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Virucidal activity of black elderberry extract (Sambucol® Original and Immuno Forte) against attenuated swH1N1 Swine Flu

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Abstract

This *in vitro* study determined the viral efficacy of 38% black elderberry extract presented as Sambucol® Original and Sambucol® Immuno Forte against Influenza NIBRG – 121 (attenuated H1N1) at concentrations that did not exhibit toxicity. Both formulations were tested and found to be non-toxic at 25% (v/v) concentration.

The virucidal activity of both formulations was measured at the 25% (v/v) concentration level over 5, 10, 30 and 60 minutes incubation period. Results showed that both formulations were able to reduce the viral titre of Influenza NIBRG-121 (H1N1) virus by 68.37% over 10, 30 and 60 minute time points when compared to the control virus titre. Additionally Sambucol® Original extract also showed significant anti-viral activity after 5 minutes. These data reinforce the established antiviral activity of black elderberry when presented as Sambucol® Original and Immuno Forte as a 38% extracted formulation.

Introduction

Swine flu is the common name given to a new strain of influenza (flu). The virus was first identified in Mexico in April 2009. It has since become a pandemic, which has spread around the globe. It has spread quickly because it is a new type of flu virus that few, if any, people have full resistance to. Flu pandemics are a natural event that occur from time to time. Last century, there were flu pandemics in 1918, 1957 and 1968, when millions of people died across the world.[1]

In most cases the virus has proved relatively mild. However, around the world hundreds of people have died and it is not yet clear how big a risk the virus is. The UK formally moved from a containment to a treatment phase for swine flu on 2nd July 2009. Intensive efforts to contain swine flu, for example through automatic school closures, ended. This was to free up capacity to treat the people who are contracting swine flu daily. As in other countries, most of the cases reported so far in the UK have been mild. Only a small number have led to serious illness, and these have often been in patients with existing health problems, such as cancer, that already weakened their immune systems. However, the government's Scientific Advisory Group on Emergencies (SAGE) believes that there is still some doubt about the risks of the virus. For example, there are reports of some cases in Argentina where young, healthy adults have become extremely ill from swine flu. While there is still this doubt, the government has decided to offer the antiviral medicines to everyone who is confirmed with swine flu.[1]

The epidemiology of viral respiratory infections is well described.[2] Influenza has been established as a serious human affliction that can cause localized epidemics and global pandemics of acute respiratory infections. Each year the Influenza virus is responsible for 20,000 to 40,000 deaths and up to 300,000 hospitalisation cases in the United States.[3] In the pandemic of 1918 it is widely believed that in excess of 40 million people died.

Although children and younger adults experience more cases of infection, severe illness is more common in the elderly, Immunocompromised individuals, or those with chronic illnesses such as asthma, diabetes, kidney failure and heart disease.[4]

The annual epidemics run from November to March in the Northern Hemisphere, and from April to September in the Southern Hemisphere.[5] The impacts of these viruses have led to the search for alternative compounds with which to minimize their effects within human populations. With the recent news of a **H1N1** pandemic the need to prevent any opportunities of transmission of the virus has risen. Disinfectants, hand sanitizers/gels and antiviral formulations that are able to inactivate the Swine flu virus are of particular importance to control the spread of the virus.

Against this current pandemic backdrop it is important to determine whether natural extracts can offer an alternative prevention or treatment capability, particularly to those people who cannot tolerate pharmaceutical interventions.

Studies have shown that elderberries are unusually rich in the phytochemicals known as flavonoids and among all fruits, elderberries are the most concentrated source of anthocyanins, a class of flavanoids that act as powerful antioxidants to maintain the immune system and protect the body's cells from harm.[6] These anthocyanins are found in the purple pigment of black elderberries. Research has shown that the anthocyanins in black elderberry can boost the production of cytokines,[6] the proteins that act as messengers within the immune system, and thereby serve to enhance the body's immune response. Sambucol® products contain extracts of black elderberry at a 38% patented formulation that provide a rich source of anthocyanins which have been shown to help maintain the body's immune system. Over the centuries, elderberry has been used to treat colds, flu, fever, burns, cuts, and more than 70 other maladies, from toothache to the plague.[8]

Aims and Objectives

The aims and objectives of this study were to determine the virucidal activity of the two formulations of Sambucol®, Sambucol® Original Formula Regular and Sambucol® Immuno Forte against Influenza NIBRG-121 attenuated sw(H1N1) virus. We also wished to establish the toxicity of the test extracts at the test concentration on the MDCK-S cells line.

Materials and Methods

Extracts of the test materials were received in original packaging from the manufacturer and were within their product specification and expiry date. Extracts were stored in conjunction with the manufacturer's recommendations. The test extracts that were used were diluted in cell culture grade water to a concentration of 25% (v/v). As the test article undergoes a 9/10 (v/v) dilution with virus in the virucidal assay, the resulting test concentrations was 22.5%

Reagents and Control Articles

The controls utilised in the virucidal assay were:

Cell only control

The cell only controls comprised of cells not infected with virus. This was a negative control for vCPE (viral cytopathic effect) and was also an indicator of cell quality. MDCK (Madin-Darby canine kidney) cell only controls were taken from an existing facility and stored at 37°C (±2°C), 5% CO, under normal safety precautions for a category II material. It was confirmed that no wells exhibited HA activity or vCPE.

