Effect of Some Compounds of American and Chinese Chestnut Inner Bark on the Growth of *Endothia parasitica*

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ABSTRACT.— A compound extracted from the inner bark of healthy Chinese chestnut demonstrated 100 percent inhibition of growth of *Endothia parasitica.* This substance was found to be a long-chain unsaturated fatty acid.

A primary hindrance to production of blight resistant American chestnut (*Gas tanea dentata* [Marsh.] Borkh.) is the lack of an efficient test for screening seedlings for resistance. Such a test will be much easier to develop when the factors determining resistance in Chinese chestnut *(Castanea mollissima* Bl.) and, hopefully, American chestnut, are known. This paper reports some of the results from an investigation of the natural compounds in both American and Chinese chestnut inner bark. Petroleum ether soluble substances were tested for their fungistatic activity and the chemical nature of the more active ones determined.

EXTRACTION

Air-dried and ground healthy inner bark of American and Chinese chestnut was soaked at room temperature with petroleum ether for ten days. The solution was filtered and then the solvent removed under vacuum. The bark was put in a Soxhlet apparatus and extracted again with petroleum ether for a week. This extract was then dried under vacuum. Thin-layer chromatography (TLC) and spectral data of both extracts indicated that the two were similar; therefore, they were combined for subsequent fractionations and bioassays. The yield of petroleum ether extractives from the American species was 0.79 percent while that from the Chinese chestnut was 3.08 percent.

The crude extract was dissolved in high boiling petroleum ether and left in a refrigerator overnight. The solution was filtered, the solvent evaporated, and the residue used in the following purification.

PURIFICATION

Nuclear magnetic resonance data obtained on the crude residue indicated the presence of long-chain unsaturated acids. In an attempt to isolate pure compounds, 200 mg of the residue were refluxed overnight with 10 percent NaOH in MeOH. This solution was then extracted with petroleum ether to give aqueous layer I and organic layer 1. The aqueous layer was made strongly acidic with 10 percent HC1 and washed five times with petroleum ether to get organic fraction II. The aqueous layer was then washed with CH_2Cl_2 to remove the more polar compounds to obtain organic layer III (Fig. 1).

Each of the organic layers was further purified by column or thick-layer silica-gel chromatography. Organic layer I had one major fraction (40 mg); layers II and III each had one major product with small amounts of three to four other compounds.

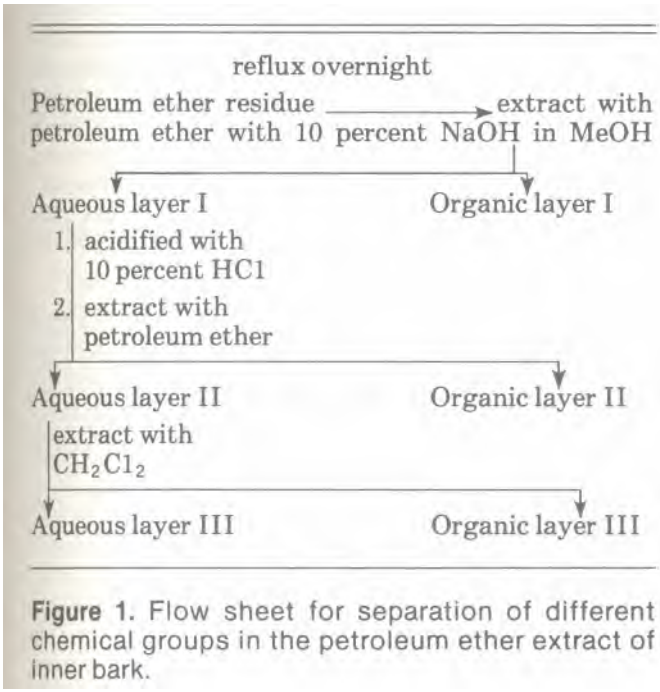

Figure 1. Flow sheet for separation of different chemical groups in the petroleum ether extract of inner bark.

BIOASSAY

The effect of some of these fractions, obtained from both the American and Chinese chestnuts, were tested on *Endothia parasitica* (Murr.) P. J. & H. W. And. growth using the methods described by Barnett (1973). One-half mg of each fraction was tested three times in the bioassay for fungistatic activity. The primary colonies of mycelia were incubated for 72 hours at room temperature then the diameters measured (Fig. 2

Analysis of variance of diameter growth indicated that some fractions significantly inhibited fungal growth. Duncan's Multiple Range test

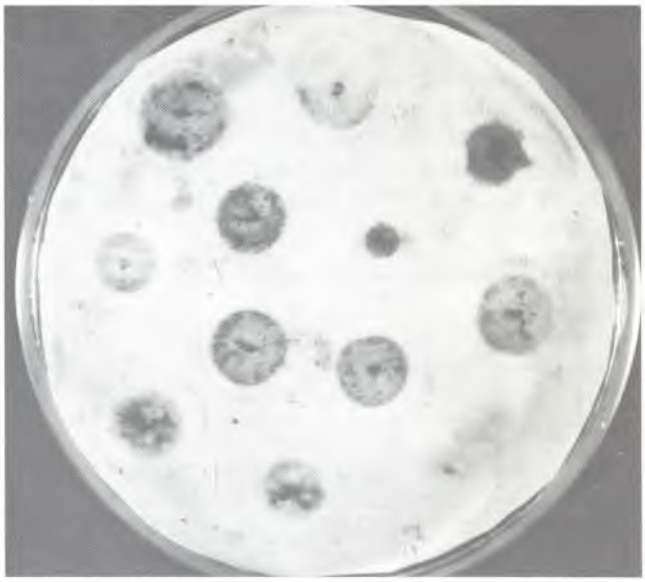

Figure 2. Growth of *Endothia parasitica* in bioassay of some fractions obtained from inner bark of American and Chinese chestnut after 72 hours of incubation. Colonies are stained with toluidene blue.

