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Anticancer Activities as well as Antiviral and Virus-enhancing Properties of Aqueous Fruit Extracts from Fifty-six European Plant Species

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Abstract—*Several plant-derived drugs are used in medical oncology today. Since only a small part of the flora has been tested for any kind of bioactivity intensive further screening may be rewarding. Fifty-six plant extracts were studied attempting to explore the feasibility of an assay that screens cytotoxic, antiviral and virus-enhancing activities in the same test. We made use of the property of an avian influenza virus replicating in a human breast cancer cell line. During the first 3 days of the test the cytotoxicity of the extracts was evaluated by phase-contrast microscopy. From the 4th day on when viral cytopathogenic effect became manifest we were able to identify antiviral and virus-enhancing activity among some of those extracts not showing cytotoxicity during the first 3 days of incubation. Aqueous extracts from the fruits of 56 plant species belonging to 22 families were screened. Twelve species exhibited cytotoxic, eight antiviral, five virus-enhancing and 31 no activity. These results show that the replication of a myxovirus in a human tumor cell offers the possibility of screening cytotoxic, antiviral and virus-enhancing activity in the same assay.*

INTRODUCTION

SEVERAL of the cytotoxic drugs used clinically in the treatment of cancer stem from plants (e.g. vinca alkaloids, podophyllotoxin derivatives). The search for further substances from plants active against cancer and viral diseases such as AIDS has been intensified recently as a small part only of the flora has been tested for any kind of bioactivity [1]. In this report we describe a procedure which screens cytotoxic, antiviral and virus-enhancing activity in the same assay making use of the property of an avian influenza virus replicating in a human breast cancer cell line [2]. Extracts from 56 plant species belonging to 22 families were tested.

MATERIALS AND METHODS

Plant extracts

All plant specimens were collected in northern Switzerland (with the exception of *Ribes alpinum*, *Sambucus racemosa* and *Vaccinium* in Zermatt, Switzerland) during the summer and fall seasons. In a first step, only aqueous extracts were tested in order to avoid artefacts of solvents or of extraction procedures; the fruits collected were limited to 56 species. In general 10 g of fruit were frozen in 50 ml Falcon tubes (Falcon Plastics, Oxnard, CA) at −20°C. After thawing the specimens were mashed in the tubes with a glass homogenizer and Eagle's minimum essential medium (MEM) containing 2% fetal bovine serum was added to a volume of 20 ml. The samples were frozen at −80°C. The thawing was then done on a vortex at maximum speed in order to homogenize the fruits further by the ice lumps. After centrifugation at 2250 ***g*** for 30 min at 4°C the supernatants were stored at −80°C in aliquots of 1 ml until used.

Cell line

The BT 20 breast cancer line [3] was used in all assays.

Virus

Avian influenza virus (AIV; A/Turkey/England/63, Hav1 Nav3, Langham strain) had been adapted to human tumor cells [4, 5] and had been shown to replicate in primary cultures of human tumors [6, 7] and in human breast [2] and lung cancer [8] cell lines. AIV replicating in the human breast cancer cell line BT 20 was used in these assays.

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Assay for cytotoxicity and antiviral activity

Freshly trypsinized BT 20 cells were seeded in 25 cm^2 Falcon plastic flasks in such a way that monolayers were formed after 5 days of incubation at 37°C containing about 2×10^6 cells. At this time samples of fruit extracts were thawed, diluted in MEM (1:12.5), and sterilized by filtration (Millipore, pore size 0.22 μm). The medium of the BT 20 monolayers was replaced by 2.0 ml of sterilized extract dilution and 2.0 ml of MEM containing 10^4 50% tissue culture infectious doses ($TCID_{50}$)/ml AIV. Incubation at 37°C was continued. Checking for cytopathogenic effect (CPE) was done daily by phase contrast microscopy and compared to virus and medium controls. Cytotoxicity due to plant extracts occurred during the first 72 h of incubation, whereas CPE due to AIV replication was only observed between 96 and 144 h of incubation. When AIV CPE was observed in the virus controls the experiment was terminated. The supernatants were assayed for hemagglutinin (HA) activity, and aliquots of 0.5 ml were put into sterile glass ampoules and stored at −80°C until infectivity titrations could be performed.

Virus infectivity assays. Hemagglutinin determinations

Virus infectivity assays were carried out in Falcon 3040 microplates containing chicken embryo fibroblasts monolayer cultures. The monolayers were infected with 0.1 ml of serial 10-fold dilutions of virus in MEM without serum. Six wells per dilution were used. After 5 days of incubation at 37°C the monolayers were checked for cytopathogenic effect. Wells with incomplete destruction of the monolayers were tested for hemagglutinin. The $TCID_{50}$ was calculated according to the method of Reed and Muench [9]. The hemagglutinin titrations were effected using WHO standard procedures [6]. Inhibition of HA formation was defined as a reduction of at least 2 log 2 and inhibition of AIV infectivity as a reduction of at least 1 log 10 over the controls; virus enhancement was defined analogically.

