ORIGINAL ARTICLE

THE ANTIVIRAL/VIRUCIDAL EFFECTS OF ALCHOOLIC AND AQUEOUS EXTRACTS WITH PROPOLIS

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Abstract

The objective of this study was to evaluate the antiviral/virucidal characteristics of two types of propolis extracts (alcoholic and aqueous) against human pathogenic DNA-viruses. In our tests three virus strains were used: HSV1 (*Herpex simplex* virus) (strain VR # 3), *Varicella zoster* (VZV) and adenovirus type 5 (ADV5). The cells used in each experiment were selected according to their sensitivity to produce high titers of infectious viruses. Non-cytotoxic concentrations of aqueous/alcoholic propolis extracts were tested so that the toxic effect does not overlap with the cytopathic effect. After treatment, the titer of each virus was determined by daily monitoring of the occurrence and evolution of cytopathic effect and by immunofluorescence. At non-cytotoxic concentrations, we have not depicted a significant reduction in the viral titre, so we concluded that propolis extract pre-/post-treatment do not provide antiviral protection, for all tested viruses. As virucid, propolis tincture showed a strong anti-HSV, VZV activity in direct contact with virus, similar to that of acyclovir, cafeic and chlorogenic acids. The propolis aqueous extract was less efficient on HSV and VZV, even in direct contact, but showed the strongest inhibitory effect in the pre-treatment of ADV5 infected cells.

Rezumat

Obiectivul acestui studiu a fost evaluarea caracteristicilor antivirale/virulicide a două tipuri de extracte de propolis (alcoolice și apoase), asupra virusurilor ADN patogene umane. În testele noastre au fost utilizate trei tulpini de virus: HSV1 (virus *Herpex simplex*) (tulpina VR # 3), *Varicela zoster* (VZV) și adenovirus tip 5 (ADV). Celulele utilizate în fiecare experiment au fost selectate în funcție de capacitatea lor de a produce titruri virale crescute pentru virusurile selectate. Au fost utilizate concentrații non-citotoxice de extract de propolis astfel încât efectul toxic să nu se suprapună peste efectul citopatic. După tratament, titrul viral a fost determinat prin monitorizarea zilnică a apariției și evoluției efectului citopatic și prin imunofluorescență. La concentrațiile non-citotoxice nu s-a observat o reducere semnificativă a titrului viral, astfel că pre- și post-tratamentul cu extract de propolis nu asigură protecție pentru virusurile testate. Ca virulicid, tinctura de propolis a prezentat o puternică activitate impotriva HSVsi VZV atunci când a fost pusă în contact direct cu virusul, similară cu cea a aciclovirului, precum și a acizilor cafeic și chlorogenic. Extractul apos de propolis a fost mai puțin eficient impotriva HSV și VZV, chiar și în contact direct, dar a prezentat cel mai puternic efect inhibitor ca pre-tratament urmat de infecţia celulelor cu ADV5.

Keywords: propolis extracts, antiviral/ virucid effects

Introduction

The antiviral therapies developed to treat viral diseases, including nucleoside analogues [7, 12], amantadine [9], or neuraminidase inhibitors [5], have limited effectiveness and also, side-effects and systemic toxicity that limit their application at immuno-compromised patients. For these reasons, the development of efficacious and low toxic antiviral compounds is needed.

Studies on natural resources capable to provide nutritional balance to human organism and anti-microbial and antiviral effects have highlighted the nutritional and therapeutic value of apiculture products. Of these, propolis is one of the greatest challenges for both nutritionists and the medical scientific world.

Propolis is a sticky, brown-to-dark-brown, resin-like product made by bees to cover and seal the internal surfaces of the hive and to create optimum survival and normal development conditions of the bee families. Propolis originates from plant exudates (in particular, from buds of tree species are the main source of propolis, such as poplar (*Populus spp.*), birch (*Betula spp.*), beech (*Fagus sylvatica*), chestnut

(*Aesculus hippocastanum*), willow (*Salix spp.*) etc.) collected by bees and blended with the wax they secrete, together with small amounts of sugars. Bees deposit propolis as a fine film on new honeycombs in cracks of the hive, to fix vertical frames, to reduce the bee entrance in cold periods of the year in temperate climates. The propolis used to apply this film is green matter harvested from plant buds, slightly modified by the addition of enzyme secretions from the pharyngeal and mandibular glands of working bees with an exclusive genetic predisposition.

In the chemical composition of the raw propolis were identified mainly resins, waxes, oils, pollen, carbohydrates, amino acids, vitamins, enzymes, mineral salts and impurities [8, 11], terpenes, oligoelements (aluminium, silver, chromium, cobalt, iron, magnesium etc.), flavonoids, flavones, free amino acids (proline, arginine), tannin substances - resulting as secretions of salivary glands from bees. Of these, chrysin, vanillin, izovanilin, caffeic acid, quercetin, routine, pinocembrin, galangin, ferulic acid, 5-oxydimethoxyflavones etc. (known to have anti-bacterial [19] and antiviral activity [3, 10, 15, 16]) together with volatile resins and oils, trace elements such as Fe, Zn, Cu, Co, Mo, Mn, Li, Mg and other flavonoid substances give propolis very complex biological properties.

