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Aflatoxin Inhibition and Fungistasis by Peanut Tannins[1]

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ABSTRACT

Three fractions of tannins were isolated from Florunner peanut testa. Both the growth of *Aspergillus parasiticus* (NRRL 2999) on PDA agar and production of aflatoxins in liquid culture were inhibited by the tannins. The ability to inhibit growth was inversely related to the ability to inhibit aflatoxin production for each fraction. Two types of tannin assays were inadequate as predictors of the biological activity of the tannin fractions.

Key Words: Aflatoxin, *Aspergillus parasiticus*, Tannin, Peanut, Fungistasis

Tannins represent a large class of heterogenous compounds. Haslam (9) suggested that the general definition of a tannin was a polyphenol with a molecular weight range of 500-3000 and having some tanning action on leather. Tannins are generally divided into two classes (9); the hydrolyzable tannins containing a carbohydrate core with phenolic carboxylic acids bound by ester linkages and the condensed tannins or condensed procyanidins (proanthocyanidins) that do not contain carbohydrates and are not as easily hydrolyzed.

Stansbury et al. (14) isolated tannin from spanish peanuts and investigated some of the chemical properties of peanut tannins. Carter (4) reported that peanut tannin extracts from colored seed coats inhibited the germination of *Aspergillus flavus* spores. Sanders and Mixon (13) isolated a crude tannin extract from peanut testa that exhibited fungistatic activity toward actively growing *A. parasiticus* (NRRL 2999) and could be related to percent colonization by *A. parasiticus*. Sanders and Mixon (13) did not attempt to fractionate the tannin extract to ascertain the role of certain tannins in the observed fungistatic assay. Sanders (12) further investigated this tannin extract and concluded that the extract contained primarily species of the condensed tannin class.

The purpose of this investigation was to examine the fungistatic properties of various condensed tannin fractions from peanut testae including their effect on aflatoxin production by *A. parasiticus*. During the course of this investigation, two types of analytical assay for tannins were adapted from other work (3, 6). The ability of these assays to predict fungistatic activity is discussed.

Although the tannins isolated from peanut seed coat are known to produce cyanidin (1) during acid hydrolysis and therefore belong to the class of condensed procyanidins, their structures have not been elucidated, therefore the term "tannin" will be used in this paper as it has been used in previous literature.

Materials and Methods

Tannins were isolated from dry, cured Florunner peanut testae obtained from commercial shelling operations. A flow diagram of the isolation procedure is presented in Figure 1. The testae were defatted with

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petroleum ether and air dried, then ground in 70% aqueous acetone in a high speed blender, and filtered. The residue was further extracted with 50% aqueous acetone and filtered, then extracted statically overnight with 50% aqueous acetone and refiltered. The combined aqueous acetone extracts were vacuum evaporated at 40 C to remove the acetone. The resulting aqueous phase was then extracted 8 x 500 mL with ethyl acetate. The combined ethyl acetate phases were vacuum evaporated to dryness at 40 C to effect the EtAC fraction. The aqueous phase was concentrated to one-half of the original volume and chromatographed on a 3.5 x 10.8 cm column packed with 80% ethanol equilibrated Sephadex LH 20 (Pharmacia). A 50 mL aliquot of the aqueous extract was layered on the gel and eluted first with 80% ethanol/water until the eluate was essentially clear (200-300 mL), followed by 50% aqueous acetone until all colored material had been removed from the column (100-150 mL). Sufficient material for the fungistatic assays was obtained by combining equivalent fractions from three separate columns after checking the elutes on thin layer chromatography (System A, Table 1) to ensure that the fractions from each column were equivalent.

