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CONSTITUENTS OF *GERANIUM THUNBERGII* SIEB. ET ZUCC.

VII*. HIGH-PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY OF HYDROLYSABLE TANNINS AND RELATED POLYPHENOLS

TAKUO OKUDA, KAZUKO MORI, KAORU SENO and TSUTOMU HATANO
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700 (Japan)
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SUMMARY

Hydrolysable tannins in plants were analysed by reversed-phase high-performance liquid chromatography (HPLC) on a hydrocarbon polymer column. Geraniin, corilagin, mallotusinic acid, mallotinic acid, chebulinic acid and chebulagic acid were separated from each other, from polyphenols of lower molecular weight and also from flavonol glycosides. Variations in both the structure and amount of these ellagitannins and their hydrolysis products during extraction were also demonstrated by HPLC. Comparisons of the results of the quantitation of these tannins in plants with those obtained by relative astringency determination indicate that tannins in some plants can be represented by peaks in HPLC.

INTRODUCTION

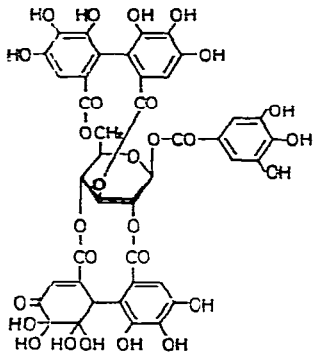
Tannins and related polyphenols are widely distributed in plants. Although many species of plants have been considered to contain "tannin", the characteristics of the tannin in each species have remained unknown for most of the plants, because of the difficulty in analysing complex mixtures of tannins. The quantitation of tannin has generally been based on the combination of tannin with proteins, *e.g.*, collagen, gelatin, casein and haemoglobin[1]. By these methods, however, polyphenols of smaller molecular size, which are often present in the plants, and which are also produced by hydrolysis of the original tannins during extraction and concentration, combine with protein to a certain extent, and are included in the determined amount of tannin.

Chromatographic analyses of tannins and related compounds have mainly been performed by paper-partition chromatography[2], and sometimes by thin-layer chromatography on cellulose powder[3] or polyamide[4]. The resolution and reproducibility with these methods are often poor. Because of the instability of tannin on storage and extraction of plants, and also seasonal variations in the tannin contents of

* For Part VI, see T. Okuda, K. Mori and R. Murakami, *Yakugaku Zasshi*, 97 (1977) 1273.

plants, the development of an analytical method of high resolution and good reproducibility has been lacking. Recently, the separation of some polyphenols with a flavonoid skeleton, which are related to condensed tannins, by high-performance liquid chromatography (HPLC) has been reported[5], although the HPLC analysis of tannin itself has not yet been described.

Of the two largest groups of vegetable tannins, *viz.*, hydrolysable tannins and condensed tannins, in several species of plant the former are known to be composed of a few tannins whose molecular weights are around or lower than 1000, whereas the latter are regarded as complex mixtures of a number of compounds produced by condensation of monomers with a flavonoid skeleton. This paper describes the analysis of hydrolysable tannins by reversed-phase HPLC. We have recently isolated several new ellagitannins (a sub-group of hydrolysable tannins) from plants which are commonly used as natural medicines in Japan, *viz.*, geraniin (I) from *Geranium thunbergii* Sieb. et Zucc.[6], and mallotusinic acid (II) and mallotinic acid together with geraniin from *Mallotus japonicus* Muel. et Arg.[7]. HPLC analysis of these ellagitannins and related polyphenols of lower molecular weight, and co-existing flavonol glycosides, has been carried out. The hydrolysable tannins of the fruit of *Terminalia chebula* Retzius[8], commonly known as Myrobalans, which is used in leather making as well as as a medicine, have also been analysed.



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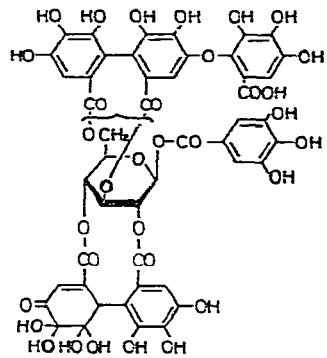

II

EXPERIMENTAL

Apparatus and column

Liquid chromatography was performed on a Shimadzu–DuPont Model LC-1 chromatograph, equipped with a gradient elution system, using an ultraviolet detector at 254 nm. A 100 cm × 7.9 mm I.D. stainless-steel column with Zipax HCP (hydrocarbon polymer; DuPont, Wilmington, Del., U.S.A.), or a 100 cm × 2.1 mm column with the same packing, was used at 30–100 kg/cm^2. The column temperature was kept at 35° in an oven.

