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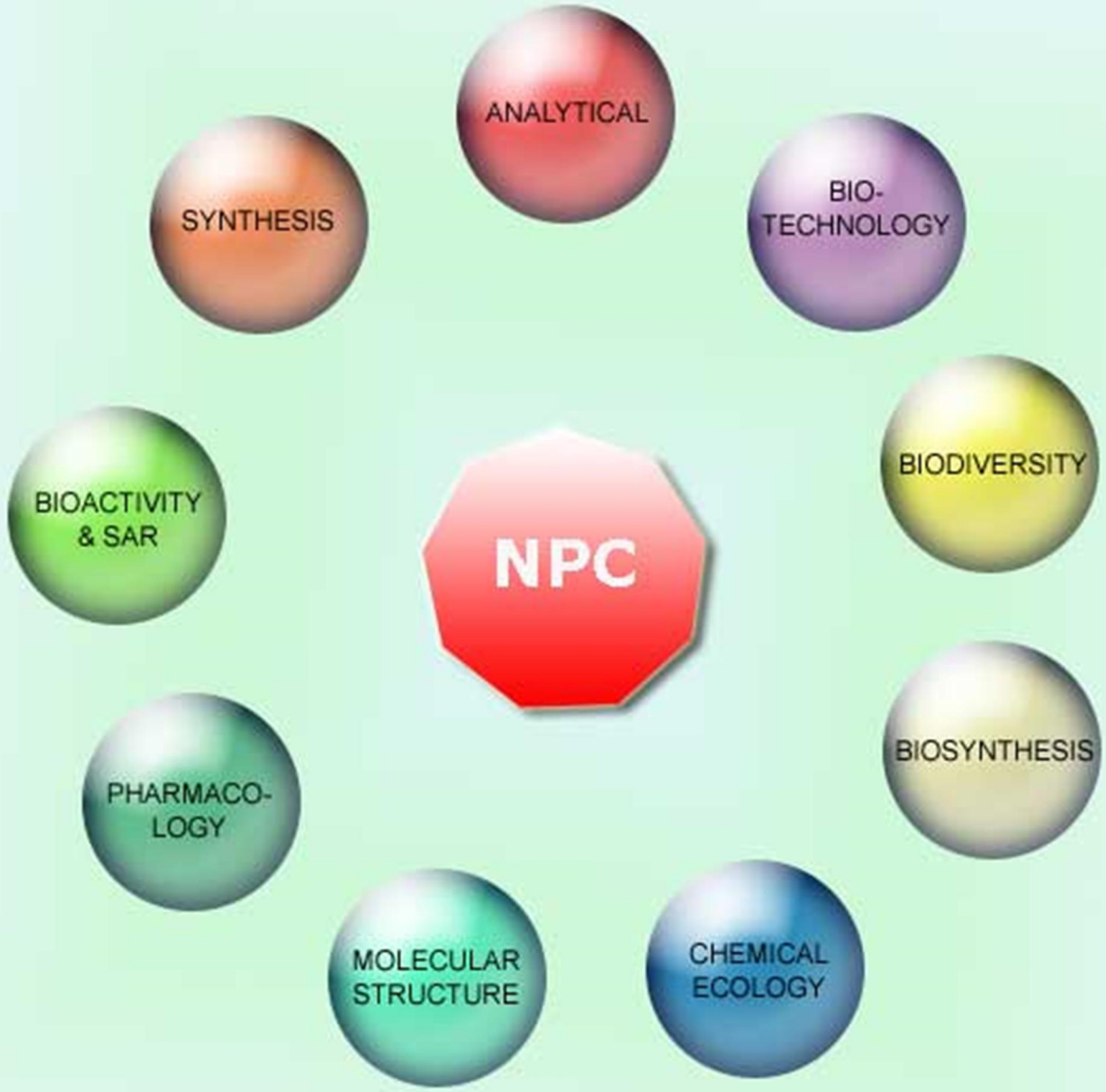

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In Vitro Antiviral Activity of a Series of Wild Berry Fruit Extracts against Representatives of *Picorna-*, *Orthomyxo-* and *Paramyxoviridae*

