

Antibiotic Substances from *Ranunculaceae*

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It has been known for quite some time that extracts of many species of the family *Ranunculaceae* exert an inhibitory effect on the growth of a wide variety of microorganisms including bacteria, fungi, yeasts, and protozoa. The literature on this seems to be somewhat inconsistent. It was partly because of this that the present investigation was undertaken and partly through the interest of the Indiana State Board of Health to find a substance which would inhibit the growth of *Candida albicans* but not that of other organisms commonly found growing together with it.

The antibiotic substance present in the flowers, stems, and leaves is a lactone, protoanemonin, which on exposure to air polymerizes to form anemonin, which also possesses antibiotic properties, although to a lesser degree.

The first known work on the antibacterial action of *Ranunculaceae* was done sometime prior to 1850 by Clarus (5). Erdmann in 1858 (5) suggested that a certain volatile oil present in these plants caused their sharp odor, and that this oil could easily be converted to two other substances which he called anemonin and anemonic acid. Two Japanese chemists Asahina and Fujita (5) have given us the structure for protoanemonin and anemonin and have synthesized protoanemonin.

The antibacterial action of these extracts was first described by Boas (1) in 1934. The most extensive study was made by Holden, Seegal and Baer (4) in which extracts of *Ranunculus* and *Anemone pulsatilla* were found to exert an inhibitory action on the growth of a number of pathogenic bacteria, fungi, and protozoa.

Plants were collected in early October and stored for a few days at a temperature of approximately 35°F. Four different extracts were prepared and tested. The extract, termed "juice", was obtained by putting the stems and leaves of plants through a meat grinder. The resulting material was mixed thoroughly with an equal amount, by weight, of distilled water. The mixture was then pressed through gauze, sterilized and stored at 35°F. From this was prepared a second extract, the "distillate", by steam distillation of part of the juice. From the distillate the two other extracts—protoanemonin and anemonin were finally obtained after a method described by Boas and Steude (2) in which a portion of the distillate was saturated with NaCl; an equal volume of ether was added and the mixture shaken thoroughly in a separatory funnel. The ether layer, containing the antibiotic substance, was drawn off, and measured portions placed in sterile petri dishes. The ether evaporated from these leaving behind the protoanemonin to which was immediately added glucose agar and the two then thoroughly mixed. Portions of the ether mixture were placed in other petri dishes and the ether allowed to evaporate. The protoanemonin thus obtained was left exposed to the air at room temperature for about two hours; during this time the protoanemonin polymerized to form anemonin. Glucose agar was then added to the plates and mixed with the anemonin. Also present

with the anemonin was a whitish substance which possibly corresponded to the isoanemonin acid described by Boas and Steude (2).

The four extracts just described—juice, distillate, protoanemonin, and anemonin, were tested for their antibiotic effects on *Candida albicans*, a species of *Penicillium*, *Rhizopus nigricans*, *Staphylococcus aureus*, *Bacillus megaterium*, *Aerobacter aerogenes*, *Sarcina lutea*, and *Mycobacterium smegmatis*. The yeast and two molds were subcultured in the agar, the bacteria were streaked on the plates. Tests were run a minimum of five times along with proper controls. All organisms were incubated for at least one week.

Amounts from 0.1 to 1.0 cc. of the juice and distillate were used. The concentrations of protoanemonin and anemonin were determined by measuring the amount of antibiotic-containing ether placed in the petri dishes, assuming that all went into solution in the ether. Both substances are very soluble in ether but only slightly so in water.

It was found that the juice was potent for about ten months. The distillate was perfectly clear and colorless and could only be recognized by the characteristic odor of the juice. The distillate was kept under the same conditions as the juice and was effective for about six months. The protoanemonin was a pale-yellow oil having a strong, putrid odor which was somewhat irritating. The anemonin was crystalline in form and odorless.

The effects of juice and distillate were studied more thoroughly on *C. albicans*, *Penicillium*, and *R. nigricans*. Both extracts were used over a period of ten months but their power of inhibition seemed to decline from month to month. Complete inhibition of the growth of *C. albicans* was obtained with as little as 0.1 cc. of freshly prepared juice. The same was true for the distillate. However, after ten days a slight amount of growth appeared on plates containing 0.10 cc. but not those containing 0.15 cc. distillate. After two weeks incubation these latter showed no growth.

