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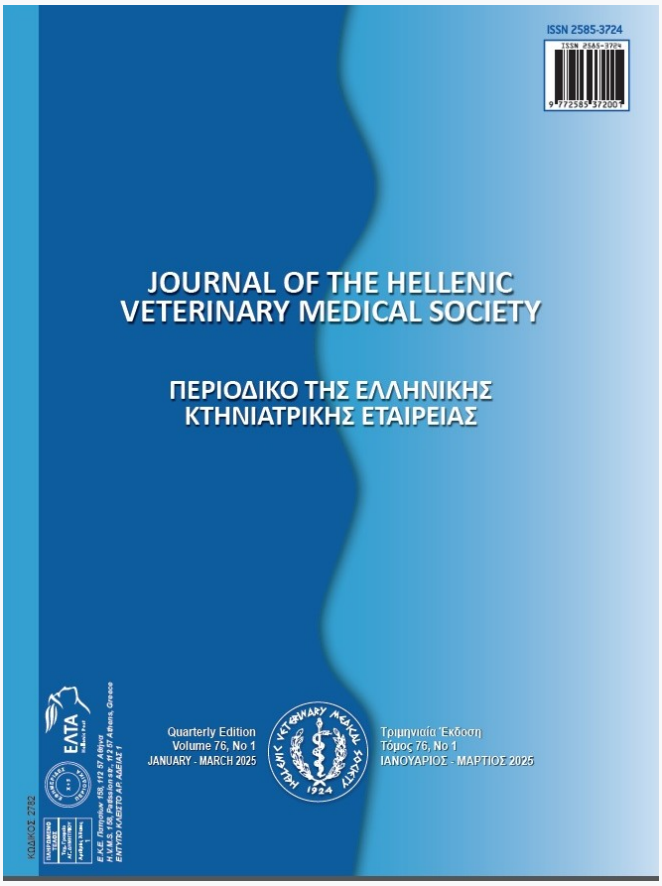

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Research article
Ερευνητικό άρθρο

In vitro investigation of the efficacy of myo-inositol (vitamin B8) as an antioxidant and preservative additive for feed safety

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ABSTRACT: The study was carried out to examine the safety and potential activities of myo-inositol as an antioxidant, preservative, hygiene enhancer, and antiviral feed additive in pet nutrition. For this purpose, the vitamin C equivalent antioxidant capacity (VCEAC of myo-inositol was measured by MTT assay. The cytotoxicity (CC_{50}) of myo-inositol was experimented on mammalian kidney and liver cell lines. The virucidal and antiviral activities of myo-inositol were determined against murine norovirus-1 (MNV-1). One milligram of myo-inositol equals the antioxidant capacity of 0.454 mg of vitamin C. The CC_{50} was determined between 280 and 966 mg/ml for kidney and liver cell cultures. Myo-inositol exhibited dose-dependently virucidal activity against MNV-1 as a surrogate for pet caliciviruses. In three different antiviral experiments, 50% inhibition concentrations were determined as 0.054, 0.084, and 0.047 mg/ml. This in vitro study presents that myo-inositol might be a potential candidate as an antioxidant, preservative, and hygiene enhancer feed additive against oxidative deterioration and microbial contamination in feed production technology for ensuring feed safety and promoting pet health.

Keywords: Antiviral activity; antioxidant capacity; cytotoxicity; feed additive; feed hygiene; virucidal activity

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INTRODUCTION

Feed additives play a crucial role in feed technology and production by enhancing nutritional value and maintaining the quality and safety of animal feeds. Nutritional feed additives, including minerals, vitamins, and prebiotics, enrich the feed content. Additionally, antioxidant, preservative, and feed hygiene enhancer additives help prevent spoilage, extend the shelf life of feeds, and protect them from various forms of deterioration such as oxidation and contamination by pathogens and toxins (European Commission, 2021). However, the selection and usage of feed additives should comply with regulatory guidelines and safety standards, taking into consideration restrictions, feed safety, and the health of the species consuming it (European Commission, 2003, 2021; Vapa Tankosić et al., 2022).

Several natural compounds, such as polysaccharides, can serve as alternatives to chemically synthesized ones in feed production for animal nutrition (Guo et al., 2004; Kiczorowska et al., 2017; Nie et al., 2022). Myo-inositol, a naturally synthesized polysaccharide found in both animal and plant cells, is an essential component of biological membranes in tissues (EFSA, 2014; Li et al., 2022). It is authorized as a nutritional feed additive like vitamins, pro-vitamins, and chemically well-defined substances having a similar effect on fish, dog, and cat nutrition (EFSA, 2014, 2016; European Commission, 2021). In addition to its nutritional benefits, few researchers have studied the antioxidant and antimicrobial activities of myo-inositol and its derivatives against contagious viruses with DNA or RNA genomes, such as norovirus, Coxsackie virus, herpesvirus and HIV (Jiang et al., 2013; Korkmaz et al., 2022; Moslehi et al., 2023). Therefore, this *in vitro* study aimed to investigate the safety, potential virucidal, and antiviral activities of myo-inositol as a technological feed additive (antioxidant, preservative, hygiene enhancer) for pet nutrition.

