International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
IJHM 2017; 5(5): 169-176
Received: 23-07-2017
Accepted: 24-08-2017

Angel S Galabov
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Lubomira Nikolaeva-Glomb
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Lucia Mukova
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Emilia Ajvazova
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Mimi Remichkova
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Irina Georgieva
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Monika Cseuz
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Correspondence
Angel S Galabov
The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Large-scale antiviral activity screening of a triplex mixed herbal extract

Angel S Galabov, Lubomira Nikolaeva-Glomb, Lucia Mukova, Emilia Ajvazova, Mimi Remichkova, Irina Georgieva and Monika Cseuz

Abstract
Effects of a combined mixture of three herbal immunologically active substances (Preparative Esberitox N, recommended in the therapy of virus-induced respiratory infections) and its ingredients, *Echinacea purpurea*, *Baptisia tinctoria* and *Thuja occidentalis* were tested against viral strains of six taxonomic groups. Experiments were conducted *in vitro*. It was established that *Echinacea purpurea* radix ethanol extract possesses pronounced inhibitory effect on influenza virus A (H3N2) replication, activity preserved in the lyophilisate extract. *Baptisia tinctoria* radix extract also showed marked activity against influenza virus A(H3N2), but *Thuja occidentalis* extract showed no antiviral effect. The combined preparative manifested distinct inhibitory effect on rhinovirus 14 replication and a moderate one against influenza virus A(H3N2). Activity against human rhinovirus 14 was preserved in the lyophilisate combination preparative, but activity against influenza virus A(H3N2) was lost. All substances tested were without distinct effects against Coxsackievirus B1, ECHO13, influenza virus B, respiratory syncytial virus, polyomavirus SV40 and human cytomegalovirus.

Keywords: Triple plant combination, antiviral activity, cell culture tests

1. Introduction
The immunostimulating activity of the perorally administered combination of three plants, *Echinacea purpurea*radix (purple coneflower; root), *Baptisia tinctoria* (wild indigo; root) and *Thuja occidentalis* (white cedar; leaf, one- to two-year-old shoots), is highly recommended and used globally for treatment mainly of acute infections of the respiratory tract with viral etiology. It is especially widely used in the treatment of influenza, as an additive component of therapy with antivirals and antioxidants [1, 2]. *Echinacea* (coneflower) is historically related with and used by North American Indians [3]. Since John King's initial data [3, 4], the application of *Echinacea* in the last century has covered a relatively wide range of infections, including viral, bacterial and mycotic, as well other human diseases [5,6]. The immunologically active substances in *Echinacea*—an arabinogalactan protein and arabinogalactans (polysaccharides) as well as glycoproteins—were isolated [7, 8]. Wild indigo (*Baptisia tinctoria*) root is also an American Indian medicinal herb, manifesting immunostimulating effects due to polysaccharides and glycoproteins [9]. The origin of the white cedar (*Thuja occidentalis*) plant's medicinal use is also related to American Indians (from Canada where it is known as *arbor vitae*). The extract of this plant's leaves was proved immunologically active based on glycoproteins [10].
The immunomodulatory effect proven for the whole extract and the individual extracts of this historical triple herb combination [2] can be summarized as follows: (i) increased unspecific resistance (phagocytosis activities of granulocytes and serum bactericidism) [11] and macrophage activation (increased phagocytosis and interleukin-1release)[12]; (ii) macrophage-dependent stimulation of Tcells (CD-4 + helper/inducer cells, and an ensuing increased production of interleukin-2)[13–15]; (iii) accelerated differentiation of B-lymphocytes to antibody-producing lymphoblasts and increased IgM production[15]; (iv) interferon induction [14, 15].
Some antiviral activity data was mentioned regarding *Echinacea* and *Thuja* components [14, 16, 17]. Wacker and Hilbig [16] noted some undetermined virucidal effects of *Echinacea purpurea* aqueous and alcoholic root extracts on influenza, herpes- and vesicular stomatitis viruses. Some fractions of *Thuja occidentalis* as well as glycoprotein-containing fractions of *Echinacea* root manifested some activity on herpes simplex virus 1 *in vitro* by the plaque reduction test [18, 19]. Concerning the antiviral action of the triple herb combination preparative, special interest should be given to its established antiviral effect on influenza A viral experimental infection in mice treated orally with the combined preparative.

A marked reduction of the lung hemagglutination titer was recorded, especially at Days 2–5 post infection, with virus titer maximum at Day 2[20].

Especially impressive was the effect of the follow-up herbal medicinal product containing *Echinacea purpurea* and *pallida*, *Baptisia tinctoria* and *Thuja occidentalis* (Esberitox®N tablets, an advanced product of the combination experimentally tested here) registered in a multicenter, randomized, double-blind placebo-controlled trial in 263 patients with acute respiratory viral infections (common cold) [21]. The results of this study manifested (i) an increased response rate in patients with at least moderate symptoms - 55.3% in the combination preparative treated group, in contrast to 27.3% in the placebo group; (ii) a markedly higher effectiveness in patients with therapy starting at the onset of the disease; (iii) a therapeutic effect registered at Day 2 and a more marked effect registered at Day 4 of the disease onset.

