

ORIGINAL ARTICLE

Sea buckthorn bud extract displays activity against cell-cultured Influenza virus

A. TORELLI¹, E. GIANCACCCHI¹, S. PICCIRELLA¹, A. MANENTI¹, G. PICCINI¹, E. LLORENTE PASTOR¹, B. CANOVI², E. MONTOMOLI^{1,3}

¹ VisMederi Srl, Siena, Italy; ² Pool Pharma - MI -Scientific Director, San Giuliano Milanese, Italy; ³ University of Siena, Italy

Key words

Sea buckthorn plant extract • Influenza prevention • Antivirals

Summary

Introduction. Vaccines and antiviral drugs are the most widely used methods of preventing or treating Influenza virus infection. The role of sea buckthorn (SBT) bud dry extract as a natural antiviral drug against Influenza was investigated.

Methods. Influenza virus was cultured in the MDCK cell line, with or without SBT bud extract, and virus growth was assessed by HA and TCID₅₀ virus titration in terms of cytopathic effect on cells. Several concentrations of extract were tested, the virus titer being measured on day 4 after infection.

Results. After infection, the virus titer in the control sample was calculated to be 2.5 TCID₅₀/ml; treatment with SBT bud extract reduced the virus titer to 2.0 TCID₅₀/ml at 50 µg/ml, while the HA titer was reduced from 1431 (control) to 178. Concentrations

lower than 50 µg/ml displayed an inhibitory effect in the HA assay, but not in the TCID₅₀ virus titration; however, observation of the viral cultures confirmed a slowdown of viral growth at all concentrations.

Discussion. Natural dietary supplements and phytotherapy are a growing market and offer new opportunities for the treatment of several diseases and disorders. These preliminary experiments are the first to show that SBT bud extract is able to reduce the growth of the Influenza A H1N1 virus in vitro at a concentration of 50 µg/ml. This discovery opens up the possibility of using SBT bud extract as a valid weapon against Influenza and, in addition, as the starting-point for the discovery of new drugs.

Introduction

Influenza is caused by RNA viruses belonging to the family of *Orthomyxoviridae*. Three types of influenza viruses can be distinguished. Among these, influenza A (IAV) viruses mainly infect birds and mammals, whereas influenza B viruses almost only infect humans. Influenza A virus displays constant evolutionary changes, defined as “antigenic drift” [1] and “antigenic shift” [2], enabling new mutant strains to emerge and spread. Influenza viruses constitute a public health problem, as they can give rise to both epidemics and pandemics, causing high rates of morbidity and mortality [1]. Influenza viruses are considered the leading cause of respiratory illness in humans and are responsible for annual seasonal outbreaks that have a serious economic impact [3]. The average global burden of influenza may be in the order of 1 billion cases of flu, 3-5 million cases of severe illness that need hospitalization and 250,000-500,000 deaths yearly [4]. The most effective means of fighting influenza is primary prevention through vaccination [5], which can prevent the disease after exposure or reduce the severity of symptoms. Antiviral drugs can also be used [6]. Indeed, in subjects who are not completely protected by vaccination strategies, an important role has been played by antiviral treatments [7].

Currently, two main classes of antiviral drugs are available; these are based on the ability to block viral M2

ion channels [8] and viral neuraminidase [9]. However, both classes of drugs have been associated with limited efficacy, adverse side effects [1] and the spread of drug resistance in circulating influenza viruses [10, 11]; this last problem has been associated with an uncontrolled administration of the drugs to humans and, sometimes, to farm animals [12]. Other classes of drugs can be used against influenza virus, some of these include: inhibitors of hemagglutinin, endosomes, lysosomes, proteases, polymerases, nucleoprotein (NP), nonstructural-1 (NS1) glycoprotein, RNA synthesis and caspase; however their efficacy in blocking Influenza virus and avoiding the development of antiviral resistances should be further investigated [13]. It is therefore necessary to develop new anti-influenza treatments that are able to inhibit the replication or cellular processes of viruses [1, 7].

