Pinto beans as a functional food for influenza prevention

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Seasonal influenza causes approximately 250,000 – 500,000 deaths every year worldwide with most of the deaths occurring in poor countries. Although influenza drugs and vaccines are available, the virus changes quickly often making them ineffective. These drugs and vaccines are also not affordable for people in poor countries. Therefore, overcoming drug resistance and the discovery of a cheap and accessible treatment option for influenza is an urgent public health need. Pinto beans (*Phaseolus vulgaris* L.) are produced globally and their consumption has been linked to the prevention of multiple diseases including cancer, diabetes, and heart diseases (1,2). These health benefits are associated with the high content of polyphenols and bioactive proteins such as lectins (3,4). Some of the phytochemicals found in pinto beans have been reported to possess antiviral properties. Kaempferol, the main polyphenol in pinto beans has demonstrated antiviral activity against herpes simplex virus, human immunodeficiency virus, cytomegalovirus and influenza virus (5,6). In this study we investigated the antiviral activity of an ethanol extract of pinto beans against human influenza viruses. The extract inhibited the growth of several strains of influenza virus in cell culture including a tamiflu-resistant clinical isolate of the 2009 H1N1 pandemic influenza virus. Further analysis of the effect of co-treatment with tamiflu and extract in cells infected with tamiflu-resistant clinical isolate demonstrated an enhanced antiviral activity of tamiflu. The 50% inhibitory activity of tamiflu decreased from >100 μg/mL in the absence of extract to <0.2% in the presence of 6.25 μg/mL of pinto bean extract, demonstrating the synergistic effect of the tamiflu/extract combination. These results highlight the possibility of using pinto beans in the prevention and management of influenza virus infections.

Methods

Preparation of pinto beans extracts

Pinto beans were pulverized into a fine powder using a blender. One gram of the powder was added to 10 mL of 80% ethanol and shaken at room temperature for overnight. The solvent was then evaporated to obtain a dry material which was reconstituted to 10 mg/mL in ethanol and used for the determination of cytotoxicity and antiviral activity.

Crystal violet assay

Cellular toxicity and antiviral activity were evaluated using crystal violet assay as described (7). Madin Darby Canine Kidney (MDCK) cells were seeded in 96-well tissue culture plates and treated with serial dilutions of extracts in minimum essential medium (MEM). Influenza virus solution of 100 $TCID_{50}$ (50% tissue culture infective dose) was added per well for the antiviral activity assay. Cells were fixed with EtOH and stained with 0.5% crystal violet after 2 days of incubation. The plates were air dried at room temperature and the optical density at 560 nm was measured with the Infinite M200 plate reader (TECAN, Switzerland). The percentage cell viability in wells treated with extract was calculated in reference to the uninfected untreated control. The 50% inhibitory concentration (IC_{50}) was calculated by linear regression analysis using Microsoft Excel software.

Plaque reduction assay

Monolayers of MDCK cells cultured in 6-well plates were washed with serum-free medium and infected with 0.5 mL of virus solution (300 pfu/mL – A/WSN/33 virus) in serum-free MEM for 1 hour at 37 °C in the presence of tamiflu or extracts. Cells were washed with serum-free MEM and overlaid with MEM containing tamiflu or extracts and 0.8% agarose, 0.1% bovine serum albumin (BSA), 1% 100 × MEM vitamin solutions, 0.03% glutamine. After 72 hours of incubation, plaques were visualized by fixing the cells with acetic acid:ethanol (1:1) for 1 hour and staining with 0.5% amido black 10B for 3 hours at room temperature.

Combination of pinto beans extract and tamiflu

Combination treatment with pinto beans extract and tamiflu was performed using checkerboard method as previously described (8), to investigate whether they have additive or synergistic effects on inhibiting the replication of a tamiflu-resistant clinical isolate of the 2009 H1N1 pandemic influenza virus. MDCK cells in 96-well culture plates were infected with 100 $TCID_{50}$ of A/Virginia/ATCC2/2009 virus per well in the presence of two folds serial dilutions of tamiflu (0.2 to 100 µg/mL) mixed with two fold serial dilutions (0.78 – 50 µg/mL) of pinto beans extract. Cells were incubated at 37 °C for 48 h and cell density was determined using the crystal violet assay. The

percentage inhibitory activity of the tamiflu/extract combination was calculated relative to the cell density in untreated controls.

