Aquatic Plants from Minnesota
Part 3 - Antimicrobial Effects

by

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FOREWORD

This Bulletin is published in furtherance of the purposes of the Water Resources Research Act of 1964. The purpose of the Act is to stimulate, sponsor, provide for, and supplement present programs for the conduct of research, investigations, experiments, and the training of scientists in the field of water and resources which affect water. The Act is promoting a more adequate national program of water resources research by furnishing financial assistance to non-Federal research.

The Act provides for establishment of Water Resources Research Centers at Universities throughout the Nation. On September 1, 1964, a Water Resources Research Center was established in the Graduate School as an interdisciplinary component of the University of Minnesota. The Center has the responsibility for unifying and stimulating University water resources research through the administration of funds covered in the Act and made available by other sources; coordinating University research with water resources programs of local, State and Federal agencies and private organizations throughout the State; and assisting in training additional scientists for work in the field of water resources through research.

This Bulletin is number 48 in a series of publications designed to present information bearing on water resources research in Minnesota and the results of some of the research sponsored by the Center. The Bulletin is concerned with the investigation of the antimicrobial activity of 22 Minnesotan aquatic plants. The investigation is part of a research project aimed at finding a nutritional, medicinal or industrial use for the unwanted aquatic plants in lake shoreline areas. It is possible that some aquatic plants may contain industrially useful gums-mucilages, or new useful antimicrobial, anticoagulant, or antineoplastic therapeutic principles. If a good industrial, medicinal, or nutritional use for aquatic plants can be discovered, the results of the research could provide an economic incentive for aquatic plant collection and control. The successful completion of the project may significantly assist the State and Nation in partially solving their lake pollution problems.

This Bulletin is related to the following research project:

OWRR Project No.: A-025-Minn.

Project Title: Alleviation of Lake Pollution by Utilization of Aquatic Plants for Nutritional, Medicinal or Industrial Purposes

Principal Investigator: E. John Staba, Dept. of Pharmacognosy, College of Pharmacy, University of Minnesota

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Publication Abstract:

In this study, the antimicrobial activity of the following 22 Minnesotan aquatic plants was investigated: Anacharis canadensis, Calla Polustris, Carex lacustris, Ceratophyllum demersum, Chara vulgaris, Eleocharis smallii, Lemna minor, Myriophyllum exalbescens, Nuphar variegatum, Nymphaea tuberosa, Potamogeton amplifolius, P. natans, P. pectinatus, P. richardsonu,

P. zosteriformis, Sagittaria cuneata, S. latifolia, Sparganium eurycarpum, S. fluctuans, Typha angustifolia, Vallisneria americana, and Zizania aquetica. Furthermore, the chemical constituents responsable for the significant antimicrobial effect were isolated and identified.

The skellysolve F, chloroform, 80% ethanol and fresh water extracts of plant species were tested for antimicrobial activity employing the qualitative filter paper disc diffusion method and reference antibiotic discs. Ethanol (80%) extracts of Myriophyllum exalbescens (activity ratio of 0.34 as compared to the 30 mcg chloramphenicol discs), Nymphaea tuberosa (leaf: 0.40, stem: 0.38) and Nuphar variegatum collected in Lake Minnetonka (leaf: 0.43, stem: 0.45) were moderately active against S. aureus. Ethanol (80%) extracts of Carex lacustris (activity ratio of 0.34 as compared to the 10 mcg streptomycin discs), Nymphaea tuberosa (leaf: 1.01, stem: 1.10) and Nuphar variegatum collected in Lake Minnetonka (leaf: 0.73, stem: 0.58) were active against M. smegmatis. All extracts were relatively inactive against E. coli except the water extract of Potamogeton natans where a low activity ratio of 0.10 as compared to the 30 mcg chloramphenicol discs was indicated. Skellysolve F stem extracts of Nuphar variegatum collected in the Pine Lake and Sparganium fluctuans showed a rather distinct action against C. albicans, the activity ratio as compared to the 100 units mycostatin discs were 2.06 and 1.08, respectively. Regarding antifungal activity, ethanol (80%) extracts of Carex lacustris (activity ratio of 1.08 as compared to 5% aq. phenol standard), Nymphaea tuberosa (stem: 0.72) and skellysolve F extract of Potamogeton zosteriformis (0.60) were active against Alternaria sp., 80% ethanol stem extracts of Nymphaea tuberosa and Nuphar variegatum were active against F. roseum with the activity ratios of 0.41 and 0.48, respectively, as compared to the 5% aqueous phenol standard. In general, the plant pathogenic fungi are more resistant than animal pathogenic organisms toward the actions of aquatic plant extract

Publication Descriptors: *Aquatic Plants/ *Antimicrobial Activity/ *Minnesota/ Bacteria/ Fungi/ Protozoa/ Pathogens/ Microorganisms/ Antifungal Activity

Publication Identifiers: *Skellysolve F/ *Filter Paper Disc Diffusion Method/ *Zones of Inhibition

I. INTRODUCTION

A. Scope of the Study

Minnesotan aquatic plants have not been previously studied for their antimicrobial activities. The two previous reports had been concerned about the chemical constituents (1) and pharmacological properties (2) of aquatic plants from Minnesota. In this study, the antimicrobial activity of those plant extracts was investigated. Furthermore, the chemical constituents responsable for the significant antimicrobial effect were isolated and identified.

B. Survey of the Literature

An antimicrobial substance is usually applied to an organic compound which in low concentration will kill or inhibit bacteria, fungi, or protozoa. Relatively complete review of antimicrobial substances from higher plants are presented chronologically by Osborn in 1943 (3), Florey in 1949 (4), Skinner in 1955 (5), Nickell in 1959 (6), Hiller in 1964 (7) and Farnsworth in 1966 (8).

1. Antimicrobial substances from terrestrial plants

a. Against animal pathogens

Numerous investigators have surveyed plants in many countries and have demonstrated the wide occurrence of antimicrobial compounds in higher plants. For instance, Atkinson (9-12) in Australia, Chang (13) in China, George (14,15) in India, Okazaki (16-23) and Hasegawa (24) in Japan, Nakanishi (25) in Malaysia, Jiu (26) in Mexico, and in the United States by Azarbwicz (27) and Hughes (28) in Southern California, Madsen (29,30) and Pates (31) in Florida, Bushnell (32) in Hawaii, Sanders (33) in Indiana, Huddleson (34) in Michigan, and Hayes (35) and Carlson (36,37) in Ohio and Oregon.

Additional works on individual family (38) or plant (39-47) are numerous.

Antimicrobial screening aimed at different classes of natural products have also been studied. Okazaki (48,49) demonstrated antimicrobial action of essential oils against pathogenic fungi and various bacteria. Perfume oils were found to possess remarkable antimicrobial property with greater activity against fungi than against bacteria (50). Some alkaloids, such as berberine (51), terpene alkaloids and delphisongorine (52) have been found to be potent antibiotics against various pathogenic organisms. Recently, chrothiomycin was found to be a new pigment antibiotic (53).

Some workers have been engaged mainly with the discovery of antitubercular antibiotics (54,55,56).

b. Against plant pathogens

Gottlieb (57) and Irving (58) successively announced the discovery of a substance in the juice of the tomato that strongly inhibited the growth of Fusarium oxysporum var. lycopersici, the organism causing Fusarium wilt of tomatoes. This substance was first named lycopersicin but is now known as tomatin. Hawley (59) and Sowder (60) tested the aqueous extracts of the woods of several trees and found they were toxic to two wood-rotting fungi, Fomes annosus and Lentinus lepideus. The growth of the latter fungus was also inhibited by the volatile constituents distilled from the wood, Thuja plicata. Antifungal activity against Fusarium was found by Little (61) in some crude plant saps and by McDonough (62) in the alcohol and water extracts of the bark of some trees. Nene et al. (63,64) in their search for antifungal agents, discovered that aqueous extracts of Erigeron linifolium and Anagellis arvensis possessed strong antifungal activity against Colletotrichum papayae. The antifungal agents in A. arvensis were further isolated and qualitatively characterized by Khanna (65). The antifungal factors, hordatines present in barley were isolated and synthesized by Stoessel (66). Additional antifungal constituents have been found in Dioscorea (67), rice bran (68), citrus plants (69) and Pterocarpans (70).

Tannin (71), saponins (72) and phenols (73) have been found to have a fungistatic effect and may play a role in the resistance of a plant toward some fungi.

