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Inhibitory effect of *Bergenia ligulata* on influenza virus A

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Methanol water extract from rhizomes of *Bergenia ligulata*, a plant used in Nepalese ethnomedicine, inhibited in vitro the replication of influenza virus in a dose dependent manner and did not show virucidal activity at effective concentration. Pretreatment of cells with *B. ligulata* extract was shown to be most effective to prevent cell destruction. The extract inhibited viral RNA synthesis and reduced viral peptide synthesis at 10 μg/ml. The virus inhibitory effect is related to the presence of condensed tannins in the extract.

1. Introduction

The current intervention against influenza virus is based on vaccines and chemotherapy. The clinical use of amantadin, an ion channel inhibitor is limited by rapid occurrence of resistant viruses selected by mutations [1]. Zanamivir, one of the two licenced neuraminidase inhibitors, has poor oral bioavailability and the clinical use is limited to topical application to the respiratory tract [2]. Additionally also induction of drug-resistant strains was observed [3].
Therefore it is necessary to initiate alternative developments with different targets. One approach used in this field is the search for viral inhibitors from natural origin. In former experiments we could show that there are several natural sources for compounds with anti-influenza virus activity, basidiomycetes [4], cyanobacteria [5–7] and plants [8]. Traditional herbal medicines have been used worldwide to treat patients with influenza virus infection as well as common cold [9–12]. However, little is known about their targets and mechanism of action.
Recently, we have reported about screening of plants which are used in Nepalese traditional medicine to treat fever and pulmonal disorders for their antiviral activity. The methanol-water extract from the rhizomes of *Bergenia ligulata* (Wall.) Engl. was found to possess strong antiviral activity against influenza A virus with an IC_{50} value of 10 μg/ml. The extract did not show cytotoxicity [13].
The presented study was undertaken to investigate the anti-influenza virus activity of *B. ligulata* in more detail on cellular and molecular level. Additionally the chemical nature of the active principle should be characterized.

2. Investigations, results and discussion

2.1. Effect of extract on virus growth in MDCK cells

The effect of the extract on virus replication was studied employing multiple cycles of virus. The virus yield determined by hemagglutination (HA) titer was strongly inhibited in a concentration dependent manner (Table).

Table: Inhibitory effect of the extracts of *B. ligulata* on replication of influenza virus A/WSN in MDCK cells

Extract concentration (μg/ml)	Hemagglutination titre* (log HA)
50	4
40	4
30	8
20	16
10	16
5	32
1	32
without extract	64

* the values are the mean of four independent experiments

The activity was further investigated in time addition experiments using the colour uptake assay. Results are shown in Fig. 1. Pretreatment of MDCK cells with the extract at a concentration of 25 μg/ml led to 60% protection of the cells against the damaging effect of viruses. The addition of extract in the range of 6.25 to 50 μg/ml after virus infection did not protect the cells. When the extract was present at the time of adsorption a slight inhibition could be observed.
To evaluate the direct inactivating effect of *B. ligulata* on influenza A virus the virus suspension was incubated for 1 h at 37 °C with the extract. At concentrations of 5 and 50 μg/ml no reduction in HA titer in comparison to the untreated control was observed (data not shown).

2.2. Effect of extract on viral RNA synthesis

When the cells were infected in presence of extract virus RNA synthesis decreased in a concentration-dependent manner (Fig. 2). At concentrations of 100 and 10 μg/ml the synthesis of RNA was completely inhibited whereas at 1 μg/ml extract RNA was detected comparable to the untreated control.

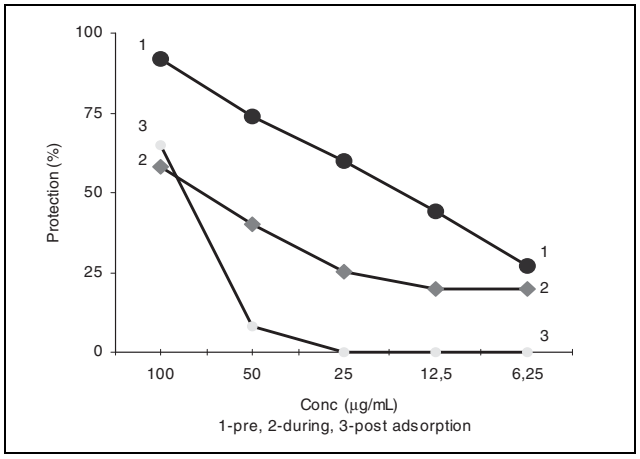

Fig. 1: Inhibitory effect of extract of *B. ligulata* on virus replication in dependence on time of addition. 1: MDCK cells were preincubated with extract for 30 min, washed with PBS and challenged with influenzavirus. After adsorption for 1 h cells were washed again and further incubated for 72 h. 2: Extract was added at the time of adsorption. 3: Extract was added after adsorption

2.3. Effect of extract on viral protein synthesis

To determine the inhibitory effect on the level of viral protein synthesis the ^{35}S-methionine labelled viral polypeptides were analysed by polyacrylamide gel electrophoresis. 100 µg/ml extract led to complete inhibition of protein synthesis (Fig. 3). The visible bands were quantified using a phospho imager. The amount of ^{35}S incorporated into the protein in the presence of extracts was compared with untreated control. 10 µg/ml of the extract of *B. ligulata* reduced the amount of virus protein to 36%.