MATERIALS AND METHODS

Materials, Virus and Cell Lines

Myo-inositol (white powder, >98% purity, food/feed grade) was supplied from Holimer Drug and Health Service Inc. For the stock solution, myo-inositol powder was solved in a cell medium to reach a concentration of 2 mg/ml. After mixed well, the stock solution was stored at 4 °C for further analysis. Murine norovirus 1 strain (MNV-1, VR-1937), murine macrophage (RAW 264.7, TIB-71), African Green Monkey kidney (Vero, CCL-81) baby hamster kidney (BHK-21, CCL-10), human hepatocellular carcinoma (HepG2, HB-8065) and bovine kidney (MDBK, CCL-22) cell lines were from ATCC, USA. RAW 264.7 and Vero were cultured and maintained with Dulbecco's Modified Eagle Medium (DMEM) and BHK-21, HepG2, and MDBK were cultured with Eagle's Minimum Essential Medium (EMEM) in the incubator with the standard condition of 37 °C and 5% CO_2. Both mediums contained fetal bovine serum (10%), L-alanyl-L-glutamine (200 mM), and 1% penicillin (10.000 unit/ml)-streptomycin (10 mg/ml)-amphotericin B (0.025 mg/ml). For virus titration in all experiments, ten-fold serial dilutions method (10^{-1}-10^{-10}) was performed by adding in microplate wells containing 100 µl host cell suspension (3×10^5 cell/ml) in four replicates. For cytotoxicity and antiviral assays, 100 µl of the stock viable cell suspension (3×10^5 cell/ml) was seeded in each well (3×10^4 cell/well) and kept in SC for 24 h to confluence at least 90% in 96-well microplates. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) was supplied from Serva, Germany to use for antioxidant capacity and cytotoxicity assays.

Vitamin C Equivalent Antioxidant Capacity (VCEAC)

Vitamin C (ascorbic acid, HPLC grade) was diluted two-fold to concentrations of 600, 300, 150, 75, and 37.5 µmol using ultra-distilled water. Similarly, a stock solution of myo-inositol was two-fold diluted to concentrations of 1, 0.5, 0.25, 0.125, 0.062, and 0.031 mg/ml, also with ultra-distilled water.

In an Eppendorf tube, 380 µl MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, 1 mg/ml) was combined with 20 µl of each dilution of Vitamin C and myo-inositol. The mixtures were then incubated at 37 °C for 4 h. After incubation, 400 µl DMSO was added to all tubes and thoroughly mixed to dissolve the blue formazan salt formed during incubation. Subsequently, 100 µl of each mixture was added in quadruplicate to a U-bottom microplate and read at 570 nm. A standard curve was generated using optic density (OD, nm) values of five vitamin C dilutions through linear regression analysis (*vitamin C equivalence* = $m\ x\ OD_{570} + b$, R^2). The equivalence of myo-inositol dilutions to vitamin C was calculated regarding the standard curve.

Cytotoxicity assay

The myo-inositol stock solution was two-fold serially diluted to concentrations of 1, 0.5, 0.25, 0.125,

0.062, 0.031, and 0.015 mg/ml in a maintaining medium. Then, 100 µl of each dilution was added to the microplates with monolayer cell cultures at six replicated wells. Fresh medium was only added to cell control wells (medium and cell) and blank wells (medium without cells). The microplates were then incubated in the incubator (5% CO_2, 37 °C) for 24 h. After the incubation period, the medium was discarded and 40 µl MTT in PBS (1 mg/ml) was added to each well. Following a 4-hour incubation in the incubator (5% CO_2, 37 °C), DMSO (100 µl) was added to the wells. The microplate was gently shaken to solubilize the formazan crystals and read at a wavelength of 570 nm (Absorbance 96, Byonoy, Germany).

Virucidal activity

The procedure of virucidal assay was adapted from the BS EN 14476:2013+A2:2019 standard method for evaluating virucidal activity in the medical area (European Commission, 2019) (Figure 1). Briefly, a stock virus suspension of 5.25 $TCID_{50}$/ml (1:10 v/v) was exposed to myo-inositol dilutions (1, 0.5, 0.25, 0.125, 0.062 and 0.031 mg/ml) (8:10 v/v), containing bovine albumin solution (BSA 0.3 g/l) as an interfering substance (1:10 v/v) at room temperature (21±2 °C) for 30 seconds. After the contact time, one milliliter of each mixture was transferred to a 9 ml ice-cold medium to stop the reaction. For the titration of the remaining virus, ten-fold serial dilutions were inoculated in 96-well microplate wells containing 100 µl host cell suspension in eight replicates and incubated in the incubator (5% CO_2, 37 °C) for 72 hours. Acetic acid (0.7% in PBS, v/v) (positive control) and PBS (negative control) were used in parallel with the virucidal test.

