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# Additive Effects of Water-Soluble Propolis (Greit 120) and Human Interferon Alfa (HuIFN-αN3) against Influenza Viruses *in Vitro*

Bratko Filipič[1], Lidija Gradišnik[2], Adriana Pereyra[3], Rok Kopinč[3], Klemen Rihar[4], Eva Ružić-Sabljić[5], Snežana Kramar[5], Damir Đermić[1, 6], Eugen Šooš[*7], Nicola Volpi[8], Alfredo Fachini[9] and Hrvoje Mazija[1]

*1. Croatian Institute of Experimental and Translation Oncology, Koledinećka 3, Zagreb 10040, Croatia*

*2. Institute of Biomedical Sciences, Medical Faculty of Maribor, Taborska 8, Maribor 2000, Slovenia*

*3. Medex D.o.o., Linhartova 49b, Ljubljana 1000, Slovenia*

*4. Chengdujska 4, Ljubljana 1000, Slovenia*

*5. Institute of Microbiology and Immunology, Medical Faculty, Zaloška 4, Ljubljana 1105, Slovenia*

*6. Division of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, Zagreb 10000, Croatia*

*7. Trg Sv. Ivana 5, Kloštar Ivanić 10312, Croatia*

*8. Department of Life Sciences, University of Modena & Reggio Emilia, Modena 41125, Italy*

*9. BNatural, Via Gran Sasso 33, Corbetta-MI 20011, Italy*

**Abstract:** 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. It was found that influenza A viruses remain resistant to amantadine and rimantadin with high level of oseltamvir resistance. 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 an antiviral activity against influenza virus. In this study we elucidated the anti-influenza activity of the mixes of water-soluble propolis (WSP) (Greit 120) and HuIFN-αN3 at different ratios. Greit 120 polyphenols, total phenol acids and bioflavonoid were characterized by HPLC-UV-ESI-MS504971 and HuIFN-αN3 by reverse-phase high-performance liquid chromatography (RP-HPLC). Influenza A and B viruses were separately added to the LLC-MK2 cells treated with WSP (Greit 120) and HuIFN-αN3 alone or in proportions 1:1, 1:2 and 2:1. Plates were incubated and cytopathic effect was determined. The best results ($ID_{50}$) were obtained with the mix of 10% WSP and HuIFN-αN3 in proportion 1:2, showing $ID_{50}$ at 12 ± 0.2 μg/mL and 19 ± 0.6 μg/mL for influenza A and B viruses, respectively. When comparing anti-influenza activity of WSP (Greit 120)/HuIFN-αN3 with that of ribavirin, it was found that 1:2 was the optimal ratio for WSP (Greit 120)/HuIFN-αN3 (0.5 and 0.6 for influenza A and B, respectively). This new formulation of WSP (Greit 120) and HuIFN-αN3, showing better anti-Influenza activity, will definitely improve its application in flu infections.

**Key words:** Water-soluble propolis, Greit 120, HuIFN-αN3, anti-influenza activity, ribavirin, $ID_{50}$.

## 1. Introduction

Influenza virus affects the respiratory tract in humans and animals leading to a range of diverse symptoms, consisting of fever, nasal secretions, cough, headache, muscle pain and pneumonia which usually could become severe [1, 2]. During influenza season, antigenic drift in the virus usually appears when the design of the year's vaccine was then established. Therefore, the vaccine became less protective and outbreaks occur [3]. The pandemic avian H5N1 and H1N1 and then changed influenza virus A (H3N2) strains have expanded worldwide, so the emergence of

**Corresponding author:** Bratko Filipič, Ph.D., research fields: apidology, imunology, virology and microbiology.

[*] Paper is devoted to the remembrance of Dr. Eugen Šooš, who passed away in December 2017.

pathogenic influenza virus strains can be anticipated [4].

It is noticed that most influenza A viruses remained resistant to amantadine and rimantadine with a high degree of oseltamivir resistance (but zanamivir sensitivity) in the seasonal wave of infection with H1N1 [5]. Therefore, a constant improvement of new anti-influenza virus drugs active against resistant influenza viruses is constantly required.

