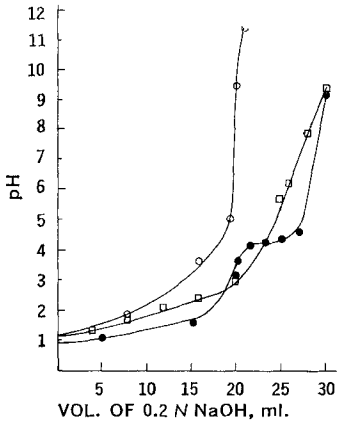

Fig. 5.—Potentiometric titrations of aluminum chloride (●), sodium tartrate (○), and aluminum chloride plus sodium tartrate (□).

both occurring at 20 ml. of alkali. However, the combination of these salts required more base for neutralization; the inflection point was displaced to the right, to about 24 ml. It is interesting to note that although the titration of aluminum chloride alone produced a precipitate of the hydroxide, no precipitate was observed in the presence of citrate or tartrate. This latter phenomenon suggests the presence of a water-soluble complex. In addition, since the system containing the aluminum complex required more alkali to produce neutralization than did either of the salts when tested separately, evidence is provided to indicate that the formation of this complex produces the release of excess protons and thereby decelerates the change of pH with respect to time. Similar results were found for the sodium tartrate–aluminum chloride potentiometric titration (Fig. 5).

In view of these findings we may conclude that a dual mechanism is operative to produce inhibition. AHDG reacts with sodium tartrate or citrate to produce a water-soluble complex with the concomitant liberation of protons. This effect coupled with flocculation causes a retardation in the rate of change of pH with time.

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Cytotoxicity of North Dakota Plants I

In Vitro Studies

By I. A. MUNI, W. H. BHATTI, L. J. SCHERMEISTER, and M. C. VINCENT

In this study, a method is presented for the direct determination of cytotoxicity and antitumor activity of plant materials *in vitro* using HeLa cells. Fifty poisonous plants representing 96 plant parts were tested by this method, out of which 25 plants showed limited cytotoxicity and six plants were found to be highly cytotoxic. This procedure can be used on a large scale prior to *in vivo* evaluation.

THE IMPORTANCE of plant screening for various chemical constituents of medical importance is well established. Plants have provided innumerable drugs which are being used for the prevention or cure of various diseases. This work was directed to the search for new drugs which may find application in the prevention or cure of cancer.

In vitro screening for cytotoxicity based on morphological evaluation was first reported by Biesele and associates (1) in 1952 using nonsynthetic media. Fjelde *et al.* (2) introduced human tumor cell lines for cancer chemotherapy.

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Eagle and Foley (3) described a technique for determination of cytotoxicity of chemical compounds for mammalian cells *in vitro* using semisynthetic media. Later they (4) reported cytotoxic activity of 180 compounds against the KB strain of human epidermoid carcinoma. Smith, Lummis, and Grady (5) determined the cytotoxic activity of various nucleic acids and vitamin antagonists, heavy metals, respiratory poisons, detergents, miscellaneous chemical solvents, and fermentation beers. Nitta (6,7) and other workers (8–11) have tested the different antibiotics for antitumor activity. A recent survey (12) indicates that about 400 plant species possess antitumor activity.

EXPERIMENTAL

Collection of Materials.—The investigational material was collected from wild growing plants throughout the State of North Dakota. Each plant under investigation was taxonomically classified and a sample of each specimen was retained in the herbarium for future reference.

The fresh plant material was air-dried at room temperature, separated into the respective plant parts, ground to a No. 40 powder, and stored in air-tight containers. When the presence of moisture interfered with grinding, the plant material was oven dried at 60°.

Preparation of Extracts.—Two extracts, aqueous and alcoholic, of all plant parts were prepared. The aqueous extract was prepared as follows. A 2-Gm. sample of dried material was macerated with 25 ml. of distilled water for 30 to 40 min. and filtered through Whatman No. 1 paper. The extracts were freeze dried and kept frozen until used. Prior to screening, each frozen extract, representing 2 Gm. of dried plant material, was dissolved in 2 ml. of isotonic saline. The pH of the solution was adjusted to 7.0 to 7.5 with 0.25 *N* HCl or 0.25 *N* NaOH. Then it was mixed with 0.5 ml. of 5X culture medium containing antibiotics and sterilized through UF-filtration. UF-filtration was accomplished by centrifuging in the closed system.

The same general procedure was used in preparing the alcoholic extracts, using 95% alcohol instead of water. However, to increase the solubility of the plant material, 2 drops of alcohol was added to the isotonic saline.