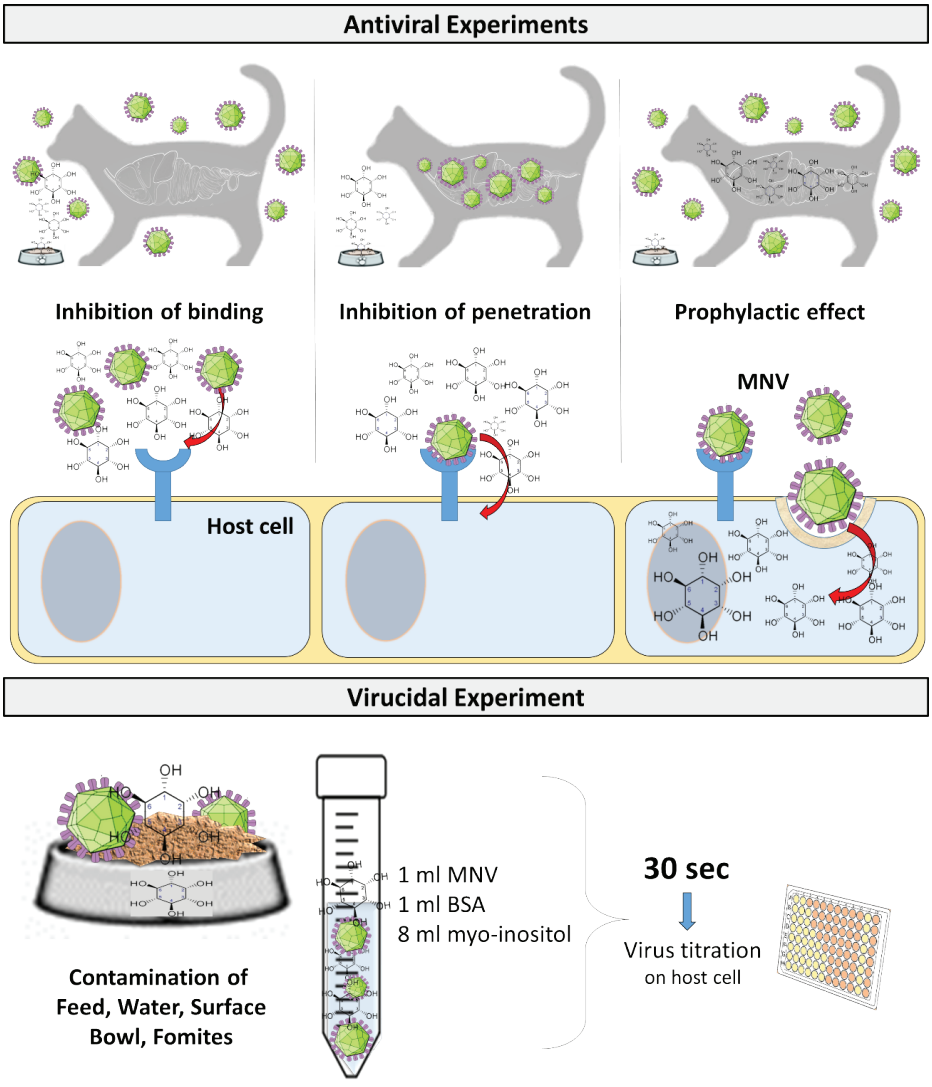

Figure 1. Summary of antiviral and virucidal experiments.

Antiviral activity

For the strategy against possible feed-borne Calicivirus infection, three experiments were conducted to determine the efficiency of myo-inositol at non-cytotoxic concentrations (CC_{50}: 0.185 mg/ml). The experiments focused on evaluating myo-inositol's effectiveness in binding (1), penetration (2) to the host cell, and prophylaxis (3) of MNV-1 as a surrogate (Figure 1). All experiments were carried out using the host cell in 24-well cell culture plates (12 x 10^4 cells/well). The plates were incubated in the incubator (5% CO_2, 37 °C) and allowed to confluence at least 90% the day before. Both negative (uninfected, untreated cell) controls and positive (infected, untreated) controls were conducted in parallel.

(1) Effect of myo-inositol on virus binding to host cell: The host cell was treated with both MNV-1 virus and myo-inositol at different concentrations during the 72-hour incubation period. Each non-cytotoxic concentration of myo-inositol (0.125, 0.062, and 0.031 mg/ml medium) in 1 ml, along 200 µl virus stock (MOI: 0.5 $TCID_{50}$/cell) was added to three replicated wells, excluding negative control wells.

