Mori Cortex Radicis extract inhibits human norovirus surrogate in simulated digestive conditions

Chae Yeon Lim1 · Hyojin Kim1 · Mi Sook Chung1

Abstract Norovirus is a major cause of acute gastroenteritis globally, resulting in enormous health and societal costs. In this study, the antiviral activities of Mori Cortex Radicis (MCR) extract and its bioactive flavonoids, morusin and kuwanon G, were tested against murine norovirus (MNV), a human norovirus surrogate, using plaque assay. The antiviral activity was confirmed in simulated digestive conditions, including simulated saliva fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). Pre-treatment of MNV with MCR extract at 1000 μg/mL showed antiviral activity with a 1.1-log reduction. Morusin and kuwanon G also demonstrated a 1.0-log and 0.6-log reductions of MNV titers, respectively, at 100 μM. MCR extract at a concentration of 2 mg/mL in SSF, SGF, and SIF markedly reduced MNV titers by 1.8, 1.9, and 1.5 logs, respectively. Therefore, these data suggest that MCR extract can be used to control norovirus infectivity.

Keywords Murine norovirus · Mori Cortex Radicis · Morusin · Kuwanon G · Simulated digestive conditions

Introduction

Morus alba (the mulberry tree) is widespread in Asia, America, and Europe. The root bark of M. alba, Mori Cortex Radicis (MCR) has been used to treat jaundice, hematemesis, edema, and pulmonary inflammatory diseases in Asia (Lee et al., 2014; Seo et al., 2013). MCR reduces lipid peroxidation, enhances tyrosinase inhibitory activity, regulates the secretion and production of airway mucin, and promotes antioxidant activity (Kim et al., 2012; Lee et al., 2014; Singab et al., 2005; Zhu et al., 2019). MCR is rich in phytochemicals such as flavonoids, benzofuran, and alkaloids (Lee et al., 2012). Morusin and kuwanon G are the biological marker compounds in MCR (Eom et al., 2017; Kim et al., 2015). Morusin, an isoprenylated flavone, reportedly possesses multifunctional bioactivities, including anti-allergic, anti-cancer, and lipogenesis inhibitory activities (Choi et al., 2020; Jin et al., 2019; Lee et al., 2018). Morusin demonstrates antimicrobial activity on Staphylococcus aureus (Pang et al., 2019). Notably, morusin is a potential inhibitor of the main protease 6M03 of severe acute respiratory syndrome coronavirus 2, which causes Coronavirus disease 2019 pandemic (Singh and Florez, 2020). Kuwanon G, another isoprenylated flavone, has antimicrobial activities on Streptococcus mutans, S. sobrinus, and S. sanguis, and Porphyromonas gingivalis (Park et al., 2003) and antiviral effect against human coronavirus 229E (Thabti et al., 2020).

Noroviruses belong to the Caliciviridae family and are a non-enveloped round virus with a positive-stranded RNA. Human noroviruses are a major cause of acute gastroenteritis globally and are estimated to cost $4.2 billion and $60.3 billion per year for direct health system costs and societal costs, respectively (Bartsch et al., 2016; Lindesmith et al., 2003). Noroviruses are rapidly transmitted...
through consumption of norovirus-contaminated food or water, contact with contaminated environmental surfaces, and person-to-person contact (Lindesmith et al., 2003). Norovirus foodborne outbreaks frequently occur in long-term care facilities, schools, hospitals, cruise ships, and the military. The most common symptoms are vomiting, watery diarrhea, and nausea. Norovirus is a leading cause of severe and chronic gastroenteritis in children younger than 5 years of age and immunocompromised persons (Bok and Green, 2012). Currently, no drugs or vaccines can effectively control and prevent norovirus infection.

Although human norovirus can grow in B-lymphocyte and stem cell-derived human enteroid culture systems, murine norovirus (MNV) shares biochemical and genetic features with human norovirus and is robust and a cost-effective means for in vitro replication. MNV’s these advantages make it widely used as a model for human norovirus infection (Ettayebi et al., 2016; Jones et al., 2014; Wobus et al., 2006).