RESULTS

Four categories can be made of the 56 plant species with respect to their cytotoxic, antiviral and virus-enhancing activity: (1) production of cytotoxicity in BT 20 breast cancer cells within 72 h of incubation: 12 plant species; (2) inhibition of AIV replication: eight plant species; (3) virus-enhancing activity: five plant species; (4) no activity: 31 plant species.

In Table 1 the 22 plant families are listed alphabetically with the results shown for cytotoxicity and AIV behavior for all the 56 species examined. The results for categories 2 and 3 (inhibition or enhancement of AIV replication) are shown in detail in Tables 2 and 3.

DISCUSSION

Previous work from this laboratory showed adaptation of an avian influenza virus to different human tumor cells [2, 4–8]. A breast cancer cell line in which AIV replicates was chosen as a test system to screen cytotoxic, antiviral and virus-enhancing activities of plant extracts. Since all but two of the most important antitumor agents originating from plants were discovered by *in vitro* cytotoxicity tests [10] further intensive screening of the flora by *in vitro* assays for anticancer and anti-AIDS virus substances is being organized [1].

In this report we describe a system, which, during the first 3 days of the assay screens cytotoxicity, and, during the next 2 days, antiviral or virus-enhancing activities. Because of the low multiplicity of AIV infection (only 0.01; i.e. 10^4 $TCID_{50}$ per 10^6 cells) the virus infected BT 20 cells show no virus-related CPE until 4 days after infection. During the first 3 days therefore, any cytotoxicity observed is due to the plant extracts. The extracts of 12 plant species exhibited cytotoxicity during the first 3 days of incubation (category 1, see above). AIV replication could never be detected here at day 5 (see Table 1: no AIV replication). The regular absence of AIV replication confirms the early cellular damage observed by phase-contrast microscopy and can be used as an additional criterion for the early cytotoxicity. The results of the extracts of *Aesculus hippocastanum* and *Convallaria maialis* prove this assay also to be useful for detecting cytotoxicity of saponins being known to exhibit antitumor activity [11]. The aqueous extracts of the *edible* fruits (*Sambucus racemosa*, *Ribes album*, *Ribes nigrum*, *Malus* cult., *Physalis alkekengi*) are of special interest in category 1. Further analysis of these extracts may be worthwhile. Cytotoxicity of edible plants may play an important role in local (e.g. mouth, pharynx, esophagus) cancer prevention as anticarcinogens in the human diet seem to play systemically [12, 13].

Antiviral activity was detected in eight extracts (category 2; Table 2). Only *Sambucus nigra* (one of the four edible extracts—the others are *Ribes rubrum*, *Ribes uva-crispa*, *Fragaria vesca*—exhibiting antiviral activity) was further analyzed with respect to the mechanism of action. AIV infectivity was found to be neutralized by a D-galactose specific lectin [14].

The third category (virus enhancing extracts) shown in detail in Table 3 may be of importance as to enhancing virus production *in vitro*, i.e. viruses otherwise difficult to grow such as the HIV virus group. The myxovirus used in our assay being also an enveloped RNA virus may be a valuable model. In two instances (*Vaccinium vitis-idaea* and *Prunus cerasus*) the infectivity is the same as in the controls, the hemagglutinin, however, is lower indicating that the proportion of infectious to incomplete virus particles is higher than in the controls.

