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ANTI-INFLUENZA VIRUS EFFECT OF SOME PROPOLIS CONSTITUENTS AND THEIR ANALOGUES (ESTERS OF SUBSTITUTED CINNAMIC ACIDS)

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ABSTRACT.—The antiviral activity of six synthetic substances, esters of substituted cinnamic acids, identical with or analogous to some of the constituents of the Et_2O fraction of propolis was studied in vitro. One of them, isopentyl ferulate, inhibited significantly the infectious activity of influenza virus A/Hong Kong (H3N2) in vitro and the production of hemagglutinins in ovo.

By the use of diverse experimental patterns, it was found that the maximal inhibition of viral reproduction was observed when test substances were present in the medium during the whole infectious process.

The literature on the experimental study of propolis (bee glue) and its antiviral activity is still sparse. Aqueous and EtOH extracts of propolis inhibited the replication of some plant viruses, virus vaccinia, virus Auezsky, herpes simplex virus, and vesicular stomatitis virus (1–4). An anti-influenza activity has been shown for an aqueous extract of propolis and for a combination of various apiarian products (5,6).

Propolis is an extremely complicated mixture of natural substances (7). In the search for some active principles of its antiviral effect, we have studied earlier the inhibitory effect of various extracts from propolis and their fractions on the reproduction of influenza viruses A/H1N1, A/H3N2, and B/Lee in vitro and in vivo (8,9). The data suggested that the inhibitory effect of propolis on the reproduction of influenza virus could be related to the petroleum ether fraction and the Et_2O fraction (EP). The EP was obtained by extraction with Et_2O of the MeOH total extract from propolis samples collected in three diverse areas. The EP decreased markedly the infectious activity of A/H1N1 (1.7 log ID_{50}/ml) and of A/H3N2 (2.3 log ID_{50}/ml) in vitro and protected white mice from experimental influenza infection by A/Aichi/2/68 (H3N2) in the dose of 20 mg/kg given orally (9). By chromatographic and spectral methods the complicated chemical composition of the EP has been studied by Bankova and co-workers (10–13). The EP contained mainly polyphenolic compounds: six flavones, six flavonones, seven phenolic acids and seven esters of phenolic acids. Among them four were described for the first time by Bankova *et al.* (13).

Some substances identical with or analogous to the constituents of the EP were synthesized (14). In order to evaluate the comparative share of the separate components of the EP in its antiviral effect, six esters of substituted cinnamic acids were studied for their inhibitory action on the reproduction of influenza virus.

EXPERIMENTAL

SUBSTANCES.—The Et_2O fraction of propolis and its preparation are as previously described (8). From propolis samples, collected in three apiarian areas (P, K, and V), total extracts were obtained by extraction with MeOH (TP, TK, and TV). The TP, TK, and TV were subjected to further extraction with petroleum ether (PP, PK, and PV) and with Et_2O (EP, EK, and EV). Esters of substituted cinnamic acids, synthesized by one of the authors, were identical or analogous to compounds, found in EP. The synthesis of the esters is described elsewhere (14). The products were used without separation of the stereoisomers, the *E*/*Z* ratio being 4:1 for all compounds. The stock solutions were prepared in sterile distilled H_2O after treatment with DMSO.

Voucher specimens of the propolis samples are deposited in the Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences.

Viruses.—Influenza viruses A/PR/8/34 (H1N1), A/Krasnodar/101/59 (H2N2), A/Hong Kong/1/68 (H3N2), and B/Lee were cultivated in embryonated hen's eggs and used as allantoic fluids. The viruses are from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences.

Test systems.—Tissue cultures from surviving chorioallantoic membranes (CAM), were prepared from 11–13-day-old embryonated hen's eggs according to Maltzeva *et al.* (15) (membranes-on-shell system) or Zakstelskaya (17) (membranes only). Maltzeva's method (15) is a modification of the method of Fazekas de St. Groth and White (16). Zakstelskaya's method (17) is a modification of the method of Tamm *et al.* (18). Embryonated 11-day-old hen's eggs were also used.

