REVIEW

Inactivation of norovirus and surrogates by natural phytochemicals and bioactive substances

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Human norovirus is the leading cause of sporadic gastroenteritis, which is responsible for more than 90% of all nonbacterial gastroenteritis outbreaks. While norovirus infections typically cause mild and self-limiting symptoms lasting 24–48 h, chronic persistent infections can cause severe symptoms. Although recent advances have been made in understanding the molecular characteristics of norovirus infection, no norovirus-specific antiviral drugs, or vaccines are available. Conventional intervention methods used to inactivate norovirus, such as treatment with disinfecting agents (e.g. ethanol, hypochlorite, and quaternary ammonium formulations), have shown a lack of efficacy against human norovirus when they are applied to foods and in food preparation processes. Therefore, alternative antiviral or inactivating agents such as phytochemicals have received attention as potential norovirus inhibitors due to their relatively low toxicity and lack of side effects, which allows them to be prepared as food-safe formulations. Evidence from studies using viral surrogates suggests that numerous phytochemicals and foods containing flavonoids and polyphenols have anti-norovirus activity, and future studies will be necessary to confirm the effectiveness of such compounds against human norovirus and the molecular mechanisms through which they produce antiviral effects.

Keywords:
Antivirals / Inactivation / Infection cycle / Natural compounds / Norovirus / Phytochemicals

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1 Introduction

Human noroviruses (HuNoVs) are the leading cause of gastroenteritis outbreaks and severe childhood diarrhea worldwide [1, 2]. Although the form of gastroenteritis caused by HuNoVs is typically mild, self-limiting, and associated with symptoms lasting 24–48 h, chronic persistent infections can be fatal in children, the elderly, and immunocompromised individuals [2–4]. HuNoV is also associated with several important clinical outcomes such as necrotizing enterocolitis, seizures in infants, encephalopathy, pneumatosis intestinalis, and disseminated intravascular coagulation [5]. In the United States, approximately 21 million cases of HuNoV infection occur annually, resulting in approximately 71 000

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Abbreviations: **FCV**, feline calicivirus; **HBGA**, histo-blood group antigen; **HuNoV**, human norovirus; **MNV**, murine norovirus; **NS**, nonstructure; **RdRp**, RNA-dependent RNA polymerase; **$TCID_{50}$**, 50% tissue culture infective doses

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hospitalizations and approximately 800 deaths, at a total cost of $493 million [6–8]. HuNoV infections are more common in developing countries, where HuNoVs are estimated to be responsible for approximately 200 000 deaths annually in children under 5 years of age [9]. Approximately 55% of NoV outbreaks occur in the winter, especially from December–February [10].

HuNoVs are transmitted by the fecal-oral route, with the predominant modes of infection being person-to-person direct contact, as well as food- and water-borne transmission [4, 11]. Outbreaks commonly originate in healthcare facilities (e.g. nursing homes, hospitals, and residential-care centers), recreational settings (e.g. cruise ships and hotels), schools, day-care centers, or restaurants or other food service establishments. The rapidly progressing nature of some NoV outbreaks, in which large groups of people become ill within a short period, suggests a common source of infection, such as food or water [12, 13]. Rapid HuNoV has been associated with consumption of contaminated food (fresh fruit, vegetables, shellfish, and bakery products) and/or water (drinking water, ice, or swimming), and contact with contaminated surfaces [14]. Although exposure to as few as a few dozen viral particles can successfully transmit HuNoV infection [15], a recent report suggests that the 50% human infectious dose can be higher than previously estimated [16]. Nonetheless, virus shedding from infected persons, including those with asymptomatic infection as well as the relatively long half-lives of virions in the environment, is particularly important in the spread of disease, and accounts for the high rate at which HuNoV infections tend to spread [17].

HuNoVs are genetically diverse and undergo error-prone replication. Although our understanding of HuNoV biology has been dramatically improved over the past 10 years by the development of Norwalk virus-replicon bearing cells and the identification of murine noroviruses (MNVs), effective antiviral compounds and vaccines have not been developed [18, 19]. While public and industrial control measures are important for limiting food-borne NoV outbreaks, testing decontamination procedures remains difficult due to the lack of viable cell culture system for propagating HuNoVs [20]. An improved understanding of NoV biology and pathogenesis remains essential for the control and treatment of highly infectious HuNoVs. Here, we describe recent progress regarding the control of NoV infections, with a special emphasis on natural substances and phytochemicals with bioactivity against NoVs, after a brief introduction to the molecular features of NoVs.

