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SYNERGISTIC EFFECT OF FLAVONES AND FLAVONOLS AGAINST HERPES SIMPLEX VIRUS TYPE 1 IN CELL CULTURE. COMPARISON WITH THE ANTIVIRAL ACTIVITY OF PROPOLIS

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ABSTRACT.—The in vitro activity against herpes simplex virus type 1 of the major flavonoids identified in propolis was investigated. Flavonols were found to be more active than flavones, the order of importance being galangin, kaempferol, and quercetin. The efficacy against HSV-1 of binary flavone-flavonol combinations has been also investigated. The synergy demonstrated by all combinations could explain why propolis is more active than its individual compounds.

Propolis (bee-glue) is a natural resinous substance gathered by honey bees, on buds of various trees: poplar (*Populus* spp.), birch (*Betula alba*), beech (*Fagus sylvatica*), horse-chestnut (*Aesculus hippocastanum*), alder (*Alnus glutinosa*), and various conifers. It has been reported to have an inhibitory effect on several viruses including influenza (1,2), vaccinia (2), Newcastle disease (2), and avian herpes (3,4). Its chemical composition was found to be a very complex mixture of compounds including benzoic acid and esters, substituted phenolic acid and esters, terpenoids, and flavonoid aglycones (5–9).

Many in vitro and in vivo studies have been published on the antiviral activity of flavonoids. Recent reviews (10–12) have emphasized the great variety of viruses tested and also the diversity of methods used which demonstrated different effects: direct inactivation or anti-replicative effects. As regards the action of flavonoids against herpes simplex virus (HSV), quercetin, procyanidin, and pelargonidin were found to be virucidal (13) whereas luteolin was inactive. The direct inactivation of HSV by quercetin, catechin, and hesperitin has been verified (14). Although the inhibition of HSV-1 multiplication in cell culture has been reported by some researchers [quercetin, dihydroquercetin, and procyanidin (15), quercetin (16), quercetin and hesperitin (14), quercetin, luteolin, and naringin (15) were inhibitors, while rutin (15), hesperidin and rutin (16), and catechin and naringin (14) were inactive], a study of 58 flavonoids, including apigenin, luteolin, tectochrysin, kaempferol, and quercetin, failed to demonstrate they had any activity aginst HSV-1 (18). Apigenin, hesperidin, kaempferol, quercetin, and rutin were also judged inactive (19).

We now report a study, using a multistep virus replication assay, of the anti-HSV-1 activity of the main flavonoids identified in a batch of propolis gathered near Rennes, France, and a comparison of their effects with those induced by the crude material. Moreover, the antiviral activity of binary flavone-flavonol combinations was investigated in order to detect a possible synergy.

RESULTS AND DISCUSSION

ANTIVIRAL ACTIVITY OF SINGLE FLAVONOIDS.—By comparison with commercial markers, tlc revealed in propolis balsam phenolic acids (caffeic and ferulic),

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flavonols (galangin, kaempferol, and quercetin), flavones (chrysin, apigenin, luteolin, and tectochrysin), and flavanones (pinocembrin and isosakuranetin). Hplc indicated galangin and chrysin were the major flavonoids, which was in agreement with Greenaway *et al.* (8), whereas pinocembrin and galangin were found to be the major flavonoids in a Bulgarian propolis sample (6). This difference might be related to the bud exudate composition of the trees from which balsam is gathered. For instance, a close similarity between a propolis balsam batch from England and the exudate of *Populus euramericana* has been reported (8). Our propolis sample was probably collected by bees on buds of *Populus nigra*, the trees which surround the hives.

The anti-HSV-1 effect of each compound identified in propolis balsam was determined as described in the Experimental.

The caffeic and ferulic acids, the flavanones pinocembrin and isosakuranetin, and the C-7 methoxyflavone tectochrysin showed little or no activity.

The antiviral activities of the other flavonoids, with the hydroxyl substituents characteristic of each of them, the solutions used for the assay, and the 50% cytotoxic concentrations, are indicated in Table 1. To be considered as active, a compound should induce at least a 2 $\log_{10}$ decrease in the virus titer in comparison with untreated virus controls (19).

