Studies on Inhibition Mechanism of Autoxidation by Tannins and Flavonoids. V.[1] Radical-Scavenging Effects of Tannins and Related Polyphenols on 1,1-Diphenyl-2-picrylhydrazyl Radical

Takashi YOSHIDA, Kazuko MORI, Tsutomu HATANO, Tomoko OKUMURA, Ikue UEHARA, Keiko KOMAGOE, Yuzaburo FUJITA and Takuo OKUDA*

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan. *Received December 5, 1988*

Radical scavenging effects of tannins and related polyphenols on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were evaluated by colorimetry. All of the polyphenols examined showed effects much stronger than that of α-tocopherol. The hydrolyzable tannins having galloyl groups in the molecule exhibited stronger effects than those having modified galloyl groups, such as hexahydroxydiphenoyl (HHDP), dehydrohexahydroxydiphenoyl (DHHDP) and chebuloyl groups. (−)-Epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin gallate and methyl gallate also showed fairly significant effects, even though they are small molecules. The predominant reaction products upon the treatment of various alkyl gallates with DPPH radical on a preparative scale were dialkyl hexahydroxydiphenates, which should be formed by mutual coupling of C-centered galloyl radicals. Evidence for the formation of the alkyl gallate radicals was also obtained by the electron spin resonance spectroscopy.

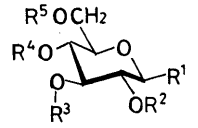
Keywords tannin; 1,1-diphenyl-2-picrylhydrazyl; radical scavenger; radical coupling; alkyl gallate; dialkyl hexahydroxydiphenate; hydrolyzable tannin

Tannins and related polyphenols have recently been found to have potent inhibitory effects on lipid peroxidation in rat liver mitochondria and microsomes,[2] and on Cu(II)-catalyzed autoxidation of ascorbic acid.[3] The antioxidative activities of each tannin were dependent on the type of phenolic groups and their numbers in the molecule.[3,4] A mechanistic study of autoxidation of methyl linoleate, as a model system of lipid peroxidation, indicated that the tannins which have a hexahydroxydiphenoyl (HHDP) group in the molecule exhibit stronger inhibitory effects than those having two galloyl groups in place of the HHDP group.[4] These activities of tannins were shown to be associated with their radical scavenging effects, by kinetic and electron spin resonance (ESR) measurements.[4] There is considerable interest in the biological significance of several active oxygen species and free radicals,[5] so we decided to confirm the radical scavenging effects of tannins in several experimental systems. We have thus investigated the reactivity of these polyphenols with a stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), to find out whether the efficiency of tannins as radical scavengers can be evaluated by using this reaction. We also investigated in detail the reactivities of methyl gallate and its analogues with DPPH, as well as the structures of the reaction products from the polyphenols after scavenging the radicals of the other coexisting compounds.

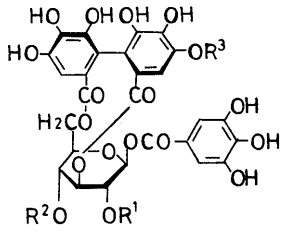
Materials and Methods

^{1}H-Nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Hitachi R22FTS (90 MHz for ^{1}H) spectrometer; the chemical shifts are given in δ values (ppm) relative to tetramethylsilane (TMS) as an internal standard. ESR spectra were recorded on a JEOL JES-FE3XG instrument (X-band, 100 kHz modulation) at room temperature.

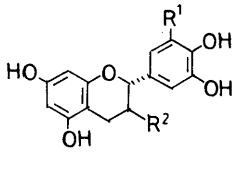
Materials DPPH, *dl*-α-tocopherol, and gallic acid were reagent grade materials, purchased from Wako Chemical Industry. (−)-Epicatechin (**13**) and (+)-catechin (**14**) were from Sigma. Hydrolyzable tannins: penta-*O*-galloyl-β-D-glucose (**1**),[6a] tellimagrandins I (**2**) and II (**3**),[6b] pedunculagin (**4**),[6b] isoterchebin (**5**),[6a] corilagin (**6**),[6c] geraniin (**7**),[6c] chebulinic acid



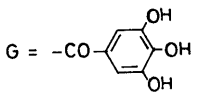
1 : R^1=OG(β), R^2=R^3=R^4=R^5=G
2 : R^1=OH(α, β), R^2=R^3=G, R^4~R^5=(S)-HHDP
3 : R^1=OG(β), R^2=R^3=G, R^4~R^5=(S)-HHDP
4 : R^1=OH(α, β), R^2~R^3=R^4~R^5=(S)-HHDP
5 : R^1=OG(β), R^2=R^3=G, R^4~R^5=(1′S)-DHHDP



