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INHIBITORY EFFECT OF TANNINS ON REVERSE TRANSCRIPTASE FROM RNA TUMOR VIRUS

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ABSTRACT.—Twenty-four tannins and related compounds were examined for inhibition against reverse transcriptase from RNA tumor virus. Hydrolyzable tannins showed a potent inhibitory effect comparable to nitidine, in the presence of polyadenylic acid-oligothymidylic acid as a template-primer. A lesser inhibitory activity was observed for the monomeric ellagitannins, gallotannins, and nitidine using polycytidylic acid-oligodeoxyguanylic acid as a template-primer, whereas the inhibitory activity of dimeric ellagitannins was almost as potent as that observed for these compounds in the polyadenylic acid-oligothymidylic acid directed reaction. Inhibition by tannins was reversed by the addition of either template-primer or enzyme, suggesting that the inhibition is due to the interaction of tannins with both of them.

Molecular biological studies on the mechanism of carcinogenesis have recently elucidated that retroviruses play an important role in the initiation of tumorigenic conversion of animal as well as human cells (1).

RNA-directed DNA polymerase (reverse transcriptase), which is found in the virion of RNA tumor viruses (2,3), is responsible for the integration of virus genes into host genomes (4). A variety of inhibitors of this enzyme has been developed from an interest in understanding the mechanism of malignant transformation of virus-infected cells and from the requirement for chemotherapeutic applications (5).

Since some Chinese crude drugs such as herbs of *Agrimonia pilosa* Ledeb. (6) and rhizoma of *Rumex patientia* L. (6), which are currently used for treatment for leukemia and sarcoma in China, are known to be rich in tannins, we investigated the inhibitory property of 24 tannins isolated from crude drugs on reverse transcriptase from avian myeroblastosis virus.

MATERIALS AND METHODS

We purchased the $(rA)_n \cdot (dT)_{12}$, $(rC)_n \cdot (dG)_{12-18}$ and unlabeled nucleoside triphosphates from Boehringer Mannheim; (methyl-^{3}H)dTTP and (8-^{3}H)dGTP were obtained from Amersham. Activated calf thymus DNA and DNA polymerase-α from calf thymus were purchased from PL Biochemicals, Inc., and AMV reverse transcriptase was obtained from Life Science Co. Ltd.

TANNINS.—Twenty-four tannins (see Figure 1 for representative structures) were isolated from the following plants according to the methods we have previously reported: trigalloylglucose and tetragalloylglucose were from the leaves of *Arctostaphylos uvae-ursi* (L.) Splengel (9); pentagalloylglucose (**1**), hexagalloylglucose, heptagalloylglucose, and octagalloylglucose were from the galls of *Rhus javanica* L. (10); chebulagic acid and chebulinic acid were from the fruit of *Terminalia chebula* Retzus (11); geraniin (**2**) and corilagin were from the herbs of *Geranium thunbergii* Sieb. et Zucc. (12,13); granatin A, granatin B, punicalin, and punicalagin were from the pericarp of *Punica granatum* L. (14); pedunculagin was from the leaves of *Psidium guajava* L. (15,16); gemin A was from the whole plants of *Geum japonicum* Thunb. (17); coriariin A was from the leaves of *Coriaria japonica* A. Gray (29); cornusiin was from the fruit of *Cornus officinalis* Sieb. et Zucc. (18); agrimoniin (**3**) was from the roots of *Agrimonia pilosa* Ledeb. [=*A. japonica* (Miq) Koidz.] (19,20); rugosin D was from the petals of *Rosa rugosa* Thunb. (21); nobotanin A was from the leaves of *Tibouchina semidecandra* Cogn. (22); (−)epigallocatechin gallate was from the leaves of *Thea sinensis*

L.; and procyanidine B-2 (4) and procyanidine B-2 gallate were from the leaves of *Saxifraga stolonifera* Meerb.

ENZYME ASSAY.—The standard reverse transcriptase assay was performed in a reaction mixture that contained the following in a final volume of 20 μl (23): 50 mM Tris-HCl (pH 8.3), 40 mM NaCl, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5 μg/ml template-primer, 0.1 mM (methyl-^{3}H) dTTP or (8-^{3}H) dGTP, and 1 unit of reverse transcriptase.[1]

DNA polymerase assay mixture of 20 μl contained the following (24): 60 ml Tris-HCl (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 200 μg/ml activated calf thymus DNA, 0.1 mM each deoxyribonucleoside triphosphate, 500 μg/ml bovine serum albumin (BSA), 12.5% glycerol, and 0.5 units of DNA polymerase alpha.[2]

The serial dilutions of samples in DMSO were added immediately before incubation. The final DMSO concentration was 10%.