Flavonols appeared to be more active than flavones. All three flavonol compounds could reduce the viral titer by 2 $\log_{10}$ or more whereas among the flavones, only luteolin showed this capability. Besides, the activity of flavonols was found to decrease in the reverse order of the number of their hydroxyl substituents, i.e., galangin>kaempferol> quercetin. For kaempferol, the anti-HSV-1 activity was in agreement with Debiaggi *et al.* (20), but discrepancies were noted for chrysin and galangin. This might be explained by differences in the methodologies used, especially a different HSV-1 strain, a lower time of incubation (24 h instead of 72 h), and a different evaluation method of activity (plaque reduction test instead of yield reduction). It has been previously shown that the plaque reduction test and the virus yield reduction might reveal contradicting results because the two tests detect different aspects of viral infection in cultured cells (21). In the same way, the anti-HSV-1 activity of quercetin is controversial since it was found to have an inhibitory effect (14–17) or to be without any significant activity (18,19). These discrepancies are also probably due to obvious differences between the methodologies used. Quercetin was found to be effective when the time of incubation was about 24 h, i.e., when the assays were performed after one multiplication cycle. It was considered ineffective when the incubation time was over 3 days. An oxidative degradation of quercetin in aqueous solution might explain the variations of the activity with the length of the experiment (22,23).

To evaluate the antiviral activity of a compound, another important criterion is the selectivity index, i.e., the ratio between the 50% cytotoxic and the active concentrations. A selectivity index of 4 or more should be appropriate (18,19). Thus, the activity of a compound was considered significant if this compound gave a 2 $\log_{10}$ decrease in virus yield at a concentration four times lower than its 50% cytotoxic concentration.

According to these criteria, only galangin, kaempferol, and propolis appeared to be really active against HSV-1. To explain the good activity of propolis balsam where active and inactive compounds are mixed, several hypotheses could be put forward. For instance, the presence in propolis of small amounts of vitamins, in particular vitamin C (24), could enhance the effect of flavonols by preventing their oxidative degradation (23). Along with the major flavonoids, other components such as minor methoxyflavones or caffeoyl conjugates may contribute to the antiviral activity, since methoxyflavones, for instance, were found to be very active against another class of viruses, the Picornavirideae.

TABLE 1. Effect of Single Flavonoids on HSV-1 Multiplication in Vero Cells.

Flavonoid	Substituent					Concentration		Inhibition
	3	5	7	3′	4′	mM[a]	μg/ml[b]	$\log_{10}$[c]
Galangin	OH	OH	OH	—	—	0.05	2.7	0.6
						0.10	5.4	0.6
						0.20	10.8[d]	2.3
						0.40	21.6	3.5
						0.80	43.2	CC_{50}[e]
Kaempferol	OH	OH	OH	—	OH	0.10	5.7	0.00
						0.20	11.4	0.70
						0.40	22.9[a]	1.50
						0.60	34.3	3.10
						0.80	45.7	3.50
						1.60	91.5	CC_{50}
Quercetin	OH	OH	OH	OH	OH	0.20	12.0	0.10
						0.40	24.1	0.75
						0.60	36.2[d]	1.25
						0.80	48.3	1.40
						1.00	60.4	2.00
						1.20	72.5	2.30
						2.50	151.0	CC_{50}
Chrysin	—	OH	OH	—	—	0.10	5.0	0.00
						0.20	10.1	0.30
						0.40	20.3[d]	0.50
						0.60	30.5	0.80
						0.80	40.6	1.10
						1.50	76.2	CC_{50}
Apigenin	—	OH	OH	—	OH	0.10	5.4	0.25
						0.20	10.8[d]	0.50
						0.40	21.6	1.00
						0.60	32.4	1.50
						1.00	54.0	CC_{50}
Luteolin	—	OH	OH	OH	OH	0.02	1.4	0.25
						0.05	2.8	0.40
						0.10	5.7[d]	0.90
						0.20	11.4	2.20
						0.40	22.9	CC_{50}
Propolis						—	10.0	1.00
						—	15.0	1.50
						—	20.0	2.00
						—	30.0	3.00
						—	72.0	CC_{50}

[a]Concentrations of the flavonoid solutions added to wells.