Preparation of Medium.—The culture medium recommended by Joseph (13) for the growth of HeLa cells was prepared as follows:

Medium 199 (10X)	8 ml.
Calf serum	20 ml.
Hank's BSS	72 ml.
Sodium bicarbonate solution, 7.5%	0.5 ml.
Potassium penicillin G	100 units/ml.
Streptomycin sulfate	100 mcg./ml.

The pH was found to be 7.0 to 7.5.

Fresh medium was prepared before running each set of samples and checked for sterility with alternative thioglycolate medium U.S.P. (14).

Cell Lines.—Stock cultures of HeLa cells were obtained from Southern Research Institute, Birmingham, Ala., and Microbiological Associates, Bethesda, Md. These cultures were maintained in Blake bottles containing culture medium 199 at 37°. Stock cultures were refed with fresh medium every 50 to 60 hr. A suspension of isolated cells was obtained by trypsinizing 75 to 80 hr. old cell cultures.

The number of cells present in the culture medium was determined in a hemocytometer. The required dilution was made with culture medium to obtain 40,000 to 50,000 cells/ml.

Screening Procedure.—Two-milliliter vinyl plastic cup trays were sterilized by immersing in 95% ethanol for 1 hr., and then dried for 4 to 6 hr. under ultraviolet light.

Cell suspensions were placed in a 125-ml. Pyrex flask, which was equipped with glass and rubber tubing connecting a 1-ml. Cornwall syringe. A three-way adapter was attached to the syringe to allow recirculation of medium that remained in the tubing between samples. The suspension was stirred either by circulation or by shaking the flask gently after every sample extraction.

Plant extracts were added to the cups in definite quantities, representing 4, 20, 40, 100, and 200 mg./ml. of dry plant material, using a syringe microburet. One-half milliliter of cell suspension was added to each cup and the volume was made up to 1 ml. with the addition of culture medium. A 30-gauge blunt-tip needle was used.

A series of controls were determined for each test series, such as sodium nitrofurantoin[1], methyl green, and Hank's basal salt solution for comparison. The plastic trays were covered with sterile plastic and sealed air-tight with strips of Scotch cellophane tape. All trays were incubated at 37°.

Evaluation of Data.—Toplin's evaluation method (15), accepted by CCNSC was used. The trays were examined after 75 to 80 hr. incubation for evidence of cytotoxicity. Cytotoxicity was evaluated by direct microscopic examination of cultures usually at 100 × magnification and, if necessary, at 430 × magnification. The cytotoxic end point (CE) and lethal end point (LE) were determined.

RESULTS AND DISCUSSION

Fifty poisonous plants, representing 96 plant parts, were tested for cytotoxicity using Gey's HeLa S-3 cells. The cytotoxic and lethal end point for all the plants tested is given for both alcoholic and aqueous extracts in Table I.

Toplin's direct microscopic method for cytotoxicity evaluation was used because it was simple, rapid, and reproducible.

The plant materials screened for cytotoxicity were used in concentrations representing 4 to 200 mg. of plant material per milliliter of culture medium. In Table II the comparison of *in vivo* results, as reported by Leiter *et al.* (16) and Hardinge *et al.* (17), with the *in vitro* cytotoxicity of the same plants at 200 mg. concentration indicated that the positive results were due to a general cellular, and not a specific, toxicity. For this reason, the highest concentration of plant material used was 200 mg./ml.

Both CE and LE values were necessary to determine the effectiveness of cytotoxic substances in the plant extracts. The effectiveness of these

[1] Marketed as Furadantin Sodium by Eaton Laboratories, Inc., Norwich, N. Y.

TABLE I.—CYTOTOXICITY SCREENING DATA[a]