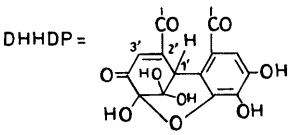
6 : R^1=R^2=R^3=H
7 : R^1~R^2=(1′R)-DHHDP, R^3=H
8 : R^1~R^2=chebuloyl, R^3=H
9 : R^1~R^2=(1′R)-DHHDP, R^3=



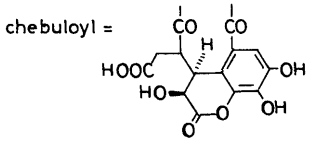
10 : R^1=OH, R^2=OG(α)
11 : R^1=OH, R^2=OH(α)
12 : R^1=H, R^2=OG(α)
13 : R^1=H, R^2=OH(α)
14 : R^1=H, R^2=OH(β)



G =



DHHDP =



chebuloyl =

Chart 1

(**8**),[6d] and mallotusinic acid (**9**),[6e] were isolated from plant extracts by the cited methods. (−)-Epigallocatechin gallate (**10**), (−)-epigallocatechin (**11**), and (−)-epicatechin gallate (**12**), were obtained from green tea (Chart 1). Alkyl gallates (methyl, ethyl, propyl, and butyl gallates) were supplied by Fuji Chemical Industry. Deuteromethyl gallate was prepared by methanolysis of methyl tri-*O*-benzylgallate with $NaOCD_3$ in CD_3OD, followed by hydrogenolysis over 5% Pd–C.

Radical Scavenging Effect on DPPH Radical An MeOH solution of a tannin or related polyphenol at various concentrations (1—14 μg/ml) was added to a solution of DPPH (1.5×10^{-4} M) in MeOH (1 ml), and the reaction mixture (total volume, 5 ml) was shaken vigorously. After storage at room temperature for 30 min in air, remaining DPPH was determined by colorimetry at 520 nm, and the radical-scavenging activity of each polyphenol was expressed by the ratio of lowering of the absorption of DPPH (%), relative to the absorption (100%) of DPPH solution in the absence of polyphenol (control). The mean values were obtained from triplicate experiments.

Preparation of Dimers of Alkyl Gallates Alkyl gallate (0.4 mM) in MeOH (5 ml) was added to a solution of DPPH (236 mg, 0.6 mM) in MeOH (20 ml), and kept at room temperature for 1 h in an N_2 atmosphere. The reaction mixture was evaporated *in vacuo*, and the residue was suspended in H_2O. The precipitates (1,1-diphenyl-2-picrylhydrazine, mp 171—172 °C) were collected, and washed with H_2O. The filtrate after concentration was subjected to column chromatography over Toyopearl HW-40C (1.1 cm i.d. × 33 cm) developed with H_2O containing increasing amount of MeOH. Dialkyl hexahydroxydiphenates, except for dibutyl hexahydroxydiphenate, were obtained from the H_2O eluate on column chromatography of the products of each reaction. Dibutyl hexahydroxydiphenate was obtained from the eluate with 40% aqueous MeOH. The starting materials were recovered from the 50% aqueous MeOH eluates in each experiment. Yield of each dialkyl hexahydroxydiphenate was calculated based on the recovered starting materials.

Results and Discussion

A methanol solution of DPPH was found to be stable for over 60 min by colorimetry at 520 nm of an 80 μg/ml solution. The radical scavenging effects of tannins and related polyphenols were then measured by colorimetry of the DPPH radical after its reduction by the polyphenols.

As shown in Table I, the polyphenols examined in this study exhibited higher reducing effects on DPPH than ascorbic acid and *dl*-α-tocopherol. Hydrolyzable tannins showed stronger effects than the samll-molecular polyphenols. This table also indicates that the reducing effects of the hydrolyzable tannins having several galloyl groups in a molecule are stronger than those of the tannins having HHDP, dehydrohexahydroxydiphenoyl (DHHDP), or chebuloyl groups, as reflected in the order of efficacies of the reducing effects: penta-*O*-galloyl-β-D-glucose (**1**) > tellimagrandin II (**3**) > isoterchebin (**5**) > chebulinic acid (**8**) ≈ tellimagrandin I (**2**) > pedunculagin (**4**) ≈ geraniin (**7**). The strong contribution of the galloyl group was also reflected in the fairly significant effects of (−)-epigallocatechin gallate (**10**), (−)-epicatechin gallate (**12**) and methyl gallate, in spite of their small molecular size. It is noticeable that these trends in the strength of radical scavenging effects of polyphenols on DPPH are somewhat different from those of their inhibitory effects on the peroxidation of methyl linoleate.[4] This difference in the scavenging effects could be interpreted in terms of the accessibility of the radical center of DPPH to each polyphenol.