[b]Final concentrations in the culture medium. Vero cells were seeded in the microtiter plates with 100 μl of medium, and in each well, 50 μl of one drug, 50 μl of virus, and 50 μl of MEM (in prevision of the second drug in combination tests) were added. Therefore, each compound was diluted five times in the wells.

[c]The viral inhibition was expressed as the $\log_{10}$ reduction of the virus titer by comparison with controls.

[d]$CC_{50}/4$.

[e]CC_{50} = 50% cytotoxic concentration.

There is also the possibility of a synergism between two or more compounds which together could provide useful antiviral activity (12).

In order to verify this hypothesis, binary combinations of flavones and flavonols

TABLE 2. Yield Reduction Assay of HSV-1 with Combinations of One Flavone (Luteolin) and Flavonols.

Flavonol		Luteolin								
		0.05 mM[a] (0.4 $\log_{10}$)[b]			0.1 mM[a] (0.9 $\log_{10}$)[b]			0.2 mM[a] (2.2 $\log_{10}$)[b]		
		Yield		Interaction	Yield		Interaction	Yield		Interaction
		observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)
Galangin	0.1 mM[a] (0.6 $\log_{10}$)[b]	0.72	0.77	−0.05 AD[c]	0.80	0.87	−0.07 AD	0.00	0.28	−0.28 SY[d]
	0.2 mM[a] (2.3 $\log_{10}$)[b]	0.53	0.50	0.03 AD	0.25	0.58	−0.33 SY	0.00	0.16	−0.16 SY
Kaempferol	0.2 mM[a] (0.7 $\log_{10}$)[b]	0.80	0.90	−0.10 SY	0.58	0.73	−0.15 SY	0.14	0.71	−0.57 SY
	0.4 mM[a] (1.5 $\log_{10}$)[b]	0.54	0.65	−0.11 SY	0.43	0.61	−0.18 SY	0.00	0.50	−0.50 SY
Quercetin	0.4 mM[a] (0.7 $\log_{10}$)[b]	0.60	0.60	0.00 AD	0.62	0.56	0.06 AD	0.28	0.61	−0.33 SY
	0.8 mM[a] (1.4 $\log_{10}$)[b]	0.66	0.73	−0.07 AD	0.50	0.66	−0.16 SY	0.14	0.50	−0.36 SY
	1.0 mM[a] (2.0 $\log_{10}$)[b]	0.60	0.60	0.00 AD	0.46	0.56	−0.10 SY	0.00	0.50	−0.50 SY

[a]Flavonoid concentrations added to wells.

[b]Reduction factor of the viral titer by comparison with untreated controls.

[c]AD = additive interaction.

[d]SY = synergistic interaction.

TABLE 3. Yield Reduction Assay of HSV-1 with Combinations of One Flavone (Apigenin) and Flavonols.

Flavonol		Apigenin								
		0.2 mM[a] (0.5 $\log_{10}$)[b]			0.4 mM[a] (1.0 $\log_{10}$)[b]			0.6 mM[a] (1.5 $\log_{10}$)[b]		
		Yield		Interaction	Yield		Interaction	Yield		Interaction
		observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)
Galangin	0.1 mM[a] (0.6 $\log_{10}$)[b]	0.71	0.80	−0.09 AD[c]	0.42	0.65	−0.23 SY[d]	0.57	0.65	−0.08 AD
	0.2 mM[a] (2.3 $\log_{10}$)[b]	0.08	0.29	−0.21 SY	0.00	0.28	−0.28 SY	0.00	0.36	−0.36 SY
Kaempferol	0.2 mM[a] (0.7 $\log_{10}$)[b]	0.76	0.85	−0.09 AD	0.64	0.72	−0.08 AD	0.35	0.72	−0.37 SY
	0.4 mM[a] (1.5 $\log_{10}$)[b]	0.09	0.52	−0.41 SY	0.00	0.55	−0.55 SY	0.00	0.60	−0.60 SY
Quercetin	0.4 mM[a] (0.7 $\log_{10}$)[b]	0.85	0.85	0.00 AD	0.28	0.72	−0.44 SY	0.14	0.72	−0.58 SY
	0.8 mM[a] (1.4 $\log_{10}$)[b]	0.59	0.63	−0.04 AD	0.18	0.60	−0.42 SY	0.14	0.60	−0.46 SY
	1.0 mM[a] (2.0 $\log_{10}$)[b]	0.45	0.45	0.00 AD	0.14	0.41	−0.27 SY	0.00	0.41	−0.41 SY

