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EFFECTS OF PROPOLIS FLAVONOIDS ON VIRUS INFECTIVITY AND REPLICATION

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SUMMARY

The effect of five propolis flavonoids on the infectivity and replication of some herpesvirus, adenovirus, coronavirus and rotavirus strains has been studied. Experiments were performed *in vitro* in cell cultures using the viral plaque reduction technique. The cytotoxicity of flavonoids, including chrysine, kaempferol, acacetin, galangin and quercetin, was evaluated on uninfected monolayers to determine their effect on cell growth and viability.

Chrysine and kaempferol caused a concentration-dependent reduction of intracellular replication of herpes-virus strains when monolayers were infected and subsequently cultured in a drug-containing medium. However, virus infectivity was not significantly affected. Acacetin and galangin had no effect on either the infectivity or replication of any of the viruses studied. Quercetin reduced infectivity and intracellular replication, but only at the highest concentrations tested.

KEY WORDS *Flavonoids, antiviral activity, virucidal activity.*

INTRODUCTION

Flavonoids are a group of substances that are widely distributed throughout the vegetable kingdom: they have been the subject of study because of their biochemical and pharmacologic properties (Havsteen, 1983; Farkas *et al.*, 1986). These natural substances have anti-inflammatory (Gabor, 1979), anti-carcinogenic (Kuhnau, 1976) and anti-allergic (Middleton *et al.*, 1981) activity.

Other authors have also studied the mutagenic properties of some flavonoids and have defined the structural characteristics of the molecule that are essential for the activity described (MacGregor *et al.*, 1978).

Furthermore, some flavonoids' inhibition of viral replication is of particular interest (Van den Berghe *et al.*, 1986; Kaul *et al.*, 1985; Mucsi *et al.*, 1985). Some 3-methoxyflavons have been

shown to be active both *in vitro* and *in vivo* in terms of the replication of picornaviruses.

Studies conducted on the mechanism of action demonstrated that these flavonoids specifically inhibit one step of viral replication between uncoating and the start of RNA synthesis (Ishitsuka *et al.*, 1982).

Some flavonoids, such as quercetin and quercetrin, have an additive or synergic effect, respectively, on herpes simplex virus type 1 and pseudo-rabie virus when administrated together with 5-ethyl-2-deoxyuridine (Mucsi *et al.* 1984).

These natural substances also increase the intracellular level of cyclic AMP (cAMP), and this effect seems to be closely connected with antiviral activity. Indeed, flavonoids have been described as powerful inhibitors of cellular cAMP phosphodiesterase, an enzyme responsabile for the breakdown of cAMP (Mucsi *et al.*, 1985). It has also been demonstrated that the antiviral activity of flavonoids, with a free hydroxyl group in position 3, is increased by ascorbate which prevents oxidative degradation in aqueous solution.

Many flavonoids are present in propolis in the form of glycosides (Farkas *et al.*, 1986). However, various extractions carried out for analytical and preparative purposes produce their corresponding aglycons instead. Therefore, the data currently available do not provide a clear, cognitive picture of the glycoside/aglycon ratio present in propolis, or better still in the various grades of propolis, which tand to be characterized as a function of their origin.

A "bouleau" type raw material, whose declared origin was Georgia, was used for extraction of the flavonoids considered in this study (chrysine, kaempferol, acacetin, galangin and quercetin). As these natural substances have been demonstrated to be extremely important, potential antiviral agents, we decided to evaluate the activity of five flavonoids extracted from propolis on some DNA and RNA viruses.

MATERIALS AND METHODS

Viruses and cells

Type 2 (MS-2 strain) and type 1 (McIntyre strain) human herpes simplex viruses (HSV) and the type 1 TK^- mutant (CGU-2 strain) were propagated on VERO cells. The titers of the stocks obtained were $5x10^5$ plaque-forming units (p.f.u.) per milliliter of HSV-2 and $1x10^6$ of HSV-1 and TK^-.

Bovine herpes virus BHV-1 was cultured on embryonic bovine lung fibroblasts and titrated at $2x10^6$ p.f.u. per milliliter. Human adenovirus (Ad-2) was grown on Hep-2 cells; the stock obtained had a titer of $1x10^6$ p.f.u. per milliliter.

Human coronavirus OC43 and bovine NCDCV were suitable for growth on embryonic bovine lung fibroblasts. Stock with titers of $2x10^5$ and $1x10^7$ p.f.u. per milliliter were used for OC43 and NCDCV, respectively. The SA-11 monkey rotavirus strain was grown on MA-104 cells as described elsewhere (Ferrari *et al.*, 1986). The stock used in this study was titrated at $2x10^4$ p.f.u. per milliliter.