As a folk medicine since 300 BC, bee propolis is used as a food supplement for maintenance or improvement of human health [6]. It is composed of resins (40%-55%), beeswax and fatty acids (20%-35%), essential oils (10%), pollen (5%) and other ingredients, such as minerals, vitamins and sugar. The chemical arrangement of propolis is complex and in it, over 180 compounds were determined. Among them, the polyphenols are most significant [7]. Its chemical composition differs, depending on origin and local plant ecology. Propolis' pharmacological properties were accounted as anti-carcinogenic [8], anti-inflammatory [9] and anti-microbial [10]. The antiviral activity against several viruses [11] was further shown, e.g., adenovirus [12], HIV [13], herpes simplex virus [14, 15] and anti-influenza activity [16, 17] both *in vitro* and *in vivo*.

Human leukocyte interferon (HuIFN-αN3) is a multi-subtype protein showing antiviral, anti-proliferative, anti-tumor, radio-protective and anti-toxic activity. There are three main classes of IFNs, designated as Types I, II and III [18]. Type I includes IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-ζ, IFN-κ, IFN-ν, IFN-τ, and IFN-ω. Type II is composed of a single cytokine, IFN-γ [19]. Types III is IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. Type I- and type III-IFNs with similar signal transduction systems stay linked to each other than type II-IFN [20]. It is applied to treat a variety of viral illnesses and cancers. Among viruses, there are influenza A and B being susceptible to low dosage of HuIFN-αN3 [21-23]. It is crucial to analyze the effectiveness of the combo of extract of propolis (EEP) and water-soluble propolis (WSP) (Greit 120) with HuIFN-αN3 and through this to improve their potential clinical usefulness.

The purpose of the performed experiments was to elucidate the anti-influenza activity against influenza viruses A and B, of the combos of WSP (Greit 120) and HuIFN-αN3 *in vitro*.

## 2. Materials and Methods

### *2.1 Cells and Viruses*

LLC-MK2 cells were cultivated in the Eagle's medium with 10% fetal calf serum (FCS) and antibiotics. Influenza A and B viruses' different clinical isolates were from Virological Department of the Institute of Microbiology and Immunology of the Medical Faculty in Ljubljana (Slovenia).

### *2.2 Compounds Employed in the Experiments*

The 10% WSP prepared from 30% WSP (Greit 120) was obtained from BNatural, Corbetta (Italy). And 10% EEP was from Medex D.o.o., Ljubljana (Slovenia). HuIFN-αN3 was from Institute of Immunology, Zagreb (Croatia).

### *2.3 Inhibition of Influenza A and B Viruses Evaluated with Plaque Reduction Assays*

Approximately $6 \times 10^3$ LLC-MK2 cells/well in Eagle's medium + 10% FCS were seeded into 96-well plates, and incubated for 24 h at 37 °C in 5% $CO_2$ atmosphere. After reaching the confluence, the Eagle's medium + 10% FCS were removed and on each plate from second to eleventh well were added 100 μL of Eagle's medium + 2% FCS. In the first well 200 μL of: 10% EEP, 10% EEP + HuIFN-αN3 (1:1, 1:2 and 2:1), 10% WSP (Greit 120), 10% WSP (Greit 120) + HuIFN-αN3 (1:1, 1:2 and 2:1), 200 μL of HuIFN-αN3 and 200 μL of ribavirin as a control. All samples were serially diluted and incubated for 8 h at 37 °C. Samples were added in two parallels and three replicates. Then, medium with samples was removed and influenza A and separately influenza B viruses

(100 μL/well) were added and plates incubated at 37 °C for 24 h in 5% $CO_2$ atmosphere, when in the control 100% cytopathic effect (CPE) with small plaques were established. The plates with treated/infected LLC-MK2 cells were rinsed with phosphate-buffered saline (PBS), fixed with 5% glutaraldehyde, washed further with PBS and to them 100 μL of crystal violet was added for twenty minutes. With PBS, washed plates were air-dried and measured at 570 nm. The effective concentrations for $ID_{50}$ were determined from a curve relating the plaque number to the concentrations of the propolis extracts and HuIFN-αN3 [24, 25]. Effect of various combinations of EEP or WSP (Greit 120) with HuIFN-αN3 in different combinations (1:1, 1:2 and 2:1) on influenza A and B viruses was further expressed as a ratio to ribavirin [26] by Eq. (1):