[a]Flavonoid concentrations added to wells.

[b]Reduction factor of the viral titer by comparison with untreated controls.

[c]AD = additive interaction.

[d]SY = synergistic interaction.

TABLE 4. Yield Reduction Assay of HSV-1 with Combinations of One Flavone (Chrysin) and Flavonols.

Flavonol		Chrysin								
		0.4 mM[a] (0.5 $\log_{10}$)[b]			0.6 mM[a] (0.8 $\log_{10}$)[b]			1.0 mM[a] (1.1 $\log_{10}$)[b]		
		Yield		Interaction	Yield		Interaction	Yield		Interaction
		observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)	observed (Yab)	expected (Yc)	(Yab − Yc)
Galangin	0.1 mM[a] (0.6 $\log_{10}$)[b]	0.73	0.82	−0.09 AD[c]	0.69	0.79	−0.10 SY[d]	0.19	0.47	−0.28 SY
	0.2 mM[a] (2.3 $\log_{10}$)[b]	0.30	0.53	−0.23 SY	0.22	0.51	−0.29 SY	0.13	0.43	−0.30 SY
Kaempferol	0.2 mM[a] (0.7 $\log_{10}$)[b]	0.80	0.76	0.04 AD	0.89	0.89	0.00 AD	0.64	0.66	−0.02 AD
	0.4 mM[a] (1.5 $\log_{10}$)[b]	0.49	0.60	−0.18 SY	0.44	0.60	−0.16 SY	0.47	0.51	−0.04 AD
Quercetin	0.4 mM[a] (0.7 $\log_{10}$)[b]	0.54	0.72	−0.18 SY	0.44	0.59	−0.15 SY	0.33	0.59	−0.26 SY
	0.8 mM[a] (1.4 $\log_{10}$)[b]	0.39	0.55	−0.16 SY	0.22	0.42	−0.20 SY	0.11	0.42	−0.31 SY
	1.0 mM[a] (2.0 $\log_{10}$)[b]	0.27	0.45	−0.18 SY	0.11	0.25	−0.14 SY	0.00	0.27	−0.27 SY

[a]Flavonoid concentrations added to wells.

[b]Reduction factor of the viral titer by comparison with untreated controls.

[c]AD = additive interaction.

[d]SY = synergistic interaction.

were added to infected Vero cells. The virus yields were then compared to controls receiving single flavonoids.

ANTIVIRAL ACTIVITY OF FLAVONOID COMBINATIONS.—The combined effects of three flavonols, galangin, kaempferol, quercetin, and three flavones, chrysin, apigenin, luteolin, are summarized in Tables 2, 3, and 4 where the yields observed for the drug combinations (Yab) were compared to the yields expected (Yc). Flavonoids were combined at concentrations inducing reductions of virus titer ranging from 0.5 to 2 $\log_{10}$ when they were used alone. In order to obtain a minimum titer reduction of 1.5 $\log_{10}$ for flavones, it was necessary to get close to the 50% cytotoxic concentration. Therefore the cells were carefully examined prior to titrations to check that no apparent cytotoxicity had appeared; combinations were Yab = 0 corresponded to cell layers without any apparent cytotoxicity or viral destruction. In this way, the enhanced antiviral effect of the flavone-flavonol combinations could be safely attributed to a synergistic interaction rather than to an increased toxicity. The data in Tables 2, 3, and 4 represent the average of four similar assays, each titration being carried out in duplicate.