In the present study the antiviral/virucidal effects of two types of propolis extracts have been investigated, an alcoholic and an aqueous one. These extracts of propolis have been tested against a panel of viruses. The objective of this study was to evaluate *in vitro* the antiviral/virucidal characteristics of both extracts against some human pathogenic DNA-viruses.

Materials and Methods

Preparation of propolis extracts. Aqueous solution of propolis (APS) was obtained by refluxing propolis (30 g and 50 g, respectively) with purified water (100 mL) during 5 - 8 hours until the colour of the solution became brownish-grey. The obtained solution was filtrated and water was added to a final volume of 100 mL. To obtain propolis tincture (PT), the residual product from propolis aqueous solution preparation was gently heated until it became a homogeneous and viscous mass and covered with 70% ethanol. The obtained mixtures were subjected to a maceration process at room temperature, away from light and humidity, and, after one week, were filtered to obtain the corresponding, 30% or 50%, propolis tincture.

Quantitative determination of propolis active ingredients (polyphenols and flavonoids). The total content of polyphenols and flavonoids was determined using as standard caffeic acid/chlorogenic acid, respectively, rutin/quercetin based on a method developed after the European Pharmacopoeia and Romanian Pharmacopoeia (FR X) and on a method of analysis developed according to the literature [14]. The total content of polyphenols and flavonoids in the tested samples was calculated using standard curves established in parallel and under the same conditions as the test solutions of caffeic acid/ chlorogenic acid or of rutin/quercetin. Using the UV-VIS spectrophotometer, the absorbance of the test solution against the clearing solution was measured and the total polyphenols content was expressed as % of caffeic acid/chlorogenic acid and total flavonoids content was expressed as % of rutin/quercetin.

Cell cultures. Adherent cell cultures (HeLa ATCC® CCL-2™, MG63 ATCC® CRL-1427™) were maintained at 37°C under 5% CO_2, in DMEM/F12 (Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12) culture medium (Sigma), supplemented with 10% foetal bovine serum (Sigma) and 400 IU/mL penicillin, 200 µg/mL streptomycin. Cell viability before freezing and thawing was determined by the tripan blue dye exclusion test. Taking into account that efficient virus replication is correlated with changes in cell morphology (cytopathic effects), in cell physiology and sequential biosynthetic events depending on cell type; the cells used in each experiment were selected according to their sensitivity to produce high titers of infectious viruses.

Cytotoxic effect assessment. In our study, non-toxic concentrations of propolis extracts were used. For this reason, to test the cytotoxic effect, the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay and trypan blue technique were used.

For CellTiter assay, the cells were grown in 96-well plates (5 x 10^3 cells/well) and maintained at 37°C in 5% CO_2 atmosphere. The treatment using different dilutions of aqueous and alcoholic propolis extracts was performed at 24 hours. The experiments were performed in triplicate. Added tetrazolium salt, MTS, was bio-reduced by cells into a soluble formazan product that was measured spectrophotometrically at 490 nm (with reference absorbance at 620 nm) directly in 96-well assay plates without additional processing. The effect of propolis extracts on living cells in culture was established as percent of viable cells related to the control.

To evaluate the cell viability with trypan blue, cells were seeded into 24 well culture dishes at a concentration of 1×10^5 cells/well. The cells treated with different propolis extracts dilutions and cells of the control group without propolis treatment were incubated under the same conditions. After 24 hours, the treated cells were detached with trypsin solution and centrifuged. The pellets were re-suspended in 1 mL PBS (phosphate buffered saline) and cell viability was determined by mixing the cell suspension 1:1 with trypan blue solution (Sigma).

Viruses. In our tests were used three virus strains: HSV (*Herpex simplex* virus) type 1 (strain VR # 3), *Varicella zoster* (VZV) and adenovirus type 5 (ADV5)

from the reference collection of the "Ştefan S. Nicolau" Institute of Virology, Bucharest, Romania. Also, in our assessments we used the cell line that gave the best results depending on the virus. As such, the experiments implying ADV5 were performed on HeLa cells, while in the case of HSV and VZV, MG63 cells were used.

Preparation of the virus stock. In order to have sufficient viral material of the same origin and titer, initially, a basic stock of virus was prepared according to the inoculation lot principle, from which, subsequently, work stocks have been obtained. Cells grown in high capacity culture vessels were inoculated with virus at multiplicity of infection (m.o.i.) when the cells reached the exponential growth phase. When the cytopathic effect (CPE) was sufficiently extensive (approx. 75%), the cells were collected, concentrated by centrifugation and the virus was released by two consecutive freeze-thaw cycles. Working virus stocks were aliquoted and stored at -70°C until use.