Lubomira Nikolaeva-Glomb[a,*], Luchia Mukova[a], Nadya Nikolova[a], Ilian Badjakov[b], Ivayla Dincheva[b], Violeta Kondakova[b], Lyuba Doumanova[a] and Angel S. Galabov[a]

[a]*Department of Virology*, *The Stephan Angeloff Institute of Microbiology*, *Bulgarian Academy of Sciences*, *Sofia*, *Bulgaria*

[b]*Agro Bio Institute*, *Sofia*, *Bulgaria*

lubomira@microbio.bas.bg

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Wild berry species are known to exhibit a wide range of pharmacological activities. They have long been traditionally applied for their antiseptic, antimicrobial, cardioprotective and antioxidant properties. The aim of the present study is to reveal the potential for selective antiviral activity of total methanol extracts, as well as that of the anthocyanins and the non-anthocyanins from the following wild berries picked in Bulgaria: strawberry (*Fragaria vesca* L.) and raspberry (*Rubus idaeus L.*) of the *Rosaceae* plant family, and bilberry (*Vaccinium myrtillis* L.) and lingonberry (*Vaccinium vitis-idaea* L) of the *Ericaceae.* The antiviral effect has been tested against viruses that are important human pathogens and for which chemotherapy and/or chemoprophylaxis is indicated, namely poliovirus type 1 (PV-1) and coxsackievirus B1 (CV-B1) from the *Picornaviridae* virus family, human respiratory syncytial virus A2 (HRSV-A2) from the *Paramyxoviridae* and influenza virus A/H3N2 of *Orthomyxoviridae*. Wild berry fruits are freeze-dried and ground, then total methanol extracts are prepared. Further the extracts are fractioned by solid phase extraction and the non-anthocyanin and anthocyanin fractions are eluted. The *in vitro* antiviral effect is examined by the virus cytopathic effect (CPE) inhibition test. The results reveal that the total extracts of all tested berry fruits inhibit the replication of CV-B1 and influenza A virus. CV-B1 is inhibited to the highest degree by both bilberry and strawberry, as well as by lingonberry total extracts, and influenza A by bilberry and strawberry extracts. Anthocyanin fractions of all wild berries strongly inhibit the replication of influenza virus A/H3N2. Given the obtained results it is concluded that wild berry species are a valuable resource of antiviral substances and the present study should serve as a basis for further detailed research on the matter.

Keywords: Antiviral, Wild berry, *Fragaria vesca*, *Rubus idaeus*, *Vaccinium myrtillis*, *Vaccinium vitis-idaea*.

Wild berries have long been used in folk and traditional medicine [1]. They are well known for their antiseptic and astringent properties, as well as their protective role in cardiovascular diseases, blood vessel disorders and some ophthalmological conditions. Berries are exceptionally rich in substances with powerful antioxidant and antimicrobial activities. Cranberry, in particular, is well known for its ability to prevent urinary tract infections [2]. On the whole, berry fruits are rich in bioactive compounds, such as phenolics and organic acids, which have a proven effect against a wide range of human pathogenic bacteria. However, while the antimicrobial activities of wild berries are quite obvious, long known and extensively studied, their antiviral capacity has not been well evaluated. Nevertheless, as early as the 1970s, Canadian researchers reported the antiviral activity of strawberry fruit juice against poliovirus type 1, coxsackievirus B5 and echovirus 7 [3]. It is also established that aqueous extracts of bilberry, cowberry and blackcurrant practically completely inactivate tick-borne encephalitis virus [4,5]. Cranberry juice and its polyphenolic constituents induce reduction of infectivity titers of rotaviruses [6] and food-borne viral surrogates such as murine norovirus and feline calicivirus [7]. Cranberry juice is also found to inhibit both influenza A and B viruses [8]. Influenza A virus replication is inhibited by extracts from a strawberry species, *Fragaria indica* L. [9] and by procyanidin, the active compound found in Canadian blueberry (*Vaccinium angustifolium* L.) [10]. Polyphenolic fractions extracted from Natsuhaze (*V. oldhamii* L.), bilberry (*V. myrtillus* L.), cranberry (*V. oxycoccos* L.) and blackcurrant (*Ribes nigrum*) also reveal high antiviral effects against influenza viruses [11]. Aqueous extracts of Korean black raspberry (*Rubus coreanus* Miq.) are able to inhibit significantly the replication *in vitro* of surrogate food-borne viruses [12] and possess, in addition, anti-hepatitis B activity [13]. Extracts from another raspberry species, *Rubus imperialis* L., show antiviral activity against the replication *in vitro* of herpes simplex virus 1 [14].