2. Antimicrobial substances from aquatic plants

a. Fresh water plants

Several Russian scientists have studied two aquatic plants for their antimicrobial activity. The alkaloid, nupharine from Nuphar luteum has in vitro and in vivo activities against 45 strains of 23 phytopathogenic bacteria. It was most effective against Corynebacterium, especially C. michiganense and less effective against Xanthomonas (74). Root extracts of Nuphar luteum had strong antibacterial activity against Gram positive bacteria (75), Protista, and Trichomonas (76). The lupine alkaloid, deoxynupharidine from Nuphar japonicum had inhibited slightly Mycobacterium tuberculosis, but was ineffective against Staphylococci and Streptococci. Okazaki (23,77) conducted thorough screening of plants living in and around the water in Japan for their activity against Staphyloccus aureus. The results concerning aquatic species of interest are shown in Table 1.

b. Marine plants

Ether, cold water and hot water extracts of many seaweeds were tested for their antibacterial activity against Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa by Pratt (78). The results showed that the algae collected in Spring and Fall possessed definite antibacterial activity whereas those collected in Winter were totally inert. Burkholder (79) also reported the presence of antibiotic activity in some marine algae of Puerto Rico. Duff (80) tested cell extracts of fourteen species of marine phytoplanktons for antibacterial activity against nine marine isolates and 14 strains of terrestrial saprophytes. Extracts of many marine sponges had inhibited the marine bacteria isolated from widely different geographic areas (81).

Table 1. Antimicrobial Activity of Aquatic Plants. Against S. aureus[1/] (77)

Plant name	I	II
Carex albata		L. R.
Carex brunnea	L. St.	Fr.
Ceratophyllum demersum		T.
Chara coronata		T.
Eleocharis japonica		T.
Elocharis plantaginea	R.	L.
Myriophyllum spicatum var. muricatun		T.
Myriophyllum verticillatum		T.
Nymphaea peltarum		T.
Nymphaea tetragona var. angusta		L. St. Fl. R.
Potamogeton franchetii		L. St.
Potamogeton malaianus		L. St. Fl. Fr.
Potamogeton Oxyphylsus		T.
Potamogeton teganumensis		L. St.
Sagittaria aginashi	St. R.	L. Fl.
Sagittaria trigolia var. typica	St. R.	L.
Sparganium glomeratum	Fl. R.	L. St.
Typha angustata		L. Fl.
Vallisneria spiralis var. asiatica		T.

1/I- Plant parts with positive activity L.- leaves; St.- stems; R.- roots; Fr.- fruits; Fl.- flowers; T.- total plant; II- Plant parts with negative activity.

Chlorophyll derivatives are believed to be responsible for the antibacterial activity observed in some algae. Jørgensen (82) observed growth-inhibition of Bacillus subtilis by photo-oxidized chlorophyllides and pheophytin-like substances obtained from three species of Chlorophyceae. Sieburth (83) reported some evidence which suggested that part of the wide-spectrum activity of the diatom Skeletonema costatum might be due to chlorophyll derivatives. Bruce (84) found several active chlorophyll-a derivatives (probably pheophytin-a and an atypical chlorophyllide-a) in Isochrysis glabana. Therefore, active chlorophyllides may play a significant antibacterial role in the microbial ecology of natural waters.

C. General Techniques

Antimicrobial screening techniques fall mainly into three groups, i.e., diffusion, dilution and bioautographic methods. Various modifications of each have been devised. Factors such as the test microorganisms and media, concentration of inoculum, incubation period and temperature, etc. all affect the screening result. Therefore, the antimicrobial screening system should be adequately standarized and controlled in order to give reproducible results and to establish a fixed reference point for comparison.

The preparation of plant materials for screening varies considerably. Earlier workers have pressed out the sap from fresh plants by means of presses (33,57,61) or by crushing the plants with sand in a mortar (3). Certain workers have used steam distillates of plants (38,60). Saline (85,37), buffered acidic and basic extracts (36) were used by Carlson et al. and aqueous

extracts were used by many investigators (14,16,42,63). Ma (54) developed a micro-scale extraction technique by which aqueous extract from 350-550 mg of dry plant material can be made. Organic solvents such as ethanol (15,62,86,87), methanol (25), acetone (17), chloroform (14), ether (88), petroleum ether (41) and benzene (87) have all been used for the extraction of antimicrobial principles. Since antimicrobial compounds can be extracted by various solvents, the sequential solvent extraction seems to be the most logical system for screening purposes. Plant extracts obtained have to be adjusted so that the pH values fall within the limits at which the test organism will grow. If this precaution is followed, inhibitory effects due to the antibiotics may be distinguished from those due to pH alone.

The results of an in vitro test by any of the generally used methods should not be interpreted in terms of absolute values, since they may show considerable variations with alterations in the conditions of test. For example, an inoculum of 100 cells/ml in a test system will be inhibited by considerably less antimicrobial agent than will 100,000 cells/ml and this discrepancy may be extreme when the inoculated cells are capable of destroying the antimicrobial agent. The presence of an antibiotic substance in a plant extract will be influenced by a number of factors such as the stability of the active principles to various treatments during preparation and testing of the extract, the solubility of the principle in the solvent or solvents used and the ability of the principle to respond to the particular test employed. The results with inhibition or noninhibition of bacterial growth can not be considered as final since the concentration of the active principle is an unknown factor. It is possible that in some instances, materials used merely appeared specific to a certain organism because the antibacterial principle was present in a concentration too low to affect the other organisms (56).

1. Diffusion methods

In the diffusion method, a substance suspected of containing an antibiotic is placed on a solid (e.g., agar) nutrient medium which has been seeded with a suitable test microorganism. During incubation of the culture, the antibiotic, if present, will diffuse out into the medium and affect the growth of the test organism. The distance to which an inhibitory compound has moved is indicated by a clear zone of inhibition of growth.

a. General procedures

Any agar medium may be used provided that it permits rapid growth of the test organism. The preparation of different kinds of media for various organisms has been discussed by Collins (89). Medium may be seeded throughout with the organism while it is still in the liquid form (bulk-seeding) or on its solidified surface. Spreading, flooding or spraying have been used as means of surface seeding. Plates prepared with seed agar containing either a light or a heavy inoculum of Staphylococcus aureus can be stored at 5°C for a maximum of twenty eight days (90).

Human and animal pathogenic bacteria are usually incubated at 37°C but rather lower temperatures are commonly used for plant pathogenic fungi (e.g., 18-28°C). The incubation period is usually 18-24 hours for bacteria and 2-6 days for plant fungi. At the termination of the incubation period, the results can be recorded in terms of diameter in millimeters of the clear zones developed.

b. Advantages and disadvantages

Diffusion methods for antimicrobial screening have long been popular because of their relative technical simplicity, the presence often of sharp margins, and their rapidity. However, their reliability has been questioned (91) because of the large number and variety of methods available and lack of uniformity in interpreting the results. Two microorganisms may have an identical sensitivity for a certain antibiotic as determined by dilution test, yet the zone sizes may vary widely.

c. Methods

1) The direct testing of plant tissues

Osborn and Harper (44) germinated seeds of Leptosyne maritima and then transferred the young seedlings to Petri dish plates of agar medium which had previously been uniformly seeded with Staphylococcus aureus. Transverse slices of stem were also made and placed on the seeded agar plates. After incubation for a suitable time, the widths of the inhibition zones caused by diffusion of the inhibitor from the tissues were recorded. Atkinson (12) used the same technique in her screening of Australian plants. Pratt (78) directly placed disks of tissue cut from algae on the seeded agar plates.

2) "Ditch" and filter paper strip methods

A "ditch" filled (92,93) or a filter paper strip (94) impregnated with an extract is positioned near the center of a Petri plate. Microorganisms are then streaked at right angles to the ditch or the filter strip and the area of inhibitions observed.

3) Gradient plate method

This method was developed by Szybalski (95) and had been applied by Braude (96). To establish the gradient, 20 ml of plain nutrient agar is allowed to harden with the plate slanted sufficiently so that the entire bottom is just covered. With the dish in the normal horizontal position, another 20 ml of agar containing the appropriate concentration of extract is added. Downward diffusion of the extract, which in this way becomes diluted in proportion to the ratio of the thickness of the agar layers, establishes an uniform, linear concentration gradient.

The gradient plate method determines an MIC (minimum inhibitory concentration) value in case of pure antimicrobial principle, and gives some indication of the nature of the inhibition of the microorganisms, as demonstrated by the sharpness of the end point at the termination of the streak. The technique permits simultaneous testing of several microbial strains against differing concentrations of extract but it requires many plates to assay a single microorganism against several extracts.

