THE WATER-SOLUBLE PROPOLIS SHOWS ANTI – INFLUENZA VIRUS ACTIVITY

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Summary

The Influenza virus affects the respiratory tract in humans, causing a range of distinct manifestations including fever, nasal secretions, cough, headaches, muscle pain and pneumonia, which could become violent and severe. Influenza A viruses remain resistant to Amantadine and Rimantadine with a high level of Oseltamivir. Therefore, there is a need for constant improvement of drugs active against resistant Influenza viruses. Propolis has anti-influenza activity both *in vitro* and *in vivo*. Human leukocyte interferon (HuIFN-αN3) is a multi-subtype protein that displays activity against Influenza A, B, and C viruses.
In this study, authors elucidated the anti - Influenza activity of the mixes of Water extract of Propolis and HuIFN-αN3 at different ratios:1:1,1:2 and 2:1. Water extract of Propolis's polyphenols and HuIFN-αN3 were characterized by RP-HPLC. Influenza A and B viruses were separately added to the LLC-MK2 cells treated with Water extract of Propolis and HuIFN-αN3 alone or in ratios 1:1, 1:2, and 2:1.Plates were incubated and cytopathic effects were determined. The best results of ID_{50} were obtained with the mix of 10% Water extract of Propolis and HuIFN-αN3 1:2, showing ID_{50} at 12 ± 0.2 μg/mL for Influenza A and 19 ± 0.6 μg/mL for Influenza B viruses. When comparing the anti-influenza activity of the Water extract of Propolis /HuIFN-αN3 with that of Ribavirin, it was found that 1:2 was the optimal ratio for Water extract of Propolis /HuIFN-αN3 (0.5 and 0.6 for Influenza A and B). This new formulation of Water extract of Propolis and HuIFN-αN3, showing better anti-influenza activity, will improve its application in children' s flu infections *in vivo*.

Keywords: Ethanol extract of Propolis, Influenza viruses, Antiviral activity, Tissue culture

Introduction

Influenza virus infects the respiratory tract in humans and animals causing a variety of different symptoms, including fever, nasal secretions, cough, headache, muscle pain, and pneumonia which often could become severe (Beilharz et al., 2007). During the influenza season, the antigenic drift in the virus occurs often when the formulation of the year's vaccine has already been made. As a consequence, the vaccine became less protective and outbreaks can occur (Dai et al., 1987). The pandemic avian H5N1, H1N1, and already changed A(H3N2) influenza virus strains have spread worldwide, so the emergence of pathogenic influenza virus strains can be predicted (Merigan et al., 1973). In general, it was found that most Influenza A viruses remained resistant to Amantadine and Rimantadine with high levels of Osletamvir resistance (but Zanamivir sensitivity) in seasonal H1N1 (Kugel et al., 2009). Therefore, the constant development of new anti-influenza virus rugs that are effective against resistant Influenza viruses is needed. Bee Propolis is used as folk medicine since 300 BC as a food supplement to maintain or improve human health (Mishima et al., 2005). It is composed of resins (40-55 %), beeswax and fatty acid (20-35 %), essential oils (10%), pollen (5%), and other components such as minerals, vitamins, and sugar. The chemical composition of propolis is complex and more than 180 compounds were identified in it. Biologically most important are polyphenols (Kumazava et al., 2000). Its chemical composition is qualitatively and quantitatively variable, depending on origin and regional plant ecology. The pharmacological properties of Propolis were reported as anticancerogenic (Armstrong, 1981), anti-inflammatory (Uruhisaki et al., 2011), and antimicrobial (Kai et al., 2011). The antiviral activity against several viruses was demonstrated, e.g. Adenovirus (Kujumgijev et al., 1999), HIV (Li et al., 2005), Herpes simplex virus (Hayakari et al., 2013) and anti-Influenza activity (Filipič et al., 2007) (Shi et al., 2007) as *in vitro* as *in vivo*. It was also characterized by the kaempferol flavonoid-related compound (AF-08) responsible for anti-influenza activity (Schanen et al., 2006). HuIFN-αN3 is a multi-subtype protein showing antiviral, antiproliferative, antitumor, radioprotective and antitoxic activity. There are three major classes of IFNs, designated as Types I, II and III (Pavlovich et al., 1990). Type I-IFNs consist of IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-ζ, IFN-κ,IFN-ν, IFN-τ, and IFN-ω. Type II-IFN is composed of a single cytokine, IFN-γ (Prix et al., 1998). Type III-IFNs are IFN-λ1, IFN-λ2,