The reaction mixture was incubated at 37° for 60 min. Ten μl of each assay mixture was applied to 2.3-cm circular Watman DE 81 cellulose paper, and the paper was washed in a batch in 3 ml of a 5% Na_2HPO_4 solution six times, followed each time with H_2O and EtOH. The cellulose paper was dried and counted in a xylene-based scintillation fluid (ACS-II, Amersham).

Control assay was performed in a 10% DMSO solution.

RESULTS

In recent years, a large number of tannins have been isolated from higher plants, and their structures have been determined (6). They can be classified, according to their structures, as condensed tannins and hydrolyzable tannins, which include gallotannins and ellagitannins.

The 24 tannins that were examined in this paper can be divided into these categories, which are gallotannins (trigalloylglucose, tetragalloylglucose, pentagal-

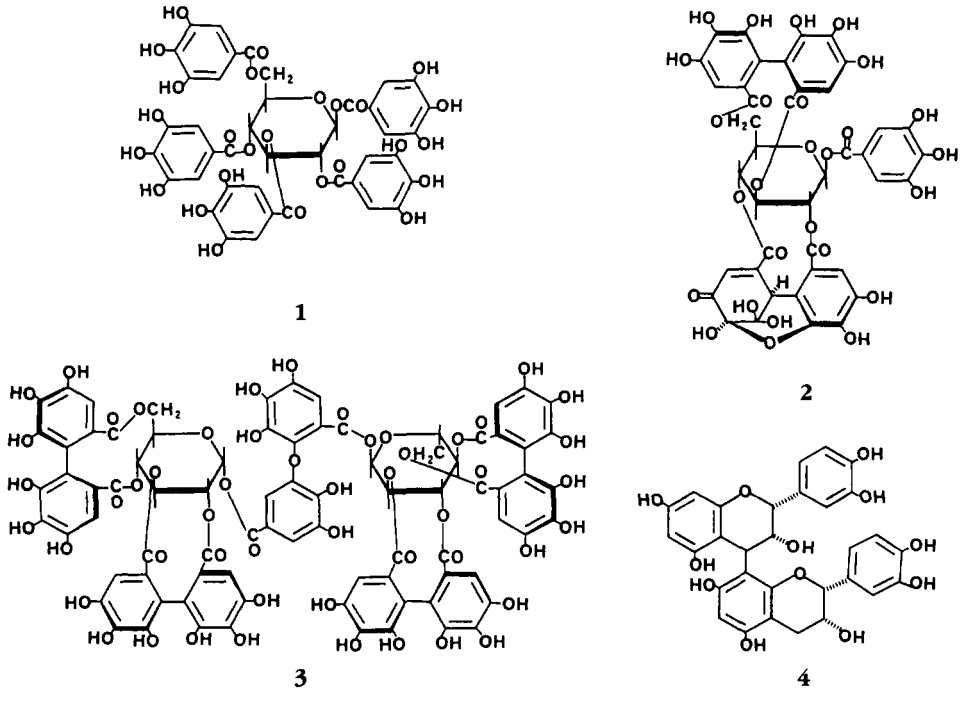

FIGURE 1. Structures of tannins.

[1]One unit of enzyme activity is expressed as the incorporation of 1 nanomole of dTMP into an acid-insoluble product with poly(rA)-oligo(dT) as the template-primer in 10 min at 37°.

[2]One unit catalyzes the incorporation of 1 nanomole of dAMP into an acid-precipitable product with poly(dT)-oligo(rA) as the template-primer in 1 h at 37°.

loylglucose, hexagalloylglucose, heptagalloylglucose, octagalloylglucose), ellagitannins (geraniin, granatin A, granatin B, chebulagic acid, chebulinic acid, punicalagin, punicalin, corilagin, pedunculagin), dimeric ellagitannins (gemin A, coriariin A, agrimoniin, cornusiin A, rugosin D, nobotanin A), and condensed tannins (procyanidin B-2, procyanidin B-2 digallate, (−) epigallocatechin gallate).

