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Inhibition of Several Strains of Influenza Virus *in Vitro* and Reduction of Symptoms by an Elderberry Extract (*Sambucus nigra* L.) during an Outbreak of Influenza B Panama

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ABSTRACT

A standardized elderberry extract, Sambucol® (SAM), reduced hemagglutination and inhibited replication of human influenza viruses type A/Shangdong 9/93 (H3N2), A/Beijing 32/92 (H3N2), A/Texas 36/91 (H1N1), A/Singapore 6/86 (H1N1), type B/Panama 45/90, B/Yamagata 16/88, B/Ann Arbor 1/86, and of animal strains from Northern European swine and turkeys, A/Sw/Ger 2/81, A/Tur/Ger 3/91, and A/Sw/Ger 8533/91 in Madin–Darby canine kidney cells. A placebo-controlled, double blind study was carried out on a group of individuals living in an agricultural community (kibbutz) during an outbreak of influenza B/Panama in 1993. Fever, feeling of improvement, and complete cure were recorded during 6 days. Sera obtained in the acute and convalescent phases were tested for the presence of antibodies to influenza A, B, respiratory syncytial, and adenoviruses. Convalescent phase serologies showed higher mean and mean geometric hemagglutination inhibition (HI) titers to influenza B in the group treated with SAM than in the control group. A significant improvement of the symptoms, including fever, was seen in 93.3% of the cases in the SAM-treated group within 2 days, whereas in the control group 91.7% of the patients showed an improvement within 6 days ($p < 0.001$). A complete cure was achieved within 2 to 3 days in nearly 90% of the SAM-treated group and within at least 6 days in the placebo group ($p < 0.001$). No satisfactory medication to cure influenza type A and B is available. Considering the efficacy of the extract *in vitro* on all strains of influenza virus tested, the clinical results, its low cost, and absence of side-effects, this preparation could offer a possibility for safe treatment for influenza A and B.

INTRODUCTION

Influenza virus A or B causes an acute, febrile illness that occurs in outbreaks of varying severity almost every winter.

Amantadine and rimantadine were shown to be mainly effective in the prevention of influenza A (Younkin et al., 1983; Reuman et al., 1989; Brady et al., 1990). They inhibit influenza B *in vitro* at such high concentration that can-

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not be achieved in patients (Douglas, 1990). Besides the high cost of these products they elicit side effects, especially in elderly people (Stange et al., 1991). Moreover, it has been reported that mutations in the influenza M2 membrane protein confer resistance to amantadine (Grambas et al., 1992). Rimantadine-resistant influenza A strains appeared during therapeutic use of this product as early as 2 days after starting treatment (Hayden et al., 1989, 1991). This could lead to rapid selection and transmission of drug-resistant influenza A viruses. Ribavirin is effective against type A and B viruses, but only when given in aerosol. This mode of administration is difficult in influenza patients suffering from respiratory diseases and is an expensive and cumbersome mode of therapy (Gilbert et al., 1986).

The black elder had been used in the folk medicine for its properties against influenza. Therapeutic indications of the elder flowers are influenzal colds and sinusitis (British Herbal Pharmacopoeia, 1983). Antiviral activity of the infusion of three plants including the elder has been reported against influenza and herpes (Serkedjieva et al., 1990).

A standardized extract, Sambucol® (SAM), is a preparation based on the berries of the black elder, used as herbal remedy against influenza virus infections. It contains a high amount of three flavonoids (Bronnum-Hansen and Hansen, 1983). The flavonoids are naturally occurring plant substances. Numerous reports have been published on the antiviral activity of polyphenols such as the flavo noids, flavonols, and flavones. Antiviral activity against herpes virus type 1, respiratory syncytial, parainfluenza, and influenza viruses was demonstrated using several plant extracts containing flavonoids or purified flavonoids (Amoros et al., 1992; Serkedjieva et al., 1992; Nagai et al., 1990; Mahmood et al., 1993).

The aim of this study was to test this extract for its antiviral properties under *in vitro* conditions in cell cultures infected by several human strains of type A and B and animal influenza viruses. In addition, its ability to reduce the duration of the illness caused by influenza viruses was tested in a double-blind clinical placebo-controlled, randomized study carried out in a group of normally healthy population that was not previously vaccinated against flu.

MATERIALS AND METHODS

In vitro tests

Cells. Madin–Darby canine kidney (MDCK) cells were grown in RPMI 1640 medium containing 10% inactivated fetal calf serum (FCS), penicillin G (100 units/ml), and streptomycin (100 μg/ml). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. For assays, 2×10^5 cells per well were plated in 24-well plastic culture plates (Nunc, Roskilde, Denmark) and used when confluent monolayers were formed.