Many research papers have been published on plant-derived natural antivirals against norovirus, including black raspberry seed extract, grape seed extract, blueberry, aged green tea, and Artemisia princeps var. orientalis essential oil (Bae et al., 2018; Chung 2017; Falcó et al., 2019; Joshi et al., 2015, 2017). However, the antiviral activity of MCR and its constituents against norovirus has not been reported. To use plant-derived natural antivirals in food, it is essential to evaluate their antiviral activity in saliva and gastrointestinal fluids in different digestive enzyme conditions, salt concentrations, pH levels, and digestion times (Falcó et al., 2019; Joshi et al., 2015). In this study, we evaluated antiviral activities of MCR extract and its bioactive flavonoids, morusin and kuwanon G, in RAW 264.7 cells against MNV and then confirmed the antiviral activity of MCR extract under simulated in vitro human digestive conditions.

Materials and methods

Materials

Dimethyl sulfoxide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and formaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) and American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM) and penicillin–streptomycin (PS) were purchased from Gibco BRL (Karlsruhe, Germany). Morusin and kuwanon G were obtained from Biosynth Carbosynth® (Compton, UK). Human saliva α-amylase (Type IX-A), porcine pepsin, porcine pancreatin, and bovine/ovine bile acid mixture were purchased from Sigma-Aldrich.

Preparation of virus and cell

RAW 264.7 cells (mouse leukemic macrophage cell line; RAW) were purchased from ATCC and grown in DMEM supplemented with 10% FBS and 1% PS at 37 °C in a 5% CO₂ incubator. Dr. Herbert Virgin (Washington University School of Medicine, St. Louis, Missouri, USA) kindly provided murine norovirus-1 (MNV). MNV was inoculated in RAW 264.7 cells at 37 °C for 1 h in a 5% CO₂ incubator, and then the viral inocula were discarded. DMEM supplemented with 10% FBS and 1% PS was added to the cells, which were incubated at 37 °C for 72 h in a 5% CO₂ incubator until we observed MNV-induced cytopathic effects. The MNV-infected cells were frozen at −80 °C and thawed on ice, and then the cell lysates were collected and centrifuged. The supernatant was filtered using a 0.22 μm syringe filter. For MNV purification, the filtered supernatant was centrifuged at 96,589 × g (Beckman OptimaTM LE-80 K Ultracentrifuge, Beckman Coulter Inc. Indianapolis, IN, USA) using a 30% sucrose solution for 3 h at 4 °C. After centrifugation, the pellets were collected and dissolved in 20% glycerol. The purified MNV suspension was aliquoted and kept at −80 °C until its use.

Preparation of the MCR extract

The dried root barks of M. alba (MCR; voucher no. DSNPL0018) were purchased from Booguk Co. (Seoul, Korea). The MCR extract was prepared as previously reported with minor modification (Eom et al., 2017). The MCR was ground into powder (< 1-mm in size). 10 g of the MCR powder was extracted with 100 mL of 70% ethanol in an ultrasonic bath (Powersonic 420, Hwashin Instrument Co., Ltd. Seoul, Korea) at 20 °C for 1 h. The extract was centrifuged for 30 min (1610 × g) at 4 °C and the supernatant was freeze-dried (IIShin BIOBASE, Seoul, Korea). The MCR extract yield (%) was calculated as follows: the dry mass weight obtained after drying was divided by the dried bark weight used in the extraction and multiplied by 100.

Cell viability

RAW 264.7 cells were grown > 90% confluent in 96-well culture plates. MCR extract underwent a tenfold serial dilution using DMEM. 10 μL of MCR extract and 90 μL of DMEM supplemented with 10% FBS and 1% PS were added to the cells. The cell plate was incubated for 12 h at 37 °C and 5% CO₂. The MTT solution was added to each well in the plate. After each plate was incubated for 1 h,
dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance at 570 nm was analyzed using a microplate reader (SpectraMax M2, Molecular Devices, San Jose, CA, USA). Cell viability (%) was calculated as follows: the absorbance obtained after sample treatment was divided by the absorbance of the untreated control and multiplied by 100. The MTT cell viability test was performed in triplicate.