Mobile phases

Mobile phase A was a mixture of 0.5 *M* KH_2PO_4 (1 l), ethanol (10 ml) and ethyl acetate (1 ml), B a mixture of 0.5 *M* KH_2PO_4 (1 l) and ethyl acetate (2 ml), and

C a mixture of 0.5 *M* KH_2PO_4 (1 l), ethanol (20 ml) and ethyl acetate (5 ml). The gradient was from mobile phase A to a mixture of 0.5 *M* KH_2PO_4 (1 l), ethanol (50 ml) and ethyl acetate (5 ml) in 20 min.

Materials

Geraniin, kaempferitrin and kaempferol 7-O-rhamnoside (from *Geranium thunbergii*), mallotusinic acid and rutin (from the leaf of *Mallotus japonicus*) and chebulinic acid and chebulagic acid (from the fruit of *Terminalia chebula*) were obtained by extraction of the plants, followed by appropriate fractionation. Corilagin[9], mallotinic acid[7], brevifolin[3] and ellagic acid were prepared by hydrolysis of geraniin, mallotusinic acid and their derivatives. Hexahydroxydiphenic acid was obtained by alkaline hydrolysis of ellagic acid, and also by hydrogenolysis of hexa-O-benzylhexahydroxydiphenic acid. Tannic acid JP (tannic acid according to the Japanese Pharmacopoeia) was prepared from Chinese nutgalls. Hyperin was extracted from *Geranium carolinianum* L.

The extract of *Geranium thunbergii* was prepared by homogenizing the fresh overground part (36.5 g) in a mixture of acetone and water (1:1, 250 ml), and evaporating the filtrate at 40° *in vacuo*. The residue was powdered and further dried *in vacuo* at room temperature overnight over silica gel to give a dry powder (3.27 g). Other species of *Geranium* and the leaf of *Mallotus japonicus* were treated in an analogous way. The extract of Myrobalans was prepared by extraction of powdered dried fruit with methanol, followed by distillation and drying *in vacuo*.

Paper-partition chromatography, with *n*-butanol–acetic acid–water (4:1:5, upper phase) as developing solvent, of a mixture of geraniin, mallotusinic acid and corilagin, etc., gave poor resolution of the components and poor reproducibility of the mobility of each tannin. Development with 7% acetic acid gave better reproducibility, but geraniin and mallotusinic acid were not separated.

Procedure

Tannins and dried extracts were dissolved in methanol or 50% aqueous methanol. The concentrations were 0.5 mg/ml for each purified tannin (geraniin was the hexahydrate of structure I, and mallotusinic acid was the nonahydrate of structure II) and 5 mg/ml for the dried powder of each plant extract. Volumes of 1–6 μl of the solutions were injected. Particular care with the column, such as washing each day and frequent exchange of the silica-wool and of the stationary phase at the top of the column, was required for the analysis of the tannin-containing plant extracts. Filtration of the sample solutions of the plant extracts through a membrane filter (Toyo Filter Paper TM-80 P) before injection reduced the contamination of the column, but some compounds, such as ellagic acid, were adsorbed to some extent on the filter. The filter was therefore not used for the quantitative analysis.

Calibration and calculation

Linear calibration graphs were obtained for geraniin, mallotusinic acid, corilagin and hyperin on plotting the ratio of the peak areas against the volume of solution of weighed samples injected into the liquid chromatograph. The calibration graph for geraniin was established daily before quantitative analyses were carried out, and was used as the internal standard for the other polyphenols. The peak

areas of polyphenols in the samples were multiplied by correction factors which were determined by comparing the slopes of the calibration graphs. The correction factors were as follows: geraniin, 1.00; mallotusinic acid, 0.84; corilagin, 1.8; and hyperin, 2.1.

