Preliminary In Vitro Study For Using Aqueous Cinnamon Extract Against Foot-and-Mouth Disease Virus

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ABSTRACT: Aqueous cinnamon extract was examined for its effect to inhibit infection by Foot-and-Mouth Disease virus (FMDV) in BHK-21 cells. The cytotoxicity studies revealed that by increasing concentration of the aqueous cinnamon extract, the toxicity increased. At concentration of 137.5ug/ml the toxicity was 100% after 24 hours post inoculation however at concentration of 34.375ug/ml the extract is non-toxic to the cells. Treatment of the virus extracellularly with the non-toxic dilution of the aqueous cinnamon extract before inoculation on the cells help in complete reduction of the virus titer. However, prophylactic effect of the different concentrations of the aqueous cinnamon extract revealed complete protection of the cells at concentration of 137.5 µg/ml while at concentration of 68.75 µg/ml the virus titer was not affected. When infected cells treated with the different concentrations of the aqueous cinnamon extract, concentration of 68.75 µg/ml showed complete treatment of the infected cells while at concentration of 34.375 µg/ml the virus titer was not affected. Aqueous cinnamon extract could be used as an inactivator agent during the production of FMD vaccine.

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INTRODUCTION

Foot-and-Mouth Disease Virus (FMDV) is a member of the *Apthovirus* genus in the *Picornaviridae* family. Foot-and-Mouth Disease (FMD) is a highly contagious viral disease of cloven-hoofed animals that can lead to serve loses. FMDV can be transmitted directly by contact between infected and non-infected or indirectly through people, contaminated fomites or aerosol and can be spread rapidly among susceptible animals. Although vaccines have been extensively be used to control FMD, there was no antiviral therapy available to treat ongoing infections with FMD virus **(Grubman and Baxt, 2004)**.

There are seven known serotypes of FMDV: A, O, C, Asia1 and Southern African Territories (SAT) 1, 2, 3 **(Knowles and Samuel, 2003).** Within these serotypes, over 60 subtypes have also been reported. Because of this diversity there are no universal vaccines thus presenting challenges in the selection of vaccine strains **(Brown, 2003)**.

Formerly, from 1940 onwards, the virus was inactivated with formaldehyde. This inactivation was relatively slow, about 0.2 - 0.3 log 10 per hour. Because formaldehyde not only reacts with the virus produced but with many other components in the medium, such as proteins and amino acids, its concentration can become rate-limiting and inactivation plots may show tailing-off, resulting in residual infectivity **(Bartling and Cassim, 2004).** The most effective FMD vaccines consisted of chemically inactivated FMDV and can only offer complete protection after 7 days of vaccination because of the time needed to trigger an immune response **(Grubman, 2005)**. It has been proposed that a combination of vaccine and antiviral agents can be more efficacious strategy to treat FMD-infected animals, limiting the spread of the disease and reduce the number of animals that need to be slaughtered during outbreaks **(James and Rushton, 2002)**. However, there are currently no approved anti-FMDV drugs for the treatment or prevention of FMD **(Vagnozzi, *et al.* 2007)**. In Egypt, FMDV was first detected in 1950 when strain SAT2 caused an outbreak, then to 1958 when outbreaks were caused by strain A. In the beginning of year 2006, FMD has taken an enzootic form caused by the new exotic strain of FMDV serotype A, where had attached susceptible animals showed more severe forms than others caused by dominant serotype O **(Ali, *et al.* 2006).**

Cinnamon (*Cinnamomum cassia)* of the family *Lauracease* is a favorilte spice around the world because of its health benefits and flavor **(Chaudhry and Tariq, 2006)**. Cinnamon is widely used in traditional medicines and its biological properties have been intensely studied. The most favorite chemical constituents of cinnamon are volatile oil (cinnamaldehyde, eugenol, cinnamic acid, and weitherhin), mucilage, diterpenes and

proanthocyanidins **(Jayaprakasha et al. 2002).** Water-based extracts of cinnamon was found to have anti-ulcer effect in rat models **(Rezq and Ellmallh, 2010).** Also it has anti-bacterial effects in clinical trials against *Helicobacter pylori,* associated with gastric ulcer **(Martin and Ernst, 2003)**. Trans-cinnamaldehyde (CA) extracted from the cinnamon bark can decrease the concentration of foreign DNA in cells and animals infected with the influenza A/PR/8 virus **(Hayashi et al. 2007).**

The aim of this work is to study the inhibitory effect of aqueous cinnamon extract against Food-and-Mouth Disease Virus *in vitro* using Baby Hamster Kidney cells (BHK-21 cells).

MATERIALS AND METHODS

1] Foot-and-Mouth Disease virus (FMDV) :

Foot-and-mouth Disease virus strain (A) was obtained from the virology department of the Animal Health Institute, Dokki, Giza. It has a titre of 5.0 $Log_{10}CCID_{50}$ / ml.