2 Molecular characteristics of noroviruses

2.1 Genomes and classification

Noroviruses belong to the family *Caliciviridae*, members of which have a small, positive-sense RNA genome. Noroviruses contain a linear, single-stranded, positive-sense RNA genome, which is approximately 7.4–7.7 kb in size. While the 3′-end is polyadenylated, the 5′-end of the genome is covalently linked to the virally encoded nonstructural (NS) VPg protein. The NoV genome typically contains three tightly packed ORFs. NoV genome ORF1 encodes a polyprotein that is cotranslationally and posttranslationally cleaved by a virally encoded protease into six nonstructural proteins, while ORFs 2 and 3 encode the major and minor capsid proteins, which are known as VP1 and VP2, respectively [18, 21]. Norwalk virus, the prototypical virus of the genus *Norovirus*, produces small (27 nm), round, nonenveloped virion particles [22].

Like other RNA viruses, NoVs possess an error-prone replication mechanism, resulting in a high degree of genetic diversity. NoVs are subdivided into at least six genogroups (GI, GII, GIII, GIV, GV, and GVI), each of which is further subdivided into several genotype subtypes, as indicated by numbering to the right of the genogroup name (e.g. GII.4) [23, 24]. The majority of HuNoVs belong to genogroups I and II, and the predominant norovirus strains associated with gastroenteritis outbreaks worldwide are the NoV GII.4 genogroup subtype, which includes GII.4/2006b, GII.4/2009, and GII.4/2012 (Sydney) [4, 9]. As an important laboratory surrogate of HuNoVs, MNVs, which belong to genogroup V, contain a fourth ORF, and are the first noroviruses that can be propagated in in vitro culture systems [18, 21]. Although there are some discrepancies in infection symptoms in vivo, MNVs share many genetic and biochemical features with HuNoVs, such as modes of transmission and acid resistance. MNVs are considered to be the best available substitute for HuNoVs, and represent an efficient laboratory model system that has advanced our understanding of HuNoV infection [25, 26]. Feline calicivirus (FCV) has also been used as a laboratory surrogate for HuNoVs, because it shares similarities in genome sequence and organization with HuNoVs [27]. However, it should be noted that FCV, a calicivirus that causes respiratory infection in cats and does not tolerate acidic conditions, exhibits significantly different characteristic from those of HuNoVs, such as the mode of transmission and the stability of virus particles [28].

2.2 Infection cycle

2.2.1 Attachment, entry, and uncoating

Norovirus utilizes cell surface molecules as mediators for binding and cellular entry. HuNoVs interact with histo-blood group antigens (HBGAs) on the surfaces of host cells, and interactions between the viral capsid and HBGAs have been characterized by structural analysis [29]. Although HBGAs play an important role in NoV entrance and susceptibility, HBGAs alone are not sufficient to enable viral entry [30], as HuNoV can bind and enter host cells independently of HBGAs [31]. Several molecules have been proposed to facilitate the entry of MNVs, but none has yet been validated as a cellular receptor for MNVs or HuNoVs. The results of

previous studies suggest that sialic acid, glycolipids, and glycoproteins are important for MNV attachment [32, 33], while cholesterol and dynamin II, but not clathrin or caveolae, are critical for receptor-mediated endocytosis of MNVs [34, 35]. However, viral entry of MNV-1 seems to be independent of low pH, whereas that of FCV, another surrogate for HuNoVs, is dependent on the acidification of endosomes [36]. The uncoating events during which noroviruses release their viral genome to allow it to become accessible to the translational machinery have not been well defined.

2.2.2 Translation

Cytosolic translation of the VPg-linked NoV RNA genome is initiated immediately following uncoating of the viral capsid. After the positive-sense NoV genomic RNA is released into the cytoplasm, it serves as a template for translation of the ORF1 polyprotein. VP1 and VP2 structural proteins (as well as VF1 in the case of MNV) are translated from subgenomic RNA, which is comprised of the 3′-end of the genomic RNA. Subgenomic RNAs are present in greater abundance than full-length genomic RNAs within NoV-infected cells, resulting in enhanced production of VP1, the major capsid protein [5].