Propolis pre-treatment followed by virus inoculation experiment. The MG63 cells and HeLa cells, respectively, were seeded in 96-well plates at 5×10^3 cells/well concentration. The cells were treated for 24 hours with aqueous and alcoholic propolis extracts, as well as with reference substances, at non-cytotoxic concentrations previously established. Thus, the following concentration were used: 1% of aqueous propolis 30%; 0.5% of aqueous propolis 50%; 0.06% of propolis tincture 30%; 0.06% of propolis tincture 50%; 0.16 mg/mL chlorogenic acid; 0.25 mg/mL cafeic acid; 0.0078 mg/mL quercetin; 0.5 mg/mL acyclovir. Experiments were performed in triplicates for each concentration and extract. After 24 hours, the culture medium was removed and, then, cellular monolayers were inoculated with virus. Basically, for each virus, decimal serial dilutions were made from 10^{-3} to 10^{-11} in DMEM/F12 supplemented with 2% FBS (foetal bovine serum). Also, each of the three viruses, HSV1 VR#3, VZV and ADV5, was titrated in parallel in triplicate, the last wells being the non-inoculated cell control. The infectivity of each virus was determined by monitoring the occurrence and evolution of cytopathic effect (CPE) and by immunofluorescence. As such, the cytopathic effect has been monitored daily for one week. The infecting titre was calculated by the Spearman Karber method and was expressed as 50% Tissue Culture Infectious Dose ($TCID_{50}$) - the amount of the pathogenic agent that induces CPE in 50% of inoculated cell cultures.

Propolis treatment after viral inoculation. In 96-well plates have been grown 5×10^3 cells/well. At 24 hours, viruses were inoculated in triplicate in decimal dilutions beginning with 10^{-4} and up to 10^{-10} in DMEM/F12 supplemented with 2% FBS. After 1 hour, the viral inoculum was removed and each well was treated with the same substance concentration (treatment at 1 hour post-infusion). The occurrence of the cytopathic effect was monitored daily for one week.

Propolis treatment at the time of viral inoculation. For the evaluation of the direct virucidal effect of the propolis extracts, 96-well plates were used and cell monolayers were obtained by inoculation of 5×10^3 cells/well. The propolis extracts and the virus were put in contact in a mixture containing 80 µL of stock propolis extract, 10 µL of stock virus and 10 µL PBS. The mixture was incubated for 15 minutes at room temperature before inoculation. Practically at this stage, the virus was diluted 10-fold, obtaining 10^{-1} dilution, and the substance is 80% of the stock. Sequential serial dilutions in DMEM/F12 supplemented with 2% FBS were then performed. With these dilutions, the monolayer was inoculated for one hour. Subsequently, the inoculum was removed and the maintenance medium (DMEM/F12 supplemented with 2% FBS) was added. The cytopathic effect was monitored for one week.

Immunofluorescence for end point viral screening. For accurate evaluation of CPE, these were verified by immunofluorescence using the kits: DFA Respiratory Viral Screening & Identification Kit and HSV1/2 Identification Kit (Light Diagnostics, USA). Thus, after the appearance and evaluation of the cytopathic effect, the plates were fixed in CH_3OH: H_2O (8:2) for 10 minutes and washed with PBS solution. In the last well in which cytopathic effect was observed, as well as the following well, the anti-adenovirus or anti-HSV1/2 monoclonal antibodies coupled with fluorescein (DFA Adeno Reagent or DFA HSV1/2 Reagent), containing 0.02% Evans Blue to counter staining, was added in sufficient quantity to cover all the cells. After incubation at 37°C for 30 minutes, the plate was washed and labelled; the wells were evaluated by fluorescence microscopy (Zeiss Observer D1) at 488 nm wavelength and photographed.

Results and Discussion

The propolis extracts and their active ingredients concentration

The chemical composition of obtained extracts was determined according to European and Romanian Pharmacopeias. The main raw materials evaluated from propolis were the total polyphenols, expressed as caffeic acid/chlorogenic acid, and flavonoids, expressed as rutin/quercetin (Table I).

Table I

Content in total polyphenols (expressed in caffeic and chlorogenic acid) and in flavonoids (expressed in rutin and quercetin) of aqueous and alcoholic extracts

No	Analysed parameter	30% APS	50% APS	30% PT	50% PT
1.	Polyphenols expressed as % of caffeic acid	0.94	1.97	2.46	3.55
2.	Polyphenols expressed as % of chlorogenic acid	0.15	0.23	1.08	1.69
3.	Flavonoids expressed as % of rutin	0.019	0.47	0.057	0.88
4.	Flavonoids expressed as % of quercetin	0.010	0.022	0.045	0.059

Cytotoxicity of propolis extracts

The cytotoxic effect of propolis extracts were tested in human HeLa and MG63 cell lines. CellTiter analysis revealed that the percentage of living cells was decreased in a time- and dose-dependent manner in both cell lines. The results obtained after 24 hours treatment on MG63 cell line are presented in Figure 1.