Some studies have indicated that certain plant extracts are able to mimic the anti-neuraminidase effect of antiviral drugs. One of these plants is sea buckthorn (SBT) (*Hippophae rhamnoides*): commonly known as sea buckthorn, it belongs to the family Elaeagnaceae [14] and it is a shrubby to bushy plant that grows also at a high altitude and is widely distributed in Eurasia. In India this plant grows predominantly in several high-altitude areas such as Sikkim, Himachal Pradesh, Kashmir, Jammu, and Uttar Pradesh but *Hippophae* is cultivated worldwide for its medicinal properties [15].

All parts of SBT contain large amounts of several active compounds [16] and these include: vitamins (folic acid, vitamin C, vitamin A, vitamin E, vitamin K, riboflavin), carotenoids (lycopene α , β , δ -carotene), phytosterols (amyrins, ergosterol, stigmasterol, lansterol), organic acids (malic and oxalic acids), polyunsaturated fatty acids and essential amino acids [17].

Several medicinal and therapeutic applications utilize the extracts obtained from the leaves of this plant, which display immunomodulatory [18] and anti-inflammatory [19] properties, as demonstrated by Padwad and colleagues [16]. Furthermore, SBT leaves also exert antiviral, anti-bacterial and anti-tumor effects [20, 21]. Preliminary results have also shown that SBT seed extracts have anti-bacterial activity (for example against *Listeria monocytogenes* and *Yersinia enterocolitica*) [22, 23] and anti-viral activities, as reported by the group of Jain against Dengue virus [24].

SBT bud extracts are present in commercial dietary supplements, such as preparations of nutrient and vitamin products [25, 26], and are formulated into Influpirin[®] (PoolPharma). However, no data are currently available on the putative antiviral activity of bud extracts when taken in the form of dietary supplements.

The aim of the present study was to evaluate the therapeutic anti-viral potential of SBT bud extracts on Influenza A/H1N1 virus infection in Madin Darby Canine Kidney cells (MDCK). In 2009, this viral strain was responsible for an Influenza pandemic that spread rapidly around the world [27]. Moreover, it currently circulates in the population, causing seasonal outbreaks, and its antigens are included in the available seasonal influenza vaccines [28].

Methods

CELLS AND CELL CULTURES

Madin Darby Canine Kidney (MDCK) cells were purchased from Sigma-Aldrich (ECACC, Public Health England, Porton Down, United Kingdom) and cultured in Minimum Essential Medium Eagle (EMEM medium) supplemented with 2mM Glutamine, 10% Fetal Bovine Serum (FBS) EU Approved (Euroclone, Pero, Italy) and 100UI/ml of penicillin-streptomycin. Sub-confluent cultures (70%-80%) were grown at 37°C in a humidified atmosphere containing 5% CO₂ and subcultures were performed every 3-4 days [29]. Except where indicated otherwise, all the above reagents were from Lonza (Verriers, Belgium).

SBT BUD DRY EXTRACT

SBT bud dry extract from PoolPharma S.r.l. (San Giuliano Milanese, Italy) was weighed on a precision scale and dissolved in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) at a final concentration of 1 mg/ml; the pH of the solution was measured (6.96). The dissolved extract was then sterile-filtered through a 0.22 μ m filter.

INFLUENZA A/CALIFORNIA/7/2009 (H1N1) VIRUS

Influenza A/California/7/2009 (H1N1) virus was obtained from the National Institute for Biological Standards and Control (NIBSC) (Potters Bar, Hertfordshire, United Kingdom) and used according to the instructions provided by the supplier. The virus was propagated in MDCK cells [30], harvested and stored at -80°C. The propagated virus had a tissue culture infectious dose (TCID₅₀) titer of 10^{3.5}.

