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ASTRINGENT TANNINS OF THE LEAVES OF *GERANIUM* SPECIES*

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Key Word Index—*Geranium*; Geraniaceae; leaves; proanthocyanidins; galloyl esters; gallotannins; ellagitannins; geraniin; astringency; chromosome numbers; geography; taxonomy.

Abstract—Fifty per cent methanolic extracts of the leaves of 70 species (including some subspecies, varieties and one hybrid) of *Geranium* have been analysed for proanthocyanidins, total galloyl esters, hexahydroxydiphenoylglucose and astringency. Proanthocyanidins are present in most species (prodelphinidin in four) but only rarely in considerable quantities and then not readily extracted. A reaction with potassium iodate characteristic of geraniin (a derivative of dihexahydroxydiphenoylglucose) is given by most species. The very considerable differences in astringency between species cannot at present be accounted for in terms of chemical differences, but a systematic review suggests that geographical location, chromosome number and annual or biennial habit are involved in the amount of tannin present in particular species.

INTRODUCTION

Since the publication in 1973 [1] of a PC survey of the flavonoid constituents of *Geranium*, methods have been developed for the determination of proanthocyanidins [2], ellagitannins [3], total galloyl esters [4] and the astringency of extracts by precipitation of blood proteins [5]. These methods have now been applied to the analysis of the leaves of the 70 species which comprise the collection now being grown in the University Botanic Garden at Cambridge by Dr. P. F. Yeo. The opportunity has been taken of evaluating the usefulness of the methods in a genus such as this in which the species vary widely in tannin content of both kinds, condensed and hydrolysable. These methods will first be described.

RESULTS

Hydrolysis with 2 N HCl

This is a necessary preliminary to the analysis of the unhydrolysed tissue, the amyl alcoholic extract of the hydrolysate being used for PC. Nearly all species of *Geranium* show at least a tinge of anthocyanidin, several a deep red. It is only the latter which contain enough proanthocyanidin to contribute to the astringency of the leaves, but the former may show a faint spot of cyanidin on the PC. This is recorded in Table 1 as a 'trace'; otherwise as 'nil'. Ellagic acid is revealed as a violet spot in UV, R_f 0.35 and gallic acid as a dark purple spot when fumed with NH_3, R_f 0.65. Other dark (absorbing) constituents may be present and identifiable.

Preparation of the extract

The fresh leaves are dried at 40° for 24 hr, ground, and sieved through a 100 mesh sieve. The powder is then extracted three times by boiling in 50% MeOH and the extract filtered through fibreglass. The MeOH is removed by distillation *in vacuo* to a volume representing *ca* 1% with respect to the powder.

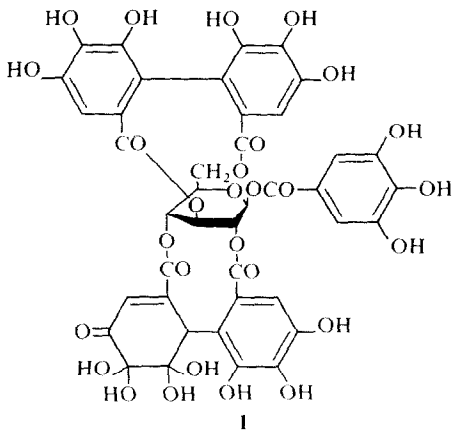
Determination of proanthocyanidin

0.5 ml of extract is heated for 2 hr at 95° with about 5 ml 5% butanolic HCl and the absorbance of the anthocyanidin produced measured in a recording spectrophotometer. Cyanidin has λ_{max} 547, delphinidin λ_{max} 558, so that the relative amounts of each can be calculated from the observed λ_{max}. The contribution to the absorption by chlorophyll can be corrected by balancing against an unheated extract. The proanthocyanidin content can only be expressed in terms of the extinction coefficient $E^{1\%}_{1\,cm}$, because the conversion to anthocyanidin varies so much with the constitution of the proanthocyanidin actually present [3]. A useful approximation can be achieved, however, by assuming an 'average' $E = 150$ for procyanidin and $E = 300$ for prodelphinidin. The extractability of the condensed tannins can be evaluated by comparing the E of the extracts with that of the powder, suspended in a small volume of 50% MeOH and similarly heated in BuOH/HCl.