The effect of tannins and reference compounds on reverse transcriptase from avian myeloblastosis virus (AMV) was measured using poly(rA)-oligo(dT) as a template-primer (Table 1). Most of the ellagitannins, both monomeric and dimeric, showed comparable inhibitory effect at a concentration of 10^{-5} M with nitidine which had been reported to be effective on reverse transcriptase from AMV, Rauscher murine virus (25) and simian sarcoma virus type 1, mouse embryo DNA polymerase, RNA polymerase, and poly(A) polymerase (26). None of the condensed tannins inhibited the AMV reverse transcriptase reaction at concentrations from 10^{-7} to 10^{-4} M.

Among the gallotannins, pentagalloylglucose and hexagalloylglucose were the most effective. The reduced activity was seen with gallotannins having a smaller number of galloyl residues, i.e., trigalloylglucose and tetragalloylglucose.

TABLE 1. Percent of dTMP Incorporated in the Presence of $(rA)_n$-$(dT)_{12}$ as a Template-Primer

Compounds	Concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
I Hydrolyzable Tannins					
a. Gallotannins					
Trigalloyl glucose	83±10[a]	87±17	70±3	29±2	12±2
Tetragalloyl glucose	86±0	66±13	39±2	28±10	6±1
Pentagalloyl glucose (**1**)	67±1	39±6	30±7	25±7	7±1
Hexagalloyl glucose	76±2	37±11	26±8	25±0	7±3
Heptagalloyl glucose	103±1	68±13	35±10	18±6	6±0
Octagalloyl glucose	87±3	57±32	39±2	25±9	8±4
b. Monomeric Ellagitannins					
Geraniin (**2**)	89±9	39±14	12±12	6±3	6±3
Granatin A	86±1	59±21	9±2	5±1	3±2
Granatin B	74±19	52±15	10±3	10±4	2±0
Chebulagic acid	106±10	90±3	48±15	31±2	6±1
Chebulinic acid	109±12	71±9	22±9	24±4	8±5
Punicalagin	85±19	22±0	19±11	11±1	2±0
Punicalin	119±18	62±3	35±5	8±1	1±0
Corilagin	88±4	92±2	88±3	25±2	3±0
c. Dimeric Ellagitannins					
Pedunculagin	88±6	51±2	15±2	17±7	4±0
Gemin A	70±2	19±4	16±11	3±0	1±1
Coriariin A	73±0	25±0	11±2	4±0	1±0
Agrimoniin (**3**)	86±0	27±1	20±6	19±3	2±1
Cornusiin A	82±5	19±7	4±0	6±1	6±2
Rugosin D	55±4	16±5	7±3	5±1	6±0
Nobotanin A	66±7	24±4	4±2	5±0	3±0
II Condensed Tannins					
Procyanidin B-21 (**4**)	102±5	116±15	97±3	98±3	55±12
Procyanidin B-2 digallate	93±2	99±1	94±0	80±0	8±0
(−) Epigallocatechin gallate	88±3	97±3	96±3	95±1	81±7
III Reference Compounds					
Nitidine	85±11	102±10	11±12	1±12	1±1
Ellagic acid	93±2	77±8	45±1	18±1	0±0
Gallic acid	84±18	93±4	75±4	75±1	78±5

[a]Average of duplicate experiments with standard error.

When poly(rC)-oligo(dG) was used as a template-primer, dimeric ellagitannins, such as gemin A, coriariin A, agrimoniin, and rugosin D, also effectively inhibited the reverse transcriptase-reaction at the concentration of 10^{-5} M. Monomeric ellagitannins and gallotannins showed reduced inhibitory activity (Table 2). The condensed tannins had no effect on this enzyme reaction.

TABLE 2. Percent of dGMP Incorporated in the Presence of $(rC)_n$-$(dG)_{12-18}$ as a Template-Primer

