Isolation of Tannins from Leaves of Some Trees and Shrubs and Their Properties

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Tannins extracted from leaves of five trees/shrubs (*Acioa barteri*, *Dichostachys cinerea*, *Guiera senegalensis*, *Quercus incana*, and *Piliostigma reticulatum*) in aqueous acetone containing ascorbic acid were isolated using Sephadex LH-20. There was no significant difference detected between ascorbic acid containing preparations and preparations in which ascorbic acid was removed, based on the vanillin-HCl (VA), butyl alcohol-HCl-Fe^{3+} (BHF), and protein precipitation assays, suggesting that the removal of ascorbic acid from the tannin preparation did not oxidize the tannins studied. The λ_{max} of the preparations free of ascorbic acid was between 270 and 280 nm. The protein precipitation capacity (PPC) was from 3 to 10 g of bovine serum albumin precipitated/g of tannins. The poor correlations of PPC with condensed tannins (r = 0.58 and 0.51, respectively) with the VA and BHF values and a high correlation (r = 0.95) with total phenolic content suggested the preparations to be a mixture of condensed and hydrolyzable tannins, which was substantiated by the detection of gallic acid on hydrolysis of these tannins. These preparations should be used as a standard with caution. The PPC of isolated tannins was almost the same as that for those present in the extract, indicating that the biological activity of the isolated tannins is similar to those present in the plant.

INTRODUCTION

Tannins are chemically diverse polyphenolics which have a variety of biological activities. Various assays are available for quantification of tannins (see reviews: Tempel, 1982; Deshpande et al., 1986; Makkar, 1989). The problems encountered in selecting appropriate standards for tannin assays have been discussed by Hagerman and Butler (1989). Tannins to be used as a standard for determining the absolute amount of tannins in a plant or for studying their nutritional, physiological, and ecological implications could be obtained by purifying the tannins from the plant of interest (Wisdom et al., 1987; Van Hoven and Furstenburg, 1992). A simple purification procedure based on Strumeyer and Malin (1975) using Sephadex LH-20 has been used by various workers for obtaining standards (Hagerman and Klucher, 1986; Van Hoven and Furstenburg, 1992). The addition of ascorbic acid to prevent oxidation of tannins during the extraction and purification procedures has been recommended (Hagerman and Klucher, 1986; Scalbert et al., 1990; Peng and Jay-Allemand, 1991). Ascorbic acid added in aqueous acetone used for elution of tannins from Sephadex LH-20 would be present in the lyophilized tannin samples as a contaminant, which makes the tannin preparation unsuitable for use as a standard. Also, ascorbic acid interferes in the redox assays of tannins. The preparation containing ascorbic acid can also not be used in various biochemical studies unless the amount of ascorbic acid present in the preparation is known. In addition, the isolated tannins with or without ascorbic acid should have activity similar to those present in the leaves. The objectives of the present study were (i) to determine the extent of ascorbic acid present in the tannin preparation, (ii) to remove ascorbic acid from the tannin preparation and compare this tannin preparation with that containing ascorbic acid using various chemical and protein precipitation assays, and (iii) to compare the activity (capability to precipitate protein) of tannins obtained after removal of ascorbic acid with that of the tannins present in the leaves.

MATERIALS AND METHODS

Isolation of Tannins. The purification procedure was essentially according to that of Strumeyer and Malin (1975). Extracts of the dried (at about 50 °C in a forced-air-draft oven) ground leaves (15–20 g) were prepared in 100 mL of 70% aqueous acetone containing 0.1% ascorbic acid using an ultrasonicator. The supernatant obtained on centrifugation was evaporated under vacuum at about 30 °C to remove acetone. The aqueous sample was lyophilized. The dried sample was dissolved in 80% aqueous methanol containing 0.1% ascorbic acid and, after filtration, added to a swollen slurry of Sephadex LH-20 prepared in 80% aqueous methanol containing 0.1% ascorbic acid. It was stirred for about 5 min and then filtered through sintered glass crucibles. The Sephadex LH-20 was washed slowly on the crucibles with 80% aqueous methanol containing 0.1% ascorbic acid under gravity without applying vacuum until absorbance of the eluate at 280 nm was <0.05. The procedure removed non-tannins from the Sephadex. The tannins remained on the Sephadex LH-20 and gave it a brown color. Tannins were eluted using 50% aqueous acetone containing ascorbic acid (1 mg/mL). The volume of eluate was recorded (x mL which contained x mg of ascorbic acid). Acetone was removed under vacuum at about 30 °C, and then, the aqueous solution containing tannins was lyophilized in a container of known weight. The lyophilized sample (y mg) had x mg of ascorbic acid, and the rest (y mg – x mg) was the tannins. In our preparations, 40–60% ($100x/y$) ascorbic acid was present (Table 1). A portion of this preparation was stored in a dessicator in the cold room, and the rest was used for removal of ascorbic acid.