VIRAL GROWTH

Influenza A/California/7/2009 (H1N1) virus was propagated in MDCK cells cultured in UltraMDCK serum-free-medium (SFM) supplemented with 0.5 μ g/ml of trypsin from bovine pancreas (TPCK) (Sigma-Aldrich, Saint Louis, MO, USA) and 100 UI/ml of penicillin-streptomycin. MDCK cells were seeded in a T25 cm² tissue culture flask at a density of 1 x 10⁶ cells/ml. After 24 hours (h), the cell medium was discarded and the cells were washed twice with sterile DPBS. After the DPBS had been discarded from the flask, the cells were treated with 500 μ l of virus inoculum (5 ml of solution contained 50 μ l of virus at 10^{3.5} TCID₅₀/ml (1:100 dilution) and 2.5 μ l of TPCK, and incubated for 1 h at 37°C in 5% CO₂. After 1 h, the inoculum was removed, the cells were washed with DPBS, and fresh UltraMDCK SFM, supplemented as previously described, was added. The cells were incubated at 37°C in 5% CO₂ and the cytopathic effect was monitored every day until post-infection day 4. The culture medium was collected and analyzed for TCID₅₀ and hemagglutination titer on the 4th day after infection.

EFFECT OF SBT BUD DRY EXTRACT ON MDCK CELLS

MDCK cells were seeded at a density of 6.5 x 10⁵ cells/ml in 6-well plates in complete EMEM medium and were incubated for 24 h at 37°C in 5% CO₂. SBT at different concentrations (1 μ g/ml, 5 μ g/ml, 10 μ g/ml, 30 μ g/ml, 50 μ g/ml, 75 μ g/ml and 100 μ g/ml) was then added to the medium in the wells. The cells were checked at 24 h, 48 h and 72 h by means of a light optical microscope to evaluate whether the extract had a cytotoxic effect on them. The experiment was repeated to confirm the preliminary results.

EFFECT OF SBT ON VIRAL GROWTH

The viral growth procedure was repeated in the conditions reported above. After infection, the inoculum was removed and the medium was replaced with fresh UltraMDCK SFM (supplemented with 100 UI/ml of penicillin-streptomycin and 0.5 g/ml of TPCK) containing different concentrations of SBT: 2.5 μ g/ml, 5 μ g/ml, 7.5 μ g/ml, 10 μ g/ml, 30 μ g/ml, 50 μ g/ml, 75 μ g/ml and 100 μ g/ml. The infection grade was observed daily for cytopathic effect, and the culture medium was harvested on day 4 to be analyzed for virus content in terms of TCID₅₀ and hemagglutination titer. In parallel, two control flasks were run: the first flask represented the cell control and

the culture medium was added with DPBS; the second flask represented the virus growth control and it was treated only with the live virus.

VIRUS TITRATION BY HEMAGGLUTINATION TEST

The ability of the influenza virus to agglutinate red blood cells from certain mammalian or avian species can be exploited to check for the presence and hemagglutinating activity of the virus in biological substrates (e.g. serum samples) [31, 32]. To evaluate the hemagglutinating capability of the virus in previously infected cell cultures, the hemagglutination test was used: 100 µl of culture medium from the flask of interest was transferred to the 1st well of 12 of a 96-well V bottom plate then 50 µl of saline solution (0.9% NaCl) (Sigma-Aldrich, Saint Louis, MO, USA) was added from well 2 up to well 12; 2-fold serial dilutions of the culture medium (contained in the 1st well) in the saline solution (wells 2-12) were performed from the 1st well up to the 12th well.

Then, 50 µl of turkey red blood cell (RBC) suspension (0.5% in saline solution) (Emozoo Snc, Casole d'Elsa, Italy) was added to each well and the plate was incubated for 45 minutes at room temperature (RT). After incubation, the plate was tilted to allow non-hemagglutinated RBCs to drip from the bottom of the wells and the result was read; the reciprocal of the highest dilution of the culture medium that was still able to cause agglutination indicated the titer of the virus in the culture medium. The experiment was repeated, this time with a 1:10 starting dilution of the culture media, and the virus titer was calculated in terms of hemagglutinating units (HAU) in 1 ml by applying the following formula: $HAU \text{ in } 1 \text{ ml} = 20 \times 10^{[\text{LOG}(\text{Dilution}_1) + \text{LOG}(\text{Dilution}_2)]/2}$.