(2) Effect of myo-inositol on virus penetration in the host cell: The host cell was treated with different concentrations of myo-inositol after a one-hour adsorbing period of MNV-1. A virus suspension (MOI: 0.5 $TCID_{50}$/cell) of 200 µl was added to 24-well cell culture plates coated with the host cell, excluding negative control wells. The plate was then incubated in the incubator (5% CO_2, 37 °C) for 1 h to adsorb the virus to host cells. Subsequently, unbound viruses were discarded by washing the wells with PBS. Following this, non-cytotoxic dilutions (0.125, 0.062, and 0.031 mg/ml medium) of myo-inositol were added to three replicated wells.

(3) Prophylactic effect of myo-inositol against the virus: The host cell was infected with an MNV-1 titer of 5.25 $TCID_{50}$/ml after a 4-hour treatment period with different concentrations of myo-inositol for prophylactic effect. Each non-cytotoxic dilution (0.125, 0.062, and 0.031 mg/ml medium) of myo-inositol was added to 24-well cell culture plates coated with the host cell in three replicates. The plate was incubated in the incubator (5% CO_2, 37 °C) for 4 h to treat the host cell with myo-inositol. Then, all mediums containing myo-inositol were discarded by washing the wells with PBS. One milliliter of maintaining medium containing virus suspension (MOI: 0.5 $TCID_{50}$/cell) was added to all wells except negative control wells.

The plate was maintained in the incubator (5% CO_2, 37 °C) for 72 hours, with daily observation of cytopathic effects (CPE) (Figure 2). At the end of the 72-hour period, the suspensions in 24-well plates were repeatedly frozen and thawed for virus titration in the 96-well microplates containing host cell suspension in the incubator (5% CO_2, 37 °C) for each myo-inositol dilution, as well as for the controls.

Data analysis

The percentage of cell inhibition was calculated using the equations as follows:

Cell viability (%) = (OD sample - OD blank) / (OD control - OD blank) × 100.

Virus titration was calculated as a tissue culture infective dose of 50% ($TCID_{50}$/ml) using the method of Spearman-Karber in all experiments (Spearman, 1908; Kärber, 1931). The 50% cytotoxic concentration (CC_{50}), virucidal concentration of 50% (VC_{50}), and inhibitory concentration of 50% for antiviral activity (IC_{50}) were calculated from concentration-based-curves after linear regression analysis using Office Excel 2016 (Microsoft, USA). The selectivity index (SI) was also determined for antiviral (CC_{50}/IC_{50}) and virucidal (CC_{50}/VC_{50}) capacities. The tables and the graphs were generated in Office Excel and PowerPoint 2016 (Microsoft, USA).

The data analysis was conducted using parametric methods by SPSS software version 15 (IBM, USA). The normality and homogeneity of variance were assessed using Kolmogorov-Smirnov, Shapiro-Wilk, and Levene's Test. Group differences were analyzed by one-way ANOVA with post hoc Tukey.

RESULTS AND DISCUSSION

Antioxidant capacity of myo-inositol

In feedstuffs and feeds, oxidation adversely affects nutritional ingredients such as proteins, carbohydrates, fats, mineral supplements, and vitamins. Most feedstuffs containing fats or oils are sensitive to oxidative deterioration causing sensorial characteristics and nutritional value of feed. So, many antioxidants including vitamin C (ascorbic acid) are authorized as feed additives in feed technology and animal nutrition for all animal species. In particular, vitamin C is permitted for its multiple functions as an antioxidant and nutritional additive without a maximum limit in feed