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

*2.4 Analysis of HuIFN-αN3 by RP-HPLC*

The HuIFN-αN3 subtype composition was analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC). Used HPLC column was Phenomenex, Aeris™ Peptide column 3.6 μm XB-C18, 250 × 4.6 mm. On it, different HuIFN-α samples (natural and recombinant) by approximately one million of antiviral units (AU/mL) in a volume of 20-40 μL were put on the column. Adsorbed, were eluted by the linear gradient of solvent A (distilled water + 0.1% of trifluoroacetic acid (TFA)) and solvent C (acetonitrile + 0.1% TFA) for 20 min with a flow rate of 0.8 mL/min and pressure of 139-140 bars. The courses of RP-HPLC chromatography of different HuIFN-αN3 samples are shown in Table 1. Temperature of the column was 40 °C. The absorbance was measured at 214 nm and 280 nm. HuIFN-αN3 species of different compositions separated agreeing to the fact that there is relative hydrophobic pattern using RP-HPLC [27, 28].

*2.5 The Detection of Pinocembrin and Galangin by RP-HPLC Method*

In 10 mL burette, 1.0 mg of pinocembrin or galangin were put and diluted to 10.0 mL with methanol. From this solution, 150 μL of the samples were 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. In the experiments HPLC column Purospher® STAR RP-18 5 μm, 150 × 4.6 mm was used. The conditions of the HPLC procedure: (a) temperature of the column: 25 °C; (b) flow: 0.7 mL/min; (c) pressure: 90-100 Bar; (d) ate: 62.5; (e) absorbance: 290 nm; (f) injection volume: 20 μL; (g) gradient: solvent A (distilled water + 1% formic acid), solvent C (acetonitrile) [29]. Steps of various HPLC runs are presented in Table 2.

*2.6 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.

**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 |

## 3. Results

### *3.1 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 its 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 [29, 30]. 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 was 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 Institute of Immunology Zagreb (Croatia) were used as standards (Fig. 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 Fig. 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) [30]. Various types of HuIFN-αN3 inductors differ in 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 in a microscope 24 h later and scored (+4, +3, +2, +1, +0 corresponding to 100% destruction, 75%, 50%, 25%, non-infected, respectively) [31] (Table 3).

### *3.2 Quantity of Caffeic Acid, Crysin, Pinocembrin and Galangin in 10% EEP*

The 1.0 mg of caffeic acid, crysin, 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 acetonitrile gradient in HPLC column (Fig. 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 acetonitrile gradient is presented in Fig. 2B [32]. The quantity of caffeic acid, crysin, pinocembrin and galangin in the experimental sample of 10% EEP was calculated in comparison to standards (Fig. 2A). Therefore, Table 4 indicates the quantity of caffeic acid, crysin, pinocembrin and galangin in the 10% EEP.

### *3.3 Molecular Composition of WSP (Greit 120) Determined by HPLC-UV-ESI-MS 504971*

Fig. 3 shows the HPLC-UV-ESI-MS504971 profile of WSP (Greit 120).

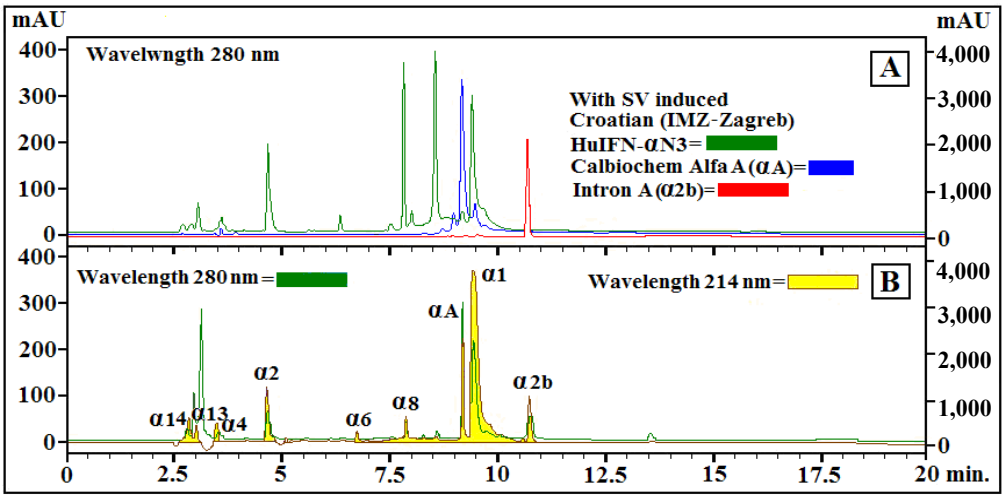


**Fig. 1 RP-HPLC profiles of the Sendai virus-induced HuIFN-αN3.**

(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).

