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Antibacterial Properties of Tannic Acid and Related Compounds against the Fish Pathogen Cytophaga columnaris

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Abstract.—Tannic acid, gallic acid, and propyl gallate exhibited inhibitory activity as demonstrated by the agar dilution assay against Cytophaga columnaris (= Bacillus columnaris, Chondrococcus columnaris, Flexibacter columnaris, or Flavobacterium columnare), a ubiquitous gliding fish pathogen, at 150, 275, and 300 µg/mL, respectively, in modified Shieh medium, at a low-bacterial-inoculum density of 10³-4 colony-forming units/mL. Methyl gallate was not effective at the highest concentration tested (500 µg/mL). The minimum inhibitory concentrations (MICs) of gallic acid, methyl gallate, and propyl gallate were lower in natural pond water than in modified Shieh medium, whereas the MIC of tannic acid was the same in both. Tannic acid, a polymeric compound with multiple hydroxyl groups, had a greater capacity for binding protein and glycogen by at least nine times that of the other test compounds. The results suggest that the hydroxyl group availability of tannins is essential for antibacterial activity.

Cytophaga columnaris (= Bacillus columnaris, Chondrococcus columnaris, Flexibacter columnaris, or most recently, Flavobacterium columnare; Bernardet and Grimont 1989; Staley et al. 1989; Bernardet et al. 1996) is a ubiquitous, gliding bacterium in the aquatic environment. Under normal conditions, C. columnaris is a saprophyte in ponds, but under stressful conditions such as a sudden temperature change, decrease in oxygen supply, dense fish population, contamination by other bacteria, eutrophic environment, or injuries of the fish body, the bacterium becomes a serious pathogen (Bullock 1972) that is regarded as responsible for columnaris disease in fish (Davis 1923). Columnaris disease is one of the most common bacterial diseases worldwide in all freshwater fish species, particularly salmonids and channel catfish (Tucker and Robinson 1990). The disease has resulted in great economic loss to the fish farming industry in the United States. Control of the spontaneous occurrence of columnaris epizootics is necessary for the healthy growth of the catfish industry. A possible means for such control may be provided by natural tannins.

Tannins are generally classified into hydrolysable or condensed tannins. Hydrolysable tannins, commonly referred to as tannic acid (gallotannic acid, gallotannin, or penta-m-digalloyl-glucose), are esters of phenolic acids and a polyol that is usually glucose (Haslam 1989; Scalbert 1991). Because these natural substances are widespread and readily available in the environment (White 1957) and as food additives, are categorized as "generally recognized as safe" (GRAS) according to the Code of Federal Regulations (USOFR 1989), the antimicrobial activities and chemotherapeutic potentials of these compounds have been the subject of increasing study. Our previous work has shown that tannic acid and related compounds were inhibitory to the growth of many microorganisms including aquatic bacteria (Chung et al. 1993, 1995b), the bloom-causing cyanobacterium Agmenellum quadruplicatum strain PR-6, and the off-flavor-producing cyanobacterium Nostoc sp. strain MAC (Chung et al. 1995a).

This paper reports the effect of tannic acid and its derivatives on the growth of the fish pathogen C. columnaris. We also measured the protein precipitation and polysaccharide binding capacities, lipophilicity, and other physicochemical properties of these compounds to understand possible mechanisms for their antibacterial action.

Methods

Bacterial strain and culture condition.—The test organism Cytophaga columnaris was obtained from the National Biological Survey (now the U.S. Geological Survey, Biological Research Service), Marion, Alabama. This representative strain was specifically isolated from channel catfish Ictalurus punctatus and maintained on modified Shieh medium at 4°C. In preparation for experiments, C. columnaris was transferred to 2 mL of modified Shieh broth medium and grown overnight at 32°C to a concentration of 2 x 10⁷ colony-forming units.