VIRUS TITRATION BY TCID₅₀

The "TCID₅₀" titer is the viral dose that gives rise to a cytopathic effect in 50% of cells in the inoculated culture. The virus titer was determined by means of the TCID₅₀ assay, using the Spearman/Karber method [33] on treated MDCK cells, as reported by Lugovtsev [34]. Twelve plastic tubes (1.5 ml) were prepared and loaded with 900 µl of cell medium (EMEM), except for the 11th tube; 100 µl of supernatants from cultures was then transferred into the first tube and serial 10-fold dilutions were performed from tube 1 to up to tube 10. The contents of the tubes were transferred into a 96-well cell culture plate: the content of the 1st tube was transferred into the 1st column of the plate, the content of the 2nd tube was transferred into the 2nd column, and so on up to the 10th tube. The 11th column was left empty. At the end of the dilution steps, 100 µl of cell suspension (in complete EMEM medium, with 0.5% FBS and 0.5 µg/ml of TPCK, at a cell density of 5×10^5 cells/ml) was added to each well. The 12th column, containing only cell medium and cell suspension, was used as a cell control. The plates were incubated for 5 days and the TCID₅₀ titer was evaluated by checking the cytopathic effect in the cell mono-layer by means of a light microscope. The results were calculated by applying the Spearman/Karber formula [33]: $TCID_{50}/100\mu\text{l} = X_0 - d/2 + d(\sum X_i/n)$,

where X_0 is the positive logarithm of the highest dilution at which all wells are positive for cytopathic effect, d represents the dose distance in log, n is the number of repeats per dilution and $\sum X_i$ is the sum of all positive wells, starting from X_0 . In the case of 10-fold dilution, $d = \log_{10} 10 = 1$ and the formula can be simplified to $TCID_{50}/100\mu\text{l} = X_0 - 1/2 + (\sum X_i/n)$. To express the results as TCID₅₀/ml, one log was added.

Results

EVALUATION OF THE CYTOTOXIC EFFECT OF SBT ON MDCK CELLS

The possible cytotoxic effect of SBT on MDCK cells previously infected with Influenza A H1N1 virus was evaluated by means of direct observation of cells under a light optical microscope. SBT treatment of MDCK cells showed no cytotoxic effect up to a concentration of 50 µg/ml. At 75 µg/ml and 100 µg/ml, SBT had a cytotoxic effect on the cells: at 75 µg/ml, the cell monolayer had a discontinuous appearance and floating cells were present in the culture medium; this effect was more evident at a concentration of 100 µg/ml (Tab. I).

Tab. I. Evaluation of the cytotoxic effect of SBT on MDCK Cells. This table shows the effect of different concentrations of SBT on MDCK cell cultures after 72 h of incubation. The concentrations of SBT tested were: 1 µg/ml, 5 µg/ml, 10 µg/ml, 30 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml. No toxic effect was observed up to a concentration of 75 µg/ml. At 75 µg/ml and 100 µg/ml of SBT, the cells in the wells showed signs of toxicity: discontinuous cell layer and floating cells in the medium.

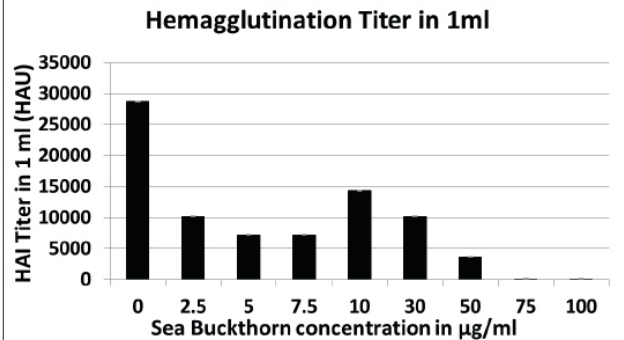
SBT concentration	Effect
Cell control	No effect
1 µg/ml	No effect
5 µg/ml	No effect
10 µg/ml	No effect
30 µg/ml	No effect
50 µg/ml	No effect
75 µg/ml	Discontinuous cell layer, floating cells
100 µg/ml	Discontinuous cell layer, floating cells

SBT EFFECT ON VIRAL GROWTH RESULTS

Hemagglutination titer results

The ability of SBT bud extract to reduce Influenza H1N1 viral growth was evaluated on post-infection day 4. MDCK cell culture supernatants were harvested from the culture plates and assayed for hemagglutination titer. Concentrations of SBT ranging from 2.5 µg/ml to 50 µg/ml markedly reduced the hemagglutination titer from 28621.6 HAU/ml (the value obtained from the viral growth control sample) (Fig. 1). The two highest concentrations of SBT used (75 µg/ml and 100 µg/ml) completely inhibited viral growth. However, they dis-