4) Cylinder-plate method

This is a technique introduced by Schmidt and Mover (97) and developed for antimicribial screenings by Irving and Fontaine (58), Little, (61), Carlson (37) and Atkinson (11).

Several sterile, short, open-ended cylinders of glass or vitreous porcelain, such as penicylinders (61) are placed on the surface of an agar plate which has previously been seeded with the test organism. Each cylinder is placed so that one end seals on to the agar to form a cup which is then filled with the solution to be tested, the dish cover restored, the plate incubated, and the zone of inhibition measured after a suitable time.

5) Cup-plate method (Hole-plate method)

The cup-plate method was originated from the Oxford cup method used in the assay of penicillin (98). Seegal et al. (38) first utilized the modified Oxford cup method (99) in their study of Ranunculaceae plants, later, many other investigators have applied this method in their screening programs (15,33,36,51,100-102). Solutions to be tested are placed in small depressions in the surface of the medium instead of within cvnlinders resting on the surface. These cavities may be made by removing small discs of the medium or by placing moulds in the dish while the medium is poured. The advantage of this method is that the presence of suspended particulate matter in the liquid being tested is less likelv to interfere with the diffusion of the antibiotics.

6) Filter paper disc method

Filter paper disc method was first introduced for use in antimicrobial screening by Sherwood (103) and Lucas (104). It was later used by Maruzzella (50), standarized by Hoette (105), and in recent vears is commonly used for antimicrobial screening (4,80,81).

The disc method consists of placing small discs of filter paper impregnated with a certain amount of plant extract on the surface of an inoculated culture plate. Several of these can be applied to a single plate at the same time. The plates are incubated for an appropriate time period and the zone of inhibition around each disc is noted. Standard sensitivitv discs are now available (Difco) with different degree of antibiotic potency. Since humidity is the most significant factor in antibiotic inactivation, storage of discs in a vacuum desiccator at -20°C was recommended for their maximal stability (106).

Quantitative significance of the disc method is doubtful, for there are a number of factors which are often difficult to control, affecting the accuracy of the method. These factors have been examined in detail bv Ericsson (107) and Erlanson (93), and were discussed bv Petersdorf (108).

Erlanson (93) stated that the size of the inoculum is onlv of minor importance in determining zone size, but Ericsson (107) and Cooper (109) differ with his point of view and emphasize that the size of the inoculum is not only extremely important in determining zone size but that it is the most difficult variable to control in routine testing. They base the importance of inoculum size on the fact that the time necessary to reach a critical bacterial population will vary with the number of organisms present at the beginning of the test. However, regardless of the multiplicity of the organisms present at the onset, the critical density of organisms will probably be reached within two to six hours after the start of the incubation, a time at which the concentration of the antibiotic near the disc is relatively constant (104). Erlanson (93) demonstrated that the logarithmic or stationary phase of growth will have no significant effect on zone size.

In spite of these many variables, Duff (80) was able to standardize conditions and quantitation of the disc method was claimed to be obtainable. With the disc method there is an approximately linear inverse relationship between the logarithm of antibiotic concentration and the size of the zone of inhibition (110,111). On the other hand, different slopes reflect different modes of action of antibiotics.

2. Dilution methods

Rammelkamp (112) first described the dilution technique in his study of the resistance of Staphylococcus aureus toward the action of penicillin. This method is often used for the detection of antimicrobial activity in selected plant species (41,87).

a. Broth tube dilution method

Briefly, this method involves exposing microorganisms to an increasing or decreasing concentrations of antimicrobial substances in liquid media, usually by serial two-fold dilution. The mixture consisting of microorganisms, nutrient medium, and antibiotic is then incubated at appropriate temperature for a certain period of time depending on the microorganism used, and the point at which no visible growth occurs (the first tube showing no turbidity to the naked eye) is defined as minimal inhibitory concentration (MIC).

The tube dilution test has several distinctive advantages: i) It permits testing of organisms with two or more antibiotics simultaneously and provides information about antimicrobial synergism and antagonism; ii) It yields the minimal inhibitory concentration in quantitative terms, enabling direct comparison of various antibiotics; iii) It provides a simple method for measuring bactericidal end points (113). In general, therefore, the tube dilution test has become the standard method in the clinic where exact microbial sensitivities are desired.

This test has some drawbacks, however, it is not suitable for identifying resistant clones within a bacterial culture and for recognizing contaminants (92). Occasionally, there is some difficulty in reading the end point (113). Dilution methods are more expensive than diffusion methods both in time and materials, and they require more technical skill. Therefore, the tube dilution techniques are seldom used for antimicrobial screening.

b. Agar plate dilution method.

Agar plate dilution method is a modification of the tube dilution method and it was first used by Waksman (114) and later by Waksman (114) and later by Okazaki (48) and Nakanishi (25) in the search for antimicrobial agents.

The principle of this test is based upon the inhibition of microorganism growth on the surface of agar plates prepared with samples in a series of increasing concentrations (92,93,107,113,115,116). An advantage of this method is that variations of sensitivity within bacterial strains can be detected and contaminants identified. Furthermore, because a number of cultures can be planted on a single plate, this technique leads itself well to large-scale studies.

3. Bioautography

Bioautography involves the use of paper partition-, thin-layer chromotographic, or electrophoretic technique coupled with a microbial indicator. An advantage of this technique is the application of a chemical spray to a duplicate chromatogram for the qualitative identification of the antibiotic (117). However, there are quantitative limitations to the procedure. Factors such as diffusion and distortion of the sample during chromatography, as well as the amount and nature of impurities, affect the shape and size of the zone of microbial inhibition.

a. Paper chromatography

Winsten (118) was the first to bring about the concept of bioautography on paper. In his work, a seeded agar layer was laid on top of hardened base agar layer, various paper chromatograms (in strips) were put parallel to each other on the agar plate and allowed to soak for a few minutes in order to transfer the compounds from the strips to the agar plate. The strips were then removed leaving their imprints on the agar surface. The zones of inhibition were measured after incubation.

b. Thin-layer chromatography

The general technique is to press the developed thin-layer plate face down to the surface of agar seeded with a microorganism. To avoid the adherence of the adsorbent to the agar surface, agar plates inoculated with sensitive microorganisms were covered with filter paper on which the thin-layer chromatograms were pressed. After diffusion of the antibiotics from the thin-layer plates, the agar plates were incubated at 37°C for 16-18 hours and the inhibition zones examined (119). Nicolaus et al. (120,121) applied TLC for the separation of tetracyclines, penicillins and rifomycins, and elaborated a technique for the bioautographic detection of these antibiotics. Brodasky (122) described a separation of the neomycins on a thin-layer of carbon. After development with a solvent system, the carbon plate was pressed on to an agar plate which was inoculated with sensitive bacteria. Sephadex thin-layer chromatography was used by Zuidweg (123) to isolate antimicrobial substances.

A difficulty which sometimes arises is the excessive contact between a heavy glass thin-layer plate and the surface of the agar, resulting in poorly defined spots. This problem can be avoided by use of a light silicic acid-glass fiber sheet (124) or Eastman chromatographic sheets (125) which can be cut into the desired sizes with a pair of scissors.

c. Electrophoretic chromatography

The supporting medium can be paper, cellulose acetate, starch, alumina, silica gel, charcoal, powdered glass, gelatin, plastic gel and different types of agar. The latter medium proved to be the most convenient and satisfactory. In Lightbown's work (126), small volume of sample was applied to the surface of the agar by means of a syringe. After electrophoresis for the pre-determined time (about three hours) at the chosen current, the separated components were tested as follows: A second layer of nutrient agar seeded with a suspension of the test organism is poured on the developed electrophoresis chromatogram and the double layer of agar incubated at 37°C for 18 hours. After incubation, the positions of the antibiotic spots were evident as zones of inhibition.

d. Preparative

Thin-layer chromatograms of antibiotics detected by the above mentioned methods can not be used for preparative purposes. Betina (127) developed a preparative bioautographic technique by modifying a method described by Mistryukov (128). Mistryukov prepared "reprints" from thin-layer chromatograms, using strips of filter paper which had been pressed on the thin-layer chromatograms while still wet. Compounds from the chromatograms diffused into paper strips where then detected by suitable color reactions or for the bioautographic detection. After the positions of antibiotics on the "reprints" were known, they were isolated from the original preparative-layer chromatograms.