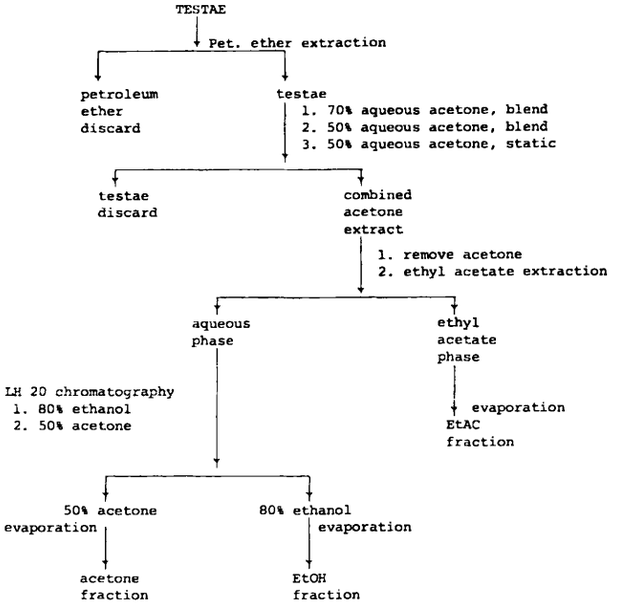

Fig. 1. Flow diagram of isolation procedure.

The 80% ethanol eluates were reduced to dryness and designated as the EtOH fraction, and the 50% acetone eluates were similarly combined and designated as the acetone fraction. Material for chemical assay and chromatographic characterization was obtained from a separate 50 mL aliquot processed in the same manner.

Fungistatic assays were performed on two separate media. Solid state assays were performed on 1 mL of 2% potato dextrose agar contained in 35 mm petri dishes. Plugs 5 mm in diameter were cut from lawns of well sporulated *A. parasiticus* (NRRL 2999) and inverted onto PDA plates, which had been treated with 0.3 mL of the various fractions dissolved in water at a concentration of 33 mg/mL. Liquid assays were done with the YES media of Davis et al. (5) amended with 5% MycoBroth (YESB). Tannin concentrations were 1% w/v and inoculations were 0.2 mL of a 6.2 x 10^6 spores/mL suspended in distilled water (1 drop Tween 20).

Production of aflatoxin in a liquid medium was determined by extracting one mL of substrate with one mL of benzene. Aflatoxin concentrations in the YESB media were determined by HPLC on a Waters instrument equipped with a radial compression module and 10 μ silica column. The eluting solvent was water saturated $CHCl_3$ plus 0.6% MeOH at a flow rate of 1.5 mL/min. A Varian Fluorichrom solid state fluorescence detector, WISP710B, and a Waters data module were used for detection, injection of 25 μL and automatic recording and system control. Thin layer chromatographic analysis of the various fractions were performed with the systems listed in Table 1.

Tannin assays by BSA precipitation were carried out according to Hagerman and Butler (6). Tannic acid (F. W. 1701.18, Fisher) was employed as the standard. Results are reported in tannic acid equivalents (TAE). The acidified vanillin method of Broadhurst and Jones (3) was also used. Catechin was employed as the standard and the results were calculated in catechin equivalents.

Results and Discussion

Isolation and Characterization

The strategy of the isolation procedure was initially based on using the ethyl acetate partition to remove the small molecular weight monomeric and dimeric catechins (10). Unexpectedly, it was found that the ethyl acetate partition also removed a sizeable quantity of highly polymeric tannins. Thin layer chromatography (System A) indicated that the EtAC fraction contained polymeric procyanidins with trace amounts of non-catechin impurities. Further TLC investigation with System B also indicated the presence of a small amount of a leucocyanidin-type compound. Analysis for benzoic acid derivatives

Table 1. Chromatographic methods

System	Substrate	First Direction		Second Direction		Detection	Reference
A	Avicel	1-Butanol	5	Water	94	p-Toluene	
	20 x 20 cm	Water	4	Acetic acid	6	Sulfonic acid	
	Analtech, Inc.	Acetic acid	1				
B	Avicel	1-Butanol	14	Water	94	Diazotized	Thompson (15)
	20 x 20 cm	Water	5	Acetic acid	6	Sulfanilic acid	
	Analtech, Inc.	Acetic acid	1				
C	Silica gel	Benzene	45	Benzene	90	Diazotized	Randerath (11)
	20 x 20 cm	Methanol	8	Dioxane	25	Sulfanilic acid	
	Whatman K-5	Acetic acid	4	Acetic acid	4		
D	Silica gel	1-Butanol	5			p-Toluene	
	5 x 10 cm	Acetonitrile	2			Sulfonic acid	
	E. Merck	Acetic acid	1				

by TLC (System C) was inconclusive because the large amount of tannin present influenced the Rf values to such an extent that no direct comparison with reference standards could be made. However, a sample submitted for microanalysis by GLC-mass spectroscopy was found to contain small amounts of protocatechuic and salicylic acids.