Thus much is clear that wild berries, although not so extensively studied for their antiviral capacity, are rich in various bioactive compounds with such properties and could be considered as an important source of new drugs and new drug leads. The purpose of this study was to reveal the potential for selective antiviral activity of total methanol extracts, as well as the anthocyanins and non-anthocyanins from the fruits of wild berries harvested in Bulgaria, against the replication of viruses belonging to different taxonomic groups and representing important human pathogens that cause diseases for which chemotherapy is indicated.

Wild berry total extracts and fractions were tested against poliovirus type 1 (PV-1) and coxsackievirus B1 (CV–B1) from the *Enterovirus* genus of *Picornaviridae*, human respiratory syncytial virus (HRSV-A2) from the *Paramyxoviridae*, and influenza virus A/Aichi/2/68(H3N2) of the *Orthomyxoviridae*. Human enteroviral infections are distributed worldwide with extremely high morbidity and although in most cases mild in their clinical course, they are manifested by a wide range of conditions and diseases including some severe illnesses of the central nervous system, the heart, endocrine pancreas, and skeletal muscles. Last but not least, the common cold caused by rhinoviruses, also members of the *Enterovirus* genus, regardless of the mild and self-limited course of the infection, has an enormous societal and economic impact due to lost labor and school days. No selective antiviral drug is registered

up to date for the infections caused by enteroviruses. In particular, as far as para- and orthomyxoviruses are concerned, several chemotherapeutic agents have been licensed and applied for therapy of influenza and infections caused by respiratory syncytial virus in humans but, due to the relatively rapid selection of resistant virus progeny and some toxicity concerns, the search for novel highly selective antivirals against these viruses is a never ending task.

The tested wild berry total extracts were found to have very low cytotoxicity and, in fact, were the least toxic among all the tested samples. Their maximal tolerated concentrations lay in the range between 3 and 10 mg/mL, and the 50% cytotoxic concentrations (CC_{50}) – between 6 and 18 mg/mL. Both fractions (non-anthocyanins and anthocyanins) had low cytotoxicity and the flavonoid fractions (the anthocyanins) were less cytotoxic than the phenolic ones (non-anthocyanins).

Each of the tested total extracts revealed a marked inhibitory effect against the replication *in vitro* of CV-B1 (Figure 1). The strongest antiviral effect was observed in the case of the fruits of bilberry (*Vaccinium myrtillis*), strawberry (*Fragaria vesca*) and lingonberry (*Vaccinium vitis-idaea*) picked in Bulgaria. Relatively high selectivity indices SI (SI = CC_{50}/IC_{50}) were determined: >50, >30 and >20, respectively. With the idea for comparing the activity of extracts from fruits picked in another country, bilberry (*V. myrtillus*) and lingonberry (*V. vitis-idaea*) originating from China, were obtained from the local market. It was established that the antiviral effect against CV-B1 replication of the total extract from the fruits of the Chinese bilberry was similar to that of the Bulgarian lingonberry and raspberry fruit extracts. The effect of the Chinese lingonberry against CV-B1 was a bit less pronounced.