Determination of ellagitannins

Usually present in plant tissues as esters of hexahydroxydiphenic acid, these are determined by reaction in absence of oxygen with nitrous acid, which forms a red product, 500 nm, rapidly changing to blue, 600 nm, then slowly to yellow. The blue product reaches a maximum after a time dependent on temperature (about 10 min at 40° [3], but the maximum reached is independent of temperature. The extinction coefficient of the absorption reaction depends somewhat on the constitution of the ester, but for hexahydroxydiphenylglucose esters the average $E^{1\%}_{1\,cm} = 51.5$, and this value is assumed in calculating the HH% of an extract (Table 1).

* Part 5 in the series "Astringency of Leaves". For Part 4 see Bate-Smith, E. C. (1980) *Phytochemistry* **19**, 982.



I

While HH are undoubtedly present in most species of *Geranium*, recent work by Okuda [6] has shown that the main hydrolysable tannin in the genus is a more complex derivative which he has named geraniin and to which he has assigned structure (**I**). Haslam (personal communication) has found that geraniin is the main constituent of *G. robertianum* tannin and has supplied a sample of this. It differs in the λ_{max} (455 nm) of the initial red product and a lower maximum of the blue absorption at 600 nm, which is reached earlier (5 min). It also differs in another respect which will be described later. This means that the HH values calculated as described above are suspect and are accordingly reported in Table 1 as 'HH'. It is interesting, however, that the values so calculated agree quite closely with those recorded for the same species by Okuda as percentage of geraniin.

Some early results with *Geranium* species published in 1972 [7] reported several species, notably *G. reflexum*, having little absorption at 600 nm but considerable absorption at 530 nm, resulting in a red colour which developed more slowly than that at 600 nm and persisted longer. It seems likely that the constituent responsible for this variation is an O-substituted HH (cf. *Cornus* spp. [8]). It probably makes some contribution to astringency, but how much it is not at present possible to say.

Total galloyl esters (TI)

The reaction upon which this method is based was first described by Haslam [9]. Galloyl esters, whether present as such or as esters of hexahydroxydiphenic acid, react in aqueous, or aqueous organic, solutions with KIO_3 to give a transient red colour. The absorption at the maximum so reached is strictly dependent on the concentration of the galloyl derivative, but varies with the constitution of that derivative. Examples are given in a description of the method in Part 1 of this series [4] dealing with the astringency of the leaves of *Acer* species. Because of its ready availability, tannic acid has been adopted as a reference substance, and the results are recorded in Table 1 as TI = the 'equivalent tannic acid concentration (iodate reaction)'.

A feature of most *Geranium* species is the formation of a blue reaction product immediately after the addition of KIO_3, reaching a maximum after a few minutes and slowly fading while the main reaction proceeds. A sample of geraniin prepared from *G. robertianum* by Dr. Haslam showed the same effect. This compound occurs widely in Geraniaceae and Euphorbiaceae [10]. A limited survey has shown that the blue reaction is also given by extracts of leaves of several species of *Acer* and several other ellagitannin containing genera, but it is not common. The survey is being continued.

Determination of astringency (TAE)

This is determined by the precipitation of blood proteins by the tannins in the extract, the result being expressed as the concentration of tannic acid required to produce the same degree of precipitation. This is measured spectrophotometrically, employing the absorption of haemoglobin at 578 nm as a standard. Blood diluted 1:100 gives a peak with the absorption conveniently at about 1.2–1.3. The precipitation is stoichiometric for tannic acid from 0.015% (up to which concentration precipitation is 0) and 0.039%, at which it is complete. A 1% extract of leaf powder having a moderate tannin content will give a measurable degree of precipitation when mixed with an equal volume of 1:50 diluted blood, but with less than usual a more concentrated extract may be needed, and with more than usual the extract will need to be diluted. When the tannin is of the hydrolysable type the value of TI will give a useful indication of the extent of dilution that will be needed to bring the precipitating range of the extract to between 20 and 80%, which is desirable for reliability. The results are reproducible with considerable accuracy.