Influenza viruses. A/Shangdong 9/93 (H3N2), A/Texas 36/91 (H1N1), A/Beijing 32/92 (H3N2), A/Singapore 6/86 (H1N1), B/Panama 45/90, B/Yamagata 16/88, and B/Ann Arbor 1/86 were obtained from Dr. J.M. Wood (National Institute of Biological Standards and Control, Potter Bar, Hertfordshire, UK).

H1N1 strains from northern European pigs and turkeys, A/Sw/Ger 2/81, A/Tur/Ger 3/91, and A/Sw/Ger 8533/91 were obtained from Prof. C. Scholtissek (Institute of Virology, University of Giessen, Germany). The viruses were grown in allantoic sacs of 10-day-old embryonated eggs for 48 h at 34°C. The allantoic fluid was harvested, clarified at 2000 rpm 10 min, and the supernatant was stored in small portions at −70°C.

The viruses were titrated on MDCK cultures in the absence of trypsin to receive a limited number of virus replication cycles (Tobita et al., 1975). The final dilution of the virus that gave a complete cytopathic effect (CPE) was used to test the protective effect of Sambucol® as well as higher concentrations in some cases. The number of $TCID_{50}$ inhibited by the elderberry extract was calculated from the titer in the MDCK.

Black elderberry extract. Sambucol® (Razei Bar Ltd, Jerusalem) is a syrup containing elderberry juice, raspberry extract, glucose, citric acid, and honey. For the *in vitro* studies,

Sambucol D®, a formulation without glucose and honey, was used. Flavonoids are measured by their absorbance at 516 nm (not less than 0.60). The extract diluted in phosphate-buffered saline (PBS) at 1:8 has a pH of 4.9. Therefore, the virus controls were performed at the same pH. Dilutions lower than 1:8 were not tested for studies in tissue culture because of their low pH. In the hemagglutination reduction test, the extract could be used at a dilution of 1:4 as well.

Hemagglutination test of the viruses. The hemagglutinin titration was effected using modified standard procedures. For this purpose, 0.1 ml of 2-fold dilutions of each of the viruses suspensions in PBS was mixed with 0.1 ml of a 1% sheep red blood cell (SRBC) suspension.

Hemagglutination reduction using SAM. Virus suspensions [8 hemagglutination units (HAU) in 0.1 ml] were incubated with an equal volume of 2-fold dilutions of SAM at room temperature for 1 h or overnight at 4°C. After incubation, 50 μl of a 2% SRBC suspension was added. In other experiments, equal volumes of virus suspensions (32–64 HAU) and SAM (final dilution 1:8) were incubated overnight at 4°C. An SRBC suspension was added to 2-fold dilutions (0.1 ml) of each virus incubated as above with SAM. Reduction of the hemagglutination titer was assessed by comparison with controls.

Inhibition of infectivity. Titration of the viruses: Confluent monolayers of MDCK cells were infected with influenza viruses at different multiplicity of infection, in 0.2 ml PBS (pH 7.4). Following 30 min adsorption, 1 ml serum-free RPMI medium was added and the cultures were further incubated at least for 48 h or until complete lysis was observed in the virus control wells. The final dilution that gave a complete lysis was determined.

Inhibition assay: The viruses [at a final concentration producing 100% CPE (2 $TCID_{50}$) and in some cases at higher dilutions] were incubated at room temperature with various concentrations of SAM 15 min before infection of the cells. The experiments for each virus were performed on triplicate samples and were repeated four times. The number of $TCID_{50}$ inhibited by SAM was calculated from the titer determined as above. Evidence of cytopathic effect was shown by staining the plates. The plates were washed with PBS to eliminate the dead cells and stained with Giemsa solution after fixation in cold methanol.

Clinical study design

A double-blind study on 40 individuals living in an agricultural community (kibbutz) in Southern Israel and visiting the dispensary was carried out. Before inclusion in the study, a description of the objectives, procedures, and benefits of participation was given to each patient, and a written informed consent was obtained from him or her. Bottles identical in appearance containing experimental medication or placebo were assigned numbers from a predetermined list kept in a sealed envelope, which resulted in random distribution. On the first visit to the dispensary patients received one bottle with the next number in sequence.