RESULTS AND DISCUSSION

All of the ellagitannins studied and hexahydroxydiphenic acid were separated from each other when HPLC was performed on a 100 cm × 7.9 mm I.D. column of

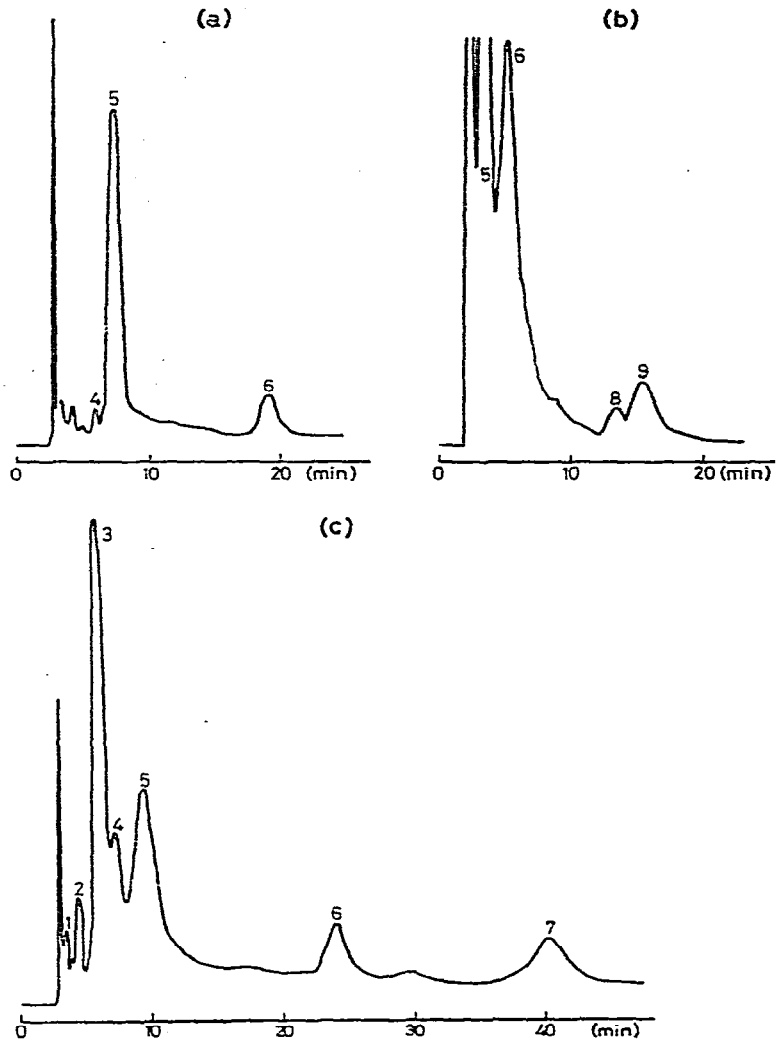

Fig. 1. Chromatograms of extracts of the overground part of *Geranium thunbergii* (a) and (b) and of the leaf of *Mallotus japonicus* (c). Conditions: (a) 100 cm × 7.9 mm I.D. column with mobile phase A, flow-rate 6.8 ml/min; (b) 100 cm × 2.1 mm I.D. column with mobile phase C, flow-rate 0.6 ml/min; (c) 100 cm × 7.9 mm I.D. column with mobile phase A, flow-rate 5.8 ml/min. Pressure, 70 kg/cm²; sensitivity, 0.04 a.u.f.s. Peaks: 1 = bergenin; 2 = mallotinic acid; 3 = mallotusinic acid; 4 = corilagin; 5 = geraniin; 6 = ellagic acid; 7 = rutin; 8 = quercetin; 9 = kaempferitrin.

hydrocarbon polymer (Zipax HCP) employing mobile phase A, although the peaks of tannic acid JP and gallic acid were overlapped by the solvent peak. The retention time of brevifolin was slightly shorter than that of corilagin.

The chromatogram obtained from the extract of the herb of *Geranium thunbergii* is shown in Fig. 1a and that of the leaf of *Mallotus japonicus* in Fig. 1c. The amount of geraniin in the fresh overground part of *Geranium thunbergii*, determined by HPLC, was 1.55%, whereas the maximum amount of crude geraniin isolated by repeated fractionation from plant material of the same origin was 1.44% and that of recrystallized geraniin was 1.13%. The difference between the two methods is attributable to losses during fractionation, partially due to hydrolysis of geraniin. The ratio of the amount of mallotusinic acid to that of geraniin in the leaf of *Mallotus japonicus* determined by HPLC was 0.86, while the ratio of these tannins upon isolation was 0.76. When a 100 cm × 2.1 mm Zipax HCP column was employed, mallotusinic acid, mallotinic acid and corilagin were hardly resolved. However, the resolution of these ellagitannins on this column was effected when it was developed with mobile phase B, although broadening of peaks and lengthening of retention times occurred. Ellagic acid appeared as a broad peak with these mobile phases.