Cell cultures were grown in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (E. MEM 10% FBS), 50 μg of streptomycin per ml and 1% glutamine. The maintenance medium varied only in terms of FBS concentration (2%).

A plaque test was used to titrate the viruses in confluent monolayers of cells grown in 96-well tissue culture microplates.

Flavonoid extraction

The method adopted was that used by A. Bilyk and G.M. Sapers for analytical purposes (Bilyk *et al.* 1985). Preparative HPLC was used to isolate and purify the flavonoids (acacetin, kaempferol, chrysine, quercetin and galangin), both under the conditions identified by the authors cited above and under those suggested by I. McMurrough, G.P. Henningan and M.J. Loughrey.

Flavonoid toxicity

Flavonoid toxicity was studied on uninfected cell growth and cell cultures by evaluating both the inhibition of cell growth and cell viability by the Trypan blue exclusion method as previously described (Dubovi *et al.*, 1981). Approximately 10^5 cell of each culture used in this study were inoculated into a 25 cm^2 cell-culture flask and incubated for 24 hrs at 37°C 5% CO_2. After incubation, the growth medium was replaced with a new medium containing various concentrations of each flavonoid (from 1 to 100 μg/ml).

Control cells were treated with a medium that did not contain test compounds. The monolayers were trypsinized 0, 24, 48 and 72 hrs after the addition of the flavonoids, and the number of whole cells and viable cells was calculated.

The dose of compound that inhibited the growth of 50% of the cells, with respect to the controls, after 72 hrs of treatment was considered to have a cytotoxic activity of 50%.

Plaque reduction assay

Approximately 10^2 p.f.u. of each virus was inoculated onto confluent monolayers of cells grown on 96-well microplates (10^2 p.f.u.: well). After an absorption period of approximately 1 h at 37°C 5% CO_2, the monolayers were washed with Hank's balanced salt solution (HBSS), and control maintenance medium (E. MEM 2% FBS) and the same medium containing various concentrations of the test compounds (100 μl/well) were added to the wells.

Each flavonoid was tested at concentrations that varied from a cytotoxic dose of 50% to 0.05 μg/ml. At least four wells were used for each concentration of the tested compounds and as controls.

After 24 hrs (for HSV-2, HSV-1, TK^- and coronaviruses) and 48 hrs (for adenoviruses and rotaviruses) of incubation at 37°C 5% CO_2 (33°C for coronaviruses), the medium was removed, and the cells were fixed for 10 min with absolute ethanol. For each well, the number of plaque of infections was determined by the indirect immunoperoxidase technique. The flavonoids' antiviral activity was expressed as ID_{50}, which was the minimum concentration of compound that reduced the number of plaques due to infection by 50% with respect to the controls.

Plaque assay

Plaque assays to determine the titration of the viruses were performed as described above for the plaque reduction assays, except that in this case serial dilutions of the virus were inoculated into each well, and after 1 h incubation, the number of infection plaques was determined using the indirect immunoperoxidase technique.

Immunoperoxidase staining technique

The immunoperoxidase technique was performed as previously described (Gerna *et al.*, 1980). We used both commercially available and positive sera with high antibody titers (hyperimmune), which had previously been tested with the same technique, for the viruses studied. Briefly, we used anti-HSV-2 or HSV-1 rabbit IgG (DAKO) for HSV-2, HSV-1 and TK^-; hyperimmune bovine serum and anti-bovine IgG peroxidase conjugated (DAKO) for BHV-1; hyperimmune human sera and anti-human IgG peroxidase conjugated (Kpl) for Ad-2 and rotaviruses, and hyperimmune bovine sera and anti-bovine IgG peroxidase conjugated (DAKO) for coronaviruses.

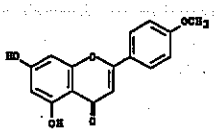
Direct virucidal activity assay

In order to evaluate possible virucidal activity, equal volumes (0.1 ml) of each virus stock and E. MEM 2% FBS containing various flavonoid concentrations (from 0 to a 50% cytotoxic dose) were mixed together and incubated for 1 h at 37°C. The viral suspensions were subsequently diluted and titrated by plaque assays. The lowest dilution tested had a flavonoid concentration of 0.01 μg/ml.

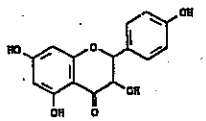
RESULTS

The effect of propolis flavonoids on virus replication

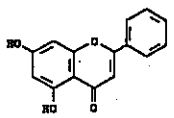
In these experiments, confluent monolayers of cell cultures were infected, and the inoculum removed with two HBSS washings 1 h after absorption at 37°C 5% CO_2 (or 33°C for coronaviruses). The monolayers were then treated with maintenance medium containing various concentrations of the flavonoids to be tested.