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IFN-λ3 and IFN-λ4 Type I-and type III-IFNs with similar signal transduction systems are phylogenetically closer to each other than type II-IFN. It is used for the treatment of a variety of different viral diseases and cancers. Among viruses Influenza A and B are being susceptible to HuIFN-αN3 (Weiss et al., 1989) (Mishima et al., 2005). It is important to analyze the effectiveness of the combination of Propolis' Ethanol and Water extracts with HuIFN-αN3 and through this to improve their possible clinical usefulness.
The purpose of the presented experiments was to elucidate the anti-influenza activity against Influenza Viruses A and B, of the combinations of Ethanol extract of Propolis and/or Water extract of Propolis and HuIFN-αN3 *in vitro*.

Materials and methods

Cells and viruses

The LLC-MK2 cells were cultivated in the Eagle's medium with 10% FCS and Antibiotics. Influenza A and B viruses were obtained from the Virological Department of the Institute for Microbiology and Immunology in Ljubljana (Slovenia).

Compounds

10% water-soluble propolis prepared from 30% water-soluble propolis that was obtained from BNatural, Corbetta, Italy. 10% Ethanolic extract of Propolis was obtained from Medex d.o.o., Ljubljana, Slovenia. HuIFN-αN3 was from the Institute for Immunology, Zagreb, Croatia.

Cell treatment

The cell treatment experiments were performed in the Interferon research laboratory of the Medical Faculty in Ljubljana, Slovenia, as follows: 100 µl of medium+2% FCS were added from the second to eleventh well on the multiwell plate. In the first well 200 µl of 10% Ethanolic extract, 10% Ethanolic extract+HuIFN-α N3(1:1,1:2 and 2:1), 10% Water extract of propolis, 10% Water extract of propolis + HuIFN- αN3 (1:1,1:2 and 2:1) and 200 µl of HuIFN-αN3 and 200 µl of Ribavirin as a control. All samples were serially diluted and incubated for 8 hours at 37 °C. Influenza A and separately Influenza B viruses were added, and plates were incubated at 37 °C for four days when in the control 100% CPE with small plaques were developed.

Detection of HuIFN-αN3 or Pinocembrin and Galangin by RP-HPLC method

The RP-HPLC analyses were performed in the Medex' HPLC research laboratory which has an accreditation to ISO 17034:2016 to perform the valid analyses. The Vaquish core HPLC sistem with up to 700 bar was used in these experiments. In 10 ml burette, 1.0 mg of Pinocembrin or Galangin are added and diluted to 10.0 ml with Me OH. From this solution, 150 µl of samples were put into the vial and filled with 1350 µl of Me OH. During the analyses 30 different samples were filtered through a 0,45 µm filter and injected 20 µl into the HPLC column. In the experiments the HPLC column Purospher® STAR RP-18; 5 µm 150 x 4,6 mm was used. Conditions of the HPLC system: (a) Temperature of the column: 25 °C, (b) Flow: 0.7 ml/min.; (c) Pressure: 90 – 100 Bar; (d) Atte: 62.5 (e): Absorbance: 290 nm; (f) Injection volume: 20 µl;(g): Gradient: Solvent A = water + 1% Formic acid; Solvent C=Acetonitrile. The Steps of the RP-HPLC run are shown in Table 1 for HuIFN-αN3 samples and in Table 2 for the detection of pinocembrin and galangin.

