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Chemical composition of Propolis Extract ACF® and activity against herpes simplex virus☆

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ABSTRACT

Propolis Extract ACF® (PPE) is a purified extract manufactured from propolis collected in a Canadian region rich in poplar trees, and it is the active substance of a topical ointment used against herpes *labialis* (cold sores or fever blisters). Aim of this study was to analyze the chemical composition of PPE in order to understand the plant origin and possible relations between compounds and antiviral activity, and to characterize the antiviral activity of the extract against herpes simplex virus *in vitro*.

Material and methods: The analysis of the propolis extract samples was conducted by Gas Chromatography–Mass Spectrometry (GC–MS). The antiviral activity was tested against herpes simplex viruses type 1 and type 2 in MDBK cell cultures by treating the cells with PPE at the time of virus adsorption, and by incubating the virus with the extract before infection (virucidal assay).

Results: Results from the GC–MS analyses revealed a dual plant origin of PPE, with components derived from resins of two different species of poplar. The chemical composition appeared standardized between extract samples and was also reproduced in the sample of topical ointment. The antiviral studies showed that PPE had a pronounced virucidal effect against herpes simplex viruses type 1 and type 2, and also interfered with virus adsorption.

Introduction

Propolis is the resinous material that honeybees collect from various plant sources and mix with wax, to use as an antimicrobial sealer in the beehive. Its chemical composition is qualitatively and quantitatively variable, depending on geographical origin and regional flora. The plant source is the main determinant of chemical composition and, as a consequence, of the biological properties of propolis from a particular region. Control of geographical origin is crucial to ensure a consistent composition of propolis and achieve the best possible standardization.

Propolis has a wide range of biological activities (Banskota et al., 2001; Burdock, 1998), and for this reason it has been used as a traditional natural remedy since ancient times. The antiviral effects of propolis have been demonstrated against various types of viruses, including the herpes simplex virus (Amoros et al., 1994; De Biaggi et al., 1990; Huleihel and Isanu, 2002; Schnitzler et al., 2010).

Propolis Extract ACF® (PPE) is a specific purified ethanol extract, developed in Canada and first patented in the 1970s. It is highly concentrated and manufactured according to Good Manufacturing Practices in a Canadian facility licensed by the NHPD (Canadian Natural Health Products Directorate), using only propolis harvested from beehives located in a specific area of Canada, rich in poplar trees. This extract, at a 3% concentration, is the active substance of a topical ointment used against *herpes labialis* (marketed under the trademark Herstat®).

The first positive effects of the topical application of this ointment were reported in clinical investigations conducted in the 1980s, showing a significant shortening of the healing time and a relief of the symptoms of cold sores. These effects have been confirmed in more recent and structured clinical studies, in both types of herpes simplex infection: cold sores by HSV-1 (Hoheisel, 2001), and herpes *genitalis* by HSV-2 (Vynograd et al., 2000). The aim of this study was to research the reasons for the effects obtained in the clinical studies, by testing samples of PPE extract and ointment for composition and *in vitro* antiviral activity. Samples of extract and ointment were analyzed by GC–MS to detect the chemical composition, to identify the likely plant source and to verify the level of standardization. For the antiviral activity, one of the samples of PPE was also tested on cultured cells against HSV-1 and HSV-2.

Abbreviations: ACF®, antiviral complex of flavonoids.

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Results show that Propolis Extract ACF is of dual plant origin and that it is markedly virucidal *in vitro* against both herpes simplex viruses, confirming the clinical results and supporting the use of its topical application in herpetic lesions.

Materials and methods

GC–MS analysis

Propolis extract samples

Three samples from three different batches of Propolis Extract ACF (SW-20, -21 and -22), manufactured in the years 2007–2010 from propolis collected in Canada, were used in the studies. The ointment sample was manufactured in 2011 and contained 3% PPE from an earlier batch.

Silylation of extracts

The ethanol extracts were silylated because most of its components were not volatile enough for GC without derivatization. About 5 mg of the extract were mixed with 50 μl of dry pyridine and 75 μl of BSTFA and heated at 80 °C for 20 min. The silylated extracts and reference compounds were analyzed by GC–MS.