Antiviral effects of MCR extract, morusin, and kuwanon G

The antiviral effects of MCR extract and its flavonoids, morusin and kuwanon G, were determined by plaque assay. A plaque assay was conducted to determine the viral titers as previously described (Bae et al., 2018). Confluent RAW 264.7 cell monolayers grown in 24-well plates were used for MNV inoculation. The MNV suspension (7–8 log PFU/mL) was incubated with MCR extract (10–1000 µg/mL) or its bioactive compound (morusin and kuwanon G, 1–100 µM) at a volume ratio of 1:1 for 3 h at room temperature. The incubated suspension was tenfold serially diluted in DMEM and then inoculated onto confluent RAW 264.7 cell monolayers for 1 h at 37 °C and 5% CO₂. The inoculum was removed after MNV absorption, and 1 mL of DMEM supplemented with 5% FBS, 0.5% PS, and 1% agarose overlay was added to each well. The plate was incubated for 48 h at 37 °C and 5% CO₂, and the cells were fixed with 500 µL of 4% formaldehyde in phosphate buffered saline (PBS) for 1 h at 4 °C. Next, the agarose overlay was discarded, and the cells were stained with 250 µL of 0.5% crystal violet solution in 25% methanol for 20 min at room temperature. The number of plaques in each well was counted under a light microscope. DMEM and 2-thiouridine (2TU) were used as untreated and positive controls, respectively (Bae et al., 2018). The plaque assay was performed in triplicate.

Antiviral effect of MCR extract under simulated digestive conditions

Antiviral effects of MCR extract in simulated digestive conditions were examined. Simulated digestive juices were prepared for the oral phase (simulated saliva fluid, SSF), gastric phase (simulated gastric fluid, SGF), and small intestinal phase (simulated intestinal fluid, SIF). The electrolyte solutions and digestive enzymes were prepared as previously reported (Minekus et al., 2014). Since each simulated digestive fluid was mixed with MNV suspension at a ratio of 1:1 (v/v), SSF, SGF, and SIF were prepared at a 2X concentration. Briefly, SSF contained salivary α-amylase (150 unit/mL) and was adjusted to a pH of 7. SGF contained porcine pepsin (4,000 unit/mL) and was adjusted to a pH of 3. And SIF had pancreatin (200 unit/mL) from porcine pancreas and bile (20 mM) from bovine and ovine and was adjusted to a pH of 7. The digestion procedures were carried out in the absence of light, and all incubations were performed in a shaking water bath at 37 °C and 200 rpm. For the oral phase, MCR extract was dissolved in 1 mL of SSF and then was mixed with 1 mL of MNV suspension. The mixture was incubated for 2 min. For the gastric and small intestine phases, MCR extract at 2 mg/mL was dissolved in 1 mL of SGF or SIF and then was mixed with 1 mL of MNV suspension (7 log PFU/mL). The mixture was incubated for 2 h, tenfold serially diluted in DMEM, and inoculated onto confluent RAW 264.7 cell monolayers for 1 h at 37 °C and 5% CO₂. The starting MCR extract concentration was 0.2 mg/mL. The remaining procedure was as described for the plaque assay method. Controls for each digestive phase were performed as described above without the MCR extract. The analysis of digestive condition was performed in triplicate.

Data analysis

The data were expressed as means ± SD and analyzed by ANOVA. The mean values at the 5% significance level were compared with a Tukey’s test for multi-group comparison using IBM SPSS Statistics (version 24, IBM Corp, New York, NY, USA).

Results and discussion

The yield of MCR extract and effects of MCR extract, morusin, and kuwanon G on cell viability

The yield of the lyophilized MCR, which was extracted with 70% ethanol, was 5.6% (w/w). It is well-known that kuwanon G and morusin are two major marker compounds of MCR, when extracted with 70% ethanol by ultrasound (Eom et al., 2017; Kim et al., 2015). Eom et al. (2017) showed that kuwanon G and morusin are contained at 0.33% (w/w) and 0.18% (w/w) in MCR, respectively. The cell viability was performed to investigate whether MCR extract, morusin, or kuwanon G exert potential cytotoxicity in RAW 264.7 cells. The viabilities of RAW 264.7 cells were ≥ 98% after incubation with the extract at 1,000 µg/mL for 12 h (Fig. 1). Morusin and kuwanon G at 100 µM showed 97% and 92% cell viabilities, respectively. The antiviral activity test of the extract, morusin, and kuwanon G was conducted at a concentration of above 90% of cell viability.
Antiviral effects of MCR extract, morusin, and kuwanon G

MCR extract showed dose-dependent inhibition at concentrations of 10–1,000 μg/mL and significant antiviral effect was achieved (a 1.1 log reduction) at 1,000 μg/mL against MNV (p < 0.05) (Fig. 2A). A positive control, 2TU at 50 μM, revealed a 0.7 log reduction of MNV. Morusin showed 0.8, 0.9, and 1.0 log reductions of MNV titers at 1, 10, and 100 μM, respectively (Fig. 2B). Kuwanon G showed 0.3 and 0.4 log reductions of MNV titers at 1 and 10 μM, respectively, and a comparable strong antiviral effect was achieved (a 0.6 log reduction) at 100 μM (p < 0.05) (Fig. 2C). These results demonstrate that the pre-treatment of MNV with MCR extract or its bioactive compounds, morusin and kuwanon G, significantly reduced MNV titers. These data suggest that the antiviral effect of MCR extract or its bioactive compounds on MNV can interfere with the attachment of MNV to the RAW cells or disrupt the virus particles.