For instance, the viral titers ($\log_{10}$ $TCID_{50}$) determined in one experiment were: virus control 5.5; apigenin (0.4 mM) 4.5 Ya = 0.81; quercetin (0.4 mM) 4.8 Yb = 0.87, yield expected Yc = 0.70; apigenin (0.4 mM) + quercetin (0.4 mM) 1.5 Yab = 0.27.

Therefore, the combination of quercetin (0.4 mM) with apigenin (0.4 mM) was more effective than the individual compounds, and the interaction Yab − Yc = − 0.43 was the proof of a significant synergy.

All combinations of flavones and flavonols demonstrated synergy against HSV-1. This was particularly obvious at concentrations inducing at least a 1 $\log_{10}$ decrease in virus titer when the compounds were used alone. At lower concentrations the interaction was more often additive.

The most interesting combinations obtained were kaempferol + luteolin; quercetin + chrysin; galangin + apigenin; kaempferol + apigenin; and quercetin + apigenin.

The theory of the effect of drug combinations suggests that only drugs with different modes of action could exhibit synergism (25). This synergy may result from a sequential blockade, concurrent inhibition, or complementary inhibition (26). As the exact action mechanism against HSV-1 of the different classes of flavonoids is at present unknown, it is very difficult to suggest an explanation for the observed phenomenon. However, this experiment confirms the possibility of synergistic interactions between two or more compounds in natural complex products such as propolis.

EXPERIMENTAL

CELLS AND VIRUSES.—African green monkey kidney cells (Vero cell line no ATCC CCL81) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum, 160 U/ml of penicillin, and 80 μg/ml of gentamicin. Cells were routinely passaged every 3 days. A virus stock of Herpes Simplex Virus type 1, strain H 29 S was prepared as follows: a nearly confluent monolayer culture was infected at a low multiplicity, incubated for 2 days, then frozen and thawed three times, before clearing the preparation by centrifugation at low speed to remove cell debris. The resulting supernatant fluid was stored at −70° until used. Virus titration was performed by the limit dilution method, using six wells of a 96-cell Nunc microplate per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses per milliliter ($TCID_{50}$/ml).

FLAVONOIDS.—Galangin, kaempferol, quercetin, chrysin, luteolin, apigenin, tectochrysin, isosakuranetin, and pinocembrin were purchased from Extrasynthese Co France. Stock solutions (0.1 M) were prepared in DMSO and distributed in 0.5-ml fractions that were stored at +4°. For the experiments, aliquots were diluted with MEM to obtain the indicated concentrations (analogous dilutions of DMSO did not interfere with the assays; data not shown).

PREPARATION OF PROPOLIS BALSAM.—Propolis (30 g) was collected from a private apiary near

Rennes (France), and a sample was deposited at the herbarium of the Faculty of Pharmacy. It was extracted with 80% EtOH (300 ml) for 18 h at room temperature with stirring. Evaporation of the solvent under reduced pressure led to a dry residue (20 g) called propolis balsam (27). This crude extract was dissolved in DMSO (10 mg/ml) previous to dilution with MEM at the appropriate concentrations.

EVALUATION OF CYTOTOXICITY.—To assess the effect of flavonoids on uninfected Vero cells, dilutions ranging from 0.05 mM to 5 mM in the maintenance medium were added to Vero monolayers (four 25 cm^2 culture flasks seeded with 2.10^6 cells per dilution). After incubation for 96 h, cytotoxicity was determined by microscopic examination of cell morphology and by counting the cell number, in treated and untreated cultures, by trypan blue dye exclusion after trypsinization (28). The concentration at which the cell number was reduced to 50%, as compared with the control, was taken as the 50% cytotoxic concentration (CC_{50}). The cytotoxicity of propolis balsam was determined in the same manner by using concentrations ranging from 10 to 100 μg/ml. The cytotoxicity of flavonoid combinations was studied by adding the compounds together, in the medium culture, in the proportions used for the determination of synergy.