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(CFU)/mL through viable plate counting with an absorbance of 0.2 at 595 nm. Modified Shieh medium, pH 7.4, supported the growth of *C. columnaris* and was used for routine cultivation. The medium (per 100 mL) contained peptone, 500 mg; sodium-acetate, 1 mg; yeast extract, 50 mg; BaCl$_2$ 1 g; MgSO$_4$7H$_2$O, 10 mg; K$_2$HPO$_4$, 5 mg; KH$_2$PO$_4$, 5 mg; Fe$_2$O$_3$ 0.1 mg; and NaHCO$_3$, 5 mg (Shieh 1980; Song et al. 1988).

All chemicals, including the test compounds gallic acid, methyl gallate, propyl gallate, and tannic acid, were purchased from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise specified. The tannin compounds were freshly prepared at specified concentrations by dissolving them in deionized water, adjusting their pH to 7.4 with 1 N NaOH, and then filter-sterilizing the solutions. These stock solutions were then used for susceptibility tests or fo biological activity tests.

**Agar dilution in Shieh medium.** The agar dilution method was employed to assay the antibacterial properties of tannic acid and related compounds (Sandven et al. 1993). Serial two-fold dilutions of gallic acid, methyl gallate, propyl gallate, or tannic acid were prepared for concentrations ranging from 0.5 to 5.0 mg/mL. A portion (1.5 mL) of each dilution was mixed with 13.5 mL of modified Shieh agar medium to give final chemical concentrations of 0.05 to 0.5 mg/mL in the agar plates. One milliliter of an overnight culture of the *C. columnaris* isolate (2 X 10$^7$ CFU/mL) in modified Shieh medium was serially diluted in sterile Butterfield phosphate buffer, pH 7.2 (FDA 1992), to approximately 2 X 10$^4$ CFU/mL of bacterial suspension. 50-µL quantity of each of the overnight culture (2 X 10$^7$ CFU/mL) and the diluted culture (2 X 10$^4$ CFU/mL) was inoculated onto separately prepared plates to obtain final concentrations of either 10$^6$ CFU/mL (high-inoculum density) or 10$^3$ CFU/mL (low-inoculum density). A plate without the test compounds was used as a control. The inoculated plates were incubated at 32°C for 48-72 h. The minimum inhibitory concentration (MIC) of test compounds was defined as the point of no growth in a serial two-fold dilution sequence.

**Agar dilution in pond water.** The same procedure as described above was performed with pond water agar instead of modified Shieh medium agar. Fresh pond water was obtained from several aquaculture ponds at different times of the day and the year (spring and summer) at Shelby Farm in Memphis, Tennessee, about 16 km from our laboratory, and agar was then made according to the methods of Chung et al. (1995b). Pond water agar without treatment with tannin compounds served as a control culture, which can support the growth of *C. columnaris*.

**Protein precipitation assay.** The Rickard (1986) method was applied to measure the protein precipitation capacities of tannins. A standard protein solution (1.0 mg/mL) of bovine serum albumin (fraction 5) was freshly prepared in distilled water, and a test compound solution (1.0 mg/mL) was prepared in either distilled water or pond water. Four milliliters of test solution were added to 16 mL of the standard protein solution. The mixed solutions were agitated on a flask shaker at room temperature for 30 min and then centrifuged at 8,000 × gravity for 15 min. The protein content of the supernatants was measured by using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, California). The results are expressed as milligrams of protein precipitated by 1 mg of test compound.

**Lipophilicity assay.** Thin-layer chromatography (Smith and Feinberg 1965; Cain et al. 1974) was used to test the lipophilicity of tannin compounds. Silica gel plates (Merck SiO$_2$ F254; Whatman International Ltd., Maidstone, UK) were used with N-BuOH-0.1 N NH$_4$Cl (1:1, volume: volume) as the solvent system. Samples (30 µL) were applied and allowed to migrate until the solvent front had moved approximately 10 cm. The migration of spots was measured, and the lipophilicity ($R_f$) values were then calculated (Smith and Feinberg 1965). The lower the $R_f$ value, the more lipophilic the compound.

