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Antiviral Activity of Natural Occurring Flavonoids *in Vitro*

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The antiviral activity of a wide range of naturally occurring flavonoids was investigated *in vitro*. Chrysosplenol B and chrysosplenol C, which are contained specifically in *Chrysosplenium* plants, and axillarin showed potent antiviral activity, especially against rhinovirus. A comparison of the activities of the compounds tested indicated that 3-methoxyl and 5-hydroxyl groups in the flavone skeleton were both necessary for antiviral activity against rhinovirus, and the activity may also be affected by various groups at other positions. The other flavonoids tested had little or no antiviral activity against herpes simplex virus, influenza virus and rhinovirus.

These results suggest that *Chrysosplenium* plants, which contain large amounts of chrysosplenol B and chrysosplenol C, may be useful as medicinal herbs against the common cold caused by rhinovirus infection. These plants have not so far been used as a medicinal herb or as a folk medicine, as far as is known.

Keywords——antiviral activity; rhinovirus; flavonoids; chrysosplenol B; chrysosplenol C; axillarin

In recent years, it has been reported that some flavonoids are effective against picornavirus infections *in vivo* or *in vitro*.[1] On the other hand, many flavonoid compounds have been isolated from various kinds of plants in our laboratories, and therefore we examined the antiviral activity of these flavonoids against a picornavirus (human rhinovirus) and other deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses (herpes simplex virus and influenza virus, respectively) *in vitro*.

It was found that 3-methoxylated flavones, chrysosplenol B and chrysosplenol C, isolated from *Chrysosplenium* plants[2] show potent specific antiviral activity against rhinovirus. The structure–activity relationship was also investigated, and the possible utility of *Chrysosplenium* as a medicinal herb for the treatment of the common cold, which is considered to be mainly due to rhinovirus infection, is discussed.

Materials and Methods

Compounds——Most of the flavonoids tested (shown in Table I) were separated and purified from various kinds of plants in our laboratories except for a few which were synthesized. 5-Iodo-2′-deoxyuridine (IDU) was purchased from Sigma Chemical Co., amantadine was purchased from Tokyo Kasei Kogyo Co., Ltd., and virazol was purchased from ICN Nutritional Biochemicals. All flavonoid compounds were dissolved in dimethylsulfoxide (DMSO) solution and diluted with DMSO as necessary before use.

Cells——Vero and HeLa (Ohio strain) cells were cultured at 37 °C in Eagle's minimum essential medium (E'MEM) containing 10% calf serum, 100 μg of streptomycin sulfate per ml and 50U of penicilin G per ml. Chick embryonic fibroblast cells (CEF cells) were obtained from secondary cultures of trypsinized chicken embryos by a

TABLE I. (1) Tested Flavonoids and Their Structures

Compound	Substituents					
	5	6	7	8	3′	4′
Flavone type						
Flavone	—	—	—	—	—	—
Acacetin	OH	—	OH	—	—	OMe
Apigenin	OH	—	OH	—	—	OH
Apiin	OH	—	OR_4	—	—	OH
Baicalein	OH	OH	OH	—	—	—
Baicalin	OH	OH	OR_1	—	—	—
5,6,7-Trimethoxyflavone[a)]	OMe	OMe	OMe	—	—	—
Cirsimaritin	OH	OMe	OMe	—	—	OH
Cirsimarin	OH	OMe	OMe	—	—	OR_1
Chrysoeriol	OH	—	OH	—	OMe	OH
Cosmosiin	OH	—	OR_1	—	—	OH
Diosmetin	OH	—	OH	—	OH	OMe
Embinin	OH	CR_2	OMe	—	—	OMe
Linariin	OH	OMe	OR_2'	—	—	OMe
Luteolin	OH	—	OH	—	OH	OH
Luteolin-7-R_1	OH	—	OR_1	—	OH	OH
5,6,7,3′,4′-Pentahydroxyflavone	OH	OH	OH	—	OH	OH
Orientin	OH	—	OH	CR_1	OH	OH
Pectolinarin	OH	OMe	OR_2	—	—	OMe
Pectolinarigenin	OH	OMe	OH	—	—	OMe
Rhoifolin	OH	—	OR_2	—	—	OH
Scutellarein	OH	OH	OH	—	—	OH
Sorbarin	OH	OH	OR_3	—	—	OH
Swertisin	OH	CR_1	OMe	—	—	OH
Tectochrysin	OH	—	OMe	—	—	—
Vitexin	OH	—	OH	CR_1	—	OH
Wogonin	OH	—	OH	OMe	—	—

a) Synthesized. R_1 = glucose. R_2 = rhamnose-glucose. $R_2' = R_2$–4‴-OAc. R_3 = rhamnose. R_4 = apiose-glucose.

usual method. The culture medium was the same as above.