Compounds	Concentration (M)				
	10^{-7}	10^{-6}	10^{-5}	10^{-4}	10^{-3}
I Hydrolyzable Tannins					
a. Gallotannins					
Trigalloyl glucose	80±6[a]	92±1	50±6	46±15	42±6
Tertagalloyl glucose	84±4	43±7	48±14	27±13	13±1
Pentagalloyl glucose (**1**)	80±4	62±9	44±4	27±5	15±13
Hexagalloyl glucose	140±4	106±5	41±10	21±11	6±4
Heptagalloyl glucose	115±11	120±19	39±6	23±6	4±0
Octagalloyl glucose	112±7	89±7	69±21	15±5	13±1
b. Monomeric Ellagitannins					
Geraniin (**2**)	103±19	93±9	62±1	15±0	12±0
Granatin A	61±2	115±4	34±4	6±0	8±0
Granatin B	90±17	79±7	40±3	6±3	5±1
Chebulagic acid	94±16	67±8	74±9	68±5	8±3
Chebulinic acid	75±4	67±13	46±8	27±4	1±1
Punicalagin	85±9	76±4	18±4	9±6	4±0
Punicalin	105±0	109±12	64±1	31±7	6±2
Corilagin	92±0	72±18	64±17	30±7	0±0
c. Dimeric Ellagitannins					
Pedunculagin	80±16	73±7	41±6	7±2	10±2
Gemin A	106±4	60±15	6±2	6±5	1±1
Coriariin A	102±9	68±13	13±6	8±4	1±1
Agrimoniin (**3**)	80±12	68±15	7±0	5±4	6±2
Cornusiin A	105±7	76±10	22±2	10±1	15±1
Rugosin D	111±19	54±6	12±0	12±1	2±1
Nobotanin A	106±19	73±1	22±5	14±1	20±4
II Condensed Tannins					
Procyanidin B-2 (**4**)	85±13	93±10	80±2	64±11	9±6
Procyanidin B-2 digallate	88±18	81±15	78±11	33±15	0±1
(−)Epigallo-catechingallate	79±1	88±12	86±10	53±7	13±8
III Reference Compounds					
Nitidine	116±18	85±7	88±0	5±5	2±0
Ellagic acid	71±3	80±13	49±15	20±3	3±1
Gallic acid	122±2	103±3	99±3	91±1	98±11

[a]Average of duplicate experiments with standard error.

Lineweaver-Burk plots were made for the kinetics of inhibition by geraniin on poly(rA)-oligo(dT)-directed reverse transcription (Figure 2). The mode of the inhibitory action of geraniin was competitive with regard to the template-primer.

When the concentration of AMV reverse transcriptase was increased, the extent of the inhibition of reverse transcription by 10^{-5} M geraniin was reduced (Table 3). Polymerization was restored up to 90% of control in the presence of three times more enzyme than the standard assay. This result suggests that geraniin could interact with not only the template-primer but with the enzyme as well.

When 10^{-4} M geraniin was added to a reaction mixture 5 min after the initiation of DNA synthesis, the polymerization was stopped almost completely, whereas the con-

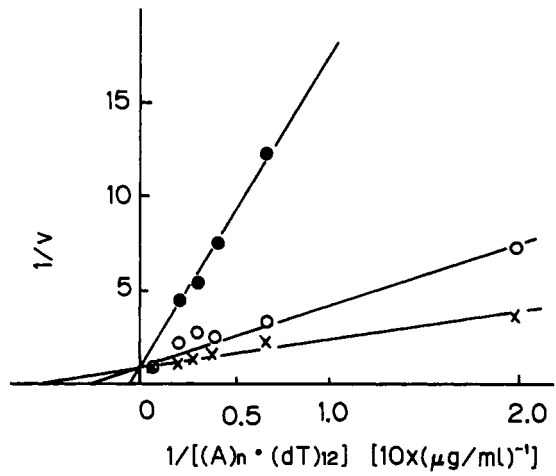

FIGURE 2. Lineweaver-Burk plots for the kinetic of inhibition of poly(rA)·oligo(dT) directed AMV reverse transcriptase reaction by geraniin. Incubation time was 15 min, v is expressed as 10^{-3}×radioactivity(CPM). Concentrations of geraniin were 10^{-5}M(●), 10^{-6}M(○), and 0 M(x).

TABLE 3. Effect of Increasing Concentration of AMV Reverse Transcriptase

RTase (unit/assay)	(^{3}H) dTTP incorporated (cpm)		% of control
	Control	10^{-5}M Geraniin	
0	134	—	0
1	6847	2265	32
3	12414	10733	86
5	12121	11148	92
17	14673	11805	81

trol reaction was still progressing. Therefore, it can be said that geraniin inhibits the elongation of polymerization (Figure 3).

The effect of the tannins on the cellular DNA polymerase of each of the categories, hexagalloylglucose, geraniin, gemin A, and epigallocatechin gallate was also examined (Figure 4). At a concentration of 10^{-5} M, none of these tannins showed remarkable inhibitory effect on the reaction catalyzed by DNA polymerase-α from calf thymus using activated calf thymus DNA as a template-primer. Gemin A inhibited 70% of polymerization at the concentration of 10^{-4} M, and hexagalloylglucose and geraniin showed inhibition at 10^{-3} M. The reaction with the condensed tannins still reached 80% of control at the concentration of 10^{-3} M.