2.4. Chemical characterization of the active fractions from the extract of Bergenia ligulata

To get first information about the chemical nature of the antiviral compound(s) of *B. ligulata* the extract was fractionated by Diaion HP 20 column chromatography. The antiviral activity was found to accumulate in the 50% methanol (F-2) and methanol fraction (F-3). The IC_{50} values were <12.5 and 12.5 µg/ml respectively without any

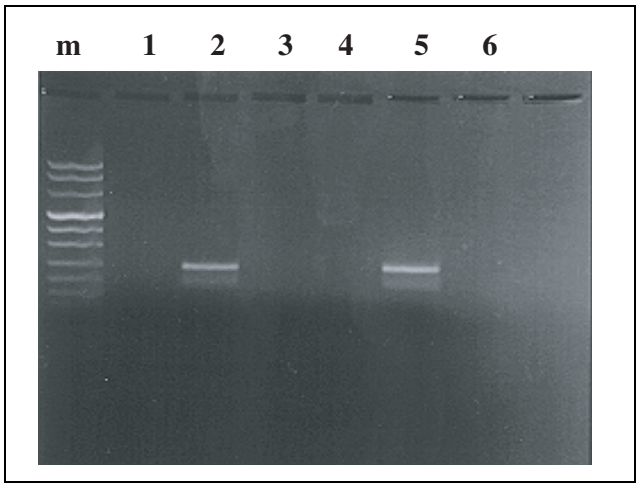

Fig. 2: Inhibitory effect of *B. ligulata* extract on the RNA synthesis of influenza virus A/WSN/33 in MDCK cells. Ethidium bromide-stained agarose gel-analysis of RT-PCR product (237 bp). Lane m: DNA marker (1114–67 bp); 1: Mock infected cells; 2: Virus infected cells, in presence of 3: *B. ligulata* 100 µg/ml; 4: 10 µg/ml; 5: 1 µg/ml; 6: amantadine 100 µg/ml

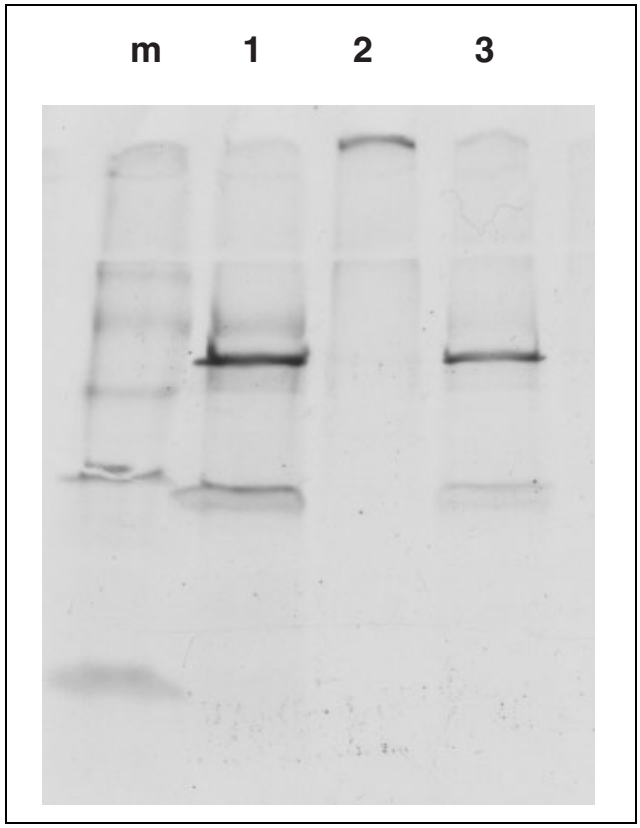

Fig. 3: Effect of extract of *B. ligulata* on polypeptide synthesis of influenza virus A/WSN/3. PAGE on 15% gel and autoradiography of ^{35}S-labelled protein in MDCK cells. Lane m: Molecular weight marker 14.3–220 kDa, 1: Virus infected cells, in presence of *B. ligulata*: 2: 100 µg/ml, 3: 10 µg/ml

toxicity on MDCK cells. The water fraction (F-1) was not active. Further fractionation of fraction F-2 by gel filtration chromatography on a Sephadex LH 20 column gave the fractions SF-2–1, SF-2–2 and SF-2–3. All showed antiviral activity.