Extraction of the preparation (ointment)

The ointment (2.2 g) was subjected to ultrasound-assisted extraction with 70% ethanol for 25 min. The extract was filtrated and evaporated to dryness *in vacuo* to yield 18 mg dry residue. Five mg of the residue was silylated and subjected to GC–MS analysis under the same conditions as the propolis extracts.

GC–MS analysis

The GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 μm film thickness HP5-MS capillary column. The temperature was programmed from 60 to 300 °C at a rate of 5 °C/min, and a 10 min hold at 300 °C. Helium was used as a carrier gas at a flow rate of 0.8 ml/min. The split ratio was 1:10, the injector temperature 280 °C, and the ionization voltage 70 eV.

Identification of compounds

The identification of the compounds was performed using computer searches on commercial libraries, comparison of mass spectra and retention times of reference compounds, and literature data, and sometimes based on the mass-spectral fragmentation. For some compounds, where no reference could be found, only tentative structures were proposed. Some constituents remained unidentified because of the lack of relevant references and information (none of them major constituents). The semiquantification of the main compounds was carried out by internal normalization with the area of each compound. The addition of individual areas of the compounds corresponds to 100% area.

Antiviral *in vitro* studies

Cell cultures

Monolayer cultures of Madin-Darby bovine kidney (MDBK) cells (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK), supplemented with 10 mM HEPES buffer (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid) (Merck, Germany) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml) in CO_2 incubator (HERAcell® 150, Heraeus, Germany), at 37 °C/5% CO_2.

Compounds tested and viruses

Canadian Propolis Extract ACF, batch SW-21, was diluted to a concentration of 100 mg/ml in dimethyl sulfoxide (DMSO), ethanol and DMEM (ratio 1:1:1) added consecutively, to give a 10% stock solution. Further serial dilutions of the PPE in maintenance medium were made from the baseline stock solution for use in the *in vitro* experiments. Acyclovir [(9-2-hydroxyethoxymethyl)-guanine] (ACV), dissolved in DMEM, was used as a reference substance for inhibition of viral replication.

Herpes simplex virus type 1, Victoria strain (HSV-1), and herpes simplex virus type 2, Bja strain (HSV-2) were received from Prof. S. Dundarov, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria and used for the experiments. The viruses were replicated in monolayer MDBK cells with a maintenance solution of DMEM (Gibco BRL) plus 0.5% fetal bovine serum (Gibco PRL). Infectious titers of stock viruses were 10^6 and $10^{5.5}$ $CCID_{50}$ (50% cell culture infectious doses) for HSV-1 and HSV-2 strains, respectively.

Cytotoxicity assay

For the cytotoxicity assay, confluent monolayer cell cultures in a 96-well plate were treated with culture medium containing increasing concentrations of PPE. Control cultures were treated only with medium without the extract. The viability of the cells after treatment with PPE was measured using neutral red uptake assay based on the initial protocol described by Borenfreund and Puerner (Borenfreund and Puerner, 1984) using ELISA reader at an optical density (OD) of 540 nm. The measurements were performed after 48 h incubation, and CC_{50} values (cytotoxic concentration of PPE that reduced viable cell number by 50%) were evaluated. Each sample was tested in triplicate with four cell culture wells per test sample.

Virus adsorption assay

MDBK cell monolayers in 24-well cell culture plates, pre-chilled at 4 °C, were inoculated with 10^3, 10^4 or 10^5 $CCID_{50}$ of HSV-1 for adsorption at 4 °C, and treated in parallel with 0.1 mg/ml of PPE. After time intervals from 15 to 120 min, cells were washed with PBS in order to remove both the extract and the unattached virus, overlaid with maintenance medium and incubated for 24 h at 37 °C. After triple freezing and thawing, the infectious virus titer of each sample was determined by the end-point dilution method. Each sample was prepared in triplicate.

Virucidal assay

Contact samples of 1 ml containing 10^5 $CCID_{50}$ of HSV-1 or $10^{4.5}$ $CCID_{50}$ of HSV-2 and increasing PPE concentrations (from 0.1 to 50 mg/ml) were prepared. Samples were stored at room temperature or at 4 °C for different time intervals (from a minimum of 15 min to a maximum of 6 h). The residual infectious virus content of each sample was determined by the end-point dilution method, and Δ logs as compared to untreated controls were calculated.