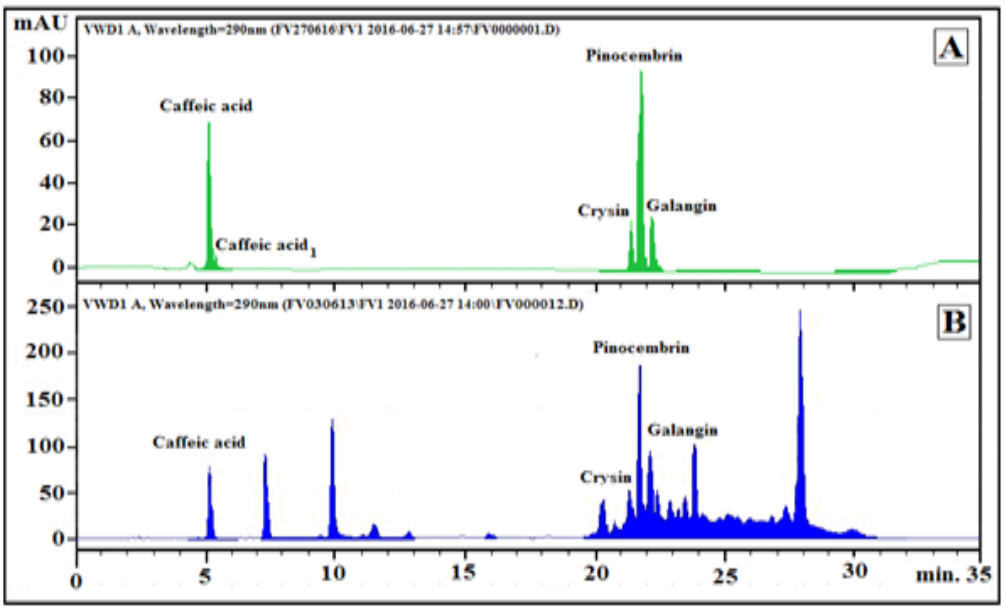


**Fig. 2 RP-HPLC profile of the bio-flavonoid's standards (caffeic acid, crysin, pinocembrin and galangin) (A); the RP-HPLC profile of 10% EEP (Medex d.o.o., 1000 Ljubljana, Slovenia) (B).**

**Table 3 The subtype composition of the Sendai virus (Cantell strain) induced HuIFN-αN3.**

| No. | HuIFN-αN3 subtype | IU/mL |
|---|---|---|
| 1 | αA | 22.430 |
| 2 | α2b | 11.000 |
| 3 | α1 | 44.280 |
| 4 | α2 | 11.750 |
| 5 | α6 | 1.280 |
| 6 | α8 | 1.800 |
| 7 | α13 | 1.800 |
| 8 | α14 | 3.500 |
| | Complete HuIFN-αN3 | 100.000 |

