Research on the virucidal activity of theaflavins-enriched tea leaf extract against influenza A virus and human norovirus surrogates

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A型インフルエンザウイルスおよびヒトノロウイルス代替ウイルスに対する茶葉由来テアフラビン濃縮抽出物の殺ウイルス効果

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Contents

Abbreviation

A	AGE	Acute gastroenteritis
	ANOVA	Analysis of variance
C	CBB	Coomassie brilliant blue
	CRFK	Crandell-Rees feline kidney
	COX-2	Cyclooxygenase 2
	CPE	Cytopathic effect
D	DMEM	Dulbecco's modified Eagle's minimal essential medium
E	EC	(-)-epicatechin
	ECG	(-)-epicatechin gallate
	EGC	(-)-epigallocatechin
	EGCG	(-)-epigallocatechin-3-gallate
	EC16	(-)-epigallocatechin-3-gallate-palmitate
F	FCV	Feline calicivirus
G	GTE	Green tea extract
H	HA	Hemagglutinin
	HRP	Horseradish peroxidase
	HuNoV	Human norovirus
I	IAV	Influenza A virus
	IBV	Influenza B virus
	ICV	Influenza C virus
	IDV	Influenza D virus
	IL	Interleukin

L	LPS	Lipopolysaccharide
M	MDCK	Madin-Darby canine kidney
	2-Me	2-mercaptoethanol
	MMP-2	Matrix metalloprotease 2
	M Protein	Matrix protein
	MNV	Murine norovirus
N	NA	Neuraminidase
	NaClO	Sodium hypochlorite
	NF	Nuclear factor
	NPs	Nucleoproteins
O	ORFs	Open reading frames
P	PBS	Phosphate-buffered saline
	PCR	Polymerase chain reaction
R	Real-time RT-PCR	Real-time reverse transcription PCR
	RNA	Ribonucleic acid
	RT-PCR	Reverse transcription PCR
S	SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
	SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T	$TCID_{50}$	50% tissue culture infectious dose
	TEM	Transmission electron microscope
	TFs	Theaflavins
	TF1	Theaflavin

	TF2A	Theaflavin-3-gallate
	TF2B	Theaflavin-3′-gallate
	TF3	Theaflavin-3,3′-digallate
	TNF	Tumor necrosis factor
V	VGM	Virus growth medium
U	USA	United States of America
W	WB	Western blotting
	WHO	World Health Organization
Z	ZIKV	Zika virus

General Introduction

Background of the viruses of study

Influenza A virus (IAV)

IAV is a single-stranded, negative-sense, enveloped ribonucleic acid (RNA) virus comprising a segmented genome, classified within the family *Orthomyxoviridae* which includes 4 genera: *Alphainfluenzavirus* [influenza A virus (IAV)], *Betainfluenzavirus* [influenza B virus (IBV)], *Gammainfluenzavirus* [influenza C virus (ICV)] and *Deltainfluenzavirus* [influenza D virus (IDV)] (Hause et al., 2014; King et al., 2012). The IAV, IBV, and ICV can be differentiated according to the antigenic variation between their matrix (M) proteins and nucleoproteins (NPs). IAV and IBV comprise 8 segmented genomes, whereas ICV and IDV consist of 7 segmented genomes (Desselberger et al., 1980; Wolff and Veit, 2021). IAV is further subdivided based on the antigenic difference of the surface glycoproteins, hemagglutinin (HA), and neuraminidase (NA) into diverse subtypes. Currently, there are 18 subtypes known on HA and 11 subtypes known on NA (Shao et al., 2017). IAV evolution proceeds through genetic reassortment and mutation, which affect the host specificity and pathogenicity of this virus (Mehle et al., 2012).

Influenza is one of the contagious diseases that infect people on a worldwide scale. In humans, the etiological agent causing influenza is IAV or IBV and, to a lesser extent ICV. IAV represents the highest threat to humans due to its frequent tendency for genetic reassortment, and its pandemic potential (Russell et al., 2018). The disease can run its course in several ways, from a mild infection or asymptomatic upper respiratory tract infections to severe sickness characterized by chills, high fever, pneumonia, muscle pain, and death (Keilman, 2019). The World Health Organization (WHO) estimates that approximately 290,000–650,000 deaths each year have been caused by seasonal

influenza (World Health Organaization, 2022). As a result, influenza constitutes a medical and economic global burden (Marbus et al., 2020; Putri et al., 2018).

Consequently, extensive research is continuously conducted to examine the directions of antigenic deviations in IAV to overcome the influenza problem. Although there are ongoing researches on vaccines (Świerczyńska et al., 2022), there is a limitation regarding the antigenic mismatching of vaccine and field strains due to the antigenic shift and antigenic drift of IAV (Scorza et al., 2016). Furthermore, there are four registered drug groups targeting IAV: NA inhibitors (zanamivir, oseltamivir), M2 proton channel antagonists (amantadine), the selective inhibitor of viral RNA-dependent RNA polymerase (favipiravir), and the cap-dependent endonuclease inhibitor (baloxavir marboxil) (Furuta et al., 2013; Shirley, 2020). Among these drugs, favipiravir and baloxavir marboxil, have recently invaded the marketplace for commercial use. Baloxavir marboxil is licensed in Europe, the United States of America (USA), Japan, Hong Kong, and Australia. Meanwhile favipiravir is approved for restricted use in Japan and is being under Phase III study for a clinical trial in the USA and Europe (Heo, 2018; Shiraki and Daikoku, 2020). Although most of those drugs were being effective, the emergence of resistant strains to the NA inhibitors, M2 ion channel inhibitor, and the cap-dependent endonuclease inhibitor eventually affected their efficacy (Uehara et al., 2020; Yin et al., 2021).

Consequently, the unpredictable risk of a novel influenza pandemic constantly exists as one of the uppermost threats to global health (Mounier-Jack and Coker, 2006; Trock et al., 2015). Therefore, preventative measures other than vaccination and therapeutic interventions are also needed to combat influenza such as disinfectants or virucidal agents. Nature affords an enormous library of novel compounds to be tested against several diseases (Denaro et al., 2020). Specfically, there is growing attention on natural plant extracts as promising virucidal agents against many viruses (Salinas et al., 2019; Takeda et al., 2020; Takeda et al., 2021a).

Human noroviruses (HuNoV)

HuNoV, known as Norwalk viruse is non-segmented, non-enveloped RNA virus, belonging to the genus *Norovirus* (NoV) of the family *Caliciviridae*. The genome length of NoV is 7.5–7.7 kb containing 3 open reading frames (ORFs) encoding 8 viral proteins, and are differentiated into 10 genogroups (GI–GX) (Chhabra et al., 2019), of which GI, GII, and GIV have been demonstrated to infect humans with GII the most frequently detected genogroup throughout the world in clinical surveillance studies (Van Beek et al., 2018). Furthermore, the genogroups are subdivided in 49 genotypes, with GII.4 is the predominant genotype causing both sporadic cases and outbreaks from HuNoV infection (Hasing et al., 2019). The lack of robust and reproducible cultivation system as well as animal models available for HuNoV represents a major barrier in the establishment of efficient interventions against HuNoV. Nevertheless, surrogate viruses such as feline calicivirus (FCV) and murine norovirus (MNV) (Bosch et al., 2018; Lacombe et al. 2017) have been widely used due to their high replicability in lab settings, in addition to their genetic or physical relatedness to HuNoV (Wobus, et al., 2006).

HuNoV is the virus most commonly related to sporadic cases and outbreaks of acute gastroentiritis (Neethirajan et al., 2017; Pires, et al., 2015), and is globally identified as a food-borne virus with the highest priority in controlling, with significant economic losses estimated at tens of billions of dollars (Ahmed et al., 2014; Bartsch et al., 2016; Havelaar et al., 2015). Specifically, in 2017 and 2018, official reports listed HuNoV infection among the most frequently reported food-borne disease cases being responsible for 211outbreaks (7.8%) in Europe and 140 outbreaks (35%) in the USA (Centers for Disease Control and Prevention, 2017; European Food Safety Authority, 2019). There are multiple transmission routes for HuNoV to establish infection such as the direct contact with infected individuals and the indirect transmission, which could be resulted from the consumption of contaminated foods and water or handling objects and surfaces contaminated with

the virus (Lane et al., 2019; Lopman et al., 2012). The possibility of HuNoV survival for weeks on diverse environmental surfaces represents a major concern for HuNoV inactivation (Djebbi-Simmons et al., 2020; Liu et al., 2009). Various virus inactivation approaches were available for the prevention of HuNoV infection, which represent an efficient way for food-borne disease control such as thermal inactivation (Bartsch et al., 2019) and irradiation (Molina-Chavarria et al. 2020). Nevertheless, the nutrient content (e.g., protein and vitamins), as well as the organoleptic characterstics (e.g., texture and color) of food products, could be altered by such approaches (Aadil et al., 2019; Molina et al., 2014). Consequently, alternative strategies to combat food-borne viruses are necessary, not only to decrease the number of infections in humans and ensure food safety, but also to diminish the direct economic costs associated with food-borne viruses (Bartsch et al., 2016). Recently, intensive research has been conducted on the properties of natural plants derived components such as tea polyphenols with health promoting potential, which may be utilized to ensure the food safety in terms of viral diseases (Amankwaah et al., 2020; Solis-Sanchez et al., 2020).

Protective effects of tea polyphenols on human health

Tea is regarded as the world's most widespread beverage, with daily consumption of more than 2 billion cups (Drew, 2019). Tea is originated from the plants of *Camellia sinensis*, an evergreen tree that belongs to the family *Theaceae*, which is home-grown in Southeast Asia and China. There are 3 main teas: black, green, and oolong tea, which could be produced form the harvested leaves of *Camellia sinensis* treated with various methods. Because of its health benefits and high nutritional value, tea beverages have been popularly consumed. For example, tea contains considerable amounts of flavonoids, mineral elements, amino acids, and organic acids (Das et al., 2019; He et al., 2020; Koch et al., 2018), which provide numerous physiological benefits to consumers. Furthermore, tea

extracts are known to have antimicrobial, anti-inflammatory, anti-diabetic, and anti-cancer activities (Li et al., 2017; Liao et al., 2016; Rha et al., 2019; Striegel et al., 2015; Xu et al., 2017), which were mainly related to its polyphenolic secondary metabolites, in particular, the theaflavins (TFs) and catechins. Therefore, these dietary and health benefits have increased the research demand for tea polyphenolic metabolites to be further tested for different therapeutic purposes such as the virucidal and anti-inflammatory effects. Specfically, Eggers et al. (2022) analyzed the virucidal efficacy of 10 mg/mL of green tea extract (GTE) against respiratory viruses such as IAV and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). They demonstrated that the titers of IAV and SARS-CoV-2 were reduced by > 99% and 80%, within 5 and 1 mins, respectively. Furthermore, the antiviral and the virucidal activities of (-)-epigallocatechin-3-gallate (EGCG)-palmitate (EC16), a polyphenolic compound extracted from green tea, were evaluated against MNV. The results showed that > 99% (> 2 $\log_{10}$) reduction of MNV titer was found after 1h incubation of 0.1% w/v EC16 with the Murine leukaemia macrophage-like cell line RAW264.7 cells either before or after MNV inoculation (Zhong et al., 2021). Additionally, Randazzo et al. (2017) showed that the 10 mg/mL of GTE inactivated MNV and hepatitis A virus within 30 mins on stainless steel and glass discs by 1.79 and > 2.8 $\log_{10}$ reduction, respectively. The results concluded that GTE had the potential to be used as a natural disinfectant to improve food quality and safety. Furthermore, for Zika virus (ZIKV), the virucidal efficacy of EGCG was evaluated by Carneiro et al. (2016). They found that EGCG at higher concentrations (> 100 μM) inhibited ZIKV entry by at least 1-log (> 90%). Recently, because of the fast spread of SARS-CoV-2 and the shortage of specific antiviral drugs against it, testing alternative medicine from natural plant extracts is considered one of the suggested approaches to strive this pandemic. Accordingly, Mhatre et al. (2020) summarized various evidence and reports supporting the use of tea polyphenols, specifically EGCG and TFs, as potential candidates in prophylaxis and treatment of SARS-CoV-2 infection. Overall, EGCG was reported to strongly bind with various

molecules in different viruses, specifically protease; therefore, EGCG interrupts their functional activities. Furthermore, EGCG affects the interaction between virus and host cells through attaching the receptors on the host cell membrane or the virion surface. In addition, EGCG could suppress viral protein expression and viral genome replication (Wang et al., 2021).

The potential anti-inflammatory activity of tea polyphenols has been widely investigated with particular explanation for the various mechanisms of action, indicating their feasibility in managing and treating inflammatory diseases (Hamer, 2007). For example, Cyboran et al. (2015) reported that GTE exhibited robust anti-inflammatory activity on red blood cells without any evident of toxicity. Furthermore, GTE demonstrated an anti-inflammatory action in primary human rheumatoid arthritis synovial fibroblasts, in which (-)-epicatechin (EC), (-)-epigallocatechin (EGC), and EGCG showed a different anti-inflammatory effect. Specifically, EGCG and EGC inhibited matrix metalloprotease 2 (MMP-2), interleukin (IL)-6, and IL-8 production and selectively suppressed the expression of cyclooxygenase 2 (COX-2); meanwhile, EC did not show any suppressive activity against these factors. These results proposed that EGCG and EGC contributed mainly to the anti-inflammatory effect of green tea, besides EGCG being the most potent catechin to suppress the downstream signaling of inflammation (Fechtner et al., 2017). Moreover, Ben Lagha and Grenier (2017) showed that black tea TFs inhibited the secretion of inflammatory factors, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, MMP-3, MMP-8, MMP-9, and chemokine C-X-C motif ligand 8, and attenuated the nuclear factor (NF)-κB signaling pathway in *Porphyromonas gingivalis*-stimulated macrophages. In this study, tea extracts and their bioactive components have robust anti-inflammatory activity, mainly through regulating inflammatory factors, like cytokines, chemokines, COX-2, and NF-κB, as well as the related signaling pathways. Overall, the use of tea polyphenols for treating different inflammatory disorders such as systemic lupus erythematosus, atherosclerosis, arthritis and sepsis could be considered a feasible approach.