Viruses——Herpes simplex virus type 1 (F strain) was propagated in Vero cells at 37 °C. Influenza virus type A (PR/8 strain) was propagated in allantoic fluid of embryonated eggs at 37 °C. Rhinovirus type 2 was kindly supplied by Dr. R. Kawana (Iwate Medical University, Morioka, Japan) and propagated in HeLa cells at 33 °C. All viruses were stored in a freezer at −80 °C until use.

Test of *in Vitro* Antiviral Activity in Culture——Confluent monolayers of Vero, CEF and HeLa cells in 96-well microculture plates (no. 3040; Falcon Plastic) were infected with 100 $TCID_{50}$ per well of herpes simplex virus (HSV), influenza virus and rhinovirus, respectively. The total volume was adjusted to 0.25 ml with E'MEM containing 2% calf serum and antibiotics. Immediately after infection, 0.001 ml aliquots of two-fold serial dilutions of test materials were added.

In the cases of HSV and rhinovirus, viral cytopathic effect (CPE) was observed microscopically at 3 d after infection. The concentration at which viral CPE was inhibited by 50% as compared with the control was taken as the minimum effective dose of compounds against HSV and rhinovirus.

In the case of influenza virus, hemagglutinin (HA) titer in the supernatants of influenza virus-infected cell cultures was measured by the method of Rabinowitz *et al.*[4)] at 3 d after infection. The concentration at which the HA titer was reduced to 1/4 as compared with the control was taken as the minimum effective dose of compounds against influenza virus.

HSV and influenza virus infections were carried out in a CO_2 incubator at 37 °C under 5% CO_2 and 95% air, but rhinovirus infection was done at 33 °C.

Cytotoxic Activity against Cells in Culture——Confluent monolayers of Vero and CEF cells were cultured with test compounds for 3 d at 37 °C under 5% CO_2 and 95% air in 0.25 ml of E'MEM (2% calf serum). HeLa cells were cultured in the same way, but at a lower temperature of 33 °C. At the termination of culture, cell numbers were

TABLE I. (2) Tested Flavonoids and Their Structures

Compound	Substituents							
	3	5	6	7	2′	3′	4′	5′
Flavonol type								
Axillarin[a),3)]	OMe	OH	OMe	OH	—	OH	OH	—
Oxyayanin A	OMe	OH	—	OMe	OH	—	OMe	OH
Chrysosplenoside A	OMe	OH	—	OMe	OR_1	—	OMe	OH
Chrysosplenol B	OMe	OH	OMe	OMe	—	OMe	OH	—
Chrysosplenoside B	OMe	OH	OMe	OMe	—	OMe	OR_1	—
Chrysosplenol C	OMe	OH	OH	OMe	—	OMe	OH	—
Chrysosplenol D	OMe	OH	OMe	OMe	—	OH	OH	—
Chrysosplenoside D	OMe	OH	OMe	OMe	—	OH	OR_1	—
Chrysosplenol E	OMe	OH	—	OMe	OH	—	OMe	OMe
Hyperin	OR_7	OH	—	OH	—	OH	OH	—
Isorhamnetin	OH	OH	—	OH	—	OMe	OH	—
Kaempferol	OH	OH	—	OH	—	—	OH	—
Kaempferol-3-R_3	OR_3	OH	—	OH	—	—	OH	—
Kaempferol-3-R_5	OR_5	OH	—	OH	—	—	OH	—
Kaempferol-3-R_6	OR_6	OH	—	OH	—	—	OH	—
Morin	OH	OH	—	OH	OH	—	OH	—
Myricetin	OH	OH	—	OH	—	OH	OH	OH
Myricitrin	OR_3	OH	—	OH	—	OH	OH	OH
Quercetin	OH	OH	—	OH	—	OH	OH	—
Quercitrin	OR_3	OH	—	OH	—	OH	OH	—
Quercetagetin	OH	OH	OH	OH	—	OH	OH	—
Rutin	OR_2	OH	—	OH	—	OH	OH	—
4′-Hydroxy-3,3′,5,6,7-pentamethoxyflavone[a)]	OMe	OMe	OMe	OMe	—	OMe	OH	—

a) Synthesized. R_1—R_4 are the same as in Table I. (1) R_5 = neohesperidose. R_6 = arabinose. R_7 = galactose.