Statistical analysis

Data on cytotoxicity and antiviral effects were analyzed statistically, and results were presented as means ± SD. Student's *t* test was applied for the comparison between PPE and ACV values, the data obtained by the GraphPad Prism4 program.

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Table 1
Chemical profile of three samples of Propolis Extract ACF and one sample of alcohol extract of ointment containing 3% PPE, obtained by GC–MS. Results are shown as % of total ion current.

Compound	PPE batch SW-20	PPE batch SW-21	PPE batch SW-22	3% PPE ointment
Aromatic acids	*36.1*	*32.5*	*31.4*	*29.7*
Benzoic acid	9.6	8.7	8.7	5.6
Cinnamic acid	2.0	1.4	1.5	1.6
Hydroxybenzoic acid	0.4	0.4	0.4	0.4
Vanillinic acid	0.3	0.3	0.3	0.6
Z-*p*-coumaric acid	1.2	1.2	0.9	1.1
E-*p*-coumaric acid	17.4	15.3	13.8	15.6
Ferulic acid	3.2	3.1	3.4	3.0
Caffeic acid	2.0	2.1	2.4	1.8
Other aromatics	*0.7*	*0.6*	*0.6*	*0.1*
Benzyl alcohol	0.6	0.5	0.5	0.1
Cinnamyl alcohol	0.1	0.1	0.1	Tr.
Fatty acids	*0.2*	*0.4*	*0.5*	*1.2*
Palmitic acid	0.1	0.2	0.2	1.0
Oleic acid	0.1	0.2	0.3	0.2
Esters	*7.0*	*8.2*	*9.2*	*7.9*
Cinnamyl caffeate	0.6	0.6	0.5	0.7
Benzyl *p*-coumarate	4.4	4.5	4.6	5.2
Benzyl ferulate	1.5	1.3	2.3	0.8
Benzyl caffeate	0.3	1.6	1.6	0.9
Caffeoyl glycerol	0.2	0.2	0.2	0.3
Flavonoids	*12.9*	*10.2*	*11.6*	*8.3*
Pinocembrin	3.1	2.2	3.1	1.0
Pinobanksin	2.0	1.2	1.3	1.0
3-O-acetyl pinobanksin	1.8	1.7	1.2	1.3
Chrysin	2.2	1.7	2.4	1.8
Galangin	2.4	2.0	2.1	2.3
Isosakuranetin	0.5	0.4	0.4	0.1
Alpinone	0.4	0.5	0.5	0.1
Kaempferol	0.5	0.5	0.6	0.7
Chalcones	*5.0*	*5.4*	*6.1*	*7.3*
Pinostrobin chalcone	0.5	0.5	0.6	0.9
Pinocembrin chalcone	4.5	4.9	5.5	6.4
Dihydrochalcones	*2.8*	*2.4*	*2.2*	*2.6*
2′,6′-Dihydroxy-4′-methoxydihydrochalcone	0.4	0.6	0.6	0.8
2′,4′,6′-Trihydroxydihydrochalcone	0.1	0.2	0.1	0.3
2′,6′-Dihydroxy-4,4′-dimethoxydihydrochalcone	0.3	0.5	0.5	0.6
2′,4′,6′-Trihydroxy-4-methoxydihydrochalcone	0.1	0.2	0.2	0.2
4,2′,6′-Trihydroxy-4′-methoxydihydrochalcone	1.9	0.9	0.8	0.7
Others				
Hexoses	17.0	12.7	11.4	7.2

Results

GC–MS analyses

Propolis extract samples

The analysis of propolis extracts was performed by GC–MS after silylation. The results of the analyses are represented in Table 1, where the percentage figures refer to percent of the Total Ion Current and are semi-quantitative.

The three extract samples are very similar in their chemical composition, both qualitative and quantitative. They display features characteristic for propolis collected from bud exudates of *Populus* species growing in Canada.