INHIBITION OF VIRUS MULTIPLICATION.—Flavonoids or propolis balsam at a range of concentrations lower than the 50% cytotoxic concentration were added to confluent 1-day-old monolayers of Vero cells grown in microtiter tissue culture plates just before inoculation, at a low multiplicity of infection: 100 $TCID_{50}$ per well. Toxicity controls, cell controls, and virus controls were run simultaneously. The assay of each drug was carried out in sextuplicate. Plates were incubated at 37° and after an incubation period corresponding to 4 cycles of multiplication (i.e., 72 h) the monolayers were observed for cytopathic effect and then the plates were frozen and thawed three times. The contents of the six identical wells were harvested, mixed, and clarified by low-speed centrifugation, and virus titrations were performed on the supernatant fluids by the limit dilution method. The antiviral activity of the compounds was determined as the reduction factor (log_{10}) of the viral titer by comparison with untreated controls.

CHROMATOGRAPHIC ANALYSIS OF PROPOLIS BALSAM.—The phenolic constituents of propolis balsam were determined by tlc and hplc. Tlc was performed on aluminium sheets precoated with Si gel $60F_{254}$ (Merck) using the solvent system C_6H_6-EtOAc-HO_2CH (40:10:5). Plates were viewed under uv light before and after spraying with $AlCl_3$. Phenolic acids and flavonoids were identified by comparison with markers purchased from Extrasynthese (France). Hplc was performed using an LDC/Milton Roy chromatograph equipped with a reversed-phase column (25 cm × 4 mm i.d.) prepacked with Spherisorb S5-ODS2-C18 and a uv detector. The eluent was H_2O-MeCN-HOAc (50:50:2) at a flow rate of 1 ml/min. Samples of propolis (1 mg/ml) and of pure phenolic compounds (2 mM solutions), prepared in the eluent solvent, were applied to the column by means of a 20 μl loop valve, and the effluent was monitored at 275 nm.

METHOD TO DETECT SYNERGY.—Flavones and flavonols were studied in binary combinations by the checkerboard technique, the concentrations of each drug being chosen according to their ability to reduce the virus titer from 0.5 to 2 log_{10} when they are used alone. Dilutions of the first drug were added to rows of wells containing confluent monolayers of cells. Dilutions of the second drug were added to the columns of wells in order to produce all possible combinations within the chosen range of concentrations (25). Controls received one drug, the second drug being replaced by an equal volume of medium. HSV-1 (100 $TCID_{50}$) was added in each well, and the plates were incubated for 72 h at 37°. Observation of the cytopathic effect and determination of virus titers were performed as described above.

DRUG INTERACTION.—The definition of drug interactions was described by Bryson and Kronenberg (29) and Schinazi *et al.* (30). The yield of control for a drug A, Ya, is defined as the titer of virus (log_{10} $TCID_{50}$) produced in the presence of drug A divided by that obtained in its absence, which corresponds to the survival fraction of virus after treatment by A. The yield of control for a drug B, Yb, is the titer of virus (log_{10} $TCID_{50}$) produced in the presence of drug B divided by that obtained in its absence, i.e., the survival fraction of virus after treatment by B. If the inhibitory activities of the two drugs are independent, the survival fraction after treatment by A will be Ya and conversely for B. In cultures treated first by A, then by B or conversely, the final survival fraction will be the product (Yc) of the two fraction Ya and Yb. When substances are added together in the culture medium, Yab is the titer of virus (log_{10} $TCID_{50}$) produced in the presence of both drugs divided by that obtained in their absence. If the inhibitory activities of drug A and drug B are independent, the observed result Yab ought to be equal to the expected result Yc ($Yc = Ya \times Yb$), and the combined effect is termed additive (AD). If $Yab < Yc$, then the interaction is termed synergistic (SY); if $Yab > Yc$ but less than the most effective agent alone, the reaction is subadditive; if Yab is greater than the most effective agent alone but less than the least effective agent, the reaction is termed interference; if Yab is greater than the least effective agent alone, the reaction is termed antagonism. The interaction that produces no inhibition greater than the most effective agent alone is called indifference. In fact, the differences Yab − Yc between +0.1 and −0.1 are considered as additive (AD) and only the differences below −0.1 are considered as synergistic (SY).

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