Among the flavonols and their glycosides, hyperin showed a sharp peak with a retention time slightly longer than that of geraniin when developed with mobile phase A. Kaempferitrin, kaempferol 7-O-rhamnoside, kaempferol and quercetin showed broad peaks with longer retention times. When developed with mobile phase C, sharpened peaks of these compounds were obtained with shorter retention times. The peaks with comparatively short retention times are shown in Fig. 1b.

The Myrobalans extract showed peaks with a wide range of retention times and gradient elution therefore was performed (Fig. 2). Three hydrolysable tannins, namely chebulinic acid, chebulagic acid and corilagin, were separated from each other within 30 min.

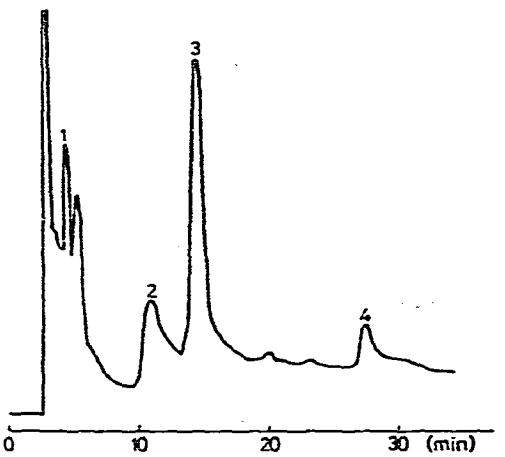

Fig. 2. Chromatogram of the Myrobalans extract. Conditions: 100 cm × 7.9 mm I.D. column; gradient, 7 min initial elution with mobile phase A, and then gradient elution programmed from this mobile phase to a mixture of 0.5 *M* KH_2PO_4 (1 l), ethanol (50 ml) and ethyl acetate (5 ml) in 20 min; flow-rate, 5.6 ml/min; pressure, 100 kg/cm^2; sensitivity, 0.02 a.u.f.s. Identity of peaks: 1 = corilagin; 2 = ellagic acid; 3 = chebulagic acid; 4 = chebulinic acid.

Attempts to perform HPLC on other stationary phases, *e.g.*, chemically bonded octadecyltrimethoxysilane (Permaphase ODS; DuPont), chemically bonded aliphatic ether (Permaphase ETH; DuPont), and chemically bonded anion-exchange resin (Permaphase AAX; DuPont) did not give better results.

Extraction of hydrolysable tannins from plants should be accompanied by hydrolysis of tannins, resulting in variation of the activities of the tannins, depending on the extent of hydrolysis. Nevertheless, virtually all of the tannin extracts have hitherto been used without examining the extent of hydrolysis of the original tannins. Structural investigations showed that hydrolysis of geraniin gives corilagin, gallic acid, hexahydroxydiphenic acid, ellagic acid, brevifolincarboxylic acid and brevifolin, etc.[10]. Hydrolysis of mallotusinic acid yields mallotinic acid, an atropisomer of valoneaic acid, and gallic acid, etc.[7]. HPLC analysis in this study distinguished most of the hydrolysis products from the original tannins, indicating that this method is useful for the determination of the extent of hydrolysis of tannins in the extracts. The variation in the amounts of geraniin and corilagin during extraction of dried overground part of *Geranium thunbergii* with water is shown in Table I. Geraniin was hydrolysed rapidly although the water temperature was raised slowly. It can be assumed that the hydrolysable tannins in plant extracts used for leather making also experience marked chemical changes during the production of the extracts.

TABLE I

VARIATION OF ELLAGITANNIN CONTENTS ON EXTRACTION OF *GERANIUM THUNBERGII* IN WATER

Dried overground part of *Geranium thunbergii* (3 g) was chopped into pieces of *ca.* 5 mm length and immersed in water (50 ml). The temperature of water was raised on a hot-plate (600 W, 50 V) to the boiling point in 26 min. Volumes of 1 μl of the solution were injected.

Time (min)	*Geraniin (mg/ml)*	*Corilagin (mg/ml)*
10	0.15	0.02
20	0.55	0.05
25	0.81	0.07
30	0.62	0.09
40	0.03	0.17
60	0.00	0.45

Comparisons of tannin contents based on relative astringency[1] with geraniin contents determined by HPLC for extracts of *Geranium thunbergii* and several species of *Geranium* are shown in Table II. The extracts were prepared by homogenizing the

TABLE II

CONTENTS OF TOTAL TANNIN AND GERANIIN IN *GERANIUM* SPECIES

Contents in the fresh overground parts.

