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

ZICHRIA ZAKAY-RONES, Ph.D.,[1] NOEMI VARSANO, M.Sc.,[2] MOSHE ZLOTNIK, M.D.,[3] ORLY MANOR, Ph.D.,[4] LIORA REGEV,[2] MIRIAM SCHLESINGER,[1] and MADELEINE MUMCUOGLU, Ph.D.[1]

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-

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 $TCID_{50}$ in-hibited 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

TABLE 1. INHIBITION OF INFECTIVITY OF INFLUENZA VIRUS STRAINS BY SAMBUCOL® (SAM)

	Number of $TCID_{50}$ of virus inhibited dilution of SAM	
Strain	1:8	1:16
A/Beijing 32/92 H3N2	40	40
A/Shangdong 9/93 H3N2	40	ND
A/Singapore 6/86 H1N1	4	ND
A/Texas 36/91 H1N1	2	ND
B/Panama 45/90	2	ND
B/Yamagata 16/88	20	10
B/Ann Arbor 1/86	7	3.5
A/Sw/Ger 2/81 H1N1	8	4
A/Tur/Ger 3/91 H1N1	2	1
A/Sw//Ger 8533/91 H1N1	8	2

and in the control group 8 out of 20 patients were excluded from the study due to inconsistence of the treatment or visits to the physician, medication with antibiotics due to streptococcal infection and tonsillitis, or because they were negative in all virological tests. The details of the 27 patients are given in Table 3. The age of the patients was 5 to 50 years (mean 23.5) in the group treated with SAM and 7–56 years (mean 19.4, median 12) in the placebo group. The male:female ratio was 9:6 in the SAM group and 9:3 in the placebo group.

In the group treated with SAM, convalescent-phase serologies showed a mean HI antibody titer of 201 to influenza B Panama/45/90 in 13 out of 15 cases and at least a 4-fold titer rise in the complement fixation test (Figs. 1 and 2). One patient was found positive to RSV and another one positive to both RSV and adenovirus. In the placebo group, convalescent-phase serologies showed a mean HI antibody titer of 171 to influenza B/Panama/45/90 in 10

TABLE 2. DISTRIBUTION OF SYMPTOMS AMONG 27 PATIENTS INCLUDED IN THE STUDY

Symptoms	% of cases
Headache	100
Fever	96.3
Fatigue	88.9
Rhinitis	85.2
Pharyngitis	74.0
Myalgia	51.8
Cough	18.5

TABLE 3. AGE, SEX, AND VIRAL INFECTIONS IN THE TREATED AND CONTROL GROUP

	Sambucol®	Placebo
Total in group	15	12
Males:females	9:6	9:3
Age (years)	5–50	7–56
Influenza B	13	10
Influenza A	—	2
RSV	1	—
Adeno/RSV	1	—

out of 12 cases (one of them was also positive to RSV). Although the differences between the antibody titers in the two groups were not significant, a clear trend in favor of the group treated with the extract was noted. The geometric mean HI titers were 154.88 in the SAM treated group and 109.06 in the control group. Two patients had serologic evidence of influenza A together with antibodies to influenza B.

Persistence of the symptoms was checked for 6 days after the onset of the treatment. The fever persisted for 4 days in the treatment group, and at least 6 days in the placebo group (Fig. 3). Mean numbers of days with fever were 2.36 ± 0.9 in the SAM group and 3.33 ± 1.5 in the placebo group. The odds for having less than 5 days of fever in the treated group were 21.3 times the odds in the control ($p < 0.01$).

Improvement of the symptoms was recorded daily (Fig. 4). In the group treated with SAM, improvement was noted in 20% of the cases one
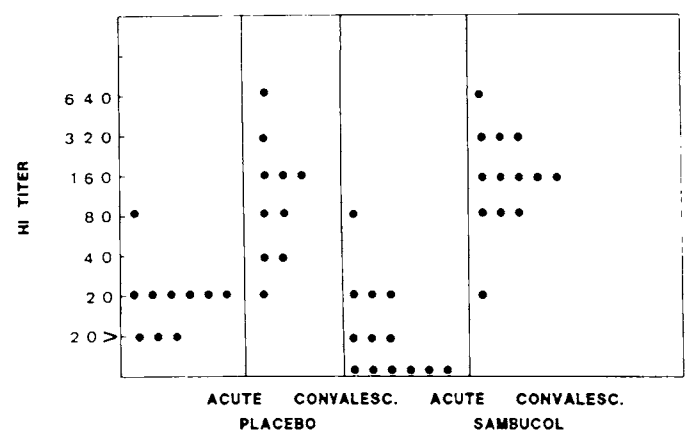