The high percentage of benzoic and p-coumaric acid, and benzyl p-coumarate are typical for poplars of Section *Leuce*, subsection *Trepidae*, such as *P. tremula* (Bankova and Kuleva, 1989) in European Boreal forests (Popravko, 1978). Its close relative quaking or American aspen *P. tremuloides* is the probable propolis source plant in Canadian Boreal forest and respectively, one of the sources of the Canadian samples studied. The samples lacked the typical compounds of *Populus nigra* (section *Aigeiros*) bud exudates: series of pinobanksin 3-O alkanoates and caffeic acid pentenyl esters (Greenaway et al., 1990).

An important group of substances found in all three samples was the one of dihydrochalcones. Dihydrochalcones are considered to be characteristic for bud exudates of poplars of the Section *Tacamahaca* but not of Sections *Aigeiros* and *Leuce* (English et al., 1991). They have been detected in propolis only rarely and mostly in low concentrations (Greenaway et al., 1990). Till now, they were found in considerable amounts only in Canadian propolis (Christov et al., 2005). Obviously, the plant source of these compounds was bud exudate of a poplar species of the Section *Tacamahaca*, different from the above-mentioned *P. tremuloides* (Section *Leuce*). Two species of the Section *Tacamahaca* are widespread throughout Canada: the black cottonwood *P. trichocarpa* [Torr. et Gray] and the balsam poplar *P. balsamifera* L. (Brayshow, 1965). Among the major components of *P. trichocarpa* bud exudates, p-hydroxyacetophenone and benzyl hydroxybenzoate have been identified (English et al., 1991) but even traces of these compounds were absent in the studied samples. Thus, the source of dihydrochalcones in the samples has to be the bud exudate of *P. balsamifera*. For this poplar species, the presence of large amounts of chalcones, pinocembrin chalcone and pinostrobin chalcone is also typical (Greenaway et al., 1989). We observed significant amounts of these two compounds in the samples.

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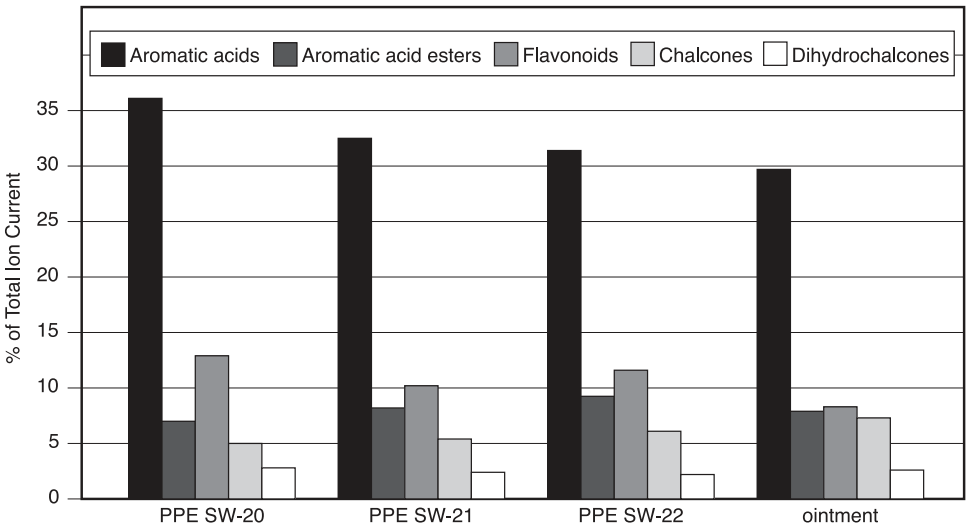

Fig. 1. Chemical profiles by groups of compounds of three samples of PPE and one sample of 3% ointment.

On the basis of this evidence, a conclusion can be drawn about the origin of the propolis samples used for production of the ointment: they are of mixed origin; their sources are bud exudates of both *P. balsamifera* and *P. tremuloides*.

Ointment sample

In order to check the presence of the whole complex of propolis constituents in the preparation, the ointment was extracted with 70% ethanol, the extract was evaporated to dryness, silylated and analyzed by GC–MS. The results are represented in Table 1, for comparison with the extracts. It is obvious that the chemical profile of propolis constituents extracted from the ointment is very similar to the profiles of the original propolis extracts. A representation of the profiles (by groups of compounds) is shown in Fig. 1.