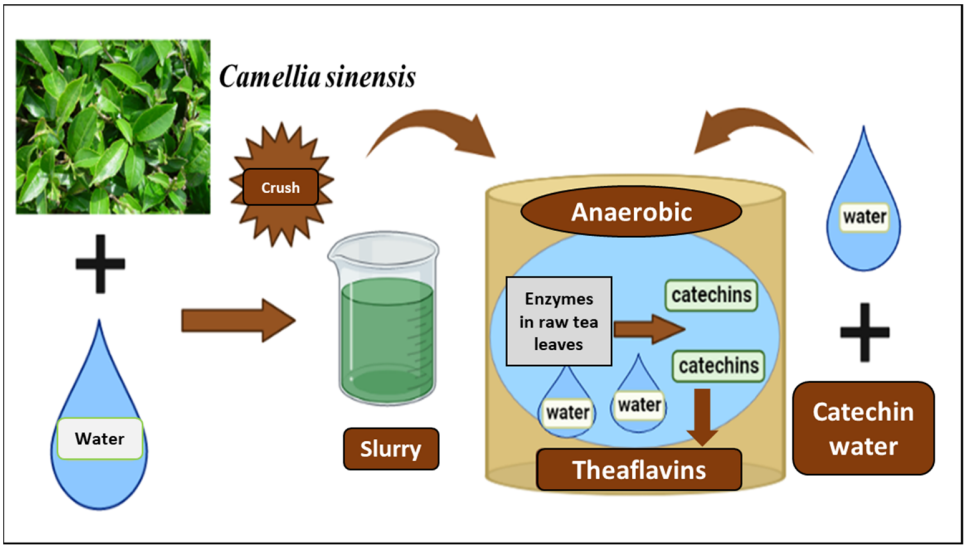
TFs-enriched tea leaf extract TY-1

In this research, the powder of TFs-enriched tea leaf extract TY-1 was provided by Yokoyama Food Co., Ltd. (Hokkaido, Japan). TY-1 was produced from the raw tea leaves according to the method described by Takemoto (2011). Briefly, the fresh tea leaves were mixed with water and then crushed into a slurry with a mixer (Figure 1a). This step liberated catechins, tannase, polyphenol oxidase, hydrolase, caffeine, peroxidase, and other compounds from the tea leaves into the water. Thereafter, the tea slurry was slightly stirred for 10 min–8 h with minimum aeration, which enhanced the interaction between the slurry components and various enzymes; hence the alteration of catechins into gallic acid and TFs took place. For sterilization, the slurry was further heated, followed by a collection of the liquid layer, which was further filtered and dried (Figure 1b). To assess the safety of TY-1, mutagenicity and genotoxicity tests were conducted through the investigation of multiple tests such as the comet assay, the ames test, and the micronucleus test. Thereafter, TY-1 components and their related yields were analyzed by Eurofins Food Testing JP., K.K (Shizuoka, Japan), and are listed in Table 1. Specfically, TFs concentration was measured by high-performance liquid chromatography; meanwhile, the amount of dietary fiber and total polyphenol were quantified by Prosky and the Folin–Ciocalteu methods, respectively. Then, the concentration of catechins, gallic acid, theanine, and caffeine was investigated by liquid chromatography–tandem mass spectrometry. TFs and their derivatives' chemical structures were illustrated according to Chen et al. (2012) and shown in Figure 2.

Table 1. Chemical constituents of 5 mg of TY-1 powder

Item		Value
Total TFs (0.083 mg)	Theaflavin (TF1)	0.056 mg
	Theaflavin-3-gallate (TF2A)	0.015 mg
	Theaflavin-3′-gallate (TF2B)	0.007 mg
	Theaflavin-3,3-digallate (TF3)	0.005 mg
Total catechins (0.034 mg)	Epicatechin (EC)	0.017 mg
	Epigallocatechin (EGC)	0.003 mg
	Epigallocatechin gallate (EGCG)	0.013 mg
	Epicatechin gallate (ECG)	0.001 mg
Caffeine		0.09 mg
Theanine		0.065 mg
Gallic acid		0.052 mg
Total polyphenols		0.8 mg
Dietary fiber		0.22 mg
Dextrin		2.5 mg

(a)



(b)

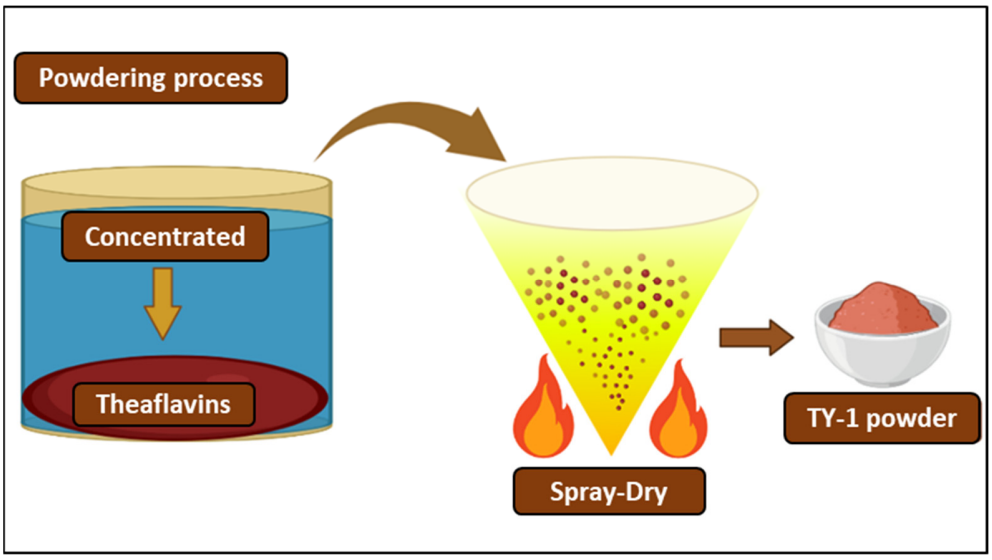


Figure 1. Schematic diagram showing the process of TY-1 powder production from raw green tea leaves.

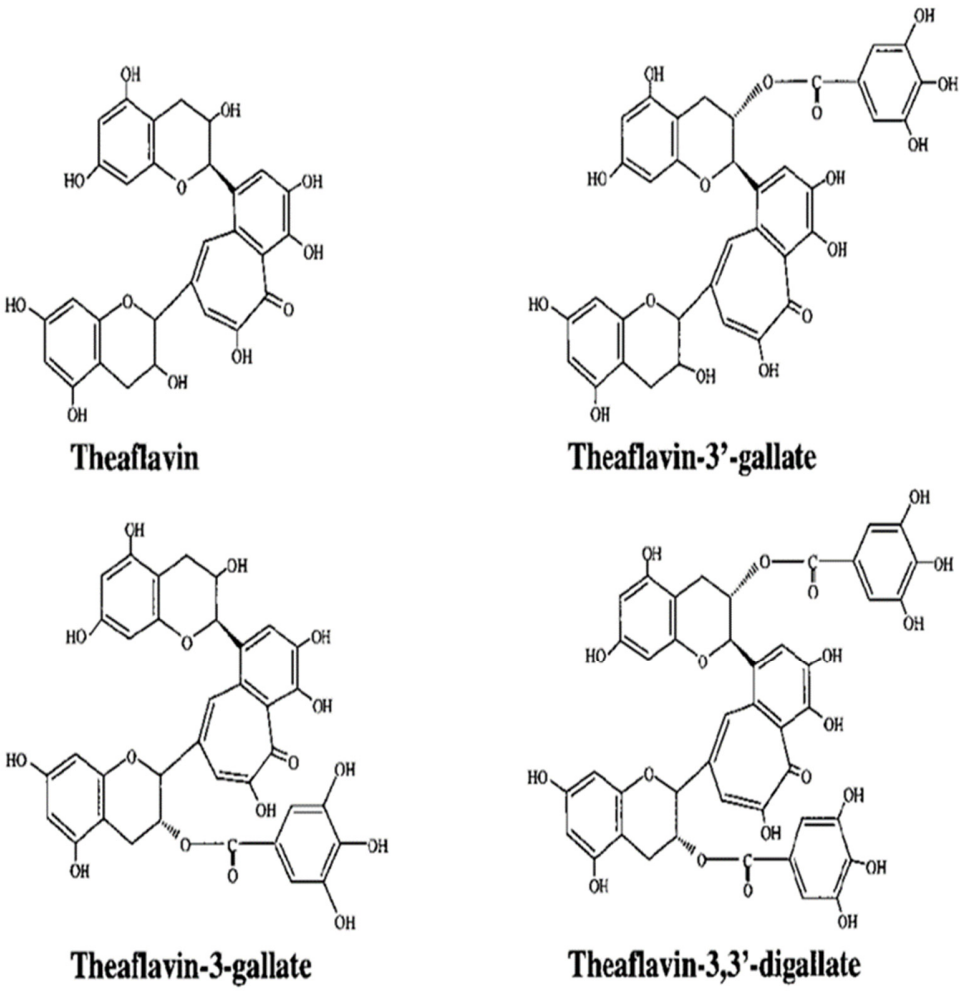


Figure 2. The chemical structures of TF1, TF2A, TF2B, and TF3 (Chen et al., 2012).

Objectives and chapter structures

The present study consists of 2 chapters:

Chapter I: Describes the *in vitro* virucidal efficacy of the TFs-enriched tea leaf extract TY- 1 against IAV as an example of enveloped-RNA viruses. Additionally, the possible mechanism of the TY-1 virucidal activity was elucidated via the hemagglutination assay, NA assay, Western blotting (WB), reverse transcription polymerase chain reaction (RT-PCR), and the observation of viral particules using transmission electron microscope (TEM).

Chapter II: Gives insight into the virucidal efficacy of TY-1 against FCV and MNV as surrogate viruses of HuNoV, as an example of non-enveloped-RNA viruses. Moreover, the inactivating action of TY-1 against FCV and MNV was investigated using WB, RT-PCR, and TEM.

Chapter I

The virucidal efficacy of the theaflavins-enriched tea extract TY-1 against influenza A virus: *In vitro* study

1.1. Introduction

The causative agent of all influenza pandemics in the past was IAV only, and the world witnessed, within the past 100 years, 4 pandemics by novel IAV strains which left 500,000–50 million deaths (Saunders-Hastings and Krewsk1, 2016). Applying the efficient prophylaxis and antiviral therapies are highly recommended for preventing and controlling such highly infectious and contagious diseases. Vaccination has been recognized as one of the most common and effective procedures for preventing IAV infection. Nevertheless, because of the antigenic drift and the antigenic shift, IAV can evade the neutralizing activity of previously established antibodies, causing them to become ineffective against next-year infection (Scorza et al., 2016). On the other hand, the therapeutic effectiveness of antiviral drugs prompted their use; however, the appearance of resistant IAV strains and the side effects of some medicines are regarded as a matter of concern (Morimoto et al., 2015; Omoto et al., 2018). Accordingly, the availability of daily intervention strategies seems to be urgent for preventing IAV infection. Such preventive strategies include commonly recommended measures such as hand washing or wearing masks (Aiello et al., 2010). Even though it is practically feasible, hand washing could only eliminate viruses from primitive contact places, and it becomes further effective when combined concomitantly with other virucidal agents. Currently, the virucidal agents stockpile markets are enriched with numerous virucidal agents (Lin et al., 2020); however, some of these chemical substances exhibit toxic effects and corrosive action causing environmental

pollution, thus limiting their practical use as virucidal agents on the human body and environmental surfaces (De Benedictis et al., 2007).

As a result of the limited efficacy of vaccination, the emergence of drug resistance against novel IAV strains, and the insufficiency of safety and durability of currently available chemical virucidal agents, having supportive safe strategies is crucial to prevent IAV infection and decrease its severity.

Tea polyphenols have gained research attention as a natural-origin and safe compound for therapeutic purposes for multiple diseases. For example, it was reported that ECG, EGC, and EGCG extracted from green tea suppressed the infectivity and replication of influenza virus and adenovirus *in vitro* (Nakayama et al.,1993; Song et al., 2005; Weber et al., 2003). Black tea, represents 78% of the consumed tea worldwide (Yang et al., 2009), and is known as a rich source of TFs, which is a polyphenol processed via the dimerization and enzymatic oxidation of catechins. The antiviral (Cantatore et al., 2013; Liu et al., 2005; Zu et al., 2012), antimicrobial (Friedman, 2007), and anti-inflammatory (Zu et al., 2012) activities of TFs have been widely investigated. Therefore, this study was focused on exploring the virucidal efficacy of TY-1, which contains abundant TFs and other polyphenols, against IAV as a representative of enveloped-RNA viruses. Thereafter, the virucidal mechanism of action of TY-1 against IAV was further invistaged aiming to introduce TY-1 as a promising virucidal supplement or alternative to the currently available virucidal agents.

1.2. Materials and methods

1.2.1. Virus and cells

H1N1 subtype IAV (A/Puerto Rico/8/1934 strain: ATCC® Catalog No. VR-95TM) was purchased from ATCC (Manassas, VA, USA). The virus was inoculated into the allantoic cavity of a 10-day-old chicken egg embryo for viral propagation. Subsequently, the allantoic fluids comprising IAV were used as viral solutions in some experiments in this study. In other experiments, the purified virus was also used. Accordingly, for the purification of IAV, sucrose gradient ultracentrifugation was done, as previously described (Imai et al., 2012). Madin-Darby canine kidney (MDCK) cells were obtained from Dr. Hideki Nagano (Hokkaido Institute of Public Health, Sapporo, Hokkaido, Japan). For MDCK cell culture, Dulbecco's modified Eagle's minimal essential medium (DMEM: Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) was used as the growth medium, and it was supplemented with 0.15% $NaHCO_3$, 10% fetal bovine serum, 2 mM L-glutamine, (FUJIFILM Wako Pure Chemical Co., Osaka, Japan), 100 μg/mL kanamycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), and 2 μg/mL amphotericin B (Bristol-Myers Squibb Co., New York, NY, USA). After viral inoculation, DMEM supplemented with 0.2% bovine serum albumin, 0.01% glucose, 2.5 mM HEPES, 0.0006% trypsin, and 0.15% $NaHCO_3$ (FUJIFILM Wako Pure Chemical Co.) was used as viral growth medium (VGM) for MDCK cell culture.

1.2.2. Preparation of a TY-1 stock solution and other test solutions

For the preparation of stock solution from TY-1 powder, 1 g of TY-1 powder was dissolved in 100 mL of phosphate-buffered saline (PBS) and centrifuged at 9000 × g for 10 min. Thereafter, using a 0.2 μm syringe filter (Sartorius AG, Gottingen, Germany), the water-soluble layer was filtered, and the filtered solution was stored at −80°C until use. As dextrin represents 50% of TY-1 powder chemical composition, 50% dextrin solution was used as a solvent control in all the experiments. This solution was prepared by dissolving 0.5 g of dextrin powder in 100 mL PBS. Thereafter, using a 0.2 μm syringe filter, the water-soluble layer was filtered and the filtered solution was stored at −80°C until use. The measured pH for TY-1 and dextrin solutions was 6.8 and 7.1, respectively. TY-1 and dextrin stock solutions' final concentrations were 10 and 5 mg/mL, respectively. To prepare 0.165 mg/mL of TFs solution, 0.010 mg of TF3, 0.043 mg of TF2B, 0.112 mg of TF1 (FUJIFILM Wako Pure Chemical Co.), and 5.000 mg of dextrin were dissolved in 1 mL PBS. To prepare 0.067 mg/mL of catechins solution, 0.067 mg of catechins mixture (FUJIFILM Wako Pure Chemical Co.) and 5.000 mg of dextrin were dissolved in 1 mL PBS. Additionally, for the preparation of 0.232 mg/mL of TFs+catechins mix solution, 0.010 mg of TF3, 0.043 mg of TF2B, 0.112 mg of TF1, 0.067 mg of catechin mixture, and 5.000 mg of dextrin were also dissolved in 1 mL PBS, and all the prepared solutions were centrifuged, filtered and stored at −80°C until use.