4. Miscellaneous methods

a. Detection of volatile antibiotics

The pulp from a crushed plant (such as garlic or clove) is placed in the lid of an inverted Petri dish, the other section of which contained nutrient agar seeded or streaked with the organisms. Inbation resulted in heavy growth of the organism except in a circular area directly above the pulp where gorwth was sparse or non-existent (5).

b. Anti-luminescent test

The test is based on the fact that many antibiotic substances and chemicals, especially quinones, quench the luminescence of species of Photobacterium. The test is quick and simple to do, but does require a 15-20°C dark room for the incubation and the reading of the results (128). The test organism, Photobacterium fischeri is cultured in an aritificial sea water broth. The inoculated flask is incubated for 48 hours with occasional

shaking to aerate the solution and to make the suspension of bacteria brightly luminescent.

II. MATERIALS AND METHODS

A. Plant Extracts

The skellysolve F, chloroform, 80% ethanol and fresh water extracts of the following plant species were tested (those in parentheses are the abbreviations used in the Tables): Anacharis canadensis (Ac), Calla palustris (Cp), Carex lacustris (Cl), Ceratophyllum demersum (Cd), Chara vulgaris (Cv), Eleocharis smallii (Es), Lemna minor (Lm), Myriophyllum exalbescens (Me), Nuphar variegatum (Nv), Nymphaea tuberosa (Nt), Potamogeton amplifolius (Pa), P. natans (Pn), P. pectinatus (Pp), P. richardsonii (Pr), P. zosteriformis (Pz), Sagittaria cuneata (Sc), S. latifolia (Sl), Sparganium eurycarpum (Se), S. fluctuans (Sf), Typha angustifolia (Ta), Vallisneria americana (Va) and Zizania aquatica (Za). The collection, identification and extraction procedures were previously reported (1).

B. Screening Methods

The qualitative filter paper disc diffusion method was used. Small sterile paper discs were dipped in the extracts to be tested and placed on agar plated previously seeded with an organism. The antimicrobial activity was observed by a definite zone of inhibition about the disc, and its diameter was compared to that formed about reference antibiotic discs.

1. Animal Pathogens

a. Microorganisms and media

The following laboratory stock strains preserved in a dormant state by drying a heavy suspension of cells in sterile bovine serum were obtained from the United States Department of Agriculture, Agriculture Research Service, Northern Utilization Research and Development Division, Peoria, Illinois: Staphylococcus aureus (NRRL B313, Gram positive), Mycobacterium smegmatis (NRRL B612, acid fast), Escherichia coli (NRRL B210, Gram negative) and Candida albicans (NRRL Y477, fungus).

The liquid growth media used were 0.8% Nutrient Broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% dextrose for S. aureus and E. coli; 3% Tryptic Soy Broth (Difco) supplemented with 6% glycerin for M. smegmatis; and 3% Sabouraud Dextrose Broth (Difco) for C. albicans. When solid media were used the corresponding liquid media were supplemented with 1.5% Bacto-Agar (Difco).

b. Standards

Difco chloramphenicol discs (30 mcg) were used for S. aureus and E. coli, Difco streptomycin discs (10 mcg) for M. smegmatis and BBL

(Cockeysville, Ma.) mycostatin discs (100 units) for C. albicans.

c. Procedures

Sterile procedures were followed throughout.

1) Inoculation

Sterilized media (10 ml) were transferred to 100 x 15 mm Optilux Petri dishes (Falcon Plastics, Division of Bio-Quest, Los Angeles, Calif.). Proper concentrations of microbial suspensions were prepared from individual stock cultures incubated on a rotary shaker (New Brunswick Scientific Co., Model G53, New Brunswick, N.J.) at 80 rpm, 20°C for 5-7 days. The turbidity of microorganisms was adjusted with a spectrophotometer (Bausch & Lomb Spectronic 20, Rochester, N.Y.) at 530 mμ to a 50% light transmittance, however, M. smegmatis was adjusted to a 25% light transmittance. A disposable serological sterile pipette (Falcon Plastics) was used to transfer approximately 0.8 ml of inoculum to each Petri dish.

2) Sample application and incubation

White filter paper discs (Difco) of 6 mm diameter were dipped into either skellysolve F, chloroform, 80% ethanol, freshly prepared water extracts or the solvents, touched momentarily to the edge of the container to remove excess solvents, and deposited on the agar surface. The discs were pressed gently with forceps to ensure the thorough contact with the media. Six discs, including one standard sensitivity disc were arranged per dish. Each extract assay was conducted in triplicate.

To assure sufficient time for diffusion of the extracts into the medium, and lessen the chance of overgrowth, the plates were kept in the refrigerator for 2 hours before transferring them to the incubator (Lab-Line Instruments, Inc., Serial No. 112 T, Melrose, Park, Ill.). Inhibition zones were observed and measured after incubating the plates for 18 hours (except 36 hours for M. smegmatis) at 37°C.

2. Plant Pathogens

A. Microorganisms, medium and standard

Cultures, kept on soil, of two plant pathogenic fungi, Alternaria sp. isolated from the wheat variety Cajeme and Fusarium roseum, were obtained from the Department of Plant Pathology, University of Minnesota, St. Paul, Minnesota, and grown on Difco potato dextrose agar (PDA) slants (2.4% potato dextrose broth with 1.5% agar) at room temperature. Skellysolve F, chloroform, 80% ethanol, and fresh cold water extracts were adjusted with the corresponding solvents to a final concentration of 1 gm of dried plant material/ml. The extracts were compared to a 5% aqueous phenol standard.

b. Procedures

Screw-cap culture tubes containing 18 ml of PDA were transferred to 100 x 15 mm Optilux Petri dishes. Approximately 3 ml of 0.1% agar was added to 40-day old Alternaria sp. and F. roseum PDA slants. The cultures were scraped lightly with a spatula and the resulting suspension transferred to a 250 ml Erlenmeyer flask containing 40 glass beads and 2 one inch magnetic bars in it. The mycelia were then subdivided by mechanical stirring (Magnestir, Scientific Products, Evanston, Ill.) at speed No. 1 for 30 minutes. The mixture was filtered through a 405 μ nylon sieve (Nitex Nylon, Tobler, Ernst Kraber Inc., N.Y.) and the concentration adjusted with 0.1% agar to 2 x 10^6 mycelia/ml for Alternaria sp. and 6 x 10^5 mycelia/ml for F. roseum on a haemocytometer. Other techniques such as inoculation, placement of discs, and refrigeration were the same as for the animal pathogens. The Petri dishes were incubated at 28°C and readings made after 40 hours.

III. RESULTS AND DISCUSSIONS

The antimicrobial effects of plant extracts against animal and plant pathogens are shown in Tables 2 and 3 respectively.

Zones of inhibition were measured to the nearest millimeter with a ruler. The activity observed was expressed as the ratio of the mean values of the corrected inhibitory zones (sample inhibition zones minus solvent inhibitory zones) to that of the standard sensitivity discs. An activity ratio below 0.01 was recorded as negative.

Ethanol (80%) extracts of Myriophyllum exalbescens (activity ratio of 0.34 as compared to the 30 mcg chloramphenicol discs), Nymphaea tuberosa (leaf: 0.40, stem: 0.38) and Nuphar variegatum collected in Lake Minnetonka (leaf: 0.43, stem: 0.45) were moderately active against S. aureus. Ethanol (80%) extracts of Carex lacustris (activity ratio of 0.34 as compared to the 10 mcg streptomycin discs), Nymphaea tuberosa (leaf: 1.01, stem: 1.10) and Nuphar variegatum collected in Lake Minnetonka (leaf: 0.73, stem: 0.58) were active against M. smegmatis. All extracts were relatively inactive against E. coli except the water extract of Potamogeton natans where a low activity ratio of 0.10 as compared to the 30 mcg chloramphenicol discs was indicated. Skellysolve F stem extracts of Nuphar variegatum collected in the Pine Lake and Sparganium fluctuans showed a rather distinct action against C. albicans, the activity ratio as compared to the 100 units mycostatin discs were 2.06 and 1.08, respectively.

In addition to the inhibitory effect of the 80% ethanol extract of N. tuberosa against M. smegmatis, a peculiar phenomenon was observed for this extract which always caused a definite white ring around the disc. The subculture of a small peice of white material failed to indicate any microbial growth, therefore, the possible stimulatory action of the 80% ethanol extract was ruled out. It is believed that tannin present in N. tuberosa reacts with agar in the medium and causes the white appearance on the media.

