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Tannic acid inhibited norovirus binding to HBGA receptors, a study of 50 Chinese medicinal herbs

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ABSTRACT

Noroviruses (NoVs) are the leading cause of viral acute gastroenteritis affecting people of all ages worldwide. The disease is difficult to control due to its widespread nature and lack of an antiviral or vaccine. NoV infection relies on the interaction of the viruses with histo-blood group antigens (HBGAs) as host receptors. Here we investigated inhibition effects of Chinese medicinal herbs against NoVs binding to HBGAs for potential antivirals against NoVs. Blocking assays was performed using the NoV protrusion (P) protein as NoV surrogate and saliva as HBGAs. Among 50 clinically effective Chinese medicinal herbs against gastroenteritis diseases, two herbs were found highly effective. Chinese Gall blocked NoV P dimer binding to type A saliva at IC_{50} = 5.35 μg/ml and to B saliva at IC_{50} = 21.7 μg/ml. Similarly, Pomegranate blocked binding of NoV P dimer to type A saliva at IC_{50} = 15.59 μg/ml and B saliva at IC_{50} = 66.67 μg/ml. Literature data on preliminary biochemistry analysis showed that tannic acid is a common composition in the extracts of the two herbs, so we speculate that it might be the effective compound and further studies using commercially available, highly purified tannic acid confirmed the tannic acid as a strong inhibitor in the binding of NoV P protein to both A and B saliva ($IC_{50} \approx 0.1$ μM). In addition, we tested different forms of hydrolysable tannins with different alkyl esters, including gallic acid, ethyl gallate, lauryl gallate, octyl gallate and propyl gallate. However, none of these tannins-derivatives revealed detectable inhibiting activities. Our data suggested that tannic acid is a promising candidate antiviral against NoVs.

1. Introduction

Noroviruses (NoVs) are recognized as the leading cause of epidemics of acute gastroenteritis and an important cause of sporadic gastroenteritis affecting people of all ages worldwide. NoVs are highly contagious often leading to large outbreaks of acute gastroenteritis in a wide variety of settings, including hospitals, schools, childcare centers, nursing homes and cruise ships and military camps, in which young children, the elderly, travelers, soldiers, and immunocompromised patients are the high-risk populations.[1,2] Recent estimation indicated that NoVs cause about 64,000 hospitalization; 900,000 clinic visits among children in developed countries as a result of NoV outbreaks and sporadic infection; and around 200,000 deaths in developing countries each year.[3]

NoVs are difficult to study owing to the lack of a cell culture and efficient small animal model for human NoVs.[4] Available data indicate that NoVs are widespread, stable in the environments, resistant to the conventional disinfectants and have very low infection dose, making NoV diseases difficult to control. Currently there is no antiviral, vaccine or other effective intervention against NoVs. Notable advancements in recent NoV research are the finding that NoVs recognize human histo-blood group antigens (HBGA) as receptors[5–9] and the establishment of an in vitro assay of NoV–HBGA interaction using NoV virus-like particle (VLP) or the protrusion (P) domain, the HBGA-binding domain of NoV capsid, as surrogate of NoVs and saliva as HBGAs.[10–15] Particularly, a recent human challenge study demonstrated that human serum blocking activity on VLP–HBGA binding correlated with the protection against NoV infection and illness.[16] This study provided direct evidence that the HBGA-binding assay mimic an important step of NoV infection and the blocking assay served as a new approach for antiviral drug development.[5,17] Compounds that are able to block NoV–HBGA interaction are likely to inhibit NoV infection and thus function as candidate antivirals against NoVs.[18]

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Many Chinese traditional medicines, such as the Chinese medicinal herbs, are frequently used to treat viral gastrointestinal infection. These medicinal herbs represent a variety of compounds that are likely to have antiviral activities.[19,20] In this study we investigated the antiviral effects of 50 frequently used Chinese medicinal herbs against NoVs through their inhibition of NoV P proteins binding to their HBGA receptors. Two medicines, the Chinese Gall and the Pomegranate, were found highly effective. Further research on their common composition showed that tannic acid may be a good candidate antiviral against NoVs.