The present work explores the antiviral activity *in vitro* of the triple combination herbal preparative and its components against a wide range of viruses representing the main taxonomic groups, including agents of infections for which antiviral chemotherapy is indicated.

2. Materials and Methods

2.1 Substances tested

N 246/95 was the retentate fraction (20 kD-cutoff) from Esberitox® N containing as active principle a mixture of three herbal substances (mixed extraction technology) (*Echinacea purpurea*, *Baptisia tinctoria* and *Thuja occidentalis*), 30% ethanolic extract;

N 231/95 was a lyophilisate analogous to N 246/95; N 243/95 was the retentate fraction (20 kD-cutoff) from *Echinacea purpurea* radix, 30% ethanolic extract; N 257/92 was a lyophilisate analogous to N 243/95; N 306/92 was the retentate fraction (20 kD-cutoff) from *Herbathujae*, 30% ethanolic extract; and N 101/92 was the retentate fraction (20 kD-cutoff) from *Baptisia tinctoria* radix, 30% ethanolic extract. These substances were supplied by Schaper & Brümmer GmbH, Germany.

Guanidine hydrochloride, Eastman Organic Chemicals (New York, N.Y., USA), MW 95.5, white crystals, soluble in water (used as a reference anti-enteroviral compound).

Oxoglaucin, synthesized by Assoc. Prof. Stephan Filipov (Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria), MW 351, yellow crystals, soluble in water (reference anti-rhinovirus compound).

Rimantadine hydrochloride, kindly supplied by Prof. V. I. Ilienko (Institute for Influenza, Saint Petersburg, Russia), MW 216, white powder, soluble in water (reference anti-influenza A virus compound).

Ribavirin, ICN (Irvine, California), kindly supplied by Prof. R. W. Sidwell (University of Utah, Logan, Utah, USA), MW 244, white powder, soluble in water (reference antiviral active versus influenza virus B, respiratory syncytial virus and human adenovirus 2).

5-Iodo-2'-deoxyuridine (IUdR), Koch-Light (U.K.), MW 354, white crystals, soluble in water, used as reference in anti-SV40 testing.

Cidofovir (1-[(S)-3-hydroxy-2-(phosphonomethoxy) propyl]cytosine dihydrate (HPMPC) was kindly supplied by Dr. E. Kern (University of Alabama School of Medicine, Birmingham, Alabama) and applied as reference in anti-human cytomegalovirus tests.

2.2 Viruses

Coxsackievirus B1 1 (Connecticut 5 strain; CVB1), from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in FL cells (maintenance solution DMEM (Gibco BRL) with 10 mmol/l HEPES (Gibco BRL), 0.5% fetal calf serum (Gibco), penicillin 100 IU/ml and streptomycin 100 µg/ml); infectious titer $10^{8.5}$ $CCID_{50}$ /ml, resp. 8.5 x 10^8 PFU/ml.

Echovirus 13 (Del Carmen strain; ECHO13), from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria), grown in FL cells (maintenance solution DMEM (Gibco BRL) with 10 mmol/l HEPES (Gibco BRL), 0.5% fetal calf serum (Gibco), penicillin 100 IU/ml and streptomycin 100 µg/ml); infectious titer $10^{8.5}$ $CCID_{50}$ /ml, resp. 1.4×10^8 PFU/ml.

Human rhinovirus type 14 (strain 1059; HRV14), supplied by ATCC, grown in MRC-5 cells (maintenance solution DMEM (Gibco BRL, Paisley, Scotland, UK) plus 2% fetal calf serum (Gibco BRL, Paisley, Scotland, UK)). Infectious virus titer $10^{6.5}$ $CCID_{50}$ /ml.

Influenza A virus [Aichi/2/68 (H3N2); IAV], from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria). The stock virus represented alantoic fluids of virus-inoculated 10-days-embryonated eggs, cultivated at 37 °C; infectious titer $10^{7.5}$ $CCID_{50}$/ml.

Influenza B virus (Lee/40; IBV]), ATCC No. VR-101 (USA). The stock virus represented alantoic fluids of virus-inoculated 10-days-embryonated eggs, cultivated at 35 °C; infectious titer $10^{7.5}$ $CCID_{50}$/ml.

Respiratory syncytial virus (Long; RSV), kindly supplied by the District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in HEp-2 cells (maintenance solution DMEM (Gibco BRL) with 10 mmol/l HEPES (Gibco BRL), 0.5% fetal calf serum (Gibco BRL) and antibiotics). Infectious titer $10^{4.5}$ $CCID_{50}$/ml.

Human adenovirus type 2 (adenoid 6 strain; HAdV2), kindly supplied by District Center of Hygiene and Epidemiology, Plovdiv (Bulgaria). The virus was grown in FL cells (maintenance solution DMEM (Gibco BRL) with 10mmol/l HEPES (Gibco BRL), 0.5% fetal calf serum (Gibco BRL) and antibiotics). Infectious titer $10^{5.3}$ $CCID_{50}$/ml.

Simian virus 40 (777 strain; SV40) from the collection of the Rega Institute of Microbiology, Leuven, Belgium, grown in CV cells (maintenance medium DMEM (Gibco BRL) without serum).Infectious titer $10^{7.5}$ $CCID_{50}$/ml.

