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Crown Gall Tumor Disc Bioassay

A POSSIBLE AID IN THE DETECTION OF COMPOUNDS WITH ANTITUMOR ACTIVITY

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ABSTRACT

Seventeen samples consisting of purified compounds and various ethanol extracts from plant sources were tested for activity on the initiation of crown gall tumors on potato discs. The results demonstrated definite correlation between the ability of these samples to inhibit the formation of crown gall tumors and their activity on the P388 leukemia system in mice. Samples showing only cytotoxic effects in KB cell cultures did not affect tumor initiation in our system. The active materials had no effects on bacterial viability or on the ability of the bacteria to attach to a tumor-binding site.

Crown gall is a neoplastic disease of plants which occurs in more than 60 families of dicotyledons and many gymnosperms. The disease is characterized by the transformation of normal plant cells into autonomous tumor cells in a short period of time. Once initiated the tumor possesses the capacity for autonomous growth independent of the normal control mechanism of the host (4). The causative agents of this disease are specific strains of the gram-negative bacterium *Agrobacterium tumefaciens* (4, 12). The relevance of the crown gall tumor system to the general cancer problem has been thoroughly reviewed (3, 5).

The use of highly specific, quantitative bioassays which require only a short period of time to obtain results are availble for studying crown gall tumor formation (1, 10). Using the potato disc bioassay we examined extracts and purified compounds of plant origin, some of which had known antitumor activity in animals, for their effect on the initiation of crown gall tumors.

MATERIALS AND METHODS

Samples used in this study were part of a screening program being conducted at the USDA Northern Regional Research Laboratory in Peoria, Ill. to detect antitumor compounds in plants. All of the samples had previously been tested in at least two tumor systems under the auspices of the National Cancer Institute (8). In this investigation the identity of the samples were unknown until the experiments had been completed.

Tumors were induced on potato discs (*Solanum tuberosum* var. Red Russett) by the method first described by Anand and Heberlein (1), and modified slightly in our laboratory (7). Twelve days following inoculation the tumors were counted with the aid of a dissecting scope. The tumors at this time, however, are clearly visible with the human eye. Each experimental sample consisted of five Petri dishes each containing five potato discs. Sterile procedures were employed throughout the experiment.

Table I. *Comparative Activity of Various Plant Materials against Initiation of Crown Gall Tumors, for Cytotoxicity in KB Cell Cultures, and for Inhibition of P388 Leukemia*

Compound Tested or Source of Ethanol Extract[a]	% Inhibition of Crown Gall Tumor Initiation (Duplicate Trials or Triplicate Trials)	Cytotoxicity KB Cell Culture ED50 μg/ml (*Active Compounds)	% Increase in Life Span of P388 Leukemic Mice. Optimal Concentration mg/kg
Eucalyptus sieberiana	0/0	7.6×10^{0}*	None
Juncus marginata	0/0/0	8.6×10^{0}*	None
Geum urbanum	0/0/0	1.1×10^{1}*	None
Sesbania punicea (crude ext.)	79/52/62	2.4×10^{0}*	105 (20)
Homoharringtonine	83/58	1.0×10^{-2}*	238 (1.0)
Conidendrin	0/0	1.0×10^{2}	None
Sesbania punicea (concentrated extract)	78/52/60	1.9×10^{0}*	78 (9)
Cephalotaxine	20/0	1.0×10^{2}	None
Trewia nudiflora	29/51/44	3.1×10^{-2}*	63 (100)
Isoharringtonine	60/82/72	1.7×10^{-1}*	172 (7.5)
Raffinose	0/0/0	1.0×10^{2}	None
Thevetia thevetioides	0/0	4.0×10^{-1}*	None
Catechin	0/0	1.0×10^{2}	None
Hyphaene turbinata	0/0/0	1.0×10^{2}	None
Akebia quinata	0/0	4.4×10^{1}	None
Digitoxigenin	0/0	3.2×10^{2}	None
Neriifolin	0/0	2.2×10^{-2}*	None

[a] Extracts were dissolved in water at a concentration of 0.2 mg/ml. Two ml of these solutions (or distilled H_2O in case of controls) were filtered through Millipore filters (0.22 μm) into tubes containing an equal volume of *A. tumefaciens* strain B6 (a 48-h culture containing 5×10^{9} cells/ml). Five-tenths ml from these tubes was used to inoculate the potato discs. Data for per cent inhibition of crown gall tumor formation represent values obtained from at least two separate experiments (using 25 potato discs per determination). KB and P388 data were obtained under the auspices of the National Cancer Institute using current protocols (8). Digitoxigenin and neriifolin were isolated from *Thevetia thevetioides* seed extracts (6), and obtained through the courtesy of Dr. Jose Iriarte of Syntex. All other extracts and purified compounds including the *Cephalotaxus* alkaloids, homoharringtonine, and isoharringtonine (14) and the *Sesbania punicea* extract (13) were prepared in our laboratory.