In vitro antiviral studies

Cytoxocity of PPE

The maximum tolerated (non-cytotoxic) concentration of PPE (sample SW-21) in MDBK cells was 0.032 mg/ml. The sample value of CC_{50} was determined as 0.13 mg/ml. The CC_{50} of acyclovir, used as reference substance in this test, was 2129 ± 49.4 μM.

Effect of PPE on HSV-1 adsorption in MBDK cells

MBDK cell monolayers were inoculated with HSV-1 and treated in parallel with the extract, at 4 °C and for a duration from 15 to 120 min. Incubation at 4 °C allows viral attachment (adsorption) but not virus entry in the cells. In these conditions, PPE suppressed the adsorption of HSV-1 at a broad scope of the viral inoculation (10^3–10^5 $CCID_{50}$), at concentration of 0.1 mg/ml (which represented its IC_{50}), and when the duration of the adsorption was equal to or more than 60 min (Fig. 2)

Virucidal effect of PPE on HSV-1 and HSV-2

The virucidal effect of PPE against HSV-1 and HSV-2 was tested at room temperature and at 4 °C with increasing concentrations of the extract (0.1, 0.32, 1, 3.2, 10, 32 and 50 mg/ml), for time intervals ranging from 15 min to 6 h (Figs. 3 and 4).

PPE manifested a pronounced virucidal effect (Δ logs exceeding 4 log) against HSV-1, at concentrations equal to or above 10 mg/ml and with a contact time equal to or above 15 min. A marked effect was observed at the PPE concentration of 3.2 mg/ml with a contact time equal to or above 15 min, and some activity was also found with 1 mg/ml but only after a contact time of 3 h.

The PPE concentration of 0.32 mg/ml was ineffective. A complete virus inactivation, observed in the control samples as well, was found after 6 h at room temperature.

PPE manifested also a pronounced virucidal effect against HSV-2 at room temperature, approximately equal to that observed against HSV-1 (Fig. 5).

The virucidal effect of PPE against HSV-1 and HSV-2 was present but lower when the contact samples were stored at 4 °C (results not shown).

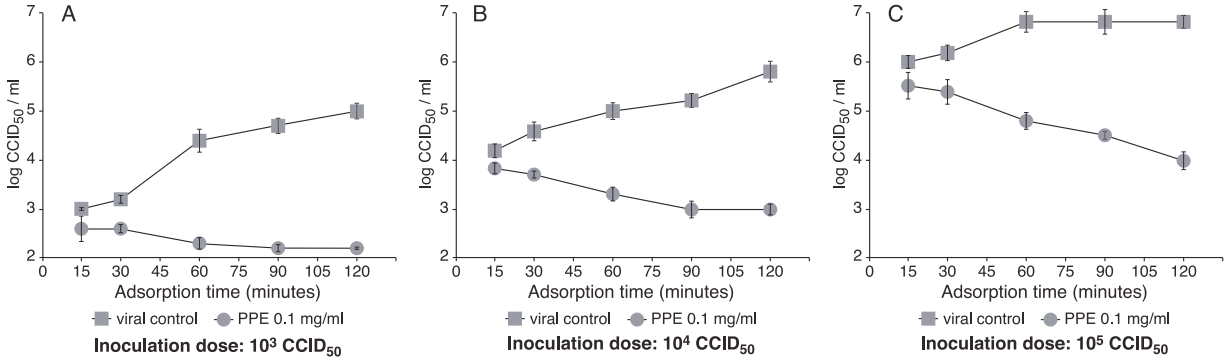

Fig. 2. Effect of PPE (0.1 mg/ml) on HSV-1 adsorption in MDBK cells with a virus inoculation dose of 10^3 $CCID_{50}$ (A), 10^4 $CCID_{50}$ (B) and 10^5 $CCID_{50}$ (C).