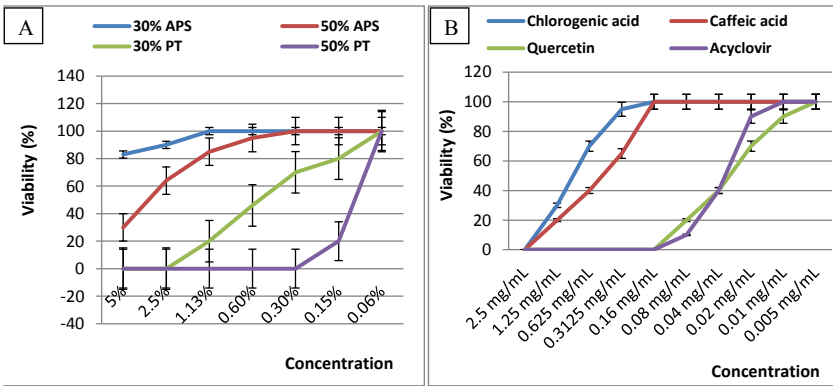

Figure 1.

The cytotoxic effect of the tested propolis extracts (A) and of the reference substances (B) on MG63 cells

To measure the antiviral effects, non-toxic concentrations of propolis extracts were used. Accordingly, the selected concentrations were as follows: 1% of APS 30%; 0.5% of APS 50%; 0.06% of PT 30%; 0.06% of PT 50%; 0.16 mg/mL chlorogenic acid; 0.25 mg/mL cafeic acid; 0.0078 mg/mL quercetin; and 0.5 mg/mL acyclovir. Also, taking into account that efficient virus replication is correlated with changes in cell morphology (cytopathic effects), in cell physiology and sequential biosynthetic events depending on cell types, in each experiment we selected a cell line according with their sensitivity to produce high titres of infectious viruses. As such, the experiments implying ADV5 were performed on HeLa cells, while in the case of HSV and VZV, MG63 cells were used.

Morphological evaluations of CPE

After infection, the viruses induced typical syncytium in cell cultures, the original cell shape was lost, cells became rounded and suspended, and the gap between cells increased. The reduction of viral infectivity by propolis extract was evaluated at morphological level through quantifying the cytopathic effect (Figure 2) and by the end point immunofluorescence evaluation.

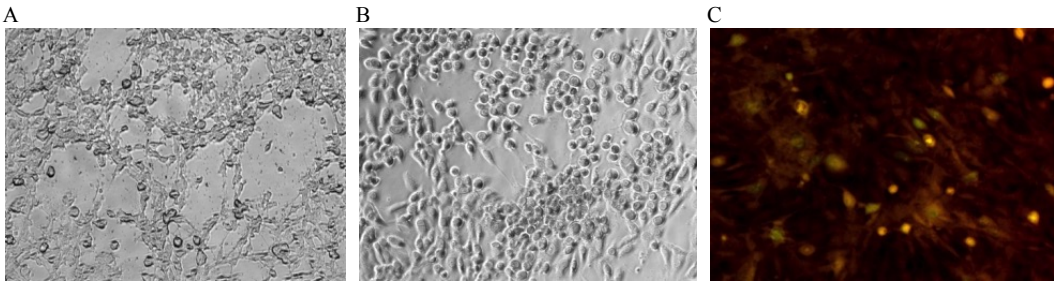

Figure 2.

The cytopathic effect of the tested virus strains: A. The cytopathic effect induced in HeLa cells by adenovirus (contrast phase microscopy (200x)); B. The cytopathic effect induced in MG63 cells by HSV1-VR (contrast phase microscopy (200x)); C. The cytopathic effect induced in HeLa cells by adenovirus (fluorescent microscopy (200x)) – immuno-detection of ADV5 in the last well with cytopathic effect. Negative cells show red staining of cytoplasm and a strong brown to black in the nucleus. Coloration in these cells is due counterstaining with Evans Blue. The positive cells show green nuclear fluorescent staining.

Pre-treatment of cells followed by virus inoculation

In order to evaluate the effects of propolis extracts pre-treatment, the MG63 or HeLa cells were previously treated with a concentration of aqueous/alcoholic propolis extracts with no cytotoxicity previously identified so that the toxic effect does not overlap with the cytopathic effect. We have not seen a significant reduction in the viral titre, so we concluded that propolis extract pre-treatment do not provide antiviral protection, for all tested viruses.

The pre-treatment with propolis extracts does not protect cells from viral infection with HSV1-VR, as opposed to control substances. The pre-treatment with acyclovir is most effective in HSV infected cells, and its effect is comparable to those of cafeic and chlorogenic acids. The most ineffective tested substances in HSV infected cells were the PT 30% and APS 30%. In VZV infected cells the less inhibitory effect was determined by both propolis tinctures, followed by acyclovir and the propolis aqueous extract. For the VZV infected cells, the most efficient substances seemed to be chlorogenic and cafeic acids. The propolis aqueous extract was most effective in pre-treatment of ADV – infected cells, more efficient than the propolis tincture (Figure 3), as well as more efficient than acyclovir, quercetin and cafeic acid that did not inhibit this virus.