Removal of Ascorbic Acid. The tannin preparation containing ascorbic acid was dissolved in 80% aqueous methanol and added to Sephadex LH-20 prepared in 80% aqueous methanol without ascorbic acid. The Sephadex LH-20 was washed to remove ascorbic acid using 80% aqueous methanol (about 300 mL for 25 g of the gel). Tannins were adsorbed on the gel, and the ascorbic acid was removed with the washings. The adsorbed tannins were eluted by 50% aqueous acetone. The rest of the procedure was the same as described above. This preparation was free of ascorbic acid. These two preparations with and without ascorbic acid were compared using the following tannin assays. For comparison of these assays, if the weight of preparation without ascorbic acid taken was a, then the corre-

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Table 1. Comparison of Tannin Preparations with (+) and without (-) Ascorbic Acid[a]

	proanthocyanidins[b] (A_{550nm})		vanillin-HCl method[c] (μg of catechin equivalent)		PPC[d] (mg of BSA pptd/mg of tannin)	
	+	-	+	-	+	-
Dichostachys cinerea (1)	0.242 ± 0.007	0.244 ± 0.006	18.58 ± 1.05	18.30 ± 0.56	9.44 ● 0.62	9.27 ± 0.81
Acioa barteri (2)	0.537 ± 0.004	0.538 ± 0.004	26.10 ● 0.70	27.20 ● 0.27	9.78 ± 0.64	10.25 ● 0.80
Quercus incana (mature) (3)	0.348 ± 0.005	0.346 ± 0.004	21.27 ● 0.61	22.80 ● 0.90	6.02 ± 0.41	5.56 ± 0.37
Quercus incana (young) (4)	0.229 ± 0.004	0.222 ± 0.003	6.01 ± 0.12	6.21 ± 0.23	8.21 ● 0.50	8.65 ± 0.43
Piliostigma reticulatum (5)	0.539 ± 0.004	0.542 ± 0.003	10.47 ± 0.50	11.00 ± 0.40	2.70 ● 0.13	2.73 ± 0.20
Guiera senegalensis (6)	0.340 ± 0.004	0.333 ● 0.003	13.20 ± 0.60	14.30 ± 0.31	3.00 ● 0.20	2.85 ± 0.15

[a] BSA, bovine serum albumin. Values are mean ± SD ($n = 3$). Ascorbic acid content (%) of the preparations was 1, 46.36; 2, 45.26; 3, 49.00; 4, 60.00; 5, 47.50; and 6, 44.60. Tannin preparations (mg/10 mL of 50% methanol) used were 1 (-), 11.15; 1 (+), 20.79; 2 (-), 16.15; 2 (+), 29.5; 3 (-), 13.21; 3 (+), 25.90; 4 (-), 13.21; 4 (+), 33.03; 5 (-), 11.51; 5 (+), 21.92; 6 (-), 11.51; 6 (+), 20.77. [b] Microliters used in the assay (see Materials and Methods) were 1, 25; 2, 12.5; 3, 25; 4, 50; 5, 50; 6, 100. [c] 50 μL used in the assay (see Materials and Methods) for all except 2 and 6 for which 25 and 100 μL, respectively, were used. [d] Microliters used in the assay for the regression: 1, 50–400; 2, 40–300; 3, 40–400; 4, 40–240; 5 and 6, 100–900.

