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Effects of Flavonoids and Alkaloids on Reverse Transcriptase

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One hundred-ninety flavonoids and 75 alkaloids were studied for their inhibitory effects on the Avian Myeloblastosis Virus reverse transcriptase (AMV-RT) by using $(rA)_n$-$p(dT)_{12-18}$ or $(rC)_n$-$p(dG)_{12-18}$ as a template-primer. Of the flavonoids tested, 6-hydroxyluteolin (**50**), pedalitin (**52**), 6-hydroxykaempferol (**109**) and quercetagetin (**129**), were the most potent inhibitors with $IC_{50} \leqq 10$ μM. Five other flavonoids, baicalein (**19**), baicalin methyl ester (**23**), scutellarein (**33**), myricetin (**126**) and gossypetin (**130**), gave IC_{50} between 35 and 200 μM. The kinetic studies on **50**, **126** and **130** gave the inhibitory constants (K_i) of 10, 94 and 90 μM, respectively, with respect to the substrate and 5, 200 and 100 μM, respectively, with respect to the template-primer. Structurally different alkaloids such as isoquinoline, indol, steroidal, quinolizidine, pyrrolizidine, pyridine and imidazole alkaloids were also investigated for the activity and berberine type alkaloids were shown to have a strong inhibitory activity. Flavonoids or alkaloids that inhibited AMV-RT did not show any significant effect on DNA-polymerase I.

Keywords——flavonoids; alkaloids; reverse transcriptase

Infectious diseases are still a concern in medical science, especially where effective therapeutic methods are not readily available. Ribonucleic acid (RNA) viruses are known to be an etiological agent of Acquired Immunodeficiency Syndrome (AIDS) and Adult-T-Cell Leukemia (ATL). They are also called retroviruses as they contain an RNA-dependent deoxyribonucleic acid (DNA)-polymerase or reverse transcriptase (RT). During the viral life cycle, RT makes double strand DNA from the viral RNA template, which is integrated into the host chromosome. This results in a viral proliferation. Since RT takes an essential part in the retrovirus life cycle, and bears different features from that of mammalian polymerases, many works for the development of effective anti-retroviral drugs have been focused on this enzyme.[1-6]

In our previous paper,[7] we reported the results of our studies on the traditional medicines for inhibiting the proliferation of Avian Myeloblastosis Virus (AMV)-RT *in vitro*. Some of Ayurvedic medicines were found to have a potent inhibitory action: arecatannins B_1 and A_2 from an *Areca catechu* extract and embelin from an *Embelia ribes* extract were shown to be potent RT-inhibitory substances. Furthermore, naturally-occurring alkaloids,[8] tannins[9] and flavonoids[10] were assayed for their inhibitory activities.

Of the alkaloids, benzo[c]phenanthridine and protoberberine type alkaloids have been known to inhibit AMV-RT by interacting with nucleic acids.[8,11-13] In regard to the flavonoids, recent works have shown that some of them are inhibitors of DNA- and RNA-dependent DNA polymerases.[14,15]

In the present paper, we report the effect of various naturally-occurring flavonoids and alkaloids on AMV-RT, with special reference to their inhibitory effect on RT.

Materials and Methods

Flavonoids and alkaloids——The following standard samples were purchased: **126**, **170**, **203**, **217**, **256**, **262**, **263** (Aldrich Chemical Co., Milwaukee, WIS, USA); **40**, **48**, **114**, **127**, **131**, **133**, **146**, **191**, **192**, **197**, **208**, **209**, **217**, **219**–

222, **228**, **232**, **238**, **242** (Carl Roth, Karlsruhe, Germany); **1**, **6–10**, **49**, **107**, **108**, **113**, **115–118**, **120**, **121**, **123**, **125**, **139**, **147**, **164**, **165** (Extrasynthese, Genay, France); **196**, **204**, **213**, **229**, **241** (Matsuura Chemicals Co., Nagoya, Japan); **173**, **215**, **216**, **255** (Nacalai Tesque, Kyoto, Japan); **96**, **169**, **137** (Sarsynthese, Merignac, France); **162**, **195**, **198–201**, **207**, **211**, **212**, **218**, **226**, **230**, **231**, **236**, **237**, **243**, **245–251**, **254**, **257–261**, **264**, **265** (Sigma Chemical Co., St. Louis, MO. USA); **110**, **163** (Tokyo Kasei Co., Tokyo, Japan); **124**, **241** (Wako Pure Chemicals Industry Co., Osaka, Japan); **193**, **202** (Yoneyama Chemicals Co., Osaka, Japan). Flavonoids **2–5**, **11–39**, **41–47**, **50–53**, **55–95**, **97–106**, **119**, **121**, **128**, **132**, **134**, **137**, **138**, **141–144**, **148–160**, **166**, **167**, **172**, **174–190** were isolated or synthesized by T. Tomimori. Gossypetin (**130**) was obtained by the hydrolysis of gossypin in 10% HCl/MeOH solution at 70°C for 4 h. Flavonoids **109**, **111** and **112** were isolated from the flower petals of *Carthamus tinctorius* L. Berberrubin (**194**) was obtained from berberine chloride by the methods of Frerichs[16] and identified by comparing the ultraviolet (UV), infrared (IR), electron impact mass (EI-MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectra with those given in the references.[17,18] Dihydroberberine (**206**) and tetrahydroberberine (**214**) were obtained by the reduction of berberine chloride with $LiAlH_4$ and $NaBH_4$, respectively. Verticine N-oxide (**252**) and verticinone N-oxide (**253**) were isolated from *Fritillaria thunbergii* Miq. Strychnine (**239**) and its analogs (**223–225**, **233–235**, **240** and **244**) were isolated from the seeds of *Strychnos nux-vomica* L.[19,20] 13-Methylberberine iodide (**210**) and dehydrocorydaline chloride (**205**) were synthesized as previously reported.[21]

Enzymes and chemicals——AMV-RT was purchased from Wako Pure Chemical Industry Co., $(rA)_n$-$p(dT)_{12-18}$, $(rC)_n$-$p(dG)_{12-18}$, DNA Polymerase I (Pol I), activated calf thymus DNA and unlabeled nucleoside 5′-triphosphates were purchased from Pharmacia (Uppsala, Sweden). [methyl-^{3}H]-Thymidine 5′-triphosphate (dTTP) (specific activity, 1.55 TBq/mmol), [8-^{3}H]-deoxyguanosine 5′-triphosphate (dGTP) (specific activity, 311 GBq/mmol) and the scintillation fluid ACS-II were obtained from Amersham-Japan (Tokyo, Japan). Adriamycin (doxorubicin hydrochloride) was purchased from Sigma Chemical Co. AMV-RT and Pol I were adjusted to 1 U/μl and 0.5 U/μl,[22] respectively, with a solution of 0.2 ml of 1.0 M phosphate buffer pH 7.2, 0.5 ml of glycerol, 0.02 ml of 0.1 M dithiothreitol (DTT), 2.0 μl of Triton X-100 and 0.28 ml of distilled water.