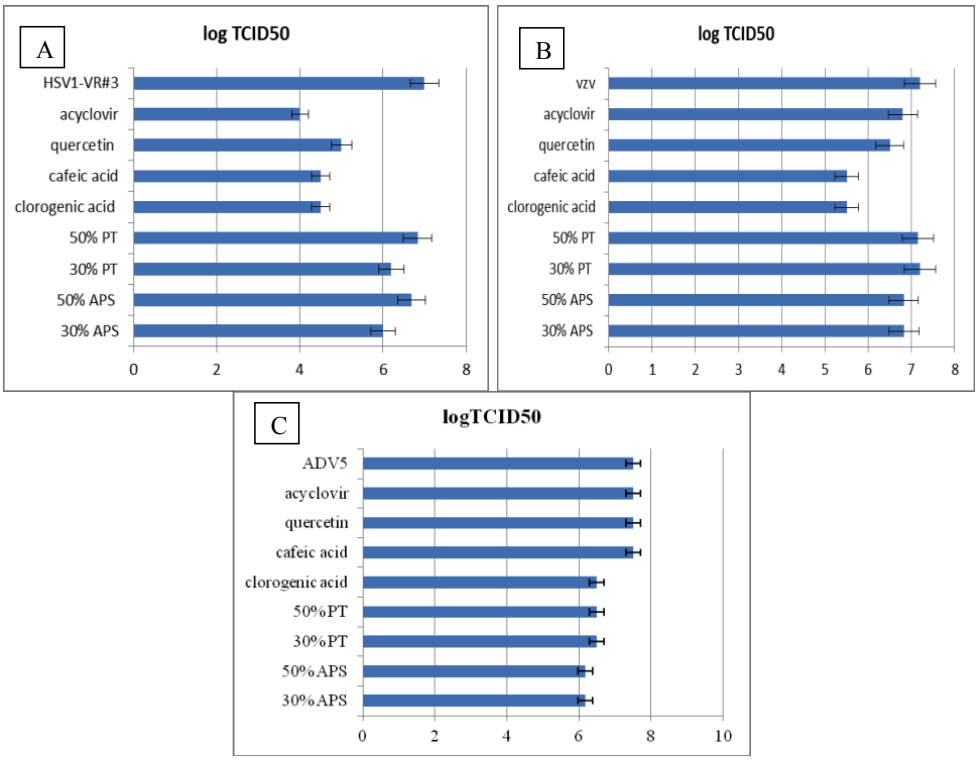

Figure 3.

The effects of alcoholic/aqueous propolis extracts pre-treatment on the viral titer of HSV1 (A), VZV (B) and ADV5 (C) presented as logarithmic values of 50% Tissue Culture Infectious Dose (logTCID50)

Post-treatment of cells following virus inoculation

In order to evaluate the antiviral effects of aqueous/ alcoholic propolis extracts by intervention in one of the stages of adsorption, internalization, decapsidation, genome transport in the cytoplasm/nucleus, genome transcription, viral protein synthesis, maturation, virion cell release, the treatment with the tested compounds was applied on cells after virus inoculation. For this purpose, MG63 and HeLa cells were inoculated with decimal dilutions of selected viruses and after 1 hour the treatment with non-cytotoxic concentrations of propolis extracts was performed.

As post-treatment for HSV infected cells the most efficient was acyclovir in 1-hour post-infection treatment, followed by PT 30% and PT 50%, while aqueous solution of propolis did not protect HSV infected cells, as well as quercetin, cafeic acid, and chlorogenic acid. As post-treatment for VZV infected cells the most efficient was quercetin in 1-hour post-infection treatment, followed by acyclovir and PT 50%, APS 30%, and APS 50%, the last three ones presenting similar results. In the case of ADV5, the post-treatment with propolis extracts of infected cells had no effect. Overall, propolis extracts were more efficient in cells infected with VZV, reducing the viral titer with 2 logs, and proved low effect against HSV and no effect against ADV (Figure 4).