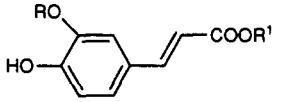
Toxicity.—The cultures of CAM according to Zakstelskaya (17) and the embryonated hen's eggs were treated with increasing concentrations of the substances and were followed for 72 h. The maximal tolerable concentration (MTC) was estimated, that is the highest dose of the substance that did not cause any morphological changes in CAM or mortality in hen's embryos.

Virucidal activity.—Tenfold dilutions of the virus A/Hong Kong were mixed with equal volumes of the substance in various concentrations. The mixtures were kept at 37° for 1 h, and the hemagglutination titer of control and treated viruses were determined.

Antiviral activity.—In CAM according to Maltzeva *et al.* (15) the viruses were cultivated with or without the substances at 37° for 48 h (B/Lee was cultivated at 33° for 72 h). The inhibitory effect was determined in one-step experiments by the difference of the infectious titers of control and treated viruses, according to Reed and Muench (19), expressed in log ID_{50}/ml. The reaction of hemagglutination was performed according to Hierholzer *et al.* (20). The hemagglutination titers were expressed in $\log_2$ HA. The minimal inhibitory concentration (MIC) was estimated as the lowest dose of the substance that inhibited significantly the viral infectivity. The inhibition was considered significant if $\geq$ 1 log ID_{50}/ml. The effective ranges were calculated by the ratio MTC/MIC. In CAM diverse experimental patterns and a two-step procedure were used: the substance **6** was applied before infection (1 h, 37°); simultaneously with infection at the time of adsorption (1 h, 4°); and after the adsorption and present in the medium for the whole period of cultivation (48 h, 37°). The viruses were collected and their infectious and hemagglutination titers were determined. Infectious titer (IT) is the reciprocal value of the last dilution of the virus which causes infection in 50% of the cells. Hemagglutination titer (HT) is the reciprocal valule of the last dilution of the virus which gives a positive reaction of hemagglutination inhibition test.

In embryonated hen's eggs, 0.2 ml of the substance **6** was inoculated 1 h before infection, and the mean geometrical hemagglutination titers were determined.

TABLE 1. Synthetic Constituents of Propolis: Their Chemical Structures, Toxicity to Chorioallantoic Membranes (CAM), and Inhibitory Activity on the Reproduction of Influenza Viruses in CAM.

Compound	Chemical structure	MTC[a] (μg/ml)	MIC[b] (μg/ml)	
			A/H3N2	A/H1N1
				
1[c]	R=H, $R^1=CH_2C_6H_5$	50	25	>50
2[c]	R=H, $R^1=CH_2CH_2C_6H_5$	50	25	50
3[d,e]	R=H, $R^1=CH_2CH_2CH(Me)_2$	50	25	25
4[c]	R=CH, $R^1=CH_2C_6H_5$	50	>50	>50
5[c]	R=Me, $R^1=CH_2CH_2C_6H_5$	50	>50	>50
6[d]	R=Me, $R^1=CH_2CH_2CH(Me)_2$	400	50	>200
EP[f]		400	50	100

[a]Maximal tolerable concentration.
[b]Minimal inhibitory concentration.
[c]Identical to a component of Bulgarian propolis.
[d]Analogous to a component of Bulgarian propolis.
[e]Found in Mongolian propolis.
[f]Et_2O fraction of Bulgarian propolis.

RESULTS AND DISCUSSION

The chemical structures of the six synthetic constituents of the Et_2O fraction of propolis, esters of substituted cinnamic acids are presented in Table 1. Four of the synthetic constituents **1**, **2**, **4**, and **5** were identical to those found in propolis. Two, **3** and **6**, were close analogues to the corresponding constituents of the natural product, and **3** in this form was found in Mongolian propolis.