Occasionally there are difficulties to be overcome. The most troublesome is the formation of a cloud which will not clear with centrifuging. This is due to the presence of saponins which also give trouble during the preparation of the extract, causing foaming and mucilaginous residues. The absorption of haemoglobin in a cloudy suspension is greater than that in clear solution so that the result, when recorded, has to be expressed as 'more than' the value so observed. A point of interest in the case of *Geranium* is that the powder of several species, when freshly prepared, gave intractable clouds but after keeping the powder for some months, the extracts were completely free from this trouble, although otherwise unchanged.

With this preamble, the results in Table 1 should now be meaningful. Both TAE and TI vary over a very wide range, and the ratio of one to the other also varies widely. This is an indication that the tannins in the genus are a very complex and variable mixture of proanthocyanidins, gallotannins and ellagitannins. Proanthocyanidins are present in most, but in quantity in only a few species, and being very inextractable contribute very little to TAE and, of course, not at all to TI. The tannin most frequently present, isolated by Haslam from *G. robertianum*, is identical with the geraniin described by Okuda. A sample supplied by Haslam has TAE/TI 0.7, which is nearly the average of the TI of the species in Table 1, but there are some species with much higher, and some with much lower, TAE/TI, indicating very different kinds of galloyl esters in these species. Some (see Table 2) do not give the blue reaction on addition of KIO_3, and presumably do not, therefore, contain geraniin. Not enough is known at present about the variation of TI with structure to be able to suggest causes for the differences observed, but chromatography is being applied to the analysis of the galloyl esters present and should provide an explanation of the values observed. In the meantime, it is only the values of TAE that can be profitably used for consideration of the systematic significance of the results in Table 1.