Human cytomegalovirus (OS323 strain; HCMV), kindly supplied by Prof. Alain le Faou, Medical Faculty, University of Nancy, France, grown in MRC-5 cells (maintenance medium DMEM (Gibco BRL) with 2% fetal bovine serum (Gibco BRL), 10 mmol HEPES buffer (Merck, Germany) and antibiotics—penicillin, 100 U/ml; streptomycin, 100 µg/ml). Infectious titer $10^{4.67}$ $CCID_{50}$ /ml.

2.3 Cells

FL cells (collection of the Stephan Angeloff Institute of Microbiology, BAS, Sofia, Bulgaria) were grown in medium containing 10% heated calf serum in DMEM (Gibco BRL, USA) supplemented with 10 mmol/l HEPES buffer (Gibco BRL, USA) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml). The cells were used for CVB1 cultivation and testing.

HEp-2 cells (National Bank for Industrial Microorganisms and Cell Cultures, No. NBIMCC-95, Sofia, Bulgaria) were grown in medium containing 10% heated calf serum in DMEM (Gibco BRL, USA) supplemented with 10 mmol/l HEPES buffer (Gibco BRL, USA) and antibiotics (penicillin,

100 U/ml; streptomycin, 100 µg/ml). The cells were used for cultivation and experiments with RSV and HAdV2.

MRC-5 cells (human embryo lung diploid cells), supplied by Collection National de Cultures de Microorganisms (CNCM, Institut Pasteur, Paris, France) and by Prof. Alian le Faou, Medical Faculty, University of Nancy, France, were cultivated in Costar plastic vessels (USA) with growth medium DMEM (Gibco BRL) containing 10% fetal bovine serum (Gibco BRL, Paisley, Scotland, UK) and antibiotics (penicillin 100 U/ml; and streptomycin, 100µg/ml). The cells were used for experiments with HRV14 and HCMV.

MDCK (Madin-Darby canine kidney) cells (NBL-2; ATCC No. CCL-34, USA) were grown in medium containing 10% fetal calf serum in DMEM (Gibco BRL, USA) supplemented with 10 mmol/l HEPES buffer (Gibco BRL, USA) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml). The cells were used for experiments with IAV and IBV.

CV cells (kindly gifted by Prof. Syed Sattar, University of Ottawa Medical Faculty, Ontario, Canada) were grown in medium containing 10% fetal bovine serum (Gibco BRL, USA) in DMEM (Gibco BRL, USA) supplemented with 10 mM/l HEPES buffer (Merck, Germany) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml). The cells were used for cultivation and experiments with SV40.

2.4 *Agar-diffusion plaque-inhibition method*

Primary antiviral screening for plaque-forming viruses was carried out through the agar-diffusion plaque-inhibition test with cylinders [22-24]. Monolayer cell cultures of FL cells for CVB1 and ECHO13 in 90 mm petri dishes (Anumbra, Czech Republic) were inoculated (adsorption at 20 ºC for 60 min for CVB1 and ECHO13) with a virus dose giving semiconfluent plaques after incubation at 37 ºC (48 h with CVB1 and ECHO13). Test compounds (0.1 ml of appropriate concentration solutions in distilled water) were added dropwise within 6-mm glass cylinders fixed in the agar overlay (1% Noble agar (Difco, USA) in Eagle's MEM (Gibco BRL, USA) medium with fetal calf serum (Gibco BRL, USA), 1.65 mg/ml sodium bicarbonate and antibiotics (100 U/ml of penicillin and 100 µg/ml streptomycin). A second overlay containing 1.5% w/v agar and 0.02% w/v neutral red (Merck, Germany) in physiological saline was added following incubation. The antiviral effect of a given compound was based on the size (diameter (Ø), in mm) of the plaque inhibition zone ($Ø_i$) and the cytotoxicity zone ($Ø_t$); four cylinders per compound, each in a separate petri dish) and designated as follows: -, $\Delta Ø \leq 5$ mm; ±, $\Delta Ø = 5–10$ mm; +, $\Delta Ø = 11–20$ mm; ++, $\Delta Ø = 21–40$ mm; +++, $\Delta Ø > 40$ mm.

2.5 Cytotoxicity test

The effect of the test compound on uninfected confluent cell monolayers and cellular morphology were traced for overt signs of cytotoxicity during the 144-h test period, and the maximum tolerated (nontoxic) concentration (MTC) value was determined. This study was carried out in parallel with the CPE inhibition test in 96-well plastic plates.

2.6 Cytopathic effect (CPE) inhibition test in a viral multicycle growth setup

Monolayer cell cultures grown in 96-well plastic microplates (Costar, USA) were used. Compounds (at subsequent 0.5 log_{10} dilution concentration ranges) were added to the maintenance medium (0.2 ml/well) immediately after virus inoculation at different viral doses. Inoculation was carried out by a 60-min adsorption (0.1 ml/well) at room temperature (for HRV14, 120 min adsorption at 33C). The plates were incubated at 37 ºC for 2–6 days (HRV14 at 33 ºC), and viral CPE was followed every day by inverted light microscope (Olympus, Japan) at 125x magnification. Four wells per test sample were used. CPE was scored on 0–4 scale, with 4 representing total cell destruction: CVB1 and ECHO13 viruses were scored at 48 h; HRV14, IAV, IBV, SV40 and HCMV at 120 h; and RSV and HAdV2 at 144 h. These data were used to obtain dose-response curves for each compound at a given viral dose. From these graphs, the minimal concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC_{50} value) was determined. The selectivity was determined by the ratio between the compound cytotoxicity (maximal tolerated concentration, MTC) and the MIC_{50}.

