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Chemical structures of the condensed tannins in the fruits of *Diospyros* species

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Abstract The structural variety of the condensed tannins (proanthocyanidins) in the fruits of 16 *Diospyros* species are reported. Eleven species contained condensed tannins mostly consisting of a mixture of catechin (CA) and gallo catechin (GCA) repeating units; the other five species did not. The GCA content in the CA-GCA total varied from 0.3% to 84.6%. The number of esterified gallic acid per one flavan repeating unit (degree of galloylation, DG) ranged from 0.01 to 0.89. The GCA content was found to be proportional to the DG values. Thus, 16 *Diospyros* species tested may be classified into five groups by the analytical data of their condensed tannins. It may be interesting to compare their structural characteristics with those of the condensed tannins in other fruits, leaves, woods, and barks from the viewpoint of their biosynthesis and function in the plants.

Key words *Diospyros* species · Ebenaceae · Persimmon fruit · Chemical structure · Condensed tannins

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Introduction

The genus *Diospyros* is widely distributed from tropical to temperate regions, with about 400 species; most are found in the humid tropics of Asia, Africa, and Central and South America.¹⁻⁵ Several fruits in these *Diospyros* species are edible.⁶ Immature fruits of persimmon (*Diospyros kaki* L.), with marked astringency, were used as the vegetable tannin sources for making tanning paper, for tanning fishing net, and for removing protein during the brewing process of the Japanese rice wine “sake.”⁶

One of the characteristics of these fruits is that they generally contain a large amount of condensed tannins. Matsuo and Ito⁷ analyzed the toluene- α -thiol degradation products of the fruit tannin, named kaki tannin, isolated from *D. kaki* L. cv. Hiratanenashi. They were the first to propose its structure in 1978, after about 50 years of structural studies initiated by Komatsu et al. during the 1920s⁸⁻¹⁰ and subsequently carried out by Ito and colleagues during the 1960s.^{11,12} The proposed structure consists of catechin (CA), CA-3-*O*-gallate, gallo catechin (GCA), and GCA-3-*O*-gallate residues as the repeating units, with a molar ratio of 1:1:2:2, respectively; these units are linked through the C-6 or C-8 with C-4 position, as shown in Fig. 1. The weight-average molecular weight (M_w) of its methylated derivative was calculated to be about 1.38×10^4 daltons using a polystyrene standard.⁷ However, no one has reported the chemical structures of *Diospyros* tannins other than the above kaki tannin.

Here, we report analytical data on the tannin contents and chemical structures of the condensed tannins for 19 fruits from 16 *Diospyros* species collected in Japan and Thailand.

Materials and methods

Plant material

Fruits from 16 species growing in Japan and Thailand were examined in this investigation. Five species – *Diospyros*

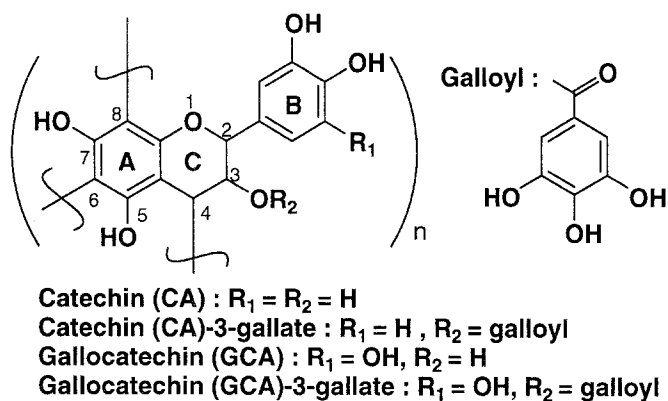


Fig. 1. Chemical structure of kaki tannin

virginiana, *D. lotus*, *D. rhombifolia*, *D. oleifera* (August 1993), *D. kaki* cultivars "fuyu," "sangokuichi," "hiratanenashi," and "kuramitsu" (July 1994) – were collected from the experimental farm of Kyoto University in Japan. Eleven species were collected in Thailand: *D. wallichii* (December 1992, Phuket Island), *D. areolata*, *D. mollis* (July 1993, Bangkok), *D. sumatrana* (July 1993, Phuket Island), *D. decandra* (July 1993, Chantha Buri), *D. glandulosa* (September 1993, mountain areas of Chiang Mai), *D. ehretioides*, *D. montana* (September 1993, Chiang Mai), *D. malabarica* var. *siamensis* (September 1993, Phitsanulok), *D. gracilis* (December 1993, Saraburi), and *D. rhodocalyx* (July 1994, Korat).¹³ All fruits were lyophilized after removing the skin and seeds and stored in a refrigerator for subsequent experiments.