Table 1. Tannin analyses of dried leaves of *Geranium* species

Species	*E*	TAE	TI	TAE/TI	'HH'	Species	*E*	TAE	TI	TAE/TI	'HH'
albanum Bieb.	nil	5.5	10	0.55	1.4	*molle* L.	nil	nil	nil	—	nil
argenteum L.	nil	7	17	0.4	5	*Xmonacense* Harz	nil	4.5	5.5	0.8	nil
aristatum Freyn. & Sint.	tr*	10	20	0.5	12	*nepalense* Sweet	nil	15	24	0.6	11
asphodeloides Burm. f.	1.2	13	20	0.65	5	*nervosum* Rydb.	nil	26.5	36	0.75	13.5
bicknellii Britt.	nil	12	18.5	0.65	5	*nodosum* L.	tr*	12.5	24	0.5	9
bohemicum L.	nil	2	14.5	0.15	2.5	*oreganum* Howell	tr	8.5	20	0.45	13.7
canariensis Reut.	nil	21	26	0.8	16	*palmatum* Cav.	nil	12	27	0.45	9
carolinianum L.	nil	11	19.5	0.55	8.4	*palustre* L.	nil	13	?	?	(3)
cataractarum Coss.						*peloponnesiacum* Boiss.	2.5	2.5	5	0.5	(2.5)
ssp. *cataractarum*	nil	13	22	0.6	5	*phaeum* L.	nil	<1.5	12	<0.1	4
ssp. *pitardii*	nil	16.5	26	0.6	7.5	*platypetalum* Fisch. & May.	nil	15	23.5	0.6	15
cinereum Cav.						*polyanthes* Edgw. & Hook.f.	tr	20	40	0.5	17
var. *subcaulescens*	tr	10.5	26	0.4	4	*pratense* L.	nil	8	16	0.5	13
var. *cinereum*	nil	11	20	0.6	3.5	var 'Kashmir Purple'	nil	13.5	26	0.5	16
collinum Willd.	nil	14.5	20	0.7	13.5	*procurrens* Yeo	nil	14	24	0.6	15
columbinum L.	nil	8	13	0.6	4.5	*psilostemon* Ledeb.	nil	26	35	0.75	24
crenophilum Boiss.	nil	7.5	14.5	0.5	3	*purpureum* Vill.	nil	12	26	0.45	9
dalmaticum DC.	nil	27.5	37.5	0.7	17.5	*pylzowianum* Maxim.	tr	15.5	26.5	0.6	12
dissectum L.	nil	6	8.5	0.7	2.5	*pyrenaicum* Burm.f.	nil	5	6	0.8	5
endressii J. Gay	tr	10	20	0.5	5	*reflexum* L.	tr	<2	5	?	(2)
erianthum DC.	tr	12.5	15.5	0.8	9	*renardii* Trautv.	2	7	16.5	0.4	8.5
eriostemon DC.	tr	12.5	19.5	0.65	8.5	*richardsonii* Fisch. & Trautv.	tr	25	31	0.8	19
farreri Stapf	tr	12.5	31	0.4	6	*robertianum* L.	nil	7.5	18	0.4	8
fremontii Torr.	nil	18	20	0.9	11	*rotundifolium* L.	nil	10	14.5	0.7	3
glaberrimum Boiss. & Heldr.	nil	20	28.5	0.7	15.5	*rubescens* Yeo	tr	10.5	14	0.75	7.5
gracile Nordm.	tr	5.5	nil	—	nil	*sagnuineum* L.	nil	7	14	0.5	6
ibericum Cav.	nil	6.5	13	0.5	5	*schiedeanum* Schlecht.	tr	8	15	0.5	5.5
incanum Burm.f.	3.2	11	21	0.5	7	*sessiliflorum* Cav.	nil	16	25	0.65	18.5
lambertii Sweet	nil	8	21	0.4	10.5	*shikokianum* Matsum.	nil	11.5	33.5	0.5	16
lanuginosum Lam.	tr	11	16.5	0.65	4	*subulatostipulatum* R. Knuth	nil	10	27	0.35	20
libani Davis	tr	9.5	24	0.4	5.5	*thunbergii* Sieb. & Zucc.	nil	15	26	0.6	12.5
lucidum L.	tr?	10	10	1.0	3.5	*traversii* Hook.f.	nil	8.5	20	0.4	12
macrorrhizum L.	nil	12.5	21	0.6	10.5	*tuberosum* L.	nil	6	19	0.3	9
maculatum L.	tr	18	27.5	0.6	10.5	*versicolor* L.	tr	8	13	0.6	nil
madarense Yeo	nil	13.5	24	0.55	12	*wallichianum* D. Don	tr	10	18	0.55	7
malviflorum Boiss. & Reid	nil	12.5	23	0.55	19	*viscosissimum* Fisch. & May	tr	24	25.5	0.9	?

* Prodelphinidin (by both chromatography and position of λ_{max}). The *E* values of the powder in these cases were *G. aristatum* 4.7; *G. gracile* 19; *G. nodosum* 14 and *G. peloponnesiacum* 6.6. Except for the last, no more than a trace of the proanthocyanidin was extracted by 50% MeOH.

DISCUSSION

In the earlier treatment of the chemotaxonomy of *Geranium* [1], Knuth's classification of the genus [11], the only one currently available, was followed. It was realized that this relied heavily on geographical considerations, with vegetative morphology having more influence than floral and seed structure. Recently Tokarski [12] has made an intensive study of seed dispersal mechanisms and Yeo [13] has suggested this might be a character on which a more natural system of classification might be based.

The commonest form of dispersal is by seed-ejection from the carpel. In this type the seed is thrown out of the mericarp to a distance of *ca* 1 m at the moment when the awn suddenly springs away from the rostrum of the fruit. So large a majority of the species are of this type that little help is afforded in the classification of the genus as a whole, but two small subgroups having particular modifications of the seed ejection mechanism can be distinguished from the large mass of species represented by *G. pratense*. This group represents about 50% of all the species examined.