FIG. 1. Influenza B/Panama/45/90 antibody titers in acute and convalescent phase sera measured by the hemagglutination-inhibition test (HI).
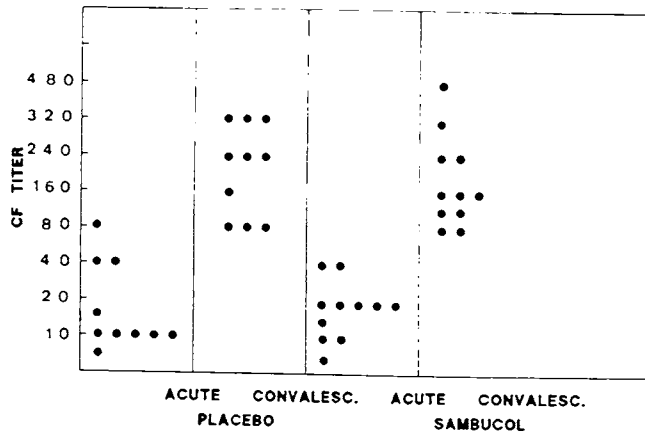
FIG. 2.   Influenza B antibody titers in acute and convalescent phase sera measured by the complement fixation test (CF).

FIG. 4.   Percentage of elderberry extract treated and control patients who showed improvement in their symptoms.

day after onset of the treatment, in 73.3% after 2 days, and in the remaining 6.7% after 3 days, compared to 8.3, 16.7, and 33.3% 1, 2, and 3 days, respectively, after onset of the treatment in the control group. In the placebo control group, improvement was recorded for more than 5 days. The odds for improvement before the fifth day in the treated group are 22.7 times the odds in the control group ($p < 0.001$).

Complete cure was observed after 2 days in 40% of patients treated with SAM and 16.7% treated with placebo. After 3 days complete cure was achieved in additional 46.7% (86.7% total) of patients who received SAM and 16.7% (33.4% total) in those who received placebo. After 4 days the rate of cure was 13.3% in the SAM group and 16.7% in the control groups. In the placebo group, complete cure was obtained after 5 days in 41.7% of the patients, and
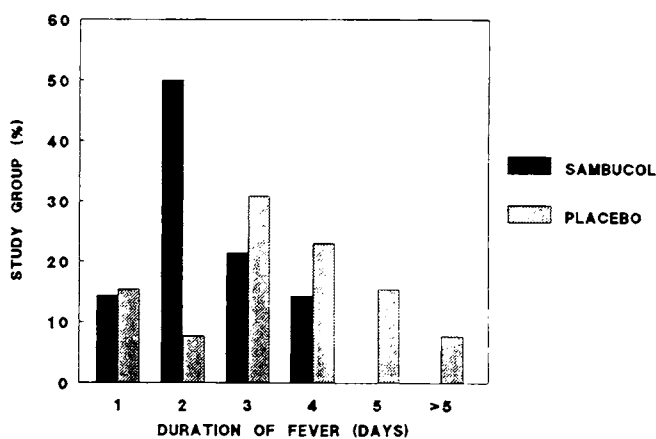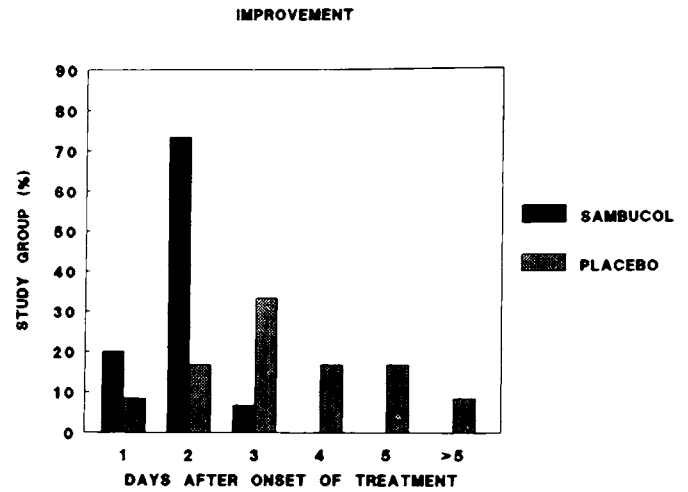
more than 5 days in 8.3% (Fig. 5). Mean duration of illness was 2.7 days in the SAM group and 4 days in the placebo group. The odds for complete cure before the fifth day in the treated group were 31.1 times the odds in the control group ($p < 0.001$).

DISCUSSION

The antiviral activity of SAM was demonstrated by *in vitro* studies, showing its ability to inhibit the hemagglutinin of several strains of influenza viruses type A and B. However, SRBC incubated with SAM were agglutinated

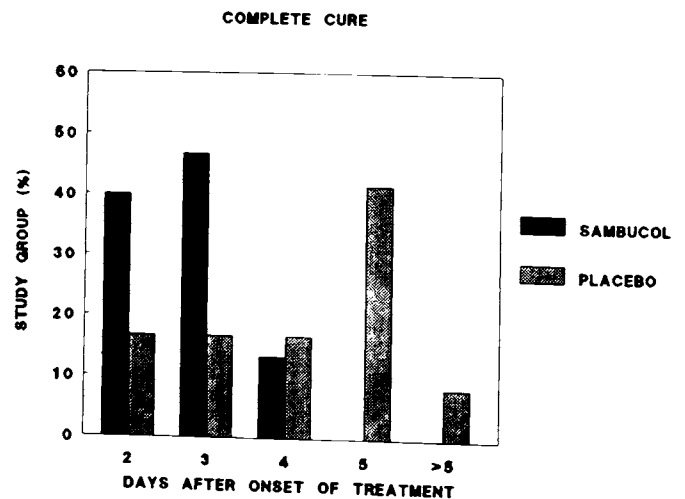FIG. 3.   Duration of fever in group treated with elderberry extract and control patients.