Assay of RT——The reaction mixture for the standard RT assay was composed of 50 mM Tris-HCl (pH 8.3), 40 mM NaCl, 10 mM $MgCl_2$, 5 mM DTT, 5 μg/ml template-primer, 0.1 mM [methyl-^{3}H]-dTTP or [8-^{3}H]-dGTP (18.5 KBq/ml) and 1 μl of RT in a final volume of 20 μl. The samples were dissolved in dimethyl sulfoxide (DMSO) and added to the reaction mixture, to which the enzyme was added immediately before the start of incubation. The control assay was performed by adding DMSO containing no samples. Adriamycin (0.5 mM) was used as a positive control for the complete inhibition of the enzyme activity. The reaction mixture was incubated at 37°C for 60 min. Ten μl of each assay mixture was applied to 2.3 cm circular Whatman DE-81 cellulose paper, which was washed batchwise with 3 ml of a 5% Na_2HPO_4 solution five times, then two times with distilled water and ethanol and once with ether. The cellulose paper was then dried and counted in a scintillation fluid.

Inhibition of the RT activity——The inhibition of RT, measured as the inhibition of the incorporation of ^{3}H-labeled substrates into the polymer fraction by alkaloids or flavonoids, was calculated as follows:

$$(\%)\ \text{Inhibition} = [1 - (\text{dpm test}/\text{dpm control})] \times 100$$

The control values were 14,000 dpm to the $(rA)_n$-$p(dT)_{12-18}$ directed reaction and 130,000 dpm to the $(rC)_n$-$p(dG)_{12-18}$ directed reaction. Adriamycin (0.5 mM) inhibited the enzyme reaction by 99.4%.

Assay of Pol I——The assay was performed by the method described above in a mixture containing 60 mM Tris-HCl (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 200 μg/ml activated calf thymus DNA, 0.1 mM of each deoxyribonucleoside 5′-triphosphate (dATP, dTTP, dGTP and dCTP; [^{3}H]-dTTP, 18.5 KBq/mmol), 500 μg/ml bovine serum albumin

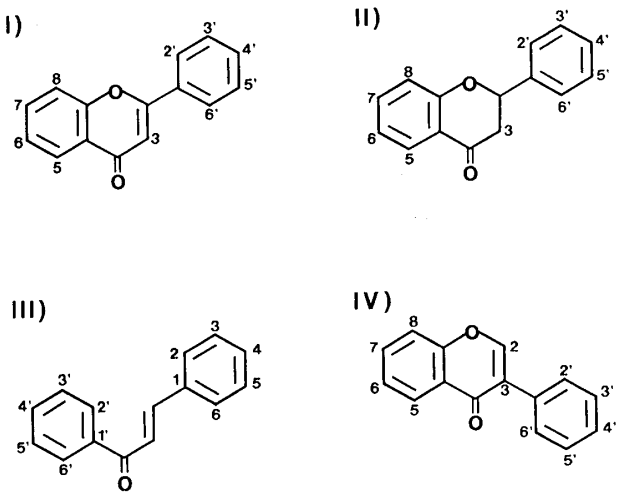


Chart 1. Structures of Flavonoids Listed in Table I

TABLE I. Inhibitory Effects of Flavonoids on AMV Reverse Transcriptase

	Compound (Trivial name)	Substituents of flavone [structure (I)] 5	6	7	8	2′	3′	4′	5′	6′	% Inhibition Mean±S.E.M.
1	(Chrisin)	OH		OH							0.5± 8.1
2		OH		OGlc$^{6}_{-}$^{1}Glc							48.5± 6.4
3		OH		OGA							4.0± 6.6
4		OPr		OPr							24.2±16.5*
5		OH		OBy							34.9± 4.0
6	(β-Naphtoflavone)	C_4H_4									4.1± 7.0*
7	(Pratol)			OH				OMe			13.6± 6.1
8	(Apigenin)	OH		OH				OH			−9.9± 8.5
9	(Acacetin)	OH		OH				OMe			48.0±10.6
10	(Fortunellin)	OH		OGlc$^{2}_{-}$^{1}Rha				OMe			19.4± 6.7
11	(Rhoifolin)	OH		OGlc$^{2}_{-}$^{1}Rha				OH			23.2± 4.1*
12		OH		OH				OPr			55.4± 6.3
13		OH		OPr				OPr			23.6± 6.2
14		OBy		OBy				OPr			36.0± 3.7
15		OAc		OAc				OAc			24.7± 2.5
16		OH		OMe				OMe			54.8± 4.6
17		OMe		OMe				OMe			45.6± 3.1
18	(Baicalein-7-*O*-Glc)	OH	OH	OGlc							99.2± 0.1*
19	(Baicalein)	OH	OH	OH							95.0± 1.2
20	(Baicalin)	OH	OH	OGA							26.4± 8.1
21		OH	OH	OGlc$^{6}_{-}$CHO							83.9± 1.9*
22	(Oroxin B)	OH	OH	OGlc$^{6}_{-}$^{1}Glc							54.6± 2.2
23	(Baicalin methyl ester)	OH	OH	OGA-ME							97.0± 0.7*
24	(Baicalein-6-*O*-Glc)	OH	OGlc	OH							12.3± 8.3
25		OAc	OAc	OAc							71.4± 3.1*
26	(Oroxylin A)	OH	OMe	OH							8.6± 9.2
27		OH	OMe	OGA							−2.6± 4.6
28		OH	OMe	OGA-ME							72.5± 9.1*
29		OH	OMe	OMe							45.9± 4.6*
30		OMe	OMe	OH							32.7± 9.9*
31		OMe	OMe	OMe							29.8± 2.2*
32		OH	OMe	OAc							18.3± 3.1*
33	(Scutellarein)	OH	OH	OH				OH			99.4± 0.1
34	(Scutellarin)	OH	OH	OGA				OH			92.1± 1.6
35		OH	OH	OPr				OPr			18.0± 2.7*
36	(Hispidulin)	OH	OMe	OH				OH			23.3± 3.1
37	(Cirsimaritin)	OH	OMe	OMe				OH			50.9± 3.7*
38	(Cirsimarin)	OH	OMe	OMe				OGlc			86.3± 4.4
39	(Embinin)	OH	Glc$^{2}_{-}$^{1}Rha	OMe				OMe			−3.1± 1.8
40	(Vitexin)	OH	OH	Glc				OH			24.7± 7.4
41	(Isovitexin)	OH	Glc	OH				OH			24.4± 3.3
42	(Swertisin)	OH	Glc	OMe				OH			6.5± 0.9
43		OAc	Glc-Ac_4	OMe				OAc			1.2± 3.1*
44		OH	Glc	OMe				OMe			−2.0± 2.1
45		OMe	Glc	OMe				OMe			−1.6± 1.0
46	(Flavocommelin)	OH	Glc	OMe				OGlc			6.0± 7.1
47	(Saponarin)	OH	Glc	OGlc				OH			−3.1± 1.6
48	(Luteolin)	OH		OH			OH	OH			79.1± 1.8