Fig. 1. Hemagglutination Titration results on day 4 after SBT treatment. Supernatants of viral cultures treated with SBT were analyzed by means of hemagglutination assay to determine whether the treatment was able to reduce the spread of Influenza virus. The values of the hemagglutination titer in 1 ml (volume assayed) are shown on the y axis; the x axis reports the concentrations of SBT (2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, 30 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml) used to treat viral cultures. No SBT was added to the virus control sample. The control sample displayed a titer of 28636; treatment with SBT dramatically decreased the hemagglutination titer. The most effective concentration of SBT was 50 µg/ml. At 75µg/ml and 100µg/ml, SBT totally inhibited viral growth, but in these cases the results could be affected by SBT toxicity. These results represent a mean of two experiments ($n = 2$).



played a toxic effect on the cells; the results obtained at these high concentrations may therefore be affected cell toxicity, as was observed after staining the cell cultures with Trypan blue.

TCID50 virus titration results

Cell culture supernatants were harvested on day 4 after infection and treatment and assayed for TCID50 virus titer to assess the number of infectious (live) viral particles released from the infected cells in the culture medium. The cytopathic effect was evaluated on day 5. The treatment of viral cultures with SBT at a concentration of 50 µg/ml markedly reduced the viral titer in terms of TCID50 (Fig. 2). At 75 µg/ml and 100 µg/ml, SBT was able to inhibit viral growth completely; however, this value could be affected by the toxicity that SBT showed on cells at such high concentrations. Direct observation of the viral cultures confirmed the results obtained from TCID50 and hemagglutination titration. The concentration of SBT that proved to have the greatest impact on viral growth while exerting little negative effect on cells was 50 µg/ml. Figure 3 reports a series of representative pictures showing the effect of SBT at 0, 10, 30, 50, 75 and 100 µg/ml on day 4 on MDCK cells infected with Influenza A/H1N1 virus.

Discussion

In recent years, since the spread of resistant viral strain towards the available pharmacologic treatments and the occurrence of unpredictable pandemics, many investigations have been carried out on new computer-designed molecules or available compounds in a search for new

Fig. 2. TCID50 Virus titration results. The graph shows the results of the TCID50 titration assay performed on supernatants from viral MDCK cultures treated with SBT. The values of the TCID50 titer in 1 ml are shown on the y axis; on the x axis the concentrations of SBT used to treat viral cultures are reported: 0 µg/ml (virus control), 2.5 µg/ml, 5 µg/ml, 7.5 µg/ml, 10 µg/ml, 30 µg/ml, 50 µg/ml, 75 µg/ml and 100 µg/ml. The virus control displayed a titer of 102.5 TCID50; the TCID50 titer was reduced when SBT was used at a concentration of 50 µg/ml. At 75 µg/ml and 100 µg/ml, SBT inhibited viral growth totally. These results represent a mean of two experiments ($n = 2$).

