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***In vitro* antiviral activity of propolis**

M Amoros ¹, F Sauvager ², L Girre ¹, M Cormier ²

¹ *Faculté de Pharmacie, Laboratoire de Pharmacognosie, 35043 Rennes Cedex;*

² *Faculté de Pharmacie, Laboratoire de Microbiologie Pharmaceutique, Rennes, France*

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Summary — The *in vitro* effect of propolis on several DNA and RNA viruses including herpes simplex type 1, an acyclovir resistant mutant, herpes simplex type 2, adenovirus type 2, vesicular stomatitis virus and poliovirus type 2, was investigated. The inhibition of poliovirus propagation was clearly observed through a plaque reduction test and a multistep virus replication assay with a selectivity index equal to 5. At the concentration of 30 µg/ml, propolis reduced the titer of herpes simplex viruses by 1 000, whereas vesicular stomatitis virus and adenovirus were less susceptible. In addition to its effect on virus multiplication, propolis was also found to exert a virucidal action on the enveloped viruses HSV and VSV.

propolis / herpes simplex virus / adenovirus / vesicular stomatitis virus / poliovirus / antiviral activity

INTRODUCTION

Propolis has been reported to possess interesting biological activities such as bacteriostatic (Villanueva *et al*, 1964, 1970; Lindenfesler, 1967; Scheller *et al*, 1977; Pepeljnjak *et al*, 1981), antimycotic (Lindenfelser, 1967; Millet-Clerc *et al*, 1987) and antiprotozoan properties (Starzyk *et al*, 1977). As regards the antiviral activity, propolis may affect the reproduction of influenza virus (Shevchenko *et al*, 1972), influenza viruses A and B, vaccinia virus and NDV (Esanu *et al*, 1981, Maximova-Todorova *et al*, 1985), and of avian herpes (König *et al*, 1985, 1988). An anti-

herpes cream (Sosnowski, 1983), an ointment against herpes zoster (Popescu *et al*, 1985) and a drug against cutaneous herpes and herpes zoster have also been described (Giurcocaneanu *et al*, 1988).

The above evidence led us to include propolis in our study of the antiviral properties of plants from Brittany (Amoros *et al*, 1977; Suganda *et al*, 1983; Amoros *et al*, 1987; Rodriguez *et al*, 1990) since a close similarity between propolis and the bud exudate collected by bees has been shown (Greenaway *et al*, 1987, 1988).

The present paper reports the *in vitro* antiviral activity of a batch of propolis gathered near Rennes (France) against a

range of viruses representative of both DNA and RNA virus groups namely for RNA viruses, poliovirus type 2, PV (non enveloped), vesicular stomatitis virus, VSV, (enveloped) and for DNA viruses, adenovirus type 2, Adeno-2 (non enveloped) and 3 strains of human herpes simplex virus, HSV-1, HSV1-R and HSV-2 (enveloped).

MATERIALS AND METHODS

Preparation of propolis balsam

Propolis (30 g) collected from a private apiary near Rennes (France) was extracted with 80% ethanol (300 ml) for 18 h at room temperature with stirring. Evaporation of the solvent under reduced pressure led to a dry residue (20 g) which, according to Ghisalberti (1979), was called propolis balsam. This crude extract was dissolved in dimethylsulfoxide (10 mg/ml) previous to dilution with medium at the appropriate concentrations.

Cells and viruses

African green monkey kidney cells (Vero) were used in all experiments. Cells 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 gentamycin. Cells were routinely passaged every 3 days. Three strains of herpes simplex virus were used: type 1 (HSV-1) strain H29S; type 1 acyclovir resistant mutant (HSV1-R) strain H29R and type 2 (HSV-2). The latter was obtained from a clinical isolate at the Pontchaillou Hospital in Rennes (France). Adenovirus type 2 (Adeno 2) was provided by Pr Kedinger, Faculty of Medicine, Strasbourg (France). Poliovirus type 2 (PV) was the vaccinal strain Sabin II propagated in our laboratory by serial passages on Vero cells. Vesicular stomatitis virus (VSV) was supplied by the virology department, where it is currently used for interferon titrations. All viruses were cultured in Vero cells. Virus stocks were

prepared as follows: nearly confluent monolayer cultures were infected at low multiplicities, incubated for 2 days, then frozen and thawed 3 times before clearing of the preparations by centrifugation at low speed to remove cell debris. The resulting supernatant fluids were stored at -70 °C until used. Virus titrations were performed by the limit dilution method, using 6 wells of a 96-cell Nunclon microplate per dilution. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses per ml (TCID₅₀).