The second largest type of seed dispersal is the carpel-projection type, in which the unit of discharge is the mericarp with the seed inside it, and this also can be divided into several subgroups on divers morphological characters. A third group, which comprises only a few species, has dispersal behaviour like that of *Erodium*, in which the mericarp remains attached to the awn but is

Table 2. Seed dispersal mechanisms, chromosome numbers and astringency

Species	2n	TAE	Species	2n	TAE
SEED-EJECTION TYPE					
Pratense group			*Asphodeloides* and *dissectum* group		
collinum	28	14.5	*asphodeloides**	28	13
endressii	28	10	*crenophilum*		7.5
erianthum	28	12.5			
eriostemon	28	12.5	*dissectum**, ‡	22	6
farreri		12.5			
fremontii		18			
*gracile**		5.5			
incanum†		11			
lambertii		8			
maculatum		18			
nepalense	28	15			
nervosum	52	26.5			
nodosum	28	12.5			
oreganum		8.5			
*palustre**	28	13			
pratense	28	8	*Tuberosum* and *platypetalum* group		
var. 'Kashmir Purple'		13.5	*ibericum*	56	6.5
procurrens		14	*libani**		9.5
psilostemon		26	*malviflorum*		12.5
pylzowianum	28	15.5	*peloponnesiacum**		25
richardsonii	52	15.5	*platypetalum*	28	15
sanguineum	84	7	*renardii*		7
schiedeanum		8	*tuberosum*		6
sessiliflorum	56	16			
shikokianum	28	17.5	*bohemicum*‡‡	28	2
subulatostipulatum		10	*lanuginosum*‡‡	48	11
thunbergii	28	15			
traversii	28	8.5			
versicolor	28	8			
viscosissimum		24			
wallichianum	28	10			
bicknellii‡‡		12			
carolinianum†, ‡	52	11			
*columbinum**, ‡	18	8			
rotundifolium‡‡	26	10			
ERODIUM TYPE					
Phaeum group			*Cinereum* group		
aristatum	28	10	*argenteum*	28	7
phaeum	14/28	1.5	*cinereum*		
*reflexum**	28	2	var. *cinereum*†	28	11
*X monacense**		4.5	var. *subcaulescens* (DC.) Knuth	28	10.5
CARPEL-PROJECTION TYPE					
Pyrenaicum group			*Polyanthes* group		
pyrenaicum	28	5	*polyanthes*	28	20
*molle**, †	26	nil			
Lucidum group			*Robertianum* group		
lucidum†	20	10	*purpureum*	32	12
			robertianum	64	7.5
			rubescens	128	10.5

Table 2. (*Continued*)

Species	2*n*	TAE	Species	2*n*	TAE
Anemonifolia			*Unguiculata*		
palmatum	68	12	*glaberrimum*	30	20
canariensis	128	21	*cataractarum*	36	
madarense‡	68	13.5	subsp. *cataractarum*		16.5
			subsp. *pitardii*		13
			dalmaticum		27.5
			macrorrhizum		12.5
	No forcible discharge mechanism:		*albanum**	28	5.5

* Blue iodate reaction negative; † blue iodate reaction weak. ‡ indicates annual or biennial.

projected by a minor explosion; and finally (in this list) there is a single species in which there is no forcible discharge.

The TAEs of the leaf extracts of the species in Table 1 are listed again in Table 2 in the groups and subgroups in which Yeo places them. Also given are their chromosome numbers, where these are known. A distinction is also made between perennial species and those which are annual or biennial in seasonal habit. It can be seen straight away that the commonest chromosome number is $2n = 28$ ($x = 14$), variations from this being most frequently seen in the annual/biennial species. Similarly the commonest values of TAE lie between 10 and 12.5, largely independent of the dispersal type, but varying most in those species with chromosome numbers differing from $x = 14$. The one distinctive subgroup is the phaeum group, the extracts of which, as was pointed out earlier, differ from those of all other species in reacting with HNO_2 to give a red colour, λ_{max} 530 nm, instead of the usual blue colour, λ_{max} 600. *G. phaeum* and *G. reflexum* appeared to have no astringency whatever (but recorded in Table 2 as less than the lowest concentration at which tannic acid itself precipitates blood protein). The result with *G. aristatum* is perplexing. Morphologically this closely resembles *G. reflexum* and on a previous occasion [7] a specimen obtained from Kew was found to have the same chemical characters as this species. It seems likely that the members of this group may vary considerably both chemically and morphologically since *X monacense*, a hybrid between *phaeum* and *reflexum* [14] has a higher TAE than that of either parent recorded in Table 2. The explanation for this may be in the variable etherification of one of the hydroxyl groups in HHDPG, which is thought [8] to be the reason for the shift in λ_{max} from 600 to 530 nm in the HNO_2 reaction.