2.2.3 Replication

Like other positive-sense RNA viruses, NoVs replicate in the cytoplasm. Due to the absence of an effective system for culturing HuNoVs, the molecular mechanisms of HuNoV replication have been mainly inferred by studying MNV replication. A virally encoded RNA-dependent RNA polymerase (RdRp, $NS7^{pol}$) performs de novo synthesis of the complementary strand of the viral RNA in a VPg-independent manner [37], and negative-sense RNA is synthesized as an intermediate. The viral RdRp ($NS7^{pol}$) utilizes VPg as a protein primer that provides a 3'-OH to initiate the synthesis of positive-sense genomic RNA from the negative-sense RNA intermediate template [37]. Vpg is also used in the synthesis of subgenomic RNA, which serves as an mRNA template for the translation of structural proteins VP1, VP2, and (in the case of MNV) VF1.

2.2.4 Assembly and release

The molecular mechanisms underlying viral particle assembly, encapsidation, and release of NoVs remain largely unknown. The major structural protein, VP1, self-assembles into virus-like particles that are similar to virions in terms of morphology and antigenicity [38]. The minor structural protein VP2 is not required for virus-like particle assembly and is instead thought to stabilize VP1/viral RNA interactions, enhancing the infectivity of nascent virions [38, 39]. Recent results suggest that HuNoVs may be released from host cells by the induction of apoptosis [40, 41].

3 Phytochemical and bioactive substances with anti-norovirus activity

HuNoVs are remarkably stable and can withstand harsh environments, including freezing temperatures, low pH, and alcohol-based disinfectants [42]. In the last decades, an extensive body of literature has reported the efficacy of various inactivation strategies tested in HuNoV and its surrogates systems. These include physical methods such as exposure to heat [43], UV radiation [44], ionizing radiation [45], filtration [46], and high pressure [47], as well as chemical methods such as exposure to alcohols [48], sodium hypochlorite [49], free chlorine- [50], and iodine-generating agents, hydrogen peroxide [51], ozone [52], aldehydes [53], quaternary ammonium compounds [54], surfactants [55], nanoparticles [56], and low pH [42]. The effectiveness of the tested inactivation methods varied depending on food matrix, temperature, humidity, contact-time, and/or tested surrogate species, and there does not appear to be a single ideal treatment that is able to inactivate HuNoVs, especially in foods with diverse properties. The advantages and limitations of traditional inactivation methods to inhibit NoVs in foods and environment are thoroughly discussed elsewhere including reviews by Nims and Plavsic [57] and Baert et al. [58], therefore, this review is not meant to describe the details of conventional NoV inactivation technologies except using phytochemical and bioactive substances with anti-NoV activity.

Several publications have demonstrated the anti-noroviral activity of natural compounds. Flavonoids are found in most biomaterials with anti-NoV activity, including grape seeds [59–61], pomegranates [62, 63], mulberries [64], black raspberries [65], cranberries [66, 67], green tea extracts [68], and persimmons [69,70]. Other compounds, such as ginseng [71], oregano essential oils [72,73], chitosan [74,75], and citric acid [76], as well as common proanthocyanidins and flavonoids from plant foods [77, 78], also effectively reduce NoV replication. In addition, Su and D'Soulza showed that myricetin, L-epicatechin, tangeretin, and naringenin produced significant anti-norovirus activity in a plaque reduction assay [78]. Pomegranates, mulberries, black berries, and cranberries contain large quantities of various types of polyphenols and anthocyanidins, grape seeds, and green tea are enriched with flavon-3-ols known as catechins, and berries and persimmons contain polymeric tannins.