Propolis balsam cytotoxicity

To assess the effect of propolis balsam on uninfected Vero cells, dilutions from 10 to 100 µg/ml in maintenance medium were added to monolayers of Vero cells (4 25 cm² cultures flasks seeded with 2.10⁶ cells per dilution). After incubation for 96 h, cytotoxicity was determined by microscopic examination of cell morphology and cell number in treated and untreated cultures by Trypan exclusion after trypsinization (Payment *et al*, 1989).

Plaque reduction assay

Confluent monolayers of Vero cells in 24-well tissue culture plates (4 x 10⁵ cells/ml/well) were infected with approximately 50 plaque-forming U/well in 0.1 ml of medium. Following 1 h of incubation at room temperature, the remaining viruses were removed by aspiration and the monolayers were washed twice with phosphate buffered saline (PBS pH 7.4) before adding fresh medium supplemented with 5% calf serum, 0.5% methyl cellulose (4 000 centipoises/s) and appropriate concentrations of balsam. Cultures were stored at 37 °C in a humidified 5% CO₂ atmosphere for 2 days (PV and VSV), or 4 days (HSV and adenovirus). The overlay medium was then removed by inverting the plate and the layer was stained with 1 ml of 0.1% Crystal violet in ethanol-water (20:80). The dye was removed after 1 h by gentle rinsing with PBS and the plaques were counted. For pretreatment experiments, MEM with or without propolis was added 24 h before infection. Thereafter, the cells were rinsed and infected with 50

PFU/well. No propolis was included in the overlay. Each assay was carried out in triplicate and each experiment was repeated 3 times.

Virus yield reduction test

Confluent 1-day-old monolayers of Vero cells in 24-well tissue culture plates were infected with viruses at an infection multiplicity within about 0.01 PFU per cell and various concentrations of propolis balsam were added to each well. Virus controls without propolis were run simultaneously. The plates were incubated at 37 °C for the duration of 4 cycles of multiplication, *ie* 28 h for VSV, 32 h for poliovirus, 72 h for HSV-1, HSV-1R and HSV-2, 132 h for adenovirus. The cultures were then frozen at -70 °C. After 3 cycles of freezing-thawing, sets of 3 cultures were harvested, cell debris were removed by low speed centrifugation and the virus yield of each supernatant was quantified by the dilution method in microtissue culture plates. Each assay was determined in triplicate.

Direct inactivation of viruses

0.2 ml of the different virus-stocks were incubated with 0.2 ml of MEM containing 100 to 5 000 µg/ml of propolis balsam (final concentrations on viruses from 50 to 2 500 µg/ml) or with 0.2 ml of MEM without propolis for 15, 30, 60, 120 min at 37 °C. Before incubation the virus titers were 10^{6.5} for HSV-1, 10⁴ for HSV-2 and 10⁶ TCID₅₀ per ml for VSV. Thereafter, the virus suspensions were serially diluted 10-fold and assayed for remaining virus in Vero cells. Propolis was primarily dissolved in DMSO then diluted in the culture medium. Analogous dilutions of DMSO in MEM were tested in order to control the direct effect of DMSO on viruses.

Search for HSV-1 resistant to propolis

Confluent monolayers in 24-well tissue culture plates were inoculated with HSV-1 to give a MOI of about 1. Viruses were adsorbed at room temperature for 60 min with gentle agitation. The inoculum was discarded and 1 ml of medium sup-

plemented with 30 µg/ml propolis balsam was added to each well. Controls without propolis were included in the experiment. When CPE occurred, the plates were frozen and thawed 3 times, and after centrifugation the culture medium of assays and controls was used to inoculate a new set of plates. This process was repeated 4 times, viral titers being determined after each passage.