Serial No.	Code No.	Scientific Name	Plant Part	Extract	Cytotoxicity Limits[b] CE	LE	Extract	Cytotoxicity Limits[b] CE	LE
1	64–146	*Achillea sibirica* Ledeb. (*Asteraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
2	63–68	*Actaea rubra Ait.* (*Ranunculaceae*)	L	Alc.	100–200	>200	Aq.	100–200	>200
3	63–62	*Apocynum androsaemifolium* L. (*Apocynaceae*)	L	Alc.	40–100	200	Aq.	100	200
4	64–108	*Apocynum sibiricum* Jacq. (*Apocynaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	100	200	Aq.	>200	>200
			R	Alc.	>200	>200	Aq.	>200	>200
5	63–32	*Ascelipas ovalifolia* Ded. (*Asclepiadaceae*)	L	Alc.	40	100–200	Aq.	200	>200
6	64–107	*Asclepias speciosa* Torr.	F	Alc.	200	>200	Aq.	>200	>200
			L	Alc.	20–40	200	Aq.	40	200
		(*Asclepiadaceae*)	R	Alc.	200	>200	Aq.	200	>200
7	64–106	*Asclepias syriaca* L.	B	Alc.	100–200	>200	Aq.	100–200	>200
		(*Asclepiadaceae*)	L	Alc.	40–100	>200	Aq.	40–200	>200
			S	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	100–200	>200
8	64–104	*Asparagus officinalis* L. (*Liliaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
9	63–31	*Astragalus caryocarpus* Ker. (*Febaceae*)	L	Alc.	20	100–200	Aq.	>200	>200
10	63–53	*Astragalus missouriensis* Nutt. (*Fabaceae*)	L-S	Alc.	100–200	>200	Aq.	>200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
11	63–34	*Astragalus tenellus* Pursh. (*Fabaceae*)	Fl-1	Alc.	20	100–200	Aq.	200	>200
			S	Alc.	40–100	200	Aq.	200	>200
12	64–116	*Celastrus scandens* L. (*Celastraceae*)	L	Alc.	100–200	>200	Aq.	>200	>200
			S	Alc.	100–200	>200	Aq.	>200	>200
13	63–29	*Cicuta maculata* L. (*Umbelliferae*)	L	Alc.	200	>200	Aq.	200	>200
			T	Alc.	>200	>200	Aq.	200	>200
14	64–126	*Eupatorium maculatum* L. (*Asteraceae*)	L	Alc.	40–100	200	Aq.	100–200	>200
			S	Alc.	100–200	>200	Aq.	200	>200
15	63–65	*Eupatorium perfoliatum* L. (*Asteraceae*)	L	Alc.	200	>200	Aq.	200	>200
16	64–124	*Euphorbia esula* L. (*Euphorbiaceae*)	L	Alc.	200	>200	Aq.	100–200	>200
			S	Alc.	40	200	Aq.	100–200	>200
17	63–80	*Fumaria officinalis* L. (*Fumariaceae*)	W	Alc.	40–200	>200	Aq.	100–200	>200
18	64–128	*Gaura coccinea* Pursh. (*Onagraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
19	62–20	*Glycyrrhiza lepidota* Nutt. (*Fabaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
20	64–121	*Gypsophila paniculata* L. (*Careophyllaceae*)	Fr-1	Alc.	40–200	>200	Aq.	40–200	>200
			R	Alc.	200	>200	Aq.	40–200	>200
			S	Alc.	40–200	>200	Aq.	100–200	>200
21	63–90	*Helenium autumnale* L. (*Asteraceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	200	>200
22	64–119	*Lactuca pulchella* Pursh. (*Asteraceae*)	L	Alc.	100–200	>200	Aq.	200	>200
			S	Alc.	40–200	>200	Aq.	100–200	>200
23	63–64	*Lathyrus ochroleucus* Hook. (*Fabaceae*)	L	Alc.	100–200	>200	Aq.	40–200	>200
24	63–72	*Lobelia spicata* Lam (*Lobeliaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
25	64–143	*Lupinus argenteus* Pursh. (*Fabaceae*)	L	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
26	63–59	*Mamillaria vivipara* Nutt. (*Cactaceae*)	L-S	Alc.	>200	>200	Aq.	>200	>200
27	63–75	*Menispermum canadensis* L. (*Menispermaceae*)	L	Alc.	40	200	Aq.	100–200	>200
			S	Alc.	200	>200	Aq.	>200	>200
28	64–100	*Nepeta cataria* L. (*Lamiaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
29	63–60	*Opuntia polycantha* Haw. (*Cactaceae*)	L-S	Alc.	100–200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
30	64–139	*Oxytropis lambertii* Pursh. (*Fabaceae*)	L	Alc.	>200	>200	Aq.	200	>200
			S	Alc.	>200	>200	Aq.	200	>200
31	63–67	*Penstemon grandiflorus* Nutt. (*Scrophulariaceae*)	L	Alc.	4	100–200	Aq.	20	200

Continued on next page.