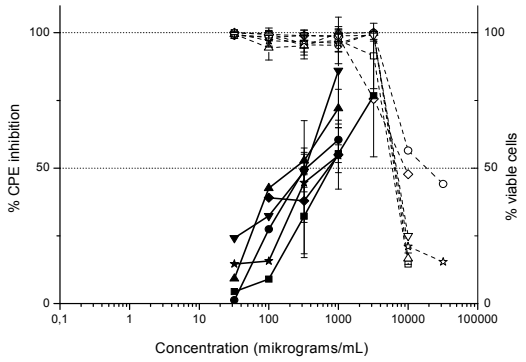

Figure 1: Effect of wild berry total extracts on the *in vitro* replication of CV-B1 in monolayer Hep-2 cell cultures in the CPE inhibition experimental set-up (dose-response curves). Thin dashed lines represent the dose-response curves of the cytotoxicity controls. (● – bilberry (*V. myrtillis*) from Bulgaria; ■ – bilberry (*V. myrtillis*) from China; ▲ – lingonberry (*V. vitis-idaea*) from Bulgaria; * – lingonberry (*V. vitis-idaea*) from China; ♦ – strawberry (*Fr. vesca*); ▼ – raspberry (*R. idaeus*).

The other representative of the *Enterovirus* genus of the *Picornaviridae*, i.e. PV-1, was inhibited to a much lesser extent by the total extracts of berry fruits. Total extracts from Bulgarian lingonberry fruits reached a SI of 8.8 and the extracts of the other fruits inhibited the virus induced cytopathic effect (CPE) by less than 50%, so IC_{50} and SI values could not be determined.

The total extracts also successfully inhibited influenza A virus (Figure 2). Its replication was inhibited to the utmost degree by the strawberry (*Fr. vesca*) extract with a SI above 100, followed by bilberry (*V. myrtillis*) fruit extracts both of Bulgarian and Chinese origin with SI >50 and >40, respectively. Raspberry (*R. idaeus*) total fruit extract also possessed a well pronounced antiinfluenza A virus effect (SI >10).

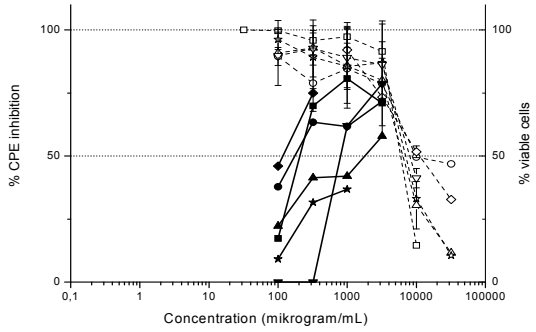

Figure 2: Effect of wild berry total extracts on the *in vitro* replication of influenza virus A/Aichi/2/68(H3N2) in monolayer MDCK cell cultures in the CPE inhibition experimental set-up (dose-response curves). Thin dashed lines represent the dose-response curves of the cytotoxicity controls. (● – bilberry (*V. myrtillis*) from Bulgaria; ■ – bilberry (*V. myrtillis*) from China; ▲ – lingonberry (*V. vitis-idaea*) from Bulgaria; * – lingonberry (*V. vitis-idaea*) from China; ♦ – strawberry (*Fr. vesca*); ▼ – raspberry (*R. idaeus*).

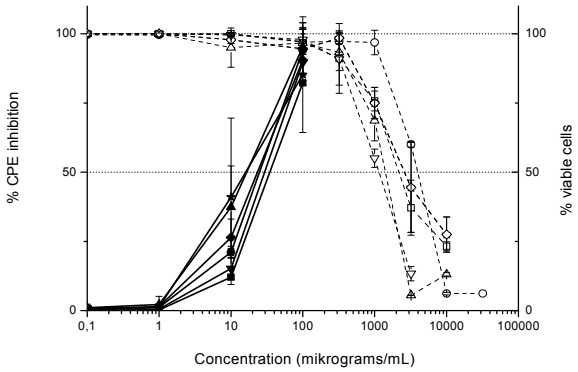

Figure 3: Effect of anthocyanin fractions on the *in vitro* replication of influenza virus A/Aichi/2/68(H3N2) in monolayer MDCK cell cultures in the CPE inhibition experimental set-up (dose-response curves). Thin dashed lines represent the dose-response curves of the cytotxicity controls. (● – bilberry (*V. myrtillis*) from Bulgaria; ■ – bilberry (*V. myrtillis*) from China; ▲ – lingonberry (*V. vitis-idaea*) from Bulgaria; * – lingonberry (*V. vitis-idaea*) from China; ♦ – strawberry (*Fr. vesca*); ▼ – raspberry (*R. idaeus*).