Compound (Trivial name)	Substituents of flavone [structure (I)] 5	6	7	8	2′	3′	4′	5′	6′	% Inhibition Mean±S.E.M.
49	OH		OGlc			OH	OH			71.2± 4.2
50 (6-Hydroxyluteolin)	OH	OH	OH			OH	OH			91.7± 0.3
51 (Eupafolin)	OH	OMe	OH			OH	OH			17.0± 2.5
52 (Pedalitin)	OH	OH	OMe			OH	OH			91.4± 1.9
53 (Cirsiliol)	OH	OMe	OMe			OH	OH			8.4± 1.5
54 (Cirsilineol)	OH	OMe	OMe			OMe	OH			−6.9± 6.9
55 (Homoorientin)	OH	Glc	OH			OH	OH			32.1± 1.2
56 (Swertiajaponin)	OH	Glc	OMe			OH	OH			21.5± 1.5
57	OMe	Glc	OMe			OMe	OMe			6.2± 3.4
58 (Norwogonin)	OH		OH	OH						36.9± 1.2
59 (Wogonin)	OH		OH	OMe						36.8± 6.0
60	OH		OBy	OH						19.0± 3.0*
61	=O		OBy	=O						8.8± 1.9
62	OH		OBy	OBy						−7.2± 1.3*
63	OH		OGA	OMe						−1.4± 0.8
64	OH		OMe	OMe						9.8± 0.8
65	OMe		OH	OMe						−2.6± 3.0
66	OMe		OAc	OMe						−7.6± 0.9
67 (Isoscutellarein)	OH		OH	OH			OH			22.0± 5.4
68	OH		OH	OGA			OH			5.6± 0.7
69	OH		OH	OH			OMe			3.0± 1.3
70	OH		OH	OMe			OH			2.3± 1.3
71	OH		OPr	OH			OPr			−1.4± 0.9
72	OH		OPr	OMe			OPr			−2.1± 0.7*
73	OH		OPr	OMe			OH			15.4± 0.7
74	OH		OPr	OMe			OMe			12.0± 3.1*
75 (Isoswertisin)	OH		OMe	Glc			OH			25.9±15.4
76	OMe		OMe	Glc			OMe			−9.3± 1.1
77	OBy		OBy	By			OPr			3.4± 3.8*
78	OMe		OMe	Me			OMe			−17.6± 1.5
79	OH		OH	By			OPr			4.1± 4.8
80 (Scutevulin)	OH		OH	OMe	OH					8.5± 2.0
81	OH		OGA	OH	OH					78.3± 4.5
82	OH		OH	OMe	OMe					−8.8± 1.1*
83	OH		OGA	OMe	OMe					−14.2± 1.8
84 (Skullcapflavone I)	OH		OMe	OMe	OH					−10.6± 2.0
85	OH		OMe	OMe	OAc					17.0± 3.0
86	OH		OH	OH		OH	OH			31.3± 2.7
87	OMe		OH	OMe		OBy	OBy			27.9± 4.8
88	OMe		OBy	OMe		OBy	OBy			7.0± 3.7*
89	OH		OH	OMe	OH				OMe	−11.7± 1.8
90 (Rivularin)	OH		OMe	OMe	OH				OMe	−8.2± 1.5
91	OMe		OMe	OMe	OMe				OMe	−4.4± 3.5
92	OH		OH	OMe	OH			OH	OMe	−0.7± 2.0
93	OH		OMe	OMe	OGlc				OMe	−7.2± 1.4
94	OH	OH	OMe	OH			OMe			39.1±10.7
95	OH	OH	OPr	OH			OPr			46.2± 0.3
96 (Tangeretin)	OMe	OMe	OMe	OMe			OMe			−8.6± 9.1
97 (Skullcapflavine II)	OH	OMe	OMe	OMe	OH				OMe	18.0± 2.3

Compound (Trivial name)	Substituents of flavone [structure (I)] 5	6	7	8	2′	3′	4′	5′	6′	% Inhibition Mean±S.E.M.
98	OH	OMe	OMe	OMe	OMe				OMe	5.4± 1.7
99	OH		OH	OH	OH					67.7± 1.6
100	OMe		OGA-ME	OMe						3.4± 1.4
101	OH		OH			OPr	OPr			12.2± 0.4
102	OBy		OBy			OPr	OPr			4.1± 1.6
103	OBy		OBy				OPr			4.5± 1.8
104	OH		OH	OBy						14.2± 4.7*
105	OH		OH	OH		OPr	OPr			17.6± 3.3
106	OH		OH	OH			OPr			9.7± 3.1

Compound (Trivial name)	Substituents of flavone [structure (I)] 3	5	6	7	8	2′	3′	4′	5′	6′	% Inhibition Mean±S.E.M.
107 (Galangin)	OH	OH		OH							42.1± 9.7
108 (Geraldol)	OH			OH			OMe	OH			52.7± 6.5
109 (6-hydroxy-kaempferol)	OH	OH	OH	OH				OH			97.2± 0.5
110 (Kaempferol)	OH	OH		OH				OH			72.6± 5.3
111	OGlc		OH	OH				OH			93.7± 0.4
112	OGlc	OH	OGlc	OH				OH			4.2± 0.7
113 (Kaempferide)	OH	OH		OH				OMe			64.6± 5.2
114	OH	OH		OGlc$^{2\text{-}1}$Rha				OH			5.2± 4.0
115 (Quercetin)	OH	OH		OH			OH	OH			41.4± 3.6
116 (Tamarixetin)	OH	OH		OH			OH	OMe			59.7± 5.0
117 (Rhamnetin)	OH	OH		OMe			OH	OH			50.8± 7.3
118 (Ombuin)	OH	OH		OMe			OH	OMe			68.1± 2.2
119 (Quercimeritrin)	OH	OH		OGlc			OH	OH			69.1± 2.6
120 (Isoquercitrin)	OGlc	OH		OH			OH	OH			25.1± 1.6
121 (Quercitrin)	ORha	OH		OH			OH	OH			21.5± 2.9
122 (Hyperoside)	OGal	OH		OH			OH	OH			38.3± 3.9
123 (Peltatoside)	OGlc$^{6\text{-}1}$Ara	OH		OH			OH	OH			14.0± 4.7
124 (Rutin)	OGlc$^{6\text{-}1}$Rha	OH		OH			OH	OH			23.2± 2.9
125 (Robinetin)	OH			OH			OH	OH	OH		87.5± 0.5
126 (Myricetin)	OH	OH		OH			OH	OH	OH		82.5± 6.3
127 (Myricitrin)	ORha	OH		OH			OH	OH	OH		62.6± 9.5
128	OAc	OAc		OAc			OAc	OAc	OAc		9.4± 2.4
129 (Quercetagetin)	OH	OH	OH	OH			OH	OH			98.4± 0.2
130 (Gossypetin)	OH	OH		OH	OH		OH	OH			85.1± 2.2
131 (Gossypin)	OH	OH		OH	OGlc		OH	OH			37.6± 3.9
132 (Trifolin)	OGal	OH		OH				OH			23.4± 1.7
133	OGlc$^{3\text{-}1}$Rha	OH		OH				OH			10.3±11.6
134 (Panasenoside)	OGal$^{2\text{-}1}$Glc	OH		OH				OH			4.3± 1.8
135 (Rhamnocitrin)	OH	OH		OMe				OMe			66.6± 4.5
136 (Robinin)	OGal$^{6\text{-}1}$Rha	OH		ORha				OH			2.0± 7.9
137 (Hyperin)	OGal	OH		OH			OH	OH			23.3± 0.8
138	OMe	OH		OMe			OMe	OMe			9.2± 2.6
139 (Datiscetin)	OH	OH		OH		OH					28.0± 4.4
140 (Morin)	OH	OH		OH		OH		OH			40.5± 4.6
141	OH	OH		OH		OH				OH	21.3± 1.0
142 (Anhydroicaritin)	OH	OH		OH	Pe			OMe			33.1± 4.4
143 (Nor-icariin)	ORha	OH		OGlc	Pe			OH			1.2± 3.6
144 (Icariin)	ORha	OH		OGlc	Pe			OMe			60.2± 2.9