In the testing towards influenza virus A the CPE inhibition test was carried out by the neutral red uptake assay[27], using an ELISA reader at OD_{540} nm. The IC_{50} concentration was identified as the concentration that inhibited development of CPE by 50%.

2.7 *Microfocus inhibition test in a viral multicycle growth setup*

This test was used to measure the antiviral effect against HCMV. Monolayer cell cultures in 96-well plastic micro plates (Costar, USA) were used. Compounds (at subsequent 0.5 log_{10} dilution concentration ranges) were added to the maintenance medium (0.2 ml/well) immediately after virus inoculation at 37 ºC (four samples per experimental group). The plates were incubated at 37 ºC, and HCMV micro focuses (micro plaques) in the cell sheet were counted at 96 h post virus inoculation under inverted light microscope (Olympus, Japan) at 125x magnification. Inhibition was computed as a percentage of the untreated control. MIC_{50} values were evaluated based on the dose-response curves. The selectivity index was determined by the ratio between the compound cytotoxicity MTC and the MIC_{50} values.

3. Results

The summarized results of the antiviral testing (screening *de facto*) are presented in Table 1. In presenting the results, only the substances manifesting an antiviral activity will be noted.

3.1 Activity against Coxsackievirus B1 in FL cells

As Table 2 shows, none of the substances studied manifested an activity. Guanidine, which was used as reference antiviral, showed a marked antiviral effect in both the agar-diffusion plaque-inhibition test (Table 2) and the CPE test: at 100 µg/ml against ≤ 3000 ID_{50}; at 32 µg/ml and 10 µg/ml versus ≤ 30 ID_{50}.

3.2 Activity against echovirus 13 in FL cells

The *Echinacea purpurea* radix lyophilisate (substance N 257/92) was markedly effective versus ECHO13 in the agar-diffusion plaque-inhibition test (Table 3); however, this level of activity was not confirmed in the CPE test, where inhibition was registered at concentrations of 320, 100 and 32 µg/ml versus comparatively low viral inoculation doses, ≤ 30 ID_{50} (inhibition at 10 µg/ml was versus 3 ID_{50} only). The MTC was 400 µg/ml, the MinIC was 32 µg/ml and the SI value was 10 (based on virus replication at 30 ID_{50}).

The retentate fraction of *Echinacea purpurea* radix (N 243/95) did not show activity in the agar-diffusion plaque-inhibition as well as in the CPE inhibition tests. The retentate fraction of *Baptisia tinctoria* (N 101/92) manifestedan effecttowards ECHO13 in the agar-diffusion plaque-inhibition test.

Guanidine recorded a marked antiviral effect at 100 μg/ml versus ≤ 5000 ID_{50} and at 32 μg/ml versus ≤ 500 ID_{50}, but it was ineffective at 10 μg/ml.

3.3 Activity against human rhinovirus 14 in MRC-5 cells

Two of the substances studied, N 246/95 and N 231/95 (the retentate fraction from the triple herbal combination preparative extract, and its lyophilisate, respectively), manifested a marked effect toward HRV14 replication in MRC-5 cells through the CPE test (Table 6). N 246/95 showed a marked antiviral effect at concentrations of 32, 10, 3.2 and 1 μg/ml versus viral inoculation doses ≤ 100 ID_{50}. The MTC for MRC-5 was 32 μg/ml. The lyophilisate N 231/95 demonstrated a marked antiviral effect at concentrations of 100, 32 and 10 μg/ml toward viral inoculation doses ≤ 1000 ID_{50} (MTCwas 100 μg/ml).

Baptisia tinctoria retentate fraction (N 101/92) demonstrated a pronounced activity against HRV14 replication at concentrations of 32 and 10 μg/ml against viral inoculation doses ≤ 1000 ID_{50} (MTC was 32 μg/ml).

Oxoglaucine (the reference antiviral) demonstrated a strong antiviral effect at 3.3 μg/ml versus virus inoculation doses ≤ 10 000 ID_{50}, at 1 μg/ml versus ≤ 1000 ID_{50} and at 0.32 μg/ml and 0.1 μg/ml versus ≤ 100 ID_{50}.

3.4 Activity against respiratory syncytial virus (RSV) in HEp-2 cells

The substance N 101/92 (the retentate fraction from *Baptisia tinctoria* radix) showed an antiviral effect in the CPE inhibition test at concentrations of 640, 320 and 100 μg/ml versus relatively low viral doses (≤ 30 ID_{50}); at 32 μg/ml it was effective against ≤ 10 ID_{50} only. The MTC for HEp-2 cells was 700 μg/ml.