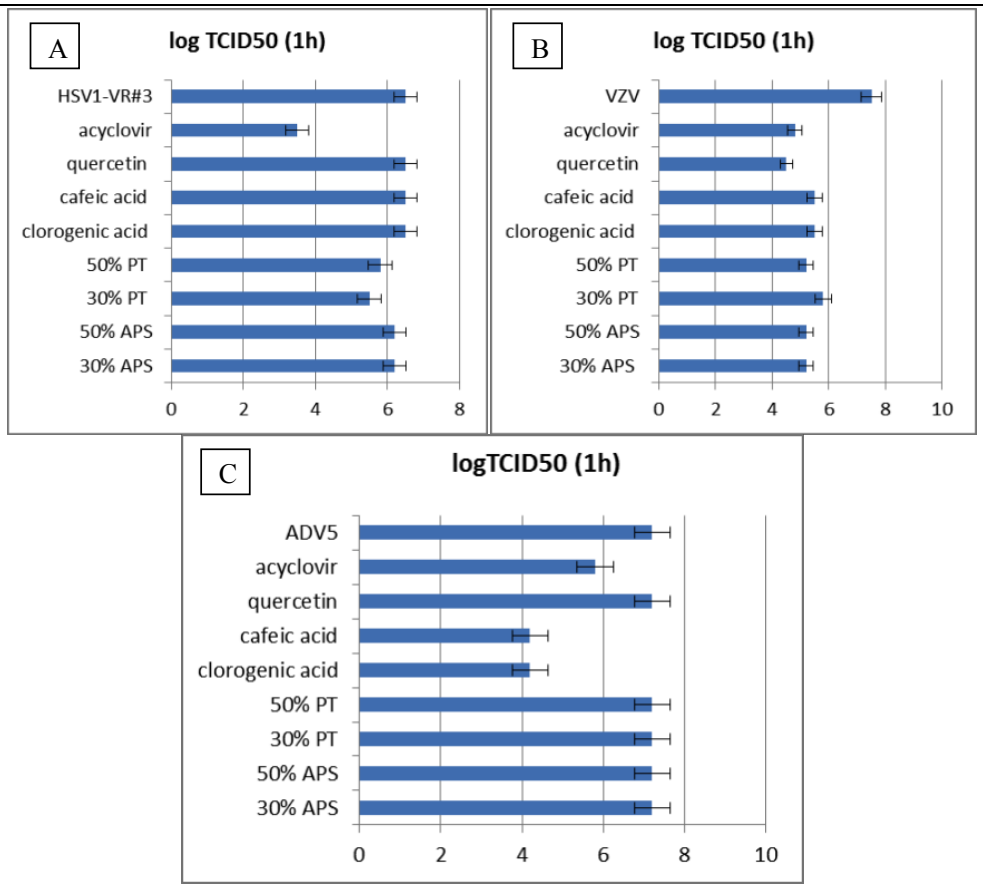

Figure 4.

The reduction of viral titer of HSV1 (A), VZV (B) and ADV5 (C) by treatment with alcoholic/aqueous propolis extracts after 1 hour post infection presented as logarithmic values of 50% Tissue Culture Infectious Dose (logTCID50)

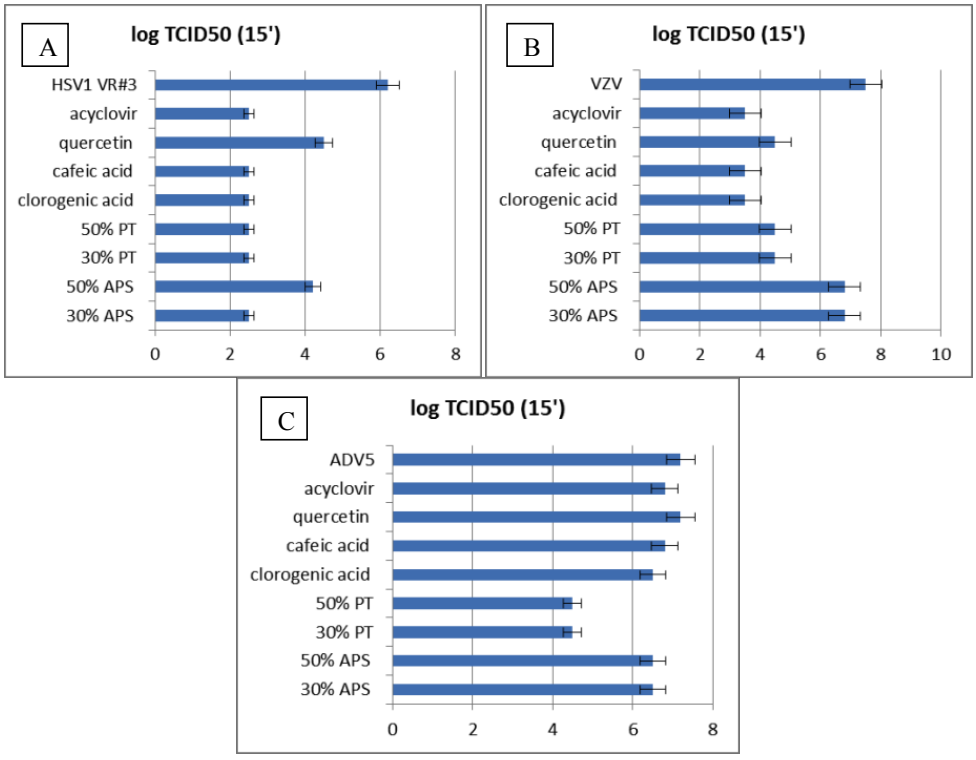

Figure 5.

The effects of the direct contact and incubation of propolis extracts with HSV1 (A), VZV (B) and ADV5 (C) virus (virucidal effects) presented as logarithmic values of 50% Tissue Culture Infectious Dose (logTCID50)

For evaluation of the virucidal effect of the aqueous or alcoholic propolis extracts, the virus incubation with the propolis extracts, and reference substances, for 15 minutes resulted in its inhibition by the corresponding compound. The aqueous/alcoholic propolis extracts have been tested in a concentration of 80% of the stock. During the incubation time, the extract/ substance may have a potentially virucidal effect which would be visible if the viral titer in the wells treated with this mixture will decrease comparing to control – cells inoculated with untreated virus. After incubation, the mixture is serially diluted, i.e. both virus and substance, unlike other experiments in which cells were treated before or post-viral infection.