The toxic effect on tissue cultures of CAM and the inhibitory effect on the reproduction of influenza viruses A/PR/8 (H1N1) and A/Hong Kong (H3N2) in CAM of the six compounds were studied. The experiments were done in parallel with EP, and the results are presented in Table 1.

Virus A/Hong Kong proved to be more sensitive to the action of the substances. Three of them, **1–3**, reduced its infectivity only when applied in high concentrations of 1/2 MTC. Compounds **4** and **5** had no effect.

The reproduction of A/PR/8 was inhibited by **2** in the dose 50 μg/ml (MTC) and by **3** in the dose 25 μg/ml (1/2 MTC). Compound **6**, isopentyl ferulate, applied in a concentration of 50 μg/ml significantly reduced the reproduction of A/Hong Kong and its effective range was 8. It was less toxic than the remaining esters. The inhibitory effect of **6** on A/Hong Kong was comparable to the effect of EP; they had equal effective ranges, and in the dose 100 μg/ml **6** inhibited the viral reproduction with 2.5 log ID_{50}/ml (Table 2) and EP with 2.3 log ID_{50}/ml (9). Compound **6** was less effective than EP against virus A/PR/8 and did not inhibit the reproduction of A/Krasnodar (H2N2) and B/Lee in CAM.

TABLE 2. Inhibitory Effect of Compound **6** on the Reproduction of A/Hong Kong (H3N2) Virus in Chorioallantoic Membranes.

Inoculation of **6** (0, 1 mg/ml)	Hemagglutination titer (log_2HA)	Infectious titer (log ID_{50}/ml)	Inhibition of infectious titer (Δ log ID_{50}/ml)
Before infection	4	3.0	—
Simultaneously with infection	3	1.5	2.0
After infection	3	1.5	2.0
Before, simultaneously, and after infection	<1	<1	>2.5
Virus control	5	3.5	

Diverse experimental patterns and a two-step procedure were used to evaluate the optimal scheme of application of compound **6** (Table 2). The pretreatment of CAM with **6** was not effective. This indicated that the substance did not reduce indirectly the viral reproduction via a cell-inhibitory function. When **6** was added at the time of adsorption or immediately after it, a reduction of 2 log ID_{50}/ml was registered. Obviously the adsorption and probably the penetration of the viral particles were inhibited (the early events in viral replication). The inhibitory effect was most pronounced when **6** in the dose 100 μg/ml was applied before, simultaneously with, and after infection. There was no production of infectious virus and hemagglutinins. The presence of **6** during the whole infectious process was essential for the maximal expression of its inhibitory effect.

The inhibitory effect of **6** on the reproduction of A/Hong Kong was confirmed in embryonated hen's eggs (Table 3).

MTC for this test system was 10 mg/ml. Compound **6** applied in concentrations of 2.5 and 5 mg/ml considerably reduced the production of hemagglutinins. The effect was dose-dependent.

TABLE 3. Inhibitory Effect of Compound **6** on the Reproduction of A/Hong Kong (H3N2) Virus in Embryonated Hen's Eggs.

Dosage of **6** (mg/ml)	Mean geometrical hemagglutination titer
1	9.4
Virus control . . .	10.4
2.5	7.35
Virus control . . .	9.2
5	6.45
Virus control . . .	10.1

In conclusion we may summarize that isopentyl ferulate [**6**], an ester of substituted cinnamic acid, a close synthetic analogue of isopent-3-enyl ferulate, the original compound in the Et_2O fraction of propolis, suppressed the reproduction of influenza virus A/Hong Kong in vitro and in ovo. The effective ranges of **6** and EP were equal. The inhibitory effect of **6** (2.5 log ID_{50}/ml) on A/H3N2 was comparable with the effect of EP itself, 2.3 log ID_{50}/ml (9).

These results offer some new information on the anti-influenza activity of propolis and contribute to the decoding of the active principles of its inhibitory effect.

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