The substance N 257/92 (*Echinacea purpurea* radix lyophilisate) applied at its MTC, 3.2 μg/ml, was active versus virus doses ≤ 20 000 ID_{50}; at 1 μg/ml it was active against ≤ 2000 ID_{50}. Nevertheless, this activity could be qualified as a borderline one, as the SI value was about 3.2 only (the lower concentrations, ≤ 0.32 μg/ml, were without effect). The retentate fraction of *Echinacea* (N 243/95) was inactive.

The MTC of ribavirin for HEp-2 cells, used as a reference inhibitor, was 1000 μg/ml. The compound demonstrated a marked effect at concentrations of 100 μg/ml versus virus doses ≤ 20 000 ID_{50}, at 32 and 10 μg/ml versus ≤ 2000 ID_{50} and at 3.2 μg/ml versus ≤ 100 ID_{50}.

3.5 Activity against human adenovirus type 2 (HAdV2) in FL cells

The activity against HAdV2 was similar to that against RSV. The CPE inhibition test manifested an antiviral effect of N 101/92 (retentate fraction from *Baptisia tinctoria* radix): at concentrations of 640, 320 and 100 μg/ml versus virus doses ≤ 500 ID_{50}; at 32 μg/ml against ≤ 10 ID_{50} only. MTC was 700 μg/ml.

Echinacea purpurea radix lyophilisate (N 257/92) was effective only at its MTCof 32 μg/ml (against virus doses ≤ 3000 ID_{50}). The retentate fraction N 243/95 was ineffective.

Ribavirin's MTC was 100 μg/ml. Applied at this concentration it demonstrated a marked effect versus virus doses ≤ 3000 ID_{50}, while effects at other concentrations were as follows: at 32 μg/ml against ≤ 200 ID_{50}; at 10 and 3.2 μg/ml against 2 ID_{50} only.

3.6 Activity against simian virus 40 in CV cells

None of the substances manifested activity toward polyomavirus SV40.

IUdR used as a reference antiviral demonstrated a marked effect at concentrations of 32 and 10 μM/l versus virus doses ≤ 50 ID_{50}.

3.7 Activity against human cytomegalovirus in MRC-5 cells

For HCMV, the multifocus inhibition test was applied. As Table 4 shows, some activity was registered with substance N 101/92 (retentate fraction from *Baptisia tinctoria* radix), which exhibited a slight antiviral effect at the MTCof 32 μg/ml and at 10 μg/ml.

Cidofovir (as an antiviral reference substance) demonstrated a strong inhibitory effect on HCMV replication in MRC-5 cells. A 50% minimal inhibitory concentration of approximately 0.1 μg/ml and an SI value exceeding 100 were registered (Table 4), with an MTCof 10 μg/ml.

3.8 Activity against influenza virus A(H3N2) in MDCK cells

Applying the CPE test by Borenfreund and Puerner (1985), marked antiviral effects (at viral inocula ≥ 300 ID_{50}) were established for the *Echinacea purpurea* radix extract N 257/92 (lyophilisate) and N 243/95 (non-lyophilisate) as well as for the *Baptisia tinctoria* radix extract (N 101/92). The mixture of the three herbal substances (N 246/95) was inactive (Table 5).

In contrast, using the routine CPE inhibition test, N 246/95 showed an antiviral effect at concentrations of 100 and 32 μg/ml versus viral inoculation doses ≤ 30 only (Table 6); lower concentrations were inactive. MTC was 200 μg/ml. A lack of antiviral effect was established for the lyophilized triple combination (N 231/95).

In full contrast, the lyophilisate of *Echinacea purpurea* radix (N 257/92) manifested a pronounced antiviral effect at concentrations of 100 and 32 μg/ml toward massive virus inocula, ≤ 300 000 ID_{50}. A marked effect was also recorded with 10 μg/ml and 3.2 μg/ml versus viral doses ≤ 300 ID_{50} (Table 6). The concentration of 1 μg/ml was ineffective. MTCwas 200 μg/ml.

A similar antiviral effect was registered with N 243/95 (the retentate fraction from *Echinacea purpurea* radix) at concentrations of 320 and 100 μg/ml against virus doses ≤ 30 000 ID_{50}, at 32 μg/ml versus ≤ 300 ID_{50} and at 10 μg/ml versus ≤ 30 ID_{50} (Table 6). The lower concentration 3.2 μg/ml was inactive. MTC was 320 μg/ml.

The substance N 101/92 (retentate fraction from *Baptisia tinctoria* radix) also showed a pronounced antiviral effect at concentrations of 320, 100 and 32 μg/ml against massive virus doses ≤ 30 000 ID_{50} (Table 6) and was inactive at lower concentrations. MTC was 320 μg/ml.

The retentate fraction from *Thuja occidentalis* (N 306/92) did not show activity at concentrations below its MTC (16 μg/ml).

The reference antiviral rimantadine manifested a strong inhibitory effect in both CPE inhibition tests (Tables 5 and 6).

3.9 Activity against influenza virus B in MDCK cells

N 101/92 (the retentate fraction from *Baptisia tinctoria* radix) showed an antiviral effect at its MTC (320 μg/ml) only versus virus doses ≤ 30 ID_{50} and was without antiviral effect at lower concentrations.