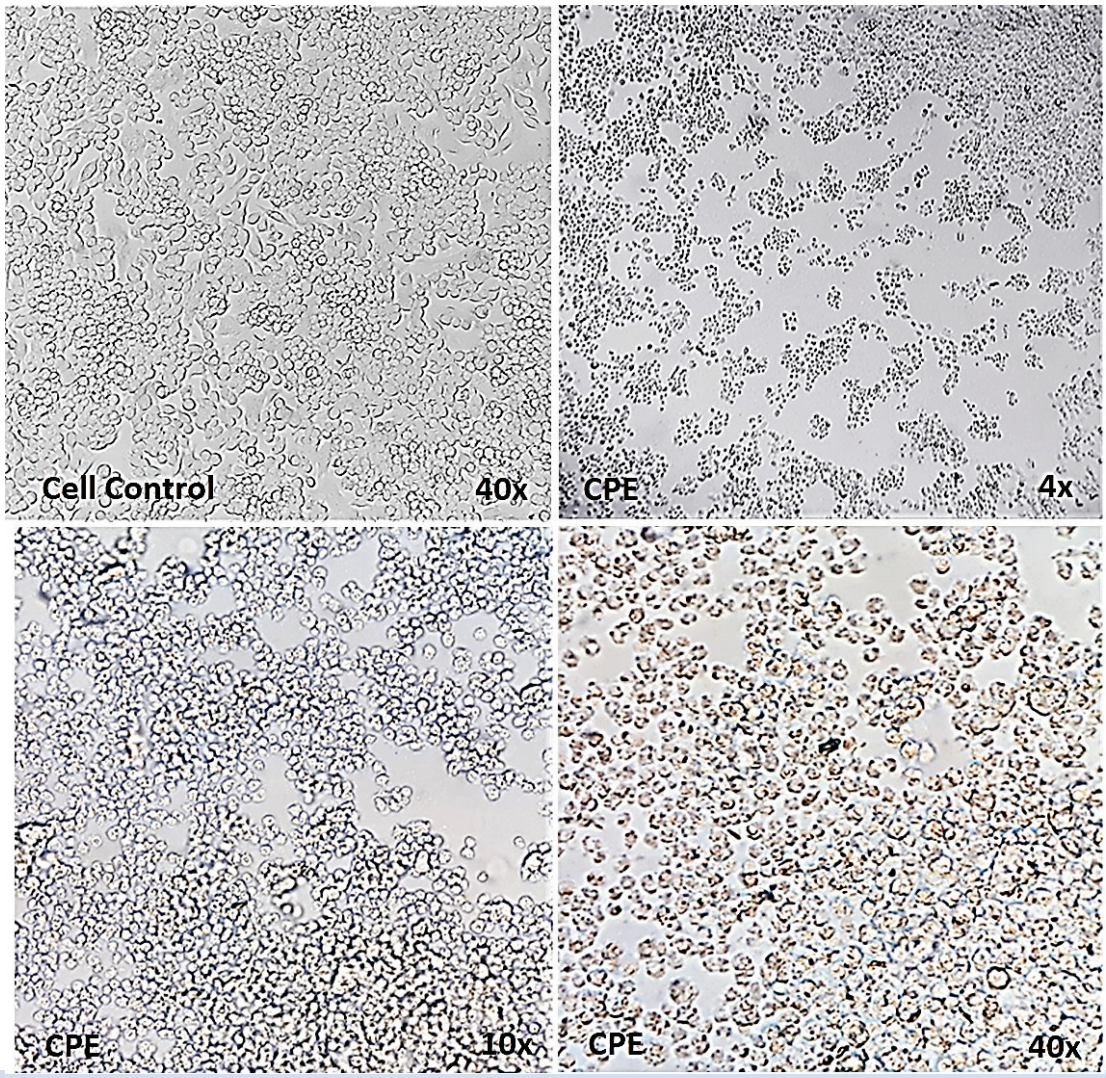

Figure 2. Cytopathic effects (CPE) of MNV-1 on host cells.

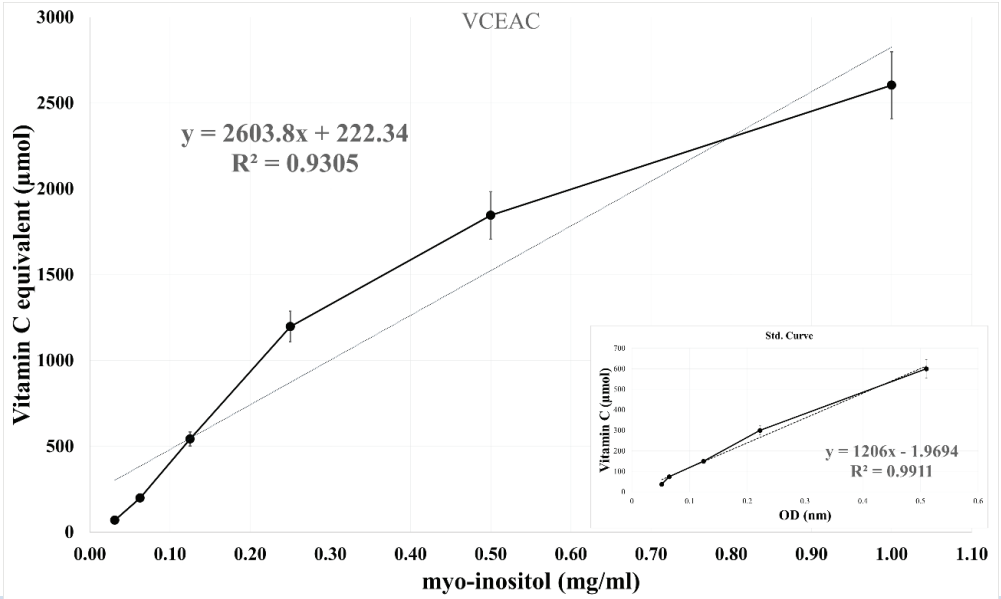

Figure 3. Antioxidant capacity of myo-inositol and Vitamin C standard curve.