When tests were run using juice and distillate, each four months old, a minimum of 0.20 cc. of juice inhibited *C. albicans*, whereas a minimum of 0.35 cc. of distillate was effective. After eleven months, 0.35 cc. of juice was effective in inhibiting the growth of the same organism, but the distillate appeared to have lost its antibiotic properties completely.

Extracts were tested on *Penicillium* four months after their preparation. Amounts of juice as large as 1 cc. had no complete inhibitory effect on this organism, although it did slow down the growth, control plates showing growth in three days as compared to seven days for test plates. However, later tests using distillate prepared by steam distillation for a relatively short time giving more protoanemonin per ml. of water, resulted in *Penicillium* being inhibited by 1 cc. of distillate.

The distillate was not tested on *R. nigricans* but the effect of the juice on this organism was somewhat surprising. Mycelia appeared on control plates within 48 hours; on plates containing 1 cc. of juice, no growth could be detected macroscopically after one week. In plates containing 0.5 cc. juice a small number of spores were seen on the surface of the agar after seventy-two hours, but mycelia were still invisible

macroscopically even after one week. It would seem that the mycelia are stunted by the substance but this has not yet been thoroughly studied.

Low concentrations of protoanemonin were effective in inhibiting the growth of *C. albicans*, *S. aureus*, *B. megaterium*, and *S. lutea*, but higher concentrations were necessary to inhibit the growth of *Penicillium* and *Mycobacterium smegmatis*. *Aerobacter* seemed not to be effected by the concentrations used. It was interesting to note that when low concentrations of protoanemonin were used on *Penicillium* and *Mycobacterium smegmatis*, growth was slowed considerably as evidenced by the fact that colonies did not appear on these plates until at least two days after growth appeared on the control plates.

The concentrations of anemonin used inhibited the growth of only *Sarcina lutea*. The growth of *C. albicans*, *Penicillium*, *Aerobacter aerogenes*, and *Mycobacterium smegmatis* was not visibly inhibited in any way. Tests on *Staphylococcus aureus* and *Bacillus megaterium* were unsatisfactory and no conclusive results were obtained.

A comparison was made between the effects of the antibiotics on the organisms and the characteristics of the organisms. There seemed to be no correlation. Only one reference made by Hill and van Heyningen (3) on the vesicant properties seemed to indicate how the antibiotics act to prevent or retard growth.

The particular species of *R. septentrionales* here studied is apparently more effective in so far as possessing antibiotic properties than may other plants of the *Ranunculaceae*. Boas (1) reported a water *Ranunculus* which appeared to be wholly ineffective, whereas other species, such as *R. acer*, *sceleratus*, *arvensis*, *bulbosus*, and others were reported to be the most effective of those studied. *R. septentrionales* could be placed along with these latter.

Summary

1. Extracts of the plant *Ranunculus septentrionales* possess antibiotic properties.
2. Two extracts may be prepared: a) the "juice", by grinding the plant thoroughly, mixing with water, straining and sterilizing; b) the distillate, by steam distillation of the juice.
3. The active substance, protoanemonin, is isolated from the distillate by extraction with ether. Another antibiotic substance, anemonin, is formed when the protoanemonin polymerizes in air at room temperature.
4. The juice and distillate lost their power of inhibition gradually from month to month, the juice retaining its potency for about eleven months and the distillate for about six months.
5. *Penicillium* and *Rhizopus* are affected less by the juice and distillate than is *C. albicans*, whose growth is inhibited by as little as 0.10 cc. of freshly prepared juice, and 0.15 cc. of freshly prepared distillate.
6. Relatively low concentrations of protoanemonin are effective in inhibiting the growth of *C. albicans*, *Staphylococcus aureus*, *Bacillus megaterium*, and *Sarcina lutea*, whereas higher concentrations are needed to achieve this effect on *Penicillium* and *Mycobacterium smegmatis*.

Aerobacter aerogenes was not affected by concentrations of protoanemonin used in this study. Anemonin was effective in inhibiting the growth of *Sarcina lutea* but of none of the other organisms studied.

Literature Cited

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