SCREENING METHODS FOR DETERMINING ANTIBIOTIC ACTIVITY OF HIGHER PLANTS[1]

H. J. CARLSON AND HARRIET G. DOUGLAS

Western Reserve University, School of Medicine, Department of Pediatrics, and the Babies and Childrens Division, Hospitals of Cleveland, Cleveland, Ohio

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Plants and their extracts have been used for centuries as remedies for the cure or alleviation of disease. Bigelow (1818) in his monographs depicts the toxicities and simple chemical reactions of numerous plant extracts. More recent investigators (Boas, 1934; Boas and Steude, 1935; Keding, 1939; Lucas and Lewis, 1944; Osborn, 1943; Schmidt, 1942; Seegal and Holden, 1945; Sanders *et al.*, 1945; Irving *et al.*, 1946; Little and Grubaugh, 1946; Heatley, 1944; Southam, 1946) have found many plants to contain substances active *in vitro* against both gram-negative and gram-positive bacteria and fungi. Carlson, Bissell, and Mueller (1946) have shown several of these plant extracts to be active *in vivo* against malarial and bacterial infections. The authorities used for classification of the plants collected were Peck (1941) and Britton and Brown (1913).

The available literature indicates that the majority of investigators have tested plant juices or water extracts on seeded agar plates in which zones of inhibition and diffusion were observed. It is the purpose of this report to relate other types of solvents which may be used to recover potential antibiotic substances from plants.

Methods of extraction. Fresh green plants were collected and stored in the cold room until prepared for testing. Those portions of the plant to be tested were macerated in a mortar or Waring blender after the addition of a volume of 0.9 per cent sodium chloride solution, equivalent to one-half the amount of plant material. Separate extractions were made of the flowers, stem, leaves, root, and seeds or buds of larger plant specimens. After the macerated plant suspension had been allowed to stand at room temperature for a period up to 1 hour, portions of it were placed in four large test tubes. To the first tube was added an equal amount by volume of 1.5 per cent sulfuric acid; to the second tube an equal volume of solution buffered at pH 4.0; to the third tube an equal volume of solution buffered at pH 9.0; and to the fourth tube an equal volume of ethyl ether. The contents of each tube were thoroughly mixed and placed in the cold room along with the saline extract for 24 hours. Just prior to being tested for antibiotic activity, the 1.5 per cent sulfuric acid extract was neutralized with 4 per cent sodium hydroxide, the supernatant being used for assay.

Removal of dissolved chlorophyll in the ether extract was accomplished by adsorption on charcoal (norite A) or kaolin. Two per cent by volume of the adsorbing agent was thoroughly mixed with the ether extract and allowed to

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stand at room temperature for 1 hour. Filtration was employed for the removal of the adsorbing agent.

Method of assay. The Oxford cup method of assay for antibiotic substances was employed throughout these studies. Freshly isolated strains of *Escherichia coli* and *Staphylococcus aureus* (hemolytic) were used as the test organisms. One ml of an 18-hour broth culture was added to 250 ml of the medium, and 20 ml of this shake culture were poured into sterile petri dishes. The lids of the petri dishes were left ajar for 30 minutes to allow drying of the agar surface. Sterile porcelain cylinders were placed on the surface of the seeded agar and filled with the aqueous plant extracts. Six drops of the ether extract, both before and after the removal of chlorophyll, were placed directly on the seeded agar and allowed to evaporate. The charcoal and kaolin used as adsorbing agents were tested in the same manner. The plates were incubated at 37 C for 24 hours.

With all aqueous solutions, zones of inhibition were measured in mm. In testing ether extracts and adsorbing agents, zones were noted as to completeness of inhibition and degree of diffusion of antibiotics. Stimulation of growth of the test organism was also recorded.

RESULTS

Table 1 summarizes the results obtained using extracts of 14 plants against *Escherichia coli* and *Staphylococcus aureus*. Saline extracts of 6 of the 14 plants contained substances which were bacteriostatic or bactericidal in character. One plant of this group, *Allium cernum*, contained antibacterial substances markedly effective against both the gram-negative and gram-positive organisms used. The other five saline extracts were observed to have only fair activity against either the gram-negative or the gram-positive organisms.

The acid-soluble fractions from seven plants (1.5 per cent sulfuric acid, neutralized) were observed to be active inhibitors of bacteria. The extract of *Humulus lupulus* was found to inhibit completely the test organisms with good diffusion of the active substances. The inhibitory activity of the six remaining acid extracts was only partial with little or no diffusion.