2. Results

2.1. Optimization of a saliva-based blocking assay using NoV P dimer and P particle for herbal extract screening

Recombinant P dimer and P particle of NoV VA387 representing the predominant GII.4 NoVs in causing epidemics worldwide were produced and used in the screening experiments. Saliva samples with defined A or B HBGAs, respectively, were used as HBGA sources.[11] To optimize the blocking assays, we titrated the key reagents used in the assays, including the P particle, the P dimer, the blocking reagent (nonfat milk), and the saliva samples. The final conditions for blocking assays were selected to produce an OD_{450} of ~1.3 in the absence of herb or compounds (Fig. 1), in which boiled saliva samples were diluted 1000-folds, nonfat milk was 5%, and the P dimer and the P particles were 5 μg/ml and 37.5 ng/ml, respectively. In addition, the primary (guinea pig anti-VA387 VLP antiserum) and the secondary (HRP-conjugated goat anti-guinea pig IgG) detection antibodies were diluted in 4000- and 5000-folds, respectively. For a reliable comparison of our daily results, eight control wells containing everything but herbs or compounds were included in each plate. The assays were considered valid when the daily OD_{450} variations in these wells were less than 20%. In fact, the OD values of the eight blank controls showed only minor variations ranging from 0 to 0.035 throughout the whole study.

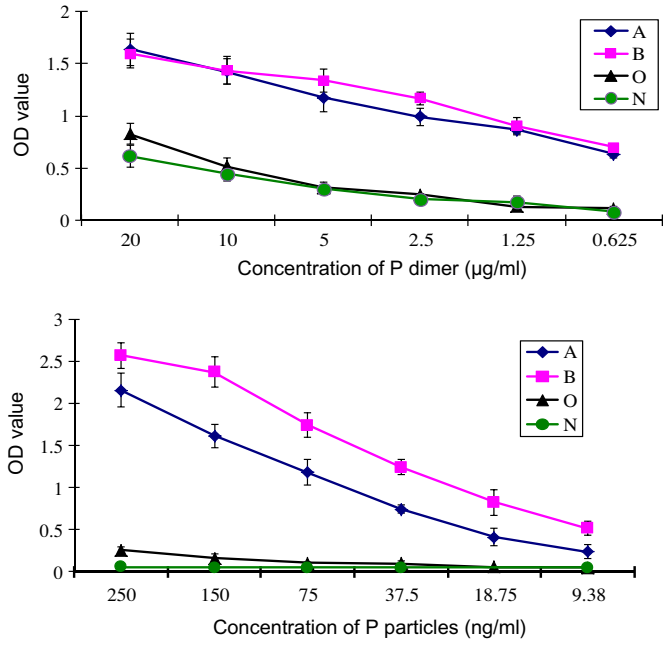

Figure 1. Binding of the P dimer and P particle to saliva sample (1:1000) with different HBGA types. *X*-Axis, the P dimer and P particle at different concentrations; *Y*-axis, binding signal intensity (OD_{450}); A, B, O, and N represents saliva samples with HBGA phenotypes of A, B, O secretor and nonsecretor, respectively. Each data point was an average of triplicated experiments.

2.2. Screening of the medicinal herbs

Primary blocking assays to screen the fifty herbal extracts by distilled water using the above optimized conditions was performed in a double blind manner. A 50% signal (OD_{450}) reduction caused by herb extracts was used as the cutoff for further characterization. Among 50 herbs studied, two herbs, the Chinese Gall and the Pomegranate, were found highly effective in inhibiting the NoV P protein binding to HBGAs receptors. Chinese Gall blocked NoV P dimer binding to type A saliva at IC_{50} = 5.35 μg/ml and to B saliva at IC_{50} = 21.7 μg/ml (Fig. 2a). Similarly, Pomegranate blocked binding of NoV P dimer to type A saliva at IC_{50} = 15.59 μg/ml and B saliva at IC_{50} = 66.67 μg/ml (Fig. 2b).

In addition, we also screened the HPLC fractions of these 50 herbs. The fractions 9, 10 and 11 of Chinese Gall and the fractions 5, 6, 7 of Pomegranate showed prominent inhibitions (Fig. 3a). Chromatograms of the two herbal extracts indicated that these fractions represented the main elution peaks of the two herbal medicines that contain the effective components (Fig. 3b and c).

2.3. Tannic acid may be the effective components of the two herbal extracts inhibiting the binding of the NoV P dimer to HBGA receptors

To determine which components may be responsible for the observed inhibition activities, we examined the ingredients of these two medicines. Previous researches[21–23] showed that tannic acid is a common composition in the extracts of both herbs, suggesting that tannic acid might be the effective compound. To further verify this, highly purified tannic acid ($C_{76}H_{52}O_{46}$, MW = 1701 Da) and different forms of hydrolysable tannins with different alkyl esters, including gallic acid, ethyl gallate, lauryl gallate, octyl gallate and propyl gallate were also studied. The result showed that highly purified tannic acid had strong inhibition effects against the binding of NoV P dimer and P particle to both A and B saliva at $IC_{50} \approx 0.1$ μM (Fig. 4a and b). In contrast, none of those tannin-derivatives revealed any detectable inhibiting activities (Fig. 4c and data not shown).