Antifungal activity was expressed as the ratio of the mean values of the sample inhibitory zones to that of the phenol standard discs. Those ratios below 0.01 were recorded as negative. Ethanol (80%) extracts of Carex lacus-

Table 2. Antimicrobial Activity Ratios of Various Extracts Against Animal Pathogens[1/].

Plant	Ext.	Activity against[2/] S. aureus	M. smegmatis	E. coli	C. albicans
Ac-1	S		0.01	0.01	
	C	0.04			0.20
	A			0.02	
	W			0.02	0.02
Ac-2	S				
	C	0.02			
	A				
	W			0.06	
Cd	S		0.03		
	C				0.02
	A		0.15		
	W				
C1	S		0.09		
	C				0.10
	A	0.26	0.34		
	W	0.05		0.07	0.07
Cp	S				
	C				0.10
	A	0.02	0.02		
	W	0.04		0.04	
Cv	S		0.09		
	C		0.16		0.13
	A	0.09	0.18		
	W			0.08	
Es	S		0.02		
	C	0.03			0.07
	A	0.20	0.29		0.11
	W			0.02	0.07
Lm	S				
	C				0.05
	A		0.06	0.02	
	W			0.09	
Me	S		0.06		
	C				0.10
	A	0.34	0.15		
	W				0.13
Nt	S		0.20		
lv	C	0.16			0.05
	A	0.40	1.01		
	W	0.02	0.01		
Nt	S		0.06		
st	C				0.18
	A	0.38	1.10		
	W	0.03			

(Table 2 continued)

Plant	Ext.	S. aureus	Activity against M. smegmatis	E. coli	C. albicans
Nv-1	S				
lv	C	0.13			
	A	0.43	0.73	0.07	0.14
	W	0.06			0.21
Nv-1	S				
st	C	0.05			0.09
	A	0.45	0.58	0.09	0.13
	W				0.21
Nv-2	S		0.35		
lv	C	0.02		0.04	0.20
	A	0.24	0.03		0.09
	W	0.04	0.13	0.03	
Nv-2	S	0.26	0.29		2.06
st	C			0.03	0.20
	A	0.17			0.05
	W		0.07	0.02	
Pa	S				
	C	0.01			0.20
	A				
	W			0.02	0.02
Pn	S	0.07	0.06	0.01	
	C				0.07
	A		0.02		
	W			0.10	
Pp	S			0.07	
	C	0.06	0.01	0.01	0.09
	A		0.02		
	W			0.06	0.13
Pr	S		0.15	0.04	
	C	0.01	0.10		0.10
	A	0.06	0.07		
	W			0.02	
Pz	S		0.06		
	C	0.09		0.03	0.12
	A				
	W			0.04	
Sc	S				
	C		0.07		0.13
	A				
	W			0.03	
Se	S		0.01		
	C				0.19
	A		0.04	0.01	
	W			0.02	
Sf	S		0.18	0.02	1.08
	C	0.04		0.04	0.17
	A		0.06	0.02	
	W				

(Table 2 continued)

Plant	Ext.	S. aureus	Activity against M. smegmatis	E. coli	C. albicans
Sl	S				
	C	0.20			0.03
	A	0.28	0.25		
	W			0.04	
Ta-1	S				
	C		0.08		0.19
	A				
	W			0.03	
Ta-2	S		0.05		
	C	0.02			0.15
	A		0.01		
	W			0.03	0.03
Va	S	0.11	0.16		0.53
	C			0.05	0.20
	A		0.11	0.05	0.09
	W			0.04	0.12
Za	S		0.07		
	C				0.17
	A	0.08	0.17		
	W			0.02	
Solvents					
Chloroform		0.08	0.05	0.08	0.02
Skellysolve F				0.01	0.13
Ethanol (20%)				0.04	0.01
Water				0.02	

1/Plant names- (refer to section "II. Materials and Methods"); Extracts S- skellysolve F; C- chloroform; A- 80% ethanol; W- water.

2/Activity was expressed as the ratio of the mean values of sample inhibitory zones to that of the standard sensitivity discs (30 mcg chloramphenicol discs for S. aureus and E. coli; 10 mcg streptomycin discs for M. smegmatis and 100 units mycostatin discs for C. albicans).

Table 3. Antimicrobial Activity of Various Extracts Against Plant Pathogens.

Plant[1/]	Ext.[2/]	Activity against[3/] Alternaria sp.	F. roseum	Plant	Ext.	Activity against Alternaria sp.	F. roseum
Ac-1	C		0.24	Nv-2	C		0.14
	A		0.12	st	A		
Ac-2	C		0.14	Pa	C		0.21
	A				A		
Cd	C		0.24	Pn	C		
	A				A	0.42	
Cl	C		0.21	Pp	C		0.30
	A	1.08			A	0.28	
Cp	C		0.19	Pr	C		0.19
	A				A		
Cv	C		0.19	Pz	C	0.60	0.16
	A				A		
Es	C		0.16	Sc	C		
	A				A		
Lm	C			Se	C		
	A	0.80	0.13		A		
Me	C		0.14	Sf	C		
	A		0.16		A		
Nt	C		0.19	Sl	C		
lv	A		0.12		A		0.16
Nt	C		0.24	Ta-1	C	0.35	0.19
st	A	0.72	0.41		A		
Nv-1	C		0.19	Ta-2	C	0.40	0.08
lv	A				A		
Nv-1	C		0.16	Va	C		0.24
st	A		0.14		A		
Nv-2	C		0.24	Za	C		0.12
lv	A				A		

1/Plant names- (refer to section "II. Materials and Methods").

2/Extracts C- chloroform; A- 80% ethanol; only the skellysolve F extracts of Nuphar variegatum (stem) and Vallisneria americana indicated some activity against F. roseum (activity ratios of 0.48 and 0.03, respectively); all the water extracts failed to show any activity against Alternaria sp. or F. roseum.

3/Activity was expressed as the ratio of the mean values of the sample inhibitory zones to that of the phenol standard disc.

tris (activity ratio of 1.08 as compared to 5% aq. phenol standard), Nyphaea tuberosa (stem: 0.72) and skellysolve F extract of Potamogeton zosteriformis (0.60) were active against Alternaria sp., 80% ethanol stem extracts of Nymphaea tuberosa and Nuphar variegatum were active against F. roseum with the activity ratios of 0.41 and 0.48, respectively, as compared to the 5% aqueous phenol standard. In general, the plant pathogenic fungi are more resistant than animal pathogenic organisms toward the actions of aquatic plant extracts.