Negative control

Cells were incubated with Influenza infection media that had undergone similar dilution steps with virus as the test extracts (i.e 360 microL infection media was added to 40 microL virus and terminated in 3.6 mL infection media). Influenza Infection Media was in liquid medium, red in colour and stored at 4°C under normal safety precautions for a category II material.

Positive control

Cells were infected with citrate buffer solution, 0.09 M [Sigma; C2488] adjusted to pH 3.5 with HCL, that had undergone similar dilution steps with virus as the test article (i.e 360 microL antiviral control was added to 40 microL virus and terminated in 3.6mL infection media. This clear liquid was also stored at 4°C.

Virus

The virus was taken from the current facility and contained in allantoic fluid with the usual safety precautions for a category II+ material. Work was conducted in a class 2 biosafety cabinet. NIBRG-121 is a reassortant virus that contains the neuraminidase and haemagglutinin surface glycoproteins of A/California/7/2009 (H1N1), which was isolated from humans. The virus was supplied by the National Institute for Biological Standards and Control (NIBSC) and propagated in allantoic fluid in an environment where full trace-ability of the virus has been maintained.

The cell line

The MDCK cell line was used for the Influenza virucidal and cytotoxicity assays. The cell line was obtained from the existing cell bank and maintained in accordance with the current standard operating procedure. Cell line was stored at 37°C (±2°C), 5% CO_2.

The cells were seeded at a' density of approximately 5 x 104 cells/ml and incubated at 37°C (±2°C), 5% CO_2, for 24 hours until 70-80% confluent. Prior to use in either the virucidal or cytotoxicity assay the MDCK growth media was removed and the cells washed twice with PBS (Gibco 10010) and replenished with Influenza infection media.

Influenza Infection Media

Constituent	Volume	Product Code
DMEM	500mL	Invitrogen 31885049
HEPES	5mL	Sigma H0887
Pen Strep	5mL	Sigma P4458
TrYPsin-treated TPCK	2.2mL(5625 Units)	Thermo Scientific Pierce 20223

Experimental methods

Cytotoxicity assay

A cellular toxicity assay was conducted prior to the main experiment in a similar manner to the virucidal activity assay. The experimental design of the cytotoxicity assay is summarized below.

a) 40 microL of the Influenza Infection media was added to 360 microL of the test article.

b) After 60 minutes (the longest timepoint being measured) had completed 3.6mL of the virus specific infection media was added to terminate the reaction.

c) 111 microL of the terminated reaction mixture was inoculated onto the first row of wells and serially diluted 10 fold (11 microL titrated from the first well to subsequent wells containing 100 microL of the virus specific infection media). The inoculated cells will be incubated for 1 hour (± 5 minutes) at 37°C (±2°C) with 5% CO2.

d) The cell line was washed twice with PBS (100 microL/well) and fresh infection media added at 100 microL/well.

e) The cells were incubated at 37°C (±2°C) with 5% CO_2 for four hours.

After incubation, visual assessment of the cell monolayer was conducted to determine any cytotoxic effects of the test compounds on the cell line. The tCPE (toxicity associated cytopathic effects) were recorded in the laboratory notebook.

Virucidal Assay

Virus (40 microL) was added to the prepared test article (360 microL) and incubated for the assay. The assay was performed as above with virus being added to the reaction mixture instead of infection media.

a) 40 microL of NIBRG-121 sw(H1N1) was added to 360 microL of the test article.

b) The timepoints measured were;

- 5 minutes
- 10 minutes
- 30 minutes
- 60 minutes

c) After each timepoint was completed 3.6mLs of the virus specific infection media was added to terminate the reaction.

d) 111 microL of the terminated reaction mixture was inoculated onto the first row of wells and serially diluted 10 fold (11 microL titrated from the first well to subsequent wells containing 100 microL of the virus specific infection media).

e) The inoculated cells were incubated for 3-days at 37°C (±2°C) with 5% CO_2.

After 3 days the plates were CPE scored and an HA performed. 50 microL of the media in each well was removed onto a 96 well V bottom plate. 50% of turkey red blood cells (TRBC's) were then added to each well. The plates were then left at room temperature for 30 minutes (±2 minutes) and then read accordingly.

Analysis of Data

Virucidal and Cytotoxicity Assays

The viral endpoint was determined by Haemagglutination assay. The qualitative HA observations were used to quantify the amount of virus present in the reaction mixture using the Karber Calculation to measure $TCID_{50}$. Similarly cell monolayers were observed for toxicity associated cytopathic effects. From this a Tissue Culture Lethal Dose 50 ($TCLD_{50}$) was calculated using the Karber Calculation, by treating a well that is positive for toxicity in a similar manner as wells that are positive for virus.