By several chemical detection methods (ferric chloride test, vanillin-hydrochloric acid test and p-dimethylaminocinnamaldehyde-hydrochloric acid test) the presence of condensed tannins was indicated in the active fractions F-2, F-3 and the subfractions. The TLC comparison of fractions with extracts from leaves of *Aesculus hippocastanum*, *Crataegus monogyna*, *Vaccinium myrtillus*, fruits from *Quercus robur* and needles from *Pinus sylvestris* containing known polyphenols did not show any agreement.

3. Discussion

It has been shown by infectious virus reduction assay that the extract of *B. ligulata* is able to inhibit the replication of influenza A virus in non cytotoxic concentrations. In addition this study has demonstrated that the effect was dose dependent and most pronounced when the preparation was added before virus infection. Extra-cellular virucidal effect was observed only at concentrations high above the effective dose. For inhibition of RNA synthesis and virus specific protein synthesis 10 µg extract were required.

The antiviral effect might be due to multiple mechanism of action, non-specific interference with virus cell interactions and specific inhibition of an early stage in viral

intracellular multiplication. The inhibition of macromolecular synthesis may be also secondary to the direct interaction of the components of extracts with the virus glycoprotein. Our results from the virus pre-treatment experiments confirm that the extract effects the early phase of influenza virus infection.

Besides the prevention of binding of the viral particles to cells the inhibitory activity may be based on post-translational cleavage of the HA which is essential for the virus adsorption [14, 15]. In former experiments for *B. ligulata* partial protease inhibitory activity was estimated [13].

The bioassay directed fractionation and TLC analysis to characterize the active principles indicated that the virus inhibitory effect of the active fractions is related to the presence of polyphenols especially condensed tannins. Their exact structure is unknown so far. Tannins are constituents of many plants with biological activity. Antibacterial and antiviral effects [16–18] among them such against influenza virus [10, 19] were reported. Mantini et al. 1999 [11] have shown that the extract of *Ephedrae herba*, an oriental traditional medicine, inhibits the growth of influenza virus A/PR8. That extract contains condensed tannins as one of its major components, which are responsible for the effect. By a vital fluorescence microscopic study it was demonstrated that the mode of inhibitory effect was associated with a disappearance of acidified endosomes and lysosomes (ELS). Acidification of intracellular compartments is essential for the uncoating step of influenza virus by triggering the viral envelope fusion activity.

In summary our data demonstrate and confirm that extract of *B. ligulata* could be a potential source of antiviral compounds. They justify further in vivo investigations based on experimental design of former studies using the pneumonia model in mice [8, 20].

4. Experimental

4.1. Cells and viruses

Madine-Darby canine kidney (MDCK) cells were passaged in Eagle's minimal essential medium (MEM) supplemented with 5% fetal bovine serum (GIBCO, Paisley, UK). Human influenza virus A/WSN/33 (H1N1) London was propagated in chorio-allantoic cavities of 10-day old embryonated hen eggs for 72 h. The yielded virus suspension was titrated in MDCK cells and stored at −70 °C until use.

4.2. Preparation of extracts

Dried and powdered plant material (5 g) of *Bergenia ligulata* was extracted successively with CH_2Cl_2 and MeOH in a soxhlet extraction apparatus followed by reflux with 50% aqueous MeOH. Evaporation of the solvent followed by drying in vacuum or by lyophilisation provided crude extracts. 5 mg of the aqueous MeOH extract was dissolved in tissue culture medium (GIBCO Life Science Technologies, Paisley, UK) and stocked at a concentration of 1 mg/ml.

4.3. Activity guided sub-fractionation

An amount of 10 g of the 50% aqueous MeOH extract of *B. ligulata* was subjected to a column (200 g of Diaion HP-20 equilibrated with H_2O). Elution was done with each 2 l of H_2O, 50% aqueous MeOH and MeOH. Three major fractions, F-1 (H_2O fraction, 3.45 g), F-2 (50% MeOH fraction, 4.5 g) and F-3 (MeOH fraction, 1.87 g) were collected.

An aliquot of 2.0 g of fraction F-2 was subjected to a Sephadex LH 20 column (20 g Sephadex LH 20), equilibrated with 50% of aqueous MeOH and eluted with the same solvent system. A volume of 20 ml of these subfractions (SF) of F2 were collected. The subfractions were pooled into two major fractions, SF-2–1 (420 mg) and SF-2–2 (290 mg). The column was then eluted with pure MeOH (SF-2–3, 810 mg). All fractions were monitored by TLC on cellulose F plates using BuOH–EtOH–MeOH (20 : 5:11) and BuOH–EtOH–MeOH–H_2O (20 : 5:0.5 : 11) as eluents.