Antiviral activity of MCR extract in simulated digestive conditions

Digestive conditions with low pH, digestive enzymes, or bile reportedly cause chemical degradation or alteration of the antiviral compounds, leading to decreased antiviral activity (Falce et al., 2019; Joshi et al., 2015, 2017). In this study, human digestive systems were simulated with a 3-step in vitro process, including the oral, gastric, and small intestine systems. The antiviral test was performed in SSF, SGF, and SIF containing MNV in the presence or absence of MCR extract (Fig. 3). MCR extract at a concentration of 2 mg/mL in SSF, SGF, and SIF significantly reduced MNV titers by 1.8, 1.9, and 1.5 logs, respectively (p < 0.05), compared to no MCR extract. Interestingly, there is no significant difference in MNV reduction titers between SSF and SGF (p < 0.05); while SSF contained salivary α-amylase with only 2 min incubation at pH 7, SGF contained porcine pepsin with 2 h incubation at pH 3. It has been reported that MNV is relatively stable at acid pH, reaching a 0.3–0.7 log reduction of MNV for a 30 min incubation (Cannon et al., 2006; Hirneisen & Kniel, 2013). Several research studies have also demonstrated that human norovirus is insusceptible to acid pH or pepsin. Dolin et al. (1972) reported that human norovirus incubated for 3 h at pH 2.7 could infect human volunteers. When greenshell mussels were incubated with human noroviruses at pH 3.8 for 4-weeks, the genome copies of human
norovirus did not decrease (Hewitt and Greening, 2004). It was recently found that the integrity of human norovirus GII.4 treated at pH 1.3 alone and pH 1.3 with pepsin did not change compared to the virus integrity after treatment at pH 8 (Chassaing et al., 2020). Therefore, MNV could mimic human norovirus’ survival at low pH levels such as gastric conditions.

On the other hand, MCR extract showed antiviral activity, reaching a 1.5 log reduction of MNV in the SIF containing pancreatin and bile at pH 7. Nelson et al. (2018) reported that bile acid facilitated MNV attachment and infection. In this study, MCR extract’s antiviral activity in SIF containing bile was significantly lower than that of MCR extract in SSF or SGF (p < 0.05). Taken together, the results of the present study demonstrate that the antiviral activity of MCR extract could be maintained in the presence of digestive enzymes, a low pH, and bile. Our results are consistent with previous findings that show antiviral effects of plant-derived extract or compounds in simulated digestive conditions. Aged-green tea extract at 5 mg/mL reduced MNV titer by 0.7, 3.1, and to an undetectable level in SSF (pH 7), SGF (pH 3), and SIF (pH 7), respectively (Falco´ et al., 2019). Joshi et al. (2015) reported that 1 mg/mL grape seed extract reduced MNV titers to an undetectable level after 6 h incubation in SGF (pH 1.5) or SIF (pH 7). At 5 mg/mL, blueberry proanthocyanidins reduced MNV titers to an undetectable level after 30 min incubation in SIF (pH 7) (Joshi et al., 2017).

The MCR is authorized for food supplement use in the European Union (European Commission 2021). The MCR extract can be added to sauces for ready-to-eat fresh salads or fresh oysters, which frequently cause norovirus outbreaks. In this context, further assessment of antiviral activities of the MCR extract in food matrices, when contaminated with human norovirus, as well as sensory evaluation of MCR extract-added foods is needed for future studies.

In conclusion, we identified the antiviral activities of MCR extract and its bioactive compounds, morusin and kuwanon G, against MNV in RAW 264.7 cells. The antiviral activity of MCR extract was also demonstrated in simulated digestive conditions, such as SSF, SGF, and SIF. Therefore, MCR extract can be used to reduce norovirus infectivity.

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Declarations

Conflict of interest None of the authors of this study has any financial interest or conflict with industries or parties.

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