Neutralizing activity of SAMBUCOL® against avian NIBRG-14 (H5N1) influenza virus

S. Balasingam*, R. Lambkin*, D. Safirman**, M. Mumcuoglu** & J.S. Oxford*

* Retroscreen Virology Ltd, St Bartholomew's & The Royal London School of Medicine & Dentistry, University of London
** Razei Bar Industries (1996) Ltd, Manhat Technology Park, Jerusalem, Israel

Abstract

The spread of avian influenza (H5N1) from the Far East to East European countries raises concerns of a possible flu pandemic and emphasizes the need to search for a preventative and/or cure. The aim of this study was to determine the antiviral activity of Sambucol®, a standardized Black Elder berry extract, against avian influenza NIBRG-14 (H5N1) virus. Antiviral assays were performed in MDCK cells using two Sambucol® dilutions at several incubation times. Results show at least 99% reduction in the titre of avian influenza NIBRG-14 (H5N1), namely 2.0 $\log_{10}$ TCID/ml. Therapeutic index calculations indicated direct influence of Sambucol® on titre reduction. Further studies will be undertaken to assess the effectiveness of Sambucol® against avian influenza (H5N1) virus in animals and humans.

Introduction

The past century has witnessed three pandemics of influenza, of which the "Spanish flu" of 1918 was the largest. The World Health Organization (WHO) and experts around the world claim that a new influenza pandemic will occur in the near future. The current global concern is the avian influenza A (H5N1) virus, which first demonstrated its ability to infect birds in China in 1997 and has since spread to other countries in South East Asia and also, via migrating birds, to East European countries. According to the WHO, the year 2005 ended with a total of 141 confirmed human cases of avian influenza A (H5N1), of which 73 were fatal (1).

Sambucol® is a branded herbal preparation, based on a standardized extract of the European Black Elder berry (*Sambucus nigra* L.). The preparation contains natural ingredients with antiviral properties (2). In vitro studies with Sambucol® have shown it to reduce hemagglutination and inhibit replication of type A and B human influenza viruses, as well as of animal strains including a type A turkey influenza virus (3). Furthermore, two double-blind placebo-controlled clinical trials confirmed the safety and efficacy of Sambucol® in the treatment of influenza A and B and its symptoms. In both trials the Sambucol® group showed pronounced improvement (including overall performance and severity of symptoms). Patients receiving Sambucol® recovered 3 and 4 days, respectively, earlier than the control groups, which achieved similar improvement on days 6 and 8, respectively, of the trials (3,4). The aim of this study was to determine the effectiveness of Sambucol® against avian influenza H5N1 in vitro.

Materials & Methods

The study was performed at the laboratories of Retroscreen Virology Ltd., St Bartholomew's & The Royal London School of Medicine & Dentistry, University of London.
All incubations were at 37^{O}C, 5% CO_2 unless otherwise specified.

Virus
The virus used was influenza NIBRG-14 (H5N1), characterized by A/PR/8 backbone, HA and NA genes from an H5N1 isolate (A/Vietnam/1194/04) with lower pathogenicity (supplied by National Institute for Biological Standards and Control).

Sambucol®
Sambucol® was supplied as a sterile filtered undiluted preparation. The test dilutions were 1/4 and 1/8 of the original preparation. In the virucidal assay each dilution of Sambucol® underwent a 9/10 dilution upon mixing with virus.

Target cells
Madin-Darby Canine Kidney Epithelial cells (MDCK). 100µl cell suspension at 5x10^4 cells/ml was seeded onto each well of 96-well plates and incubated for 24h. Prior to use in the assays, plates were washed twice with PBS (100µl/well) and 100µl standard MDCK infection media was added to each well..

Cytotoxicity assay – Crystal violet
Serial dilutions (1:10) of Sambucol® were added, in duplicate, to wells (100µl/well) that had been seeded with MDCK cells as above. Cells were incubated for 4 hours. A crystal violet test was performed to assess cell viability, measured by optical density. Data are represented as percentages of cell only controls.