Ribavirin demonstrated a pronounced effect against 5 ID_{50} IBV at a concentration of 10 μg/ml (41 μM/l), with MTC being 244 μg/ml (1 mM/l).

Table 1: Antiviral activity *in vitro* of the triple plant combination preparative (Esberitox® N) and its components.

Substances tested	Virus								
	CVB1	ECHO13	HRV14	IAV(H3N2)	IBV	RSV	HAdV2	SV40	HCMV
N 246/65	-	-	+	+	-	-	-	-	-
N 231/95	-	-	+	-	-	-	-	-	-
N 257/92	-	±	-	+	-	±	±	-	-
N 243/95	-	-	-	+	-	-	-	-	-
N 306/92	-	-	-	-	-	-	-	-	-
N 101/92	-	±	+	+	±	±	+	-	±
Reference antivirals	+ Guanidine	+ Guanidine	+ Oxoglaucine	+ Rimantadine	+ Ribavirin	+ Ribavirin	+ Ribavirin	+ IUdR	+ Cidofovir

+, marked antiviral effect; ±, borderline/weak antiviral effect; -, lack of antiviral effect at the tested dose

Table 2: Testing the activity of the triple plant combination preparative (Esberitox® N) and its components against CVB1 in FL cells by the agar-diffusion plaque- inhibition test.

Substance Tested	Inhibition zone Ø, mm	Toxicity zone Ø, mm	Antiviral effect
N 246/95	0	0	-
N 231/95	0	0	-
N 257/92	8.5	0	-
N 243/95	8.7	0	-
N 306/92	0	0	-
N 101/92	6.7	0	-
Guanidine.HCl	44.7	0	+

Concentration of the test substances: 2% (20 000 µg/ml)
External diameter of glass cylinders: 6.5 mm
+, marked antiviral effect; -, lack of antiviral effect

Table 3: Testing the activity of the triple plant combination preparative (Esberitox® N) and its components against ECHO virus 13 in FL cells by the agar-diffusion plaque-inhibition test.

Substance Tested	Inhibition zone Ø, mm	Toxicity zone Ø, mm	Antiviral effect
N 246/95	11.3	9.3	-
N 231/95	0	0	-
N 257/92	64.0	7.7	+
N 243/95	0	0	-
N 306/92	0	8.2	-
N 101/92	46.5	0	+
Guanidine.HCl	59.3	0	+

Concentration of the test substances: 2% (20 000 µg/ml)
External diameter of glass cylinders: 6.5 mm
+, marked antiviral effect; -, lack of antiviral effect

Table 4: Effect of the triple plant combination preparative (Esberitox® N) and its components on HCMV replication in MRC-5 monolayer cell cultures through the multifocus inhibition test.

Substance tested	Substance conc. (µg/ml)	MTC conc. (µg/ml)	Inhibition (% of the control)
N 246/95	32	32	0
	10		0
	3.2		0
	1		0
	0.32		0
N 231/95	100	100	20.5
	32		26.3
	10		16.4
	3.2		9.0
	1		2.5
	0.32		0
N 257/92	100	>100	27.1
	64		27.1
	32		0
	10		0
	3.2		0
N 243/95	3.2	3.2	14.6
	1		6.3
N 306/92	16	10	29.3
	10		12.3
	3.2		0
	1		0
	0.32		0
N 101/92	32	32	56.4
	10		40.1
Cidofovir	32		96.9
	16		95.4
	10		95.7
	3.2		90.8
	1		76.6
	0. 32		69.3
	0.1		53.7

Table 5: Antiviral effects of some components of the triple plant combination Preparative (Esberitox® N) on replication of influenza virus A/Aichi/2/68(H3N2) in MDCK cells (using Borenfreund and Puerner's [26] CPE inhibition test).

Substance tested	Virus inoculation dose, ID_{50}[a]	IC_{50}[b], µg/ml	SI[c]
N 246/95	3	19.4	10.3
	30	22.6	8.8
N 257/92	3	1.4	146.0
	30	2.4	83.3
	300	3.0	66.7
	3000	3.2	62.5
	30 000	20.3	9.8
	300 000	26.0	7.7
N 243/95	3	<3.2	>100.0
	30	10.0	32.0
	300	10.0	32.0
	3000	51.4	6.2
	30 000	75.3	4.2
N101/92	3	<1.0	>320.0
	30	6.6	48.5
	300	14.9	21.5
	3000	14.0	22.8
	30 000	13.5	23.7
	300 000	32.0	10.0
Rimantadine	3	<0.032	>781.2
hydrochloride	30	<0.032	>781.2
	300	<0.032	>781.2
	3000	0.052	480.8

[a]Virus infectious dose 50%
[b] Inhibitory concentration 50%
[c] Selectivity index, MTC^d/IC_{50}
[d] Maximal tolerated concentration

Table 6: Summary of data on the effects of herbal substances toward influenza A virus and human rhinovirus 14.