Apart from this group, the variation in TAE is similar in all groups, with values between 10 and 12.5 being common throughout. It is therefore the species with exceptionally high or exceptionally low values which will most reward further attention. For this purpose species with TAE over 20 and those with TAE below 6 have been considered.

The most striking feature of the five species with TAE over 20 is that three of them are N. American. It has already been noted that a number of N. American species have the dysploid (? tetraploid) number $2n = 52$, which is true of two at least of the three N. American species examined. The impression given by these results is that there is some

Table 3. *Geranium* species with astringency values (TAE) over 20 or 6 or less

TAE	*Geranium* species	Geographical location	Seasonal habit	2*n*
Over 20	*canariensis*	Macaronesia	Perennial	128
	dalmaticum	Yugoslavia	Perennial	—
	nervosum	N. America	Perennial	52
	psilostemon	Armenia	Perennial	28
	richardsonii	N. America	Perennial	52
	viscosissimum	N. America	Perennial	—
6 or less	*albanum*	Armenia	Perennial	28
	bohemicum	Mediterranean	Biennial	28
	dissectum	Widely Eurasian	Annual	22
	gracile	Armenia	Perennial	—
	molle	Mediterranean	Annual	26
	peloponnesianum	Greece	Perennial	28
	pyrenaicum	Mediterranean	Perennial	28

correlation between high TAE, polyploidy and N. American geographical location, but none of these criteria is in itself a determining factor in high or low astringency.

Several of the species with low TAE are distinctively individual. *G. gracile* has much proanthocyanidin and apparently no galloyl ester. *G. peloponnesiacum* also has much procyanidin, but in neither case is this easily extracted from the powder and contributes little to astringency. *G. molle*, with $2n = 26$, is an annual, having no galloyl ester. Several other species with low TAE are annual or biennial. Geographically these are all essentially European or W. Asian. There appears, therefore, to be some correlation between low TAE, annual/biennial seasonal habit, and W. Eurasian geographical location.

These characteristics of high and low TAE respectively are to some extent reflected in the members of the *pratense* group generally. Thus several more of the N. American species, e.g. *G. maculatum* and *G. fremontii*, have higher than average TAE. *G. sessiliflorum* is mainly Andean, but extends into Australasia. But the general impression within this large group is one of a normal statistical distribution of TAE values about an average of 12, with particular individual circumstances determining the wider deviations from this average. Among these, the presence in some species of large amounts of proanthocyanidin is worthy of remark. The *potential* for proanthocyanidin production is present in most, if not all, species. *G. phaeum*, for instance, has a high concentration in its roots, and root extracts of many species are used medicinally as astringents. It is the aerial growth which exhibits the character of extreme diversity of tannin content, and this must presumably be associated with the ecological niche which the species is adapted to occupy.

EXPERIMENTAL

Plant material. The leaves, harvested at maturity, were obtained from the Cambridge University Botanic Garden, in some cases from different plants of the same species at different times of the season. Some differences were observed, but not such as to affect significantly the conclusions drawn. Values in the tables are representative.

Methods of analysis are described and discussed in the text, or in greater detail in ref. [4].

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NOTE ADDED IN PROOF

At the request of Dr. Yeo, *G. pusillum* Burm. f., acquired since, has been examined. The extract had a trace of procyanidin, TAE 4.7%, TI 11%, nitrite reaction spectrophotometrically increasing steadily with only a slight maximum after 5 min at 600 nm, HH 3%. The species is annual, its chromosome number 26, its range European. Yeo places it with *G. pyrenaicum* and *G. molle* in his *pyrenaicum* group (Table 2). These data agree with that placing. It is to be included with the species in Table 3 with TAE 6 or less, thereby supporting the conclusions reached on pp. 215 and 216.