2.4. Validation of receptor-blocking activities on a panel of types A saliva samples and synthetic type A and B oligosaccharides

A single dosage of Chinese galls, Pomegranate and tannic acid at their IC_{50s} was used to inhibit NoV P particle (VA387, GII.4) binding to a panel of saliva samples from six randomly selected types A individuals. Significant inhibition was observed for all samples in the presence of the herbal extracts/compounds. The inhibition rates of Chinese galls, Pomegranate and tannic acid were comparable to those found in the primary screening with narrow variations (Table 1), indicating that the IC_{50s} determined in the primary screening are highly reproducible. In addition, the synthetic oligosaccharide-based blocking assays confirmed that tannic acid have the similar inhibition effects against NoV P particle binding to HBGAs (Table 1).

2.5. The cytotoxicity of the herbs and tannic acid

Human cervical carcinoma (HeLa) cells and LLC-MK2 cells were used to test the cytotoxicity in this study (see Section 5.6). The CC_{50} of Chinese Gall and Pomegranate were 420 and 750 μg/ml, respectively, while the CC_{50} of tannic acid is about 16 μM. Selectivity index (SI) was calculated from the ratio of CC_{50}/IC_{50}. The SI value of tannic acid against blocking NoV P protein to HBGAs was about 160.

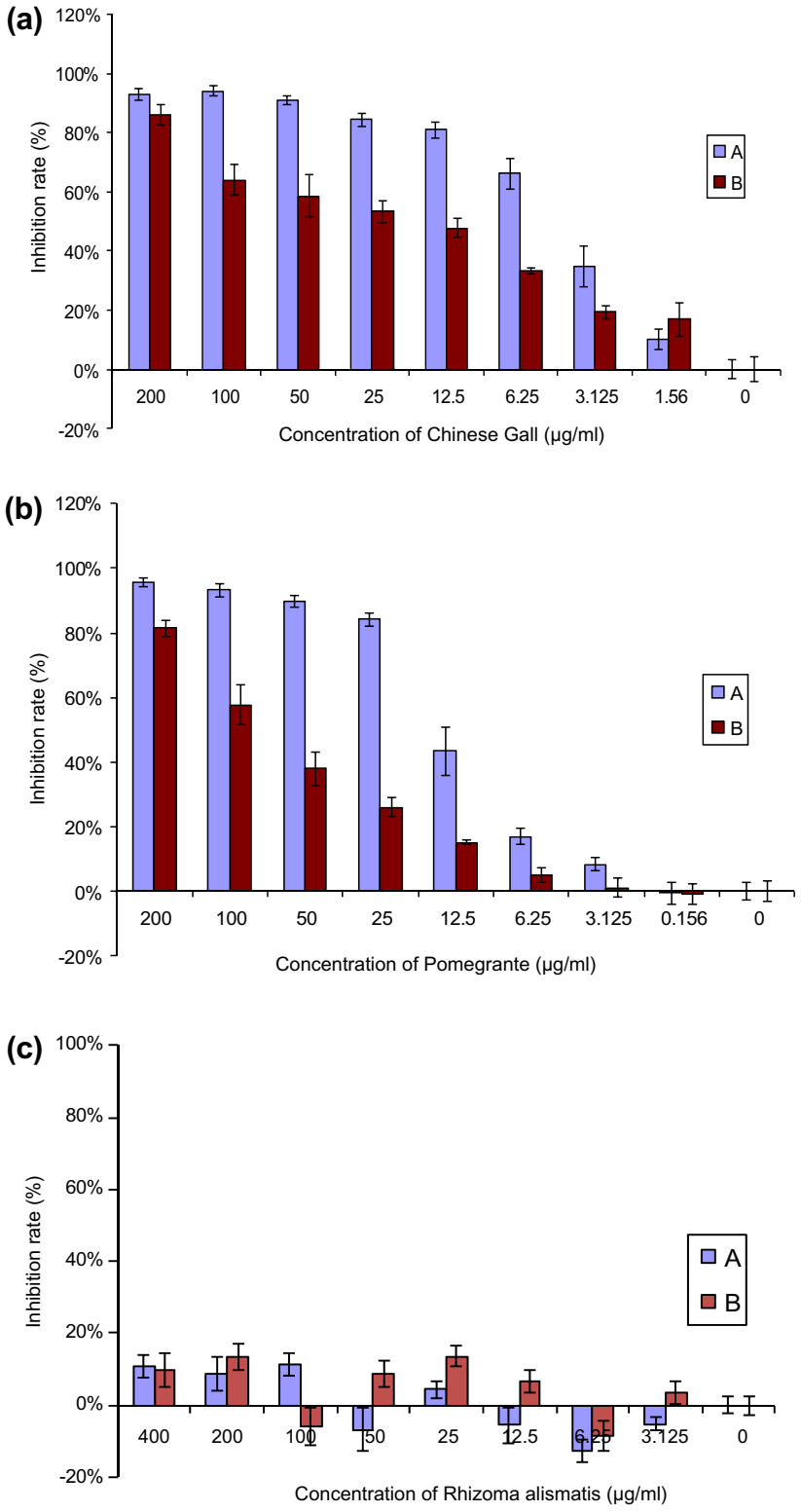

Figure 2. The water extracts of the Chinese Gall (a) and Pomegranate (b) showed strong inhibition on P dimer binding to type-A and type-B saliva. Water extracts of *Rhizoma alismatis* (c) and other herbs did not show inhibition. *X*-Axis, the water extracts of the medicinal herbs at different concentrations; *Y*-axis, inhibition rate; A and B represents type A and B saliva samples, respectively. Each data point was an average of triplicated experiments.

3. Discussion

NoVs are recognized as the leading cause of epidemics of gastroenteritis which also cause sporadic gastroenteritis in children. The prevention of NoV outbreaks has been extremely challenging because outbreaks that begin with a single common exposure to contaminated food or water can rapidly spread by person to person transmission. Currently, there is no vaccine and antiviral available

