ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN—POLONIA
VOL. XL, 3 SECTIO C 1985

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Antiviral Activity of Propolis

Aktywność przeciwwirusowa propolisu

Антивирусная активность прополиса

Studies on the activity of propolis extracts are carried out chiefly on various bacteria. Attemps are also made to use propolis in the form of nutritive preparations as well as a medicine (6, 8, 9). In the present study we have investigated the action of organic solvent extracts of propolis against vesicular stomatitis (VSV) and vaccinia viruses.

MATERIALS AND METHODS

Propolis. Samples of propolis collected from areas of Lublin and Warsaw regions were used in experiments. 10% ethanol solutions of propolis were prepared by extraction of crude propolis samples, separated from mechanical impurities and wax, with 10, 25, 60 and 96% solution of ethanol. After three days of extraction at room temperature propolis solutions were filtered on Whatman No 4 blotting paper. The activity of ethanol extracts was tested in relation to *Mycobacterium smegmatis*. Single samples active against *M. smegmatis* were evaporated to dryness. From those samples appropriate solutions of propolis were prepared by dissolving them in absolute ethanol or dimethyl sulfoxide (DMSO). Extract of propolis in 96% ethanol after evaporation to dryness was dissolved in ether: benzene (1:1) and filtered. The remaining insoluble residues were extracted subsequently with benzene, tetrachloromethane, chloroform and acetone. All extracts were evaporated to dryness, dissolved in 96% ethanol and examined for antiviral activity

Cell cultures. Cell cultures of chick embryo fibroblasts (CEF) were obtained by the standard method of trypsinization of 9-day-old chicken embryos. The cells were cultivated in Parker's medium with 10% calf serum.

Viruses. In the experiments vaccinia virus from the commercial vaccine and vesicular stomatitis virus (VS), Indiana type were used. The viruses were multiplied in cell cultures of chick embryo fibroblasts and stored at −20°C.

Agar diffusion method. The cell cultures of chick embryo fibroblasts on Petri dishes were infected with VS virus, 40 PFU per dish, and vaccinia virus 400 PFU per dish. After one hour of virus adsporption the cells were washed with Parker's medium and covered with 0.7% agar medium with Parker's concentrate and 10% calf serum. After solidification of agar, filter paper disks 10 mm in diameter saturated with proper concentration of propolis and dried were disposed on its surface. The cell cultures were incubated at 4°C for 24 hrs, 37°C for 48 hrs and then stained with neutral red. The results were read, measuring the toxicity zone round the disk with propolis and the inhibition zone of VS and vaccinia viruses plaques.

Virucidal activity of propolis. Virus VS or vaccinia virus in Parker's medium were mixed in appropriate proportions with propolis dissolved in DMSO, so as to obtain the final propolis concentration of 100 or 1000 μg/ml. The control was solution of DMSO in Parker's medium, mixed with VS or vaccinia virus. The mixtures were incubated at 4°C for 1 hr or 24 hrs and then the virus titres were determined in the cell culture of chick embryo fibroblasts.

Protective activity of propolis. Grown, 48-hrs-old CEF cell cultures were incubated with nontoxic concentrations of propolis at 37°C for 24 hrs. After removing propolis form the cell cultures, the cells were infected with VS virus 100 $TCID_{50}$/test tube. The cytopathic effect of the virus was read in the microscope.

The effect of propolis on replication of viruses. CEF cells at the density of 10^6 cells par 1 ml, suspended in Parker's medium with 2% calf serum were infected with VS or vaccinia viruses — 10 $TCID_{50}$ of viruses per one cell. Following one hour adsorption and removal of nonadsorbed viruses, propolis solutions at a concentration of 10 μg/ml were added to the cell cultures. After 24 hrs the yield of viruses from the control cell cultures and those treated with propolis was determined.

RESULTS AND DISCUSSION

All the results presented are the mean from three independent experiments. Table 1 shows the results of studies carried out by the method of agar diffusion, which express antiviral activity of propolis from various apiaries in the districts of Lublin and Warsaw. Small differences in antiviral activity of propolis coming from various places have been found, but all propolis samples inhibited the formation of plaques of VS and vaccinia viruses. All propolis samples in the dose of 1000 μg/disk were also toxic for CEF tissue cultures.