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Table II. *Activity of Various Plant Materials on Induction of Crown Gall Tumors on Potato Discs*

Experiment 1		Experiment 2	
Additions to potato discs[a]	Mean no. tumors/ disc[b]	Additions to potato discs[a]	Mean no. tumors/ disc[b]
B6 + (5×10^9 cells/ ml)	49.7 ± 4.0[c]	B6 + (5×10^9 cells/ ml)	52.2 ± 5.3[c]
B6 + *Hyphaene turbinata*	42.2 ± 3.0 (—)[d]	B6 + *Sesbania punicea*	25.4 ± 2.6 (52)[d]
B6 + *Eucalyptus sieberiana*	44.0 ± 1.8 (—)	B6 + Homoharringtonine	22.5 ± 2.4 (58)
B6 + *Juncus marginata*	49.4 ± 2.4 (—)	B6 + *Trewia nudiflora*	26.2 ± 3.4 (51)
B6 + *Geum urbanum*	45.9 ± 1.8 (—)	B6 Conidendrin	49.6 ± 3.1 (—)

[a] Five-tenths ml of inoculum containing 2.5×10^9 cells of *A. tumefaciens* strain B6, and 0.05 mg of the experimental material were added to the potato discs. See Table I for more complete explanation.

[b] Twenty-five discs were used for determination.

[c] Standard error.

[d] Per cent inhibition.

Table III. *Activity of Three Plant Samples on Crown Gall Tumor Initiation When Added to Potato Discs at Various Times after Bacterial Inoculation*

Additions to Potato Discs[a]	Mean No. of Tumors/disc[b]		
	Time elasped prior to addition of plant extract to the potato discs which have been previously inoculated with *A. tumefaciens* strain B6		
	0	15 min	30 min
B6 control (5×10^9 cells/ml)	27.2 ± 2.3[c]	-	
B6 + *Sesbania punicea* extract	10.9 ± 2.1 (62%)[d]	12.3 ± 2.1 (55%)	9.7 ± 6.2 (64%)
B6 + *Trewia nudiflora* extract	15.3 ± 1.2 (44%)	16.2 ± 1.4 (41%)	16.0 ± 1.9 (42%)
B6 + isoharringtonine	7.4 ± 1.2 (72%)	8.3 ± 0.9 (69%)	7.0 ± 1.3 (74%)

[a] Five-tenths ml of inoculum containing 2.5×10^9 cells of *Agrobacterium tumefaciens* strain B6, and 0.05 mg of the experimental material were added to the potato discs. See Table I for more complete explanation.

[b] Twenty-five discs were used per determination.

[c] Standard error.

[d] Per cent inhibition.

RESULTS AND DISCUSSION

Table I lists the effects of all of the samples tested on the initiation of crown gall tumors on potato discs, and their corresponding activity on the P388 mouse leukemia system and KB cell cultures. The samples are listed in the experimental order in which they were assayed. A definite correlation exists between the antileukemic activity of these samples, and their ability to inhibit crown gall tumor formation on potato discs. Samples which were cytotoxic in the KB cell culture but not antileukemic did not affect the initiation of crown gall tumors. Data from a typical experiment are shown in Table II. Each sample was assayed in at least two separate experiments.

The initial step in the formation of crown gall tumors involves the attachment of the bacterium to a tumor-binding site (9, 11). This attachment on the potato disc system is complete within 15 min following inoculation (9). To test the possibility that any of the active test materials were in any way preventing bacterial attachment, they were added at various times after the inoculation of the bacteria to the potato discs (Table III). The amount of inhibition obtained with the active samples is consistent whether these extracts are added to the potato discs simultaneously with the bacteria, or 15–30 min following bacterial inoculation. These results eliminate any possible effects of these samples on bacterial attachment. These active samples did not affect bacterial viability when added to the bacterial growth medium.

To rule out the possibility that these results are unique to tumor induction by *A. tumefaciens* strain B6, several of the experimental compounds were assayed for their activity on tumor initiation by TT-107, another virulent strain of *A. tumefaciens*. The results were similar to those found when B6 was used as the initiating strain.

The results of this study show a definite correlation between the antitumor activity of these samples on the P388 leukemia system and their ability to inhibit crown gall tumor initiation on potato discs. Although further studies are necessary, it appears that the crown gall tumor bioassay could be used as an aid in the screening of potential antitumor compounds. The advantages of such a system with respect to cost, time, and ease of preparation are obvious. In a related study, Richardson and Morré (15) observed that D-glucosamine, an inhibitor of several animal tumor systems (2), caused the regression of crown gall tumors on bean leaves. We are currently investigating whether our active samples can affect the growth of crown gall tumors in the potato disc system.

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