Compound (Trivial name)	Substituents of flavanone [structure (II)] 2	3	5	6	7	8	2′	3′	4′	5′	6′	% Inhibition Mean±S.E.M.
145 (Liquiritin)					OGlc				OH			13.1± 4.7
146 (Eriodyctiol)			OH		OH			OH	OH			2.8± 9.2
147 (Isosakuranetin)			OH		OH				OMe			2.3± 5.5
148 (Alpinetin)			OMe		OH							−5.4± 2.3
149 (Dihydrobaicalein)			OH	OH	OH							51.5± 1.9
150 (Dihydrooroxylin A)			OH	OMe	OH							3.8± 1.3
151			OMe	OMe	OMe							−3.5± 2.4
152	H,OEt	H, I	OMe	OMe	OMe							2.1± 2.1
153			OH	OH	OGA-ME				OH			9.6± 2.7
154			OMe	OMe	OMe		OMe	OMe	OMe	OMe		3.1± 1.2
155			OH		OH	OMe			OH			3.6± 2.0
156			OH		OH	OMe	OH					4.6± 2.3
157			OH		OMe	OMe	OH				OMe	9.8± 2.6
158			OH		OMe	OMe	OGA				OMe	11.4± 1.1
159			OH		OMe	OMe	OGA-ME				OMe	5.5± 2.6
160 (Astilbin)	H, ORha		OH		OH			OH	OH			3.6± 1.2

Compound (Trivial name)	Substituents of chalcone [structure (III)] 2	3	4	5	2′	3′	4′	6′	% Inhibition Mean±S.E.M.
161 (Isoliquiritigenin)			OH				OH	OH	34.4± 3.9
162 (Phloretin)			OH		OH		OH	OH	3.0± 3.5
163 (Phlorizin)			OH		OH		OH	OGlc	−2.6± 4.5
164 (Butein)		OH	OH		OH		OH		17.5± 1.2
165 (Homobutein)		OMe	OMe		OH		OH		−13.1± 0.8
166	OMe	OMe	OMe	OMe	OMe	OMe	OMe	OH	2.8± 0.9
167	OMe	OMe	OMe	OMe	OMe	OMe	OMe	OMe	6.7± 2.4

Compound (Trivial name)	Substituents of isoflavone [structure (IV)] 5	7	4′	% Inhibition Mean±S.E.M.
168 (Daidzin)		OGlc	OH	32.3±12.9
169 (Daidzein)		OH	OH	4.3± 7.2
170 (Biochanin-A)	OH	OH	OMe	17.4± 4.4
171 (Formononetin)		OH	OMe	15.7± 6.0

The assay was carried out as described under "Materials and Methods", by using $(rA)_n$-$p(dT)_{12-18}$ as the template primer. The concentration of the samples was 1.0 mM and the results are expressed as Mean±S.E.M. of 4 experiments.

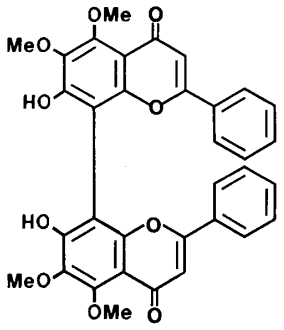
The abbreviations used are: GA, glucuronic acid; Pr, isopropyl; By, benzyl; Rha, rhamnose; Glc, glucose; Ac, acetyl; Me, methyl; ME, methyl ester; Gal, galactose; Ara, arabinose; Et, ethyl; Pe, isopentenyl; *, precipitation occurred in the reaction mixture.

(BSA), 12.5% glycerol, and 1 μl of Pol I in a final volume of 20 μl. Adriamycin (0.5 mM) was used as a positive control which gave inhibition of 80.4%.

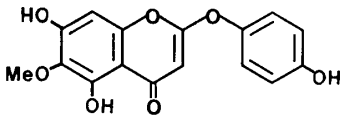
Results

The inhibitory effects of 190 flavonoids and 75 alkaloids on RT are shown in Tables I–III.

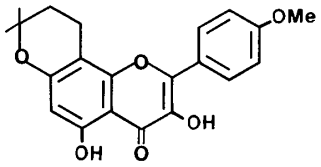
Two types of template-primers, $(rA)_n$-$p(dT)_{12-18}$ and $(rC)_n$-$p(dG)_{12-18}$, were used for the test of the inhibitory effects on the RT reaction. Only the results of poly (rA)·oligo (dT)-directed reactions are shown, because the other template-primer was not significantly affected by most of the samples: only gossypetin (**130**), quercetagetin (**129**), coralyne chloride (**203**) and 1-dodecylpyridinium chloride (**256**), inhibited the poly (rC)·oligo (dG)-directed reactions more than 90% at a concentration of 10^{-3} M.