Sephadex LH 20 chromatography was used to fractionate the remaining tannin into two components. The first fraction was obtained by elution with 80:20 ethanol-water (v/v) until no further colored material eluted from the column. The remaining tannin was then eluted by 50% aqueous acetone. Thin layer analysis of the two fractions (Systems A and B) indicated that they contained essentially equivalent proanthocyanidin species. Microanalysis by GLC-MS indicated that the ethanol fraction was contaminated by minute amounts of protocatechuic acid. Further analysis of the fractions was resumed after the apparent lack of inhibition with the EtOH fraction was observed during radial growth inhibition studies. A rather polar solvent system was developed (System D), which confirmed the presence of three previously undetected compounds in the ethanol fraction (Rf: 0.43, 0.51, 0.71). The compounds were non-fluorescent, but exhibited a quickly fading yellow fluorescence after spraying with p-toluene sulfonic acid and heating. No reaction was observed with either diazotized sulfanilic acid or anisaldehyde-sulfuric acid sprays, indicating that the compounds were not phenolics or carbohydrates. An attempt to isolate the compounds by re-chromatographing an aliquot of the ethanol fraction on LH 20 (3.5 x 10.8 cm column) with a step gradient elution from ethanol to water (10% increments x 300 mL) located the compounds in the 0-85 mL ethanol fraction (EtOH 2). Tannins were also located in the 134-510 mL fraction and the 680-1020 mL fraction (EtOH 4 and EtOH 8, respectively). The EtOH 2 fraction gave the same reactions and Rf values (TLC System D) as those in the original EtOH fraction. Ninhydrin detection spray for amines gave a very faint red-pink reaction for the 0.43 Rf spot, but no reaction was observed for the 0.51 and 0.71 Rf spots.

Fungistatic Activity

The fungistatic properties of the tannin fractions are shown in Table 2. The radial growth inhibition exhibited by the EtAC and acetone fractions is within the range predicted by data (13) for the crude tannin extract taking into account the difference in incubation times and concentrations of tannins. The extremely low value of 12.1% inhibition for the EtOH fraction indicates the presence of some mitigating principle. The exact nature of this principle is undetermined; however, re-chromatography of the EtOH fraction gave three fractions: EtOH 2, EtOH 4, and EtOH 8. Submission of these ethanol fractions to radial growth assay gave inhibition values of -42.4, 35.4, and 15.5% after 48 hours incubation for EtOH 2, EtOH 4, and EtOH 8, respectively. The negative inhibition of -42.4% is equivalent to a growth promotion of 29.8%.

Not shown in Table 2 are the percent inhibition values for the three fractions at the 24- and 48-hour incubation times. The relative order exhibited in Table 2 was maintained throughout the entire experiment, but changes in the magnitude of inhibition with incubation time varied greatly. The EtAC fraction percent inhibition at 24 hours was 85.1%, dropping to 69.8% at the 48-hour mark. The EtOH fraction also exhibited similar decreases with 36.2% and 25.1% at the 24- and 48-hour marks, respectively. The acetone fraction gave a 53.2% inhibition at 24 hours and 46.4% inhibition at 48 hours. The relative decrease of inhibition with incubation time indicates that these fractions may be dissimilar in their chemical components or basic structure.

Table 2. Radial growth inhibition by peanut tannins.