Experimental results and discussion

Cellular toxicity and antiviral activity of pinto beans extract

The pinto beans used in this study were purchased from a local supermarket in Nagasaki, Japan. After pulverization, the powder was extracted in 80% ethanol and used to investigate cytotoxicity and antiviral activity in cell culture. To determine the cytotoxic level of the extract, MDCK cells were treated with various concentrations and incubated for 48 hours before staining by crystal violet assay. The extract did not show cytotoxic effect up to a concentration of 200 µg/mL (Figure 1). To test for antiviral activity, MDCK cells treated with the extract were infected with A/WSN/33 virus, a laboratory strain of influenza virus. As shown in Figure 1, extract concentrations of more than 50 µg/mL inhibited the replication of the virus. Plaque assays using an overlay medium containing drugs is one of the most reliable method for detecting anti-influenza virus activity, we therefore used plaque assay to examine the antiviral activity of the pinto bean extract. In Figure 2, in the absence of any drug many plaques (white circles with black centers) were observed. Tamiflu and the extract (50 and 150 µg/mL) suppressed the formation of the plaques exhibiting the inhibition of virus replication. These results demonstrate that the pinto bean extract inhibits the replication of influenza virus at non-cytotoxic concentrations.

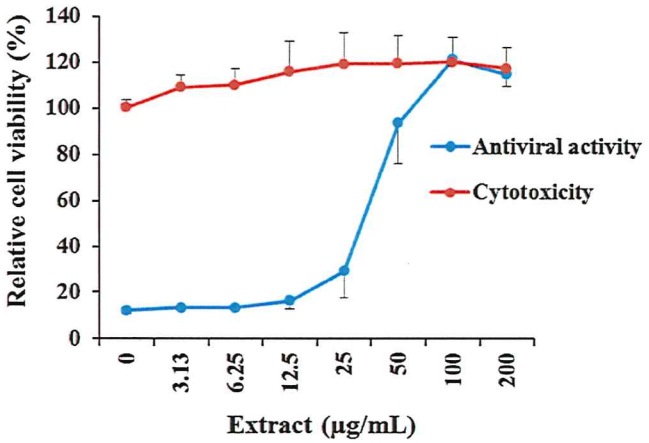

Figure 1: Cytotoxicity and antiviral activity of pinto bean extract

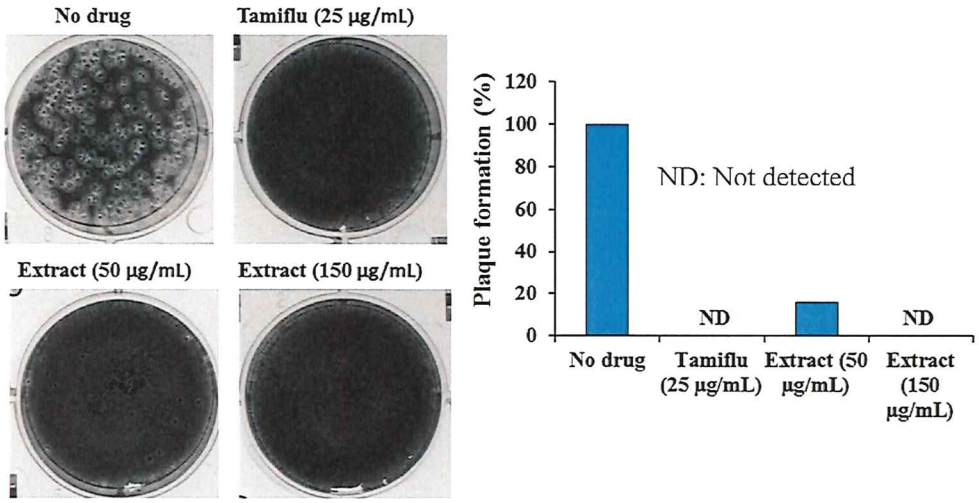

Figure 2: Inhibition of A/WSN/33 plaque formation by pinto bean extract

Sensitivity of several strains of influenza A virus to pinto bean extract

We investigated whether the pinto bean extract would inhibit the replication of various strains of influenza A virus. The extract potently suppressed the replication of all viruses tested with a IC_{50} range of 15.4 – 31.8 µg/mL (Table 1). Kaempferol, a constituent of pinto beans which was previously reported to inhibit influenza virus replication was used as a control. Notably, the pinto bean extract and kaempferol were potent against A/Virginia/ATCC/2/2009 (H1N1), a clinical isolate of the 2009 H1N1 pandemic influenza virus which showed resistance to tamiflu. Tamiflu is the widely used antiviral drug for treatment of influenza virus, thus, these results depict the applicability of pinto beans in the management of tamiflu-resistant influenza virus.