The direct contact was very efficient for viral inhibition in almost all substances tested in case of HSV-strain VR, except quercetin and APS 50%. For VZV, the most efficient substances were acyclovir, cafeic and chlorogenic acids, afterwards quercetin and propolis tinctures (30% and 50%) while in the case of ADV5 most of the substances tested were not so efficient, except for both concentrations of propolis tincture (Figure 5).

Overall, in our experiments, propolis tincture showed a strong anti-HSV activity in direct contact with virus, similar to that of acyclovir, cafeic and chlorogenic acids, and a weak inhibitory effect in pre- or post-infection treatment. Regarding PT action on VZV, the direct contact propolis tincture – VZV had a greater inhibitory effect on viral activity than PT used in pre- or post-infection treatment on VZV-infected cells. The pre-treatment with propolis tincture for cells consequently infected with VZV had no effect. Still, in direct contact, the inhibitory effect on VZV strain was weaker than on HSV-VR (2 log difference). The inhibitory effect of propolis tincture on adenovirus in direct contact was the strongest from all substances tested and was as strong as in direct contact with VZV. In pre-treatment of adeno-infected cells, propolis tinctures showed a small inhibitory effect.

The propolis aqueous extract was less efficient on HSV and VZV, even in direct contact, but showed the strongest inhibitory effect in pre-treatment of ADV5 infected cells.

Regarding the reference substances used, cafeic and chlorogenic acid showed the same strong inhibitory effect when put in direct contact with HSV, an efficiency similar to that of acyclovir. In the case of the other two types of treatment on HSV infected cells, cafeic and chlorogenic acids were more efficient as pre-treatment than post-infection treatment, with 1 log difference. Cafeic and chlorogenic acids showed a greater efficiency (with almost 1 log difference) on HSV than on VZV. These substances were also the most efficient in direct contact with VZV; and in pre- and post-treatment on VZV infected cells presented same inhibitory effect. Also, the cafeic and chlorogenic acids have some efficiency effects on ADV5, in 1-hour post-treatment experiments. On the other side, at tested concentration, quercetin had poor inhibitory effect on HSV in direct contact and no inhibitory action on ADV, all treatments, and in HSV post-treatment infected cells. On VZV, in direct contact, quercetin showed the same inhibitory effect as propolis tincture. As post-treatment on VZV infected cells, quercetin showed similar to greater inhibitory effect compared to acyclovir.

Herpes simplex, *varicella-zoster* viruses and adeno-viruses have high infectivity potential and give many clinical issues. In immunocompetent persons, these problems are usually mild and can be cured, while in immunocompromised patients these pathogens can lead to life-threatening diseases. Drugs used in clinical field to treat the diseases given by these viruses have a lot of side effects and can lead to resistant strains. That is why different natural compounds or active ingredients may offer an elegant and safe alternative to the standard treatment.

Plant bud exudates contain several hundreds of phenolic compounds and each species of exudate possesses a characteristic mixture of components that can be considered as a specific "fingerprint" that can differentiate and identify the origin. Generally, each component identified in the bud exudate in a specific geographical area is most likely to be found in the propolis produced by bees in that region because most components are incorporated into propolis without enzymatic or altered changes. Sometimes, glycosidic forms of flavones (found in plants) may be subjected to enzymatic hydrolysis by bees, both during propolis collection and when mixed with wax, so that flavonoid substances present in propolis can be found as aglycone [17]. It should be noted that although the chemical composition of propolis is variable (depending on the geographical location, the variety of trees and of other plant species used by bees for collection), there is still a certain group of substances (flavonoids, phenols and terpenes) continuously present in its structure that condition its physico-chemical attributes and, especially, the pharmacodynamic ones. Numerous studies have revealed that the bioactive components that confer beneficial properties of propolis, though variable depending on the source plant and the collection period, are found in most types of propolis collected from different geographical areas. Therefore, both the vegetable source and the raw propolis resulting from different geographical regions often have a similarly qualitative composition. Thus, in order to obtain preparations with similar biological activities and the elimination of some differences in the quantitative composition in active principles, it is necessary to use samples of propolis from the same geographic area.

In order to highlight its biological and therapeutic properties, some original technological solutions have been developed to obtain standardized extracts with applicability in medical practice. The vast majority of them are based on the preparation of highly concentrated hydroalcoholic extracts or aqueous extracts, by applying mild, non-aggressive technologies to active components that allow the use of bioactive fractions in various therapeutic regimens [4]. In most studies related to the use of solvents, it has been observed that the most active and most used solvent is the ethyl alcohol. It is used in various concentrations for the preparation of tinctures, extracts or hydro-alcoholic solutions - generally made for internal use. Schnitzler P. *et al.* have shown that both aqueous and hydroalcoholic extracts from European propolis exhibit antiviral activity *in vitro* due to the presence of polyphenols, flavonoids and phenylcarboxylic acids, identified as major components in the composition of propolis [18].