TABLE I. Effects of Tannins and Related Polyphenols on DPPH

Compound	50% reduc.[a] (μM)	Number of Galloyl	HHDP	DHHDP
Pentagalloylglucose (**1**)	3.2	5		
Tellimagrandin II (**3**)	4.2	3	1	
Tellimagrandin I (**2**)	5.2	2	1	
Pedunculagin (**4**)	5.6		2	
Isoterchebin (**5**)	5.0	3		1
Mallotusinic acid (**9**)	4.8	1	(1)[b]	1
Geraniin (7)	5.9	1	1	1
Chebulinic acid (**8**)	5.3	3		(1)[c]
Corilagin (**6**)	6.8	1	1	
(−)-Epigallocatechin gallate (**10**)	6.7			
(−)-Epigallocatechin (**11**)	11.0			
(−)-Epicatechin gallate (**12**)	2.0			
(−)-Epicatechin (**13**)	31.0			
(+)-Catechin (**14**)	34.0			
Gallic acid	16.0			
Methyl gallate	15.0			
Ethyl gallate	14.0			
Propyl gallate	16.0			
n-Butyl gallate	17.0			
Ascorbic acid	39.0			
dl-α-Tocopherol	37.0			

a) Amount required for 50% reduction of DPPH after 30 min. *b*) Valoneoyl group. *c*) Chebuloyl group.

Polyphenol radicals are generally known as highly reactive species, which undergo a variety of reactions to give dimers through C–C and C–O couplings, and also quinones, *etc.* by oxidation.[7] We previously observed that gallic acid gives an ESR signal ascribable to a dimer (HHDP) radical, instead of a galloyl radical, upon ESR measurement in alkaline aqueous dimethyl sulfoxide (DMSO) solution.[3] This finding, coupled with the considerable reactivity of methyl gallate toward DPPH, as revealed in the present study, prompted us to characterize the products formed from the galloyl radical during the reaction with DPPH.

Upon the treatment of methyl gallate and its congeners (ethyl and propyl gallates) with DPPH at room temperature for 1 h on a preparative scale, dialkyl esters of hexahydroxydiphenic acid were produced as main products, in addition to the hydrogen adduct of DPPH (DPPH-H, diphenylpicrylhydrazine), and were separated by column chromatography over Toyopearl HW-40C after the removal of DPPH-H by filtration. Their physico-chemical properties and the isolation yields are summarized in Table II. Unlike the other alkyl gallates, *n*-butyl gallate required a

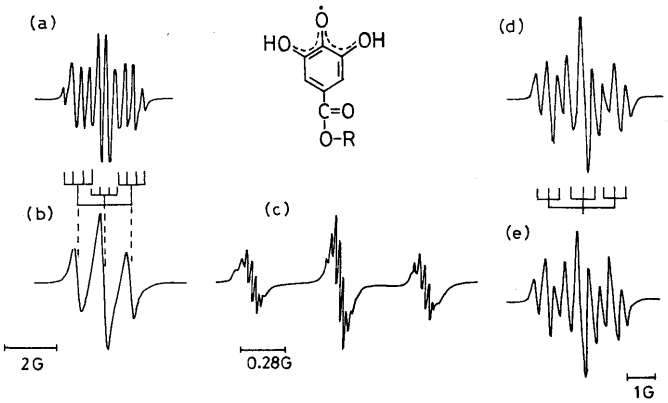


Fig. 1. ESR Spectra of Alkyl Gallates

(a) methyl gallate; (b) deuteromethyl gallate; (c) simulated spectrum of deuteromethyl gallate; (d) ethyl gallate; (e) *n*-butyl gallate. Sample solution (0.1 ml, 2 mg/ml) was added to 0.1 ml of 0.3 M KOH in DMSO–H_2O (9 : 1), and the ESR signal was recorded.

TABLE II. Physico-chemical Properties of HHDP Esters

	HHDP ester (alkyl)			
	CH_3	CH_3CH_2	$CH_3CH_2CH_2$	$CH_3CH_2CH_2CH_2$[a]
Yield (%)	32	30	41	17
mp (no mp <280 °C)				
Softens	180	221	190	172
Resolidifies	210	223	193	182
Molecular formula	$C_{16}H_{14}O_{10}$	$C_{18}H_{18}O_{10}$	$C_{20}H_{22}O_{10}$	$C_{22}H_{26}O_{10}$
EI-MS m/z (M^+)	366	394	422	450
^{1}H-NMR	7.13 (2H, s)	7.13 (2H, s)	7.15 (2H, s)	7.15 (2H, s)
(Acetone-d_6)	3.48 (6H, s)	3.91 (4H, q, J=7)	3.82 (4H, t, J=7)	3.86 (4H, t, J=7)
		0.96 (6H, t, J=7)	1.35 (4H, tq, J=7)	1.26 (8H, m)
			0.76 (6H, t, J=7)	0.83 (6H, t, J=7)

a) Reaction time: 15 h.