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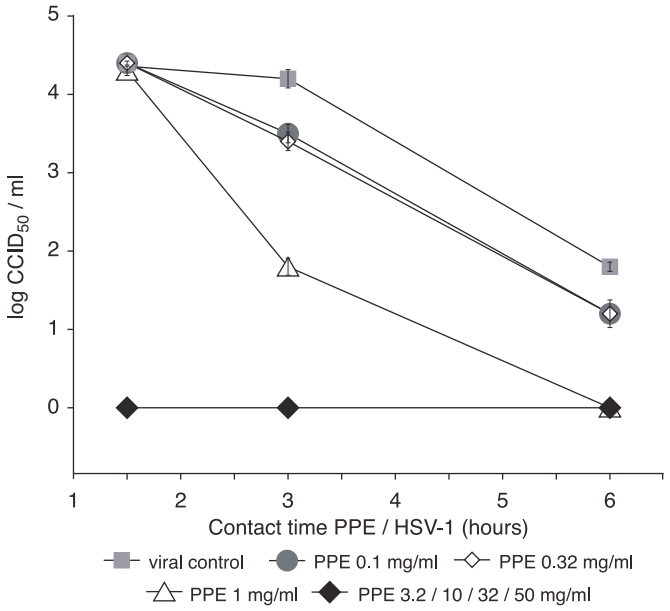

Fig. 3. Virucidal activity of various concentrations of PPE on HSV-1 (Victoria strain) at room temperature.

Discussion

Composition–activity relationship

Propolis from different phyto-geographic regions and from diverse source plants displays very different chemical profiles. Major constituents of European propolis are flavonoid aglycones, phenolic acids and their esters. Mediterranean propolis typically contains diterpenic acids, while Green Brazilian propolis is characterized by C-prenylated p-coumaric acids and Red South American propolis contains predominantly isoflavonoids. Despite this chemical diversity, all types of propolis possess significant antimicrobial activity and, in most cases, the responsible compounds are the phenolic constituents of propolis.

Many of the compounds identified in the Canadian propolis extracts in this study have been reported to possess activity against herpes simplex virus (HSV). The major constituent, p-coumaric acid, was found to have only weak antiherpetic action (Chiang et al., 2002; Schnitzler et al., 2010). For caffeic acid, consistent results showing anti-HSV activity have been reported by different laboratories (Chiang et al., 2002; Khan et al., 2005), and its mode of action against HSV-2 was found to be at multiplication stages. It is interesting to note that another aromatic acid present in the samples, cinnamic acid, produced negative results in the virucidal activity test. However, it demonstrated reduction in the viral titer at 1 and 0 h in the time of addition test, suggesting that this compound has an effect on cell receptors, and possibly also an effect at the viral adsorption stage (Gravina et al., 2011). Concerning the flavonoids identified in the Canadian samples, there are data about the anti-herpes virus potential of chrysin, galangin and kaempferol. Different research groups have reported a strong antiviral activity against HSV for galangin and kaempferol (Amoros et al., 1992b; Meyer et al., 1997; Lyu et al., 2005). Chrysin demonstrated moderate inhibitory effects against HSV-1 (Lyu et al., 2005). Amoros and colleagues (Amoros et al., 1992b) observed synergistic action of binary flavone–flavonol combinations.

Chalcones are a specific class of flavonoids and they also are known to possess different bioactivities, among them antiviral (Dimmock et al., 1999). However, for the two chalcones found in our samples, 2′,4′,6′-trihydroxychalcone (pinocembrin chalcone) and 2′,6′-dihydroxy-4′-methoxychalcone (pinobanksin chalcone) no specific data for antiviral activity could be found.

Dihydrochalcones form a small group of flavonoid compounds with relatively limited distribution in nature and their biological activities are not studied in detail. These compounds are known mainly for their antioxidant properties (Bentes et al., 2011; van der Merwe et al., 2010), and there are also data about their antibacterial and cytotoxic potential (Orjala et al., 1994). No information concerning antiviral activity of dihydrochalcones could be detected in the scientific literature. However, it is interesting to note that dihydrochalcones were among the major constituents of the extracts of apple pomace, which inhibited both HSV-1 and HSV-2 replication (Suárez et al., 2010).