1.2.3. Evaluation of TY-1 virucidal efficacy

The virucidal efficacy of TY-1 against IAV was evaluated as follows. The viral solutions were mixed with 5 different concentrations of TY-1 and 1 concentration of dextrin solution. The final concentrations in the mixture were 0.3, 0.6, 1.3, 2.5, and 5.0 mg/mL of TY-1 and 2.5 mg/mL of

dextrin. Additionally, the viral solution was mixed with 1 concentration of each TFs solution, catechins solution, and TFs+catechins solution. The final concentrations of the TFs, catechins, and TFs+catechins were 0.083, 0.034, and 0.116 mg/mL, respectively in the mixture. The IAV titer in all the mixtures was approximately 7.45 $\log_{10}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL. Then, these mixtures were incubated at 25°C for various reaction times (10 min–24 h). Subsequently, 10-fold serial dilutions were performed following inoculation of the mixtures into MDCK cells. Three days later, by detecting the cytopathic effects (CPE) on the cells, the viral titers of test solution-treated virus were evaluated and calculated using the Behrens-Karber method (Karber, 1931). The virucidal activity of each test solution represents the difference in viral titer between each test solution-treated group and the dextrin-treated group. Furthermore, the detection limit of viral titers in all groups was calculated based on the cytotoxicity in a virus-free condition of each test solution in MDCK cells. Briefly, MDCK cells were cultured in trypsin-free VGM in the presence of various concentrations of TY-1 and one concentration of each of TFs, catechins and dextrin. The final concentrations of TY-1 in the medium were ranged from 0.3 to 5.0 mg/mL. Meanwhile, the final concentration of TFs, catechins, and dextrin in the medium were 0.083, 0.034, and 2.5 mg/mL, respectively. After 3 days incubation at 37℃, the cytotoxic concentrations of the tested solutions were estimated using Cell Titer-Glo® Luminescent Cell Viability Assay (Promega Co., Madison, WI, USA). The luminescence was analyzed with Glomax®-Multi+ Detection System (Promega Co.). Bsed on the cytotoxic concentration of each test solution, the detection limit of the viral titers in the TY-1 groups of 2.5 and 5.0 mg/mL was set to be 2.25 $\log_{10}$ $TCID_{50}$/mL. Meanwhile, the detection limit of the viral titers in the 0.3, 0.6, and 1.3 mg/mL groups of TY-1, as well as the detection limit of the viral titers in the 0.083 mg/mL of TFs, 0.034 mg/mL catechins, 0.116 mg/mL TFs+catechins, and 2.5 mg/mL of dextrin groups was set to be 1.25 $\log_{10}$ $TCID_{50}$/mL.

1.2.4. SDS-PAGE

The mixtures containing purified IAV (6.58 $\log_{10}$ $TCID_{50}$/mL) and either 5.0 mg/mL of TY-1 or 2.5 mg/mL of dextrin were placed at 25°C for 48 h. After that, the one-third volume of 4 × SDS sample buffer with and without 2-mercaptoethanol (2-Me) (FUJIFILM Wako Pure Chemical Co.) was combined with the mixtures, then SDS-PAGE was performed on these samples, followed by either coomassie brilliant blue (CBB) staining or WB, as previously mentioned (Takeda et al., 2020). Briefly, rabbit anti-H1N1 (A/Puerto Rico/8/34) HA polyclonal antibody (Sino Biological Inc., Beijing, China: Catalog No. 11684-T62) and mouse anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich, Inc., St. Louis, MO, USA: Catalog No. A1949, Clone: RG-96) were utilized as the primary and secondary antibodies, respectively to detect IAV HA protein in WB. Meanwhile, rabbit anti-H1N1 NA (Gentex Inc., Zeeland, MI, USA: Catalog No. GTX125974) and mouse anti-rabbit IgG peroxidase conjugate were used as the primary and secondary antibodies, respectively, to detect NA of IAV in WB.

1.2.5. Hemagglutination and NA assays

The mixtures containing purified IAV (7.25 $\log_{10}$ $TCID_{50}$/mL), and 5.0 mg/mL of TY-1 or 2.5 mg/mL of dextrin were prepared and kept at 25°C for 48 h. Then, the hemagglutination and NA assays were performed according to the WHO manual on animal influenza diagnosis and surveillance (World Health Organization, 2002).

1.2.6. RT-PCR analysis

The mixtures of purified IAV (7.25 $\log_{10}$ $TCID_{50}$/mL) mixed with either 5.0 mg/mL of TY-1 or 2.5 mg/mL of dextrin were prepared and kept at 25°C for 48 h. Following the incubation, extraction of RNA using ISOGEN-LS (Nippon Gene Co., Ltd., Tokyo, Japan) was performed. Thereafter, using the FastGene cDNA Synthesis 5 × ReadyMix OdT (NIPPON Genetics Co, Ltd., Tokyo, Japan), the extracted RNA was reverse transcribed, and the PCR using the GoTaq® Green Master Mix (Promega Co.) was performed as previously described (Takeda et al., 2020). The primer sequences targeting the M gene of IAV and each PCR condition used in this experiment are listed in Table 2.

1.2.7. TEM observation

The mixtures of purified IAV (6.58 $\log_{10}$ $TCID_{50}$/mL) mixed with either 5.0 mg/mL of TY-1 or 2.5 mg/mL of dextrin were prepared and kept at 25°C for 48 h. Thereafter, a two-step protocol for negative staining was performed as described by Imai et al. (2012). Specifically, the TEM samples were loaded on a 400-mesh carbon-coated collodion grid (NISSHIN EM Co., Ltd., Tokyo, Japan), and the treated IAV was negatively stained using 2% phosphotungstic acid (pH 6.5) for 2 min. The samples were observed under TEM (HT7700; Hitachi High-Tech Co., Tokyo, Japan).

1.2.8. Statistical analysis

In each experiment performed, a Student's *t*-test was conducted to determine statistically significant differences among the TY-1 and dextrin groups to evaluate the viral and hemagglutination titers. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted to determine statistically significant differences among the viral titers of the TY-1, TFs, catechins, TFs+catechins, and dextrin groups. P values less than 0.05 specified the significant difference among the chosen items. Student's *t*-test analyses were conducted using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA). One-way ANOVA followed by Tukey's post hoc test was conducted using Graphpad Prism version 7 (GraphPad Software, La Jolla, CA, USA).

1.3. Results

1.3.1. TY-1 virucidal efficacy against IAV

First, the virucidal efficacy of TY-1 against IAV was evaluated using different concentrations of TY-1. Within 24 h of reaction time, all the concentrations of TY-1 tested (0.3, 0.6, 1.3, 2.5, 5.0 mg/mL) showed statistically significant IAV-inactivating activities, which were concentration- and time-dependent. Moreover, 5.0 mg/mL of TY-1 exhibited a 1.33 $\log_{10}$ $TCID_{50}$/mL reduction of the viral titer within 30 min compared to the dextrin group. In 6 h, the reduction in the viral titer was ≥ 5.17 $\log_{10}$ $TCID_{50}$/mL, which led to below the detection limit in this group (Figure 3a). The concentration of TY-1 that did not exhibit a significant reduction in the viral titer at certain contact times was not evaluated for the subsequent shorter reaction times. The 5.0 mg/mL of TY-1 contains 0.034 mg/mL of catechins and 0.083 mg/mL of TFs (Table 1). To evaluate the contribution of catechins and TFs, which are two of the main virucidal components of TY-1, in IAV inactivation, the virucidal activity of 5.0 mg/mL TY-1 was compared to that of 0.034 mg/mL catechins solution, 0.083 mg/mL TFs solution, and 0.116 mg/ mL TFs+catechins solution. As a result, the viral titers of dextrin, catechins, TFs, TFs+catechins, and TY-1 groups were 6.88, 6.56, 5.88, 5.88, and ≤ 3.06 $\log_{10}$ $TCID_{50}$/mL, respectively, after 3 h treatment. Additionally, the viral titers of the dextrin, catechins, TFs, TFs+catechins, and TY-1 groups were 6.13, 5.94, 4.88, 4.38, and ≤ 2.25 $\log_{10}$ $TCID_{50}$/mL, respectively, after 24 h treatment. With this reaction time, the viral titer of the TFs group was partially lower than that of the dextrin group, but that of the TY-1 group was below the detection limit. Although the viral titer of the catechins group was comparable to that of the dextrin group, that of the TFs+catechins group was slightly but significantly lower than that of the TFs group (Figure 3b). The

5.0 mg/mL TY-1 treatment, which exhibited the strongest virus-inactivating activity, was further tested against IAV to elucidate the mechanism of its virucidal activity.

1.3.2. Impact of TY-1 on IAV proteins

The impacts of the TY-1 treatment on the functions and structures of IAV structural proteins were analyzed. Here, a purified IAV was used to eliminate the influence of nonviral proteins. After treatment with 5.0 mg/mL TY-1 for 48 h, the viral titer decreased to below the detection limit compared to that in the dextrin group (Figure 4a). The impact of the TY-1 treatment under the same conditions on the hemagglutination activity of the HA protein was evaluated by a hemagglutination assay. The hemagglutination titer of IAV treated with 5.0 mg/mL TY-1 for 48 h was significantly lower than that of the dextrin group (Figure 4b). Subsequently, the impacts of TY-1 on the structures of viral proteins were analyzed with SDS-PAGE. As a result, CBB staining showed the reduction of intensity for some viral protein bands in TY-1-treated IAV. Such changes were observed for the viral HA0 protein detected in the absence of 2-Me and for HA1 and HA2 in the presence of 2-Me, and all the changes were tentatively recognized according to their apparent molecular mass. Contrarily, the intensities of bands which were presumed to be the M1 and NP proteins were not affected by TY-1 exposure (Figure 4c). To further evaluate the impact of TY-1 on viral HA proteins, WB was performed using a primary antibody that detects the HA protein of IAV. In line with the CBB staining results, TY-1 treatment strongly reduced the intensity of the ~60 kDa HA0 protein band. Additionally, the intensities of additional > 75 kDa bands/ladder were stronger after the TY-1 treatment than the dextrin treatment (Figure 4d-left). While the intensities of the HA1 and HA2 bands were weaker following the TY-1 treatment than the dextrin treatment, the intensities of additional bands/ladder

with a higher molecular mass were stronger after the TY-1 treatment than the dextrin treatment (Figure 4d-right). Meanwhile, the effect of TY-1 on the NA activity and the structure of the NA protein were analyzed by NA assay and WB, respectively. TY-1-treated IAV displayed substantially lower NA activity than dextrin-treated IAV (Figure 5a). Accordingly, the results of WB using an antibody against the NA protein of IAV revealed disappearance of the NA protein band in TY-1-treated IAV, whereas it was clearly identified in dextrin-treated IAV (Figure 5b).

1.3.3. Impact of TY-1 on the viral genome and viral particle morphology of IAV

The impact of TY-1 on the genome of IAV was evaluated with RT-PCR, in which three diverse regions of the IAV M gene were amplified to produce amplicons of various lengths using the designated primers for this study (Table 2). As a result, no PCR products were detected following the 48 h treatment with 5.0 mg/mL TY-1; meanwhile, the PCR products were clearly detected in the dextrin-treated IAV (Figure 6). Additionally, TEM obserbation of IAV particles treated with 5.0 mg/mL TY-1 for 48 h was performed. The results revealed that the number of IAV particles was drastically reduced after TY-1 treatment compared to the IAV treated with dextrin group. Nevertheless, TY-1-treatment did not induce clear morphorogical abnormalities in IAV particles compared to the dextrin-treated group (Figure. 7a, b).

1.4. Discussion

In recent decades, natural products have been widely investigated as alternatives to conventional treatments for several diseases. Medicinal plants were an example of these natural products, which could provide an effective and safe therapeutic option for many diseases (Dattner, 2003; Pan et al., 2013). Specifically, among medicinal plant extracts, tea might be the most consumed beverage worldwide with historical background (Martin and Cooper, 2011). In this study, the virucidal efficacy of TY-1, a natural tea leaf extract, against IAV was evaluated. TY-1 exhibited concentration- and time-dependent IAV-inactivating efficacy, in line with SARS-CoV-2-inactivationg activity (Takeda et al., 2021b). TY-1 not only contains considerable amounts of TF derivatives, gallic acid, and catechins but also an abundant amount of other polyphenols. Although TY-1 contains theanine and caffeine (Table 1), the virucidal activity of caffeine against any enveloped virus has not been reported (Utsunomiya et al., 2008), and there have been no reports proving obvious virucidal activity of theanine. On the contrary, polyphenols, particularly TFs and catechins, are extensively investigated and found to exhibit the virucidal efficacy against several types of viruses (Chowdhury et al., 2018; Clark et al., 1998; Lee et al., 2018; Nakayama et al., 1993; Ohba et al., 2017; Ohgitani et al., 2021). Results obtained in this study showed that TFs partially contributed to the virucidal potential of TY-1. Inrerestingly, the TFs+catechins treatment exhibited the additive inactivation of IAV compared to that of the TFs treatment alone. However, catechins' contribution to the inactivation of IAV was limited (Figure 3b). Accordingly, these finding suggests that not only TFs but also catechins and other polyphenols included contribute to the comprehensive virucidal activity of TY-1 against IAV. From an economic point of view, the possible application of TY-1 could be a feasible approach to control IAV infection as it will be expensive to prepare large quantities of pure TFs and other compounds with similar bioactive levels of TY-1. The data obtained in the hemagglutination assay, NA assays

and SDS-PAGE showed the destruction of both HA and NA proteins of IAV by TY-1 treatment (Figures 4 and 5). Specifically, the target sites of TY-1 in the HA and NA proteins likely included regions that are involved in hemagglutination activity and NA activity, which may be related to the virucidal activity of TY-1. Moreover, the impact of TY-1 on the HA protein is consistent with previous reports in which TFs, ECG, and EGCG inhibited the hemagglutination activity of IAV (Nakayama et al., 1993; Song et al., 2005; Zu et al., 2012). Likewise, the effect of TY-1 on the NA protein is also consistent with previous reports in which TFs, ECG, and EGCG inhibited NA activity (Muller and Downard, 2015; Song et al., 2005; Zu et al., 2012). As a result of WB targeting HA protein, it was found that TY-1-induced reduction of the band intensity of HA0, which consists of HA1 and HA2 subunits, and seemed to be greater than the reduction of the band intensities of individual HA1 and HA2 subunits. Furthermore, it was observed that TY-1 treatment enhanced the presence of high molecular mass ladders/bands of the HA proteins (Figure 4d). These ladders/bands are considered as HA aggregates or HA multimers. Similarly, high molecular mass ladders/bands of some viral spike proteins have been detected with the treatment by other polyphenolic compounds (Takeda et al., 2021a). Additionally, it has been reported that polyphenols could interact with proteins in either covalent or non-covalent interactions, which enhances the cross-linking of proteins and hence the development of a high molecular mass complex (Ozdal et al., 2019). Accordingly, our data proposes that the polyphenolic compounds present in TY-1 may interact with some regions of both HA1 and HA2 subunits, hence the aggregation of these proteins was induced. Furthermore, the reduction of the band intensity of HA0, which possesses multiple sites of action present in both HA1 and HA2 subunits, was stronger than those of separate subunits. On the contrary, the result of WB targeting IAV NA protein revealed that TY-1 treatment did not induce the appearance of such high molecular ladders/bands despite the complete disappearance of the NA band (Figure 5b). These differences in the TY-1-treated HA and NA proteins band patterns may be explained due to the

differences in the amino acid sequences and secondary/tertiary constructions of both HA and NA proteins. TEM observation performed in this study revealed a substantial decrease in the number of IAV particles after TY-1 treatment, suggesting that TY-1 might damage the virions and subsequently inhibited viral adsorption onto and penetration into host cells. Moreover, it was also observed that TY-1 treatment disrupted IAV genomes. This result was consistent with other polyphenol-enriched plant extracts that induced the destruction of the RNA of various virus species (Takeda et al., 2020; Takeda et al., 2021a; Takeda et al., 2021b).