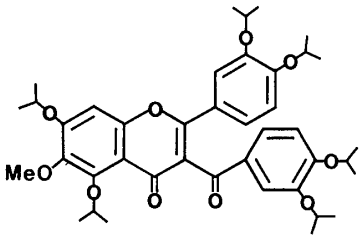
172



173 (Capillarisin)



174 (β-Anhydroicaritin)



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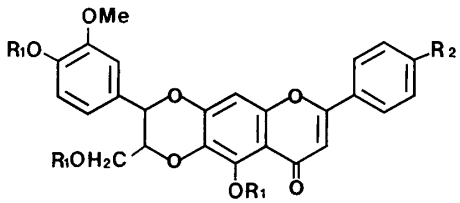
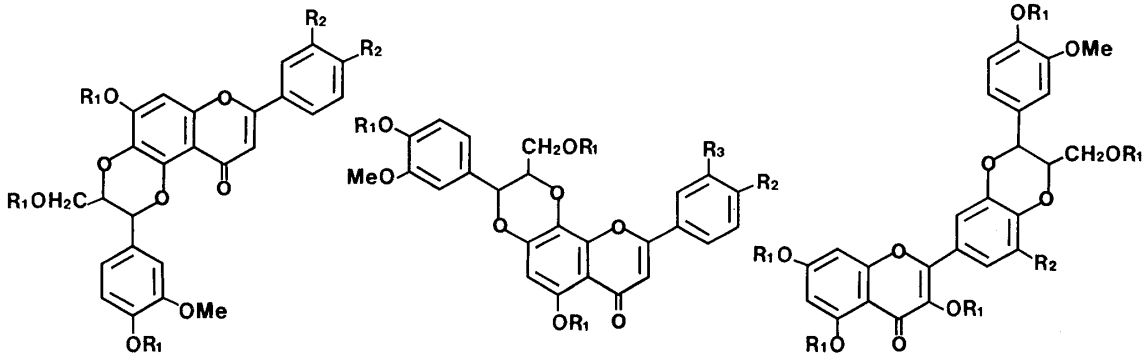


176: $R_1=R_2=H$

179: $R_1=Ac$; $R_2=OAc$



177: $R_1=R_2=H$

178: $R_1=Ac$; $R_2=H$

189: $R_1=H$; $R_2=OH$

190: $R_1=Ac$; $R_2=OAc$

180: $R_1=R_2=R_3=H$

181: $R_1=Ac$; $R_2=R_3=H$

182: $R_1=R_3=H$; $R_2=OH$

183: $R_1=Ac$; $R_2=OAc$; $R_3=H$

184: $R_1=H$; $R_2=R_3=OH$

185: $R_1=R_2=H$

186: $R_1=Ac$; $R_2=H$

187: $R_1=H$; $R_2=OH$

188: $R_1=Ac$; $R_2=OAc$

Chart 2. Structures of Flavonoids and Related Compounds Listed in TABLE II

1. Effect of flavonoids

Of the flavonoids tested, baicalein-7-*O*-glucoside (**18**), baicalein (**19**), **21**, baicalin methyl ester (**23**), scutellarein (**33**), scutellarin (**34**), cirsimarin (**38**), 6-hydroxyluteolin (**50**), pedalitin (**52**), 6-hydroxy-kaempferol (**109**), **111**, robinetin (**125**), myricetin (**126**), quercetagetin (**129**), gossypetin (**130**), **176**, **185** and **187** showed a potent inhibition of RT at a concentration of 10^{-3} M. Compounds **25**, **28**, luteolin (**48**), **49**, **81**, **99**, kaempferol (**110**), kaempferide (**113**), ombuin (**118**), quercimeritrin (**119**), myricitrin (**127**), rhamnocitrin (**135**) and icariin (**144**), showed a moderate inhibition. Most of the active compounds were aglycones with substituents at the positions 5, 6 and 7 or at 3, 3′, 4′ and 5′. Flavanones, isoflavones or chalcones did not show any inhibitory activity. In some flavonoids, the standard assay

TABLE II. Inhibitory Effects of Other Flavonoids and Related Compounds on AMV Reverse Transcriptase

Compound (Trivial name)	% Inhibition Mean±S.E.M.
172	11.1±5.5
173 (Capillarisin)	21.5±6.5
174 (β-Anhydroicaritin)	4.3±1.4
175	−1.2±2.8
176	87.5±1.0
177	6.3±5.5
178	−6.9±1.6
179	−5.6±1.8
180	2.7±3.2
181	−4.6±2.7
182	33.5±0.7
183	−2.5±0.7
184	52.7±2.6
185	80.4±1.7
186	−6.4±3.9
187	96.0±1.6
188	−3.5±3.0
189	25.0±1.9
190	−3.5±3.5

was performed at various concentrations to show the potency of their inhibitory activity (Fig. 1), and to determine the concentration that inhibits 50% of the enzyme activity (IC_{50}) (TABLE IV). The IC_{50} values of 6-hydroxyluteolin (**50**), pedalitin (**52**), 6-hydroxykaempferol (**109**) and quercetagetin (**129**) were $\leqq$10 μM, those of baicalein (**19**), baicalin methyl ester (**23**), scutellarein (**33**), myricetin (**126**) and gossypetin (**130**) were from 35 to 200 μM and those of the others were higher than 200 μM.

The kinetic studies of the potent flavonoid inhibitors were carried out and the time course of the reaction with 6-hydroxykaempferol (**109**) is shown in Fig. 2. In this experiment, a standard assay was carried out by adding **109** at or 2.5 min after the start of the reaction. Figure 2 shows that no incorporation of the substrate took place when **109** was added at the start and that the polymerization stopped immediately when **109** was added 2.5 min after the start when the reaction was at the logarithmic phase. The results suggest that this flavonoid inhibitor interferes with both of the steps of initiation and elongation of the polymerization. Similar results were obtained with 6-hydroxyluteolin (**50**) and **187**. The inhibitory constants (K_i) of 6-hydroxyluteolin (**50**), myricetin (**126**) and gossypetin (**130**), calculated by the Lineweaver-Burk equation, are shown in TABLE V. 6-Hydroxyluteolin (**50**), myricetin (**126**) and gossypetin (**130**) presented the K_i values of 10, 94 and 90 μM, respectively, with respect to dTTP and 5, 200 and 100 μM, respectively, with respect to the template-primer. The inhibitory effect of these flavonoids was non-competitive with respect to dTTP or the template-primer, but that of gossypetin (**130**) was of a competitive type inhibition with respect to the template-primer.