Qualitative analyses of condensed tannins

Condensed tannin was qualitatively analyzed by the *n*-BuOH-HCl method. A freeze-dried fruit sample (50 mg) was extracted with MeOH (20 ml) for 15 min under ultrasonic waves. The supernatant solution obtained after centrifugation (3000 rpm, 15 min) was used for the *n*-BuOH-HCl test carried out under reaction conditions using 5% HCl in *n*-BuOH at 90°C for 20 min; the ultraviolet (UV) spectrum was then recorded.

Quantitative analyses of condensed tannins

Each ground freeze-dried sample (50 mg) was extracted three times with 20 ml of MeOH for 15 min under ultrasonic waves, and the methanol suspension was centrifuged for 15 min (3000 rpm) to obtain the supernatant, which contained condensed tannin. The combined MeOH solution was used for quantitative determination of the condensed tannin carried out by the vanillin-HCl method of Broadhurst and Jones.¹⁴ The calibration curve was obtained using (+)-catechin as a standard treated under the same reaction conditions.

Determination of the esterified gallic acid content

The esterified gallic acid content was determined by subtracting the free gallic acid content extracted with H₂O/1,4-dioxane from the content of that after hydrolysis. Free gallic acid in freeze-dried fruits (30 mg) was extracted with a mixed solvent consisting of 1.6 ml H₂O and 3 ml 1,4-dioxane at 25°C for 40 min. Esterified gallic acid in freeze-dried fruits (10 mg) was hydrolyzed with 1.6 ml concentrated HCl in 3 ml 1,4-dioxane at 100°C for 40 min. Gallic acid in the extracts and hydrolyzates was analyzed by high-performance liquid chromatography (HPLC) carried out under the following conditions: column, Cosmosil 5C₁₈ reversed-phase column (4.6 × 100 mm; Nakarai Tesque, Japan); eluent, 0.1% aqueous TFA; flow rate, 1 ml min⁻¹; detector, UV 254 nm; retention time, 4.3 min (gallic acid). The gallic acid content was determined quantitatively using the calibration curve for gallic acid.

Determination of CA and GCA contents

The CA and GCA contents were determined from the quantitative analyses of catechol and pyrogallol released from the B-ring of the CA and GCA in the flavan repeating unit, respectively, by a modified phenyl-nucleus exchanging method of Abe and others.¹⁵

A freeze-dried fruit (50 mg) was reacted with 1.6 ml of a PhOH-BF₃ reagent consisting of a phenol – xylene – BF₃–phenol complex (19:10:3, v/v/v) in a sealed glass ampul at 130°C for 3 h. The reaction mixtures were extracted with EtOAc. The organic layer was washed with brine, dried over Na₂SO₄, and concentrated. The residual oil was acetylated with Ac₂O/pyridine (1:1, v/v) at 25°C for 12 h, and the resulting acetates of catechol and pyrogallol were analyzed by gas chromatography (GC). GC was carried out using the following conditions: column, OV-17, High Cap-17 capillary column (0.25 mm × 30 m, Shimadzu, Japan); carrier gas, helium at 1 kg/cm²; column temperature, 180°C for 12 min, 180–220°C at 20°C min⁻¹, 220°C for 6 min, 220–250°C at 30°C min⁻¹, 250°C for 30 min; detector, FID; retention time, catechol acetate (7.2 min), bibenzyl (10.2 min) as an internal standard, pyrogallol acetate (15.5 min). Both catechol and pyrogallol were found to be fairly unstable (i.e., the recoveries were 62 and 44% for catechol and pyrogallol, respectively, when both compounds were treated under the phenyl-nucleus exchanging reaction conditions). Values of 0.62 for catechol and 0.44 for pyrogallol were used as the survival factors when calculating the quantitative determinations of catechol and pyrogallol released by this reaction. Furthermore, the quantitative value of pyrogallol was also corrected for the pyrogallol released from esterified gallic acids in 26.3% yield under the phenyl-nucleus exchanging reaction conditions: Pyrogallol was released in a yield of 26.3% under the same reaction conditions from the condensed-tannin gallate model compound synthesized by cationic polymerization of 3',4',5,7-tetrakisbenzyloxyflavan-3,4-diol 3-*O*-tribenzyl gallate and subsequent debenzylation according to the modified method reported previously.¹⁶