Anti-noroviral activity was assessed in many studies using common approaches (plaque assays or measuring 50% tissue culture infective doses ($TCID_{50}$)), it should be noted that these studies were conducted with varying experimental conditions in terms of treatment temperatures, the number of time points collected, and the overall experimental durations. The antiviral properties of phytochemicals and natural substances were mainly evaluated through time-of-addition

experiments to cells or viruses, wherein cells were exposed to test substances either before infection, at the time of infection, or following infection, or viruses were pretreated with test compounds before infection was initiated. Pretreatment of cells with the test compounds was performed primarily to examine the ability of the compounds to inactivate cellular receptor(s), and thus to prevent viral attachment and internalization. Pretreatment test methods may also produce anti-noroviral activity resulting from immune enhancement of the host cells. Studies involving pretreatment of viruses with test compounds were performed mainly to investigate the effects of the compounds on cellular attachment mediated by the VP1 ligand. Cotreatment of cells at the time of viral infection can assess combined effects of test compounds on both viruses and host cells during the viral attachment, entry, and internalization steps. Posttreatment of cells with test compounds after viral infection was used to identify compounds that inhibit viral replication following entry.

In addition, most studies discussed below used surrogates such as FCV (FCV-F9) and MNV (MNV-1) cells, because sufficient quantities of infectious HuNoVs were difficult to obtain. From a physical standpoint, similarities in capsid structure, genomic organization, and replication cycle between MNV-1 and HuNoV [25] makes MNV-1 an appropriate surrogate for HuNoV. FCV-F9 is also a popular surrogate in NoV studies that can be cultured, and it is not transmissible to humans. Various studies have identified phytochemicals and bioactive substances with anti-NoV activity, but detailed mechanistic studies have not been performed to determine exactly how they inhibit NoV replication.

3.1 Grape seed

Grape (*Vitis vinifera*) seed is a by-product generated during the production of juices and wines and contains large amount of phenolic compounds including gallic acid and monomeric catechins, monomeric anthocyanin, and dimeric, trimeric, and polymeric proanthocyanidins [79]. Grape seed extracts have been shown to exert a range of biological effects, including antimicrobial and antiviral activities, with no apparent toxicity in humans at doses up to 100 mg per day [80]. Three studies have reported anti-noroviral activity attributed to grape seed extracts. In the first study, the grape seed extract contained >95% flavonols, with 82% oligomeric proanthocyanidins and 12% monomeric proanthocyanidins, and the antiviral effects of this extract were examined by incubating the MNV-1 and FCV-F9 surrogates with 0.5, 1, and 2 mg extract/100 mL for 2 h at room temperature or at 37°C [60]. Surrogate virus infectivity was reduced by the grape seed extract in a dose-dependent manner, as demonstrated in plaque assays. Specifically, the titers of the FCV-F9 and MNV-1 viruses were reduced by 3.6–4.6 $\log_{10}$ PFU/mL and 0.8–1.7 $\log_{10}$ PFU/mL, respectively, when pretreated with increasing concentrations of grape seed extract at 37°C. The suggested anti-NoV effects of grape seed extract was by direct inactivation of viral particles.

In the second study, two model foods (lettuce and jalapeno peppers) were inoculated with MNV-1 and FCV-F9 for periods ranging from 30 s to 5 min in the presence of grape seed extract (0.25, 0.5, and 1.0 mg/mL) [61]. FCV-F9 infectivity after exposure to lettuce was reduced by 2.3–2.7 $\log_{10}$ PFU/mL following treatment with grape seed extracts for 1 min, and similar grape seed extract treatment in lettuce and peppers moderately decreased the infectivity of MNV-1. These results suggested that grape seed extract may be a viable option for reducing transmission of foodborne norovirus.

The third study investigated the effects of grape seed extract on HuNoV as well as MNV-1 surrogate in plaque assays. The grape seed extract used in this study contained proanthocyanidins, catechin, and epicatechin as the major constituents, and viral particles were preincubated with grape seed extract at 37°C for 1 h [59]. In the MNV-1-based plaque assays, viral titers were reduced by >3 $\log_{10}$ PFU/mL following –1 treatment with grape seed extract (0.2 mg/mL). The effects of grape seed extract on the cell- and saliva-binding abilities of human NoV GII.4 were also quantified using ELISA and RT-PCR. In the ELISA-based saliva-binding assay, the binding of HuNoV GII.4 P particles to salivary carbohydrates was reduced significantly following treatment with grape seed extract. Similarly, RT-PCR experiments revealed that infection of NoV GII.4 was decreased by >80% following treatment with grape seed extract. Finally, to assess the potential mechanisms underlying reductions in viability and binding produced by grape seed extract, the authors used transmission electron microscopy to examine human NoV GII.4 virus-like particles before and after treatment with grape seed extract. Treatment resulted in dramatic inflation and deformation of virus-like particles, suggesting that denaturation of the viral capsid protein occurred. These results suggest that grape seed extracts damage the NoV capsid protein, which could reduce viral binding affinity and infectivity.