Table 2. Total Phenols (TP, Grams of Tannic Acid Equivalent/Gram of Tannins), Condensed Tannins by Vanillin-HCl Assay (VA, Milligrams of Catechin Equivalent of 1% Tannin in the Assay) and Butyl Alcohol-HCl-Fe^{3+} Assay (BHF, $E^{1\%}_{550nm}$), Protein Precipitation Capacity (PPC, Grams of Bovine Serum Albumin Pptd/Gram of Tannin), Relative Degree of Polymerization (DP), and λ_{max} of Isolated Tannins Free of Ascorbic Acid

		CT		PPC			
	TP	VA	BHF	isoltd	calcd[a]	DP	λ_{max}
Dichostachys cinerea	0.93	8.21	157.5	9.27	8.64	2.67	275.5
Acioa barteri	0.84	16.84	479.7	10.25	7.11	5.62	272.5
Quercus incana (mature)	0.73	8.63	188.6	5.56	4.86	4.12	270–272
Quercus incana (young)	0.81	2.35	60.5	8.65	na	5.21	*b*
Piliostigma reticulatum	0.53	4.78	169.5	2.73	3.04	7.10	278.5
Guiera senegalensis	0.55	3.11	52.1	2.85	2.66	2.41	274.5

[a] na, not available. From the data in the first column of Tables 1 and 2 of Makkar et al. (1993a), (value in first column of Table 2 × 1000)/(value in first column of Table 1). [b] No defined peak from 224 to 400 nm.

sponding weight for the preparation with ascorbic acid was $a/((y - x)/y)$ or $ay/(y - x)$ (Table 1).

Analysis on Isolated Tannins. The condensed tannins were determined essentially by the vanillin-HCl method of Broadhurst and Jones (1978). In short, to a 0.25-mL aliquot of tannin solution (in 50% methanol) were added 1.5 mL of the vanillin-HCl reagent and 0.75 mL of concentrated HCl. Absorbance was recorded at 500 nm after 20 min. The condensed tannins were also determined by the butyl alcohol-HCl-Fe^{3+} method of Porter et al. (1986), except that the assay volume was reduced 4-fold, i.e., to 0.25 mL of 50% methanol containing tannins were added 1.5 mL of the butyl alcohol-HCl reagent and 0.05 mL of the iron reagent. The test tubes containing this mixture were heated for 40 min in a heating block adjusted to 95 °C. After the tubes were cooled, absorbance was recorded at 550 nm using proper blanks (unheated mixture; there was no pinkish-red color suggesting absence of flavan-4-ols). The protein precipitation capacity was determined using the dye-binding method of Asquith and Butler (1985) as described in Makkar et al. (1993a). The relative degree of polymerization was determined essentially by the method of Butler et al. (1982), except that the absorbance was recorded at 510 nm after 60 min for the vanillin assay in acetic acid, as the absorbance increased with time for all the tannins studied. The rate of increase of absorbance was higher up to 40 min; thereafter, it decreased and almost plateaued at 60 min.

All the above assays were performed after dissolving tannins in 50% methanol. The nitrogen content of the tannin preparation free of ascorbic acid was determined using the methods of Grimble et al. (1988).

The tannin preparations (3–4 mg) were hydrolyzed using 1.5 mL of 2 N H_2SO_4 as described by Inoue and Hagerman (1988). The pH of the hydrolysate was brought between 5 and 6 using about 1 N NaOH. The volume was made to about 10 mL and loaded on to the HPLC (Charrier et al., 1992). The gallic acid peak was identified by two procedures: (i) comparison of retention time of the sample gallic acid peak with that of the authentic gallic acid standard (Sigma Chemical Co.) using the same HPLC operating conditions and (ii) cochromatography of the extract with the standard gallic acid.

RESULTS AND DISCUSSION

Comparison of Tannin Preparations. Table 1 presents comparison by three methods of tannin preparations with and without ascorbic acid. The condensed tannin content using butyl alcohol-HCl-Fe^{3+} and vanillin-HCl reagents was the same in both the preparations for all the tannins studied. The protein precipitation capacity (PPC), which is considered as a measure of potential biological activity, also did not differ between the preparations. The two preparations can not be compared by the redox assays like in the Folin–Ciocalteu and Folin–Denis methods as ascorbic acid interferes in these assays. The results obtained suggested that the removal of ascorbic acid from the tannin preparations did not inactivate by oxidation the tannins from the leaves we studied.