For futher studies propolis designated P7, showing a low toxicity and a high antiviral activity was chosen. It has been demonstrated, using agar diffusion method, that the lowest effective dose of propolis was 80 μg/disc (Table 2). In further experiments it was found, that antiviral

Table 1. Antiviral activity of propolis from various apiaries

Propolis symbol	Toxicity	Activity against viruses	
		VS	vaccinia
P1	17	22	21
P2	17	21	2*
P3	15	17	17
P5	12	13	14
P6	15	17	17
P7	14	19	16
P8	13	16	15
Ethanol control 96%	0	0	0

Explanation: the diameter of zones of toxicity and antiviral activity are given in mm; propolis concentration — 100 µg/disc.

activity of propolis was related to the concentration of ethanol used in extraction of crude, dry propolis (Table 3).

When propolis was extracted with 10% ethanol, such solution did not show toxicity or antiviral activity. Propolis extracted with 25% alcohol was toxic for CEF cell cultures, however, it did not show any antiviral

Table 2. Dependence of antiviral activity on propolis dose

Propolis concentration µg/disc	Toxicity	Activity against virus	
		VS	vaccinia
1000	14	19	16
100	12	13	13
80	11	12	12
60	0	0	0
40	0	0	0
20	0	0	0
-	0	0	0

Explanation: the diameter of zones of toxicity and antiviral activity are given in mm.

Table 3. Antiviral activity of ethanol — water extracts of propolis

Ethyl alkohol %	Toxicity	Activity against viruses	
		VS	vaccinia
10	0	0	0
25	12	0	0
60	13	16	15
96	12	15	14

Explanation: the diameter of zones of toxicity and antiviral activity are given in mm; propolis concentration 100 µg/disc.

activity against VS and vaccinia viruses. It was found that 60 and 96% ethanol was the suitable solvent for extraction of propolis. Those propolis extracts obtained after extraction with 60 and 96% ethanol, showed a high activity against SV and vaccinia viruses. Among the known antiviral substances of natural origin a great interest has been attached to flavonoids, because of their broad biological activity (4). Flavonoids exert antiviral activity against enveloped viruses (2), especially a potential virucidal activity and a slight inhibitory effect on the viral multiplication. In our experiments (Table 4), organic solvents extracts of propolis exhibited antiviral activity against VS and vaccinia viruses. In all extracts flavonoids were present as demonstrated by the chemical methods, however most of these compounds were extracted with ether: benzene (1:1) and with benzene, and these extracts exhibited a higher antiviral activity. In further experiments attemps were made to determine the mechanism of antiviral activity of propolis. Because of virucidal effect and toxicity of ethanol itself for the cell culture, propolis sample P7 extracted with 60% ethanol was evaporated to dry and dissolved in DMSO.

Table 4. Antiviral activity of organic solvents extract of propolis

Propolis symbol	Organic solvents	Concentration of flavonoids mg/ml of extr.	Toxicity	Activity against viruses	
				VS	vaccinia
E-B	ether: benzene /1:1/	1.94	0	18	18
B	benzene	1.93	0	17	16
CCl_4	tetrachloromethane	1.46	0	14	14
Chl^4	chloroform	1.48	0	14	14
A	acetone	1.67	0	14	14
K	ethanol 96%	1.68	12	14	14

Explanation: the diameter of zones of toxicity and antiviral activity are given in mm; concentration of extracts 100 µg/disc; concentration of flavonoids were estimated according to the method of Christ-Müller.

Table 5 presents the results of studies of virucidal action of propolis. Doses of 100 or 1000 µg/ml of propolis were incubated at 4°C with VS and vaccinia viruses for 1 hr and 24 hrs. As compared with the controls, where instead of propolis appropriate amounts of DMSO were added, the titre of VS was observed to decrease by 0.5 log after 1 hr and by over 5 log after 24 hrs of incubation. Vaccinia virus was less sensitive to propolis and its titre decreased only by 0.5 log after 24 hrs of incubation with propolis. Some of the known antiviral substances act by interferon induction (1, 3, 5, 7). Their activity is highest if they are administred prior to virus.