Obviously, the extracts and the preparation obtained from these extracts contain a number of compounds with known antiherpetic action, such as caffeic acid, galangin and chrysin, although they are not the major constituents. The most abundant component, p-coumaric acid, was reported to have mild activity against HSV. In addition, the extracts contain also many constituents with unknown and untested antiviral potential, and some of them could possess valuable anti-HSV activity, too. It is important to stress that investigations on the antiviral action of individual substances isolated from propolis showed that no individual constituents had an activity greater than that of the total extract (Schnitzler et al., 2010). Schnitzler and colleagues suggested that the antiviral impact of propolis is not only due to single compounds, but also to a mixture of different constituents and therefore the propolis complex in the galenic preparation as extract is more effective. It seems that the chemical properties of propolis have general pharmacological value as a natural mixture (Kujumgiev et al., 1999), and there are many reports in the literature about propolis synergism with selected antibiotics. The combination of Propolis and mupirocin was successful against methicillin-resistant *Staphylococcus aureus* (MRSA) in nasal carriage in rabbits (Onlen et al., 2007), and the topical

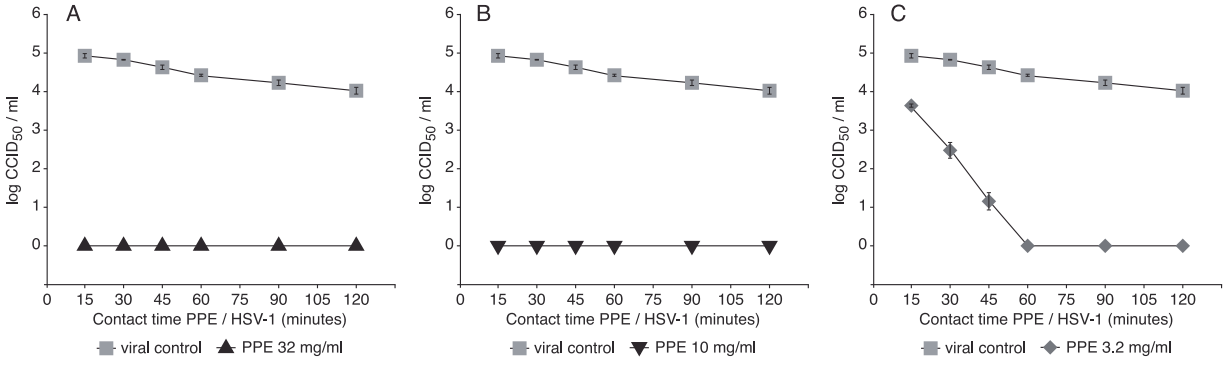

Fig. 4. Virucidal activity of PPE 32 mg/ml (A), 10 mg/ml (B) and 3.2 mg/ml (C) on HSV-1 (Victoria strain) at room temperature.

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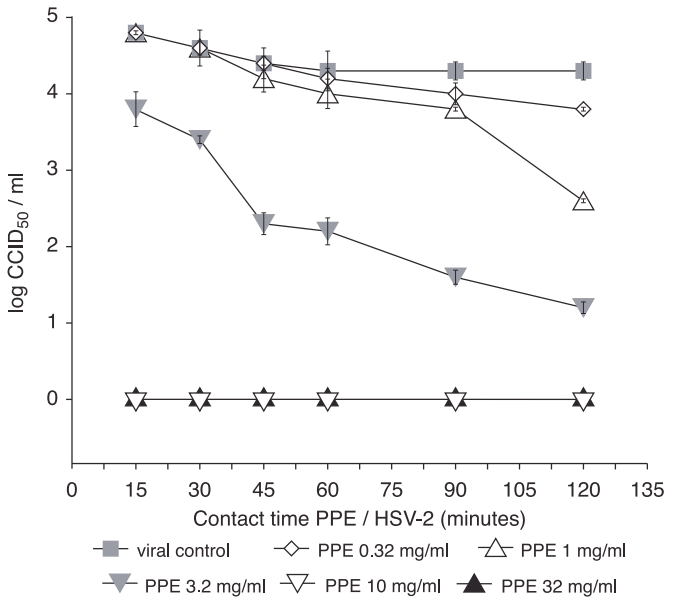

Fig. 5. Virucidal activity of various concentrations of PPE on HSV-2 (Bja strain) at room temperature.

administration of ciprofloxacin plus propolis produced significantly better results in experimental *Staphylococcus aureus* keratitis in rabbits compared to those obtained with the single compounds (Duran et al., 2006). The Canadian propolis is of dual plant origin and combines the defensive metabolites of two different tree species, quaking aspen *Populus tremuloides* and balsam poplar *P. balsamifera*. It could be expected that the biological activity of these different chemical structures would be the result of different mechanisms of action, and that such a combination would be especially effective against infections, including viral infections.