In conclusion: with this screening procedure a

Table 1. Cytotoxic and antiviral activities of aqueous fruit extracts

Plant family	Scientific name (botanical authority)	Cytotoxicity due to extracts*	AIV behavior HA†	AIV behavior Infectivity‡
Aquifoliaceae	*Ilex aquifolium* (L.=Linné)	−	−	−
Araceae	*Arum maculatum* (L.)	−	−	−
Berberidaceae	*Berberis vulgaris* (L.)	−	−	−
	Mahonia aquifolium (Nutt.)	−	−	−
Caprifoliaceae	*Sambucus nigra* (L.)	−	D	D
	Sambucus racemosa (L.)	+	No AIV replication	
	Symphoricarpus albus (L.)	−	−	−
	Viburnum lantana (L.)	−	−	−
	Viburnum opulus (L.)	−	D	D
Celastraceae	*Euonymus europaeus* (L.)	−	D	D
Cornaceae	*Cornus mas* (L.)	−	−	−
	Cornus sanguinea (L.)	+	No AIV replication	
	Cornus sibirica (L.)	−	−	−
Elaeagnaceae	*Hippophae rhamnoides* (L.)	−	−	−
Ericaceae	*Vaccinium myrtillus* (L.)	−	−	−
	Vaccinium vitis idaea (L.)	−	D	−
Grossulariaceae	*Ribes album* (L.)	+	No AIV replication	
	Ribes alpinum (L.)	−	−	−
	Ribes nigrum (L.)	+	No AIV replication	
	Ribes rubrum (L.)	−	D	D
	Ribes uva-crispa (L.)	−	D	D
Hippocastanaceae	*Aesculus hippocastanum* (L.)	+	No AIV replication	
Leguminosae	*Pisum sativum* (L.)	−	−	−
Liliaceae	*Convallaria maialis* (L.)	+	No AIV replication	
Loranthaceae	*Viscum album* (L.)	+	No AIV replication	
Moraceae	*Morus nigra* (L.)	−	−	−
Oleaceae	*Ligustrum vulgare* (L.)	−	−	−
Onagraceae	*Fuchsia* (cult.)	−	−	E
Rhamnaceae	*Frangula alnus* (Mill.)	−	−	−
Rosaceae	*Chaenomeles japonica* (cult.)	−	−	−
	Cotoneaster salicifolius (cult.)	+	No AIV replication	
	Crataegus crus-galli (cult.)	−	D	D
	Crataegus lavallei (cult.)	−	−	−
	Crataegus oxyacantha (L.)	−	−	−
	Fragaria (cult.)	−	−	−
	Fragaria vesca (L.)	−	D	D
	Malus (cult.)	+	No AIV replication	
	Prunus cerasus (L.)	−	D	−
	Prunus (cult. black cherry)	−	−	−
	Prunus domestica (L.)	−	−	−
	Prunus laurocerasus (L.)	−	−	E
	Prunus padus (L.)	+	No AIV replication	
	Prunus spinosa (L.)	−	−	−
	Pyracantha coccinea (M.J. Roemer)	−	−	−
	Rosa canina (L.)	−	−	−
	Rubus fruticosus (L.)	−	−	E
	Rubus idaeus (L.)	−	−	−
	Sorbus aria (L.)	−	−	−
	Sorbus aucuparia (L.)	+	No AIV replication	
Solanaceae	*Atropa belladonna* (L.)	−	−	−
	Physalis alkekengi (L.)	+	No AIV replication	
	Solanum nigrum (L.)	−	D	D
Taxaceae	*Taxus baccata* (L.)	−	−	−
Tiliaceae	*Tilia platyphyllos* (Scop.)	−	−	−
Vitaceae	*Parthenocissus tricusp.* (Sieb., Zucc.)	−	−	−
	Vitis vinifera (Pinot noir, gris, cult.)	−	−	−

*+ Cytotoxicity within 72 h of incubation; − No cytotoxicity within 72 h of incubation.
†HA: − no influence on HA production: D: decreased HA production (see Tables 2 and 3).
‡Infectivity: − as in controls; D: decreased HA or infectious AIV production (see Table 2); E: enhanced infectious AIV production (see Table 3).

Table 2. Inhibition of hemagglutinin formation and infectivity of avian influenza virus by aqueous fruit extracts

Plant family	Scientific name	HA* Extract	HA* Control	$TCID_{50}$† Extract	$TCID_{50}$† Control
Caprifoliaceae	*Sambucus nigra*	Neg	64	5.5	7.4
	Viburnum opulus	Neg	32	5.5	7.5
Celastraceae	*Euonymus europaeus*	Neg	32	4.0	7.6
Grossulariaceae	*Ribes rubrum*	1	64	5.0	7.0
	Ribes uva-crispa	4	64	5.5	7.0
Rosaceae	*Crataegus crus-galli*	Neg	32	6.25	7.6
	Fragaria vesca	Neg	64	2.0	7.0
Solanaceae	*Solanum nigrum*	Neg	32	5.75	7.5

*Hemagglutination titer; neg, negative, i.e. no hemagglutin detected.
†Log 10 $TCID_{50}$/ml of culture medium.

Table 3. Increase of infectious *avian influenza virus production by fruit extracts*

Plant family	Scientific name	HA* Extract	HA* Control	$TCID_{50}$† Extract	$TCID_{50}$† Control
Ericaceae	*Vaccinium vitis-idaea*	1	64	7.0	7.0
Onagraceae	*Fuchsia* cult.	64	64	8.16	6.5
Rosaceae	*Prunus cerasus*	8	64	7.0	7.0
	Prunus laurocerasus	64	64	8.5	6.75
	Rubus fruticosus	128	64	8.0	6.6

*Hemagglutination titer.
†Log 10 $TCID_{50}$/ml of culture medium.

high proportion of aqueous fruit extracts (44%) from native plants exhibits some biological activity. Further analysis like the one we started already with *Sambucus nigra* [14] may be rewarding.

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