TABLE I. (3) Tested Flavonoids and Their Structures

Compound	Substituents							
	5	6	7	8	2′	3′	4′	5′
Isoflavones								
Genistein	OH	—	OH	—	—	—	OH	—
Iridin	OH	OMe	OR_1	—	—	OH	OMe	OMe
Sophoricoside	OH	—	OH	—	—	—	OR_1	—
Flavanones								
Hesperidin	OH	—	OR_2	—	—	OH	OMe	—
Naringin	OH	—	OR_2	—	—	—	OH	—
Naringenin	OH	—	OH	—	—	—	OH	—
Flavanonol								
Rovinin	—	—	OR_1	—	—	OH	OH	OH
Biflavone								
Amentoflavone	—	—	—	apigenin$^{5'-8''}$apigenin				

R_1 and R_2 are the same as in Table I. (1)

counted microscopically by using a nucleus staining method. The concentration at which the cell numbers were reduced to 50% as compared with the control was taken as the 50% cytotoxic dose.

Determination of Therapeutic Ratio——Therapeutic ratios (TR) were determined as TR = 50% cytotoxic dose/minimum effective dose.

Results and Discussion

Antiviral Activity against HSV

Generally, viruses can be classified into two groups, DNA viruses and RNA viruses. To examine the antiviral activity of flavonoids, we chose HSV (type 1, F strain) as a model of DNA virus. Most known antiherpetic agents are within the category of nucleic acid analogues, *i.e.*, acycloguanosine (acyclovir), IDU, adenine arabinoside (ara-A) and so on. The tested flavonoids, which are not nucleic acids analogues, showed no significant antiviral activity against HSV.

A compound can be judged to have antiviral activity if its therapeutic ratio is higher than 1. This was observed only in the case of pectolinarigenin (50% cytotoxic dose = 5 μg/ml, minimum effective dose = 2.5 μg/ml), although this therapeutic ratio (=2) was much lower than that of the positive control of IDU (50% cytotoxic dose = 25 μg/ml, minimum effective dose = 0.5 μg/ml). Since we chose a therapeutic ratio of 4 or more as a criterion for an antiviral agent, pectolinarigenin was judged not to have significant anti HSV activity.

Antiviral Activity against Influenza Virus

We chose influenza virus (PR/8 strain) as a model of enveloped RNA viruses, because infection with it is common all over the world and no highly effective drug against it has yet been found. Amantadine (1-adamantanamine hydrochloride) is used in the U.S.A., but its use has not been approved in Japan because of adverse effects[5] (TR = 8 in our experiment).

Apigenin and oxyayanin A showed slight anti influenza virus activity (50% cytotoxic dose = 40 and 20 μg/ml, respectively, and minimum effective dose = 20 and 10 μg/ml, respectively), but we did not regard these compounds as true antiviral agents against influenza virus because of the low therapeutic ratios (=2).

Antiviral Activity against Rhinovirus

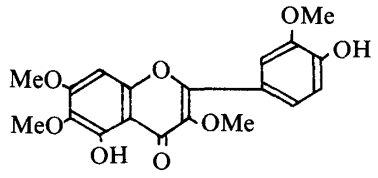
As shown in Table II, some 3-methoxylated flavones, chrysosplenol B, chrysosplenol C and axillarin, were found to have anti rhinovirus activity (the therapeutic ratios were approximately 16), whereas flavonoids without a methoxyl function at the 3-position had no antiviral activity (the test compounds not included in Table II all showed no significant activity). It appears that a methoxyl group at the 3-position in the flavone skeleton is essential

Table II. Anti Rhinovirus and Cytotoxic Activities of Flavonoids *in Vitro*[a]

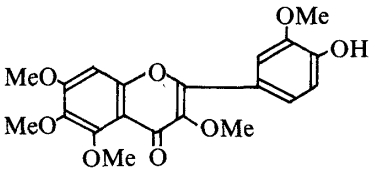
Compound	50% cytotoxic dose (μg/ml)[b]	Minimum effective dose (μg/ml)[c]
Axillarin	10	0.63
Chrysoeriol	10	10
Diosmetin	10	10
Isorhamnetin	2.5	2.5
Kaempferol	2.5	2.5
Oxyayanin A	2.5	10
Chrysosplenoside A	>40	>40
Chrysosplenol B	1.25	0.08
Chrysosplenoside B	>40	>40
Chrysosplenol C	20	1.25
Chrysosplenol D	0.16	0.08
Chrysosplenoside D	>40	>40

a) Assays were carried out as described in Materials and Methods. All the other flavonoids tested (see Table I) had no clear antiviral activity. *b*) Against HeLa cells. *c*) Against rhinovirus.