TABLE I.—(*Continued.*)

Serial No.	Code No.	Scientific Name	Plant Part	Extract	Cytotoxicity Limits[b] CE	LE	Extract	Cytotoxicity Limits[b] CE	LE
32	63–50	*Penstemon nitidus* Dougl. (*Scrophulariaceae*)	L	Alc.	20	100–200	Aq.	>200	>200
33	63–33	*Polygonatum commutatum* Schutt. (*Liliaceae*)	L	Alc.	100–200	>200	Aq.	200	>200
			S	Alc.	200	>200	Aq.	200	>200
34	62–8	*Psoralea argophylla* Pursh. (*Fabaceae*)	L	Alc.	40–100	200	Aq.	200	>200
35	64–103	*Rhus radicans* L. (*Anacardiaceae*)	L	Alc.	200	>200	Aq.	200	>200
			Rh	Alc.	100–200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	100–200	>200
36	64–144	*Rudbeckia hirta* L. (*Asteraceae*)	L	Alc.	40–100	200	Aq.	40	200
			S	Alc.	20–40	200	Aq.	>200	>200
37	64–105	*Rumex* sp. (*Polygonaceae*)	L	Alc.	40–200	>200	Aq.	>200	>200
			S	Alc.	200	>200	Aq.	>200	>200
38	64–109	*Rumex venosus* Pursh. (*Polygonaceae*)	L	Alc.	4–40	100–200	Aq.	40	200
			S	Alc.	20–40	100–200	Aq.	20	40–200
39	63–66	*Sanguinaria canadensis* L. (*Papaveraceae*)	L	Alc.	200	>200	Aq.	200	>200
			Rh	Alc.	200	>200	Aq.	200	>200
			R	Alc.	200	>200	Aq.	200	>200
40	64–115	*Silene cseri* Baum (*Caryophyllaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
			R	Alc.	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
41	64–102	*Silene noctiflora* L. (*Caryophyllaceae*)	Fl	Alc.	100–200	>200	Aq.	200	>200
			L	Alc.	100–200	>200	Aq.	100–200	>200
			R	Alc.	100–200	>200	Aq.	100–200	>200
			S	Alc.	200	>200	Aq.	200	>200
42	63–78	*Solanum carolinense* L. (*Solanaceae*)	W	Alc.	200	>200	Aq.	100–200	>200
43	64–140	*Stachys palustris* L. (*Laminaceae*)	Fl	Alc.	200	>200	Aq.	200	>200
			L	Alc.	200	>200	Aq.	200	>200
44	64–141	*Stipa comata* Trin. and Rupr. (*Graminaceae*)	W	Alc.	>200	>200	Aq.	>200	>200
45	64–142	*Thermopsis rhombifolia* Nutt. (*Fabaceae*)	L	Alc.	200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200
46	64–101	*Triglochin maritima* L. (*Juncaginaceae*)	L	Alc.	200	>200	Aq.	200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
47	63–83	*Veronica americana* Schwein. (*Scrophulariaceae*)	W	Alc.	>200	>200	Aq.	200	>200
48	64–147	*Vicia americana* Muhl. (*Fabaceae*)	L	Alc.	>200	>200	Aq.	>200	>200
49	63–76	*Xanthium italicum* Mor. (*Asteraceae*)	L-S	Alc.	100–200	>200	Aq.	200	>200
			R	Alc.	100–200	>200	Aq.	200	>200
50	63–37	*Zygadenus elegans* Pursh. (*Liliaceae*)	Fl	Alc.	100–200	>200	Aq.	200	>200
			L	Alc.	>200	>200	Aq.	>200	>200
			R	Alc.,	>200	>200	Aq.	>200	>200
			S	Alc.	>200	>200	Aq.	>200	>200

[a] Alc, alcoholic extract; Aq, aqueous extract; B, buds; CE, cytotoxicity end point; F, fruit; Fl, flower; Fr-1, fruit and leaves; Fl-1, flowers and leaves; L, leaves; LE, lethal end point; L-S, leaves and stems; R, roots; Rh, rhizome; S, stems; T, tuber; W, whole plant. [b] Cytotoxicity limits expressed in mg. of dry plant material/ml. of culture.

TABLE II.—COMPARISON OF *In Vivo*[a] AND *In Vitro* RESULTS

Serial No.	Scientific Name	*In Vitro* CE	*In Vitro* LE
1	*Asparagus officinalis* L.	200	>200
2	*Eupatorium perfoliatum* L.	200	>200
3	*Glycyrrhiza lepidota* Nutt.	200	>200
4	*Lupinus argentius* L.	200	>200
5	*Nepeta cataria* L.	200	>200
6	*Sanguinaria canadensis* L.	200	>200
7	*Stachys palustris* L.	200	>200

[a] Reported in literature and in each case was tumor non-inhibitory.

extracts in inhibiting HeLa cells was increased as the CE and LE values approached one another.