4.4. Antiviral assay

4.4.1. Colorimetric assay

Confluent monolayers of MDCK cells were preincubated with serial dilutions of the plant extract (100, 50, 25, 12.5 μg/ml) in triplicate for 30 min at 37 °C. A suspension of influenza virus A containing 30 $TCID_{50}$ was added and further incubated for 72 h at 37 °C. Antiviral activity was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric test [21]. 20 μl of MTT (7.5 mg/ml) in phosphate-buffered saline (PBS) were added to each well and incubated for 4 h at 37 °C. After incubation, the crystallized formazan was dissolved by addition of 100 μl of 20% sodium dodecyl sulfate/50% *N,N*-dimethyl formamide. Optical density was measured at 540 nm. The percent protection was calculated by the formula:

$$[(OD_t)_{virus} - (OD_c)_{virus} / (OD_c)_{mock} - (OD_c)_{virus} \times 100\ (\%)$$

t: with test compound, c: without test compound

4.4.2 Infectious virus reduction assay

MDCK cells were pre-treated with extracts of different concentrations ranging from 1 to 50 μg/ml for 30 min and infected with influenza virus A containing 30 $TCID_{50}$. The culture supernatants from the plates, after incubation for 72 h at 37 °C, were tested for the presence of infectious viruses by HA. 50 μl of supernatant from each well of the plate were serially diluted two-fold in PBS and 50 μl of 0.5% human erythrocyte suspension was added. After incubation at room temperature for 3 h the reduction in HA titer was determined.

4.5. Time of addition studies

Confluent monolayers of MDCK cells were incubated with medium containing *B. ligulata* extract (100, 50, 25 and 12.5 μg/ml) at several stages of infection, before, during and post infection (30 $TCID_{50}$). Incubation was performed for 72 h at 37 °C in a humidified atmosphere. The effect of drug was determined by colorimetric assay.

4.6. Viral RNA synthesis

Influenza virus A infected MDCK cells were incubated for 22 h at 37 °C in the presence of 100, 10 and 1 μg/ml of *B. ligulata* extract, respectively. Mock infected cells, drug untreated virus infected cells and amantadine (reference compound) treated cells were included. RNA was extracted using a QIAamp® Blood MiniKit (250) according to the manufacturer's instruction. The cDNA synthesis and first round PCR was carried out using Superscript ™One-Step ™RT-PCR System (Gibco, Eggenstein, Germany) after the manufacturer's instructions.

Two oligonucleotide primer pairs, derived from the highly conserved region of the influenza A/PR/8/34 NS gene sequence [22] were used. Primer NS3 (GGTGATGCCCCATTCCTTGA, position 108–127) and NS4 (ATTTCGCCAAC-AATTGCTCC, position 486–505) were used in the first round PCR (25 pmol of each) and primers NS1 (GAGGCACT-TAAAATGACCAT, position 249–268) and NS2 (CTCTTC-GGTGAAAGCCCTTAG, position 465–485) in the nested PCR reaction. Samples were incubated in a thermocycler (Perkin Elmer): 1 cycle at 50 °C for 30 min (involving cDNA synthesis), followed by a two step profile with 30 cycles at 94 °C for 1 min and 62 °C for 20 s. The amplification product, which was expected to yield a 237 bp product was analysed on ethidiumbromide-stained 3% agarose gel.

4.7. Viral protein synthesis

MDCK cell monolayers in 24-well plates were pre-treated for 30 min with *B. ligulata* extract at a concentration of 100 and 10 μg/ml respectively and challenged with influenza A virus. After adsorption for 1 h at 37 °C, the cells were washed twice with PBS and drug-containing medium was added. After incubation for 18 h at 37 °C the monolayers were labelled for 3 h at 37 °C with 5 μCi of ^{35}S-methionine/well and lysed with RIPA buffer. Virus polypeptides were analysed by 15% SDS polyacrylamide gel electrophoresis and visualised by autoradiography. The resulting bands were quantified with a phospho-imager. The dried gel was exposed to storage phosphor screens for three days and scanned with a STORM 840 Phosphoimager (Molecular Dynamics).

4.8. Chemical analysis

The extract and the fractions obtained from *B. ligulata* as well as catechin and tannic acid as standard compounds were dissolved at a concentration of 5 mg/ml in 50% aqueous MeOH or water. Polyphenols were analysed by ferric chloride test. Ellagitannins were detected by use of the sodium nitrite-acetic acid reagent, condensed tannins by the ferric ammonium sulphate-hydrochloric acid test, vanillin-hydrochloric acid test and *p*-dimethylamino-cinnamaldehyde-hydrochloric acid test.

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