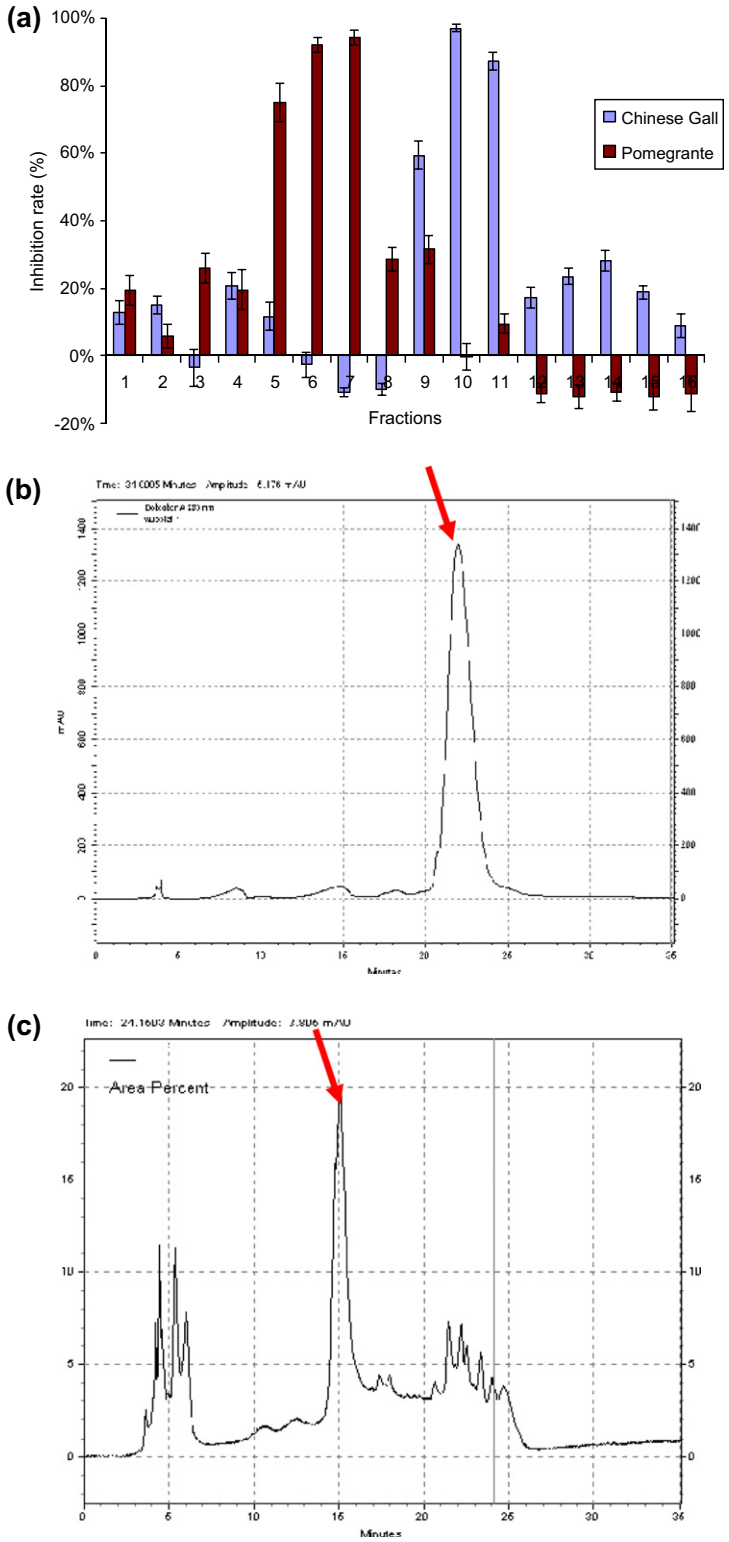

Figure 3. Inhibition of the HPLC fractions of the two herbal extracts, Chinese Gall and Pomegranate, on P dimer binding to HBGA receptor. (a) Inhibitions of the 16 HPLC fractions the two herbal extracts on P dimer binding to type A saliva sample. Fractions 9, 10, and 11 of Chinese Gall and fractions 5, 6, and 7 of Pomegranate showed predominant inhibitions. Each data point was an average of triplicated experiments. (b) and (c) HPLC chromatograms of Chinese Gall (b) and Pomegranate (c). *X*-Axis indicates the elution fractions in different retention times. *Y*-Axis show the absorbent values at 280 nm wavelength. The arrow-indicated peaks represent the fractions that showed strong inhibition in (a) (fractions 9, 10, and 11 for Chinese Gall; fractions 5, 6, and 7 for Pomegranate).

for prevention of NoV infection. Hence, our study to develop anti-NoV agents from medicinal plants is rational which are less toxic, more efficacious and cost-effective.

Previous studies demonstrated that medicinal plants used for centuries against different diseases including viral diseases become an important source of new drugs against different diseases.