Results and discussion

Analytical data of the condensed tannins in 19 fruits from 16 *Diospyros* species are shown in Table 1. Fruits of five species (samples 15–19) – *D. mollis*, *D. montana*, *D. rhombifolia*, *D. sumatrana*, *D. wallichii* – did not give anthocyanidin pigments on *n*-BuOH-HCl reactions, indicating that these fruits do not contain condensed tannins. These results have been supported by microscopic observations of the specimens¹³ (i.e., cells containing tannins were not observed in the specimens of these species). The fruits of *D. mollis*, *D. montana*, and *D. wallichii* have been used for poisoning fish and as medicine against tapeworms, in villages of Thailand.¹³

The other 14 fruits in the 11 species had typical anthocyanidin pigments with absorption maxima at wavelengths ranging from 545 to 558 nm. The results suggested that these tannins consist mainly of CA and GCA with a phloroglucinol-type A-ring because the pigments from flavan units consisting of resorcinol-type A-ring, *p*-hydroxyphenyl-type B-ring, or both, with absorption maxima between 485 and 530 nm.^{17–19}

The vanillin-HCl color reaction is a simple, useful method for qualitative and quantitative determinations of condensed tannins.^{14,20,21} In this reaction, the λ_{\max} of the pigments depends only on the substitution pattern of the A-ring of the flavan repeating units. The λ_{\max} 500 nm is expected for the phloroglucinol type A-ring but a λ_{\max} at a longer wavelength for the resorcinol type A-ring.^{21,22} All samples yielded the pigments with a symmetrical peak with a λ_{\max} at 500 nm, suggesting that all these samples have mostly phloroglucinol nuclei as the A-ring, in agreement with the expectation from the results of BuOH-HCl reactions described above.

(+)-Catechin was used as a standard material for the quantitative analysis of tannin contents in the samples by the vanillin-HCl color reaction. Thus, the obtained values are expressed as the weight % (w/w) of the repeating catechin unit for the dried weight of the sample. The tannin contents of the samples are shown in Table 1. Wide distribution in their condensed tannin contents is observed; that is, the maximum content is about 30% of the dried weight of *D. decandra* fruit.

The characteristic *D. kaki* tannin involves gallic acid esterified at the C-3 hydroxyl group in the repeating flavan-3-ol unit.⁷ Free gallic acid also has been reported to exist in young fruits of *D. kaki*.²² However, almost all of the gallic acid was found to be esterified in the present chemical analysis, except for *D. decandra*, which has a low gallic acid content.

The degree of galloylation (DG) is defined in this paper as the number of esterified gallic acids per repeating flavan-3-ol unit. Thus, the DG is calculated from the following equation based on the data shown in Table 1.

$$DG = \frac{[\text{Esterified gallic acid content (\%)/170}]}{[\text{tannin content (\%)/290}]}$$

Here, the numbers 170 and 290 are molecular weights of gallic acid and catechin, respectively. The DG values are distributed from a maximum 0.89 of *D. malabarica* var. *siamensis* to the minimum 0.01 of *D. decandra*, but they are usually about 0.2–0.6.

The phenyl-nucleus exchange method developed by Abe et al.¹⁵ is a simple, reliable method for obtaining information about the hydroxylation patterns of the B-ring in condensed tannins. Catechol and pyrogallol are released from the B-rings of CA and GCA units by this degradation reaction,

Table 1. Chemical analyses of condensed tannins of *Diospyros* species

No.	<i>Diospyros</i> species	BuOH-HCl λ_{\max} (nm)	Tannin content(%) ^a	Free gallic acid (%) ^a	Esterified gallic acid (%) ^a	DG	CA content (%)	GCA content (%)
1	<i>D. decandra</i>	545	27.6	0.09	0.24	0.01	99.7	0.3
2	<i>D. rhodocalyx</i>	548	21.8	0.05	3.64	0.28	83.8	16.2
3	<i>D. gracilis</i>	558	21.1	0.11	5.14	0.41	24.1	75.9
4	<i>D. lotus</i>	558	17.8	0.34	5.79	0.55	15.5	84.5
5	<i>D. oleifera</i>	558	17.5	0.09	2.98	0.29	24.3	75.7
6	<i>D. virginiana</i>	556	17.2	0.19	4.38	0.43	23.9	76.1
7	<i>D. malabarica</i> var. <i>siamensis</i>	555	16.5	0.08	8.69	0.89	22.9	77.1
8	<i>D. areolata</i>	558	15.0	0.25	3.72	0.42	15.4	84.6
9	<i>D. glandulosa</i>	558	12.4	0.03	2.44	0.33	25.7	74.3
10	<i>D. kaki</i>							
	cv. Hiratanenashi	555	12.0	0.24	3.29	0.46	40.2	59.8
	cv. Sangokuichi	555	8.3	0.06	2.05	0.42	33.6	66.4
	cv. Kuramitsu	555	16.2	0.12	3.19	0.33	33.2	66.8
	cv. Fuyu	555	7.2	0.01	1.11	0.26	62.5	37.5
14	<i>D. ehretioides</i>	555	1.6	trace	0.17	0.18	77.1	22.9
15	<i>D. mollis</i>	–	0					
16	<i>D. montana</i>	–	0					
17	<i>D. rhombifolia</i>	–	0					
18	<i>D. sumatrana</i>	–	0					
19	<i>D. wallichii</i>	–	0					