HRSV-A2 was inhibited to a much lesser extent. Only the total extract of fruits of the Bulgarian bilberry (*V. myrtillis*) was able to inhibit virus replication showing a SI > 10. Total extracts from the rest of the berries revealed a weak and rather marginal antiviral activity against HRSV-A2. The non-anthocyanin fractions did not reveal an antiviral effect against the replication of the tested viruses.

On the contrary, the anthocyanins (flavonoids) showed a marked antiviral activity against the replication of influenza virus A/Aichi/2/68(H3N2) (Figure 3). The strongest effect was observed when the anthocyanin fraction from Chinese lingonberry fruits (*Vaccinium vitis-idaea*) and Bulgarian bilberry fruits (*Vaccinium myrtillis*) were applied. The other tested viruses were not significantly inhibited by the anthocyanin fractions.

The experimentally obtained data concerning the chemotherapeutic characteristics of the tested samples against the replication of PV-1, CV-B1, HRSV-A2 and influenza virus A are presented in Table 1. The results obtained reveal a promising antiviral capacity of wild berries. These viruses are important human pathogens, some of them causing devastating diseases and for which chemotherapy is indicated. Strawberry juice was first reported for its antiviral effect against several enteroviruses [3]. Another enterovirus, namely CV-B1, could be added to the group of susceptible species thanks to the findings presented here. The antiinfluenza A virus effect of the flavonoid (anthocyanin) fractions of the studied wild berry fruits

Table 1: *In vitro* antiviral effect against CV-B1, PV-1, HRSV-A2 and influenza virus A/Aichi/H3N2, cytotoxicity against relevant cells and selectivity index.

Sample		Cytotoxicity CC_{50}[a] (µg/mL)		CPE inhibition							
				CV-B1		PV-1		HRSV-A2		Influenza A	
		Hep-2 cells	MDCK cells	IC_{50} µg/mL	SI	IC_{50} µg/mL	SI	IC_{50} µg/mL	SI	IC_{50} µg/mL	SI
Total extracts	I	18 300	10 000	336.7	54.3	-	-	1297.0	14.1	170	58.9
	II	5 900	9 700	220.7	26.7	-	-	-	-	206	47.1
	III	6 400	6 400	230.8	27.7	723.79	8.8	-	-	-	-
	IV	6 600	6 900	743.4	8.9	-	-	-	-	-	-
	V	9 100	11 100	275.2	33.1	-	-	-	-	100	111
	VI	6 800	8 100	294.6	23.1	-	-	-	-	803	10.1
Non-anthocyanins	1	29.20	57.28	-	-	-	-	-	-	-	-
	5	31.37	33.88	-	-	-	-	-	-	-	-
	2	27.81	35.23	-	-	-	-	-	-	-	-
	6	28.33	>100	-	-	-	-	-	-	-	-
	3	30.55	>100	-	-	-	-	-	-	-	-
	4	29.65	>100	-	-	-	-	-	-	-	-
Anthocyanins	7	6 350	3 950	-	-	-	-	-	-	25.2	156.5
	11	3 070	2 255	-	-	-	-	-	-	34.7	64.9
	8	3 590	1 400	-	-	-	-	-	-	16.3	85.7
	12	2 390	3 000	-	-	-	-	-	-	14.9	201.1
	9	4 420	870	-	-	-	-	-	-	23.3	37.3
	10	1 780	1 150	-	-	-	-	-	-	30.0	38.3
Oxoglaucine		18.2	n.a.	0.42	43.3	0.1	182	n.a.	n.a.	n.a.	n.a.
Disoxaril		8.5	n.a.	0.68	12.5	0.5	17	n.a.	n.a.	n.a.	n.a.
Ribavirin		13.0	n.a.	n.a.	n.a.	n.a.	n.a.	0.41	31.7	n.a.	n.a.
Rimantadine		n.a.	6.91	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.048	147

deserves further and more detailed investigation due to the high selectivity indices revealed.