Table 1. Time course of RP-HPLC chromatography of different HuIFN-αN3 samples

Step: chromatography of different IFN samples in step	Time (min)	Solvent A (%)	Solvent C (%)
0	0	91	9
1	3	80	20
2	6	50	50
3	12	50	50
4	15	91	9
5	20	91	9

Table 2. The steps of the RP-HPLC detection of pinocembrin and galangin

Step: Chromatography of pinocembrin and galangin step	Time (min)	Solvent A (%)	Solvent C (%)
0	0	70	30
1	5	60	40
2	15	60	40
3	20	35	65
4	25	35	65
5	30	70	30
6	35	70	30

The analyses: Plates were washed with PBS, fixed with 5% Glutaraldehyde, washed with PBS and 100 µl of crystal violet was added for 20 minutes. The plates were washed with PBS, and air dried and the OD was measured at 570 nm. The effective concentrations for 50% plaque reduction (ID_{50}) were determined from a curve relating the plaque number to the concentrations of the propolis extracts and huIFN-αN3. The effect of different combinations of Propolis' ethanol or water extract with HuIFN-αN3 in various combinations (1:1, 1:2, and 2:1) on Influenza A and Influenza B virus was also expressed as Eq (1):

$$\text{Ribavirin } ID_{50} \text{ index} = \frac{\text{Propolis' extract and/or HuIFN-αN3(1:1,1:2,2:1) } ID_{50}}{\text{Ribavirin } ID_{50}} \quad (1)$$

Statistical Analysis

The ID_{50} based on the mean plaque number was calculated on the raw data of an in-triplicate assay by regression analysis using Probit (SPSS statistical software package), determining the concentration of drug required to reduce the number of plaques by 50%. Statistical analysis of the experimental data was performed with a two-tailed Student's *t*-test for paired samples with a $p = 0.05$ as the smallest level of significance.

Results

RP-HPLC Analyses of Sendai Virus (Cantell Strain) Induced Interferon (HuIFN-αN3)

HuIFN-αN3 subtypes in different samples (natural or recombinant) are separated according to their relative hydrophobicity using HPLC column Purospher® STAR RP-18 5 µm. The separation of different HuIFN-αN3 subtypes in the samples was achieved by increasing acetonitrile concentration (Kumazava et al., 2000) (Kumazava et al., 2000b). The least hydrophobic interferon subtypes were eluted as early peaks and the most hydrophobic Interferon subtypes eluted as later. As standards, different human recombinant interferons α were used: HuIFN-αA, HuIFN-α2a, and HuIFN-α2b. Their chromatograms and the chromatograms at 280 nm of the Russian HuIFN-αN3 (NDV induced) and HuIFN-αN3 of the Institute of Immunology Zagreb (Croatia) (Sendai virus-induced) were used as standards (Figure 1A). The positions of different HuIFN-αN3 subtypes were determined according to the 214 nm chromatogram in comparison to the protein profile measured at 280 nm. The predominant components of the Sendai virus-induced HuIFN-αN3, are shown in Figure 1B, and are natural IFN subtypes: α1, α2, αA, α2b, and α14. The most important is the relative ratio between α1 and α2 (values of mAU relative units). Various types of HuIFN-αN3 inductors differ in the induction capacity of IFN subtypes: α1, α2, αA, α2b, and α14. The HuIFN-αN3 subtype's antiviral activity in IU/mL was determined by the detection of their' antiviral activity according to the standard procedure: Monolayer received interferon dilution at two-fold increasing levels overnight. The following morning, the medium was removed and 100 µL of challenge virus (Vesicular Stomatitis Virus) in Eagle's medium + 2% FCS were added, and the cell layers were examined under the microscope 24^h later and scored (+4, +3, +2, +1, +0 corresponding to 100% destruction, 75%, 50%, 25%, non-infected, respectively) (Armstrong et al., 1981).