Calculation of Virus Reduction

The reduction in virus titre for the test compounds was calculated by subtracting the virus obtained with the test compound from the virus titre obtained with the virus control on the same plates as the test compound. The log reduction in viral titre was calculated by the following formula;

$TCID_{50}$ (virus only control) – $TCID_{50}$ or $TCLD_{50}{}^{A*}$ (Test Compound)

The log reduction was converted to a percentage virus reduction using the following formula; 100% -10 $^{(2-(T1-T2))}$

Where;

Tl = $TCID_{50}$ (virus only control)

T2 = $TCID_{50}$ or $TCLD_{50}$ ^{A*} (Test Compound)

Results are reported as both log reduction and percentage virus reduction.

Results

Cytotoxicity Assay

The cytotoxicity assay was used to determine if the test substances had any toxic effects on the MDCK cell line. The cells were observed for tCPE to determine if there were any cytopathic effects caused by the test substances. The Tissue Culture Lethal Dose 50 ($TCLD_{50}$) was determined using the Karber Calculation where the $TCLD_{50}$ is the quantity of test substance that will produce a cytotoxic effect in 50% of the cultures inoculated.

The $TCLD_{50}$ for the test articles are shown in Table 1.

A* TCLD50 was used to calculate virus reduction where the observed cytotoxicity was greater than the virus titre (i.e. if $TCLD_{50}$>$TCID_{50}$)

Table 1: *Calculated Tissue Culture Lethal Dose 50 for test articles.*

Treatment	**Log_{10} $TCLD_{50}$/mL**
Sambucol®Immuno Forte	**None observed**
Sambucol® Original Formula Regular	**None Observed**

Virucidal Assay

The Tissue Culture Infective Dose 50 ($TCID_{50}$) was determined using the Karber

Calculation and the TCID50 is the quantity of virus that will produce a cytopathic effect in 50 per cent of the cultures inoculated. The results of the virucidal assay are indicated in Tables 2 and 3.

Table 2: *Influenza NIBRG-12I (HINI) virus recovery, calculated log and percentage reduction following treatment with Sambucol Original Formula Regular*

Test Article	Time Point (minutes)	Virus titre recovered (log_{10} $TCID_{50}$/mL		Reduction in virus titre	
		Virus Control	Test substance	(-log_{10} $TCID_{50}$/mL	%
Sambucol® Original Formula Regular	5	3.00	2.50	0.50	**68.37**
Sambucol® Original Formula Regular	10	3.00	2.50	0.50	**68.37**
Sambucol® Original Formula Regular	30	3.00	2.50	0.50	**68.37**
Sambucol® Original Formula Regular	60	3.00	2.50	0.50	**68.37**
Citrate Buffer	5	3.00	1.50	≥1.50	**≥96.84**

Table 3: *Influenza NIBRG-121 (H1N1) virus recovery, calculated log and percentage reduction following treatment with Sambucol® Immuno Forte*

Test Article	Time Point (minutes)	Virus titre recovered (log_{10} $TCID_{50}$/mL		Reduction in virus titre	
		Virus Control	Test substance	(-log_{10} $TCID_{50}$/mL	%
Sambucol® Immuno Forte	5	3.00	3.00	0.00	**00.00**
Sambucol® Immuno Forte	10	3.00	2.50	0.50	**68.37**
Sambucol® Immuno Forte	30	3.00	2.50	0.50	**68.37**
Sambucol® Immuno Forte	60	3.00	2.50	0.50	**68.37**
Citrate Buffer	5	3.00	1.50	≥1.50	**≥96.84**

Discussion

The Sambucol® Original Formula Regular at a concentration of 25% (v/v) reduced the viral titre of Influenza NIBRG-121 (H1Nl) virus by 0.5 – log_{10} $TCID_{50}$/mL (68.37%) over all time points when compared to the control virus titre . The Sambucol® Immuno Forte at a concentration of 25% (v/v) reduced the viral titre of Influenza NIBRG-121 (H1Nl) virus

by 0.5 - 10g_{10} $TCID_{50}$/mL (68.37%) over all time points except the 5 minutes point, when compared to the control virus titre. The data above suggests significant virucidal efficacy of both Sambucol® Original Formula Regular and Sambucol® Immuno Forte against Influenza NIBRG-121 (H1Nl). These results confirm previous work carried out using the same standardized elderberry extract, which also reduced hemagglutination and inhibited replication of human influenza viruses type A[4]. A possible mechanism of action appears to be the ability of this 38% black elderberry extract to produce four inflammatory cytokines (interleukin-1 beta, tumor necrosis factor alpha, and IL-6 and IL-8) and one anti-inflammatory cytokine (IL-10) and this was tested using blood-derived monocytes from 12 healthy donors.[5] This potential could therefore have immunostimulatory properties when administered to patients suffering from influenza. It is clear that this simple black elderberry extract has some interesting virucidal properties that warrant further investigation and that it could certainly be utilized as an alternative in preventing and perhaps treating active viral infections including the currently well described "swine flu".

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