After penetration into the host cell, the virus (DNA or RNA) can become active under the action of lysosomal enzymes which assure the digestion of the viral protein coating. The bioactive components of propolis, flavonoids (quercetin, rutin, kaempferol, galangin) and aromatic acids (caffeic acid and its derivatives), protect the lysosomal lipid membranes, the action of liposomal enzymes being blocked which prevents virus entering into the cells. This effect is still not well understood, but it seems the amino groups from the protein coating are neutralized through a phosphorylation process, hypothesis that supports the capacity of viral particles to penetrate lysosomal lipid membranes.

In our experiments, the best inhibition of HSV in direct contact has been obtained with cafeic acid, chlorogenic acid, and propolis tincture, which proved similar effects to acyclovir, used as reference substance. This indicates the substances which can prevent infectious HSV contact. As pre-treatment for HSV infected cells, the best substances seemed to be caffeic and chlorogenic acids. This means that propolis tincture or aqueous extracts of propolis enriched in caffeic acid or chlorogenic acid may have much powerful antiviral effect. This hypothesis is sustained by the chemical composition of the tested propolis extracts. As such, propolis tinctures, which are characterised by a higher content of polyphenols, were more effective against HSV infected cells compared to the aqueous solutions of propolis, characterized by a lower content of polyphenols.

Our results are convergent with that of Yildirim *et al.* who showed that a propolis treatment at the same time with HSV infection of cell culture leads to an inhibition of HSV replication [20]. The propolis aqueous extract efficiency in direct contact with HSV is sustained by Huleihel and Isanu who tested its inhibitory effect on HSV when added on cells before, after and at the time of infection. Contrary to Huleihel and Isanu, we found that the pre-treatment of cells with propolis aqueous extract has poor inhibitory effects. This can be caused by the fact that cell lines used were different. Their *in vitro* experiments were on Vero cells, while our group used HeLa and MG63 cells. This little difference can say that propolis aqueous extract has a protective effect on Vero cells for HSV infection, but not on HeLa and MG63 [13].

Amoros *et al.* also studied the activity of propolis, as a propolis balsam obtained by extraction with 80% ethanol for 18 hours at room temperature and concentrated to a dry residue, on herpes viruses [1]. The obtained balsam reduces the titer of herpes viruses (HSV-1 and HSV-2) by 1000, and inhibits HSV replication by 99.5%, and has a virucidal activity on the HSV and vesicular stomatitis viruses (VSV). Still, the inactivation of HSV-1, HSV-2 and VSV by the propolis extract is time and dose dependent [1].

It was suggested that flavonoids (galangin, quercetin, chrisin, rutin) and aromatic acids (caffeic acid and its derivatives), that are predominant in the analysed propolis, act synergistically to produce antiviral effects. In a more comprehensive study about flavonoid inhibitory action on herpes viruses, Amoros *et al.* observed individual and binar synergistic effects of flavonols (galangin, kaempferol and quercetin), flavones (chrysine, apigenin and tectocrysine) and flavanas (pinocembrin) on type 1 HSV infection, these compounds stopping *in vitro* HSV replication in monkey kidney cells. In addition, a significant reduction in HSV infectivity titer occurs when two different flavonoids are administered together, having a significant synergism, even acting through different mechanisms [2].

For VZV, the best inhibitory substances in direct contact appeared to be acyclovir, cafeic acid and chlorogenic acid, followed by propolis tincture, with 1 log difference. For the pre-treatment of VZV infected cells, the cafeic and chlorogenic acids were the most efficient. And as post-treatment in VZV infected cells quercetin, acyclovir and propolis aqueous extract were the most efficient substances, followed by propolis tincture (0.5 log difference).

In case of ADV5 the inhibitory action in all experiments with all substances was quite poor. Propolis tincture showed the best inhibitory activity in direct contact, the propolis aqueous extract and chlorogenic acid - in the pre-treatment experiment on ADV infected cells and the cafeic and chlorogenic acids – in post-treatment experiment. Chiang L.C. *et al.* showed that chlorogenic acid has a strong inhibitory activity on ADV11, and caffeic acid mainly on HSV and ADV3 [6].

Conclusions

Our study showed that the pre-/post-treatment with propolis extracts at non-cytotoxic concentrations do not provide antiviral protection, for all tested viruses. But, as virucidal agent propolis tincture showed a strong anti-HSV and anti-VZV activity in direct contact with the viruses, similar to that of acyclovir, cafeic and chlorogenic acids. The propolis aqueous extract was less efficient on HSV and VZV, even in direct contact, but showed the strongest inhibitory effect in pre-treatment of ADV5 infected cells.

All results obtained indicate propolis tincture as a possible preventive agent for HSV/VZV infectious contact, propolis aqueous extract as a possible preventive agent for ADV5 infection, and, indirectly, that alcoholic/aqueous extracts of propolis enriched in caffeic and chlorogenic acids may have a much powerful antiviral effect.

That is why the characterization of the chemical composition of propolis from different regions of the country and of the synergic actions of its components is extremely useful for continuing the research in order to find efficient alternative antiviral substances.

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