It is not surprising that especially the total extracts of berry fruits have revealed strong and varied antiviral effects. Each plant contains a variety of diverse chemical constituents which may have the ability to inhibit the replication of different viruses by different modes of action, irrespective of the fact that in many cases that is not yet explored and hence, unknown. The intrinsic feature of viruses to develop resistance towards antiviral agents enhances the continuous need for new effective compounds against viral infections. The present results indicate that berries are a real and valuable resource of promising antiviral compounds and they deserve further in depth investigation. Along with the current increase of the proportion of the global population that prefers use of natural products for treating and preventing diseases either alone or in combination with licensed drugs, experimental work in this field merits the efforts.

Experimental

Plant material: Strawberry fruits (*Fragaria vesca*, *Rosaceae*), raspberry fruits (*Rubus idaeus*, *Rosaceae*), bilberry fruits (*Vaccinium myrtillus*, *Ericaceae*) and lingonberry fruits (*V. vitis-idaea*) were collected in 2012, in the West Rhodope mountain in Bulgaria (GPS: 41°55'51.73"N; 23°46'14.98"E; altitude: 1600 m). Fruits were identified by Dr Ilian Badjakov and voucher specimens 107545, 107544, 107543 and 107542 were deposited in the herbarium collections of Sofia University "St. Kliment Ohridski" (SO). Bilberry (*V. myrtillus*) and lingonberry (*V. vitis-idaea*) originating from China were ordered from the local market. All samples were freeze-dried, ground and stored at – 80°C prior to extraction.

Sample descriptions: **I** and **II** – total extracts from bilberry (*V. myrtillus*), picked in Bulgaria and in China, respectively; **III** and **IV** – total extracts from lingonberry (*V. vitis-idaea*), picked in Bulgaria and in China, respectively; **V** and **VI** – total extracts from strawberry (*Fr. vesca*) and raspberry (*R. idaeus*), respectively, picked in Bulgaria; **1**, **2**, **3** and **4** – the non-anthocyanin fractions from bilberry (*V. myrtillus*), lingonberry (*V. vitis-idaea*), strawberry (*Fr. vesca*) and raspberry (*R. idaeus*), respectively, all picked in Bulgaria; **5** and **6** – non-anthocyanins from bilberry and lingonberry, respectively, picked in China; **7**, **8**, **9** and **10** – the anthocyanin fractions from bilberry, lingonberry, strawberry and raspberry, respectively, picked in Bulgaria; **11** and **12** – anthocyanins from bilberry and lingonberry, respectively, picked in China.

Chemicals: All solvents were of HPLC grade (Sigma-Aldrich, St. Louis, MO), and water was of Milli-Q (18 MΩ/cm) quality (Millipore Corp., Bedford, MA).

Extract preparation: To each 50 g of lyophilized powder, 500 mL of MeOH: 0.1% HCOOH in water (80:20, v/v) solution was added and the mixture was sonicated for 30 min at room temperature in the dark. The samples were filtered through paper filter. The extraction steps were repeated 3 times. The filtrates of each sample were collected and dried under vacuum below 40°C.

Fractionation by solid phase extraction: Water extracts were fractionated by Solid Phase Extraction (SPE) using Giga tubes 2 g/12 mL, C18-E units (Strata, Phenomenex®). The columns were activated with 0.1%, v/v, formic acid in acetonitrile followed by ethyl acetate and 0.1%, v/v, formic acid in water. The extracts were applied to the columns. Anthocyanins and other phenolics were adsorbed onto the columns. Water-soluble compounds (fraction 'A'): sugars, organic and amino acids, were removed with 2 volumes (2 x 12 mL) of 0.1%, v/v, formic acid in water. The non-anthocyanin components (fraction 'B') were eluted from the columns with 2 volumes (2 x 12 mL) of ethyl acetate. The anthocyanins (fraction 'C') were subsequently eluted with 2 volumes (2 x 12 mL) of 0.1%, v/v, formic acid in acetonitrile. Fractions 'B' and 'C' were then dried using a BÜCHI R-124/B-481 rotavapor.

