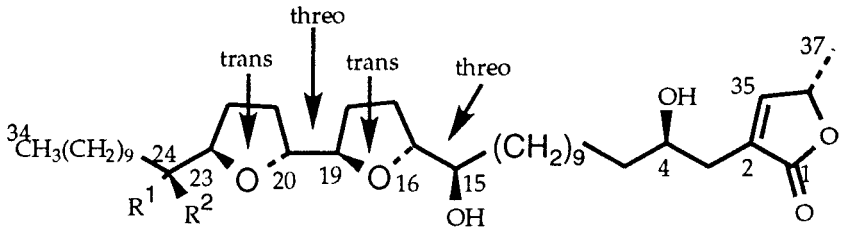

FIGURE 2. Structures of a simicin (1) and bullatacin.

edly treated with either pure azadirachtin or a refined neem seed extract (at equivalent concentrations), developed a nine-fold resistance to azadirachtin but developed no resistance to the neem extract. Such evidence helps to establish a good case for the use of crude, chemically unrefined, plant extracts, containing mixtures of the bioactive plant components, rather than the use of the pure individual components. Insect resistance is much less likely to develop, the environmental load of individual components is lessened, and the products of crude extracts are cheaper to prepare. Thus, the authors have concentrated their efforts on the pesticidal evaluation of the heterogeneous extracts (F005, F020) of the plant biomasses. The seeds of guanabana are readily available as a byproduct of its juice industry in the tropics, and the F005 extracts are quite effective against potato beetles, aphids, and white flies; the paw paw extracts show remarkable synergistic effects with pyrethrins and neem extracts (11,12).

Various plant parts of the paw paw tree were extracted into standardized pesticidal extracts (F005), and the extracts were analyzed with the brine shrimp (BST) bioassay to determine their relative potencies (29). The small twigs (0–0.5 cm diameter) yielded the most potent extracts (LC_{50} values as low as 0.04 ppm); the stem wood (LC_{50} 4.9 ppm) and leaves (LC_{50} 53.7 ppm) yielded the poorest activities. The unripe fruits, seeds, root wood, root bark, and stem bark were notably potent ($LC_{50} < 0.2$ ppm) and, generally, yielded less than 2% of their dry weight as F005. The smaller diameter stems were more potent than the larger stems. It was concluded that, by pollarding or coppicing to collect the small branches and twigs, this biomass could be dried and processed to produce a potent acetogenin mixture. Such a biomass could initially be collected from the wild and eventually could be produced from plantations of trees grown as a new crop. Quantities of biomass needed for commercialization of the mixture of acetogenins as a new pesticidal product would be sustainable and renewable through regrowth from the parent trunk and larger branches.

In this work, it was noticed that the bioactivity of extracts from paw paw biomass collected in November was apparently lower than that from biomass collected in July. This prompted a study of the monthly variation of bioactivity of twigs collected from the same trees. For this study (30), a more rapid extraction method was developed, and the extracts were, as usual, evaluated in the BST. The results were astonishing and suggested a dynamic flux in acetogenin concentration depending on the season. At this location (Amherst, NY), May, June, and July are optimum for biomass collection, and the winter months (when insects are less of a problem to the trees) are poorest for biomass collection. The potency differences were more than 25 times (February vs. May) in one of the trees. In a second tree at the same location the bioactivity flux was not as dramatic, but the twig samples of this tree were generally more potent. This observation suggested that genetic differences are likely to exist between paw

paw trees with some trees concentrating acetogenins more than others. A current project is underway to assay twig samples that are collected on the same day from 670 trees growing in a paw paw plantation in Maryland. Preliminary results show variations in bioactivities as high as 130 times from one tree to the next; however, most trees produce and maintain a useful level of potency (BST LC_{50} ca. 0.5 ppm). Growers of future plantations intended for biomass production of the paw paw pesticide will, thus, be advised to propagate only vegetative clones selected from the tree genotypes that give the highest potencies in their extracts.

Ongoing experiments at XenoBiotics Labs (Plainsboro, NJ), using the F005 extracts described above, are now confirming that the extracts with highest BST activities are also those with the highest levels of a heterogeneous mixture of the acetogenins; these analyses are made possible by liquid chromatography-mass spectrometry (LC/MS/MS) using the electrospray positive ion mode [LC(C+)ESI-MS] and requiring only 2 µg of F005. This new technique is predicted to become indispensible in the future for rapid analyses of crude heterogeneous mixtures of bioactive botanical components, and the authors are now using this method as well, for the discovery of new actogenins (31,32).

CONCLUSIONS

Four simple "bench top" bioassays are described which are useful in the detection of biologically active components of botanical extracts. These bioassays have enabled the authors' laboratory to discover over 300 bioactive compounds of which the Annonaceous acetogenins are the most promising. The bioassays permit the convenient and rapid evaluation of various plant parts, ontogenic and seasonal variations within individual plants, and highly bioactive genotypes within the infraspecific variations. In cases where crude botanical extracts, containing mixtures of bioactive compounds, will be effective, these simple procedures may permit their convenient standardization. Subsequent analyses with liquid chromatography/mass spectrometry (LC/MS/MS) can quickly confirm that the activities are due to the desired components.

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