RESULTS

Drug cytotoxicity

As determined by microscopic examination, balsam at concentrations up to 30 µg/ml was well tolerated by Vero cells over a 96-h exposure. A slight cytotoxicity was observed with concentrations from 30 to 60 µg/ml, and, at the upper concentrations of 80 and 100 µg/ml, rounding and shrinkage of the cells appeared. These preliminary observations were confirmed by counting the cells after trypsinization. The results are expressed as percentages of the values obtained in control cultures without drug and plotted against the increasing concentrations (data not shown). The concentration which caused cytotoxic effect in 50% of the cultured cells (CC₅₀), determined graphically, was found to be 72 µg/ml. However, the concentration of 30 µg/ml appeared to be the highest dose without any discernible toxic effect on cell growth; we therefore used this dose as maximum concentration throughout all experiments.

Virucidal activity

Exposure of polio and adenovirus to propolis balsam at concentrations up to 2 500 µg/ml for 2 h resulted in no loss of infectivity, whereas enveloped viruses HSV-1,

HSV-2 and VSV were susceptible. In the same experimental conditions, DMSO did not exert any virucidal effect against these viruses. As shown in table I virus inactivation was time and dose-dependent and the infectivity of all 3 viruses was reduced to zero within 120 min by a dose of 500 µg/ml propolis. However, the antiviral activity of propolis observed in the *in vitro* assays could not be attributed to a direct virus inactivation, since the concentrations used for plaque inhibition or yield reduction assays were much lower (maximum 30 µg/ml).

Effect of propolis balsam on virus plaque formation

The activity of balsam on replication of each virus in Vero cell cultures was initially studied by examining its ability to interfere with virus plaque formation. Propolis appeared to be a potent inhibitor of poliovirus since plaque formation was totally inhibited at a concentration of 30 µg/ml. For a lower range of concentrations, the resultant plaque counts were expressed as percentages of the counts obtained with un-

treated virus control cultures and the values were plotted against the concentration logarithm (graph shown in fig 1). The concentration of propolis required to reduce the number of plaques by 50% was determined as 14 µg/ml. The selectivity index computed by dividing the drug concentration which causes cytotoxic effects in 50% of the cultured cells (CC_{50}) by this 50% effective dose (ED_{50}) was found to be equal to 5 which may be compared to antiviral indexes determined for some therapeutic agents: 6 to 12 for IUdR, 1 to 2 for ara-C and 5 to 6 for ara-A (De Clercq *et al*, 1978). For the other viruses tested, the plaque number reduction was not significant. However, the size of the plaques produced by HSV-1, HSV-1R, HSV-2, Adeno-2 and VSV was greatly reduced when propolis was incorporated in the overlay medium. For instance, from the concentration of 10 µg/ml the diameter of HSV-1 plaques was reduced by half in comparison with controls and at the upper concentration of 30 µg/ml, it was found to be 4 times lower. At the same concentration of 30 µg/ml the size of VSV plaques was also 4 times lower than the controls, whereas the size of adenovirus plaques was reduced by half.

Table I. Effect of increasing concentrations of propolis balsam on the infectivity of HSV-1, HSV-2 and VSV.

Propolis (µg/ml)	HSV-1					HSV-2					VSV				
	0	50	100	250	500	0	50	100	250	500	0	50	100	250	500
Time of exposure (min)															
15	6.5	6.5	6.5	6	5	4	4	3	3	0	6	6	6	5	5
30	6.5	6.5	6.5	6	0	4	4	3	3	0	6	6	6	5	4
60	6	6	6	5.5	0	4	4	3	1	0	5	5	5	5	1
120	4	4	3.5	2.5	0	2	2	1	0	0	5	5	4	2	0

Virus-propolis suspensions were incubated at 37 °C for 15, 30, 60, 120 min prior to determination of infectivity. Infectivity was expressed as \log_{10} TCDI₅₀/ml.