Restoration of samples: Lyophilized samples were restored in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL and further diluted in a maintenance medium.

Reference compounds for antiviral tests: Oxoglaucine, an aporphinoid alkaloid from *Glaucium flavum* Cranz, and disoxaril, the latter supplied by Sanofi Winthrop Inc. (Malverne, PA, USA), served as reference antipicornavirus compounds. The reference compound for HRSV-A2 was ribavirin, a kind gift of Prof. Robert W. Sidwell, Logan, USA and rimantadine was used as a reference for the inhibition of influenza A virus.

Cells and viruses: Poliovirus type 1 (PV-1) (strain LSc-2ab), coxsackievirus B1 (CV–B1), human respiratory syncytial virus A2 (HRSV-A2) and influenza virus A/Aichi/2/68(H3N2) were used for the antiviral tests. PV-1, CV-B1, and HRSV-A2 were grown in Hep-2 cell line, and influenza virus A – in MDCK cells. Cells and viruses were from the cell culture collection of the Stephan Angeloff Institute of the Bulgarian Academy of Sciences, Sofia, Bulgaria. Cell lines were grown at 37°C in 5% CO_2 in Dulbecco modified Eagles' medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 5% fetal bovine serum and supplemented with antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamycin), and 20 mM HEPES buffer (Gibco BRL). When harvesting viruses and performing antiviral assays, maintenance medium was used, in which serum was reduced to 0.5%. Maintenance medium for influenza virus contained, in addition, 3 µg/mL trypsin (Gibco BRL).

Cellular toxicity: Monolayer cell cultures in 96-well plates (Cellstar®, Greiner Bio-one, GmbH, Frickenhausen, Germany) were inoculated with 0.1 mL/well maintenance medium containing different concentrations of the samples in 0.5 L/g intervals. After 24 and 48 h of incubation at 37°C and 5% CO_2, cells were monitored microscopically for detectable cytotoxic effects. The highest concentration at which no visible cytotoxic effect was recorded was considered as the maximal tolerated concentration (MTC). After 48 h cells were subjected to the neutral red uptake procedure [15], and the 50% cytotoxic concentration (CC_{50}) was calculated. The optical density (OD) of each well was read at 540 nm in a microplate reader (Organon Teknika reader 530, Oss, Netehrlands). The CC_{50} values were determined by regression analysis.

Antiviral activity: The CPE inhibition assay was used for evaluating the antiviral effects. Monolayer cells in 96-well plates were inoculated with 0.1 mL virus suspension containing 100 $CCID_{50}$. Mock-infected wells were left for toxicity and cell controls. After 1 h for virus adsorption (2 h for HRSV-A2), excessive virus was discarded, and cells were inoculated with 0.1 mL of maintenance medium containing different nontoxic concentrations (in 0.5 L/g intervals) of the test samples. Cells were further incubated and the CPE was scored daily till the appearance of its maximum in the virus control wells. Then viable cells were stained according to the neutral red uptake procedure and the percentage of CPE inhibition for each concentration of the test sample was calculated using the following formula: % CPE = $[OD_{test\ sample} - OD_{virus\ control}]/[OD_{toxicity\ control} - OD_{virus\ control}]x100$, where $OD_{test\ sample}$ is the mean value of the ODs of the wells inoculated with virus and treated with the test sample, $OD_{virus\ control}$ is the mean value of the ODs of the virus control wells and $OD_{toxicity\ control}$ is the mean value of the ODs of the wells not inoculated with virus but treated with the corresponding concentration of the test sample. The concentrations that inhibited 50% of the virus induced CPE, the 50% inhibitory concentrations (IC_{50}), were determined by regression analysis. The selectivity index (SI) was calculated as the ratio between CC_{50} and IC_{50} ($SI = CC_{50}/IC_{50}$).

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