Overall, the results in this study propose that the virucidal mechanism of TY-1 could be attributed to numerous factors rather than a single action. Some specific chemical structures of compounds contained in TY-1 could contribute to the virucidal activity of TY-1; for example, it has been reported that the number of galloyl groups of TFs and catechins seems to have a direct relation to their virucidal efficacy (Liu et al., 2005; Quosdorf et al., 2017; Song et al., 2005).

For the future application of TY-1, it may be recommended to be applied as a virucidal troche or mouthwash. This recommendation is supported by previous reports suggesting that gargling with black tea and GTE reduced the incidence of influenza (Ide et al., 2014; Iwata et al., 1997; Yamada et al., 2006). Furthermore, Lee et al. (2012) found that water and diet comprising green tea components suppressed influenza virus replication in animal experiments.

1.5. Conclusion

This study showed TY-1, a tea leaf extract enriched with TFs and other polyphenols, exhibited a concentration- and time-dependent IAV virucidal efficacy. Of note, TY-1 destroyed the spike proteins and genome of IAV in addition to destruction of viral particles. In advance, these findings suggest the potential contribution of TY-1 as a virucidal agent for the prevention and control of IAV infection.

Table 2. The primers sequences and the RT-PCR conditions utilized in this study targeting M gene of IAV

Primers mane	Primer sequences	RT-PCR condition
IAV-Primer set 1 (982 bp)	Fwd: 5′-ATGAGTCTTCTAACCGAGGTC-3′ Rev: 5′-GTCAGCATAGAGCTGGAGTAA-3′	95°C for 5 min ↓ [95°C for 30 sec, 52°C for 30 sec, 72°C for 1min] x 25 times ↓ 72°C for 10 min
IAV-Primer set 2 (253 bp)	Fwd: 5′-AAGACCAATCCTGTCACCTC-3′ Rev: 5′-CAGTTGTATGGGCCTCATATAC-3′	95°C for 5 min ↓ [95°C for 30 sec, 52°C for 30 sec, 72°C for 1min] x 22 times ↓ 72°C for 10 min
IAV-Primer set 3 (320 bp)	Fwd: 5′-ACAGATTGCTGACTCCCA-3′ Rev: 5′-TGATCCTCTCGCTATTGCC-3′	95°C for 5 min ↓ [95°C for 30 sec, 52°C for 30 sec, 72°C for 1min] x 20 times ↓ 72°C for 10 min

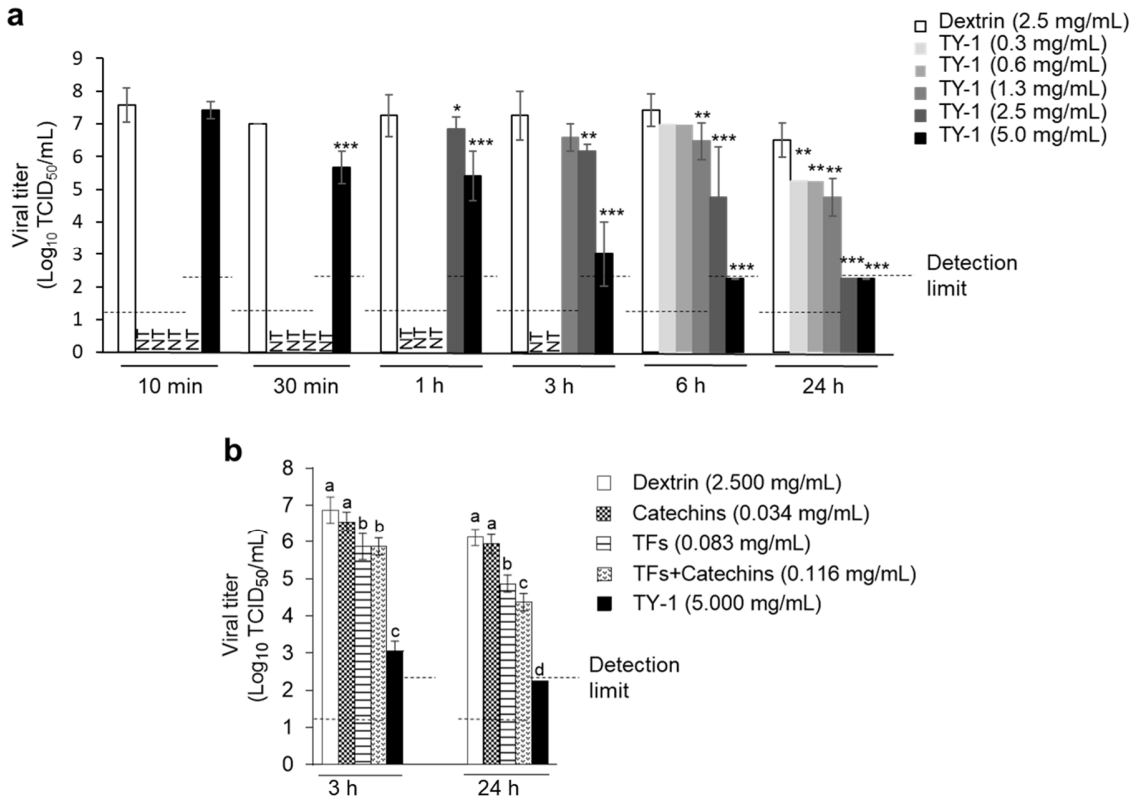


Figure 3. Evaluation of TY-1 virucidal impact at several concentrations and contact times. (a, b**)** Dextrin (2.5 mg/mL) was used as a solvent control. The results are shown as the mean ± SD ($n = \geq 4$ per group). (a) Student's *t*-test was done to analyse the statistical differences between each TY-1 group and the dextrin group: *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$. NT: not tested. (b) To analyze the statistical differences between all test solution groups, ANOVA proceeded by Tukey's post hoc test was done; test solution groups of different letters were considered significantly different (*$p < 0.05$).

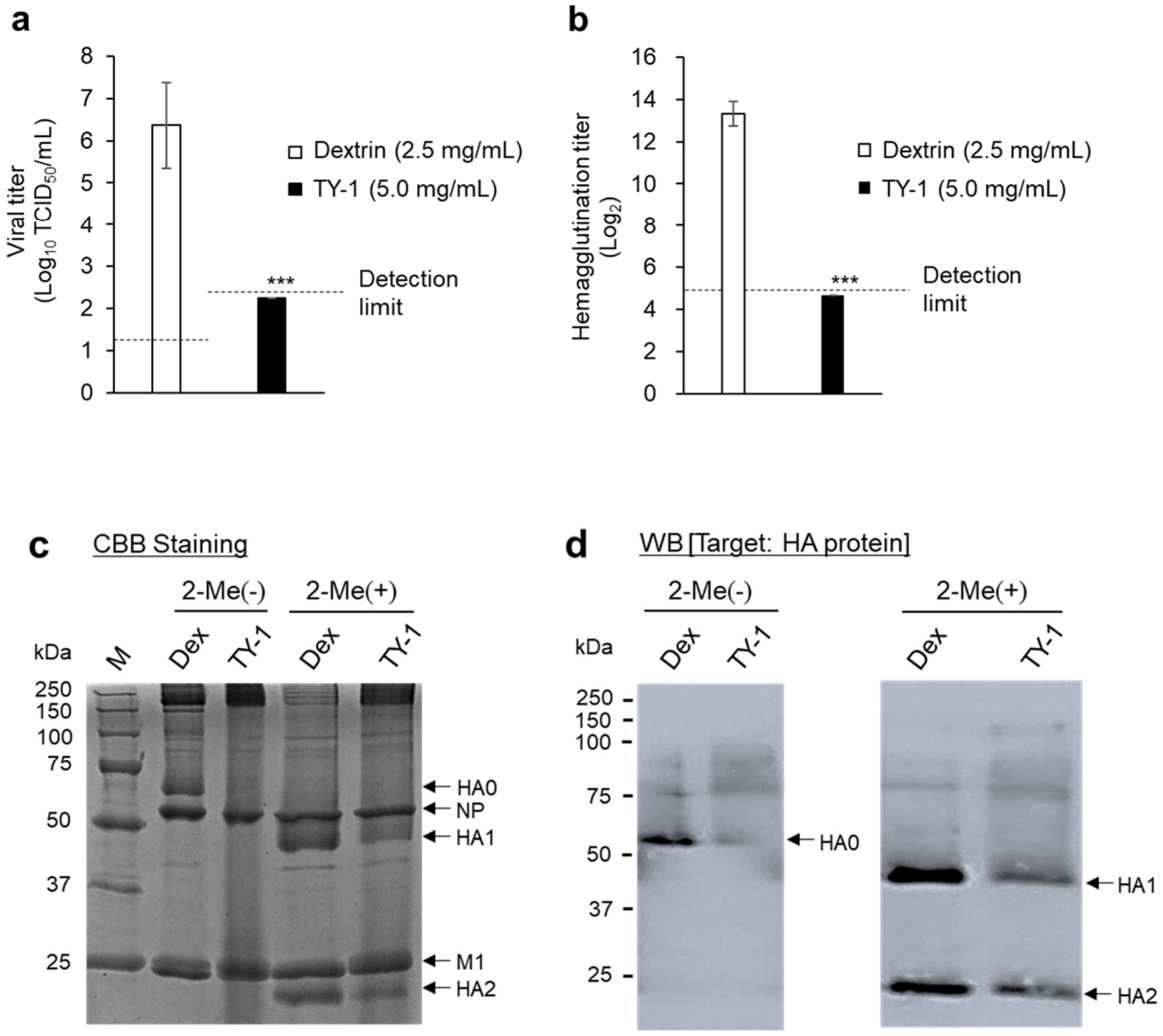


Figure 4. Evaluation of the impact of TY-1 on IAV HA protein. TY-1 and dextrin solutions were mixed with the purified IAV solution and kept at 25°C for 48 h. (a) The viral titer of the mixture was evaluated. The results are presented as the mean ± SD (n = 3 per group). (b) The mixtures' hemagglutination titers were evaluated. The results are presented as the mean ± SD (n = 3 per group). Student's t-test was achieved to analyze statistical differences between the TY-1 and dextrin groups: ***$p < 0.001$. (c) CBB staining of the gel from SDS-PAGE. Dex: dextrin, 2-Me: 2 mercaptoethanol, M: Marker. (d) Results of WB to detect IAV HA proteins. (c, d) SDS-PAGE without or with 2-Me was achieved to evaluate the bands of HA0 or HA1/HA2. The results are representative of three individual experiments.

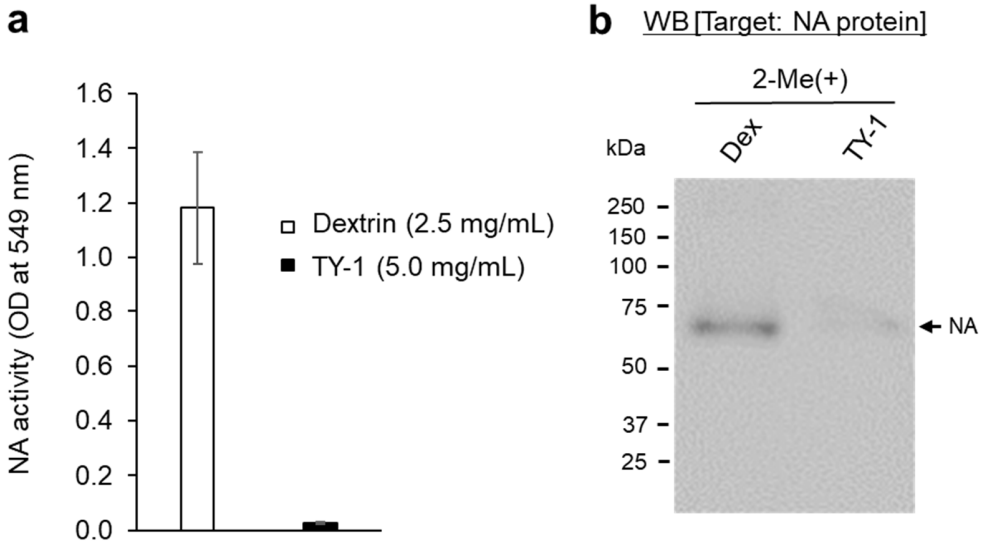


Figure 5. Evaluation of the impact of TY-1 on IAV NA protein. TY-1 and dextrin solutions were mixed with the purified IAV solution and kept at 25ºC for 48 h. (a) The NA activity of the mixture was evaluated. The results are presented as mean ± SD (n = 3 per group). (b) The result of WB for IAV NA detection is shown. Dex: dextrin, 2-Me: 2 mercaptoethanol. The results represent two independent experiments.

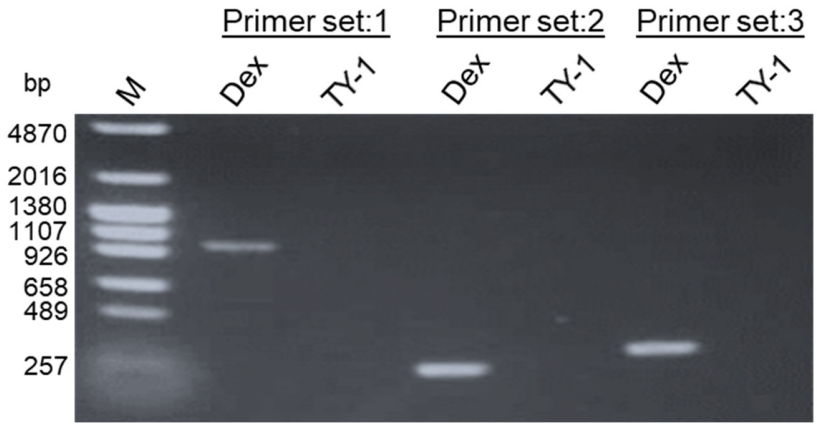


Figure 6. Impact of TY-1 on the IAV genome. TY-1 and dextrin solutions were mixed with the purified IAV solution and kept at 25°C for 48 h. The extracted viral RNA from the mixtures was used for the RT-PCR. Using IAV-Primer sets 1, 2, and 3. RT-PCR was performed to amplify 982, 253, and 320 bp of the M gene of IAV, respectively. The results are representatives of more than two individual experiments. M: marker, Dex: dextrin.