technology (European Commission, 2015, 2021). Similar to vitamin C, myo-inositol is categorized as a nutritional additive in the functional additive group of "vitamins, pro-vitamins, and chemically well-defined substances having a similar effect" (European Commission, 2021). Also, *in vivo*, studies suggested that myo-inositol supplementation had antioxidant activity, inhibited oxygen radical generation, and increased antioxidant enzyme activities against oxidative stress in humans, rats, and aquatic animals (Jiang et al., 2013; Formoso et al., 2019; Pan et al., 2022; Moslehi et al., 2023). In this study, the VCEAC of myo-inositol was calculated concerning the L-ascorbic acid standard curve by linear regression analysis (vitamin C equivalence, µmol =1206 x OD_{570} of myo-inositol - 1.9694, R^2 = 0.9911). The VCEAC of myo-inositol increased by increasing the concentrations of myo-inositol with linearity of R^2=0.9305 (vitamin C equivalence, µmol =2603.8 x myo-inositol mg/ml + 222.34) (Figure 3). The result indicated that one milligram of myo-inositol equals the antioxidant capacity of 0.454 mg ascorbic acid. In addition to nutritional value, myo-inositol might be a candidate antioxidant additive in feed production technology to maintain the stability of feedstuffs and complete feed.

Safety concentrations of myo-inositol

The cytotoxic level and safe concentration of additives, which are whether chemically synthesized or naturally extracted, are generally tested with vital cell lines of the mammalian kidney and liver. Previous studies determined 50% cytotoxic concentrations (CC_{50}) of several myo-inositol derivates as 12-50 µg/ml for HeLa (uterine melanoma cells), higher than 50 µg/ml for MDCK (dog kidney cells) and GMK (green monkey kidney cells), 10-20 µg/ml for human lung cancer cell and 2 mg/ml for fish spleen cell (Zhan et al., 2006; Tuchnaya et al., 2008; Liu et al., 2020). In this study, cytotoxicity of myo-inositol experimented on the viability of five cell lines originating from mammalian macrophage (RAW 264.7), hamster, bovine, and monkey kidneys (BHK-21, MDBK, Vero) and liver (HepG2). CC_{50} was calculated as 185, 966, 813, 280, and 563 mg/ml for RAW 264.7, BHK-21, MDBK, Vero and HepG2 respectively (Figure 4).

In previous studies, the CC_{50} was calculated between 12 µg/ml and 2 mg/ml for various derivates of inositol. And, lower cytotoxicity was presented in kidney cells. Similarly, myo-inositol showed higher toxicity on macrophages with the lowest CC_{50} values, as it was shown as safer on kidneys and liver with higher CC_{50}. When compared with previous studies (except fish spleen cells), It was suggested that myo-inısitol used in this study had low cytotoxicity on mammalian cell line cultures.

The cell viability rates of RAW 264.7 as a virus-host cell were upper than 50% at the concentrations of 0.125, 0.062, and 0.031 mg/ml (Figure 4). Therefore, 0.125, 0.062, and 0.031 mg/ml concentrations of myo-inositol were used to evaluate the antiviral activity assays.

Virucidal activity

In feed technology, several biodegradable substances such as organic acids (sorbic, malic, lactic, acetic, propionic, and formic acids) and their salts were authorized as preservative and hygiene condition enhancer feed additives without a maximum content limit in feed. They are used against feed-borne infection and to maintain feed quality or hygienic characteristics against deterioration caused by micro-organisms or their metabolites (European Commission, 2003, 2021). This was the first study reporting the virucidal activity of myo-inositol against norovirus tested with the perspective of a preservative and hygiene enhancer feed additive against virus contamination of water, feed, feed contact surfaces, and fomites. The test was valid with 0.7% acetic acid authorized as a preservative feed additive for all species without a limit (European Commission, 1985, 2021). It caused a logarithmic reduction of ≥4 $TCID_{50}$/ml in BSA soiling conditions at least (European Commission, 2019). Also, a reduction of 0.75 $TCID_{50}$/ml was measured in the negative control. The two-fold dilutions of myo-inositol presented a titer reduction between 2.5 (47.6%) and 3.75 (71.4%) $TCID_{50}$/ml (Figure 5). No concentrations of myo-inositol tested caused a reduction of 4 $TCID_{50}$/ml. $VC_{50,}$ $VC_{90,}$ and IS for virucidal activity were calculated as 0.036 mg/ml, 1.746 mg/ml, and 9.7 with the linearity of R^2=0.9571 (y = 23.39x + 49.152). This first study presented the effective concentrations of myo-inositol as a preservative and hygiene enhancer feed additive for feed production and technology.

Antiviral activity

In this study on myo-inositol as a feed additive, the three pathways of MNV-1 were investigated to set a control model for caliciviruses cause significant diseases in cats and dogs (Figure 1). Non-cytotoxic concentrations of myo-inositol (0.125, 0.062, and 0.031 mg/ml) reduced the virus titer for three pathways. The