indicated that two of the 11 fractions tested were significantly more inhibitory than all other fractions and controls. Fraction #Ch-3 from organic layer II completely inhibited fungal growth at the concentration used (0.5 mg); and fraction #Ch-5 from organic layer III showed 75 percent inhibition of fungal growth (Table 1). Both fractions, giving a significant reduction in growth rate, were obtained from extracts of Chinese chestnut inner bark.

Table 1
Effect of some fractions from American and Chinese chestnut inner bark on *in vitro* growth of *E. parasitica*.

Fraction Number	Mean Diam. Growth (mm)	Percent of Control
Ch-3	0.0	0[a]
Ch-5	4.0	25
Am-10	13.5	68
Am-11	14.2	71
Am-7	14.7	74
Ch-1	15.2	76
Am-8	16.1	81
Am-9	16.1	81
Ch-4	18.1	87
Ch-2	19.7	99
Control	20.0	100
Oleic acid	21.1	106

[a] Figures connected by the same line are not significantly different at the 5 percent level.

The most active fraction #(Ch-3) was further tested to obtain a concentration-activity curve in the bioassay. Figure 3 illustrates that growth inhibition was related logarithmically to the concentration of this compound. At about 0.1 mg/ml50 percent inhibition of fungal growth occurred while complete inhibition was obtained with a concentration of 0.5 mg/ml.

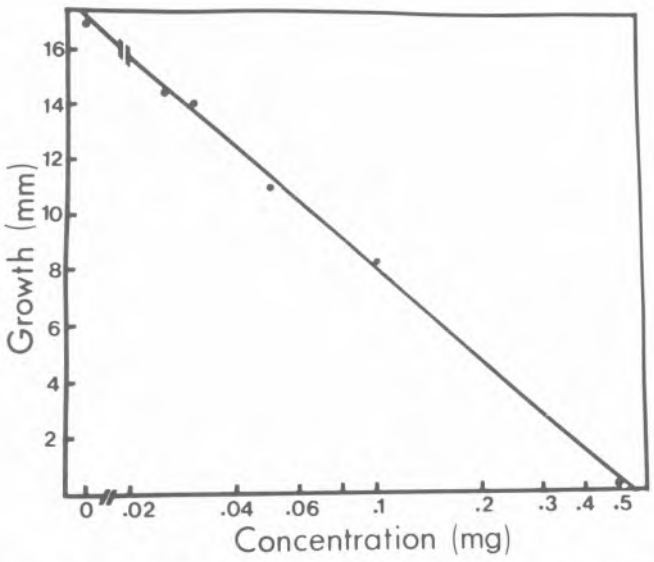

Figure 3. The growth of *E. parasitica* in bioassay with different concentrations of the active fraction (Ch-3) from Chinese chestnut.

IDENTIFICATION

The active fraction (Ch-3) was separated from organic layer II in the purification scheme. The infrared spectrum of this fraction displays a carbonyl absorption at 1710 cm^{-1}, OH stretching absorption at 3,300-2,500 cm^{-1}, and olefinic and alphatic CH stretching absorptions at 3,040 and 2,950-2,850 cm^{-1}, respectively. This indication that the principal component(s) of the fraction are unsaturated fatty acids is supported by ^{1}H and ^{13}C nuclear magnetic resonance spectra. Thus, the proton spectrum contains a broad triplet at (50.88 typical of methyl groups on hydrocarbon chains, an intense broad singlet at *61.26* typical of unbranched methylene chains, and a complex but symmetrical, olefinic absorption at (55.35. The carbon spectrum of this fraction is likewise indicative of an unsaturated fatty acid. Most absorptions due to the principal component(s) correspond closely to chemical shifts reported (Wenkert *et al.,* 1976) for oleic acid. However, intensity differences in the "unperturbed" methylene region ((529.0-29.5) and the allylic methylene region ((527.0) clearly indicate that the substance is not oleic acid. The principal methylene absorptions in the oleic acid spectrum appear at (529.0 and have been assigned (Wenkert *et al.,* 1976) for carbons 4, 5, 14, 15, while those methylene positioned closer to the double bond C - 6, 13 and C - 7, 12 absorb at 629.3 and (529.5, respectively. The principal methylene absorption in this active fraction appear at (529.5, with somewhat less absorption at (529.0. This suggests that we are dealing with a branched chain or a multiple unsaturated material. If the latter is correct, the olefinic bands must be well insulated from one another since their carbon resonances appear at ß 130 which is typical of isolated double bonds (i.e., in oleic acid at (5129.6, (5129.8). Unfortunately, due to lack of material, reliable integration of the carbon spectrum was not possible even with Forier Transform Techniques, hence, an accurate estimate of olefin content is not possible at this time. Furthermore, the low signal/noise likewise prevents complete confidence that all meaningful absorptions have been identified.

Work is continuing with this fraction to get mass spectral data and comparison with authentic samples of other fatty acids so that a definite determination of the structure can be made.

Acknowledgement

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