**Table 4 Quantity of caffeic acid, crysin, pinocembrin and galangin in 10% EEP.**

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

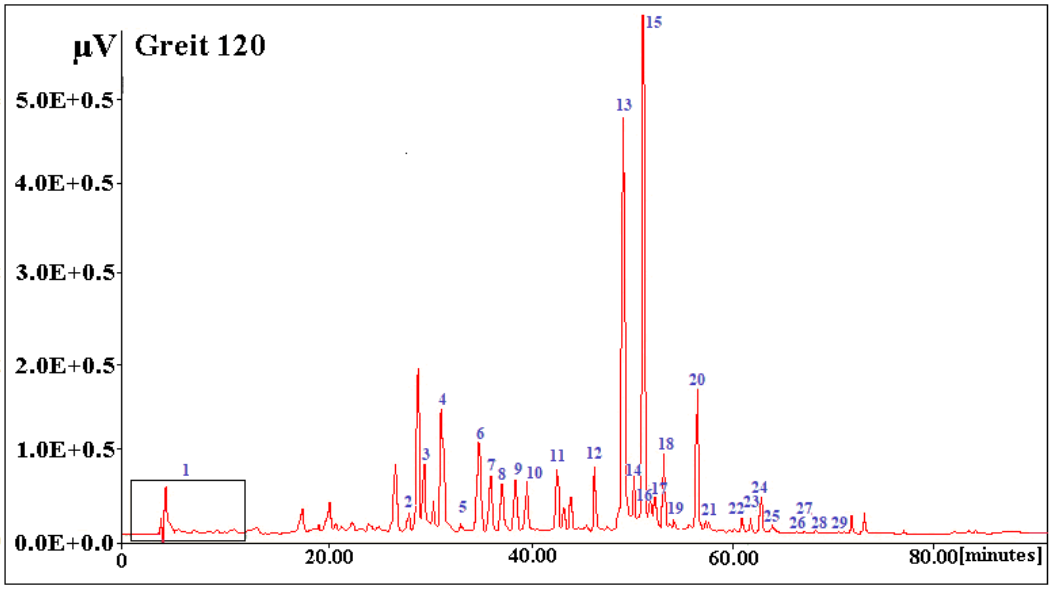


**Fig. 3 HPLC-UV-ESI-MS504971 of flavonoid's profile of an average sample of WSP (Greit 120).**
1 = phenolic acids (caffeic, coumaric, ferulic, isoferulic); 2 = quercetin; 3 = pinobanksin 5-methyl ester; 4 = quercetin 3-methyl ester; 5 = pinobanksin; 6 = apigenin; 7 = kaempferol; 8 = isorhamnetin; 9 = 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.

In Table 5, the molecular composition of WSP (Greit 120) in a very precise way is shown.

### *3.4 Antiviral Activity of Combinations of 10% EEP, 10% WSP (Greit 120) and HuIFN-αN3*

The experiments were performed to analyze the anti-influenza activity of 10% WSP (Greit 120) and 10% EEP in combination with HuIFN-αN3 in various proportions (1:1, 1:2 and 2:1). Ribavirin alone was a control. The results in Table 6 showed that the best results for $ID_{50}$ were, when the combination of 10% WSP (Greit 120) and HuIFN-αN3 in ratio 1:2 was used ($ID_{50}$ 12 ± 2 μg/mL for influenza A and 19 ± 6 μg/mL for influenza B). With 10% EEP and HuIFN-αN3, the best ratio was the same 1:2, where it was 22 ± 7 μg/mL for influenza A and 15 ± 4 μg/mL for influenza B (Table 7).

### *3.5 Ribavirin $ID_{50}$ Index*

Ribavirin $ID_{50}$ index was calculated to compare the $ID_{50}$ (antiviral activity) of WSP (Greit 120) or 10% EEP in combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1 in comparison to ribavirin. The results are in Tables 8 and 9 and Figs. 4 and 5. The lower is, the better it is. The ratio 1:2 was still the best with WSP (Greit 120) 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 1:2 (0.7 for influenza B and 1.3 for influenza A virus).

**Table 5 The molecular composition of WSP (Greit 120) determined by HPLC-UV-ESI-MS504971.**

| Number on chromatogram | Polyphenols species | Greit 120 (% w/v) |
|---|---|---|
| 1 | Phenolic acids (caffeic, coumaric, ferulic, isoferulic) | 0.5 |
| 2 | Quercetin | 1.5 |
| 3 | Pinobanksin 5-methyl ester | 1.4 |
| 4 | Quercetin 3-methyl ester | 5.0 |
| 5 | Pinobanksin | 4.0 |
| 6 | Apigenin | 1.1 |
| 7 | Kaempferol | 3.8 |
| 8 | Isorhamnetin | 3.3 |
| 9 | Luteolin 5-methyl ester | 2.1 |
| 10 | Quercetin 5-7-dimethyl ester | 1.7 |
| 11 | Galangin 5-methyl ester | 1.1 |
| 12 | Quercetin 7-methyl ester | 3.2 |
| 13 | Chrysin | 5.0 |
| 14 | Pinocembrin | 2.9 |
| 15 | Galangin | 7.5 |
| 16 | Pinobanksin-3-O-acetate | 9.5 |
| 17 | CAPE | 0.3 |
| 18 | Metoxychrysin | 1.1 |
| 19 | Pinobanksin-3-O-propionate | 1.2 |
| 20 | Caffeic acid cinnamyl ester | 0.2 |
| 21 | Pinobanksin-3-O-butyrate | 5.7 |
| 22 | Pinobanksyn-3-O-pentenoate | 2.6 |
| 23 | Other pinobanksin derivative | 0.7 |
| 24 | Pinobanksin-3-O-hexanoate | 0.3 |
| 25 | Other pinobanksin derivative | 0.4 |
| 26 | Other pinobanksin derivative | 3.3 |
| 27 | Other pinobanksin derivative | 0.5 |
| 28 | Other pinobanksin derivative | 0.2 |
| 29 | Other pinobanksin derivative | 0.7 |
| Total identified polyphenols | | 70.8 |
| Phenolic acids and derivatives | | 1.0 |
| Flavones and flavonols | | 36.4 |
| Flavanones and dihydroflavonoles | | 33.4 |