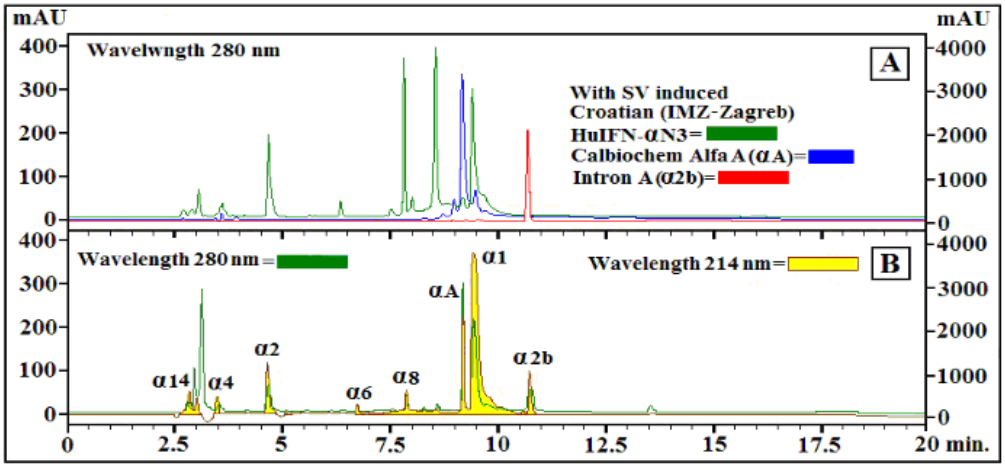

Figure 1. RP-HPLC profiles of the Sendai virus-induced HuIFN-αN3:
(A) SV=Sendai virus (Cantell strain). Protein profiles of the various IFNs at 280 nm
(B) Protein profile at 280 nm ( ) and IFN profile at 214 nm ( ) of
HuIFN-αN3 induced with 100 HA/mL of Sendai virus (Cantell strain)

Quantity of Pinocembrin and Galangin in 10% Propolis' ethanol extract and in 10% Propolis water extract

The 1.0 mg of caffeic acid, chrysin, pinocembrin, and galangin were put and diluted to 10.0 mL with methanol. From this solution, 150 µL of the sample was transferred into a vial and loaded with 1.350 µL of methanol. Samples filtered through a 0.45 µm filter were injected by 20 µL into the HPLC column Purospher® STAR RP-18 5 µm. Their separation was achieved with an acetonitrile gradient in the HPLC column (Figure 2A). The 10% EEP was analyzed under the same conditions in the Purospher® STAR RP-18 5 µm HPLC column. Its separation measured at 290 nm, with an acetonitrile gradient, is presented in Figure 2B (Uruhishaki et al., 2011). The quantity of caffeic acid, chrysin, pinocembrin, and galangin in the experimental sample of 10% EEP was calculated in comparison to standards (Figure 2A). Figure 2C shows the RP-HPLC profile of 10% Water soluble Propolis. Therefore, Table 3 indicates the quantity of caffeic acid, chrysin, pinocembrin, and galangin in the 10% EEP. Table 4 shows a comparison of 10% EEP and 10% Water soluble Propolis in regard to the content of Pinocembrin and Galangin.