2. Effect of alkaloids

Of the alkaloids tested, isoquinoline alkaloids and other alkaloids such as indol, steroidal, quinolizidine, pyrrolizidine, pyridine and imidazole alkaloids containing no quaternary nitrogen did not show any significant RT inhibitory activity. At a concentration of 1.0 mM, berberine chloride (**193**), coptisine chloride (**202**), coralyne chloride (**203**) and 3,6-diamino-1-methylacridine (**262**) showed more than 98 % of inhibition and berberrubine (**194**), dehydrocorydaline chloride (**205**), dihydroberberine (**206**), harmaline (**230**), 1-dodecylpyridinium chloride (**256**) and 13-methylberberine iodide (**210**) showed moderate to high effects. Some alkaloids which contain no quaternary nitrogen but are known to have an anti-leukemia or cytotoxic activity, such as camptothecin (**195**), cepharanthine (**196**), matrine (**215**), vin-

TABLE III. Effect of Alkaloids on AMV Reverse Transcriptase

Compound (Trivial name)	% Inhibition Mean±S.E.M.
ISOQUINOLINE ALKALOIDS	
191 (α-Allocryptopine)	7.8± 4.2
192 (Berbamine)	26.0± 3.1
193 (Berberine chloride)	99.7± 0.1
194 (Berberrubine)	51.9± 7.9
195 (Camptothecin)	16.9± 2.8
196 (Cepharanthine)	37.7±12.0
197 (Chelidonine)	18.6± 4.4
198 (Cinchonidine)	22.5± 5.9
199 (Cinchonidine hydrochloride)	34.6± 3.2
200 (Cinchonine)	16.1± 4.4
201 (Cinchonine hemisulfate)	22.6± 4.4
202 (Coptisine chloride)	98.3± 0.9
203 (Coralyne chloride)	100.0± 0.0[a)]
204 (Corydaline)	7.7± 5.0
205 (Dehydrocorydaline chloride)	76.3± 3.1
206 (Dihydroberberine)	64.3± 1.7
207 (Emetine dihydrochloride)	13.6± 2.3
208 (DL-Laudanosine)	15.8± 1.2
209 (DL-Laudanosoline)	27.1± 6.1
210 (13-Methylberberine iodide)	89.5± 0.8
211 (Noscapine chloride)	9.1± 1.2
212 (Papaverine)	4.8± 7.5
213 (Sinomenine)	10.4± 2.4
214 (Tetrahydroberberine)	24.0± 9.8
QUINOLIZIDINE ALKALOIDS	
215 (Matrine)	28.4± 9.7
216 (Oximatrine)	20.6± 5.1
IMIDAZOLE ALKALOIDS	
217 (Ergothioneine)	−0.2± 1.8
218 (Pilocarpine)	19.2±15.9
INDOL ALKALOIDS	
219 (Ajmalicine)	28.5± 2.0
220 (Ajmalicine chloride)	12.4± 1.8
221 (Ajmaline)	11.7± 3.1
222 (Ajmaline chloride)	−0.1± 0.8
223 (Brucine)	19.1± 5.3
224 (Brucine N-oxide)	4.1±15.0
225 (β-Colubrine)	22.1± 9.8
226 (Corynanthine)	52.4±10.1
227 ([−]-Eburnamonine)	−29.1± 2.3[a)]
228 ([−]-Ergotamine tartarate)	−4.0± 2.0
229 (Evodiamine)	32.2± 3.2
230 (Harmaline)	59.4± 5.9
231 (Harmine)	15.5± 7.6
232 (Ibogaine chloride)	9.4± 3.4
233 (Icajine)	−2.1±15.6

Compound (Trivial nema)	% Inhibition Mean±S.E.M.
234 (Isostrychnine)	33.1±10.4
235 (Pseudostrychnine)	29.3± 7.3
236 (Rescinamine)	37.1± 2.1
237 (Reserpine)	12.8± 6.7
238 (Sarpagine)	29.4± 6.6
239 (Strychnine)	26.2± 4.7
240 (Strychnine N-oxide)	3.9±15.4
241 (Vinblastine sulfate)	1.4± 2.9
242 (Vincamine)	10.0± 3.4
243 (Vincristine sulfate)	−15.6±11.6
244 (Vomicine)	32.2±16.4
245 (Yohimbine)	19.8± 9.7
STEROIDAL ALKALOIDS	
246 (α-Chaconine)	1.4± 4.0
247 (Solanidine)	18.8± 7.8
248 (α-Solanine)	16.2± 4.5
249 (Solasodine)	28.7± 3.2
250 (Tomatidine)	26.3± 6.2
251 (Tomatine)	39.8± 4.7
252 (Verticine N-oxide)	38.0± 3.2
253 (Verticinone N-oxide)	40.8± 2.7
PYRIDINE ALKALOIDS	
254 (Anabasine)	16.8± 8.3
255 (Arecoline chloride)	28.9± 9.1
256 (1-Dodecylpyridinium chloride)	79.5± 2.2
257 (L-Lobeline)	19.1± 6.5
258 (D-Pipecolic acid)	0.6± 0.9
PYRROLIZIDINE ALKALOIDS	
259 (Crotaline)	22.3± 4.8
260 (Retrorsine)	5.5± 5.5
261 (Retrorsine N-oxide)	25.5± 7.3
OTHER ALKALOIDS	
262 (3,6-Diamino-1-methylacridine)	100.0± 0.0[b]
263 (Betaine hydrochloride)	3.9± 1.9
264 (Demecolcine)	24.4± 2.1
265 ([−]-Norpseudophedrine)	1.8± 2.5

[a] The concentration of the sample was 0.5 mM; [b] the concentration of the sample was 0.1 mM.

The concentration of the other samples was 1.0 mM and the results are expressed as Mean±S.E.M. of 4 experiments.

blastine (**241**) and vincristine (**243**) were also tested. However, of them, only cepharantine (**196**) and matrine (**215**) slightly reduced the incorporation of [^{3}H]-dTMP by RT, while others did not show any effect on it.

Dehydrocorydaline chloride (**205**) and 13-methylberberine iodide (**210**) had a reduced inhibitory activity when compared with berberine chloride (**193**), coptisine chloride (**202**) and coraline chloride (**203**), whose chemical structures are shown in Chart 3. For the purpose of studying the effects of methoxyl groups and unsaturated rings on the protoberberine type compounds, berberine was demethylated

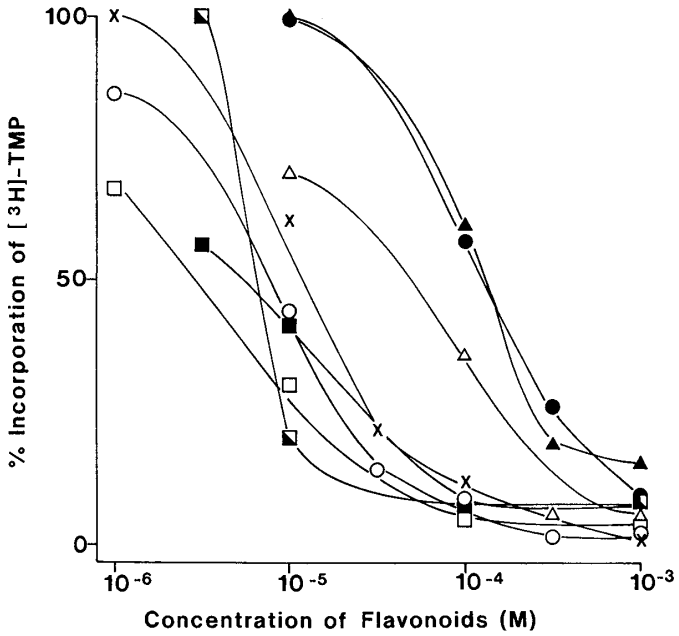


Fig. 1. Effects of Flavonoid Concentration on AMV Reverse Transcriptase

Standard assay was performed as described under "Materials and Methods," by using a reaction mixture containing $(rA)_n$-p$(dT)_{12-18}$ as a template-primer. ○: quercetagetin (**129**), □: 6-hydroxykaempferol (**109**), △: baicalein (**19**), ●: myricetin (**126**), ▲: gossypetin (**130**), ■: 6-hydroxyluteolin (**50**), ×: scutellarein (**33**), ◪: pedalitin (**52**).