Source: Data were kindly provided by Prof. Dr. Nicola Volpi (Department of Life Sciences, University of Modena & Reggio Emilia, Modena 41125, Italy).

**Table 6 Antiviral activity of 10% WSP (Greit 120) and HuIFN-αN3 in the ratios 1:1, 1:2 and 2:1 expressed as $ID_{50}$ in µg/mL.**

| Viruses | 10% WSP (Greit 120) | $ID_{50}$ inhibition | + HuIFN-αN3 (1:1) | $ID_{50}$ inhibition | + HuIFN-αN3 (1:2) | $ID_{50}$ inhibition | + HuIFN-αN3 (2:1) | $ID_{50}$ inhibition | Ribavirin | $ID_{50}$ inhibition |
|---|---|---|---|---|---|---|---|---|---|---|
| Influenza A | 31 ± 9 | - | 22 ± 2 | 9.7 | 12 ± 2 | 19.7 | 25 ± 6 | 6.3 | 20 ± 2 | 11.7 |
| *T*-test | | | 0.54 | | 1.08 | | 0.31 | | 0.63 | |
| Influenza B | 31 ± 3 | - | 29 ± 2 | 2.1 | 19 ± 7 | 11.6 | 30 ± 2 | 1.10 | 28 ± 3 | 3.1 |
| *T*-test | | | 0.08 | | 0.26 | | 0.05 | | 0.08 | |

$ID_{50}$ = Concentration of sample needed to reduce the virus-induced CPE to 50%.

**Table 7 Antiviral activities of 10% EEP and HuIFN-αN3 in the ratios 1:1, 1:2 and 2:1 expressed as $ID_{50}$ in μg/mL.**

| Viruses | 10% EEP | $ID_{50}$ inhibition | + HuIFN-αN3 (1:1) | $ID_{50}$ inhibition | + HuIFN-αN3 (1:2) | $ID_{50}$ inhibition | + HuIFN-αN3 (2:1) | $ID_{50}$ inhibition | Ribavirin | $ID_{50}$ inhibition |
|---|---|---|---|---|---|---|---|---|---|---|
| Influenza A | 82 ± 11 | - | 35 ± 7 | 46.41 | 22 ± 8 | 59.31 | 42 ± 4 | 39.71 | 20 ± 2 | 61.91 |
| *T*-test | | | 0.67 | | 0.87 | | 0.58 | | 0.97 | |
| Influenza B | 62 ± 6 | - | 31 ± 6 | 31.00 | 15 ± 6 | 47.00 | 31 ± 7 | 30.90 | 28 ± 2 | 34.40 |
| *T*-test | | | 0.51 | | 0.83 | | 0.49 | | 0.62 | |

$ID_{50}$ = Concentration of sample needed to reduce the virus-induced CPE to 50%.

**Table 8 Ribavirin $ID_{50}$ index: Comparison of $ID_{50}$ activity of 10% WSP (Greit 120) in combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1 to ribavirin.**

| Viruses | 10% WSP (Greit 120) | + HuIFN-αN3 (1:1) | + HuIFN-αN3 (1:2) | + HuIFN-αN3 (2:1) |
|---|---|---|---|---|
| Influenza A | 1 ± 0.05 | 1 ± 0.09 | 0.6 ± 0.04 | 1 ± 0.02 |
| *T*-test | | -0.45 | -0.9 | 0.73 |
| Influenza B | 1 ± 0.10 | 1 ± 0.03 | 0.6 ± 0.09 | 1 ± 0.06 |
| *T*-test | | 0.76 | -0.83 | 0.39 |