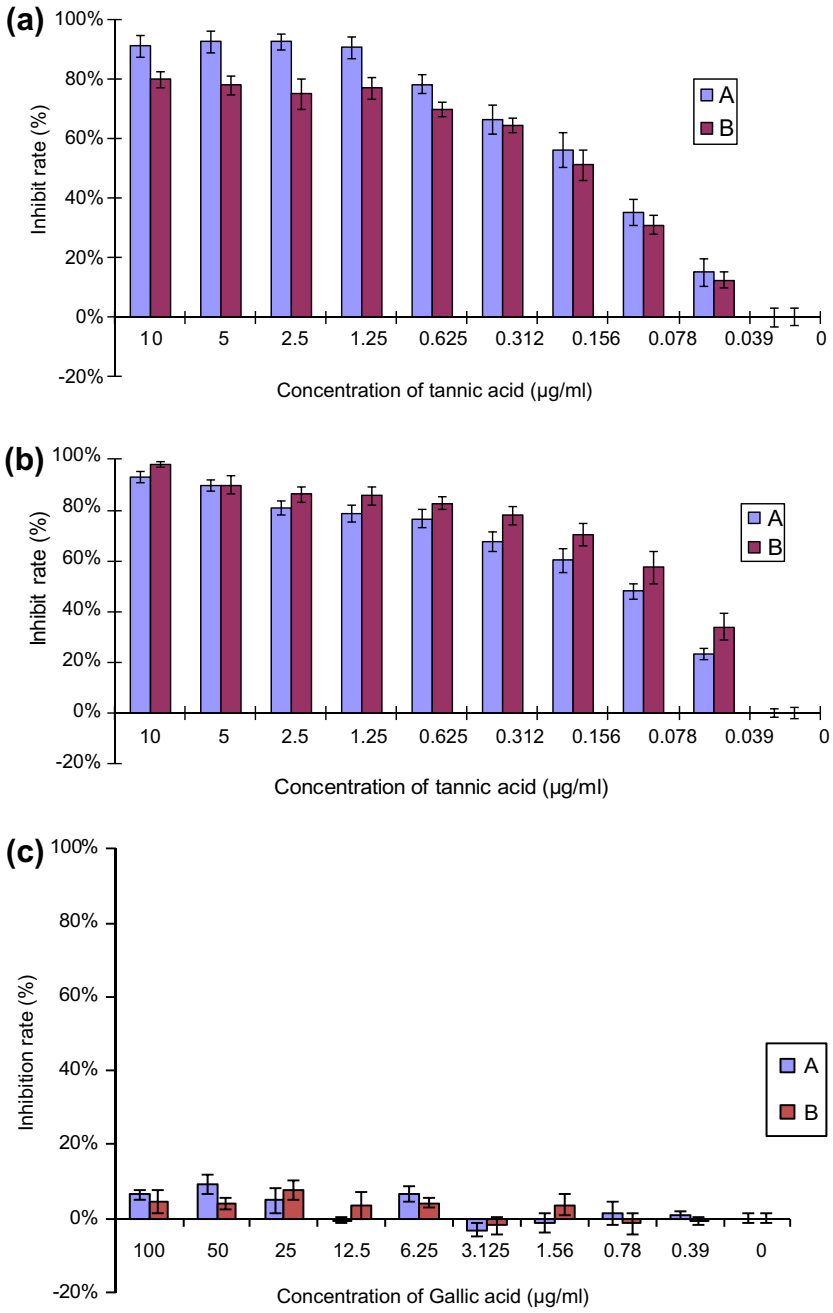

Figure 4. The inhibitory effects of tannic acid on binding of NoV P dimer (a) and P particle (b) to type-A and type-B HBGA receptors. Tannic acid blocked binding of NoV P dimer to type A saliva at IC_{50} = 0.165 μg/ml and B saliva at IC_{50} = 0.255 μg/ml, while blocked NoV P particle to type A saliva at IC_{50} = 0.118 μg/ml and B saliva at IC_{50} = 0.05 μg/ml. Gallic acid (c) and other compounds did not show very weak inhibition. Each data value was an average of triplicated experiments.

According to an estimate, 25% of the commonly used medicines contain compounds isolated from plant origin. Medicinal compounds derived from plant extracts are also of great interests to the pharmaceutical industry.[24,25] Basic theories and clinical practices have shown that some Chinese traditional herbs are frequently used against infectious diseases including viral gastroenteritis.[26–29] Recently, we summarized the most widely used core herbs in viral gastroenteritis from clinical literature data based on their frequency.[30] The 50 traditional medicinal plants can be grouped into 3 categories base on gastrointestinal clinical features or 10 categories based on their functions against human diseases. The present study attempted to lay a foundation for screening potential anti-NoV agents from these medicinal plants. For this reason, the 50 core traditional medicinal plants were processed to prepare extracts by distilled water and/or by HPLC before being used for antiviral screening and toxicity analysis. Thus, such information is useful in the theoretical design of drugs with improved specificity and efficacy.

In our previous studies we used a saliva-based receptor binding/blocking assays for drug screening against virus-like particles (VLPs) binding to HBGAs receptors.