Tannin Fraction	Colony Diameter (mm) at 24 hrs	48 hrs	72 hrs	72 hours % Inhibition
Control	9.7 ± 0.47	22.9 ± 0.85	29.8 ± 0.96	
EtAC	5.7 ± 0.48	10.4 ± 1.11	15.2 ± 0.76	58.9
EtOH	8.0 ± 0.36	18.4 ± 0.65	26.8 ± 1.72	12.1
Acetone	7.2 ± 0.63	14.6 ± 1.34	19.4 ± 0.75	41.9

Tannin concentration = 1% w/v PDA plate

Values are means of four replications with two measurement/replication

All means are significantly different at 5% level, Duncan's New Multiple Range Test.

The results of the aflatoxin inhibition study in YESB medium are shown in Table 3. The high aflatoxin inhibition value of 76% for the EtOH fraction was an unexpected result in view of the low radial growth inhibition exhibited by this fraction. The aflatoxin inhibition values for the EtAC and acetone fractions, 35% and 56%, respectively, are also reversed in relation to the magnitudes of radial growth inhibition of these fractions. The reversed order of aflatoxin inhibition to growth inhibition for the tannin fractions indicates the possibility of a selective interaction by the tannin fractions for various proteins in the media (8). Neither PDA nor YESB medium is a chemically defined medium, thus, the variety of proteins for which a selective interaction might occur is large. A precipitate formed in the YESB medium with the tannins which was insoluble in aqueous acetone and aqueous ethanol. This prevented accurate measurement of the mycelial mat weights and a direct comparison of the growth inhibition in each medium.

Table 3. Tannin inhibition of aflatoxin production in YESB medium.

Tannin Fraction	Aflatoxin Production (mg)[1] B_1	B_2	G_1	G_2	Total	% Inhibition	B/G Ratio
Control	4.42	0.25	18.42	0.33	23.42		0.249
EtAC	6.25	0.33	8.42	0.17	15.17	35	0.766
EtOH	3.58	0.25	2.00	0.00	5.83	76	1.915
Acetone	4.50	0.08	5.67	0.08	10.33	56	0.797

Tannin concentration = 1% w/v media

Seven day incubation at 27 C

[1]Means of four replications

The ratio of aflatoxins B_1 and B_2 to aflatoxins G_1 and G_2 also varied significantly for the treatments in relation to the control ratio. The change in produciton of G aflatoxins with respect to the B aflatoxins for the tannin fractions indicates that not only can a general fungistatic activity be asscribed to the peanut tannins, but also a selective inhibition mechanism may be present. Hagerman and Butler (7,8) found that sorghum tannins preferentially bind proteins rich in proline. It is possible that a similar

mechanism is present in peanut tannins that would result in differences in mycelial growth and aflatoxin production due to the decreased availability of a necessary nutrient.

Prediction of Fungistatic Activity

The ability of tannins to complex with proteins is well known. Bate-Smith (2) proposed a method of measuring the astringency of tannins by haemanalysis. However, hemoglobin is not always readily available and Hagerman and Butler (6) proposed a method based on bovine serum albumin (BSA) precipitation. This method was applied to the tannin fractions of peanut testae utilizing tannic acid as a standard. Since the dry weights of the peanut tannin fractions were known, it was possible to calculate the tannic acid equivalents (TAE) per gram of tannin. The results were 0.632, 0.496, and 0.754 TAE/g for the EtAC, EtOH, and acetone fractions, respectively. Comparison of the TAE values with radial growth inhibition and aflatoxin production inhibition indicates that the TAE value was not an accurate predictor of the fungistatic properties of the peanut tannin fractions isolated in this study.

The reaction of acidified vanillin with catechin-containing compounds has been the basis of several quantitative chemical analyses for condensed tannins. The method of Broadhurst and Jones (3) was chosen for this study. Catechin was used as a standard since it is readily available in pure form. Calculation of catechin equivalents (CE) per gram of tannin for each tannin fraction was done analogous to TAE values. The results of this assay were 0.209, 0.107, and 0.117 CE/g for the EtAC, EtOH, and acetone fractions, respectively. Comparison of CE values with the fungistatic properties of the isolated fractions indicated that these values were also not accurate predictors of activity. However, the values do show that the EtOH and acetone fractions contain essentially equivalent tannins in terms of their catechin content.

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