Cytotoxicity assay – cell observation
Serial dilutions (1:10) of Sambucol® were added, in duplicate, to wells (100µl/well) that had been seeded with MDCK cells as above. Cells were incubated for 4 hours and observed for toxic cytopathic effects that may not have been registered by the crystal violet assay.

Reduction of viral titre assay
40µl of virus (total titre in reaction 4.0 -$\log_{10}$ $TCID_{50}$/ml) was added to 360µl test sample and incubated at room temperature for 0.5, 5, 10, 30 and 60 minutes. The reaction mixture was added to the target cells and titrated across the 96-well plate following a 10-fold dilution series. The plate was then incubated for 60 minutes. Supernatants were then discarded and plates washed twice with PBS before 100µl standard infection media was added to each well. Cells were incubated for 3 days at which point viral titre was measured.
The antiviral positive control consisted of a 5 minute pre-treatment of virus with citrate buffer at pH 3.5 (known incubation time point of citrate buffer that exhibits antiviral activity against influenza A viruses – unpublished data).

Results

Cytotoxicity – Crystal violet assay
The crystal violet assay performed was used to calculate the viability of MDCK cells after incubation with various concentrations of Sambucol®. The results are shown in Fig 1. Dilution of Sambucol to 1:80 or more completely eliminated any toxic effect on MDCK cells.

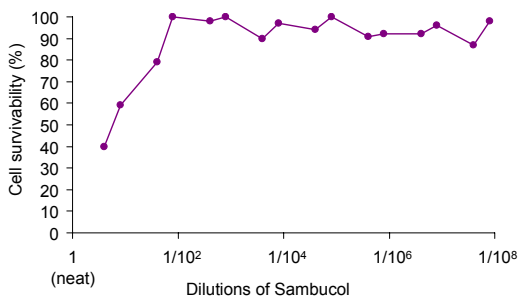

Fig 1. Moderately diluted Sambucol® is non-toxic to cells.
MDCK cells were incubated with serial dilutions of Sambucol® for 4 hours. After this time, a crystal violet test was performed and cell survival measured by optical density. Data are expressed as percentage survival as compared to positive (cell only) controls.

Cytotoxicity – cell observation
The data from cell observations have not been presented as there were toxic effects noted in some of the 'cell only' control wells. From experience and from limited successful observations, the survivability threshold for toxicity in this assay was considered to be 90%.

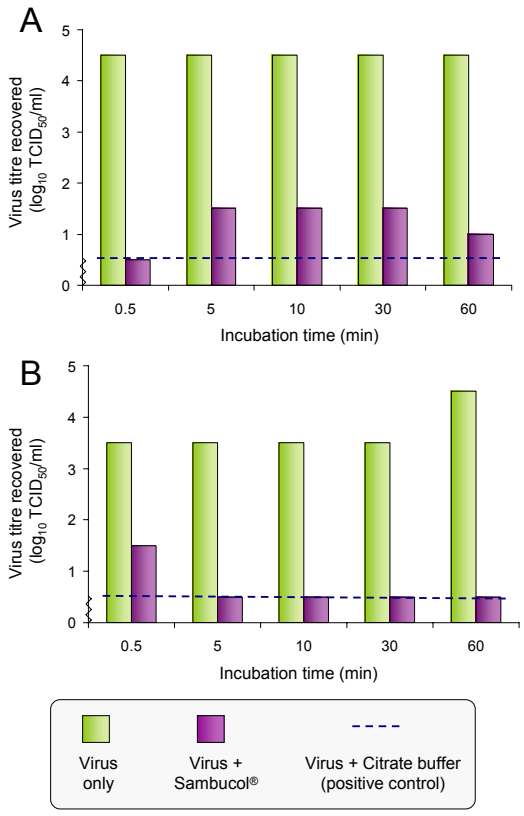

Fig 2. Sambucol® dramatically reduces NIBRG-14 titre.
Avian influenza virus NIBRG-14 was incubated with 1/4 (A) and 1/8 (B) dilutions of Sambucol®, no Sambucol®, or citrate buffer (antiviral control) for different lengths of time before virus was allowed to infect MDCK cells. 3 days later, viral titre was assessed. Graphs display results as $\log_{10}$ $TCID_{50}$/ml. The virus titre recovered from the citrate buffer antiviral control is overlaid. The lower detection limit of this assay is 0.5 -$\log_{10}$ $TCID_{50}$/ml as indicated on y-axis.