IV. FURTHER STUDIES ON NYMPHAEA TUBEROSA

A. Isolation and Purification of Active Principles Against Mycobacterium smegmatis

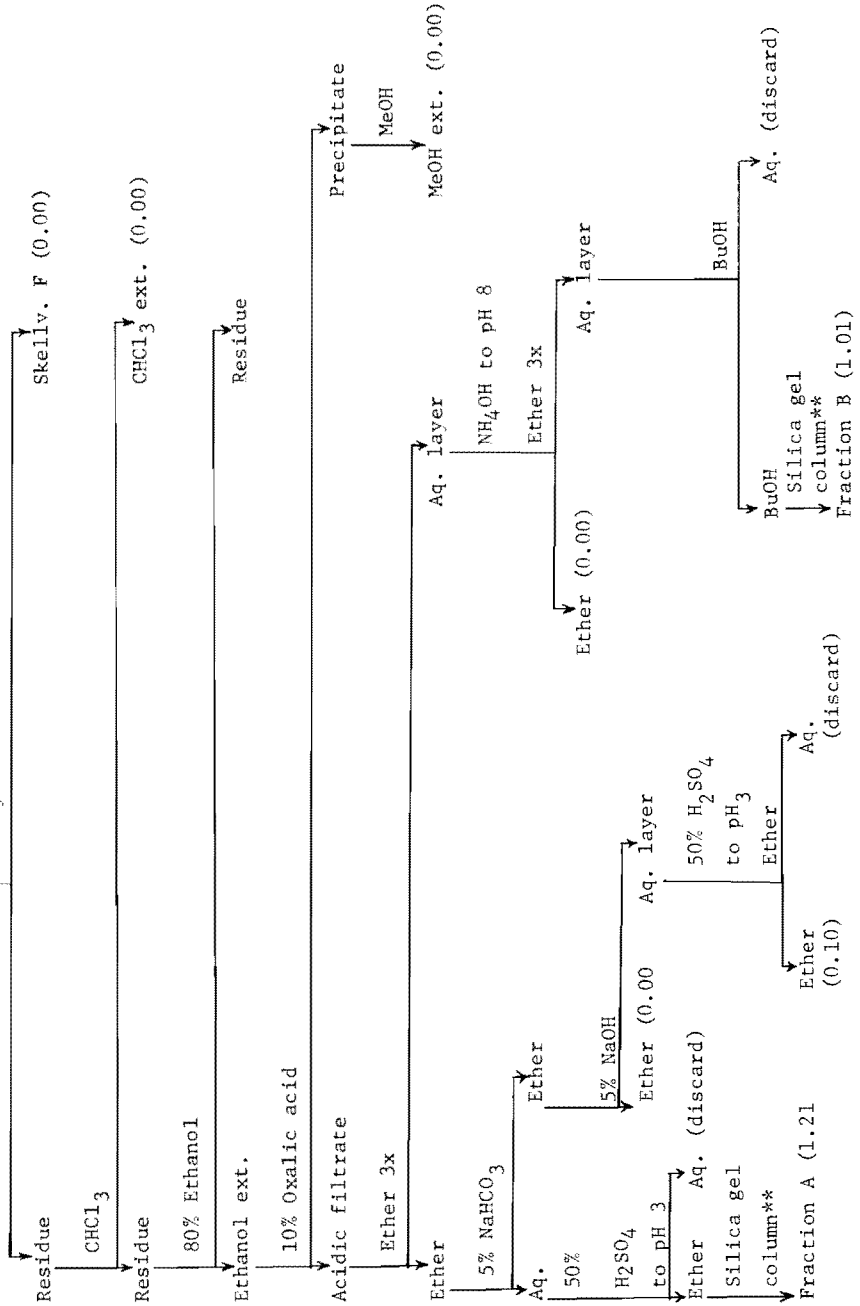
From the privious antimicrobial screening results (cf. Table 2), the ethanol (80%) extract of Nymphaea tuberosa was found to be most promising and worthy of further investigation for the isolation of the constituents against Mycobacterium smegmatis. The activity ratios of the leaf and stem ethanol extracts when compared to streptomycin sensitivity discs (10 mcg) were 1.01 and 1.10, respectively. The procedures used for the isolation of the components responsible for the activity are shown in Figures 1 and 2. The filter paper disc diffusion method was used to monitor the activity of each fraction.

The concentrated fraction B from the silica gel column was further purified by the caffeine method (130): An aqueous solution (1.5%) of caffeine monohydrate was added slowly to the aqueous solution of fraction B. The gummy precipitates (caffeine-tannate) were separated by centrifugation and the addition of caffeine solution repeated on the supernatant. The gummy precipitates were combined, dissolved in a minimum amount of methanol, diluted with water and extracted repeatedly with chloroform to remove the dissociated caffeine. The purified compound B in the aqueous layer was then freeze-dried and stored in a desiccator for chemical analysis.

The qualitative and TLC data shown in Table 4 indicated that fractions A and B were both present in fraction C. Isolation method shown in Fig. 2 was utilized to isolate more of fraction A because of its comparative simplicity and fraction B was isolated according to isolation procedure shown in Fig. 1. Preparative TLC was used to further fractionate fraction C into compounds C_1 and C_2.

The TLC procedure used to isolate C_1 and C_2 is as follows: cellulose plates (1 mm) were prepared from an aqueous cellulose suspension (25%, Avicel-PH101, microcrystalline alpha-cellulose, FMC Corporation, American Viscose Div., Marcus Hook, Pa.) by homogenizing 80 gm of cellulose and 320 ml of water in a hand homogenizer for two minutes. The plates were air dried and used directly without activation. Fraction C was then applied as thin lines (5 mm wide) through a microcapillary pipette with the tip bent at a right angle to the plates. The plates were developed first in 6% HOAc and then in BAW (14:1:5). The two bands with antimicrobial activity, compounds C_1 and C_2, fluorescent reddish purple under UV (254 mμ). Compound C_1 had the Rf values of 0.43-0.53 in 6% HOAc and 0.62-0.71 in BAW (14:1:5). Compound C_2 had the Rf values of 0.53-0.69 in 6% HOAc and 0.79-0.91 in BAW (14:1:5). The bands were collected separately, eluted with acetone, and evaporated to almost dryness under nitrogen.

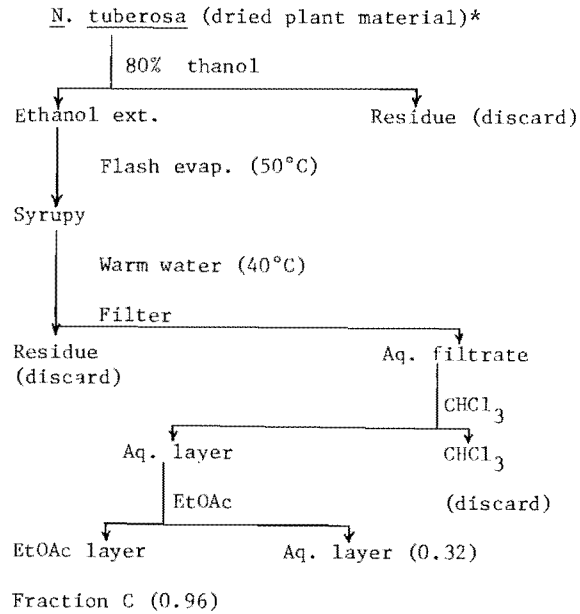
Fig. 1. Isolation of active principles from defatted 80% ethanol extract of N. tuberosa against M. smegmatis.



*The numbers in parentheses indicate the relative activity ratio as compared to the 10 mcg streptomycin sensitivity discs.

**Silica gel column was prepared from the chloroform slurry of silicic acid, eluted continuously with mixtures of 5% MeOH/EtOAc, 10% MeoH/EtOAc, etc.). The fractions (15 ml/fraction) were collected with the Gilson Fraction Collector at a rate of 10 min/tube. Fractions with identical Rf values (detected by iodine) in BuOH:HOAc:water (5:2:6) and showed the positive activity against M. smegmatis were combined and concentrated.

Figure 2. Isolation of active principles from 80% ethanol extract of N. tuberosa against M. smegmatis.



*The numbers in parentheses indicate the relative activity ratios as compared to the 10 mcg streptomycin sensitivity discs.

Table 4. Cellulose TLC Patterns of Fractions A, B and C[1/].

Fraction	6% HOAc					BAW (14:1:5)				
	Rf	UV[2/] a	UV[2/] b	$FeCl_3$-$K_3Fe(CN)_6$[3/] a	$FeCl_3$-$K_3Fe(CN)_6$[3/] b	Rf	UV a	UV b	$FeCl_3$-$K_3Fe(CN)_6$ a	$FeCl_3$-$K_3Fe(CN)_6$ b
A	0.23-0.40	gr bl	++	bl	t	0.62-0.70	pu	++	bl	++
	0.43-0.53	re pu	+	bl	++	0.71-0.79	bl gr	+	bl	++
	0.53-0.67	re pu	+	bl	++	0.79-0.90	pu re	++	bl	++
B	0.00-0.41	re pu	+	bl	++	0.59-0.90	pu	+	bl	++
C	0.00-0.40	re pu	+	bl	+	0.62-0.71	pu	t	bl	++
	0.43-0.53	re pu	+	bl	++	0.71-0.79	bl pu	+	bl	++
	0.53-0.69	re pu	+	bl	++	0.79-0.91	gr gy	t	bl	+

1/TLC of fractions A, B and C was developed in 6% HOAc and BAW (n-butanol: acetic acid:water, 14:1:5) on Chromagram sheets (Kodak 6064).
2/UV-Ultraviolet fluorescent patterns were observed at 254 mμ; a- Color; bl- blue; gr- green; gy- gray; pu- purple; re- red; ye- yellow; b- Intensity +++= high; ++= medium; += low; t= trace.
3/$FeCl_3$-$K_3Fe(CN)_6$ spray- Equal volumes of 3% $FeCl_3$ and 3% $K_3Fe(CN)_6$.

B. Identification of Compounds C_1, C_2 and B

1. Chemical properties

The poly-hydroxy phenolic nature of C_1, C_2 and B was established via the following qualitative tests listed in Table 5.

2. Solubilities

Compounds C_1 and C_2 were soluble in absolute ethanol, acetone, moderately soluble in water, slightly soluble in ether and insoluble in chloroform or benzene, whereas compound B differ from compounds C_1 and C_2 in being very soluble in water.