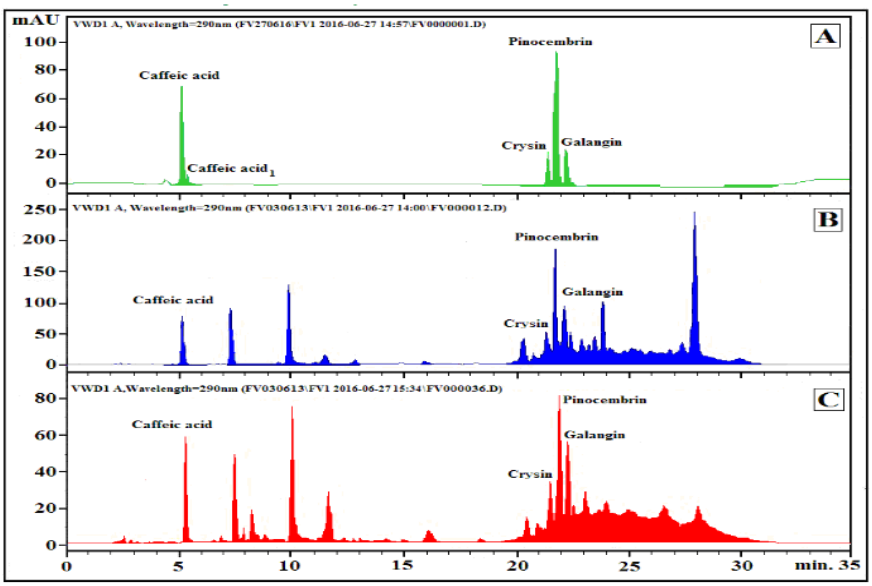

Figure 2. A = The RP-HPLC profile of the Bio-Flavonoid's standards (Caffeic acid, Crysin, Pinocembrin and Galangin); B = The RP-HPLC profile of 10% Ethanol extract of Propolis; C = The RP-HPLC profile of 10% Water soluble Propolis

Table 3. Quantity of caffeic acid, crysin, pinocembrin and galangin in 10% EEP

Extract	Caffeic acid (μg/mL^{-1})	Crysin (μg/mL^{-1})	Pinocembrin (μg/mL^{-1})	Galangin (μg/mL^{-1})
10% EEP	19 ± 0.18	5.4 ± 0.48	0.32 ± 0.08	0.29 ± 0.11

Table 4. Quantity of Pinocembrin and Galangin in 10% Propolis' ethanol extract and 10% Propolis' water extract

Extracts:	Pinocembrin (μg / mL^{-1})	Galangin (μg / mL^{-1})
10% Propolis' ethanol extract	0.32±0.08	0.29±0.11
10% Propolis' water extract	0.05±0.01	0.06±0.02