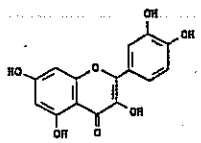
ACACETIN



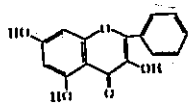
KAEMPFEROL



CHRYSINE



QUERCETIN



GALANGIN

FIGURE 1 - The structure of propolis flavonoids analyzed for antiviral activity.

After the time necessary for a complete viral replication cycle, the monolayers were fixed, and the plaques of infection determined using the indirect immunoperoxidase technique.

Two of the flavonoids studied, chrysine and kaempferol, proved to be highly active in inhibiting the replication of herpes-viruses HSV-2, HSV-1, TK⁻ HSV and BHV-1. The effect of chyrisne on herpes-virus, adenovirus, coronavirus and rotavirus replication is shown in Figure 2; the ID_{50} is approximately 0.15 μg/ml for BHV-1; 0.2 μg/ml for HSV-2; 0.5 μg/ml for HSV-1, and 0.8 μg/ml for TK⁻ HSV-1. In comparison with the other viruses studied, chrysine inhibited replication at significantly higher concentrations: the ID_{50} for adenovirus-2 is 10 μg/ml. At the same concentration, replication was reduced by approximately 45% for NCDCV, 30% for OC43 and 25% for the rotavirus SA-11.

In the case of kaempferol, the most sensitive viruses were herpes: the ID_{50} was approximately 3.5 μg/ml for BHV-1; 1 μg/ml for HSV-2, and approximately 2 μg/ml for HSV-1 and TK HSV-1. At kaempferol concentrations of 10 μg/ml, the reduction in replication of the other viruses studied was approximately 65% for NCDCV, 50% for OC43 and adenovirus-2, and 35% for rotavirus SA-11.

The flavonoids acacetin and galangin were not active in the viruses studied here, even at concentrations of 20 and 15 μg/ml respectively (cytotoxic dose) 50% of herpes-virus replication while its activity is not significant in the other viruses.

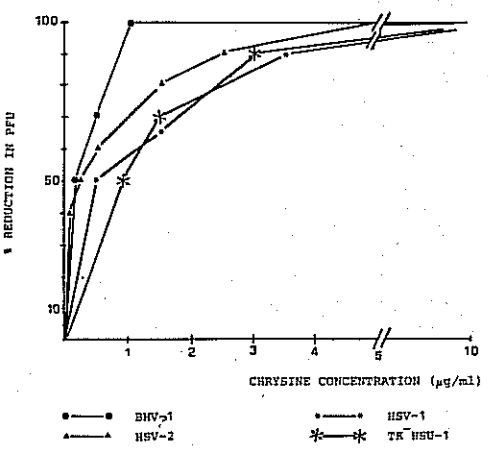


FIGURE 2 - The effect of chrysine on the replication of herpesviruses. Tissue culture cells were exposed to viruses for 1 h at 37°C and then overlayed with medium containing various concentrations of chrysine.

Flavonoid toxicity

The effect of propolis flavonoids on the replication and viability of unifected cell cultures was also studied. VERO, PFB and MA 104 cells were used. After 24 hrs of incubation at 37°C 5% CO_2, cell cultures obtained through inoculation of approximately $5x10^5$ cells into 25 cm^2 flasks were treated with various concentrations of each flavonoid. The total number of cells and viable cells in each culture was determined after 0, 24, 48, and 72 hrs, using the Trypan blue technique. Toxic effects, in relation to dose, were observed for each flavonoid tested. Table 1 lists the 50% cytotoxic dose for each substance in relation to the type of cell culture used. Results concerning cell viability also indicate that this parameter is not significantly altered. Indeed, over 95% of the cells were still viable after flavonoid treatment with a 50% cytotoxic dose.

Virucidal activity

We evaluated the effect of flavonoids on the direct inhibition of viral infectivity. In these experiments, various flavonoid concentrations (from 0 to 50% cytotoxic dose) were mixed with the viral stock and incubated for one hour at 37°C. Serial dilutions of each mixture were then inoculated into confluent monolayers of cells cultivated in 96-well microplates. At concentration of 10 μg/ml, chrysine reduced the infectivity of HSV-2, HSV-1 and TK^--HSV-1 by approximately 45% and it had a negligible effect on BHV-1. In addition, at concentrations equal to or less than 5 μg/ml, chrysine did not significantly alter viral infectivity.