Eight of the pH 4.0 extracts of the plants were found to be active against the test organisms. *Rudbeckia lacinata* and *Allium cernum*, pH 4.0 buffered extracts, inhibited both test organisms with good diffusion of the active agent.

Using a buffered solution of pH 9.0 as an extractive, it was observed that eight of the plants inhibited the growth of the test organisms in varying degrees. The extract of *Rudbeckia lacinata* inhibited both organisms with 3 plus activity. The extracts of *Physalis subglabrata* and *Humulus lupulus* showed greater inhibitory activity against the gram-positive organism than against the gram-negative organism.

The ether extracts of ten of the plants were observed to contain active substances, as indicated by the inhibition of the test organisms. Five of these extracts were inhibitory to both test organisms, with the remaining five having substances active against only the gram-positive organism. It was noted that this

solvent revealed the presence of active substances in *Hypericum multilum*, leaf, stem, and root of *Abutelon abutelon*, and stem of *Silphium perfoliatum*, whereas the aqueous solutions were negative.

TABLE 1

Activity of plant extracts

PLANT	pH OF SALINE	EXTRACTS INHIBITION									
		Saline		1.5% H_2SO_4		4.0 Buffer		9.0 Buffer		Ether	
		Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli
Cannabinaceae											
Humulus lupulus	6.1	−	−	++++	+++	+	++	+++	++P	C-E	C-E
Compositae											
Chrysopsis mariana	6.0	++P	++	+P	+P	++P	+P	++	+	P-E	C-E
Helianthus gigantens	6.4	−	−	−	+	−	−	−	−	−	−
Rudbeckia lacinata											
Stem	5.7	−	+P	−	−	+++	+++	+++	+++	−	−
Leaves	5.3	−	+P	+P	−	−	−	−	−	−	−
Flower	5.2	−	−	+P	+P	−	−	−	−	−	−
Silphium perfoliatum											
Stem	6.3	−	−	−	−	−	−	−	−	P-A	−
Leaves	6.3	++	−	−	−	−	−	−	−	−	−
Flower	4.4	+	−	−	−	−	−	−	−	−	−
Xanthium americanum	6.7	−	−	+	+	−	−	++	−	C-G	−
Corniaceae											
Cornus nutallii Aud.											
Stem	5.6	−	−	−	−	+	+	−	−	−	−
Leaves	6.2	−	−	−	−	−	−	++	++	−	−
Seed	5.2	−S	−S	−	−	−	−	−	−	−	−
Fabaceae											
Mubomia canadense	6.1	−	+	−	−	+	−	+P	++	P-A	P-A
Hypericaceae											
Hypericum multilum	6.6	−	−	−	−	−	−	−	−	C-E	−
Malvaceae											
Malva rotundifolia											
Stem	6.0	−	−	−	+	++	−	+	−	P-A	−
Leaves	6.3	−	++	−	−	−	−	−	−	P-A	−
Root	5.7	−	−	−	−	−	−	−	−	P-A	−
Liliaceae											
Allium cernum	5.8	++++	+++	−	−	+++	+++	−	−	C-F	P-F
Smilacina racemosa	5.1	−	−	−	−	−	−	−	−	−	−
Solanceae											
Physalis subglabrata	−	−	−	−	+	+	−	−	++++P	+P	C-A	C-A

Legend: −, no inhibition; ++++, 26 mm or better zone of inhibition; +++, 19–25 mm zone of inhibition; ++, 14–18 mm zone of inhibition; +, 9–13 mm zone of inhibition; P, partial inhibition; E, excellent diffusion; G, good diffusion; F, fair diffusion; S, growth stimulated; C, complete inhibition; A, inhibition in area exposed.

To rule out the activity of chlorophyll, which might be the inhibitory substance in the ether extracts, several were adsorbed on charcoal or kaolin. The resultant clear ether extracts were tested and the results are depicted in table 2.

Eight plants among those investigated were observed to contain inhibitory

substances in the original ether extracts. After adsorption of chlorophyll on charcoal, *Hypericum perforatum, Tovara virginiana, and Helianthus annuus* ether extracts were found to retain their active inhibitory substances against the test organisms. The charcoal did not contain any of the active substances when tested on a seeded agar plate. *Ribes bracteosum* Dougl., *Madia elegans* Dougl., and *Ceanothus velvitimus* (var. *laevigatus* Dougl. T. G.) ether extracts were also observed to retain the active substances after adsorption with charcoal and kaolin. When the chlorophyll was adsorbed by charcoal and kaolin, the active agents in the ether extracts of *Bidens frondosa* L. (leaf) and *Xanthium pennsylvanicum* (leaf and stem) were removed from solution. Further tests confirmed the presence of the active agents in the charcoal and kaolin.