Study group. Patients who were admitted to the study had at least three of the following symptoms of less than 24 h duration: fever >38°C, myalgia, nasal discharge, and cough. In the presence of streptococcus A (tested with Biosign strep. A, Princetown Biomeditech Corp., Princeton, NJ), patients with a sore throat were excluded from the study. None of the patients had been vaccinated against influenza.

Treatment. Children received two, and adults four tablespoons of either SAM or its placebo daily for 3 days.

Follow-up of the patients was performed by recording over a period of 6 days the presence of the following symptoms: fever, rhinitis with flow (thick, liquid, frequent, rare), headache, pharyngitis, cough, malaise, fatigue, and myalgia. Feelings of improvement or complete cure were also noted.

Serological studies. Samples of sera were obtained from the patients on their first visit to the dispensary and in the convalescent phase. The sera were tested for the presence of antibodies to influenza A and B by two independent tests. Antibodies to RSV and adenoviruses were tested by complement fixation test.

Complement fixation test (CFT). A micromethod technique of CFT was used as described by Taylor et al. (1970). Antigens were extracted as follows: RSV and adenovirus antigens from human kidney infected cells and influenza A and B from chorioallantoic membranes of 10-day-old embryonated eggs inoculated with influenza A and B.

Antibody titers were determined as the highest dilution giving maximum 50% hemolysis. A 4-fold and over increase in antibody titer between the first and the second sample was indicative of active infection.

Hemagglutination Inhibition Test (HI). HI is a subtype-specific serological test. Antibodies were evaluated using a known concentration of hemagglutinin and a chicken red blood cell suspension. The following influenza antigens were provided by the WHO collaborating Influenza Center, London: A/Taiwan/1/86, A/Beijing/353/89, B/Victoria/2/87, and B/Panama/45/90. Sera were treated to remove nonspecific inhibitors by receptor destroying enzyme (provided by the WHO collaborating Influenza Center, London) and by heat (56°C, 30 min). The test was performed by microtiter method using four units of antigen. The HI titer of each serum was the highest dilution causing a complete inhibition of agglutination.

Statistical Analysis. The Fisher exact test was used to test for a difference between the treated group and the control group. An odds ratio was used as a summary measure.

RESULTS

Inhibition of virus hemagglutination

Short incubation (1 h) of 8 HAU of influenza virus with SAM at the dilution of 1:4 inhibited hemagglutination for A/Beijing 32/92 (H3N2), A/Singapore 6/86 (H1N1), B/Panama 45/90, and B/Yamagata 16/88. Higher dilutions of SAM (1:8 to 1:16) inhibited hemagglutination when the duration of the incubation with the extract was increased to 16 h.

In other experiments, the viruses were incubated overnight with SAM at the final dilution of 1:8. Hemagglutination titer of the viruses was reduced 4-fold for A/Beijing, 16-fold for A/Singapore, and 8-fold for B/Panama and B/Yamagata strains.

The hemagglutination titer of the viruses was not affected when using SRBC previously incubated for 16 h with SAM.

Antiinfluenza virus activity of the elderberry extract in cell cultures

The effect of SAM on replication of influenza viruses was studied on human influenza viruses type A/Shangdong 9/93 (H3N2), A/Texas 36/91 (H1N1), A/Beijing 32/92 (H3N2), A/Singapore 6/86 (H1N1), type B/Panama 45/90, B/Yamagata 16/88, B/Ann Arbor 1/86, and on new animal strains from northern European swine and turkeys, A/Sw/Ger 2/81, A/Tur/Ger 3/91, and A/Sw/Ger 8533/91. The inhibition of replication of these strains was observed when the virus inoculum was left in contact with the elderberry extract before infecting the cell cultures. This inhibition was dose-dependent. SAM completely inhibited viral CPE at the dilution of 1:8 [final dilution during incubation with the virus was 1:16 and approximately 1% (1:96) in the culture medium]. SAM at initial dilution of 1:16 (final concentration in culture medium 0.5%) could only partially inhibit the cytopathic effect produced by the viruses at the same concentration. The number of $TCID_{50}$ inhibited by SAM is shown for each strain in Table 1.

No changes were observed in cell controls in the presence of SAM, undiluted and at different dilutions in the same conditions of the experiment.

Clinical study

Before the beginning of the study, SAM was tested for the absence of side-effects on 35 healthy individuals from Jerusalem who received 4 tablespoons daily for 3 days. No side-effects were recorded.

The symptoms of the patients that were observed during the first visit to the dispensary are summarized in Table 2. Headache, myalgia, fever, malaise, fatigue, and rhinitis were uniform complaints and, more rarely, cough.

In the treatment group 5 out of 20 patients