3.2 Pomegranate

Epidemiological evidence suggests that pomegranate (*Punica granatum*) has activity against foodborne viruses [81–83], leading Su et al. to investigate the effects of pomegranate juice on MNV-1. Pomegranate juice contains a high concentration of polyphenols, including ellagitannins, punicalagin, and punicalin, as well as low concentrations of tannins, ellagic acid, and anthocyanins (delphinidin, cyanidins, and pelargonidin) and their glycosides [84]. MNV-1 was preincubated with pomegranate juice or polyphenols at room temperature for 1 h [62], and this preincubation step significantly decreased the FCV-F9 and MNV-1 titers by 2.56 and 1.32 $\log_{10}$ PFU/mL, respectively (an equal volume of juice was mixed with virus solution). Pomegranate-derived polyphenols exhibited greater activity against MNV-1, and reduced plaque formation of MNV-1 by 1.30–3.61 $\log_{10}$ PFU/mL.

In a second study, the time-dependent effects of pomegranate juice and its polyphenolic components were examined [63]. Pomegranate juice or its constituent polyphenols (4 and 8 mg/mL) were incubated with viral surrogates at room temperature for up to 1 h. In plaque assays, viral titers were reduced by approximately 50% within the first 20 min of treatment, indicating that viral titer reduction may be achieved rapidly by exposure to pomegranate juice and its constituent polyphenols. The reduction of FCV-F9 titer was most dramatic when the virus was incubated concurrently with pomegranate juice and its constituent polyphenols. However, the mechanisms underlying the effects of pomegranate on NoV infectivity were not investigated.

3.3 Mulberry

Mulberries (*Morus alba*) possess antioxidant and antimicrobial activities, and both mulberry juice and mulberry seed extracts exhibit anti-NoV activity [64]. Mulberry is rich in flavonoids such as quercetin, rutin, and cyanidin glycosides [85]. In this study, mulberry extract was added at different steps in the plaque assays. Mulberry extract was added to cells (precell treatment) or viral samples before infection (previral treatment), during viral adsorption (cotreatment), or following viral adsorption (posttreatment). In the cotreatment experiments, the minimum concentrations of mulberry juice required to produce a 50% reduction in MNV-1 and FCV-F9 viral titers were 0.005 and 0.25% v/v, respectively, suggesting that mulberry juice inhibits viral replication at a step(s) preceding gene expression. Two predominant compounds in mulberry juice (cyanidin-3-glycoside and cyanidin-3-rutinoside) showed anti-NoV activity following a 1 h cotreatment. These results suggest that mulberry juice and its anthocyanin glycoside components may inhibit MNV-1 during internalization or an early replication step. In this study, the effects of two major anthocyanins in mulberry juice were further investigated, and results showed that treatment with yanidin-3-glucoside and cyanidin-3-rutinoside during viral adsorption produced anti-norovirus activity.

In addition to mulberry juice, the effects of mulberry seed extract were examined in plaque assays and RT-PCR experiments under similar experimental conditions. The major compounds identified in mulberry seed extract included caffeic acid, 3,4-dihydroxybenzoic acid, rutin, and cyanidin-3-rutinoside. Maximum plaque reduction was observed following cotreatment of cells at the time of MNV-1 viral challenge (47% reduction). Despite moderate inhibition by mulberry seed extract, this effect was confirmed using RT-PCR. Among the individual compounds comprising mulberry seed extract, cyanidin-3-rutinoside resulted in the greatest reduction in MNV-1 RNA expression. Collectively, these data suggest that both mulberry juice and its seed extracts effectively inactivate HuNoV surrogates at an early step of the viral life cycle.