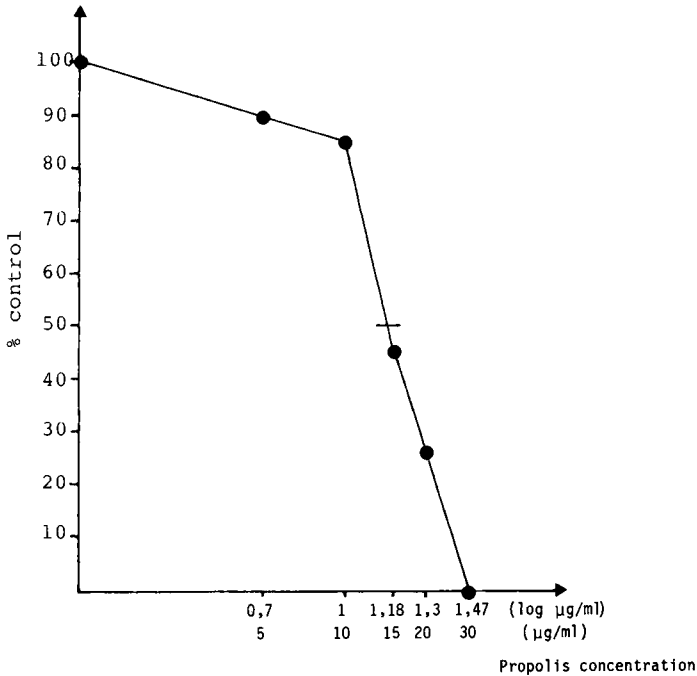


Fig 1. Effect of increasing concentrations of propolis on the replication of poliovirus. The sensitivity of the virus was measured by a plaque reduction assay in Vero cell cultures. Plates were counted 48 h after infection. The values represent the mean of 3 experiments.

In one set of experiments the cells were pretreated with propolis for 24 h at 37 °C prior to virus infection without addition in the overlay. In these experimental conditions, no inhibition was observed.

Yield reduction

The yield of infectious viruses produced in the presence of varying concentrations of propolis balsam following 4 rounds of multiplication is shown in table II. The greatest reduction in virus yield was obtained with poliovirus. This result was in full agreement with the plaque inhibition assay.

HSV-1, HSV-2 and the HSV-1 acyclovir resistant mutant appeared to be less susceptible. However, treatment of cultures infected by one or the other HSV with propolis balsam partly inhibited viral replication. For instance, when the medium was supplemented with propolis 30 µg/ml, HSV replication was inhibited by 99.9% which appeared a promising activity and could explain the reduction of the plaque size observed in the previous test. Relating to adenovirus and VSV the yield of infectious virus was reduced by approximately 90% at the upper concentration of propolis, the lower doses being virtually inactive. This result could also explain the reduction in plaque size, although a mathematic corre-

Table II. Effect of propolis on poliovirus, vesicular stomatitis virus, herpes simplex viruses and adenovirus multiplication, in the yield reduction test.

Propolis concentration ($\mu\text{g/ml}$)		Virus titer (\log_{10} TCID ₅₀ /ml)				
PV	VSV	HSV-1	HSV-1R	HSV-2	Adeno	
None	8	7.5	6.5	7	4.5	2
5	8	7.5	6.5	7	4	2
10	6	7.5	5.5	7	3	2
15	5	7.5	5	5.5	2.5	2
20	3	7	4.5	5.5	1	2
30	2	6.5	3.5	4	1	0.5

Increasing concentrations of propolis were added to Vero cells inoculated at a MOI of about 0.01; virus titrations were performed after 4 cycles of multiplication. Each assay was determined in triplicate.

lation between the reduction of plaque size and the reduction of virus titer did not appear.

DISCUSSION

The results presented here provide evidence that propolis is very active *in vitro* against poliovirus and herpes viruses, whereas vesicular stomatitis virus and adenovirus are less susceptible.