3. Identity of compounds C_1, C_2 and B

Elucidation of the structures of purified C_1, C_2 and B were obtained by various spectrophotometric methods (Ultraviolet, Infrared, Nuclear Magnetic Resonance and Mass Spectroscopy) and by other physical methods such as the melting point determination and the elementary analysis. Fraction C_1, C_2 and B were identified as gallic acid (3,4,5-trihydroxy benzoic acid), ethyl gallate (ethyl-3,4,5-trihydroxy benzoate) and tannic acid (penta-galloyl-alpha-D-glucopyranose), respectively, according to the physical data listed in Table 6 and Table 7. The mass spectra for compounds C_1 and C_2 are shown in Fig. 3 and Fig. 4, respectively.

Table 5. Qualitative Tests for Compounds C_1, C_2 and B.

Test[1/]	Reactions of compounds		
	C_1	C_2	B
$FeCl_3$	blue-purple	blue-purple	blue-black
Gelatin-salt	cloudy	cloudy	ppt.
Caffeine	cloudy	cloudy	ppt.
Acetic-lead acetate	ppt.	ppt.	ppt.

1/$FeCl_3$-9% Aqueous $FeCl_3$; Gelatin-salt reagent- Dissolve 1 gm of gelatin and 5 gm of NaCl in 100 ml of acid phthalate buffer (pH 3.0); Caffeine- 1.5% aqueous caffeine; Acetic-lead acetate reagent- 10% HOAc and lead acetate (2:1).

Table 6. Comparison of the Spectrometric Data of Compound C_1 vs. Gallic Acid, C_2 vs. Ethyl Gallate and B vs. Tannic Acid[1/].

Compound	UV EtOH λmax(mu)	IRνmax(in KBr)[2/] cm^{-1}	NMR [3/]	MS[4/]
C_1	216	3350-3490 bs 1275bm 1625ss 1150sm 1650ss 1025ss 1600ss 900sm 1549ss 826ss 1450ss 760ss 1425ss 735ss 1350ss	7.8 2.9	170
Gallic acid	216	(Identical as C_1)	2.9	170
C_2	218 277	3300-3450bm 1710ss 1190sm 1620ss 1030sm 1530sm 860sm 1470sm 760sm 1310bm 750sm 1250sm 725sm	8.7 5.8 3.0	198
Ethyl gallate		(Identical as C_2)		
B	218 278	3350-3450bs 1200bs 1702ss 1080sm 1602ss 1025sm 1530sm 860sm 1450sm 752sm 1370-1300bs	7.5 4.9 2.6	170?
Tannic acid	218	(Identical as B)	3.0	

1/UV- Ultraviolet absorption measurements were made using a Cary 14 Recording Spectrophotometer; IR- Infrared spectra were determined for KBr pellets on a Perkin-Elmer 125 Spectrometer and calibrated with a film of polystyrene at 1604 cm^{-1}; NMR- Nuclear magnetic resonance spectra were measured on a Varian Model V-60A Spectrometer; MS- Mass spectra were recorded by the Mass Spectrometry Service Laboratory, University of Minnesota, Mpls., Minn.) at 200°C, 180°C and 250°C for compound C_1, C_2 and B, respectively, the energy used was 50 eV.

2/Nature of IR bands bm- broad medium; bs- broad strong; sm- sharp medium; ss- sharp strong.

3/ values- Chemical shift values with reference to the internal standard of tetramethylsilane = 10.

4/Only those masses with respect to parent peaks were listed.

Table 7. Comparison of the Melting Points and Elementary Data of Compounds C_1 vs. Gallic Acid, C_2 vs. Ethyl Gallate and B vs. Tannic Acid[1/].

Compound[2/]	M.P. (°C)	Elementary analysis (%)[3/] C	H	O
C_1	232-238 (decomp.)	50.68	4.06	45.26
Gallic acid	235-240 (decomp.)	49.41	3.53	47.06
C_2	156-158	58.13	5.75	36.12
Ethyl gallate	158	54.54	5.06	40.40
B	196-205 (decomp.)	50.23	3.55	46.22
Tannic acid	198-206 (decomp.)	53.65	3.08	43.26

1/M.P.- Melting points were determined on a Fisher-Johns Melting Point Apparatus and are uncorrected; Elementary analysis- Samples were dried at 90°C high vacuum for 3 days before they were sent to the M-H-W Laboratories, Garden City, Michigan.

2/Gallic acid- From Merck's Laboratory Chemicals, N.Y.; Tannic acid- According to U.S.P.; Ethyl gallate- Prepared from gallic acid Merck: 1 mole of gallic acid + 10 moles of ethanol + 0.5 mole of sulfuric acid and reflux for 6 hours. Purification of ethyl gallate was obtained by preparative cellulose TLC.

3/Those for gallic acid ($C_7H_6O_5$), ethyl gallate ($C_9H_{10}O_5$) and tannic acid ($C_{76}H_{52}O_{46}$) were calculated theoretical data.

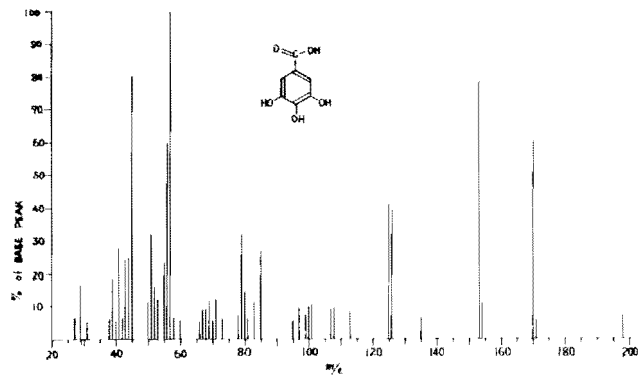


Figure 3. The mass spectrum for compound C_1.

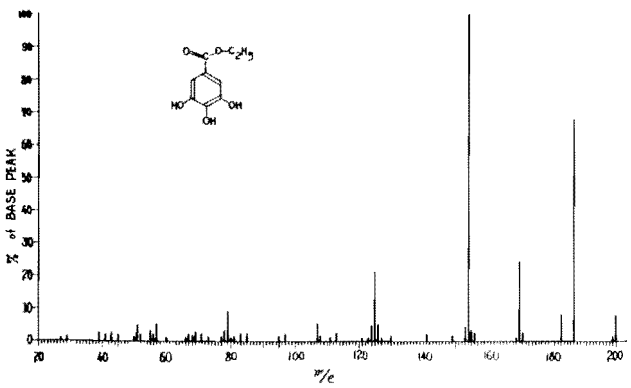


Figure 4. The mass spectrum for compound C_2.

To further confirm the identity of compound B, and O-methyl derivative was prepared as follows: compound B (80 mg) in methanol (5 ml) and ether (10 ml) was mixed with diazomethane in ether (10 ml), and the mixture was set under a well ventilated hood at room temperature for three days. At the end of the reaction period, the solvents were distilled off and the O-methyl derivative was recrystallized from methanol and water. Diazomethane was prepared from N-nitrosomethyl urea in 40% aqueous KOH and ether (131). The physical data for O-methyl derivative of compound B were: m.p. 76-78°C; λ_{max}^{EtOH} 268 mμ; major ir bands at 3400, 1724, 1712, 1600, 1500, 1470, 1340, 1125, 860, 766, 758 and 727 cm^{-1}; 5.99, 2.52; M.W. (from mass spectrometric analysis) 266; Anal: calc. for methyl-3,4,5-trimethoxy benzoate ($C_{11}H_{14}O_5$): C, 55.93; H, 5.93; O, 38.14; found C, 54.89; H, 5.93; O, 39.16%.

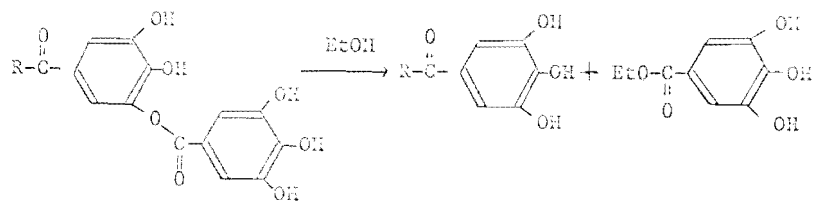
C. Antimicrobial Spectra of Compounds C_1, C_2 and B

Antimicrobial activities of compounds C_1, C_2 and B were re-examined against Staphylococcus aureus (NRRL B313), Mycobacterium smegmatis (NRRL B612), Escherichia coli (NRRL B210), and Candida albicans (NRRL Y477) by the filter paper disc diffusion method (cf. p. 107). Standards used were Difco chloramphenicol discs (30 mcg) for S. aureus and E. coli, Difco streptomycin discs (10 mcg) for M. smegmatis and BBL mycostatin discs (100 units) for C. albicans. The activity observed was expressed as the ratio of the mean values of the corrected inhibitory zones (sample inhibition zones minus solvent inhibition zone) to that of the standard discs. The results are shown in Table 8. Fig. 5 shown the clear zones of inhibition of compounds C_2 and B against M. smegmatis as detected by the filter paper disc diffusion method (cf. p.).