DG, degree of galloylation; CA, catechin; GCA, gallic acid

^aPercent (w/w) for dry weight of sample

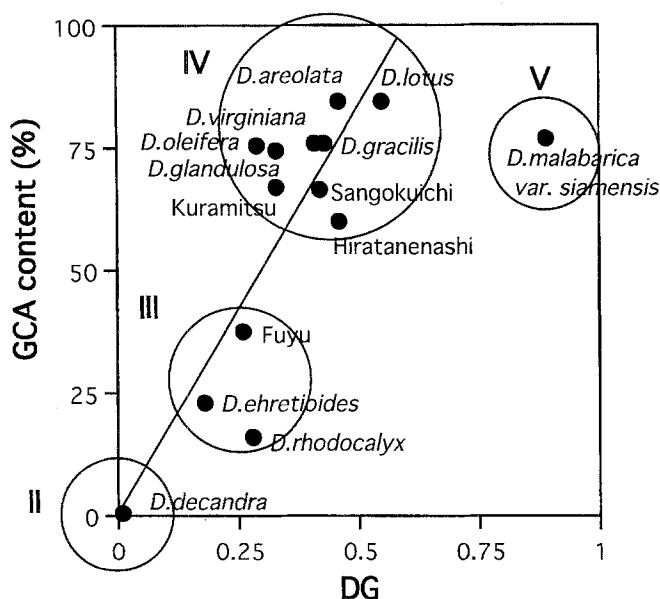


Fig. 2. Gallic acid (GCA) content (%) versus degree of galloylation (DG) Groups II–V represent localized clusters

respectively. Thus, the CA and GCA contents are determined by the quantitative analysis of catechol and pyrogallol. The results are shown in Table 1. The CA and GCA units are widely distributed in the tested samples. The CA content of *D. decandra* is 99.7%, and its DG is only 0.01, suggesting that the chemical structure of tannin in *D. decandra* is interestingly almost the same as those of typical coniferous wood-bark tannins.²⁴ On the other hand, the GCA contents of *D. lotus* and *D. areolata* are 84.5% and 84.6% with DGs 0.55 and 0.42, respectively. The highest DG 0.89 was obtained from *D. malabarica* var. *siamensis*. The relations between DG and GCA content are shown in Fig. 2. There is a linear relation between them ($r = 0.842$, based on the results except for the data of *D. malabarica* var. *siamensis*). The present findings may give an interesting suggestion about the biosynthesis of gallic acid in *Diospyros* species. That is, gallic acid in *Diospyros* fruits may be mainly synthesized via 3,4,5-trihydroxy cinnamic acid, not directly from 3-dehydroshikimate.²⁵

Consequently, 16 *Diospyros* species tested may be classified into five groups based on the chemical analyses of the condensed tannins: group I, without condensed tannin (e.g., *D. mollis*); group II, LL (low GCA/low DG, e.g., *D. decandra*); group III, MM (moderate GCA/moderate DG, e.g., *D. ehretoides*); group IV, HM (high GCA/moderate DG, e.g., typical *D. kaki*); group V, HH (high GCA/high DG, e.g., *D. malabarica* var. *siamensis*) as shown by the circles in Fig. 2. Mostly, *Diospyros* species belong to group IV with about 75% GCA content and about 0.5 DG value.

There may be an interesting relation between the chemical structures and genetic revolutions of *Diospyros* species; probably the species have evolved from group I toward group V, considering lignin biosynthesis and its evolution.^{26,27}

Thus, the obtained data may be useful from the viewpoints not only of phylogenetic relations and the chemo-

taxonomy of *Diospyros* species but also their structure-function relations comparing with those of condensed tannins in other fruits, leaves, woods, barks, and roots.

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