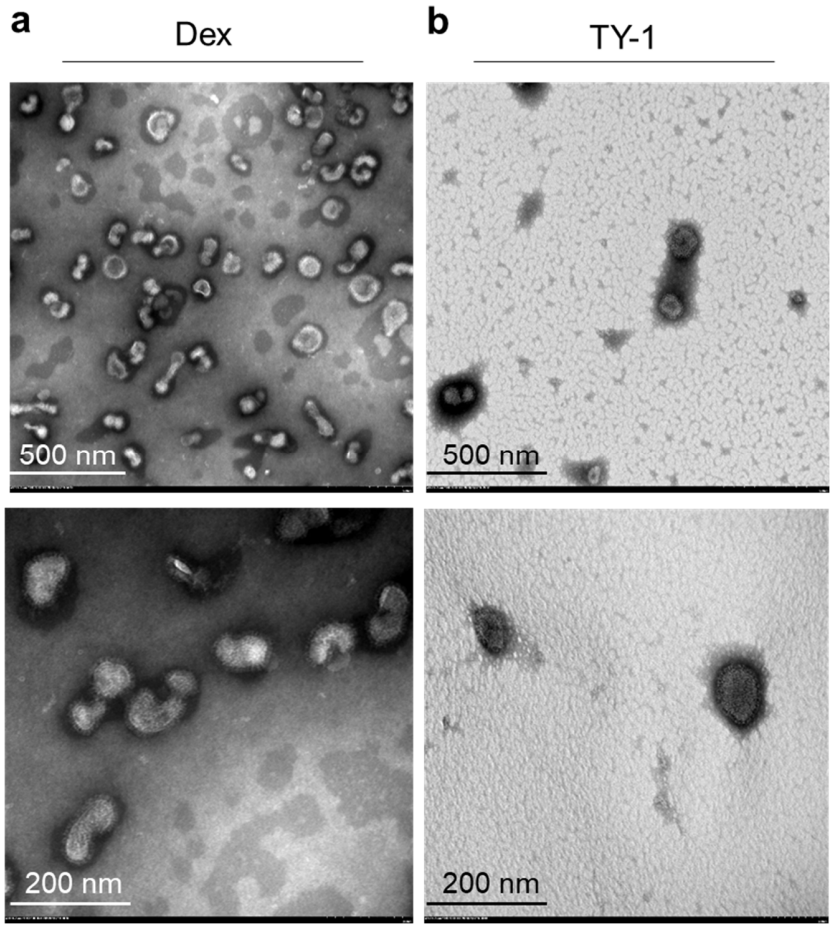


Figure 7. TEM of TY-1-treated IAV virions. Dextrin (a) and TY-1 (b) solutions were mixed with the purified IAV solution and kept at 25°C for 48 h. Subsequently, the IAV particles were observed under TEM. The results are representative of TY-1- and dextrin-treated viral particles within a single 6.25 μm^2 field and 0.52 μm^2 field of the upper and lower panel, respectively. Dex: dextrin.

Chapter II

In vitro virucidal efficacy of theaflavins-enriched TY-1 tea leaf extract against human norovirus surrogates

2.1. Introduction

In humans, infection with enteric food-borne viruses represents the primary cause of acute gastroenteritis (AGE) (Bányai et al., 2018). Amongst the food-borne viruses, in low- and high-income countries, HuNoV causes about one-fifth of AGE cases, leading to over 200,000 deaths per year between all age categories (Ahmed et al., 2014; Atmar et al., 2018; Bányai et al., 2018). Moreover, the economic losses resulting from HuNoV outbreaks cost approximately 4.2 billion USD annually (Bartsch et al., 2016). HuNoV likely could persist on hands, environmental surfaces, and inanimate objects for a considerable time. Specifically, after 2 h of incubation, the RNA of HuNoV can be detected on the finger pads of humans (Liu et al., 2009), and after ≥ 28 days on environmental surfaces (Djebbi-Simmons et al., 2020; Lamhoujeb et al., 2009). Accordingly, this feature could contribute to the outbreaks and the high prevalence of HuNoV infection detected in closed environments, such as schools, cruise ships, long-term care facilities, and food service foundings (Cook et al., 2016; Lopman et al., 2012). Currently, no approved antiviral drugs or vaccines are available for HuNoV. However, several virucidal chemical compounds have been tested on environmental surfaces for disinfection of HuNoV surrogate viruses (Girard et al., 2016; Magulski et al., 2009; Zonta et al., 2018). Nevertheless, the residual property in the environment and the adversative health impacts of some of these virucidal compounds accounted for their limited application as a daily-use sanitizer. Therefore, it is

necessary to develop effective and environmentally-friendly strategies to inhibit and prevent HuNoV infection.

The difficulty in HuNoV propagation and cultivation in major cell culture systems represents a significant concern that hindering the research on this virus. Meanwhile, HuNoV was successfully cultivated in some recent studies using human enteroids, B cells, and zebrafish larvae model (Duizer et al., 2004b; Ettayebi et al., 2016; Van Dycke et al., 2019). However, some limitations hindered the use of these methods (e.g., low reproducibility, complicated procedures, or lower virus amplification efficiency). The quantitative RT-PCR is regarded as the gold standard for HuNoV detection due to its high sensitivity and specificity for detecting and evaluating the RNA of HuNoV in different clinical and environmental samples (Vinjé, 2015). However, to distinguish between infectious and non-infectious HuNoV, genome-based molecular approaches like quantitative RT-PCR assay cannot be used. Altogether, the deficiency of a well-established cell culture model for HuNoV has hindered the evaluation of the virus infectivity by the plaque or $TCID_{50}$ assays. Accordingly, the assessment of inactivation activities of disinfectants against HuNoV remains mainly dependent on the use of readily available cultivable surrogates with comparatively close genetic and structural similarities to HuNoV, such as the FCV, MNV, and Tulane virus (Duizer et al., 2004a; Kamarasu et al., 2018; Tree et al., 2005; Wobus et al., 2006). FCV belongs to the *Vesivirus* genus, infecting domestic cats and causing respiratory disease. Even though the disease signs are dissimilar from those of HuNoV, FCV was approved for a long time as a surrogate virus by the U.S. Environmental Protection Agency to evaluate the effectiveness of various virucidal agents against HuNoV (United States Environmental Protection Agency, 2017). Meanwhile, in 2003, MNV was first detected when researchers recognized sporadic deaths in severely immunocompromised mice due to the virus infection (Karst et al., 2003). In addition, a further study identified MNV as a new member of the

Nov genus with genetic and structural features similar to those of HuNoV (Wobus et al., 2006). Therefore, in virus inactivation studies, both FCV and MNV have been commonly used as surrogate viruses of HuNoV (Cromeans et al., 2010; Fraisse et al., 2011; Kahler et al., 2010; Nowak et al., 2011; Predmore and Li., 2011).

Many herbal plants are found to be harmless to humans and the environment. As a result, their potential use as hand sanitizers against different pathogens has become a research focus (Daverey and Dutta, 2021; Kalaivani et al., 2018; Malabadi et al., 2021). Thus, the ability of these herbal plants to prevent HuNoV infection should be tested. Accordingly, in this study, the virucidal efficacy of TY-1 against HuNoV surrogates FCV and MNV, as representatives of non-enveloped-RNA viruses, was evaluated. In addition, the virucidal mechanism of TY-1 was investigated, and the possible application of TY-1 to prevent HuNoV transmission and infection was discussed.

2.2. Materials and methods

2.2.1. Viruses and cells

Dr. Ken Maeda (Yamaguchi University, Yamaguchi, Japan) kindly provided the FCV F9 strain, and Crandell-Rees feline kidney (CRFK) cells. Dr. Yukinobu Tohya (Nihon University, Tokyo, Japan) kindly provided the MNV S7 strain. The RAW264 cells were purchased from the RIKEN BRC (Ibaraki, Japan). After FCV and MNV inoculation, CRFK and RAW264 cells were cultivated in the VGM, as previously mentioned (Takeda et al., 2020). In some experiments, purified FCV and MNV solutions were used. For the preparation of purified virus solutions, the supernatant of the virus-inoculated cells were layered on 30% sucrose (Nacalai Tesque Inc., Kyoto, Japan.) in an ultracentrifuge tube (Hitachi Koki Co., Ltd., Tokyo, Japan), and ultracentrifugation was performed for 3 h at 100,000 × g. After that, the pellets of viruses were resuspended in PBS and these purified virus solutions were stored till use at −80ºC.

2.2.2. Preparation of test solutions

The chemical structure of the TY-1 powder, the process of producing of TY-1, the yields of compounds in TY-1, and the preparation methods of test solutions were specifically described in the general introduction and Chapter I.

2.2.3. Evaluation of TY-1 virucidal efficacy against FCV and MNV

The virucidal efficacy of TY-1 against FCV and MNV was investigated by mixing equal volume of the unpurified viral solutions with either 0.6 to 10.0, or 50.0 mg/mL of TY-1 or 5.0 or 25.0 mg/mL of dextrin. The final concentrations of TY-1 and dextrin were 0.3 to 5.0, or 25.0 mg/mL and 2.5 or 12.5 mg/mL in the mixtures, respectively. Furthermore, the virucidal efficacy of the different chemical components present in TY-1 was evaluated by mixing 0.165 mg/mL of TFs, 0.067 mg/mL of catechins, and 0.232 mg/mL of TFs+catechins solutions with equal volume of FCV or MNV solutions. The final concentrations of TFs, catechins, and TFs+catechins were 0.083, 0.034, and 0.116 mg/mL, respectively in the mixture. The viral titers of FCV and MNV were approximately 6.6 and 5.3 $\log_{10}$ $TCID_{50}$/mL in the mixture, respectively. Thereafter, all the mixtures were incubated at 25°C for 10 s to 24 h contact times, and the viral titers ($\log_{10}$ $TCID_{50}$/mL) were measured as mentioned in Chapter I. The cytotoxicity of each test solutions in CRFK and RAW264 cells under a virus-free condition was measured. Briefly, CRFK and RAW264 cells were cultured in VGM in the presence of various concentrations of TY-1, TFs, catechins and dextrin. The final concentrations of TY-1 in the medium were ranged from 0.3 to 5.0 mg/mL or 25.0 mg/mL, and the final concentration of TFs and catechins were 0.083, 0.034 mg/mL, respectively. Meanwhile, the final concentration of dextrin were 2.5 or 12.5 mg/mL. After that the cytotoxic concentrations of the tested solutions were estimated as mentioned in Chapter I. Based on the result of the cytotoxicity test, the detection limit of the viral titers was determined as 1.25 $\log_{10}$ $TCID_{50}$/mL for the solutions containing TFs, catechins, TFs+catechins, dextrin, or 0.3, 0.6, and 1.3 mg/mL TY-1. Meanwhile, the detection limit of the viral titers was 2.25 $\log_{10}$ $TCID_{50}$/mL for the solutions containing 2.5 or 5.0 mg/mL TY-1, and 3.25 $\log_{10}$ $TCID_{50}$/mL for the 25.0 mg/mL TY-1 solution.

2.2.4. Evaluation of TY-1 virucidal efficacy against FCV and MNV on a dry surface

The volume of 0.06 mL of unpurified FCV and MNV solutions were added to six-well plates (Nunc, Rochester, NY, USA). The viral titers of FCV and MNV solutions were set approximately to 6.9 and 5.3 $\log_{10}$ $TCID_{50}$/mL, respectively. Subsequently, 0.1 mL of 5.0 mg/mL TY-1 or 2.5 mg/mL dextrin were applied after complete dryness of the virus solutions over the entire surface of each well, and the plates were kept at 25°C for 10 min. Then, the solutions were harvested, and the viral titers were measured as mentioned in Chapter I.

2.2.5. WB analysis

WB was performed to evaluate MNV VP1 as previously described (Takeda et al., 2020). Briefly, a purified MNV solution (5.6 $\log_{10}$ $TCID_{50}$/mL) was mixed with an equal volume of the TY-1 or dextrin solution to achieve the final TY-1 and dextrin concentration of 5.0 and 2.5 mg/mL, respectively. Thereafter, the one-third volume of a 4 × SDS sample buffer containing 2-Me was added to the mixtures immediately (0 h contact time) or after 24 h incubation at 25°C (24 h contact time). Then, SDS-PAGE was performed, followed by WB analysis using a mouse anti-norovirus (MNV-1) antibody (Merck & Co., Inc., Kenilworth, NJ, USA, Catalog No. MABF2097, Clone: 5C4.10,) and a goat anti-mouse IgG2b cross-adsorbed secondary antibody, Horseradish peroxidase (HRP)-labeled (Catalog No. M32407, Thermo Fisher Scientific Inc. Waltham, MA, USA) to detect the MNV VP1 protein.

2.2.6. RT-PCR analysis

RT-PCR was performed to investigate the TY-1 impact on FCV and MNV genomes as previously mentioned (Takeda et al., 2020). In summary, an equal volume of the TY-1 or dextrin solution was mixed with the purified FCV (7.3 $\log_{10}$ $TCID_{50}$/mL) or MNV (7.3 $\log_{10}$ $TCID_{50}$/mL). The final concentrations of TY-1 and dextrin were 5.0 and 2.5 mg/mL, respectively. For RNA extraction, ISOGEN-LS was added either immediately (0 h contact time) or following 24 h incubation at 25ºC (24 h contact time) to the mixtures. Thereafter, using the FastGene cDNA Synthesis 5 × ReadyMix OdT, the extracted RNA was reverse transcribed, and the PCR was performed using various primers sequences, and the ues of GoTaq® Green Master Mix under specific PCR conditions (Table 3).

2.2.7. Observation of the morphology of FCV and MNV virions using TEM

An equal volume of the TY-1 or dextrin solutions was mixed either with the purified FCV (7.3 $\log_{10}$ $TCID_{50}$/mL) or the purified MNV (7.3 $\log_{10}$ $TCID_{50}$/mL). The final concentrations of TY-1 and dextrin were 5.0 and 2.5 mg/mL, respectively. The mixtures were kept for 6 h at 25ºC. Then, the viral particles in the negatively stained samples were observed using TEM as mentioned in Chapter I.

2.2.8. Statistical analysis

In each experiment performed, a Student's *t*-test was conducted to determine statistically significant differences between the TY-1 and dextrin groups. Furthermore, the Student's *t*-test with Bonferroni correction for multiple comparisons was conducted to determine statistically significant differences among the viral titers of the TY-1, TFs, catechins, TFs+catechins, and dextrin groups. P values less than 0.05 specified the significant difference among the chosen items. These analyses were completed using Microsoft Excel 2013.

2.3. Results

2.3.1. Virucidal efficacy of TY-1 against FCV and MNV

The virucidal efficacy of TY-1 at various concentrations against FCV and MNV was investigated. TY-1 showed the virucidal activity against both viruses in a concentration- and -time dependent manner. Specifically, at 1 min to 24 h contact times, the viral titers of the 1.3, 2.5, and 5.0 mg/mL TY-1-treated FCV solution were significantly reduced compared with that of the dextrin-treated FCV solution. Furthermore, at the contact times of 6 h and 24 h, the viral titers of the FCV in either 2.5 or 5.0 mg/mL TY-1-terated group were below the detection limit. Specifically, at the 6 h, compared with the dextrin group, the reductions in viral titer by 2.5 and 5.0 mg/mL TY-1 were ≥ 3.8 $\log_{10}$ $TCID_{50}$/mL. Meanwhile, the significant reductions in viral titer in the FCV solutions treated with either 2.5 or 5.0 mg/mL TY-1 were 0.5 and 1.3 $\log_{10}$ $TCID_{50}$/mL, respectively at the 10 s contact time (Figure 8a). On the other hand, the significant reductions in the viral titre of MNV solution treated with 0.3–5.0 mg/mL TY-1 were observed at 1–24 h contact times. Specifically, at the contact time of 24 h, the reduction of the viral titer of 5.0 mg/mL TY-1-treated MNV solution was below the detection limit (reduction by $\geq$ 2.7 $\log_{10}$ $TCID_{50}$/mL). Additionally, compared with the dextrin group, the treatment with 5.0 mg/mL TY-1 caused a significant decrease in the viral titer by 0.5 $\log_{10}$ $TCID_{50}$/mL at the 10 min contact time (Figure 8b). The concentration of TY-1 that did not exhibit a significant reduction in the viral titer at certain contact times was not evaluated for the subsequent shorter reaction times. Afterward, the virucidal activities of TY-1 at a higher concentration (25.0 mg/mL) against FCV and MNV were assessed at 1 min contact time. As a result, the 25.0 mg/mL TY-1 significantly reduced the viral titer of FCV and MNV with $\geq$ 3.44 and 0.88 $\log_{10}$ $TCID_{50}$/mL reduction, respectively (Figure 8c, d).