**Polysaccharide binding assay.** Nelson's test (Wharton and McGorty 1982) was performed to assess the concentration of reducing sugar after acid hydrolysis of glycogen (bovine liver type 9). Briefly, after tannin compounds were mixed with glycogen, 2 mL of the supernatant was vortexed with 2 mL of 4 M HCl. An aliquot of 0.5 mL of the mixture was transferred to a test tube containing 2.5 mL 1 M K$_2$HPO$_4$, and the volume was brought to 10 mL with distilled water. One milliliter of the hydrolysate was mixed with 1 mL of Nelson's reagent C, a mixture of 25 mL of reagent A (containing 12.5 g of Na$_2$CO$_3$, 12.5 g of NaK tartrate, 10 g of NaHCO$_3$, and 10 g of Na$_2$SO$_4$ in 500 mL of distilled water), and 1 mL of reagent B (containing 15 g of CuSO$_4$5H$_2$O and 1 drop of H$_2$SO$_4$ in 100 mL of distilled water). The mixture was placed in a boiling water bath for 20 min. After the mixture cooled, 1 mL of arsenomolybdate reagent (containing 25 g of ammonium molybdate, 21 mL of concentrated H$_2$SO$_4$, and 3 g of Na$_2$HASO$_4$ 7H$_2$O in 475 mL of distilled water)
was added to the tubes, which were allowed to stand for 2 min before the volume was brought to 10 mL with distilled water. The absorbance of the samples was measured at 510 nm with a Spectronic 20 colorimeter. The amount of reducing equivalents was calculated from the d-glucose standard curve (Wharton and McGorty 1982). Results were expressed as milligrams of glycogen precipitated by 1 mg of test compound. All the assays were repeated two or three times and the results were averaged.

Results

With the agar dilution assay method, gallic acid, propyl gallate, and tannic acid were found to inhibit the growth of C. columnaris on both modified Shieh medium and pond water agar plates (Table 1). At low-inoculum density (10^3-4 CFU/mL), the MICs of gallic acid, propyl gallate, and tannic acid were 275, 300, and 150 µg/mL, respectively, on modified Shieh medium agar. Methyl gallate was not effective up to the highest concentration tested (500 µg/mL). At high-inoculum density (10^6-7 CFU/mL), the MICs for gallic acid and tannic acid were 425 and 225 µg/mL, respectively. Neither methyl gallate or propyl gallate was effective at concentrations up to 500 µg/mL.

In pond water agar, the antibacterial activity of gallic acid, methyl gallate, and propyl gallate increased dramatically, but that of tannic acid remained the same as in modified Shieh medium. At low-inoculum density, the inhibitory activity ranged from 75 to 200 µg/mL, whereas at high-inoculum density, it ranged from 200 to 350 µg/mL. Compared with the inhibitory effects exerted in modified Shieh medium, the anticytophagal activity of gallic acid, methyl gallate, and propyl gallate increased by at least 50%. Tannic acid, however, did not show any demonstrable increase in its inhibition of C. columnaris in pond water agar (Table 1). Our experimental results were consistent in pond water tests with different pond water samples.

The results of the lipophilicity assay as determined by Rf values (Table 2) indicated that gallic acid and tannic acid were more lipophilic than methyl gallate and propyl gallate. Tannic acid was significantly stronger in protein-binding capacity than gallic acid, methyl gallate, or propyl gallate. One milligram of tannic acid precipitated 3.184 mg of protein (approximately 10 times the protein precipitation of methyl gallate, 11 times that of propyl gallate, and 17 times that of gallic acid). Tannic acid also had a significantly greater capaci-
ity for binding glycogen (approximately 9 times the glycogen-binding capacity of gallic acid and 41 times that of methyl gallate). Propyl gallate had no detectable glycogen-binding capacity. The MIC of tannic acid was 40–240 µg/mL against eight other aquatic bacteria and C. columnaris (Chung et al. 1995a, 1995b).