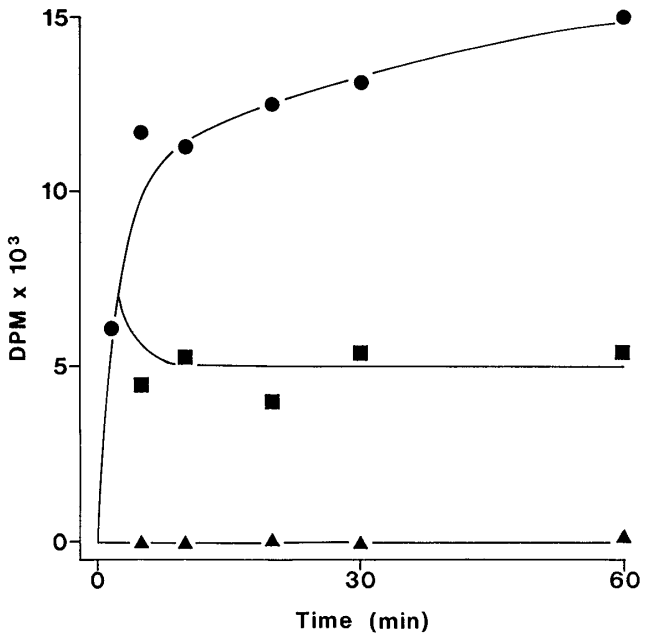


Fig. 2. Time Course of Polymerization by AMV Reverse Transcriptase when $(rA)_n$-p$(dT)_{12-18}$ was used as a template-primer. Flavonoid (**109**) was added to the standard reaction (●) at 0 min (▲) or at 2.5 min (■) as an inhibitor. Aliquotes were taken at indicated time to determine the incorporation of [^{3}H]-dTMP.

at position 9 to give berberrubin (**194**): berberrubin showed a weak inhibitory effect on the enzyme activity. Hydrogenated products of berberine, dihydroberberine (**206**) and tetrahydroberberine (**214**), also showed a reduced inhibitory effect.

To study the effect of quaternary nitrogens of alkaloids on the RT inhibition, a synthetic alkaloid bearing a quaternary nitrogen in its structure of three aromatic rings, 3,6-diamino-1-methylacridine

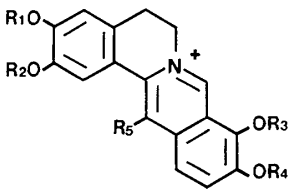
TABLE IV. IC_{50} of Flavonoids

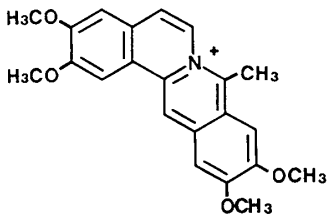
Compound (Trivial name)	IC_{50} (μM)
18 (Baicalein-7-*O*-glucoside)	250
19 (Baicalein)	50
20 (Baicalin)	250
23 (Baicalin methyl ester)	200
33 (Scutellarein)	35
34 (Scutellarin)	380
50 (6-Hydroxyluteolin)	7
52 (Pedalitin)	10
109 (6-Hydroxykaempferol)	8
125 (Robinetin)	360
126 (Myricetin)	150
129 (Quercetagetin)	8
130 (Gossypetin)	160
187	250

TABLE V. Inhibitory Constants (K_i) of Flavonoids in Regard to dTTP or $(rA)_n$-$p(dT)_{12-18}$

Compound (Trivial name)	K_i (μM)	
	dTTP	$(rA)_n$-$p(dT)_{12-18}$
50 (6-Hydroxyluteolin)	10 (non-competitive)	5 (non-competitive)
126 (Myricetin)	94 (non-competitive)	200 (non-competitive)
130 (Gossypetin)	90 (non-competitive)	100 (competitive)

The K_i values were calculated by Lineweaver-Burk plot and the mode of inhibition with respect to dTTP or $(rA)_n$-$p(dT)_{12-18}$ is indicated in parentheses. The experiment was carried out in the standard reaction mixture described under "Materials and Methods." The reaction time was five minutes. 6-Hydroxyluteolin (7.0 μM), myricetin (200 μM) or gossypetin (100 μM) was used with a variable concentration of dTTP (5–100 μM) or $(rA)_n$-$p(dT)_{12-18}$ (2–10 μg/ml).





193: R_1-R_2=CH_2; R_3=R_4=CH_3; R_5=H

194: R_1-R_2=CH_2; R_3=OH; R_4=CH_3; R_5=H

202: R_1-R_2=CH_2; R_3-R_4=CH_2; R_5=H

205: R_1=R_2=R_3=R_4=R_5=CH_3

210: R_1-R_2=CH_2; R_3= R_4=R_5=CH_3

203

Chart 3. Structures of Protoberberine Alkaloids

(262), was tested for its activity: it was a potent inhibitor with IC_{50} of 40 μM. The inhibitory activity of various alkaloids at different concentrations are shown in Fig. 3.

Both flavonoids and alkaloids shown to have RT inhibitory properties in this experiment were also

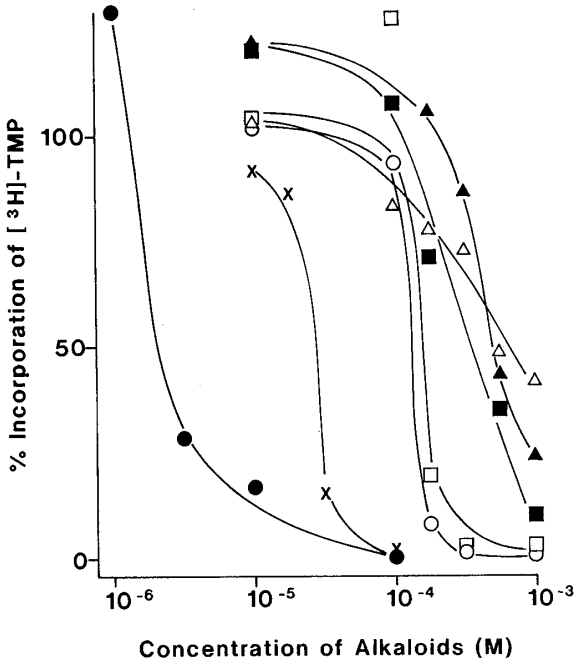


Fig. 3. Effects of Alkaloid Concentration on AMV Reverse Transcriptase
Standard assay was performed as described under "Materials and Methods" by using a reaction mixture containing $(rA)_n$-p$(dT)_{12-18}$ as a template-primer. ○: berberine chloride (**193**), △: berberrubine (**194**), □: coptisine chloride (**202**), ▲: dehydrocorydaline chloride (**205**), ■: 13-methylberberine iodide (**210**), ●: coralyne chloride (**203**), ×: 3,6-diamino-1-methylacridine (**262**).

tested for their effect on DNA polymerase I (TABLE VI). Only 3,6-diamino-1-methylacridine (**262**) affected this enzyme significantly at 1.0 mM: no other flavonoid and alkaloid inhibitors tested in this report affected the enzyme.