As shown in Table I, a CE value of 100 or lower was produced by 66 out of 192 extracts. Eight of these extracts had close CE and LE values and were considered highly inhibitory to HeLa cells. The most effective alcoholic extracts were obtained from the leaves of *Ascelpias ovalifolia, Astragalus caryocarpus, Penstemon grandiflorus, Penstemon nitidus,* and *Rumex venosus,* the stems of *Rumex venosus,* and the flowers and leaves of *Astragalus tenellus.* In the case of aqueous extracts, only the stem of *Rumex venosus* was included.

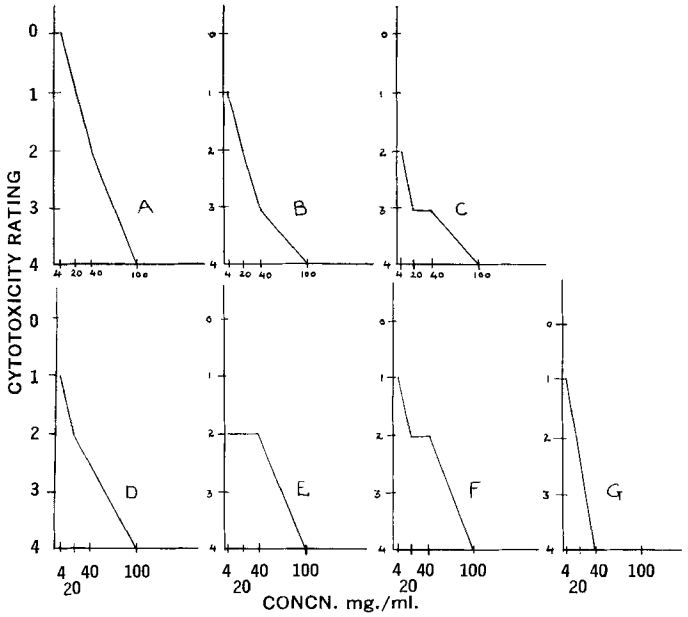

Fig. 1.—Graphical representation of cytotoxicity. Key: A, *Asclepias ovalifolia* leaves (alc.), B, *Astragalus tennellus* flowers and leaves (alc.) and *Astragalus caryocarpus* leaves (alc.); C, *Penstemon grandiflorus* leaves (alc.); D, *Penstemon nitidus* leaves (alc.); E, *R. venosus* leaves (alc.); F, *R. venosus* stem (alc.); G, *R. venosus* stem (aq.).

In order to determine which of the above plant extracts were more effective as cytotoxic agents, Toplin's plotting method was employed. These plots are presented in Fig. 1. From this figure it can be observed that plants which have greater cytotoxicity have a sharper slope. The sharpest slope was obtained with the aqueous extract of stems of *R. venosus*. All other effective extracts were alcoholic, and the decreasing order of cytotoxicity was as follows: leaves of *Ascelepias ovalifolia* and *Astragalus caryocarpus*, flowers and leaves of *Astragalus tenellus;* leaves of *Penstemon grandiflorus* and *Penstemon nitidus;* stems of *R. venosus* and the leaves of *R. venosus*.

The data in Table I indicate that the naturally occurring tumor inhibitory substances in these plants were more generally alcohol soluble. These alcohol soluble substances were more frequently found in leaves than in stems, while none were present in roots of those plants tested. Only the stems of *R. venosus* contained inhibitory substances which were soluble in both alcohol and water.

Of the 23 families of native plants tested, only four families contained species having cytotoxic constituents. They were *Polygonaceae, Asclepiadaceae, Fabaceae,* and *Scrophulariaceae.*

A slight inhibitory effect on HeLa cells was shown by 58 plant extracts. If the moderately cytotoxic plant extracts were further concentrated, other effective cytotoxic activities might be indicated.

The limitations of all *in vitro* studies are numerous and the correlation between antitumor activity in experimental animal tumors *in vivo* and cytotoxicity to mammalian cells *in vitro* can only be established by experiments.

SUMMARY

1. Gey's HeLa cells were used to determine *in vitro* cytotoxicity and antitumor activity of plant extracts.

2. Evaluation of cytotoxicity was based on microscopic observation of the cells after incubation with plant samples for 75 to 80 hr.

3. Fifty-eight out of 192 extracts tested were moderately cytotoxic in the range of 4 to 100 mg. of dry plant material per milliliter of culture media.

4. Eight plant extracts exhibited marked human cancer cell inhibitory activity.

5. More alcoholic extracts were found to be cytotoxic than the aqueous extracts for the plants tested.

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