It is necessary to note that there is a lack of data concerning the antiviral activity against HSV for the chalcones and dihydrochalcones, characteristic for this specific type of Canadian propolis. As a next step of the research activity, these compounds could be isolated (or synthesized) and their antiviral activity could be determined. Such a research would add to our knowledge about propolis and add value to Propolis Extract ACF and its related ointment preparation.

Antiviral activity

Propolis Extract ACF showed some significant activity against herpes simplex virus in two conditions – during the adsorption phase of the viral infection, and when incubated in direct contact with the virus.

When the cells were treated in parallel with both the extract and the virus, the extract markedly inhibited the adsorption of HSV-1 to the cells when the duration of the adsorption was at least equal to 60 min.

A pronounced virucidal effect of PPE (Δ logs exceeding 4 log) against HSV-1 was observed at concentrations equal to or more than 10 mg/ml and with a contact time of at least 15 min, but also with 3.2 mg/ml. Some activity was found with 1 mg/ml, but only after a contact of 3 h. As expected, the virucidal effect was lower at 4 °C, and the extract exerted a pronounced virucidal effect also against HSV-2, approximately equal to that against HSV-1.

Overall, the results obtained in this study indicate that PPE is able to directly interfere with both types of herpes virus and seems to impair the ability of the virus to adsorb or to penetrate the host cells. The results of the virucidal assay suggest that the herpes virus outside the host cell is quite vulnerable to propolis, and that the direct contact triggers changes that somehow affect the infectivity of the virus. This is also confirmed by the results obtained in the adsorption test, as the propolis extract is added to the cell cultures in the phase of infection characterized by virus still outside the cells.

Our results are in line with those of Amoros et al. (1992a), who reported a virucidal effect after pre-treatment of the herpes virus with propolis, and of Schnitzler et al. (2010), who investigated the effect of aqueous and ethanolic propolis extracts against herpes virus and obtained a marked antiviral effect when HSV-1 was pre-incubated with the extracts before being inoculated in the cells.

Schnitzler found no significant effects when the extracts were added to the cultured cells after penetration of the virus into the host cells. Similarly, PPE was not able to inhibit the progress of the viral infection when added to the cells after adsorption and penetration (results not shown). Schnitzler and Amoros did not detect any effect after cell pre-treatment with propolis, and PPE showed a weak effect on HSV-1 replication when the cells were treated prior to infection (results not shown). This is in contrast with results obtained by Huleihel and Isanu (2002), who observed instead a marked inhibitory effect when an aqueous extract of propolis was added to the cells before or at the same time of the viral inoculation and also showed an effect when propolis was added to the cells 2 h after infection, indicating the possibility of an intracellular activity of propolis. Overall, this confirms the possibility of different antiviral mechanisms of propolis, and this is not surprising if we take into consideration the high number of compounds that have been identified in propolis to date.

The results from the GC–MS analyses reveal the dual plant origin of Propolis Extract ACF and the good level of standardization achieved through geographical selection, with a chemical composition practically unvaried between different extract samples, and reproduced in the topical ointment. The extract contains components derived from resins of two different species of poplar, and additional synergy in the mechanisms of action of the extract could be expected by this combination. The results obtained *in vitro* against herpes virus show that the antiviral activity of PPE can be partly due to interference of the viral adsorption to the cells. The direct contact with propolis seems to mostly damage the herpes virus, and it has been suggested that propolis might interfere with specific components of the viral envelope (glycoproteins) that mediate adsorption and allow attachment and penetration of the virus into the cells.

The results obtained *in vitro* with PPE are in accordance with those of two randomized, placebo-controlled clinical studies, where the topical ointment containing 3% Propolis Extract ACF was used to treat cold sores (Hoheisel, 2001) and herpes *genitalis* (Vynograd et al., 2000), and support the usefulness of a topical preparation with Propolis Extract ACF against herpes simplex virus lesions.

Conflict of Interest

Barbara Di Perri is a director of Enzpharma (UK) Ltd. and works as a consultant to Lisoma International Ltd. for medical and regulatory issues.

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