Additionally, the virucidal impact of 0.034 mg/mL catechins, 0.083 mg/mL TFs, and 0.116 mg/mL TFs+catechins compared with that of 5.0 mg/mL TY-1 against FCV and MNV was evaluated. The concentrations of catechins, TFs, and TFs+catechins solutions were equal to their concentrations in the 5.0 mg/mL TY-1 solution. When each test solution and FCV were mixesd, TFs, TFs+catechins, and TY-1 significantly reduced the viral titer by 3.1, 3.0, and ≥ 4.3 (below the detection limit) $\log_{10}$ $TCID_{50}$/mL at the 3 h contact time, respectively. Meanwhile, no statistical difference was exhibited in the viral titres between catechins and dextrin groups at that contact time. Furthermore, at the contact time of the 24 h, the viral titer reduction by catechins, TFs, TFs+catechins, and TY-1 were 0.9, 3.3, 3.7, and ≥ 3.3 (below the detection limit) $\log_{10}$ $TCID_{50}$/mL, respectively (Figure 9a). When each test solution and MNV were mixed, the viral titer reduction by catechins, TFs, TFs+catechins, and TY-1 were 1.4, 2.1, 2.5, and 2.1 $\log_{10}$ $TCID_{50}$/mL at the 3 h contact time, respectively. Meanwhile, the viral titer reduction by catechins, TFs, TFs+catechins, and TY-1 were 1.1, 2.4, 2.7, and ≥ 2.4 (below the detection limit) $\log_{10}$ $TCID_{50}$/mL at the contact time of 24 h, respectively (Figure 9b).

2.3.2. Virucidal efficacy of TY-1 against FCV and MNV on a dry surface

The virucidal efficacy of TY-1 on a dry surface against FCV and MNV was investigated. At the contact time of 10 min, the significant viral titers reductions by 5.0 mg/mL TY-1 were 2.75 and 1.5 $\log_{10}$ $TCID_{50}$/mL against FCV and MNV, respectively (Figure 10a, b).

2.3.3. TY-1 impact on the structural protein of MNV

The purified MNV solution was mixed with the TY-1 or dextrin solution. Subsequently, the impact of TY-1 on the VP1 protein of MNV was analyzed by WB. Accordingly, at the 0 h contact time, there was no difference in protein band patterns in both the dextrin- and TY-1-treated MNV. Meanwhile, the VP1 band was observed in the dextrin-treated MNV solution but not in the TY-1-treated MNV solution at the contact time of 24 h (Figure 11).

2.3.4. TY-1 impact on the genomes of FCV and MNV

The impact of TY-1 on the FCV and MNV genomes was evaluated by RT-PCR at the 0 and 24 h contact times. As a result, at 0 h contact time, there was no observed change in the band intensities of the specific PCR products in both TY-1-treated and dextrin-treated viruses. Meanwhile, after 24 h, the PCR products were detected only in the dextrin-treated viruses and were hardly observed in the TY-1-treated viruses (Figure 12a, b).

2.3.5. TY-1 impact on the viral particles of FCV and MNV

The morphological changes in FCV and MNV particles induced by 5.0 mg/mL TY-1 were observed using TEM. At the contact time of 6 h, the FCV and MNV particles appeared to preserve typical structures, which were observed in the dextrin-treated sample (Figures 13a, c, e, g). In contrast, several TY-1-treated FCV and MNV particles exhibited abnormal structures (e.g., loss of cup-shaped

depressions on the capsid, reduction in diameter of viral particles), and the aggregation of viral particles was detected. Moreover, there was a noticeable reduction in the number of intact viruses in the TY-1 treatment group (Figures 13b, d, f, h).

2.4. Discussion

Using cultivable surrogate viruses is a proposed procedure for HuNoV research due to the unavailability of the reproducable and practical cell cultivation system. To prevent outbreaks of food-borne HuNoV infection, the establishment of virucidal agents became crucial. Sodium hypochlorite (NaClO) is one of the recommended disinfectants for HuNoV. Duizer et al. (2004a) reported that sufficient concentrations (around 100–3,000 ppm) of NaClO showed the virucidal efficacy against FCV and MNV both on surfaces and in solution within a few minutes, and proposed that the degree of virus inactivation was proportional to the concentration of NaClO. Unrelatedly with its rapid and broad virucidal activity, the main disadvantage of using NaClO is that it is not always appropriate or approved for application on food, skins, and environmental surfaces due to its corrosiveness and toxicity. On the other hand, there have been many reports demonstrating the virucidal effects of plant extracts against HuNoV or its surrogate (D'Souza, 2014; Elizaquível et al., 2013). It has been found that TY-1 showed multiple modes of virucidal activity against enveloped-RNA viruses like IAV as described in Chapter I, and SARS-CoV-2 (Takeda et al., 2021b). In this study, the virucidal efficacy of TY-1 against non-enveloped HuNoV surrogates, FCV and MNV, was further evaluated. TY-1 exhibited concentration- and time-dependent virucidal efficiency against FCV and MNV. In addition, a potent and rapid TY-1-mediated inactivation of FCV compared with MNV was observed, where 2.5 mg/mL TY-1 inactivated FCV in 10 s, while 5.0 mg/mL TY-1 took more than 10 min for the inactivation of MNV (Figure 8a, b). However, at the contact time of 1 min, the higher concentration of TY-1 (25.0 mg/mL) exhibited a statistically significant virucidal efficacy against MNV (Figure 8d). Moreover, unlike FCV on a dry surface, MNV was less sensitive to TY-1 (Figure 10). These results correspond to the previous findings that FCV was more sensitive than MNV to plant-based polyphenols (Su et al., 2010a; Su et al., 2010b; Su et al., 2011). Furthermore, Ueda et al. (2013)

evaluated the virucidal efficacy of GTE against FCV and MNV. They described that both viruses were inactivated by the GTE at 3 min contact time, with FCV being more affected by the virucidal impact of the GTE than MNV. An additional study identified that a chitosan-based film containing ≥ 10% of GTE inactivated MNV solutions at 3 h contact time (Amankwaah et al., 2020). Besides, on clean stainless steel and glass surfaces, 10 mg/mL of GTE exhibited a reduction in viral titer of MNV by 1.42 and 1.96 $\log_{10}$ $TCID_{50}$/mL at 15 min contact time, respectively (Randazzo et al., 2017). In these studies, the GTE exhibited concentration- and time-dependent virucidal efficacy, similarly detected in our finding. Thereafter, the TY-1 virucidal mode of action against FCV and MNV was investigated. In the WB analysis, the disappearance of the VP1 capsid protein band in the TY-1-treated MNV was observed (Figure 11). This result is reliable with the previous report in which *Saxifraga spinulosa*, a flavonoid-enriched medicinal herb, affected the structural proteins and viral capsids of non-enveloped and enveloped-RNA viruses (Takeda et al., 2020). Particularly in non-enveloped viruses, the capsid protein importantly protect the viral RNA inside and enhances viral adsorption to the host cell, and facilitates infection establishment (Cliver, 2009). Subsequently, the impact of TY-1 on viral RNA was investigated using RT-PCR. As anticipated, the specific bands disappeared in TY-1-treated FCV and MNV (Figure 12). Furthermore, the TEM analysis showed that TY-1 treatment induced abnormalities in virion morphology (Figure 13). These results suggest that TY-1 could induce conformational changes in viral proteins or possibly destruction of the capsid protein, and eventually disruption of the viral genome. These findings are similar to the virucidal mechanism of action of allspice oil, which induced the disruption of the viral genome and destruction of the capsid protein of MNV (Gilling et al., 2014). Overall, in this study, TY-1 showed the similar mode of virucidal action against FCV and MNV as that against IAV mentioned in Chapter I and SARS-CoV-2 (Takeda et al., 2021b). As described earlier, 5.0 mg/mL TY-1 contains 0.034 mg/mL catechins and 0.083 mg/mL TFs. As described in Chapter I, the contribution of catechins to IAV

inactivation by 5.0 mg/mL TY-1 was partial, and that of TFs was stronger. In this study, limited but confirmed FCV/MNV-inactivation was accomplished under catechins treatment as well as TFs treatment (Figures 9a, b), which is consistent with the previous findings explained the virucidal impact of catechin derivatives on FCV/MNV (Cliver, 2009; D'Souza, 2014; Oh et al., 2013; Randazzo et al., 2017). These findings propose that both TFs and catechins may contribute to the virucidal efficacy of TY-1. Although the MNV-inactivating activity of 5.0 mg/mL TY-1 and 0.083 mg/mL TFs was comparable (Figure 9b), the FCV-inactivating activity of TY-1 with this concentration was stronger than the 0.116 mg/mL TFs+catechins (Figure 9a). The result of the experiment targeting IAV in Chapter I and targeting FCV in this Chapter may propose that TY-1 contains additional virucidal compounds besides TFs and catechins.

Even though these results afford important information regarding the virucidal efficacy of TY-1 against HuNoV surrogates, there are some limitations. The results obtained from HuNoV surrogates have been constantly debated and challenged, and such surrogates do not always behave in the same way as HuNoV. Therefore, their results should be carefully interpreted (Cromeans et al., 2014; Kniel, 2014; Richards, 2012). This study used FCV and MNV, which are different surrogate viruses with diverse characteristics. Additional experiments targeting other HuNoV surrogates (e.g., Tulane virus) could contribute to a better prediction of the virucidal efficacy of TY-1 against HuNoV.

Furthermore, the viability-quantitative RT-PCR and TEM analysis of HuNoV particles treated by virucidal agents (Takahashi et al., 2015) may also contribute to the evaluation of the impact of TY-1 on virus capsids. This study focused only on testing TY-1 as a virucidal disinfectant in solutions and on environmental surfaces against HuNoV surrogates. However, another point that should be considered is the frequent incidence of HuNoV food-borne transmissions by ingesting uncooked or partially cooked virus-infected bivalve shellfish (Gorji et al., 2021). Currently, there are

no affordable disinfectants to remove HuNoV inside the bivalve shellfish. Hence, it may be feasible to investigate the possible application of TY-1 regarding this condition. Moreover, the results described by Falco et al. (2020) suggested the possible synergistic virucidal activity of a gentle heat treatment with plant-derived components such as TY-1, which may contribute to controlling foodborne HuNoV transmission. Overall, future studies targeting whether TY-1 can efficiently deactivate HuNoV in foods, bivalve shellfish, and food contact surfaces are highly warranted.

2.5. Conclusion

In this study, TY-1 showed the virucidal efficacy against HuNoV surrogate viruses, one of the most important food-borne viruses. TY-1 exhibited a concentration- and time-dependent virucidal efficacy against the both viruses in solution. Additionally, it also deactivated the two surrogate viruses on a dry surface. Furthermore, TY-1 promoted the profound destruction of virion structures, including the genome and capsid proteins. Hence, our results suggested that TY-1 can be potentially applied as a harmless virucidal agent that can be applied to the disinfection of HuNoV both in solution and on various environmental surfaces.

Table 3. The PCR conditions and primer sequences targeting the VP1 gene of FCV and the nonstructural polyprotein gene of MNV used in this study

Primers name	Primer sequences	RT-PCR condition
FCV-Primer set (264 bp)	Fwd: 5'-TCCACACTAGCGTCAACTGG-3' Rev: 5'-GACGAGCGTCAAACAGAACA -3'	95 °C for 5 min ↓ [95 °C for 30 sec, 49 °C for 30 sec, 72 °C for 1 min] x 22 times ↓ 72 °C for 10 min
MNV-Primer set (549 bp)	Fwd: 5'-GCCCACTGGATTCTGACTCT-3' Rev: 5'-GGTCTCAGCATCCATTGTTCC-3'	95 °C for 5 min ↓ [95 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1min] x 35 times ↓ 72 °C for 10 min

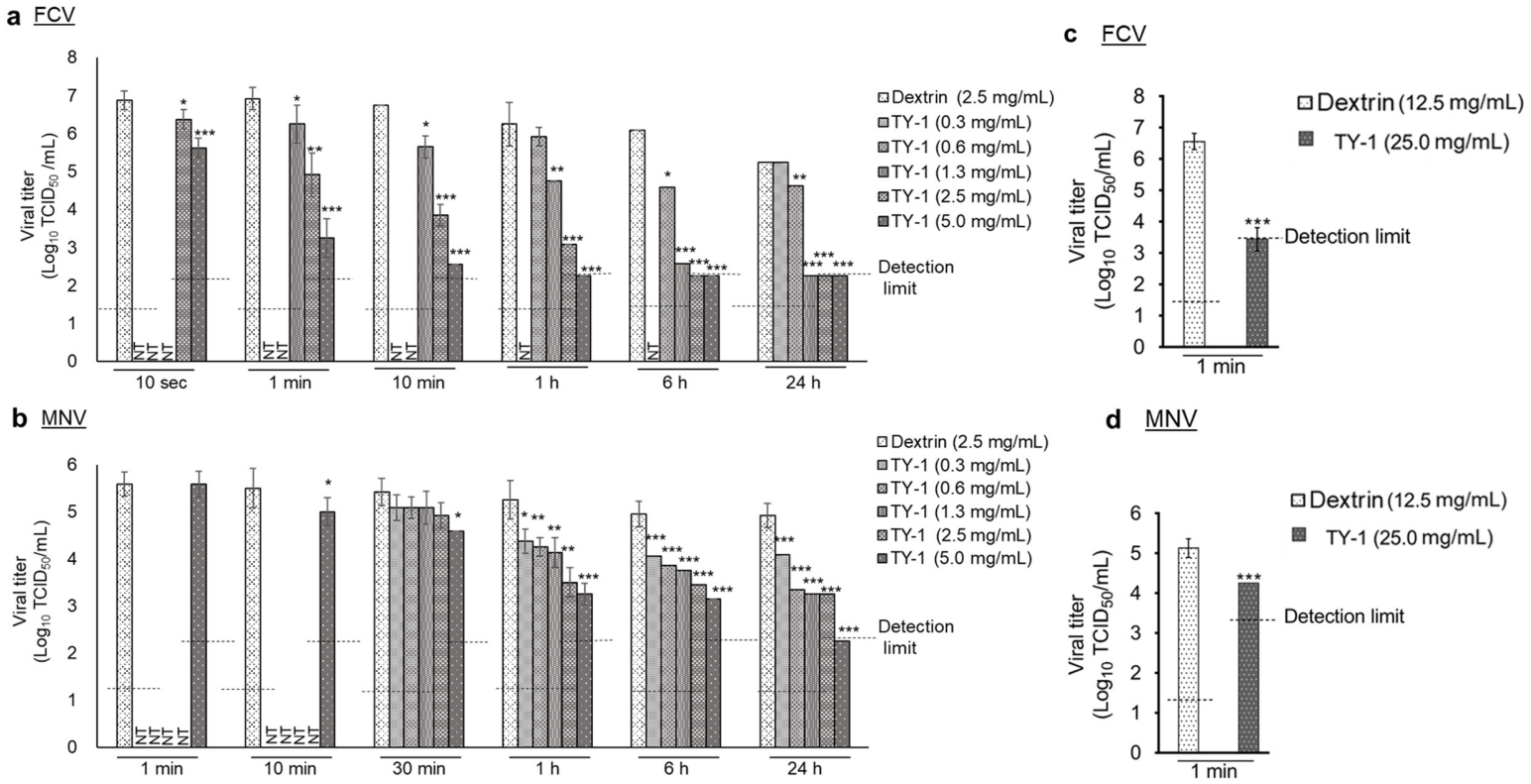


Figure 8. Evaluation of the virucidal efficacy of TY-1 on FCV and MNV. FCV (a,c) or MNV (b,d) solutions were mixed with TY-1 [final concentration: 0.3–5.0 mg/mL (a,b) or 25.0 mg/mL (c,d)] or dextrin [2.5 mg/mL (a,b) or 12.5 mg/mL (c,d)]. The mixtures were kept at 25°C from 10 s to 24 h. The data are expressed as mean ± SD ($n = \geq 6$ per group). The Student's *t*-test was performed to evaluate the statistically significant differences between the dextrin group and TY-1 group with each concentration; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NT: not tested.