[18,31] The study described here used NoV P dimers or P particles as NoV surrogates. Compared with VLPs that are produced using the baculovirus expression system, the P dimers and P particles are more easily produced in *Escherichia coli*. These P protein complexes, particularly the P particles, are highly stable, immunogenic and bind to HBGAs receptors and therefore good alternative for VLPs. The P dimer is also suitable for primary screening because the binding affinity of the P dimers

Table 1
Inhibitory activities of Chinese Gall, Pomegranate and tannic acid against the binding of P particle of VA387 to HBGAs

Herb or compound	Concentration	% Inhibition of binding to HBGA type A saliva samples						Avg. % inhibition (mean ± S.D.)
		1	2	3	4	5	6	
Chinese Gall	5 μg/ml	61.8	51.6	50.8	49.6	49.8	54.9	53.1 ± 4.7
Pomegranate	15 μg/ml	53.4	55.1	61.9	50.8	43.2	47.6	52.0 ± 6.5
Tannic acid	0.1 μM	55.1	47.3	49.6	50.1	49.2	48.7	50.0 ± 2.7
		% Inhibition of binding to synthetic oligosaccharides containing human HBGA epitopes						
		Oligosaccharide A			Oligosaccharide B			
Tannic acid	0.1 μM	45.9	40.2	43.2	44.9	45.6	46.8	44.4 ± 2.4

to HBGAs is lower than that of P particles and VLPs, making the P dimer a highly sensitive model for finding an antiviral candidate even with low affinity.[32] In conclusion, our approach to use a combination of different P protein complexes is highly effective and low cost for rapid finding of antiviral candidates for NoVs.

In this study, we found that the binding/blocking assay in screening herb extracts is sensitive to low pH value. For example, *Prunus mume* and *Crataegus pinnatifida* showed strong blocking activities against NoV P protein binding saliva when the original extracts were tested without dilution, in which an extremely low pH value (pH 3.65–6.4) of the extracts were found. However, after adjusted the pH value to 7.4, neither of the two herbs showed blocking effect, even with a high concentration of extract material (data not shown). In our later confirmation experiments, all extracts were diluted with PBS (pH 7.4) before being tested, which avoid the problem of low pH effects.