DISCUSSION

Tannins are defined as polyphenols, which can precipitate with proteins and alkaloids. However, the biological properties are very different among the group. In the case of the inhibitory effect on lipid peroxidation in the mitochondria or microsome of liver, for instance, most of the hydrolyzable tannins show higher inhibitory potency than those of the condensed tannins (27). Furthermore, gallotannins inhibit the glucan synthesis catalyzed by glucosyltransferase from a cariogenic bacterium, *Streptococcus mutans*, more effectively than ellagitannins (28).

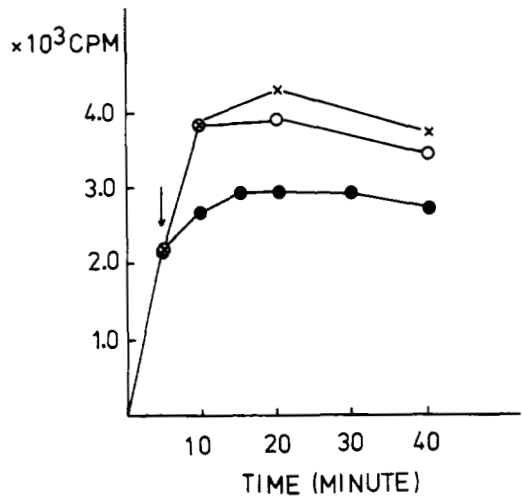

FIGURE 3. Effect of geraniin on poly(rA).oligo(dT) directed AMV reverse transcriptase reaction, when geraniin was added after 5 min (as indicated by arrow) during incubation of the assay mixture. Concentrations of geraniin were 10^{-4}M(●), 10^{-5}M(○) and 0 M(x).

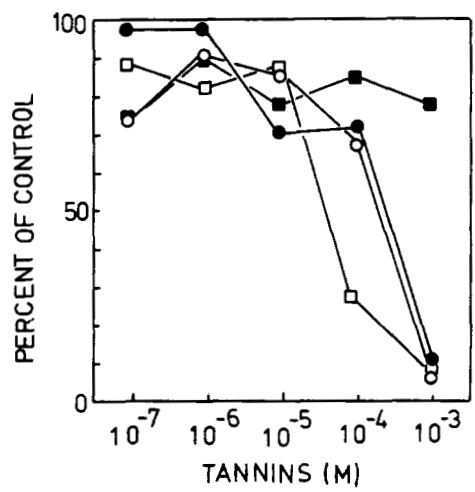

FIGURE 4. Effect of tannins on DNA polymerase alpha from calf thymus. In a standard assay mixture containing serial dilutions of hexagalloylglucose(○), geraniin(●), gemin A(□), and epigallocatechin gallate(■).

The present studies demonstrated that hydrolyzable tannins inhibited the polymerization catalyzed by the reverse transcriptase from retrovirus. Moreover, dimeric ellagitannins were more effective inhibitors of the reaction, especially with poly(rC)-oligo(dG) as a template-primer, than monomeric ellagitannins, and trigalloylglucose and tetragalloylglucose were less effective than the gallotannins with a larger number of galloyl residues. These findings suggest that the higher molecular

weight tannins with a number of functional groups can strongly interact either with nucleotides or with proteins. In contrast to the inhibitory effect on glucosyltransferase from *S. mutans*, ellagitannins were generally more effective than gallotannins on the reverse transcriptase reaction.

The inhibitory aspect was also affected by the nature of template-primers. The reverse transcription was found to be more sensitive to tannin inhibition with poly(rA)-oligo(dT) as a template-primer than with poly(rC)-oligo(dG). The same finding reported in the case of benzophenanthridine alkaloids has been explained as the result of strong binding affinity of the alkaloids with the rA-dT base pair (25,26). Since the tannins, bulkier molecules, are unlikely to intercalate between base pairs. The tannins are presumed to attach to the grooves of the double-stranded chain.

The tannin inhibition was restored by the addition of either enzyme or template-primers. This observation suggests that the tannins inhibit the formation of a template-primer-enzyme-nucleotide complex.

The hydrolyzable tannins also inhibited the cellular DNA polymerase but at higher concentrations than were necessary to inhibit reverse transcriptase. Tannins may have a more specific affinity for the reverse transcriptase than for the cellular DNA polymerase; or the template-primer of DNA polymerase, activated calf thymus DNA, may be more resistant to inhibition by tannins, since the double-stranded DNA is more stable than poly(rA)-oligo(dT) or poly(rC)-oligo(dG).

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