Propolis balsam is a very complex mixture of compounds including benzoic acid and esters, substituted phenolic acid and esters, terpenoid and flavonoid aglycones (Vanhaelen *et al*, 1979; Bankova *et al*, 1987). In our propolis sample, flavonoids were found to be the main constituents. High-performance liquid chromatography indicated galangin and chrysin as the major flavonoids, which was in agreement with the findings of Greenaway *et al* (1988), whereas pinocembrin and galangin were found to be the major flavonoids in a Bulgarian propolis sample (Bankova *et al*, 1982). The properties of propolis might be

explained by this high content in flavonoids. Quercetin and luteolin were shown to be virucidal (Mucsi *et al*, 1977; Kaul *et al*, 1985), the sensitive viruses being only those with membranes. Therefore, the extracellular inactivation of enveloped viruses might be due to the flavonoids present in propolis. Also, the good activity against picornaviruses of flavones, particularly methoxy-flavones has been reported (Ishitsuka *et al*, 1982; Van Hoof *et al*, 1985; Castrillo *et al*, 1986); the target is an early stage in the replication cycle since the viral RNA synthesis was drastically reduced provided 3-methoxyflavones were present between 1 and 2 h pi. Therefore, the remarkable inhibition of poliovirus in propolis treated cultures could result from its high content in flavonoids. Among these, a 3-methoxyflavone named ermanine has been particularly pointed out by Vanhaelen *et al* (1979). For the other tested viruses, virus yields were reduced by a 1 \log_{10} or more, whereas a reduction in the plaque size was noted in the plaque reduction test. According to Streissle *et al* (1981) the growth rate of plaques depends on the number of infectious units (IU) which are released from the

infected cells to infect adjacent cells. Plaque formation may not take place or cease at an early stage when the average number of IU which determines the spreading of infection is small. In propolis treated cultures, the reduction of plaque size could be explained in this way, but only if a few infectious units have been released after one multiplication cycle, the viral titer should be low after 4 cycles. To explain the partial reduction in virus titer found for HSV, Adeno and VSV, we suggest that during a longer period of incubation these particles spread more easily in liquid medium and replicate, allowing the rise of the viral titer. This might occur if the active compound is degraded during the incubation as has been reported for nucleoside analogues (Reefschläger *et al*, 1982). To account for this possibility, a propolis solution was maintained at 37 °C for 72 h before being added to infected cells. In comparison with a fresh solution, no differences in viral titer reductions were noted. This also might occur if the active compound is in too poor a concentration in the medium to be maintained at a sufficient level, for instance if this active compound is a minor flavonoid. To verify this hypothesis it would be necessary to increase propolis concentration in the medium 4-fold but this is difficult to do without approaching the toxic dose. Other compounds such as benzoic esters or caffeic acid esters might also act, since the interest of caffeoyl conjugates against HSV-1 (Konig *et al*, 1985) and VSV (Cheminat *et al*, 1988) has been emphasized. These compounds are present in our propolis sample at low levels, but so far we have failed to isolate them and could not verify their activity. Another possibility would be the emergence, under multistep conditions, of a virus progeny resistant to propolis. This hypothesis has been discarded after experiment, as viral titers were non significant after 4 passages in propolis-treated cultures. Finally,

the present study showed the interesting *in vitro* effect of propolis against a range of DNA and RNA viruses. Propolis being such a complex product, several compounds could be responsible for the activity against HSV viruses, adenovirus and vesicular stomatitis virus, whereas flavonoids are probably the inhibitory compounds of poliovirus.

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Résumé — Activité antivirale *in vitro* de la propolis. L'activité antivirale de la propolis a été étudiée *in vitro* contre une gamme de virus représentative des virus à ADN (adénovirus type 2, herpès simplex virus type 1 et 2) et des virus à ARN (virus de la stomatite vésiculaire et poliovirus type 2).

La propolis exerce un effet virucide sur les virus enveloppés HSV-1, HSV-2, VSV. Le tableau I indique les réductions de titre infectieux (en \log_{10}) en fonction de la concentration en propolis et du temps d'incubation, comparativement aux témoins-virus. L'effet virucide est net aux concentrations élevées 250 et 500 $\mu\text{g/ml}$ mais il n'y a pas d'inactivation directe à la concentration maximale utilisée pour tester l'activité antivirale (30 $\mu\text{g/ml}$).

L'activité antivirale de la propolis a été déterminée par le test de réduction du nombre de plaques. La figure 1 indique l'inhibition des plages de poliovirus en fonction de la concentration en propolis. La concentration réduisant de 50% le nombre de plages est de 14 $\mu\text{g/ml}$, l'inhibition étant

totale à la concentration de 30 µg/ml. Pour les autres virus, le nombre de plages n'est pas diminué; par contre, leur diamètre est considérablement réduit (au 1/4).