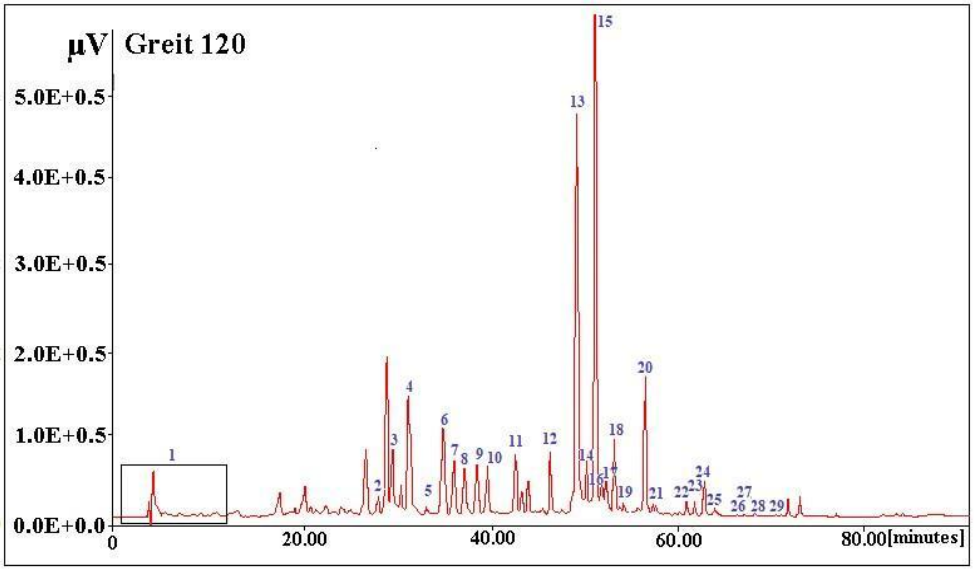

Figure 3. Molecular Composition of Water extract of Propolis Determined by HPLC-UV-ESI-MS 504971
1 = phenolic acids (caffeic, coumaric, ferulic, isoferulic); 2 = quercetin; 3 = pinobanksin 5-methyl ester;
4 = quercetin 3-methyl ester;5 = pinobanksin; 6 = apigenin; 7 = kaempferol; 8 = isorhamnetin; 9 = luteolin 5-methyl ester; 10 = quercetin 5-7-dimethyl ester; 11 = galangin 5-methyl ester; 12 = quercetin 7-methyl ester;
13 = chrysin; 14 = pinocembrin; 15 = galangin; 16 = pinobanksin-3-O-acetate;17 = CAPE; 18 = metoxychrysin; 19 = pinobanksin-3-O-propionate; 20 = caffeic acid cinnamyl ester; 21 = pinobanksin-3-O-butyrate;
22 = pinobanksyn-3-O-pentenoate; 23 = other pinobanksin derivative; 24 = pinobanksin-3-O-hexanoate;
25 = other pinobanksin derivative. (Obtained by kind help of Dr. Nicola Volpi from Department of Life Sciences, University of Modena & Reggio Emilia, Modena 41125, Italy)

Figure 3 shows the HPLC-UV-ESI-MS504971 profile of the Water extract of Propolis. The used ID_{50} 12 ± 2 μg/mL for influenza A and 19 ± 6 μg/mL for influenza B are shown. With 10% EEP and HuIFN-αN3, the best ratio was 1:2, where it was the ID_{50} 22 ± 7 μg/mL for influenza A and 15 ± 4 μg/mL for influenza B.

Ribavirin ID_{50} Index

The Ribavirin ID_{50} index was calculated to compare the ID_{50} (antiviral activity) of Water extract of Propolis or 10% EEP in combination with HuIFN-αN3 in ratios 1:1, 1:2, and 2:1 in comparison to Ribavirin. The results are presented in Table 5 and Figures 4 and 5. The lower is, the better it is. The ratio 1:2 was still the best with WSP in combination with HuIFN-αN3 (0.5 for influenza B and 0.6 for influenza A virus). With EEP in combination with HuIFN-αN3, the best was the same ratio of 1:2 (0.7 for influenza B and 1.3 for influenza A virus).

Antiviral activity of combinations of Propolis and HuIFN-αN3

Table 5. The antiviral activity of 10% Ethanolic extract of Propolis, 10% Water extract of Propolis and HuIFN-αN3 in the ratios: 1:1, 1:2 and 2:1 expressed as ID_{50} in µg/mL

Sample:	Bioflavonoids as Caffeic acid (mg mL $^{-1}$)	Influenza A ID_{50}(µg mL^{-1})[1]	Influenza B ID_{50}(µg mL^{-1})[1]
10% Ethanolic extract of Propolis	19±0.18	82±11	62±6
10% Ethanolic extract of Propolis + HuIFN-αN3 1:1	9±0.59	35±7	31±6
10% Ethanolic extract of Propolis + HuIFN-αN3 1:2	6±0.39	22±8	15±6
10% Ethanolic extract of Propolis + HuIFN-αN3 2:1	12±1.78	42±4	31±7
10% Water soluble Propolis	14±1.20	31±9	29±2
10% Water soluble Propolis + HuIFN-αN3 1:1	7±1.10	22±2	31±3
10% Water soluble Propolis + HuIFN-αN3 1:2	4±0.73	12±2	19±7
10% Water soluble Propolis + HuIFN-αN3 2:1	9±0.46	25±6	30±2
Ribavirin		20±2	28±4

[1]ID_{50} = is the concentration of the sample needed to inhibit virus induced CPE (Cytopathogenic effect) on 50%