FIG. 5.   Percentage of elderberry extract treated and control patients completely cured of flu.

by influenza viruses, suggesting that the extract inhibits the hemagglutinin of the viruses by binding to the virus itself and does not interfere with the glycoconjugate receptor on the erythrocytes. Moreover, the replication of human influenza viruses type A and B, including the strains (Shangdong, Singapore, Panama) of the vaccine proposed for the winter 94/95 as well of new strains of animal influenza viruses from turkey and swine, could be prevented in cell cultures by previous incubation of the virus inoculum with SAM. To avoid any nonspecific interaction due to its low pH, SAM was used in most of the experiments at the dilution of 1:8. It could be assumed that SAM at higher concentrations would have been an even more potent inhibitor. To remove any doubt on the efficacy of the pure elderberry extract itself, a number of experiments were performed with this extract alone, e.g., without the presence of additives such as citric acid. Influenza A and B viruses express two envelope glycoproteins: hemagglutinin and neuraminidase. The hemagglutinin is known to mediate the attachment of the virus to the host cells via sialic acid residue in glycoconjugate receptors and the subsequent fusion of viral and host cell membranes (Wieley and Skehel, 1987). The sialidase catalyzes cleavage of terminal sialic acid residue from the sialoconjugate receptors (Gottschalk et al., 1972). It can be assumed that the inhibitory effect on influenza virus in tissue culture is mediated by inactivation of viral glycoproteins, which prevent the initial stage of reproduction. The inhibition of the hemagglutinin was clearly demonstrated. The presence of flavonoids (Bronnum-Hansen and Hansen, 1983) in SAM could be responsible for blocking of the virus sialidase since flavonoids are known to have potent antiinfluenza activity. Further experiments are underway to test this hypothesis and preliminary results indicated that SAM may partially block the sialidase of influenza viruses.

Influenza vaccine is useful for prophylaxis of influenza virus infection, but antigenicity of influenza viruses is often alterable by antigenic shift and antigenic drift on their two antigens, hemagglutinin and neuraminidase. In this study we have shown that SAM inhibits the hemagglutinin of all strains of influenza viruses tested. Moreover, fresh pandemic may come from an influenza virus that infects animals but also infects humans under favorable conditions. Data have accumulated that indicate that genetic reassortment occurs *in vivo* in mixed infections in swine and turkeys. They may also arise from a hybridization of an animal strain and a human strain. Mutant type A swine flu viruses may have been responsible for the widespread epidemics in 1918 and 1957 (Scholtissek et al., 1978; Hinshaw et al., 1978; Kilbourne et al., 1971; Webster et al., 1973). SAM was shown to inhibit strains isolated from turkeys and swine that under favorable circumstances could produce such pandemic epidemics to which people lack immunity. The eventuality of a new pandemic has been raised lately and seems quite likely (Hannoun, 1994).

The antiviral properties of SAM were further tested in a double-blind, placebo-controlled study. The comparison of 15 patients who received SAM with 12 patients who received a placebo showed that in the treatment group a significant improvement of the symptoms of flu, including fever, was seen in 93.3% of the cases within 2 days. In the control group 91.7% of the patients showed an improvement within 6 days. A complete cure was achieved within 2 to 3 days in nearly 90% of the group treated with SAM and within at least 6 days in the placebo group ($p < 0.001$).

Most of the patients showed laboratory documented influenza B infection. In the control group, the two patients who showed evidence for influenza A had also antibodies to influenza B. This could be the result of a heterotypic antibody response to influenza A as reported in 25% persons infected with influenza B (WHO, 1991–1992). Convalescent-phase serologies showed higher mean and geometric mean HI antibody titer to influenza B in the elderberry extract group. Administration of SAM seems to enhance the immune response, whereas amantadine suppresses the serologic response to influenza A (Reuman et al., 1989).

These preliminary results should be confirmed by a study on a larger number of patients, which should also include more influenza A-infected individuals. Although the laboratory data documented the diagnosis of influenza, it would be of interest to isolate the

virus in the nasal and throat secretions of the patients. Treatment with SAM could result in a decrease of transmissibility of influenza viruses, resulting in fewer secondary cases of infection in communities such as the homes for the elderly, army camps, and university residences.

Vaccination with influenza B induces a poor antibody response, particularly in elderly patients, since influenza B is less immunogenic (Peters et al., 1988). In the absence of any proper medication against influenza B virus, and considering the efficacy of the Sambucol® against all strains tested and its absence of side effects, this preparation could offer a possibility for a safe treatment for influenza, and especially in the eventuality of a new pandemic.

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Address reprint requests to:
Prof. Zichria Zakay-Rones
Department of Virology
Hebrew University-Hadassah Medical School
POB 12272
91120, Jerusalem, Israel