A plot of the logarithm of the different concentrations of each active compound (C_2 or B) vs. diameters of inhibition zones on M. smegmatis showed a linear relationship (Table 9 and Fig. 6).

D. Discussion

Tannic acid is noted for its lability towards hydrolytic agents (tannase, hot water, acids or alkali), therefore, the presence of free gallic acid found in Nymphaea tuberosa may in part have resulted from enzymic or chemical decomposition of the labile tannic acid during extraction. Ethanolysis of the digalloyl derivative of tannin (I) during 80% ethanol extraction may account for the presence of ethyl gallate (II) found.



(I) (II)

Table 8. Antimicrobial Spectra of Compounds C_1, C_2 and B[1/].

Compound Conc. (mg/ml)	Activity against			
	S. aureus	M. smegmatis	E. coli	C. albicans
C_1	0.17	0.33	0.15	0.08
C_2	0.41	1.91	0.25	0.08
B	0.45	1.00	0.37	0.33

1/Activity was expressed as the ratio of the mean values of the corrected inhibitory zones (sample inhibition zones minus solvent inhibition zone) to that of the standard discs (30 mcg chloramphenicol discs for S. aureus and E. coli; 10 mcg streptomycin discs for M. smegmatis and 100 units mycostatin discs for C. albicans).

Figure 5. Detection of the inhibitory actions of compounds B (1- 300 mg/ml; 2- 150 mg/ml; 3- 75 mg/ml; T- U.S.P. tannic acid 200 mg/ml) and C_2 (I- 100 mg/ml; II- 50 mg/ml; III- 25 mg/ml; S- Standard 10 mcg 10 mcg streptomycin disc) on M. smegmatis by the filter paper disc method.

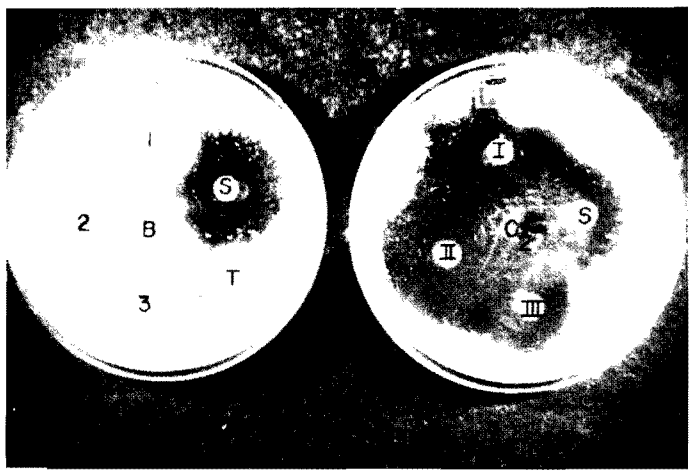
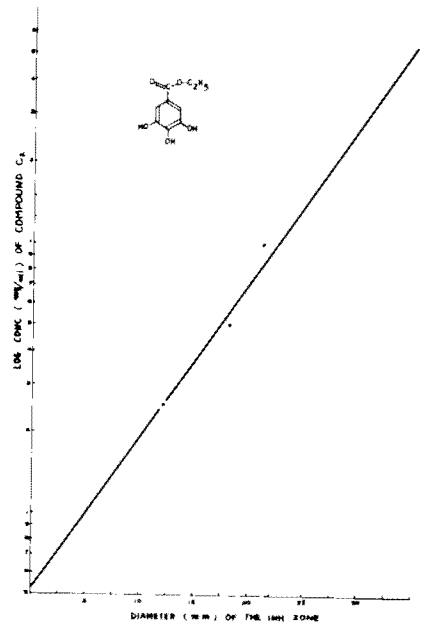


Table 9. Response of M. smegmatis Against Compounds C_2 and B.

Compound	Conc. (mg/ml)	Mg/disc[1/]	Inh. zone (mm)	Act. ratio[2/]
C_2	100	15.00	42	1.91
	50	7.50	36	1.63
	25	3.75	24	1.09
B	300	45.00	24	1.00
	150	22.50	18	0.75
	75	11.25	14	0.58

1/Each paper disc takes up 0.15 ml of sample solution.
2/Activity ratio was expressed as the ratio of the mean values of the corrected inhibitory zones (sample inhibitory zones minus solvent inhibitory zone) to that of the standard streptomycin disc (10 mcg).

Figure 6. Logarithm of the concentration (mg/ml of compound C_2 vs. diameters (mm) of inhibition zones against M. smegmatis.



The presence of an aromatic ring in compounds C_1, C_2 and B is indicated by the ultraviolet absorption at ca. 277 mμ and a strong end absorption at ca. 216 mμ. The infrared spectra of compounds C_1, C_2 and B are marked by the intense absorptions in the 3500-3300, 1710-1600, 1300-1200 and 860-700 cm^{-1} regions. Except for the first region where the absorption is broad and strong, others are quite sharp with either strong or medium intensities. The broad band at 3500-3300 cm^{-1} is assigned to hydroxyl-stretching, and the strong and sharp bands at 1724-1620 cm^{-1} are due to the skeletal vibrations of the ring. The peaks at 1200-1125 cm^{-1} is assigned to C-OH deformation. The strong to medium intensity band at 860 cm^{-1} is due to the out-of-plane bending of the isolated hydrogen in the benzene ring. Tannic acid is known to be the pentagalloyl-alpha-D-glucopyranose, therefore the occurrence of characteristic absorption in the 900-800 cm^{-1} region is due to the pyranose ring vibration. The mass spectra of compounds C_2 (ethyl gallate) and B (tannic acid) gave a similar breakdown pattern typical of compound C_1 (gallic acid) since the gallic acid is the basic unit for both ethyl gallate and tannic acid. The unusually low molecular weight (170) obtained for tannic acid (M.W. ca. 1700) may be due to its very low volatility. The singlets at 2.52-3.00 in the MNR spectra are due to the aromatic protons, the quadruplet and triplet at 5.78 and 8.75 of C_2 NMR spectrum are due to the $-CH_2-$ and CH_3- of the ethyl moiety, respectively.

Compounds C_2 and B have a strong antimicrobial activity against Mycobacterium smegmatis, but are only moderately active against S. aureus and E. coli and least active against C. albicans. Tannic acid had been noted for its antiseptic action in as early as 1920's (132). Tannic acid in a concentration of 0.03% had been reported to be bacteriostatic against three strains of E. coli (133), a 10 to 20% tannin solution completely destroyed E. coli, S. albus, S. citreus (134), and it is also responsible for the antibacterial action of chocolate milk (135). Gallic acid had been reported to inhibit S. aureus only slightly (136). The action of ethyl gallate upon M. tuberculosis strain 607 had been found (137-139) to be not only bacteriostatic but also bactericidal in a somewhat greater concentration, the minimum concentration inhibiting this organism was 39.6 mcg/ml determined by the turbidometric method. The approximate minimum inhibitory concentration for compound C_2 against M. smegmatis when extraporated from Fig. 6 was 5.5 mg/ml (5500 mcg/ml). The application of different techniques (dilution vs. diffusion method used in our study) and against different organisms (M. tuberculosis vs. M. smegmatis) render us hard to compare the MIC data. It was reported that ethyl gallate antagonize the utilization of shikimic acid by M. tuberculosis and thus inhibited the growth of the organism (140). Gallic acid is only slightly active against M. smegmatis, S. aureus, E. coli and C. albicans, the activity was markedly increased by esterification (cf. the activity of gallic acid vs. that of ethyl gallate).

Tannic acid causes liver damage and is toxic for mice and rate when given intravenously or subcutaneously (141,142), whereas gallic acid (143) and ethyl gallate (144) produce no hepatoxicity in rats. A preliminary trial on the tannin treatment of tuberculosis was reported by Loumos and Rosenblum (145). In that study, a 0.5% solution of sodium tannate showed a more or less marked improvement in 13 out of 19 cases of pulmonary tuberculosis and in one case of lymphnode tuberculosis, the discharging lymph nodes were improved quickly.

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