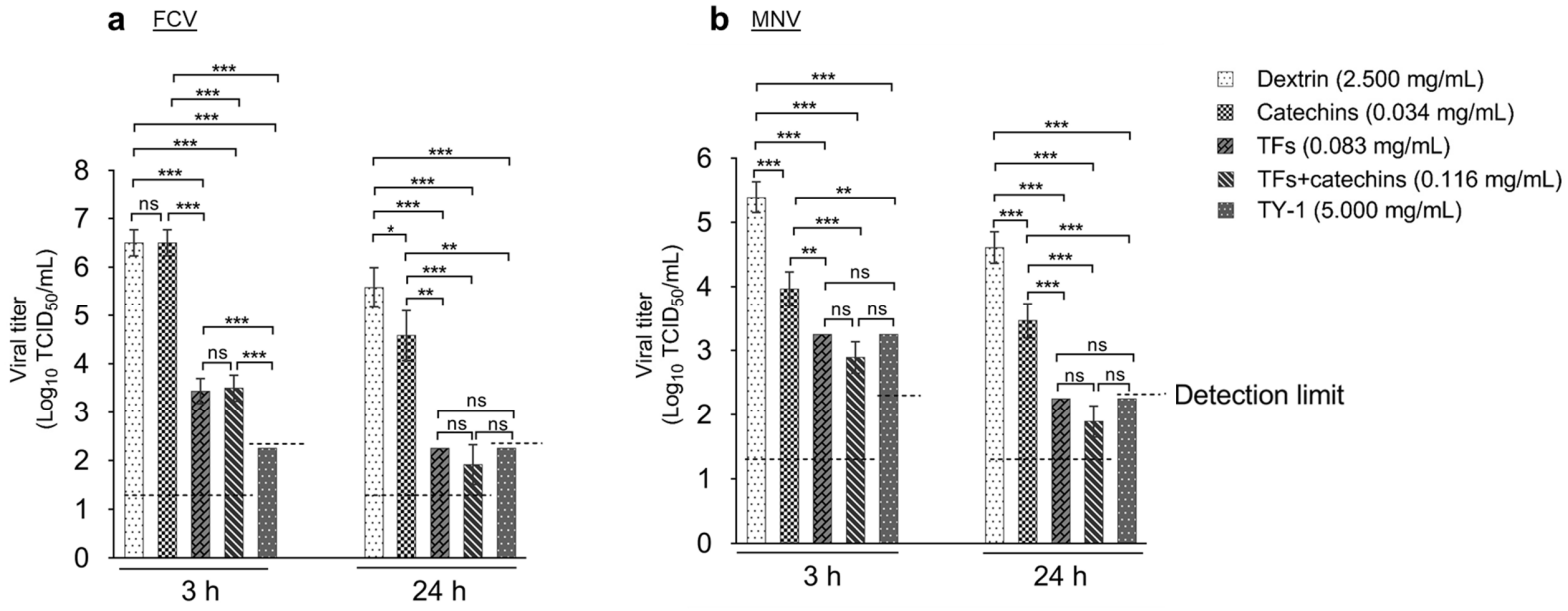


Figure 9. Evaluation of the virucidal efficacy of TY-1, TFs, catechins, and TFs+catechins, on FCV and MNV. FCV (a) or MNV (b) solutions were mixed with dextrin (final concentration: 2.5 mg/mL), and catechins (0.034 mg/mL), TFs (0.082 mg/mL), TFs+catechins (0.116 mg/mL), or TY-1 (5.0 mg/mL). Then the mixtures were kept at 25°C for 3 and 24 h. The data were expressed as mean ± SD ($n = \geq 6$ per group). Student's *t*-test with Bonferroni correction for multiple comparison was performed to evaluate the statistical significance of the differences among the different groups; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns: not significant.

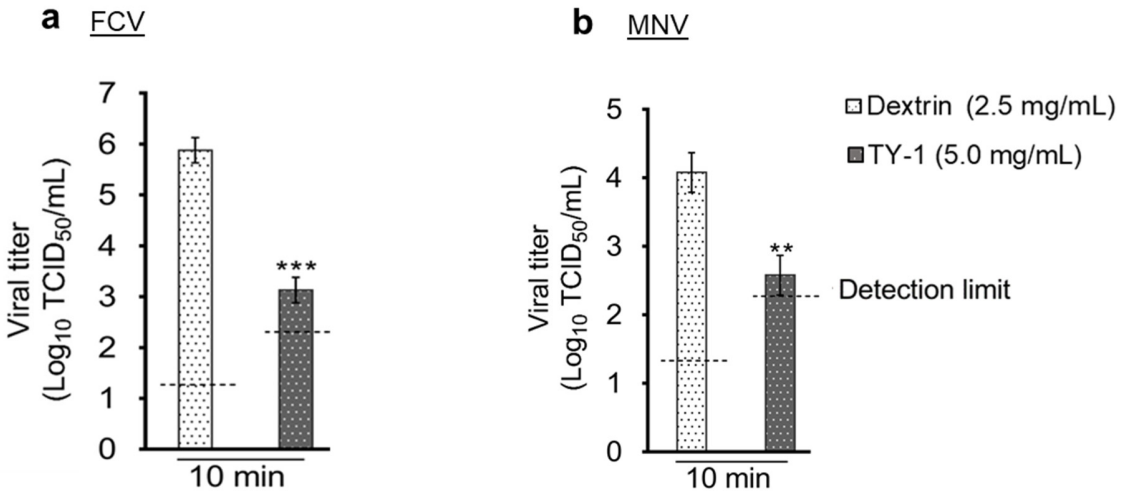


Figure 10. Evaluation of the virucidal efficacy of TY-1 against FCV and MNV on a dry surface. TY-1 (final concentration: 5.0 mg/mL) or dextrin (2.5 mg/mL) was applied on the FCV (a) or MNV (b) on a dry surface, and kept at 25°C for 10 min. The data were expressed as mean ± SD ($n = \geq 3$ per group). Student's *t*-test was performed to evaluate the statistical significance of the differences among the dextrin group and TY-1 group; $^{**}p < 0.01$; $^{***}p < 0.001$.

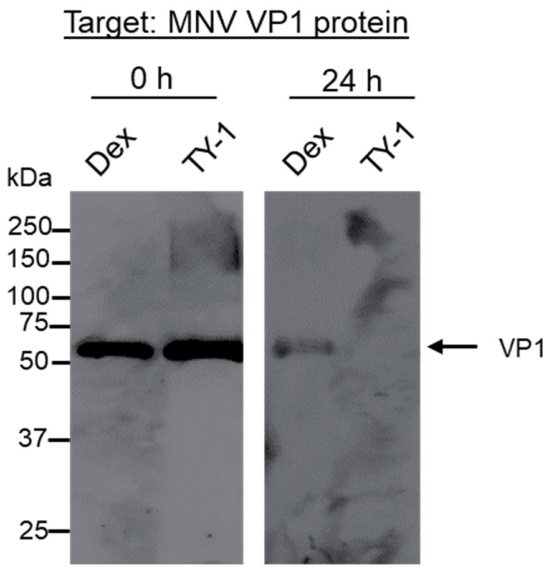


Figure 11. Impact of TY-1 on MNV VP1 structural protein. The purified MNV solution was either mixed with TY-1 (final concentration: 5.0 mg/mL) or dextrin (2.5 mg/mL) and kept at 25°C for 0 or 24 h. Then, WB was achieved to detect the MNV VP1 structural protein. The results were representative of 2 individual experiments. Dex: dextrin.

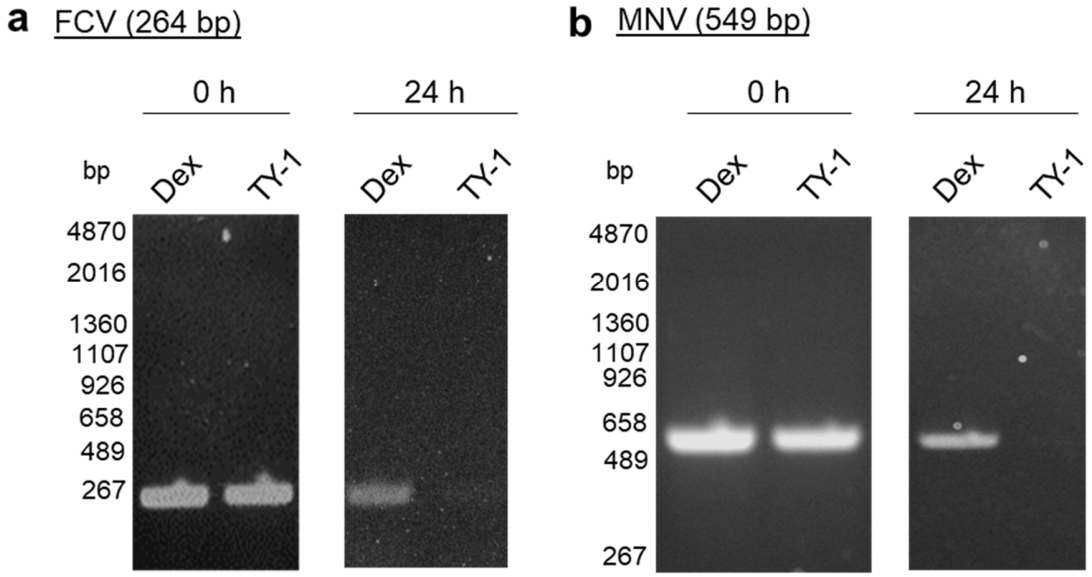


Figure 12. Impact of TY-1 on the genomes of FCV and MNV. The purified FCV (a) or MNV (b) solutions were mixed with TY-1 (final concentration: 5.0 mg/mL) or dextrin (2.5 mg/mL) and kept at 25°C for 0 or 24 h. Then, RT-PCR was performed to analyze the extracted viral RNA from the treated FCV and MNV. RT-PCR was done using the primer set to amplify the region on the gene encoding FCV VP1of 264 bp (a) or the primer set to amplify the region on the gene encoding the MNV nonstructural polyprotein of 549 bp (b). Dex: dextrin.

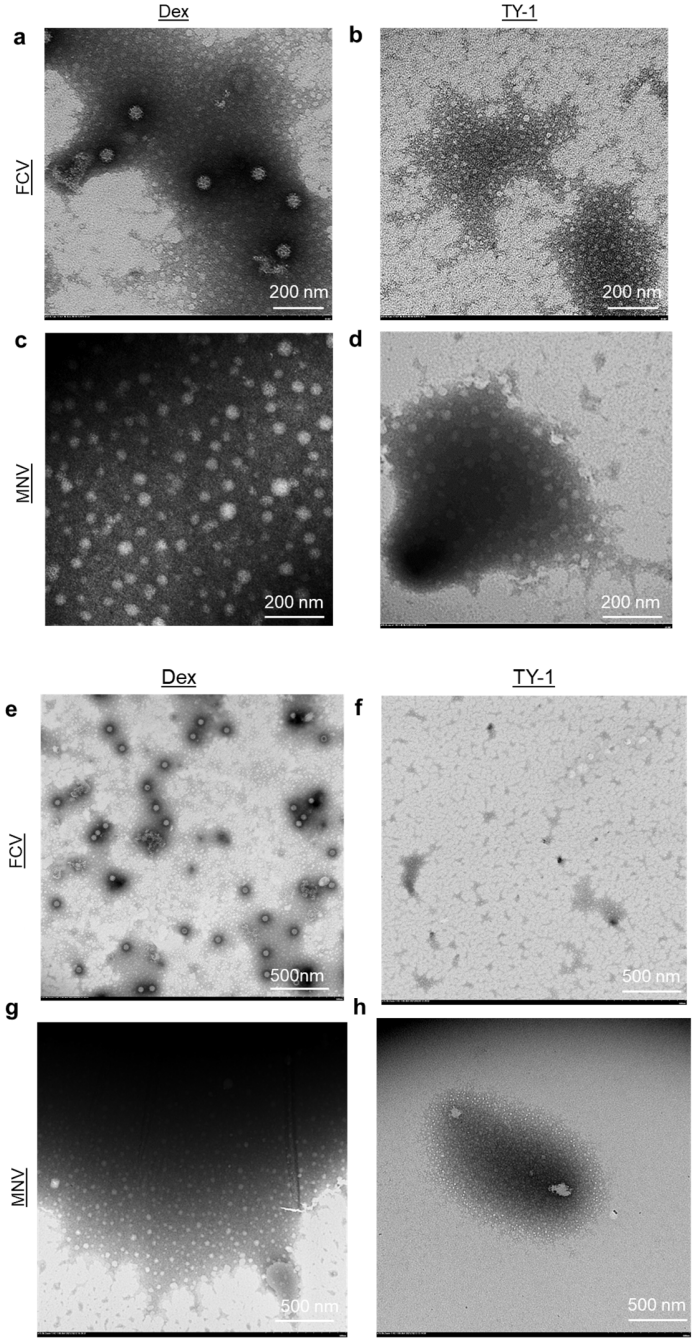


Figure 13. Morphology of dextrin- or TY-1-treated FCV and MNV particles under TEM at higher (a, b, c, d) and lower (e, f, g, c) magnifications. The purified FCV or MNV solutions were mixed either with dextrin (final concentration: 2.5 mg/mL) (a, c, e, g) or TY-1 (5.0 mg/mL) (b, d, f, h) and kept at 25°C for 6 h. Then, the viral particles were detected using TEM.

General Discussion

To prevent viral infection, the use of an efficient natural virucidal substance may be a powerful approach. In the present study, the virucidal efficacy of TFs-enriched tea leaf extract TY-1, which comprises a considerable amount of polyphenols including TFs and other catechins, against enveloped and non-enveloped-RNA viruses was evaluated. In Chapter I, the IAV-inactivating activity of TY-1 as a representative of enveloped-RNA virus was investigated. TY-1 exhibited concentration- and time- dependent virucidal efficacy against IAV. Specifically, 5.0 mg/mL TY-1 reduced the viral titer by 1.33 and $\geq$ 5.17 $\log_{10}$ $TCID_{50}$/mL within the reaction time of 30 min and 6 h, respectively. TY-1 treatment decreased the band intensity of HA and enhanced the appearance of additional ladders/bands with high molecular mass. In addition, the band intensity of NA was also reduced after TY-1 treatment which was revealed with WB. Meanwhile, the hemagglutination and NA assay showed that TY-1 reduced hemagglutination and NA activities. These results specified that TY-1 induced structural abnormalities in the spike proteins of IAV. Moreover, RT-PCR targeting the IAV genome and TEM analysis of viral particles revealed that upon application of TY-1, the viral genes were damaged, and the number of intact viral particles were drastically reduced. The virucidal action of TY-1 was accredited to the combined additive activities of various virucidal compounds including TFs and catechins

In Chapter II, the virucidal efficacy of TY-1 against the HuNoV surrogates was evaluated as representatives of non-enveloped-RNA virus. The viral titer of both surrogate viruses FCV and MNV was reduced in a concentration- and time-dependent manner by TY-1 treatment. Specifically, statistically significant reductions of viral titers in FCV treated by 5.0 mg/mL TY-1 and MNV treated by 25.0 mg/mL TY-1 were recognized in 10 sec and 1 min, respectively. Additionally, TY-1 reduced FCV and MNV viral titers on a dry surface within 10 min reaction time.