3.4 Black raspberry

The *Rubus coreanus* species of black raspberry is native to Korea, China, and Japan and is known as *bokbunja* in Korea. *R. coreanus* is commonly used for wine and beverage production and contains high concentrations of polyphenols and flavonoids, including tannins, proanthocyanidins, and anthocyanidins [86]. The effects of black raspberry juice on viral replication were investigated by plaque assays using the surrogate viruses MNV-1 and FCV-F9 [65]. As with mulberry juice extracts, maximum plaque reductions were observed following treatment of cells at the time of viral challenge. Black raspberry juice (0.64 mg of total polyphenols/mL) decreased MNV-1 plaque formation by 75.3 $\pm$ 6.6% and 92.7 $\pm$ 1.9% at concentrations of 3 and 6%, respectively. Pretreating cells or viruses significantly reduced viral titers. Gallic acid and quercetin did not show antiviral activity on either tested virus, suggesting that polyphenols other than gallic acid and quercetin may be the primary active compounds in black raspberry. These results suggest that black raspberry juice may primarily block viral entry into the cell by inhibiting its binding and internalization, or through direct effects on viral particles or host cell receptors.

3.5 Cranberry

Cranberry juice from the *Vaccinium macrocarpon* species contains proanthocyanin, a major polyphenol known to possess both antibacterial and antiviral properties together with several other pharmacological activities. HPLC studies showed that fresh cranberry contains cyanidin-galactoside, cyanidin-arabinoside, peonidin-galactoside, and peonidin-arabinoside as major flavonoids [87]. Su et al. investigated the antiviral effects of cranberry juice and its proanthocyanidins using MNV-1 and FCV-F9 viruses as HuNoV surrogates. The viruses were directly incubated with cranberry juice and proanthocyanidins for 0–1 h at room temperature and then studied in plaque assays [66, 67]. The most significant reductions were seen in FCV-F9, which showed a reduction of 5 $\log_{10}$ PFU/mL within 30 min of exposure to cranberry juice proanthocyanin (0.15 mg/mL). MNV-1 titers were reduced by 1 $\log_{10}$ PFU/mL following incubation in cranberry juice for 1 h [66]. In general, approximately 50% of the inhibitory effect of cranberry juice was achieved within the first 10 min of treatment. Transmission electron microscopy analyses of FCV-F9 virions treated with cranberry juice under acidic conditions revealed significant structural and morphological damage, which likely led to reduced overall viral infectivity. Further studies are necessary to determine the mechanism underlying the anti-noroviral activity of cranberry juice.

3.6 Green tea extract

Green tea (*Camellia sinensis*) is enriched with polyphenolic catechins, of which (-)-epigallocatechin gallate is a major

compound, and has anti-viral properties [68]. Activity-guided fractionations of green tea methanolic extracts followed by plaque assays with FCV-F9 were used to identify compounds in green tea with anti-noroviral activity. Fractionated green tea extracts were added to cell cultures immediately following viral infection, and the cells were incubated at 37°C in 5% CO_2 for 24–48 h. The ethyl acetate-soluble fraction of green tea resulted in the greatest reduction of FCV-F9. Further analysis of this fraction by HPLC enabled the identification of catechins as the major antiviral compounds, and epigallocatechin gallate exhibited the best combination of antiviral activity (IC_{50}, 12 mg/mL) and low cytotoxicity.

3.7 Red ginseng

Ginseng (*Panax ginseng*) is a popular and well-studied herbal medicine that is used in East Asia because of its wide range of biological activities. Red ginseng is produced by steam heating of peeled ginseng, which is followed by a drying process. Gingeng saponins known as ginsenosides, including ginsenoside Rb1, Rb2, Rc, Rd, and Rg1, are bioactive compounds [88]. The antiviral activities of ginseng have been reported in several previous studies, and the strongest effects were produced by red ginseng. Anti-NoV activity was studied with Korean ginseng extract and two component ginsenosides, Rb1 and Rg1. MNV-1 titers were significantly reduced in cells pretreated with red ginseng extract or ginsenosides, but not in cells that were treated at, or following, the time of viral challenge [71]. FCV-F9 titers were significantly reduced by 0.23–0.83 $\log_{10}$ TCID/mL in the pretreatment group (5–10 μg/mL of red ginseng extract), while MNV-1 titers in this group were reduced by 0.37–1.48 $\log_{10}$ TCID/mL. Rg1 was more effective than Rb1 in the MNV-1 and FCV-F9 plaque assays. These data suggest that red ginseng extract and ginsenosides Rb1 and Rg1 may possess significant anti-NoV activities. A more detailed investigation into the antiviral mechanisms of red ginseng has not been conducted.