In the present study, from the 50 medicinal plants tested, two plants, the Chinese Gall and Pomegranate, were most effective against NoV P protein binding to their receptors based on water extracts of these herbs. These effects were further confirmed by their HPLC fractions. Interestingly, these two herbs belong to the same group of astringent therapy herbs. Chinese galls have been widely used as an astringent medicine for their antidiarrheal, hemostatic, and antibacterial properties.[33,34] It was reported that they contain high levels (50–70%) of Gallotannins.[21] Pomegranate has been extensively consumed by people in different countries for thousands of years. Pomegranate contains rich of tannins (10.4%) which is believed to have a strong astringent effect.[22,23,35] The major property of pomegranate hulls exploited in folk medicine is their strong astringency, making them a popular remedy throughout the world, such as China and Greek, in the form of an aqueous decoction (i.e., boiling the hulls in water for 10–40 min), for dysentery and diarrhea, and also for stomatitis. The decoction can be drunk, used as a mouthwash, douche or enema. Recently, some studies showed pomegranate juice and pomegranate polyphenols extracts have antiviral effects on murine norovirus and feline calicivirus.[36–38]

Tannins are a unique group of phenolic metabolites with molecular weights between 500 and 30,000 Da, which are widely distributed in almost all plant foods and beverages. Condensed tannins and hydrolysable tannins are the two major groups of these bioactive compounds by their chemical structures. Hydrolysable tannins are polyesters of a sugar moiety and organic acids. If the acid component is gallic acid, the compounds are called gallotannins. Esters with hexahydroxydiphenic acid are called ellagitannins.[39] The tannins from traditional Chinese medicine such as Chinese Gall, Pomegranate and Sumac are hydrolysable tannins mostly. Some studies found that tannic acid are effective inhibitors against SARs $3CL^{Pro}$ and HIV reverse transcriptase; and used as prophylactic treatment against influenza.[40,41] So we speculate that tannic acid, a common composition in Chinese Gall and Pomegranate, might be the effective compound in blocking NoV P protein binding to their HBGA receptors. We performed further studies on commercially available, highly purified tannic acid and confirmed that the tannic acid acted as a strong inhibitor in binding of NoV P protein to both A and B saliva ($IC_{50} \approx 0.1$ μM). The SI value of tannic acid in blocking NoVs binding to HBGAs was about 160, indicating that the tannic acid is a potential good candidate antiviral against NoVs.

Upon hydrolysis of gallotannins or ellagitannins by acid, bases, or certain enzymes, glucose and gallic acid (GA) or ellagic acid (EA) can be released.[42] Some researchers reported that gallic acid has anti-HSV-2 effect[43]; some gallate can inhibit influenza virus replication[44]; while octyl gallate has antiviral effect against DNA and RNA viruses.[45] In the present study, we further tested different forms of hydrolysable tannins with different alkyl esters, including gallic acid, ethyl gallate, lauryl gallate, octyl gallate and propyl gallate. However, none of these tannins-derivatives revealed any detectable inhibiting activities. Whether there are other compounds present in the Chinese Gall and Pomegranate that also block NoV P protein binding to HBGAs receptors remain unknown. Future studies to address this question by retesting these herbs following various spectroscopic techniques are needed. Furthermore, the mechanism of tannic acid in blocking NoV P protein binding to HBGAs receptors need to be elucidated in our future study.

4. Conclusion

In the present study, we developed a herb screening model based on saliva-based binding/blocking assays using the NoV P protein, and have identified Chinese Gall and Pomegranate from 50 Chinese medicinal herbs as highly effective candidates to inhibit NoV binding to HBGA receptors. Moreover, we have identified tannic acid, the common composition of the two medicinal herbs, as a strong inhibitor in the binding of NoV P protein to both A and B saliva. Though the evaluation of antiviral drugs for NoV is still a challenge because of the lack of cell cultures or small animal models, our data provided an important start with the tannic acid as a potential candidate for development of antivirals against NoVs.

5. Experimental

5.1. Herb materials

Fifty clinically effective Chinese medicinal herbs against gastroenteritis were included in this study. According to their potential functions against gastroenteritis symptoms, these herbs were divided into three major groups based on gastrointestinal clinical symptom (or ten group based on the herbs' effective function): (1) bellyache and diarrhea (astringent therapy herbs, dissipating dampness herbs, water-disinhibiting damp-percolating medicinal, herbs for restore deficiency, and herbs for inner-warming); (2) nausea and vomiting (expectorant-cough suppressant, Qi-regulating drugs and digesting drugs); (3) fever and headache (heat-clearing drugs and herbs that release the exterior).[30] All medicinal herbs were purchased from Nanfang Hospital (Guangdong Yifang Pharmaceutical Co., Ltd, China).