Thereafter, the impact of TY-1 on viral capsid proteins and viral genome was investigated with WB, RT-PCR, and TEM. Accordingly, TY-1 induced the profound destruction of capsid proteins, genomes, and viral particles. The various compounds present in TY-1, including TFs and catechins, seemed to attributed to the overall virucidal efficacy of TY-1.

Altogether, our findings indicate that the virucidal mechanism of TY-1 could be attributed to multiple factors rather than a single action. Furthermore, the virucidal efficacy of TY1 could be attributed to the chemical structures of compounds in TY-1. For example, it has been reported that the number of galloyl groups of TFs and catechins seems to have a direct relation to their virucidal efficacy (Liu et al., 2005, Quosdorf et al., 2017; Song et al., 2005). Meanwhile, Ohba et al. (2017) investigated that the hydroxyl groups of TFs are more significant than the galloyl groups for the virucidal efficacy against caliciviruses. Overall, these findings suggests the potential usage of TY-1 as a nature-derived virucidal agent in healthcare settings and food processing facilities to reduce IAV and HuNoV transmission.

Even though our objectives in these studies focused only on the direct virucidal efficacy of TY-1, additional experiments were also performed to evaluate the anti-IAV activity of TY-1 in infected cells and lab animal models. However, TY-1 did not show antiviral activities in those studies, which could be explained as most of the polyphenols, particularly TFs and catechins, are extensively informed to have mainly the virucidal efficacy against several types of viruses (Chowdhury et al., 2018; Clark et al., 1998; Lee et al., 2018; Nakayama et al., 1993; Ohba et al., 2017; Ohgitani et al., 2021). Accordingly, additional studies evaluating the anti-IAV and HuNoV activities of TY-1 in different experimental settings such as in infected cells, animal models, and patients are essential to further evaluate the antiviral efficacy of TY-1 against IAV and HuNoV. Moreover, as previously mentioned, tea polyphenols have been investigated for numerous physiological benefits to have

antimicrobial, anti-inflammatory, anti-diabetic, and anti-cancer activities (Li et al., 2017; Liao et al., 2015; Rha et al., 2019; Striegel et al., 2015; Xu et al., 2017). Activating immune cells including macrophage could play an essential role in the inflammatory response. Lipopolysaccharide (LPS), which is a constituent of cell wall of gram-negative bacteria, induces the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and other inflammatory mediators, including prostaglandin E2 and nitric oxide (Agarwal et al., 1995; Yun et al., 2008). Therefore, additional experiments were also conducted *in vitro* to evaluate the anti-inflammatory potential of TY-1. Briefly, the anti-inflammatory effect of TY-1 (70 μg/mL) on the LPS-stimulated RAW264 cells was investigated. The suppressive activity of TY-1 against the increase of gene expression levels of IL-6, IL-15, IL-10, TNF-α, and COX-2 induced by LPS (0.25 μg/mL) was evaluated. The results revealed that TY-1 tended to suppress the gene expressions of these infllamatory mediators but did not exhibit highly significant effect to conclude the possible ant-inflammatory efficacy of TY-1. Accordingly, to judge the potential anti-inflammatory effect of TY-1 and clarify its mechanism of action, future studies with modifications, such as changing the cell line or using other inflammatory stimulator other than LPS are required.

General Conclusion

Influenza is a highly contagious disease that disturbs human life on a universal scale. There are ongoing researches on antiviral drugs and novel vaccines, which are reconfigured yearly. Nevertheless, it remains a challenge to establish a complete strategy against influenza. HuNoV, which is highly contagious and durable, is also a important agent causing the outbreaks of foodborne diseases, namley AGE worldwide. Unfortunately, no approved HuNoV vaccine or antiviral drugs are currently available. The main barrier is the lack of a reproducible and robust *in vitro* cultivation system for the virus. Accordingly, establishing effective virucidal agents to compete against such highly infectious viruses could support the communal health sector. Medicinal plants can be useful natural resources for virucidal agents, for instance tea extracts have promising health benefits, mainly through a high concentration of their polyphenolic constituents. In the present study, research was performed to evaluate the virucidal efficacy of TFs and other polyphenols-enriched tea leaf extract TY-1 against enveloped and non-enveloped-RNA viruses, IAV and HuNoV surrogates, respectively. As a result, TY-1 exhibited a concentration- and time-dependent virucidal efficacy against these viruses in solutions. In addition, TY-1 also inactivated the HuNoV surrogate on a dry surface. Interestingly, TY-1 promoted the profound destruction of virion components, including the genome and structural proteins, and futher virion structures.

As the recommended application of TY-1, TY-1 can be potentially applicable as a natural-derived virucidal agent that can be used against viruses in solution and on environmental surfaces. Specifically, TY-1 may be applied as troche, mouthwash, nasal mask, food additive, and supplement with virucidal activity. Furthermore, to approve the validity of implementing the antiviral and anti-inflammatory effect of TY-1, it will be essential to take on broader and deeper analyses using different animal models and cell lines to evaluate the usefulness for TY-1 application. In addition, human

clinical studies are essential to confirm the antiviral, anti-inflammatory and other health-promoting potentials of TY-1. This could be in the path of synergistic treatments via integrating natural extracts with heat treatment, ozone, gamma-irradiation, or synthetic chemical therapeutic agents like nanoparticles.

In summary, this study introduced the tea leaf extract TY-1 enriched with TFs and other polyphenols as a promising candidate of natural-derived virucidal agent for preventing and controlling IAV and HuNoV infection.

要約

インフルエンザは、ヒトに感染するインフルエンザウイルスによって引き起こされる伝播性の高い感染症である。数種類のインフルエンザウイルスのうち、過去に起きたインフルエンザパンデミックは全てA型インフルエンザウイルス(IAV)によって引き起こされている。この約 100 年間に4回、新型の IAV の出現によってパンデミックが引き起こされ、その度に 50 万人～5,000 万人が亡くなっている。ワクチン投与は IAV 感染を防止する代表的な対策の一つである。しかしながら、抗原ドリフトに加えて抗原シフトを引き起こす遺伝子変異が起きる結果、過去の感染によって産生された中和抗体が十分に作用しない変異ウイルスが生じる可能性があり、その様な変異ウイルスに対しては現行ワクチンによる感染防御は不十分となる。一方、IAV 感染に有効ないくつかの抗ウイルス剤が開発され使用可能となっている。しかし、それら薬剤に対して耐性をもつウイルスの出現が問題となる。従って、ワクチンと治療剤以外に、殺ウイルス効果をもつ物質をウイルス不活化剤として活用することなどが、インフルエンザ対策には必要である。

ヒトノロウイルス (HuNoV) は、世界的に急性胃腸炎の原因ウイルスとして最も重要なウイルスであり、その感染によって、毎年、全年齢のヒトで 20 万人以上の死者が出ている。HuNoV は、食品媒介ウイルスとして世界的に最も重要なウイルスとされ、引き起こされる疾病によって生じる経済損失は数百ドルに及ぶと推定されている。現在のところ、HuNoV に対して有効なワクチンも抗ウイルス剤も開発されていない。一方、いくつかのウイルス不活化剤が、環境表面の HuNoV を不活化するのに有効であることが確認されている。しかし、それら薬剤の中には環境中への残留や健康被害の問題などから使用が限定されるものも多く存在する。そこで、有効でかつ環境にやさしい手法によって HuNoV 感染を防止する戦略が求められている。しかしながら、万人が容易に実施可能であり、かつ再現性のある *in vitro* の HuNoV 培養法が未だ確立されていないことが HuNoV の研究を難しくしている。そのため、HuNoV に遺伝的および構造的に類似し、培養細胞での増殖が可能なネコカリシウイルス (FCV) およびマウスノロウイルス (MNV) を代替ウイルスとして用い、HuNoV に対して有効な可能性のある殺ウイルス物質の探索、およびその活性評価が行われている。

さまざまな疾病に対して行われる補足的あるいは慣例的な治療において、薬用植物は広く活用されてきている。それらのうち、茶葉ポリフェノールは、複数の病原ウイルスに対する天然由来殺ウイルス物質として研究が進められてきた。そこで、本研究では、緑茶 *Camellia sinensis* 由来のポリフェノール豊富な茶葉抽出物である TY-1 (横山食品株式会社、札幌市、北海道) について、その殺ウイルス活性を調べることとした。本研究の内容は 2

つの Chapter に述べられている。まず Chapter I の研究では、エンベロープを保有する RNA ウイルスの代表である IAV に対する TY-1 の殺ウイルス活性の評価を行った。その後、TY-1 の IAV に対する殺ウイルス活性の作用機序について解析を行った。Chapter II では、エンベロープを保有しない RNA ウイルスとして、HuNoV の代替ウイルスである FCV および MNV に対する TY-1 の殺ウイルス活性を評価した。さらに、TY-1 の FCV および MNV に対する殺ウイルス活性の作用機序について解析を行った。TY-1 に含まれる物質単体のうち IAV、FCV および MNV に対して殺ウイルス活性を示す化合物単体の解明を目指した研究も行った。

目的とした研究を行うため、以下の方法で TY-1 粉末からストック液を作製した。すなわち、1 g の TY-1 粉末を 100 mL のリン酸緩衝生理食塩水に溶解させた後に遠心分離を行い、得られた可溶性画分を濾過した後、使用時まで−80 度で保存した。TY-1 粉末成分の 50%は dextrin であるため、50% dextrin 溶液を溶媒対照として実験に用いた。すなわち、TY-1 および dextrin のストック溶液としてそれぞれ 10 mg/mL、5 mg/mL を準備した。

Chapter I では、以下の方法で、TY-1 の IAV に対する殺ウイルス活性の評価を行った。まず、5 段階の濃度の TY-1 および 1 濃度の dextrin をウイルス液と混和した。混和液中の TY-1 の終濃度は 0.3~5.0 mg/mL、dextrin の終濃度は 2.5 mg/mL であった。その混和液を 25℃で様々な反応時間（10 分〜24 時間）静置した。その後、各濃度の TY-1 液の殺ウイルス活性について、各試験液で処理したウイルスと dextrin 液で処理したウイルスの力価を比較することで評価した。その結果、TY-1 は IAV に対して濃度および時間依存的な殺ウイルス活性を示すことが明らかとなった。5.0 mg/mL の TY-1 は、30 分間および 6 時間の反応により、dextrin 群に比較して、それぞれ 1.33 $\log_{10}$ 50%組織培養感染量 ($TCID_{50}$)/mL および ≥ 5.17 $\log_{10}$ $TCID_{50}$/mL のウイルス力価の低下を引き起こした。

続いて、TY-1 の IAV に対する殺ウイルス機序の解析を行った。ウェスタンブロッティング（WB）によって、TY-1 処理によりウイルスのスパイクタンパク質であるヘマグルチニンのバンドの濃さが減弱し、高分子量の複数のバンドが出現する結果が得られた。また、もう一つのスパイクタンパク質であるノイラミニダーゼ（NA）のバンドの濃さも減弱していた。赤血球凝集試験によって TY-1 処理によってウイルスの赤血球凝集活性が低下すること、さらに NA 試験によって NA 活性も低下することが確認された。これらの結果から、TY-1 は IAV のスパイクタンパク質の構造変化と機能異常を引き起こし、粒子構造の破壊へと導く可能性が示唆された。TY-1 で処理したウイルスの遺伝子を標的とした逆転写 PCR（RT-PCR）によって、PCR 産物が著しく減少することが明らかとなり、TY-1 はウイルスゲノムに傷害を与える可能性が示された。さらに、透過電子顕微鏡（TEM）によ

るウイルス粒子の観察により、TY-1 処理によって正常なウイルス粒子数が劇的に減少することが明らかとなった。

TY-1 の主要成分である theaflavins (TFs) 及び catechins の IAV 不活化活性への寄与について評価するために、ウイルス液を TFs、catechins、TFs+catechins、TY-1 及び dextrin 溶液と混和させた。混和液中の TFs、catechins、TFs+catechins、TY-1 及び dextrin の終濃度は、それぞれ 0.083、0.034、0.116、5.0、2.5 mg/mL であった。TFs、catechins 及び TFs+catechins 溶液の濃度はそれぞれが 5.0 mg/mL の TY-1 溶液中に含まれる濃度に匹敵する。混和液を 25℃で 3 時間または 24 時間静置した。その後、それぞれの試験液処理群と dextrin 処理群のウイルス力価を比較することにより、各試験液の殺ウイルス活性を評価した。得られた結果より、TY-1 の殺ウイルス活性の一部は TFs に起因する一方、catechins の果たす役割は限定的であることが明らかとなった。しかし、TFs+catechins 処理では、TFs 単独処理に比べて IAV 不活化効果は増強されることも確認された。得られた結果から、TY-1 の殺ウイルス活性を担う主な化合物は TFs であるものの、catechins を含むその他のポリフェノール類も役割を果たしている可能性が示唆された。

Chapter II では、TY-1 の HuNoV 代替ウイルスである FCV 及び MNV に対する殺ウイルス活性を、溶液中及び乾燥表面にて評価した。まず、ウイルス液と TY-1 及び dextrin 溶液を混和した。混和液中の TY-1 及び dextrin の終濃度は、それぞれ 0.3〜5.0 または 25.0 mg/mL、及び 2.5 または 12.5 mg/mL であった。それら混和液を 25℃で 10 秒から 24 時間静置した後、ウイルス力価（$\log_{10}$ $TCID_{50}$/mL）を測定した。その結果、TY-1 は濃度及び処理時間依存的に、FCV 及び MNV に対して殺ウイルス活性を示した。なお、2.5 mg/mL の TY-1 溶液と 10 秒間反応させることにより FCV は不活化されたのに対し、MNV の不活化には、5.0 mg/mL の TY-1 溶液で 10 分以上反応させることが必要であった。この結果より、TY-1 は MNV よりも FCV に対してより強く迅速な不活化作用を示すと判断された。一方、高濃度の 25.0 mg/mL の TY-1 を用いることにより、1 分間の反応時間で MNV のウイルス力価を有意に低下させられることが確認された。さらに、TY-1 は、乾燥表面においても 10 分の反応で FCV 及び MNV を不活化することが確認された。TY-1 の FCV 及び MNV のウイルスタンパク質及びゲノムに対する作用について、それぞれ WB 及び RT-PCR を用いて解析した。その結果、TY-1 は、ウイルス粒子成分であるカプシドタンパク質及びウイルスゲノムを著しく損傷させることが明らかとなった。TEM 観察により、ウイルス粒子構造の異常が引き起こされていることも確認された。また、限定的ではあるものの、catechins 及び TFs による FCV 及び MNV に対する不活化作用が確認された。以上より、TFs 及び catechins が TY-1 の FCV 及び MNV に対する不活化活性に寄与することが示唆されたものの、他の成分も関与することが示唆された。

本研究は、TFs や catechins といったポリフェノール類を豊富に含む茶葉抽出物 TY-1 が、天然物由来の殺ウイルス物質であることを明らかにした。TY-1 の応用として、様々な場所で、トローチ、口腔洗浄、鼻マスク、食品添加物への含有、また環境表面に適用する殺ウイルス性スプレーへの適用により、IVA 及び HuNoV の感染制御に活用される可能性が考えられる。

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