Substances tested	Conc. µg/ml	IAV grown in MDCK cells		HRV14 grown in MRC-5 cells	
		Effect vs. MTC virus dose µg/ml scopes (MDCK cells)		Effect vs. MTC virus dose µg/ml scope (MRC-5 cells)	
Triple mixture retentate					
(N 246/95)	100	≤30		200	
	32	≤30		≤100	32
	10	inactive		≤100	
	3.2			≤100	
	1.0			≤100	
Triple mixture lyophilisate					
(N 231/95)	100	inactive	10	≤1000	100
	32			≤ 1000	
	10			≤ 1000	
	3.2			inactive	
Echinacea purpurea retentate	320	≤30 000			
(N 243/95)	100	≤30 000			
	32	≤300			
	10	≤30			
	3.2	inactive			
Echinacea purpurea lyophilisate	100	≤300 000	200	inactive	
(N 257/92)	32	≤300 000			
	10	≤300			
	3.2	≤300			
	1.0	inactive			
Baptisia tinctoria retentate	320	≤30 000	320		
(N 101/92)	100	≤30 000			
	32	≤30 000		≤ 1000	32
	10	inactive		≤ 1000	
Thuja occidentalis retentate	10	inactive	16	inactive	32
(N 306/92)					

4. Discussion and Conclusion

In summarizing the experimental data, it should be noted that the retentate (high-molecular) fraction of *Echinacea purpurea* aradix extract (N 257/95) possessed a pronounced inhibitory effect on influenza virus A/Aichi/2/68 (H3N2) replication, even at massive virus inocula. This activity was preserved in the lyophilisate extract (N 243/92) (Tables 1 and 6). N 257/92 demonstrated weak to borderline antiviral effects toward ECHO13 virus, RSV and HAdV2, which were absent in the non-lyophilized fraction, N 243/95.

The retentate fraction of *Baptisia tinctoria* radix extract (N 101/92) also showed a marked activity against influenza virus A(H3N2), but also against HRV14 (Table 6). It was active towards HAdV2 as well. Borderline effects (SI 3.2) were registered against influenza B virus, ECHO13, HCMV and RSV.

The retentate fraction of *Herbathujae* extract (N 306/92) did not manifest antiviral effects toward any tested virus.

Interestingly, the retentate fraction of the ethanol extract of the total triple combination preparative (N 246/95) and its lyophilisate (N 231/95) showed a pronounced antiviral effect against HRV14 replication. This effect was registered at a wide concentration range: for N 246/95, 1–32 μg/ml, and a marked selectivity ratio (MTC/MinIC of 32 at viral 100 ID_{50}); for N 231/95, 1000 ID_{50} included (Table 6). Moreover, the retentate non-lyophilisate substance manifested a moderate activity against influenza virus A/Aichi/2/68 (H3N2), which was not registered in the lyophilisate N 231/95. The total preparative extract (N 246/95 and N 231/95) was inactive against all other viruses tested.

This loss of anti-influenza A virus activity in the lyophilisate of the triple combination could be explained by a decrease in the active structure content and/or the lack of a favorable combination effect of the individually active substances contained in *Echinacea purpurea* and *Baptisia tinctoria*. Moreover, the anti-flu activity was probably affected by the fact that the three individual herbal substances are not present in an equal weight ratio in the combined preparation (Esberitox® N). These details make it difficult to precisely interpret the results obtained. However, the overall antiviral activity is obvious based on the data presented here.

Alternatively, the observed anti-rhinovirus activity of the combined preparation could be explained as the synergistic effect of some of its preparative ingredients, along with the presence of *Baptisia tinctoria*.

All substances tested were inactive or had weak to borderline activity against enteroviruses (CVB1 and ECHO13), IBV, RSV, polyomavirus SV40, HAdV2 and HCMV. The lack of a distinct antiviral effect against HCMV was in contrast to the observed favorable influence of the combination preparative (Esberitox® N) on labial herpes simplex, which is caused by another virus from the same taxonomic group [26].

The antiviral effects of the active ingredients are obviously not due to an action on the host cell machinery and are instead based on an effect toward virus-specific targets, most probably viral proteins.

The anti-influenza A virus effects of the herbals in Esberitox® N established in this investigation agree with previously reported data [20]from experiments in mice treated orally with the combined preparative. We should also stress once again the favorable results obtained by the double-blind trial in patients with viral acute respiratory tract infections [21]. Undoubtedly, the distinct antiviral effect of the combined preparative toward viruses proven to be respiratory tract infections agents, recorded in the present study, could be considered a component of the activity seen in that earlier trial. Here, we have in mind the combined preparative' sinhibitory effect on human rhinovirus replication. It is well known that rhinoviruses are the main agents of the common cold.

5. Acknowledgements

We would like to thank Schapper & Brümmer Ltd., Salzgitter, Germany, for the financial support of this study.