Discussion

Various plants containing flavonoids and/or alkaloids are widely used in traditional medicine, and *Evodia rutaecarpa* (JUSS.) BENTH.,[23a] *Corydalis bungeana* TURCZ.,[23b] *Polygonum cuspidatum* SIEB. et ZUCC.[23c] *Bupleurum chinense* DC., *B. scorzonerifolium* WILLD.,[23d] *Scutellaria baicalensis* GEORGI[23e] and *Coptis chinensis* FRANCH.[23f] are known to have an antiviral activity.

Previously we reported on the traditional crude drugs and their components such as tannins and alkaloids having AMV-RT inhibitory activities. In this paper, we describe our works on the naturallyoccurring compounds that might also act as AMV-RT inhibitors.

The results of the AMV-RT inhibition test using structurally different flavonoids, showed that free hydroxyl groups are important for the inhibitory effect. When the hydroxyls are substituted by sugars, methoxy, acetoxy, isopropoxy or benzyloxy groups, the activity was reduced and the reduction was proportional to the size of the substituents: when the substituents were one methoxy group or one sugar (commonly at position 7), the reduction in the inhibitory effect was not much, whereas when they were bulky substituents such as benzyloxy and isopropoxy groups or two or more sugars, the reduction was quite significant. It was also found that, not only the number of free hydroxyl groups present in the molecule but also their positions were also important for the potency of inhibition. Many flavonoids with five or six hydroxyls showed a strong inhibition, while **86** and **141** with five hydroxyls did not show any significant inhibition. Thus, the positions of the hydroxyl groups in the molecule may also be equally important for the activity. By comparing baicalein-6-*O*-glucoside (**24**) and oroxylin A (**26**) with baicalein (**19**), for example, or hispidulin (**36**) and isovitexin (**41**) with scutellarein (**33**), we may conclude that the methoxy and glucosyl groups at position 6 reduce the inhibitory effect. The presence of hydroxyl groups in the galloyl type structure seems to give a potent inhibitory activity to flavones or flavonols, as exemplified by 6-hydroxyluteolin (**50**), 6-hydroxykaempferol (**109**), myricetin (**125**), robinetin (**125**) and quercetagetin (**129**).

TABLE VI. Effects of Flavonoids and Alkaloids on DNA-Polymerase I

Compound (Trivial name)	% Incorporation of [^{3}H]-dTMP Mean±S.E.M.
19 (Baicalein)	99.2±2.2
23 (Baicalin methyl ester)	107.7±1.4
33 (Scutellarein)	88.8±1.8
34 (Scutellarin)	94.7±1.3
50 (6-Hydroxyluteolin)	78.5±2.3
52 (Pedalitin)	106.6±3.2
109 (6-Hydroxykaempferol)	63.5±2.2
125 (Robinetin)	78.4±3.8
126 (Myricetin)	69.9±1.7
129 (Quercetagetin)	95.2±1.4
130 (Gossypetin)	85.6±1.7
187	89.9±2.0
191 (α-Allocryptopine)	90.7±4.1
193 (Berberine chloride)	102.0±4.4
194 (Berberrubine)	96.5±5.5
196 (Cepharanthine)	104.3±2.6
202 (Coptisine chloride)	106.5±4.9
203 (Coralyne chloride)[a)]	96.5±0.9
205 (Dehydrocorydaline chloride)	113.2±0.7
206 (Dihydroberberine)	104.8±3.7
210 (13-Methylberberine iodide)	114.1±0.7
214 (Tetrahydroberberine)	89.9±4.2
230 (Harmaline)	109.2±2.0
241 (Vinblastine sulfate)	99.1±1.9
256 (1-Dodecylpyridinium chloride)	102.7±1.9
262 (3,6-Diamino-1-methylacridine)	24.9±1.1

The concentration of the samples was 1.0 mM except for coralyne chloride[a)] which was carried out at a concentration of 0.5 mM. Results are the Mean± S.E.M. of 4 experiments.

Representative alkaloids of different structures were also submitted to the RT assay. Primarily chosen for the screening were those compounds known to interact with nucleic acids or interfere with the cell division, such as berberine (**193**) which binds to nucleic acids and has antimicrobial and other pharmacological activities, vinblastine (**241**) and vincristine (**243**), having antimitotic properties. Among the compounds shown to be inhibitors in this trial were protoberberine type and other type alkaloids with a quaternary nitrogen in the flattened aromatic ring system. In fact, all the compounds containing a quarternary nitrogen, tested in the present experiment, inhibited the enzyme. However, the activity was not always strong: at concentrations below 10^{-4} M only coralyne chloride (**203**), a protoberberine type compound having aromatic B ring, and 3,6-diamino-1-methylacridine (**262**) showed a strong RT inhibitory effect.

Sethi reported that the methylenedioxy group in the protoberberine type alkaloids reduced the inhibitory activity[24)] but our result did not agree with theirs, because coptisine chloride (**202**) with methylenedioxy groups at positions 2–3 and 9–10 was shown to be a strong inhibitor in the present work. In protoberberine type alkaloids, methyl groups seem to affect the activity: the inhibitory activity of dehydrocorydaline chloride (**205**) and 13-methylberberine (**210**), was lower than that of berberine (**193**). However, apparently, methyl group gives less influence when it is at position 8 as in coralyne chloride (**203**).

Since berberine and some flavonoids did not reduce the activity of DNA polymerase I, we may conclude that they inhibit RNA-dependent DNA polymerase (RT) selectively. This paper shows also that

the inhibitory activity of RT was found in both flavonoids and alkaloids containing aromattc-ring systems which confer them a flattened structure.

Protoberberine alkaloids are very common compounds occurring in Berberidaceae, Menispermaceae and Papaveraceae plants. Protoberberine alkaloids and flavonoids are also present in many plants which are used frequently in the phytotherapy. Various pharmacological activities of these compounds, such as tumor cytotoxicity and antimicrobial effects, have been published.[25,26]

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