**Table 9 Ribavirin $ID_{50}$ index: Comparison of $ID_{50}$ activity of 10% EEP in combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1 to ribavirin.**

| Viruses | 10% EEP | + HuIFN-αN3 (1:1) | + HuIFN-αN3 (1:2) | + HuIFN-αN3 (2:1) |
|---|---|---|---|---|
| Influenza A | 4 ± 0.65 | 1 ± 0.76 | 1 ± 0.12 | 2 ± 0.9 |
| *T*-test | | -0.08 | 0.9 | 0.94 |
| Influenza B | 2 ± 0.22 | 1 ± 0.12 | 0.5 ± 0.05 | 1 ± 0.12 |
| *T*-test | | 0.48 | -0.53 | 0.48 |

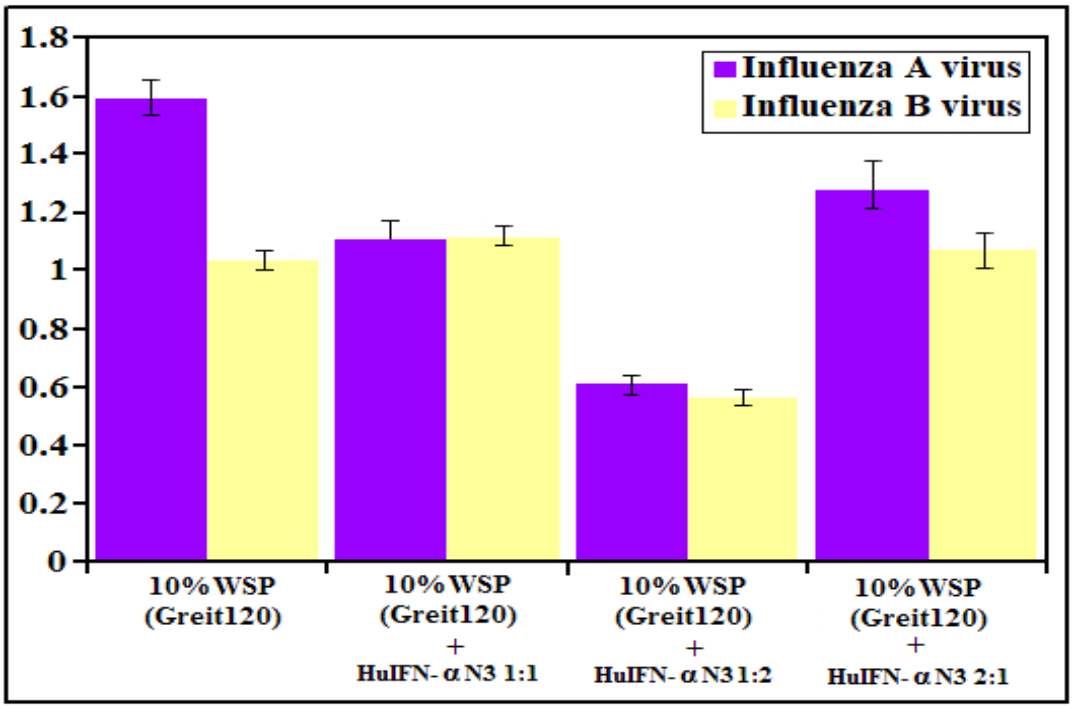


**Fig. 4 Ribavirin $ID_{50}$ index of WSP (Greit 120) and/or combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1.**