Ribavirin ID_{50} index

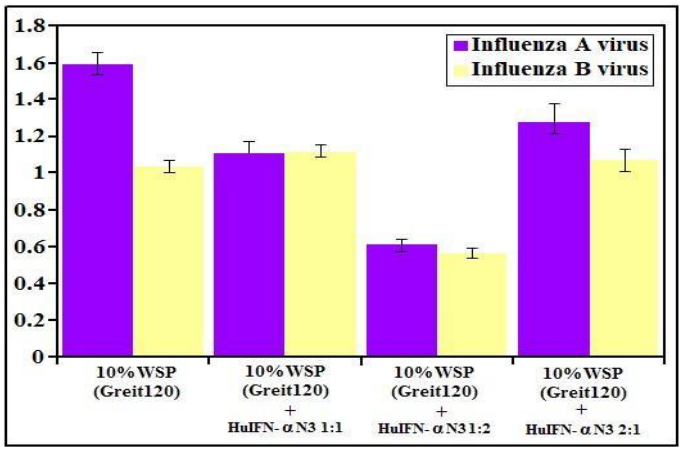

Figure 4. Ribavirin ID_{50} index of Water extract of Propolis and/or combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1

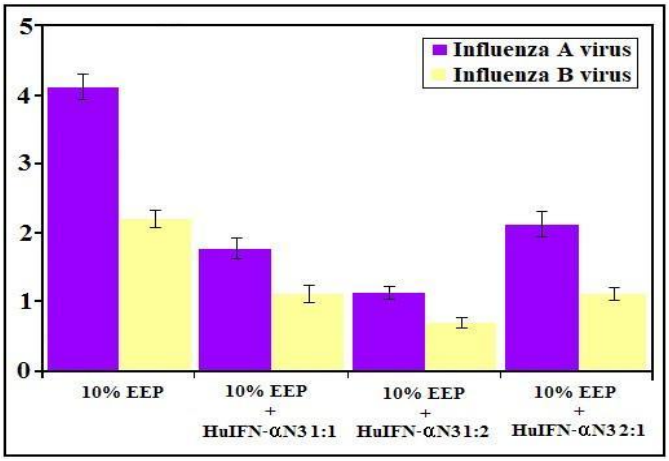

Figure 5. Ribavirin ID_{50} index of EEP and/or combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1