2] Baby hamster kidney cell line (BHK-21):

For detection of the antiviral and cytotoxic effect of extract, we used BHK-21 (Baby Hamster Kidney cells). Cells were cultivated at 37°C with 5% CO2 in Eagle's MEM medium (Gibco) supplemented with 8% fetal calf serum (FCS), 100 IU /ml penicillin, and 100 µg/ml streptomycin. The FMDV (Foot and Mouth Disease virus) was used to induce a cytopathic effect (CPE). Virus dilutions were performed in Eagle's MEM supplemented with 2% FCS.

3] Preparation of the aqueous extract of cinnamon:

Dried cinnamon powder (*Cinnamomum cassia*) was purchased as crude dried material from the market after it was identified microscopy and chromatography according to their monographs in Eur. Ph. Aqueous extract was prepared by adding 100 ml of boiling distilled water to 10 grams of the dried leaves, then incubated for 15 min., subsequently filtered and cooled down. The resulting extract was sterile filtered, aliquoted and stored at -20°C according to **(Nolkemper, *et al.* 2006).** The aqueous extract was freeze-dried to know the weight of the dried active principle found in the used extract.

4] Determination of the cytotoxic concentration of aqueous cinnamon extract on BHK-21 cells:

BHK-21 cells were seeded in 96–well micro-titre plates then incubated over night at 37°C in 5% CO2. Two-fold serial dilutions of the aqueous cinnamon extract in fresh medium containing 2% fetal calf serum was added to the confluent cell monolayer and incubated for 24 h . After over night incubation, cytotoxicity was determined by examining cellular morphology *in situ (***Freshney, 1983)** after cell staining with crystal violet stain (**Doyle, *et al.* 1995).**

5] Extracellular treatment of the FMD virus:

Equal amounts of the 100 $CCID_{50}$ / ml of the FMD virus and 1/20 of the aqueous cinnamon extract were mixed together and incubated at 37°C for 4 hours then ten-fold serial dilutions were prepared and put on a previously seeded monolayer sheets of BHK-21 cells. After 24 hours when the virus control showed CPE, the drop in the virus titer was calculated regarding to the titer of the positive virus control. This method was done according to **(**Dragana **Šmidling, *et al.* 2008).**

6] Intracellular protective effect of the aqueous cinnamon extract against infection with the FMD virus:

Two- fold serial dilutions of the aqueous cinnamon extract were put on a previously seeded BHK-21monolayer cells starting from dilution 1/20. After 90 min, cell cultures were rinsed with PBS and a virus suspension (100 $CCID_{50}$/ml) was added to each well of the treated cell monolayer (100 µl / well). The incubation was done for 24-36 hours 37°C in 5% CO2, when the presence of 100% CPE in the virus infectivity control cultures was confirmed, the cultures were fixed and stained. Back titration was done to evaluate the intracellular protective effect of the aqueous cinnamon extract against FMDV. This method was done according to **(Dragana Šmidling, *et al.* 2008).**

7] Evaluation of the antiviral activity of the aqueous cinnamon extract:

A virus suspension (100 $CCID_{50}$/ml) was pre-incubated with a previously seeded monolayer sheets of BHK-21 cells. After 90 min, the virus was removed and the infected cell cultures were rinsed with PBS. Two-fold serial dilutions of the aqueous cinnamon extract were added to the infected BHK-21 monolayer cells. The incubation was done for 24-36 hours at 37°C in 5% CO2, the presence of 100% CPE in virus infectivity control cultures was confirmed and the cultures fixed and stained. Back titration was done to evaluate the antiviral activity of the aqueous cinnamon extract against FMDV. This method was done according to **(**Dragana **Šmidling, *et al.* 2008).**

RESULTS

Table (1): **Cytotoxicity of the aqueous cinnamon extract:**

Dilution of the cinnamon extract	Concentration of cinnamon extract (µg / ml)	Cytotoxicity %
1/20	137.5	100%
1/40	68.75	50%
1/80	34.375	0% (non-toxic)
1/160	17.188	0% (non-toxic)

These results showed that there was a direct relationship between increasing concentration of the aqueous cinnamon extract and the cytotoxicity of the treated cells. At concentration of 137.5µ g/ml, the cytotoxicity was about 100% and when the concentration was decreased till reaching 34.275 µg/ml there was no cytotoxicity found in the treated cells.

Table (2): **Extracellular treatment of the FMD virus:**

Dilution of the cinnamon extract	Concentration of cinnamon extract (µg / ml)	Treated virus titer ($Log_{10}CCID_{50}$/ml)	Positive control virus titer ($Log_{10}CCID_{50}$/ml)
1/40	68.75	0	5.0

These results indicated that the complete reduction in the virus titer was achieved by incubating the virus with the aqueous cinnamon extract at concentration of 68.75 µg/ml for 4 hours at 37°C compared with the non-treated virus (positive virus control).