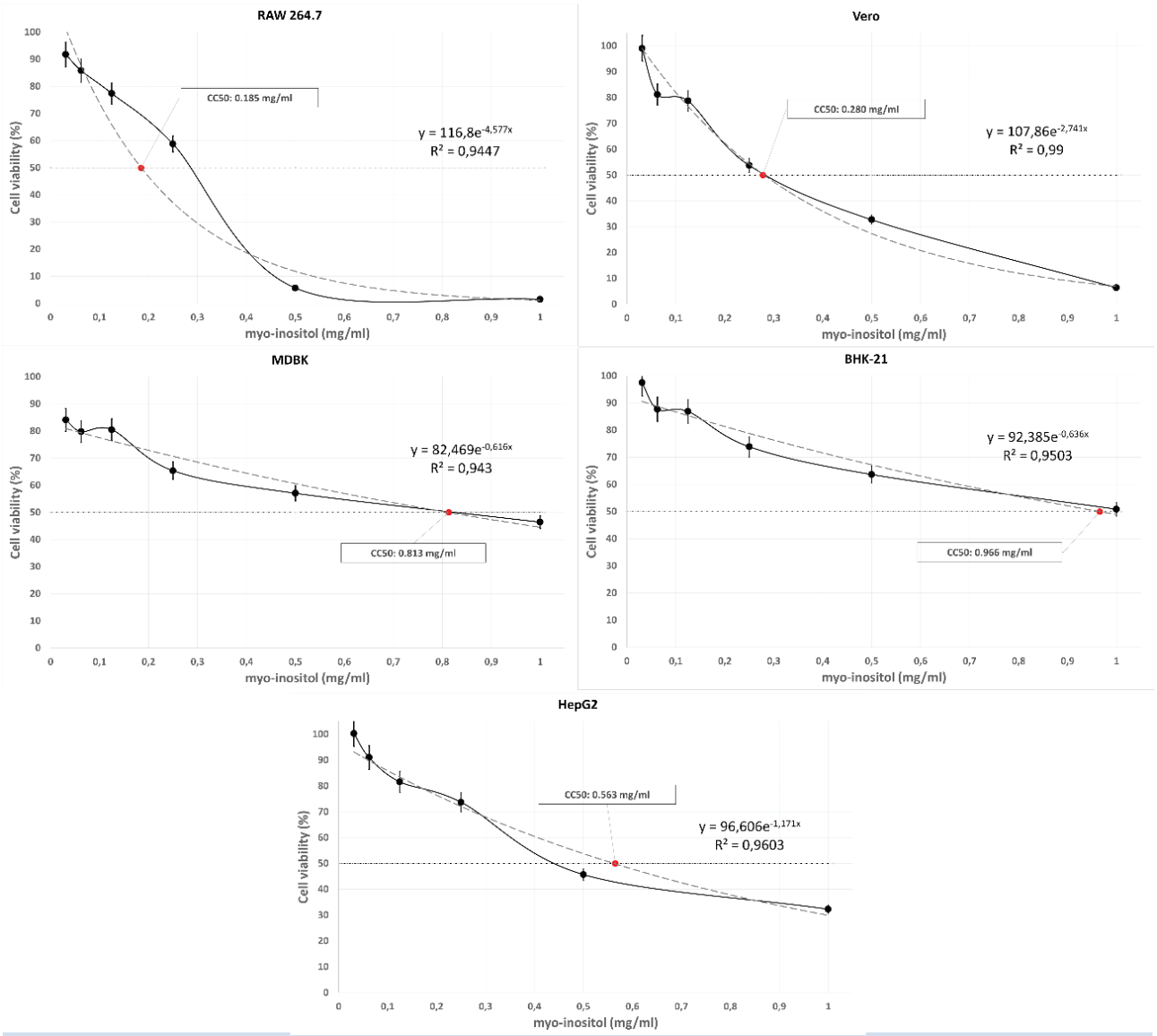

Figure 4. The effects of myo-inositol on the viability of mammalian cells.

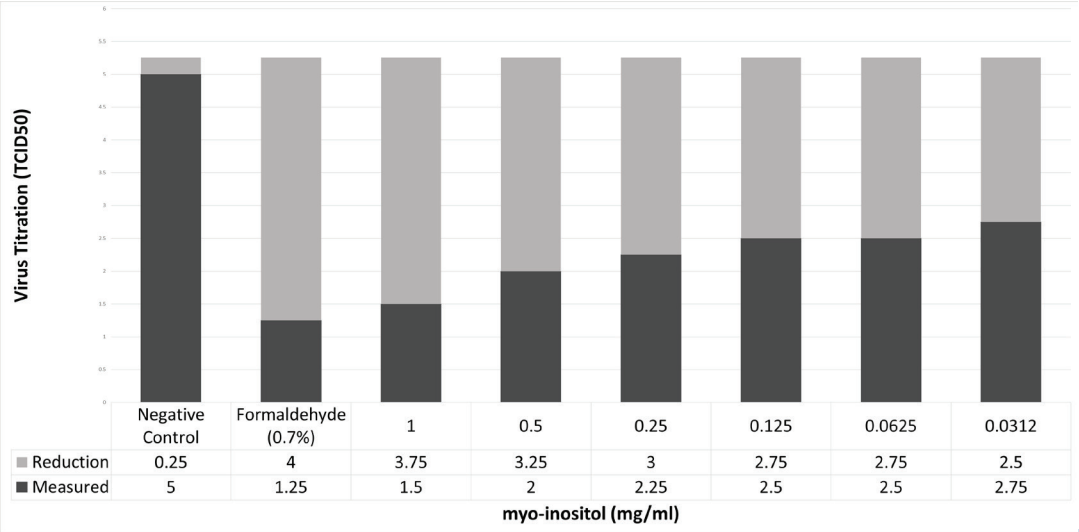

Figure 5. Virucidal activity of myo-inositol.