In our experiments propolis did not protect the CEF cell culture against VS virus. The nontoxic propolis doses of 25, 10 and 1 µg were incubated with CEF cell cultures at 37°C for 24 hrs. Then, after removing

Table 5. Virucidal activity of propolis P7

Time of incubation at 4°C hrs	Propolis concentration µg/ml	Virus titre /$TCID_{50}$/ml/	
		VS	vaccinia
1	0 /DMSO control/	$10^{7.0}$	$10^{6.0}$
	1000	$10^{6.5}$	$10^{6.0}$
24	0 /DMSO control/	$10^{5.8}$	$10^{5.5}$
	100	$10^{4.5}$	$10^{5.5}$
	1000	0	$10^{5.0}$

Explanation: $TCID_{50}$ (tissue culture infectious dose) 50% — dilution of virus that cause cythopatic effect in 50% of cell cultures; propolis was dissolved in DMSO.

propolis the cells were infected with VS virus at a dose of 100 $TCID_{50}$ per test tube. In comparison with control no protection of cell culture from cythopatic action of VS virus was found (data not presented). Propolis (10 µg/ml) exerted only a slight inhibition of replication of VS virus (Table 6). As in our experiments propolis only slightly inhibited the replication of VS virus, and showed a certain virucidal activity, we suppose that antiviral activity of propolis may be connected with the presence of flavonoids in propolis.

The attempt to separate the substances with antiviral activity from propolis will be continued.

Table 6. Effect of propolis P7 on replication of virus in CEF cell cultures

Propolis concentration µg/ml	Virus titre /$TCID_{50}$/ml/	
	VS	vaccinia
/DMSO control/	$10^{6.0}$	$10^{5.55}$
10	$10^{5.31}$	$10^{5.55}$

Explanation: dose of viruses 10 $TCID_{50}$/cell; harvest time 24 hrs; propolis was dissolved in DMSO.

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STRESZCZENIE

Badano *in vitro* przeciwwirusową aktywność propolisu, stosując dwa wirusy testowe: wirus *stomatitis vesicularis* (VSV) oraz wirus krowianki. Aktywność propolisu zależała nie tylko od pochodzenia (rejonu kraju), ale także od metody ekstrakcji surowego propolisu. Ekstrakcja 60 i 96% alkoholem etylowym dawała preparaty wykazujące aktywność przeciwwirusową. Z kolei rozpuszczenie takiego preparatu w mieszaninie eter:benzen (1:1) ekstrahowało więcej aktywnych przeciwwirusowo substancji niż stosowanie innych rozpuszczalników organicznych. Mechanizm przeciwwirusowego działania propolisu polegał głównie na jego wirusobójczej aktywności, chociaż w niewielkim stopniu propolis hamował również replikację wirusa VS. Przypuszcza się, że za aktywność przeciwwirusową odpowiedzialne są głównie flawonoidy zawarte w propolisie.

РЕЗЮМЕ

Антивирусную активность прополиса исследовали *in vitro* с применением двух тестовых вирусов: *stomatitis vesicularis* (VSV) и вируса оспенного детрита. Активность прополиса зависела не только от происхождения (района страны), но и от метода экстракции сырого прополиса. Благодаря экстракции 60%-ым и 90%-ым этиловым спиртом получали препараты, обладающие антивирусной активностью. В свою очередь, при растворении такого препарата в смеси эфир: бензол (1:1) экстрагировали больше антивирусно активных веществ, чем при применении других органических растворителей. Механизм антивирусного действия прополиса заключался, главным образом, в его вирусоцидной активности, хотя прополис также незначительно тормозил репликацию вируса VS. Предполагают, что за антивирусную активность ответственны главным образом содержащиеся в прополисе флавониды.