3.8 Persimmon

Persimmon (*Diospyros kaki*) is a common fruit that contains high levels of tannins, and two studies have demonstrated NoV reduction by persimmon using HuNoV surrogates [69, 70]. In one study, several pathogenic food-borne viruses were incubated with various plant-derived tannins [70]. Persimmon tannins are condensed tannins containing proanthocyanidins, and the chemical structure of persimmon tannins is not well understood. The persimmon extracts consisting of 22% tannins produced the greatest reduction in MNV-1 titer. MNV-1 was pretreated with persimmon extracts at a concentration of 0.25% for 3 min and added to cells for 1 h. Persimmon extracts reduced MNV-1 titers by 4.3 $\log_{10}$ PFU/mL, suggesting that tannin compounds block viral attachment or internalization, thereby inhibiting infection. A second study used HuNoV GII.4 that was pretreated with persimmon extract and its tannins to assess the effects of these agents on replication by qPCR [69]. The results demonstrated that extracts containing more than 0.11 mg/mL of persimmon tannin reduced noroviral genome replication by >70%. Persimmon tannins did not show cytotoxicity at the concentrations used, indicating that persimmon tannins are nontoxic anti-noroviral agents.

3.9 Herbal essential oils

Herb-based essential oils, which are enriched in plant-derived volatile aromatic compounds, have long been used by practitioners of traditional medicine, and more recent analyses of these oils have identified several associated pharmacological effects, including antibacterial, antifungal, and antioxidative activities [89, 90]. A study showed that oregano (*Origanum vulgare*) essential oil decreased FCV-F9 and MNV-1 titers in a dose-dependent manner when the viruses were pretreated with the oils before infection was initiated [73]. In this study, incubation of virus with 2% oregano essential oil for 2 h at 37°C reduced FCV-F9 titers by 3.75 log $TCID_{50}$/mL. In addition, Gilling et al., reported that oregano essential oil and its primary component carvacrol inactivated MNV-1 within 1 h of exposure [72]. Both oregano oil (4%) and its major volatile compound carvacrol (0.5%) significantly reduced MNV-1 infectivity within 15 min of exposure (0.95 and 1.28 log $TCID_{50}$/mL, respectively). Of particular interest was carvacrol (0.5%), which produced a dramatic MNV-1 reduction as the time of exposure increased. Furthermore, both oregano essential oil and carvacrol caused the viral capsid to expand and lose integrity, as determined by transmission electron microscopy experiments. To investigate the mechanism underlying this antiviral effect, the authors used an RNase I protection assay followed by qPCR to quantify the amount of viral RNA protected by the capsid. Interestingly, viral RNA was reduced with and without RNase I treatment ($p < 0.05$) relative to untreated controls at all-time points tested (30 min, 6, and 24 h) for both oregano oil and carvacrol. Together, these results indicate that degradation of the viral capsid occurred, and that the anti-NoV compounds present in oregano essential oil may act directly on the exposed RNA.

3.10 Chitosan

Some polysaccharides exert anti-viral activity by inhibiting viral entry into host cells. Chitosan is a biopolymer produced by the deacetylation of chitin derived from the exoskeletons of crustaceans. Two studies have investigated the anti-noroviral activity of chitosan using standard NoV surrogates. Su et al. reported that the incubation of water-soluble chitosan (molecular weight of 53 000, 0.7%) with viruses at 37°C for 3 h reduced FCV-F9 titer by 2.8 $\log_{10}$ PFU/mL, but had no effect on the MNV-1 titer [74]. Davis et al. examined purified chitosan

with different molecular weights by pretreating viral samples for 3 h at 37°C [75], and found that water-soluble chitosan with an average molecular weight of 53 kDa was the most effective of the tested chitosans against FCV-9 replication; however, this chitosan was only marginally effective against MNV-1 replication. The authors suggested that chitosan might interact with NoV capsid proteins, thus interfering with virus attachment and entry.