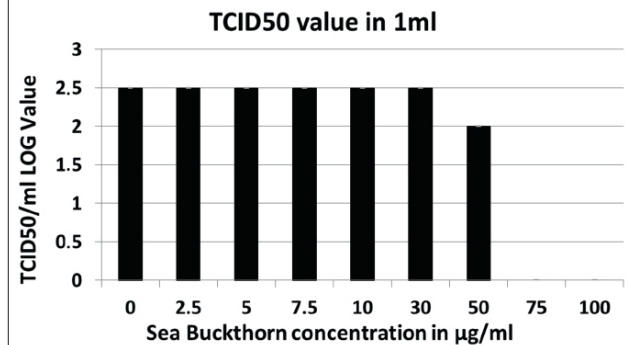
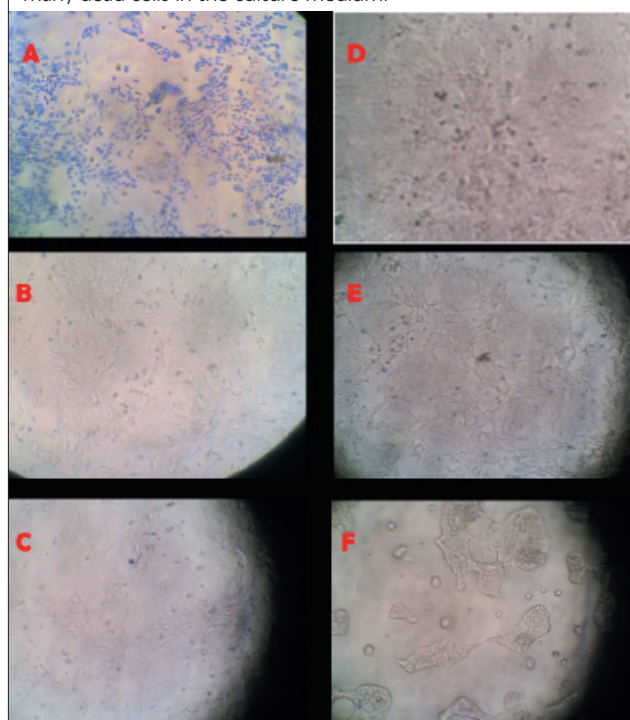


Fig. 3. Cell viability of MDCK cells infected with Influenza H1N1 upon treatment with SBT. The figure shows a series of representative pictures from the light optical microscope (Trypan blue-stained; original magnification 100X) showing MDCK cell cultures infected with Influenza virus H1N1 A/California/7/2009 and treated with different concentrations of SBT: A, virus control; B, 10 µg/ml; C, 30 µg/ml; D, 50 µg/ml; E, 75 µg/ml; F, 100 µg/ml. Cells were stained with Trypan blue to observe dead cells. The virus control displayed a large number of dead cells (blue spots); 10 µg/ml and 30 µg/ml of SBT induced a reduction in the number of dead cells, and the cellular monolayer was more compact. Picture D represents the 50 µg/ml SBT concentration: no cytopathic effect and a limited number of dead cells. The 75 µg/ml and 100 µg/ml SBT concentrations showed a discontinuous cell layer with many dead cells in the culture medium.



anti-influenza drugs. In the present study, the antiviral activity of SBT bud extract (contained in Influpirinviral®) against influenza H1N1 A/California/7/2009 was evaluated *in vitro* on MDCK cells infected with this virus. A previous study conducted by Jain and colleagues [24] demonstrated that SBT leaf extract at a concentration of 50 µg/ml exerted an antiviral activity against Dengue virus. We found that SBT bud extract had an antiviral activity on influenza virus H1N1, especially at 50 µg/ml (the same concentration used by Jain's group). Specifically, after 4 days the viral titer was evaluated in terms of TCID50 titer and hemagglutination titer upon treatment with SBT bud extract; both methods revealed that, at 50 µg/ml, the viral titer was reduced in comparison with the virus control. Direct observation of the virus cultures confirmed the antiviral activity at 50 µg/ml, although an antiviral effect was also visible at lower concentrations, starting from 2.5 µg/ml. When SBT was used at high concentrations (75 µg/ml and 100 µg/ml), viral growth was completely inhibited. However, the treatment had an adverse effect on cell cultures, and this could have affected the results obtained at these concentrations.

Conclusions

The data obtained from this preliminary study confirmed that SBT bud extract has an antiviral activity on influenza H1N1 A/California/7/2009 *in vitro*, supporting its potential use as an anti-influenza agent. Nevertheless, further investigations are needed in order to generate more data, to evaluate the preventive role of SBT treatment against Influenza infection, and to understand the specific mechanism of action of this extract both *in vitro* and *in vivo*.

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■ Conflicts of interests: Dr. Brenno Canovi is the Scientific Director of Pool Pharma, the company that distributes the Inlupiriniviral®, the dietary supplement containing sea buckthorn bud extract.

■ Correspondence: Alessandro Torelli, VisMederi Srl, via Fiorentina 1, 53100 Siena, Italy - Tel. +39 0577 231254 - Fax +39 0577 43444 - E-mail: torelli@vismederi.com