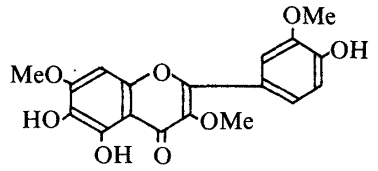
TABLE III. Correlation between Anti Rhinovirus Activity and Structure[a)]



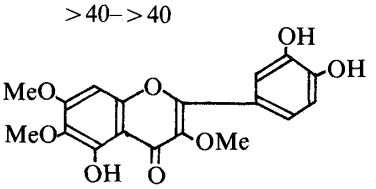
chrysosplenol B
1.25–0.08



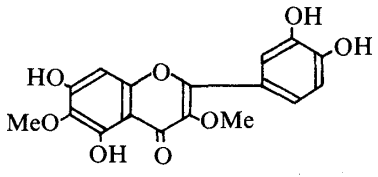
4′-hydroxy-3,3′,5,6,7-pentamethoxyflavone
>40–>40



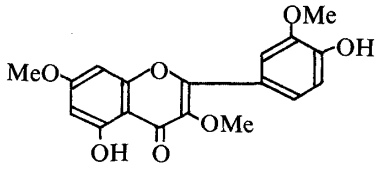
chrysosplenol C
20–1.25



chrysosplenol D
0.16–0.08



axillarin
10–0.63



Ro-09-0179[a,b)]

a) The left value and right value under the name of each compound are the 50% cytotoxic dose (μg/ml) against HeLa cells and the minimum effective dose (μg/ml) against rhinovirus, respectively.

for anti rhinovirus activity.

It is interesting that chrysosplenol B lost its antiviral and cytotoxic activities when it was glycosylated at its 4′-hydroxyl group, namely converted to chrysosplenoside B. Reduced cytotoxicity was also found in cases of Vero and CEF cells.

Collelation between Anti Rhinovirus Activity and Structure

Among all the flavonoids tested, only the 3-methoxylated flavones expressed anti rhinovirus activity. Therefore, we studied the structure–activity relation in 3-methoxylated flavones (Table III).

A comparison of chrysosplenol B with 4′-hydroxy-3,3′,5,6,7-pentamethoxyflavone showed that the antiviral and cytotoxic activities were greatly reduced when the substituent at the 5-position was converted to a methoxyl group from a hydroxyl group. This indicates that the hydroxyl group at the 5-position is also important for antiviral activity, as well as the methoxyl group at the 3-position. Chrysosplenol B, chrysosplenol C, axillarin and Ro-09-0179, which is known to have anti rhinovirus activity,[1a,b)] fulfil these conditions.

It is not clear what other groups are important, but the antiviral and cytotoxic activities may be differently affected by structural changes. For example, chrysosplenol D, in which only the substituent on the 3′-position (–OH) is different from that of chrysosplenol B (–OMe), has stronger cytotoxicity and its therapeutic ratio is reduced. On the other hand, in spite of having the same substituents as chrysosplenol D on the 3′,4′-positions (–OH on both), axillarin has potent anti rhinovirus activity and its cytotoxicity is not strong. In addition, the structures of chrysosplenol B, chrysosplenol C and Ro-09-0179 differ only at the 6-position (–OMe, –OH and free, respectively), and all of them have antiviral activity. Thus, the substituent on the 6-position does not appear to be related to the antiviral activity directly, although it affects the cytotoxicity.

In summary, it is suggested that both 3-methoxyl and 5-hydroxyl groups of the flavone skeleton are necessary for specific antiviral activity against rhinovirus, but the effects of functional groups at other positions on the antiviral and cytotoxic activities remain to be fully elucidated.

It is estimated that approximately half of all cases of common cold are due to rhinovirus infection.[6] Among the flavonoids tested, chrysosplenol B, chrysosplenol C and axillarin were found to have anti rhinovirus activity. These are all 3-methoxylated flavones, and such flavonoids are very rare in nature. However, chrysosplenol B and chrysosplenol C are contained in *Chrysosplenium* in large amounts.[2] This plant has not previously been reported as a medicinal herb. Many problems remain, however, before *Chrysosplenium* can be established as clinically useful; for example, no assay system in experimental animals has yet been established for rhinovirus infection, it is not known how other components in this plant affect the activity, and so on. Further studies are required.

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