Results obtained with quercetin are substantially in agreement with data published elsewhere (Kaul *et al.,* 1985). At a concentration of 60 μg/ml, quercetin reduced the infectivity of HSV-2, HSV-1 and TK^--HSV-1 by approximately 65%, BHV-1, Ad-2, OC43 and NCDCV by approximately 50%, and rotavirus SA-11 by approximately 70%. Starting at concentration of 20 μg/ml, quercetin did not affect viral infectivity at all.

Results obtained with kaempferol, galangin and acacetin show that these substances do not produce significant alterations in viral infectivity at 50% cytotoxic concentrations (30, 15 and 20 μg/ml, respectively).

DISCUSSION

The results obtained confirm previous observations (Kaul *et al.,* 1985; Van der Berghe *et al.,* 1986), indicating that some flavonoids inhibit the replication of certain viruses.

The substances that we studied were mainly flavonoids extracted from propolis. While there is no data in the literature about the possible antiviral activity of chrysine, acacetin and galangin, various studies have been conducted on the inhibitory effects of quercetin (Kaul *et al.,* 1985; Mucsi *et al.,* 1985; Mucsi, 1984; Vrijsen *et al.,* 1988), and some data are available on kaempferol (Van den Berghe *et al.,* 1986). Our results obtained on the activity of quercetin were completely in line with those obtained by other authors (Kaul *et al.,* 1985). On the other hand, there are descrepancies between our results on kaempferol and those presented in an earlier work (Van den Berghe *et al.,* 1986). As in the case of quercetin, differences can be explained by the fact that kaempferol may undergo oxidative degradation in aqueous solution, since it has a free hydroxyl group in position 3 (Vrijsen *et al.,* 1988).

This structural characteristic has also been considered essential for flavonoids' mutagenic activity. Furthermore, kaempferol, quercetin and galangin appear to have mutagenic activity in *Salmonella thyphimurium* (MacGregor *et al.,* 1978) which clearly precludes their possible therapeutic application.

TABLE 1

The effect of a 50% cytotoxic dose of Propolis flavonoids on three different cell cultures after 72 hours of treatment

Flavonoids	CYTOTOXIC DOSE 50%*		
	VERO	PFB	MA-104
Chrysine	10	10	10
Kaempferol	20	30	20
Acaceztin	20	15	20
Galangin	15	15	10
Quercetin	40	60	40

* A 50% cytotoxic dose in expressed as μg/ml.

Acacetin and galangin did not reduce the intracellular replication of the viruses studied, while chrysine proved to be effective in herpes-viruses (HSV strains and BHV-1). Indeed, the ID_{50} determined by plaque reduction assays varied from 0.15 to 0.8 μg/ml, depending on the type of herpes-virus. The 50% cytotoxic dose (the dose of substance that inhibits 50% of cell growth after 72 hrs of treatment) was 10 μg/ml.

Furthermore, chrysine has not virucidal effect except at cytotoxic concentrations. At a concentration of 10 μg/ml, the infectivity of the herpes-viruses is reduced by 50%, while at lower concentrations (5 μg/ml) the effect is negligible. Therefore, this flavonoid does not interact with the virus in an extracellular environment.

With reference to the studies on mutagenicity mentioned above, a few comments may be made about chrysine's chemical structure. This molecule does not have the structural characteristics considered to be essential for mutagenicity.

According to J.T. MacGregor (MacGregor *et al.*, 1978), the following structural characteristics are essential in order for a flavonoid to exhibit mutagenic activity: 1) a free hydroxyl in position 3; 2) a double bond in position 2-3; 3) a ketone group in position 4; 4) a structure that allows the proton in the hydroxyl group in position 3 to tautomerize into ketone in position 2. Chrysine does not have a hydroxyl group in position 3, and tautomerization into ketone in that position is not possible.

On the basis of the results obtained, chrysine's activity on herpes-viruses multiplication should be investigated further, especially in terms of the mechanism of action and evaluation of the specificity of antiviral activity. The data contained in the literature about antiviral activities of flavonoids are encouraging in this sense.

For example, the synthetic flavonoid 4,6-dichloroflavan inhibits the RNA synthesis of rhinoviruses (Bauer *et al.*, 1981), and the compound 4,5-dihydroxi-3,3',-7-trimethoxyflavon shows virus-specific antiviral activity towards rhinoviruses and coxsackieviruses (Ishitsuka *et al.*, 1982). These data clearly show that some flavonoids specifically interfere with the intracellular cycle of viral multiplication.

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