Species	*Total tannin* (%)*	*Geraniin** (%)*
G. thunbergii	2.3	1.55
G. carolinianum	2.4	1.13
G. sibiricum	1.1	0.80
G. wilfordii var. *hastatum*	1.0	0.50

* Determined by relative astringency measurement[1]. The data presented were obtained with the same samples as used for HPLC, which are different from those in ref. 1.

** Determined by HPLC.

green part of the plants in a mixture of acetone and water. The rare instances of plants in which the main component of the tannin is a single compound can clearly be seen. Simple calculation indicates that in *Geranium thunbergii* the amount of geraniin corresponds to 67% of the total amount of tannin. These results show that the tannin in certain plants can be represented by a single peak or a few peaks on HPLC. However, analogous comparisons of the relative astringency data with HPLC data for other plants have shown that in most plants tannins are complex mixtures.

The distribution of ellagic acid in nature has been investigated for use in chemotaxonomy, employing paper-partition chromatography[11]. The reproducibility of the mobility by this method is often poor. An improved analysis by gas chromatography of the trimethylsilyl derivative at high temperature has been reported[12]. HPLC analysis without derivatization in this study gave good qualitative and quantitative reproducibility. This method permitted concurrent analysis of tannins and ellagic acid.

We investigated whether hydrolysable tannins such as geraniin are present in the plant cells without any further chemical bonding. Fresh leaves of *Geranium thunbergii* were frozen immediately after collection to inhibit enzymatic cleavage of chemical bonds, and then homogenized in aqueous acetone. The resulting suspension was centrifuged and the supernatant solution was injected on to the HPLC column. The chromatogram showed the peak of geraniin. The peak area was identical with that observed on extraction without pre-treatment of the leaves. This result indicates that geraniin is present in the plant cells without any further chemical bonding, although the occurrence of some interactions of this tannin with protein and other materials of high molecular weight or basic properties cannot be excluded.

In addition to their usefulness in leather manufacture and as medicines, tannins could also be useful markers in chemotaxonomy. The HPLC analysis of geraniin and mallotusinic acid in the plants of Geraniaceae and Euphorbiaceae showed the usefulness of this method.

CONCLUSIONS

The analysis of hydrolysable tannins, particularly ellagitannins, and of related polyphenolic compounds by reversed-phase HPLC has the following advantages over other methods such as paper-partition chromatography:

(1) component tannins in the mixture can be separated from each other and from compounds of other types with good resolution and reproducibility;

(2) quantitation of each tannin can be achieved;

(3) co-existing flavonoids and their glycosides can be analysed simultaneously; and

(4) ellagic acid and other polyphenols produced by hydrolysis of tannins can be analysed simultaneously with good resolution and reproducibility.

REFERENCES

1 T. Okuda, K. Mori and K. Aoi, *Yakugaku Zasshi*, 97 (1977) 1267.
2 O. Th. Schmidt and R. Lademann, *Justus Liebigs Ann. Chem.*, 571 (1951) 41.
3 W. Mayer, A. Görner and K. Andrä, *Justus Liebigs Ann. Chem.*, (1977) 1976.
4 P. Stadler and H. Endres, *J. Chromatogr.*, 17 (1965) 591.

5 A. C. Hoefler and P. Coggon, *J. Chromatogr.*, 129 (1976) 460.
6 T. Okuda, H. Nayeshiro and K. Seno, *Tetrahedron Lett.*, (1977) 4421.
7 T. Okuda and K. Seno, *Tetrahedron Lett.*, (1978) 139.
8 J. C. Jochims, G. Taigel and O. Th. Schmidt, *Justus Liebigs Ann. Chem.*, 717 (1968) 169.
9 T. Okuda, T. Yoshida and K. Mori, *Phytochemistry*, 14 (1975) 1877.
10 T. Okuda, T. Yoshida and H. Nayeshiro, *Chem. Pharm. Bull.*, 25 (1977) 1862.
11 E. C. Bate-Smith, *Chem. Ind. (London)*, (1956) 32; *J. Linn. Soc. London, Bot.*, 58 (1962) 95.
12 W. E. Hillis, A. N. Rozsa and L. S. Lau, *J. Chromatogr.*, 109 (1975) 172.