Table 1: Inhibitory activities of pinto bean extract against strains of human influenza A virus

	50% inhibitory concentration (µg/mL)		
	Pinto bean extract	**Kaempferol**	**Tamiflu**
A/WSN/33 (H1N1)	31.8±4.9	30±4.1	3.7±0.2
A/Puerto Rico/8/34 (H1N1)	15.7±8.2	Not determined	7.1±7.5
A/Virginia/ATCC/2/2009 (H1N1) (Clinical isolate of A/H1N1/2009 pandemic)	15.6±7.1	7.5 ±5	>100
A/California/7/2009 (H1N1) (Clinical isolate of A/H1N1/2009 pandemic)	17.3±1.9	0.9±0.4	0.6±0.09
A/Aichi/2/68 (H3N2)	15.4±2.9	Not determined	1.3±0.5

Synergistic effect of tamiflu and pinto bean extract combination against tamiflu-resistant influenza virus

Our results demonstrated that pinto bean extract could potently suppress the replication of tamiflu-resistant clinical isolate of the 2009 H1N1 pandemic. We thus investigated the effect of combining the extract and tamiflu against the tamiflu-resistant virus. MDCK cells in 96-well tissue culture plates were infected with virus in the presence of increasing concentrations of tamiflu mixed with various concentrations of the extract. In figure 3A, the cells infected with

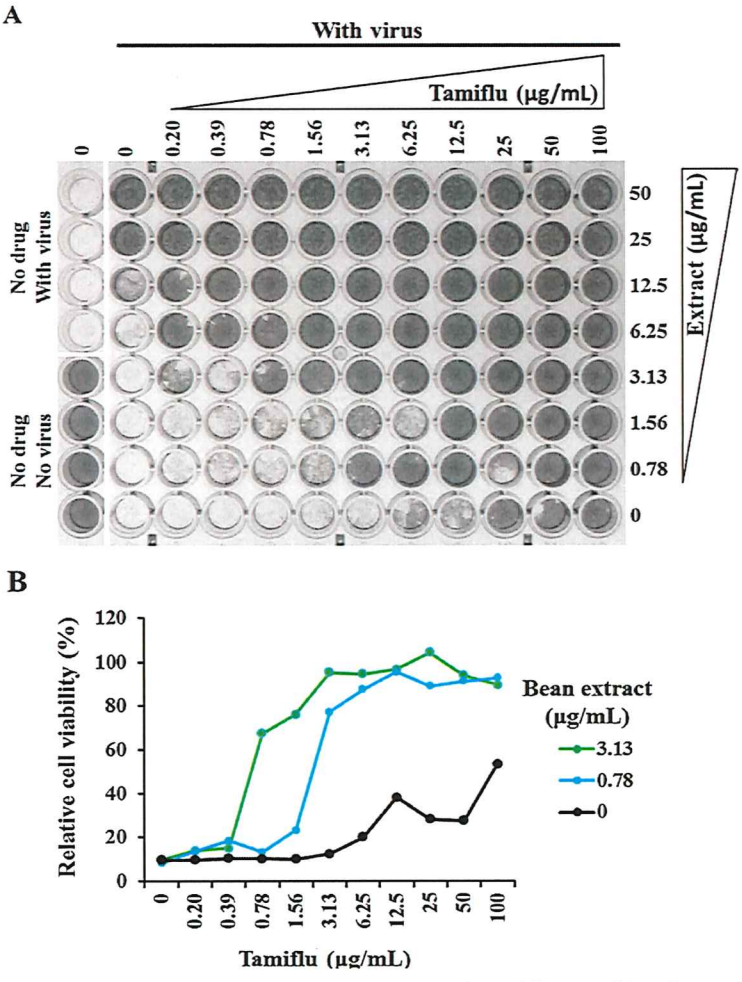

Figure 3: Effect of pinto bean extract and tamiflu combination

virus in the absence of a drug were detached from the bottom of the well hence a clear appearance of the wells after crystal violet staining. On the other hand, the uninfected cells remained attached on the bottom of the wells showing a dark staining appearance. The cells treated with tamiflu only were partially attached at 25- 100 μg/mL but in the presence of 0.78 μg/mL of the extract cells were attached even in wells treated with 3.13 μg/mL of tamiflu. In the presence of 6.25 μg/mL of extract cells remained attached in all wells with tamilfu co-treatment. As demonstrated in Figure 3B, the cell viability was greatly increased in the presence of tamiflu/extract co-treatment (blue and green graphs) when compared to only tamiflu treatment (black graph). This data demonstrate that pinto beans can be used to augment the antiviral activity of tamiflu for the management of influenza virus infections.

Conclusion

Our data demonstrates that pinto beans and kaempferol potently suppresses the replication of influenza virus including a tamiflu-resistant clinical isolate of the 2009 H1N1 pandemic. Interestingly, a combination of the extract and tamiflu greatly enhanced the antiviral potency of tamiflu against the tamiflu-resistant influenza virus. This highlights the applicability of pinto beans in the management of influenza especially in poor countries because they are produced and consumed globally. Since influenza virus changes quickly leading to loss of sensitivity to medicines, the reduced use of the medicines will help fight against drug resistance. Thus, important medicines can be stockpiled for emergency cases and most serious influenza cases. Experiments are ongoing to determine the mechanism of action of the pinto bean extract against influenza virus.

Acknowledgement

This study was funded by a grant from Takano Life Science Research Foundation. We are grateful to the foundation for the generous support. Also, we thank Prof. Noriyuki Nishida and Dr. Ken Watanabe of Nagasaki University for supporting this project.

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