Some tannins may be labile and undergo inactivation. For such tannins, the values obtained after removal of ascorbic acid would be lower than those for the ascorbic-acid-containing preparation (after accounting for the ascorbic acid present). Should this be the case, the preparation containing ascorbic acid can be used in various biochemical and ecological studies and the approach described here would enable one to know the amount of tannin present in the preparation.

Some Properties of the Isolated Tannins Free of Ascorbic Acid. Table 2 shows the contents of total phenols, λ_{max}, PPC, relative degree of polymerization of tannins, and condensed tannins as absorbance at 550 nm of 1% tannin in the butyl alcohol-HCl-Fe^{3+} assay ($E^{1\%}_{550nm}$) and as mg of catechin equivalent of 1% tannin in the vanillin-HCl assay. The λ_{max} of the tannins was between 270 and 280 nm. There was a large difference in the PPC of the tannin preparations, which varied from 3 to 10 g of bovine serum albumin precipitated/g of tannins, suggesting that different tannins even at the same level could have different biological effects. The PPC was highly correlated ($r = 0.95$) with total phenolic content. On the

other hand, correlation of PPC with condensed tannins was lower (r = 0.58 and 0.51 with mg of catechin and absorbance at 550 nm, respectively). This poor correlation is due to the presence of hydrolyzable tannins in the preparations. Some of the leaves studied are known to contain both condensed and hydrolyzable tannins (Makkar et al., 1988; Dawra et al., 1988). The gallic acid has also been detected by HPLC after hydrolysis of all the tannins isolated (free gallic acid was not detected before hydrolysis). Both condensed and hydrolyzable tannins contribute to the PPC. Similarly, there was very poor correlation (r = −0.04) of the PPC and the relative degree of polymerization, which is obvious as the method (Butler et al., 1982) measures the degree of polymerization of condensed tannins. The tannin preparations which are a mixture of condensed and hydrolyzable tannins should not be used as a standard for the determination of condensed tannins in the plant extract. Similarly, the extinction coefficients obtained for condensed tannins (Table 2) are not of any use in determination of condensed tannins in the plant as these would overestimate the condensed tannins.

The nitrogen content of the tannin preparation in the present study was very low (<0.3%) even without the phenol treatment used by Hagerman and Butler (1980) for sorghum and beans to remove proteins from the isolated tannins. It appears that the proteins do not come along in any significant amount in the tannins isolated from leaves. The isolated tannins do not appear to contain any significant amount of other contaminants, as the weight of the residue following drying of the aqueous acetone eluate was nil on subjecting a sample of grass hay (free of tannins) to the chromatographic conditions for isolating tannins. The tannin preparations were also free of ascorbic acid as judged by HPLC using the procedure of Mueller-Harvey et al. (1987), except that the gradient time was reduced from 50 to 15 min (under these conditions the authentic ascorbic acid standard gave a peak at about 3 min).

Tannins determined gravimetrically in a plant source and their PPC as determined in Makkar et al. (1993a) allow one to compare the PPC of the isolated tannins with that of those present in the plant. The PPC of the isolated tannins was slightly higher than the calculated values for the leaves (Table 2). For the gravimetric determination of tannins, the plant extracts were prepared in 70% aqueous acetone, and for the PPC, the extracts were in 50% aqueous methanol, as acetone interferes in the protein precipitation assay (Makkar et al., 1993a). Methanol (50%) extracted less tannins from these leaves compared to 70% acetone (Makkar et al., 1990, 1993b), which led to lower values of PPC/g of tannins present in the leaves. The results obtained for PPC of the isolated tannins and those present in the leaves (Table 2) suggest that the isolated tannins have not undergone any change during the isolation procedure and these can be considered as true representatives of those present in the leaves. The biological activity of these isolated tannins would be expected to be similar to that of those present in the leaves. The tannins thus isolated can be used for studying the nutritional, physiological, and ecological implications of tannins in biological systems. The above approach for comparison of PPC of isolated tannins and those present in the plant could also be applied for isolated tannins containing ascorbic acid after accounting for the weight of ascorbic acid present in the preparation.

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