When biological activities of test compounds were measured in natural pond water, significantly different values for protein- and glycogen-binding capacities were obtained (Table 3). Natural pond water did not affect the lipophilicity \( R_f \) of these compounds. However, the protein-binding capacity of gallic acid and methyl gallate increased in pond water, that of tannic acid decreased dramatically, and that of propyl gallate decreased slightly. The glycogen-binding capacity of methyl gallate and propyl gallate increased, but that of tannic acid decreased in natural pond water. Glycogen was not bound by gallic acid.

### Table 3—The biological activity of tannic acid and related compounds in the presence of natural pond water; \( R_f \) is lipophilicity. All the results were the average of two or three repeated experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_f )</th>
<th>Protein binding</th>
<th>Glycogen binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gallic acid</td>
<td>0.81</td>
<td>0.387</td>
<td>0</td>
</tr>
<tr>
<td>Methyl gallate</td>
<td>0.90</td>
<td>0.510</td>
<td>0.359</td>
</tr>
<tr>
<td>Propyl gallate</td>
<td>0.89</td>
<td>0.159</td>
<td>0.301</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>0.82</td>
<td>0.041</td>
<td>0.808</td>
</tr>
</tbody>
</table>

### Discussion

The 3,4,5-trihydroxy benzene ring (Figure 1) is the core structural moiety of all hydrolysable vegetable tannins. The addition of a 1-carboxyl group onto this core structure yields a typical monomeric hydrolysable tannin. Esterification with other chemical groups such as alkyls or sugars provides the diversity and complexity of tannin compounds (Table 2).

Our results suggest that the protein precipitation ability and polysaccharide-binding capacity of tannin compounds are attributable to the availability of the hydroxyl groups on the core structure of the tannin molecules. These hydroxyl groups may produce hydrogen bonds. Experimental evidence shown in Table 2 supports the notion that tannic acid, a polymer, was more effective than monomers such as gallic acid, methyl gallate, and propyl gallate in forming complexes with macromolecules in biological systems (Field et al. 1989). Such binding may interfere with many enzymatic functions in bacteria and with the surface patterns of cells, which may be crucial for control of cellular growth (Pate and Ordal 1967).

This is also supported by the fact that the anti-C. columnaris activity of tannic acid was not found to have any demonstrable increase in pond water as contrasted with gallic acid, methyl gallate and propyl gallate. The pond water strongly reduced the ability of tannic acid to bind protein and glycogen (Table 3), resulting in the partial or full loss of the anti-cytophagal activity of tannic acid. Of course, there are many aquaculturial factors such as temperature, pH, light, dissolved oxygen, total alkalinity and hardness, other metal elements (iron and copper), and nutrients (e.g., casein and vitamin C) in pond systems, which have significance and influence the availability of tannic acid and related compounds (unpublished data).

Although gallic acid has relatively little binding capacity towards protein and glycogen, our results have shown that gallic acid was superior to the other test compounds in bacterial toxicity towards C. columnaris and cyanobacteria (Chung et al. 1995a). This suggests that another mechanism for tannins' bacterial toxicity is a possibility. The 1-carboxyl group of gallic acid may become charged by protonation or uncharged by deprotonation at physiological pH. The uncharged species may accelerate both the rates of diffusion and the final equilibrium distribution of the compound across the membrane bilayer of some bacteria (Nikaido and Thanassi 1993). It should also be noted that the various types of ester functionality on the tannin molecule are postulated to play a role in bacterial toxicity. Our preliminary work showed that those compounds with alkyl groups strongly inhibited the gliding motility of C. columnaris (data not shown). This suggests that action on the structural elements of the bacterial membrane by ester linkages may be relevant.

In general, it is concluded that a close relation-
ship appears to exist between chemical structure and bacterial toxicity. The hydroxyl groups of tannins are considered to be essential for antibacterial activity.

Regardless of the possible mechanisms of action, tannic acid and related compounds seem to be strong inhibitors of the growth of *C. columnaris*. Further study, such as investigation of tannin concentration in pond systems and its impact on fish, is necessary for assessing the commercial application potential of these compounds. In the natural aquatic environment, tannins may be important indigenous factors in the control of fish diseases.

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**References**