6. References

1. Bauer R. Pflanzliche Immunstimulanzien in der Selbstmedikation. Pharm uns Zeit. 1998; 27:144-157.
2. Teuscher E, Bodinet C, Lindequist L, Freudenstein J. Investigation of active substances in herbal immunostimulants and their mode of action after peroral administration. J Phytotherapy. 2004; 25:11-20.
3. Bauer R, Wagner H. *Echinacea* – Handbuch für Ärzte, Apotheker und andere Naturwissenschaftler, Wissenschaft. Stuttgart:Verlagsges, 1990.
4. Lloyd JU. History of *Echinacea angustifolia.* Pharm Review. 1904; 22:9-14.
5. ESCOP Monograph: *Echinacea purpurea*radix. In: European Scientific Cooperative on Phytotherapy, Exeter, 1999a.
6. ESCOP Monograph. *Echinacea pallidae*radix. In: European Scientific Cooperative on Phytotherapy, Exeter, 1999b.
7. Wagner H, Stuppner H, Schäfer W, Zenk MA. Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. Phytochemistry. 1988; 27:119-126.
8. Egert D, Beuscher N. Studies on antigen specificity of immunoreactive arabinogalactan proteins extracted from *Baptisia tinctoria* and *Echinacea purpurea*. Planta Medica. 1992; 58:163-165.
9. Hänsel R, Keller R, Rimpler H, Schneider G. In:Haggers Handbuch der Pharmazeutischen Praxis, Drogen A – D (*Baptisia*), Bd. 4. Springer-Verlag, Berlin, 1992:461-467.
10. Schrum S, Probst P, Fleischer B, Zipfel PF. Synthesis of the CC chemokines MIP-1 alpha, MIP-1 beta, and RANTES is associated with a type 1 immune response. JImmunol. 1996; 157:3598-3604.
11. Beuscher N. Über die medikamentöse Beeinflussung zellulärer Resistenzmechanismen in Tierversuch. III. Steigerung der Leukozytenmobillisation bei der Maus durch pflanzlichen Reizkörper. Arzneim-Forsch/Drug Res. 1980; 30:821-825.
12. Beuscher N. Über die medikamentöse Beeinflussung zelluläre Resistenzmechanismen in Tierversuch. Aktivierung von Peritonealmakrophagen der Maus durch pflanzlichen Reizkörper. Arzneim-Forsch/Drug Res. 1982; 32:134-138.
13. Gohla SH, Neth RD. Gesielte Immunstimulation durch eine Polysaccharidfraktion aus *Thuja occidentalis* L. Therapeutikon. 1988; 2:717-725.
14. Beuscher N, Bodinet C, Willigmann I, Egert D. Immunodulierende Eigenschaften von Wurzelextrakten verschiedener *Echinacea*-Arten. Z Phytother. 1995; 16:157-166.
15. Bodinet C. Immunopharmakologische Untersuchungen an einem pflanzlichen Immunmodulator [dissertation]. Greifswald: Universitätbibliotek, 1999.
16. Wacker A, Filbig W. Virushemmungmit. Echinacea purpurea. Planta Med. 1978; 33:89-102.
17. Beuscher N, Kopanski L. Reinigung und biologische Charakterisierung von antiviralen Substanzen aus Thuja occidentalis. Plantamedica. 1986; 52:555-556.
18. Beuscher N, Scheit KH, Bodinet C, Egert D. Modulation der körpereigenen Immunabwehr durch polymere Substanzen aus *Baptisia tinctiria* und Echinacea purpurea (Modulation of host resitance by polymeric substances from *Baptisia tinctiria*and *Echinacea purpurea*).In: Immunotherapeutic Prospects of Infectious Diseases. Masihi KN, Lamge W, editors. Springer-Verlag Berlin, 1990.

19. Bodinet C, Beuscher N. Antivirale und immunologische Aktivität von Glykoproteinen aus *Echinacea-purpurea*-Wurzel. In: 39th Annual Congress of Medicinal Plant Research, Planta Medica, special issue 1991.
20. Bodinet C. Esberitox® N – herbal combination in pharmacological and toxicological investigations. Schaper&Brümmer Workshop ESBERITOX® N, Salzgitter-Ringerheim, 2002.
21. Henneicke-von, Zepelin H-H, Hentschel C, Schnitker J, Kohnen R, Kohler G *et al*. Efficacy and safety of a fixed combination phytomedicine in the treatment of common cold (acute viral respiratory tract infection): results of randomized, double blind, placebo controlled, multicenter study. Curr Med Res Opinion 1999; 15:214-227.
22. Rada B, Závada J. Screening test for cytostatic and virostatic substances. Neoplasma (Bratislava) 1962; 9:57-65.
23. Galabov AS. Application of the agar-diffusion plaque-inhibition method for testing combination antiviral effects: PTU-23 and guanidine hydrochloride interactions and combined effects on the replication of poliovirus 1. Acta Microbiol Bulg. 2016; 32:47-55.
24. Galabov AS, Galabov BS, Neykova NA. Structure-activity relationship of diphenylthiourea antivirals.J Medic Chem. 1979; 23:1048-1051.
25. Borenfreund E, Puerner JA. Toxicity determination *in vitro* by morphological alterations and neutral red adsorption. Toxicol. Lett. 1985; 24:119-124.
26. Bockhorst H, Gollnick N, Guran S. Therapie des Herpes simplex in der Praxis –Ein Berichtzur Behandlung des Herpes simplex labialismit Esberitox® [The treatment of herpes simplex in practice - A report about the treatment of herpes simplex labialis with Esberitox®]. Zeitschrift für Allgemeinmedizin. 1982; 32:1795-1798.