3.11 Citric acid

NoV is resistant to low pH; however, some organic acids, such as citric acid, produce viral inhibition. Citric acid is a weak acid that is found at high concentrations in many citrus fruits. Extensive studies of the biological activities of citric acid show that it chelates divalent cations and inhibits lipogenesis. Hansman et al. performed x-ray crystallography and saturation transfer difference nuclear magnetic resonance studies to determine the structure of the HuNoV coat protein and the role of citric acid in viral entry [76]. HuNoVs bind HBGA saccharides using their VP1 protruding 2 (P2) domain, and this interaction is considered critical for viral entry into epithelial cells of the gastrointestinal tract. Structural analyses showed that citrate also binds to the P2 domain of VP1. As citrate and water form a chemical structure similar to that of fucose, a key HBGA sugar molecule in viral recognition, citrate may act as a competitive inhibitor of HuNoV binding to HBGA, thereby inhibiting viral attachment and entry.

4 Discussion

Current reports suggest that several phytochemicals and bioactive substances have anti-noroviral activities. These substances can be grouped into several categories, including flavonoids, aroma compounds, polysaccharides, and organic acid, and they are frequently consumed in the daily diet as foods (pomegranates, mulberries, raspberries, cranberries, persimmons), food additives (grape seed extract and citric acid), herbs and tea (green tea and oregano), and functional foods (ginseng and chitosan). Among substances that show significant anti-norovirus activity with potential application in food industry, plant polyphenols such as anthocyanins, proanthocyanins, and catechins are the best characterized. In general, the anti-NoV effects of natural phytochemicals and biomaterials are much milder than those produced by conventional physical and chemical inactivation methods; however, there are advantages to the use of natural substances. All substances described in this review have been consumed as a part of the daily diet for a long period of time, and are thus considered to have a safety profile better than that of conventional chemical antivirals. For example, several systematic reviews suggest that ginseng generally has a good safety profile and a low occurrence of side effects, whereas ginseng produces beneficial biological effects in humans, including anti-diabetic, anti-cancer, and anti-obesity effects [91–93]. Meanwhile, there is limited data on the anti-norovirus effects of phytochemicals directly on food items. Further studies regarding decontamination procedures on food items such as fresh produce and shellfish using phytochemicals and natural substances will assess their efficacy. To acquire consistent and comprehensive data, experimental protocols, including the initial virus titer measurement, inoculation procedure, and treatment ratio, must be integrated and conducted in a consistent manner.

While research on the anti-noroviral activity of bioactive phytochemicals is an emerging field, the studies discussed here holds some limitations and future researchers should consider several factors that can improve the quality of such research. While most studies examined the effects of extract mixture substances, they did not thoroughly investigate minor compounds in the extracts that may have had potent activity. Thus, activity-guided fractionation analysis could identify novel compounds with anti-noroviral activity other than well-known bioasubstances. In addition, the antiviral effects of phytochemicals and natural substances should be validated against HuNoVs. Many studies exploring the antiviral activity of natural extracts and phytochemicals discussed here relied on the use of laboratory surrogates such as MNV-1 and FCV-F9, and only few studies used HuNoV GII.4. Studies using HuNoVs indicate that the effects of bioactive phytochemicals on HuNoVs were less potent than their effects on MNV-1 or FCV-F9, and thus it may be necessary to optimize treatment conditions. Besides, FCV-F9 was shown to be more sensitive to substances containing plant polyphenols [60,62,65,67] and chitosan [74,75], which may be, in part, due to the relative instability of FCV-F9 in comparison with MNV-1 or HuNoVs. However, we cannot yet conclude whether the effects of different substances on different surrogate viruses are valid, because of the limited number of studies and the heterogeneity of study settings. Furthermore, the mechanisms of action through which the antiviral effects of phytochemicals are mediated are not fully understood, and elucidation of detailed mechanisms will be necessary for such compounds to be used in industrial and practical applications, including the development of anti-NoV drugs from natural compounds.

In this report, we discussed studies showing that various phytochemicals are known to exhibit potent anti-NoV effects. Given the inherent advantages of natural substances in terms of toxicity, side-effects, and food-safe formulations, the use of such substances should be expanded, which may help to prevent transmission of HuNoVs, and thus improve the quality of life of all susceptible individuals, particularly those at high risk of infection, such as infants, the elderly, and the immunosuppressed [94].

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