Discussion

The samples of very detailed analyzed Water extract of Propolis containing different polyphenols: apigenin (ID_{50} 8.1 ± 4.7 μg/mL), chrysin (ID50 > 100 μg/mL), kaempferol (ID_{50} 24.8 ± 4.3 μg/mL) quercetin (ID_{50} > 100 μg/mL) and caffeic acid (ID_{50} 49.7 ± 5.0 μg/mL) already showed anti-Influenza activity *in vitro* (Kai et al., 2014). The anti-influenca A and B virus activity of complete Water extract of Propolis molecule is: ID_{50} 31 ± 0.9 μg/mL for influenza A virus and ID_{50} 29 ± 0.2 μg/mL for influenza B virus, what is a bit lower, but comparable with ribavirin, having ID_{50} 20 ± 0.2 μg/mL for influenza A and ID_{50} 28 ± 0.4 μg/mL for influenza B. When HuIFN-αN3 is added to Water extract of Propolis in a ratio of 1:1, the ID_{50} 22 ± 0.2 μg/mL for influenza A and 31 ± 0.3 μg/mL for influenza B are found. When this ratio is 1:2, the ID_{50} is 12 ± 0.2 μg/mL for influenza A and 19 ± 0.7 μg/mL for influenza B virus. The ratio 2:1 shows the ID_{50} 25 ± 0.6 μg/mL for influenza A and 30 ± 0.2 μg/mL for influenza B. The highest increase was found when Water extract of Propolis was combined with HuIFN-αN3 in a ratio of 1:2. To elucidate the mechanisms of anti-influenza activity of Water extract of Propolis it was found that caffeic acid from it could restore the viability of cells infected with influenza virus in a dose-dependent manner (Kujumgijev et al., 1999). To find working mechanisms of this anti-influenza activity, it was measured the relative value of influenza virus RNA in cultured cells with and without antiviral compounds. It was found that the relative value of influenza virus RNA/viable cells was not significantly different between groups with different compound concentrations. So it is possible that Water extract of Propolis has no direct influence on an influenza virus or does not interact with influenza virus components, although Li et al. (2005) reported that caffeoylquinic acid from Water extract of Propolis binds to the gp120 of RSV (respiratory syncytial virus) and inhibits virus-cell fusion events in the early stage of the replication cycle. Thus, the anti-influenza activity of Water extract of Propolis is not derived from an inhibition of virus replication, as is true for a neuraminidase inhibitory drug, but may be due to another mechanism, such as an enhancement of cell resistance. As to the effect on antiviral executor genes, Water extract of Propolis enhanced myxovirus resistance 1 (Mx1) expression (Hayakari et al., 2013). Different specificities in antiviral effects of HuIFN-αN3 against influenza A and B viruses were reported as *in vitro* and *in vivo* (Schanen et al., 2006). They share the same specific cell receptor, interferon type I receptor (IFN-αR) composed of two subunits, IFN-αR1 and IFN-αR2, and interact with its different regions (Cook et al., 1996). Antiviral activity of HuIFN-αN3 against influenza A, B, and C viruses is mediated, at least in part, by the induction of intracellular antiviral proteins, such as MxA protein. It is induced by HuIFN-αN3 as a whole and inhibits the replication of various influenza viruses (Pavlovich et al., 1990) (Zurcher et al., 1992). Water extract of Propolis enhances the anti-influenza activity of HuIFN-αN3 in a dose-dependent ratio *via* enhanced resistance of Mx1 expression and MxA induction of influenza virus replication inhibition.

Conclusions

Pinocembrin and Galangin are probably the main antiviral components of Propolis that interact with HuIFN-αN3. The 10% Ethanol extract of Propolis and 10% Water extract of Propolis were analyzed by RP-HPLC. The findings can be seen in Figure 3 and Table 4. The results show a lower amount of Pinocembrin and Galangin in 10% Water extract of Propolis, as in 10% Ethanol extract of Propolis, even here the higher antiviral activity against Influenza A and Influenza B viruses *in vitro* alone and combination with HuIFN-αN3 can be found. The experiments were performed to analyze the anti-influenza activity of 10% Ethanolic extract of Propolis and 10% Water extract of Propolis in combination with HuIFN-αN3 in different ratios (1:1, 1:2 and 2:1). Ribavirin alone was used as acontrol. The results in Table 4 show that the best results (ID_{50})were obtained when the combination of 10% Water extract of Propolis and HuIFN-αN3 in a ratio of 1:2 was used. (ID_{50}12±2 μg/ml for Influenza A and 19±6 μg mL-1 for Influenza B). In the case of 10% Ethanolic extract and HuIFN-αN3, the best ratio was 2:1, where 22±7 $\mu g/mL^{-1}$ for Influenza A and 15±4 $\mu g/mL^{-1}$ for Influenza B. The Ribavirin ID_{50} index was calculated to compare the ID_{50} (AV) activity of Water extract of Propolis or Ethanol extract of Propolis in combination with HuIFN-αN3 in ratios: 1:1, 1:2 and 2:1 in comparison to Ribavirin. The lower is, the better it is. In the case of Ethanol extract of Propolis in combination with HuIFN-αN3, the best was the ratio 1:2 (0.6 for Influenza B and 1.3 for Influenza A). The same ratio was also the best in the case of Water extract of Propolis in combination with HuIFN-αN3 (0.5 for Influenza B and 0.6 for Influenza A). In the future experiments it will be necessary to extend this new formulation to children flu infection.

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Conflict of Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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