L'effet de la propolis sur la multiplication virale a également été recherché par la mesure, après 4 cycles de multiplication, du titre infectieux de cultures additionnées de propolis à des concentrations variant de 0 à 30 µg/ml. Les réductions de titre infectieux par rapport à ceux des témoins-virus sont indiquées tableau II. Le poliovirus est le plus fortement inhibé mais l'activité de la propolis sur les 3 souches de virus herpétique est également intéressante, l'inhibition atteignant 99,9% à la concentration de 30 µg/ml. L'adénovirus et le virus de la stomatite vésiculaire sont moins sensibles.

Le rôle éventuel, dans l'activité antivirale, des différents composés identifiés dans la propolis est discuté par comparaison avec les résultats d'autres auteurs. La haute teneur en flavonoïdes semble responsable de l'activité contre le poliovirus tandis que d'autres composés, comme des esters de l'acide caféique, pourraient également jouer un rôle dans l'activité antiherpétique.

propolis / herpès simplex virus / adénovirus / virus de la stomatite vésiculaire / poliovirus

Zusammenfassung — Die antivirale Aktivität von Propolis im Experiment. Die antivirale Aktivität von Propolis wurde im Experiment gegen Verdünnungsreihen von Viren des Typs ADN (Adenovirus Typ 2, Herpes simplex-Virus Typ 1 und 2) und des Typs ARN (Vesikuläres Stomatitis-Virus, Poliovirus Typ 2) geprüft. Propolis übt eine viroide Wirkung auf Viren mit Hülle vom Typ HSV-1, HSV-2 und VSV aus. Tabelle I zeigt die Reduktion des in-

fektiösen Titers (in log₁₀), im Verhältnis zu einem Kontrollvirus, in Funktion der Propolis-konzentration und der Inkubationszeit. Der viroide Effekt ist in höheren Konzentrationen von 250 und 500 µg/ml eindeutig, aber es wurde keine direkte Inaktivierung bei der benutzten Maximalkonzentration (30 µg/ml) in dem Versuch über die antivirale Aktivität beobachtet.

Die antivirale Aktivität des Propolis wurde mit dem Test der Reduktion der Zahl der Plaques bestimmt. Abbildung zeigt die Hemmung der Poliovirus-Plaques in Funktion der Propolis-konzentration. Die Konzentration, welche die Zahl der Plaques um 50% reduziert, belief sich auf 14 µg/ml, die Konzentration mit einer totalen Hemmung betrug 30 µg/ml. Bei den anderen Viren wurde die Zahl der Plaques nicht verringert, aber ihr Durchmesser wurde beträchtlich vermindert (um 1/4).

Außerdem wurde die Wirkung von Propolis auf die Vermehrung der Viren untersucht, indem nach vier Vermehrungszyklen der Infektionstiter von Kulturen bestimmt wurde, denen man Propolis in verschiedener Konzentration, von 0 bis 30 µg/ml, zugesetzt hatte. Die Reduktion des Infektionstiters im Verhältnis zum Kontrollvirus ist in Tabelle II angegeben. Das Poliovirus wird am stärksten gehemmt, aber die Aktivität auf die drei Stämme des Herpesvirus ist ebenfalls beträchtlich: die Hemmung erreicht 99.9% bei einer Konzentration von 30 µg/ml. Das Adenovirus und das Virus der Vesikulären Stomatitis sind weniger empfindlich.

Durch Vergleich mit den Resultaten anderer Autoren wird die mögliche Rolle der verschiedenen bisher bestimmten Komponenten des Propolis diskutiert. Der hohe Gehalt an Flavenoiden scheint für die Aktivität gegen das Poliovirus verantwortlich zu sein, aber auch andere Komponenten, wie die Ester der Kaffeesäure, könnte bei der Wirkung gegen Herpes eine Rolle spielen.

Propolis / Herpes simplex-Virus / Adenovirus / Virus der Vesikulären Stomatitis / Poliovirus

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