Reduction of viral titre
Each Sambucol® concentration was tested against avian influenza NIBRG-14 (H5N1) virus at five different incubation times (Fig 2). Results of the positive control (virus only) show that the titre recovered for untreated virus was, on average, 4.16 $\log_{10}$ $TCID_{50}$/ml. The antiviral control (citrate buffer, pH 3.5) titre recovered was <0.5 $\log_{10}$ $TCID_{50}$/ml. Both 1/4 and 1/8 dilutions of Sambucol effectively reduced the viral titre to a similar level at all incubation times tested. This equates to a reduction in viral titre at both concentrations and all incubation times tested of over 99% (Table 1).

Therapeutic index
The therapeutic index is an indication of the specificity of the toxicity of a substance to the virus, as opposed to the host cells, and it is expressed as a ratio of the reduction in viral titre to the reduction in cell viability. The calculations show that the reduction in viral titre is directly due to the presence of Sambucol®.

Time of incubation (min)	Sambucol 1/4 dilution		Sambucol 1/8 dilution	
	(-$\log_{10}$$TCID_{50}$/ml)	(%)	(-$\log_{10}$$TCID_{50}$/ml)	(%)
0.5	>4.0	>99.90	2.0	99.00
5	3.0	99.90	>3.0	>99.90
10	3.0	99.90	>3.0	>99.90
30	3.0	99.90	>3.0	>99.90
60	3.5	99.97	>4.0	>99.99

Table 1. Sambucol® is over 99% effective at reducing viral titre.
Data from the reduction of viral titre assay were analysed. Sambucol® reduced the viral titre recovered from infected MDCK cells by over 99% at both concentrations and all timepoints tested.

Discussion

This study aimed to determine the antiviral activity of Sambucol® against the avian NIBRG-14 (H5N1) influenza virus. Both 1/4 and 1/8 dilutions were at least 99% effective and reduced the titre of avian influenza NIBRG-14 (H5N1) by at least 2.0 -$\log_{10}$ $TCID_{50}$/ml. A reduction of 1 -$\log_{10}$ $TCID_{50}$/ml has previously been deemed significant (5). Sambucol® was therefore effective at significantly reducing the titre of avian influenza NIBRG-14 (H5N1) virus. Sambucol® was as effective as a citrate buffer positive control at reducing viral titre. The therapeutic index calculated for both dilutions of Sambucol® indicate that the reduction in titre was due to the action of Sambucol® on the avian influenza NIBRG-14 virus.
Sambucol®, which has previously been shown to be effective against human influenza viruses A and B *in vitro* and *in vivo*, has now been demonstrated to have anti-viral properties against avian influenza H5N1 virus. This should be confirmed by further work in animal models and clinical studies.

References

1. http://www.who.int/csr/disease/avian_influenza/en/index.html
2. Flos Sambuci (2002) *WHO Monographs on Selected Medicinal Plants* 2:269-275.
3. Zakay-Rones Z., Varsano N., Zlotnik M., Manor O., Regev L., Schlesinger M. & Mumcuoglu M. (1995) 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 *J Altern Complem Med* 1(4):361-369.
4. Zakay-Rones Z., Thom E., Wollan T., Wadstein J. (2004) Randomized study of the efficacy and safety of oral elderberry extract in the treatment of influenza A and B virus infections. *J Int Med Res* 32(2):132-140.
5. Oxford J.S., Zuckerman M.A., Race E., Dourmashkin R., Broadhurst K. & Sutton P.M. (1994) Sodium deoxycholate exerts a direct destructive effect on HIV and influenza viruses *in vitro* and inhibits retrovirus-induced pathology in an animal model. *J Antimicrob Chemother* 45(5):617-621.