DISCUSSION

In the search for antibiotic material from higher plants, the possible active substances cannot be foretold, thus a screening technique devised to test various types of extractives would seem indicated. An essential step in extractions should be the breaking up of plant cells. This may be accomplished through alternate freezing and thawing, enzymatic hydrolysis (autolysis), or maceration (grinding with sand, Waring blender, ball mills, hydraulic press, etc.). After cell rupture, several general methods of extraction have been used. The following have been tried in this laboratory:

(1) Extract with water (or 0.9 per cent saline). This will remove inorganic compounds and a few enzymes. It will also remove most albumin, histones, protamines, proteases, peptones, and similar amino acids.

(2) Dilute weak acids (acetic, etc.). This will remove glutelins, several enzymes, and possibly metaproteins and albuminoids.

(3) Dilute weak base (sodium bicarbonate, etc.). This will remove principally acidic compounds and glucosides.

(4) Weak solutions of strong acids (1.5 to 5 per cent sulfuric acid). This will remove all alkaloids and similar substances of a basic nature.

(5) Ether extraction. This will remove chlorophyll, waxes, and sterols. It will also denature proteins and enzymes.

The foregoing five solvents have been used by the authors to test over 300 plants involving more than 1,500 extracts. The solvents were selected to yield extracts of varied types of material in which potential inhibitory substances might exist. The results of the use of multiple extractions are shown in table 1. If only saline had been used, one plant of the 14 used would have yielded a substance that merited further study, though several of the other aqueous extracts did show slight to fair inhibition. By using the other solvents in conjunction with saline, evidence of the presence of some type of inhibitory substance was observed in all plants except one.

The use of ethyl ether as a solvent brings up the question whether the dissolved chlorophyll acts as the inhibitory agent. This has not proved to be the case, as many plants tested have shown no activity from the ether solutions containing chlorophyll. Further evidence to substantiate this observation is shown

in table 2. Several ether extracts of plants were adsorbed on charcoal and kaolin. Chlorophyll was removed by the adsorbing agents, and simultaneously in several instances the active principles were partially or completely adsorbed. These adsorption experiments appear to indicate the presence of two or more active substances, though at this writing the authors are not able to furnish further information.

Sanders, Weatherwax, and McClung (1945) found the juice of *Xanthium pennsylvanicum* to show only fair activity against the gram-negative test organism. When using the five suggested solvents, extracts of *Xanthium pennsylvanicum* were found to be inhibitory against both gram-positive and gram-

TABLE 2

Activity of ether extracts and adsorption agents

PLANT	INHIBITION							
	Ether Extracts				Charcoal		Kaolin	
	Before*		After*					
	Staph.	Coli	Staph.	Coli	Staph.	Coli	Staph.	Coli
Compositae								
Bidens frondosa L. leaf	C-G	C-F	N	N	N	P-A	P-A	P-A
Helianthus annuus	C-F	C-F	C-F	C-F	N	N		
Madia elegans D. Don.	C-A	C-A	C-G	C-G	P-A	P-A	P-A	C-A
Xanthium pennsylvanicum	P-A	C-P-A	N	N	P-A	P-A	C-P-A	P-A
Hypericaceae								
Hypericum perforatum	C-G	C-E	C-E	C-E	N	N		
Polygonaceae								
Tovara virginiana	C-E	C-G	C-E	C-E	N	N		
Rhamnaceae								
Ceanothus velutinus (var. *laevigatus* Dougl.)	C-A	C-A	C-G	C-G	P-A	P-A	P-A	P-A

Legend: C, complete inhibition; P, partial inhibition; A, area exposed; F, fair diffusion; G, good diffusion; E, excellent diffusion; N, no inhibition.

* Adsorption.

negative organisms. The active substances were soluble in saline, ether, and alkaline (pH 9.0) solutions. The use of other solvents brought out further evidence of the antibiotic substances present in this plant which were probably not active or in a diffusible state in the pressed juice.

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SUMMARY

The screening of plants for the presence of antibiotic substances would seem to require the testing of extractives prepared by the use of more than one solvent. Extractions with saline, strong acid, ether, weak acid (buffered at pH 4.0), and weak alkali (pH 9.0) were found to be necessary before a plant could be discarded as having no antibiotic activity. The results of the use of various solvents in the extractions of a small series of plants are presented.

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