Table 1. Antiviral activity of myo-inositol on the infectivity pathways of a virus.

Myo-inositol (mg/ml)	Pre-treatment (log TICD50/ml)	Inhibition of virus binding		Inhibition of virus penetration		Prophylactic effect	
		Measured (log TICD50/ml)	Reduction %	Measured (log TICD50/ml)	Reduction %	Measured (log TICD50/ml)	Reduction %
0.125	5.25	1.25±0.25b	76.19±4.76	0.50±0,25b	90.48±4.76	1.50±0.25c	71.43±4.76
0.062	5.25	1.25±0.25b	76.19±4.76	4.00±0,00a	23.81	1.50±0.25c	71.43±4.76
0.031	5.25	4.00±0.00a	23.81	5.00±0,25a	4.76±4.76	3.50±0.50b	33.33±9.52
Pos. Control	5.25	5.00±0.25a	4.76±4.76	5.00±0.00a	4.76	5.25±0.00a	0
P value		0.025		<0.01		0.039	
IC50 (mg/ml)		0.054		0.084		0.047	
CC50 (mg/ml)*		0.185		0.185		0.185	
IS		3.43		2.20		3.94	

*for RAW 264.7 as host cell; Measured was expressed as log $TICD_{50}$/ml

highest reduction rates were at the concentrations of 0.125 mg/ml for the virus penetration experiment and the concentrations of 0.125 and 0.062 mg/ml for both virus binding and prophylaxis experiments (Table 1). IC_{50} was 0.054, 0.084, and 0.047 mg/ml for the inhibition of virus binding, penetration, and prophylaxis effect respectively. IS was calculated as 3.43, 2.20, and 3.94 for all three pathways of MNV-1 on host cells respectively. A higher SI value indicated that myo-inositol had lower cytotoxicity and higher antiviral activity. The virus was tittered in the positive control (non-treated with myo-inositol), but not in the negative control (non-treated with the virus).

In previous studies, the antiviral capacity of inositol and its derivatives was experimented against several serious and highly contagious viruses with DNA or RNA genomes. These in vitro studies suggested that inositol at various concentrations was effective against Coxsackie (non-enveloped, RNA), Herpesviruses (enveloped, DNA), HIV (enveloped, RNA), and Iridovirus (enveloped, RNA) which are serious for humans and animals (Tuchnaya et al., 2008; Baranova et al., 2014; Korkmaz et al., 2022). Especially, its phosphate and sulfate derivatives showed antiviral activity against the Coxsackie virus, Herpes Simplex virus, and Iridovirus with IC_{50} concentrations of lower than 0.05 mg/ml (Tuchnaya et al., 2008). Also, plant-based inositol presented an inhibition of up to 90% on Iridovirus (Liu et al., 2020). In previous studies, several inositol derivates were effective against serious viruses with envelope or non-envelope and RNA or DNA genomes (Tuchnaya et al., 2008; Baranova et al., 2014). Despite higher concentration than previous studies, myo-inositol showed activity against norovirus, a non-enveloped virus with RNA genome, in this study.

On the European Food Safety Authority panel of additives and products or substances used in animal feed (FEEDAP), inositol was categorized as a nutritional additive (functional group: vitamins, pro-vitamins, and chemically well-defined substances having a similar effect) in feed. The FEEDAP Panel also declared that inositol is safe up to 3000 mg free inositol/kg dry complete feed (88% dry matter) in commercial diets for pets (EFSA, 2016). When myo-inositol was supplemented in dry cat food which is one of the suspects for feed-borne COVID-19, the SARS-CoV-2 viral load was decreased dose-dependently at room temperature without any changes in physicochemical characteristic of feed. The IC_{50} for SARS-CoV-2 was determined as 36.64-58.15 mg myo-inositol/kg dry cat food (Korkmaz et al., 2023). These results suggested that myo-inositol might have the potential to prevent serious diseases while used as a feed additive in feed technology and animal nutrition concerning the limitation of feed legislation.

CONCLUSION

In conclusion, myo-inositol (vitamin B8) could be a potential candidate as an antioxidant, preservative, and feed hygiene enhancer to prevent and control oxidative deterioration and possible feed-borne diseases at safe concentrations regarded to its natural polysaccharide structure in animal nutrition.

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CONFLICT OF INTEREST

The authors have no declaration of competing interests.

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