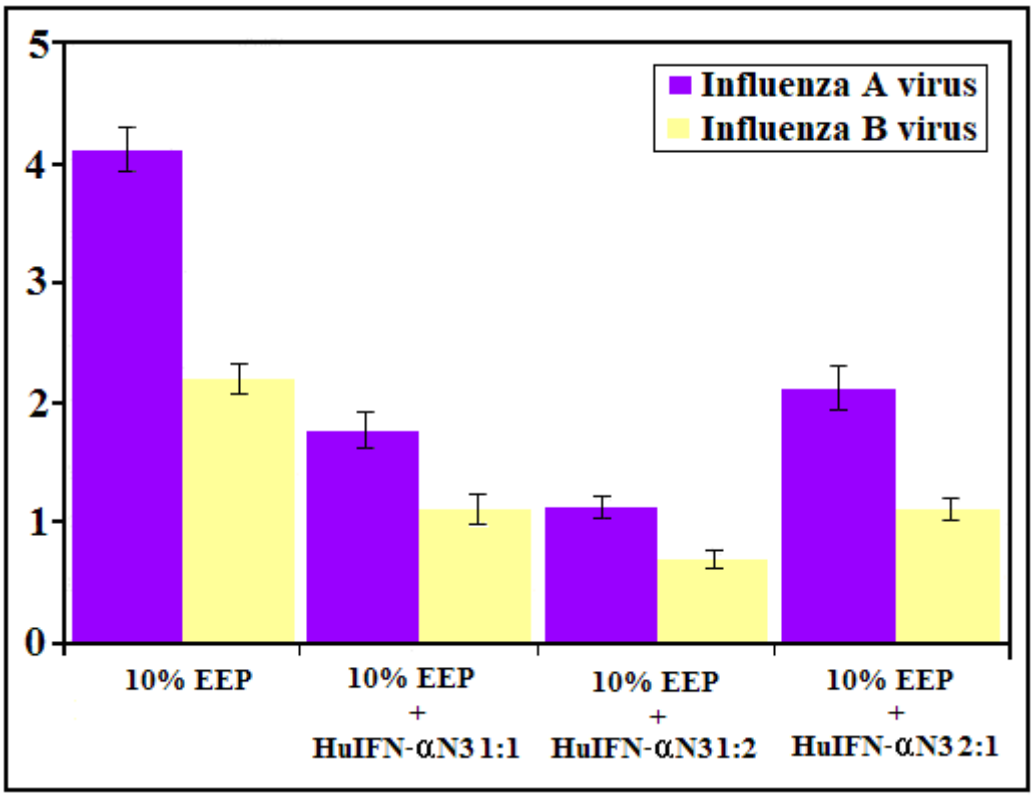


**Fig. 5 Ribavirin $ID_{50}$ index of EEP and/or combination with HuIFN-αN3 in ratios 1:1, 1:2 and 2:1.**

## 4. Discussion

The samples of very detailed analyzed WSP (Greit 120) (Table 4) containing different polyphenols: apigenin ($ID_{50}$ 8.1 ± 4.7 μg/mL), chrysin ($ID_{50}$ > 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* [33]. The anti-influenza A and B virus activity of complete WSP (Greit 120) 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 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 Greit 120 in ratio 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 WSP (Greit 120) is combined with HuIFN-αN3 in ratio 1:2. To elucidate out the mechanisms of anti-influenza activity of WSP (Greit 120) it was found that caffeic acid from it could restore the viability of cells infected with influenza virus in a dose dependent manner [34]. 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 concentration. So it is possible that WSP (Greit 120) has no direct influence on an influenza virus or does not interact with influenza virus components, although Li et al. [35] reported that caffeoylquinic acid from WSP (Greit 120) 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 WSP (Greit 120) 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, WSP (Greit 120) enhanced myxovirus resistance 1 (Mx1) expression [36]. Different specificities in antiviral effects of HuIFN-αN3 against influenza A and B viruses were reported as *in vitro* and *in vivo* [37]. 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 [38]. Antiviral activity of HuIFN-αN3 against influenza A, B and C viruses is mediated, at the 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 [39, 40].

WSP (Greit 120) enhances the anti-influenza activity of HuIFN-αN3 in dose-dependent ratio via enhanced resistance 1 (Mx1) expression and MxA induction of influenza virus replication inhibition.

## 5. Conclusions

From the performed experimental study, the following conclusions may be drawn. The anti-influenza A and B activity of WSP (Greit 120) is $ID_{50}$ 31 ± 0.9 μg/mL for influenza A virus and $ID_{50}$ 29 ± 0.2 μg/mL for influenza B. It is 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.

Combining WSP (Greit 120) with HuIFN-αN3 in ratio 1:2 caused the highest increase of anti-influenza A and B viruses' activity *in vitro*.

Anti-influenza activity of the combination of WSP (Greit 120) with HuIFN-αN3 is higher against influenza B than against influenza A virus.

This new formulation of WSP (Greit 120) and HuIFN-αN3 showing better anti-influenza activity, will definitely improve its application in flu infections.

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## Conflict of Interest

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

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