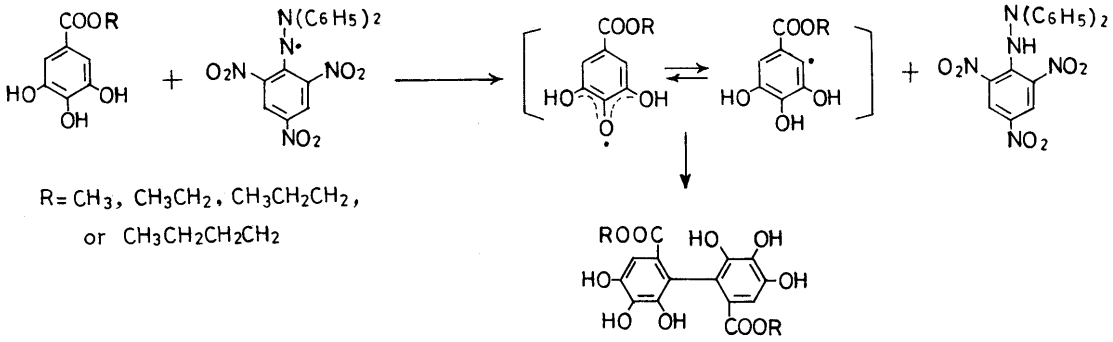


Chart 2

prolonged reaction time for the formation of a significant amount of the corresponding dimer. Although the reason for this difference will have to be investigated in a future study, it is most likely that the coupling reaction of galloyl radicals is dependent on the length of the alkoxyl group, which may cause steric hindrance when alkyl gallates approach the radical center of DPPH, or each other. This steric hindrance may be important for alkoxyl groups larger than a propyl group.

Alkyl gallate radicals have now been demonstrated by ESR measurement, although gallic acid radical was not detected because of its instability.[3] The ESR signal of methyl gallate obtained by air-oxidation in alkaline aqueous DMSO solution is shown in Fig. 1. The triple quartet in the hyperfine structure is attributable to the couplings of the unpaired electron with hydrogens on the ring and those on the α-carbon of the alkyl substituent. This assignment was supported by collapsing of the signal of deuteromethyl gallate to a triplet, and also by the triple triplet signal of higher alkyl gallates (Fig. 1). The ESR signals of these gallate radicals decayed with half-lives of about 20 min.

Production of dimers from alkyl gallates in the presence of DPPH is thus proposed to occur as illustrated in Chart 2.

These results indicate that the stronger inhibition of lipid peroxidation by large-molecular tannins than that by small-molecular polyphenols might be due to formation of stable radicals from tannins. As shown by the experiments on the reactions with DPPH radical in the present study, and on inhibition of methyl linoleate autoxidation,[4] the strength of radical-scavenging effects of tannins and related polyphenols is influenced by the nature of the radical species (*i.e.*, DPPH radical or peroxy radical) under consideration. Comparison of the effects of tannins in biological systems, therefore, should be performed for each radical species.

References

1) Part IV: Y. Fujita, K. Komagoe, I. Uehara, Y. Morimoto and R. Hara, *Yakugaku Zasshi*, **108**, 625 (1988).
2) T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda and S. Arichi, *Chem. Pharm. Bull.*, **31**, 1625 (1983).
3) Y. Fujita, K. Komagoe, Y. Sasaki, I. Uehara, T. Okuda and T Yoshida, *Yakugaku Zasshi*, **107**, 17 (1987).
4) Y. Fujita, K. Komagoe, Y. Niwa, I. Uehara, R. Hara, H. Mori, T. Okuda and T. Yoshida, *Yakugaku Zasshi*, **108**, 528 (1988).
5) H. Sies, "Oxidation Stress," Academic Press, London, 1985.
6) *a*) T. Okuda, T. Hatano and T. Yasui, *Heterocycles*, **16**, 1321 (1981); *b*) T. Okuda, T. Yoshida, M. Ashida and K. Yazaki, *J. Chem. Soc., Perkin Trans. 1*, **1983**, 1765; *c*) T. Okuda, T. Yoshida and T. Hatano, *ibid.*, **1982**, 9; *d*) T. Yoshida, T. Okuda, T. Koga and N. Toh, *Chem. Pharm. Bull.*, **30**, 2655 (1982); *e*) T. Okuda and K. Seno, *Tetrahedron Lett.*, **1978**, 139.
7) H. -I. Joschek and S. I. Miller, *J. Am. Chem. Soc.*, **88**, 3273 (1966).