Table (3): **Intracellular protection of the BHK-21 cells before infection with the FMD virus :**

Dilution of the cinnamon extract	Concentration of cinnamon extract (µg / ml)	The virus titer ($Log_{10}CCID_{50}$/ml)	The positive control virus titer ($Log_{10}CCID_{50}$/ml)
1/20	137.5	0	5.0
1/40	68.75	5.0	
1/80	34.375	5.0	
1/160	17.188	5.0	

The results showed that at concentration of 137.5 µg/ml of the aqueous cinnamon extract there was complete protection of the cells from being infected with the virus. The intracellular protecting effect of the treated cells with the aqueous cinnamon extract was affected by decreasing the concentration of the active principles where the virus is not affected by the extract.

Table (4): **Antiviral evaluation of the BHK-21 cells infected with the FMD virus :**

Dilution of the cinnamon extract	Concentration of cinnamon extract (µg / ml)	The virus titer ($Log_{10}CCID_{50}$/ml)	The positive control virus titer ($Log_{10}CCID_{50}$/ml)
1/20	137.5	0	5.0
1/40	68.75	0	
1/80	34.375	5.0	
1/160	17.188	5.0	

The results revealed that the aqueous cinnamon extract at concentration of 68.75 µg/ml had antiviral effect on the infected cells (previously infected with the virus) and showed complete inhibition of the virus replication while by decreasing the concentration of the active principles in the extract, the virus was not affected by the extract.

DISCUSSION

There are currently no FDA-approved drugs for the treatment or prevention of FMDV. Hence, control of the disease relies on slaughter of the exposed animals and vaccination with chemically inactivated FMD vaccines. However, these vaccines typically provide protection against one or few of the 60 different FMDV serotypes. Moreover, they are unable to induce protection prior to 7 days post vaccination. Following the acute phase of FMDV infection in ruminants, some animals may experience a long asymptomatic persistent infection **(Eble, *et al.*2006).** In addition, animals which have been successfully vaccinated may also become persistently infected if exposed to infectious virus. These animals are referred to as carrier animals and the carrier state is a complication which can occur during outbreak situations **(Grubman and Baxt 2004).** There had been several attempts to develop antiviral drug therapy that affect specific viral protein targets. In this study we tried to find a natural antiviral herbs that could be used simply in the field to reduce the carrier state of the animals and to reduce the number of infected animals during outbreaks .

According to (**Vanden Berghe, *et al.* 1986),** when screening plant extract for antiviral activity *in vitro* one is looking for non-specific action of antiviral agents on infected cells. Regarding the results found in table (1) it was showed clearly that the cytotoxicity of the cells was directly proportional to the concentration of the aqueous cinnamon extract. Increased concentration of the extract was accompanied by changes in cell morphology, at concentration 68.75 µg/ml cells became round and nuclei were more prominent and the cells were found to float in the medium. At 137.5 µg/ml concentration, cells were found to be similar to apoptotic cells and these results were agree with **(Dragana Šmidling, *et al.* 2008).** However, at concentration 34.375 µg/ml the cells appear normal and there was no cytotoxicity in the cells. This observation deserves further study as apoptosis-induced aqueous cinnamon extract are potentially useful in chemoprevention.

Concerning the results in table (2) it was noticed that the aqueous cinnamon extract at concentration of 34.375 µg/ml revealed 100 % reduction in the virus titre after treatment of the virus with the extract externally before infection of the cells compared to the non-treated virus and this result is agreed with that results obtained by **(Yutaka, *et al.* 2008)** who studied the antiviral activity of the cinnzeylanine (the main active principle of the aqueous cinnamon extract) against HSV-1 in Vero cells.

By using the aqueous cinnamon extract with different concentrations either before infection of the virus or after infection of the cell line with the virus, the results investigated that concentration of 68.75 µg /ml has not prophylactic effect for the cells before infection with the virus but when this concentration was used after adsorption of the virus to the cells it exhibited a 100% reduction in the virus titer meaning that this concentration had an antiviral effect against infection of FMDV. These results were agreed with that investigated by **(Yutaka, *et al.* 2008)** who proposed that cinnzeylanine (the main active principle of the aqueous cinnamon extract) inhibited the proliferation of HSV-1 in Vero cells and considered a good candidate antiviral agent against HSV infection. Also the obtained results were agreed with **(Hayashi, *et al.* 2007)** who showed that an extract from the cinnamon bark can decrease the concentration of foreign DNA in cells and animals infected with the influenza A/PR/8 virus.

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