5.2. Preparation of crude extracts from herbs

5.2.1. Extraction with distilled water

Each herb (10 g) was cut into small pieces, and powdered by a mixer grinder. The herbal powder was extracted by soaking in 100 ml distilled water for 8 h at 4 °C, repeat twice. The pooled extract was condensed to 1 mg/ml of herb under reduced pressure at a low temperature less than 40 °C. The extract was sterilized with high pressure for 20 min, then divided into small aliquots, and stored at −80 °C until further use.

5.2.2. Separation of the herbal extraction by high-performance liquid chromatography (HPLC)

Approximately 50 mg of herbs powder were soaked with 25 ml ethanol/water (95:5, v/v) for 24 h. The mixture was sonicated for 60 min and filtered through 0.45 μm filters. The remaining herb was extract again with 25 ml ethanol/water (50:50) for 24 h. The mixture was sonicated for 60 min and then filtered with the same filter. Both filtrates were combined and then lyophilized after removal of the solvent by rotary evaporation. The components of the herb filtrates were then separated by HPLC system Prostar 230 (Varian Co., Palo Alto, USA) using column YWG-C18 (4.6 × 200 mm, 10 μm, Elite Co., Dalian, China).The HPLC fractions were collected. The mobile phase was a continuous gradient of water (eluent A) and methanol (eluent B). The gradient program was as follows: 5% eluent B (2 min), 5–95% eluent B (30 min) and 95–100% eluent B (3 min). Total run time was 35 min, and the first 3 min fractions were discarded. Then 16 fractions were collected every 2 min, and kept at −80 °C until further use.

5.3. Preparation of NoV P proteins

E. coli-expressed recombinant P proteins in forms of P dimer and P particle of VA387 strain was used in this study. Production of P Particle and P dimer have been published previously.[13,14,46] Briefly, the construct containing NoV P protein-encoding sequences was expressed in *E. coli* strain BL21 at room temperature overnight with an induction of 0.5 mM IPTG. Purification of the recombinant glutathione S-transferase (GST)-P fusion protein from bacteria was performed with Glutathione Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions.[32] The fusion protein was eluted by glutathione (Amersham Biosciences, Piscataway, NJ), and the P proteins were released from GST by thrombin (Amersham Biosciences, Piscataway, NJ) cleavage at room temperature for 16 h. The purity of P particles and P dimer used in this study was more than 90% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoretic (SDS–PAGE) analysis.

5.4. Saliva-based EIA and the blocking assay to screen herbal medicine for inhibiting activity on NoV binding to human HBGAs

Boiled saliva samples with defined HBGAs[11] were used to coat 96-well microtiter plates at 4 °C overnight, after blocking with 5% nonfat milk, P dimer or P particles of NoV were added. The bound P particle/P dimer was detected using a Guinea pig antiserum against VA387 VLP, followed by the addition of HRP-conjugated goat anti-Guinea pig IgG. The bound horseradish peroxidase conjugates were detected by the TMB kit (Kirkegaard & Perry Laboratories), and the OD_{450} was read using an EIA spectrum reader (Tecan). For each step, the plates were washed five times with PBS containing 0.5% Tween 20. The blocking effects of the Chinese medicinal herbs extracts at serial dilutions on binding of NoV to HBGAs were determined by incubation with the P dimer and/or P particle for 30 min before adding them to the coated saliva. All the materials were diluted in phosphate buffered saline (PBS) (pH 7.4). Type A and B saliva were used as HBGAs for the blocking assay.[11,31]

The herb extracts with clear blocking effects from the primary screening were retested with serial dilutions, and the blocking activity of the extract was defined as inhibitor concentration yielding 50% inhibition (IC_{50}) by comparison of the signals (OD_{450}) in wells with the extract or compound with the signals in negative-control wells without the extract or compound after subtraction of the background noise (the blank control wells that contain all assay components except compounds and P particle and P dimer). Statistical analysis was performed using Probit regression analysis by using SPSS statistical software version 13.0 (SPSS, Chicago, IL).

5.5. Tannins-derivatives for blocking NoV binding to human HBGAs receptors

Tannins and tannins-derivatives, including gallic acid, ethyl gallate, lauryl gallate, octyl gallate and propyl gallate were purchased from Sigma Company (USA) (Fig. 5). The blocking assays methods were consistent with the herbs extract, just replaced herb extracts by the tannins-derivatives compounds.

An assay to confirm the inhibition effects were performed by using Synthetic oligosaccharides containing human HBGA epitopes A and B with the same protocol as the blocking assay above, with the following exception: oligosaccharide-PAA-biotin conjugates

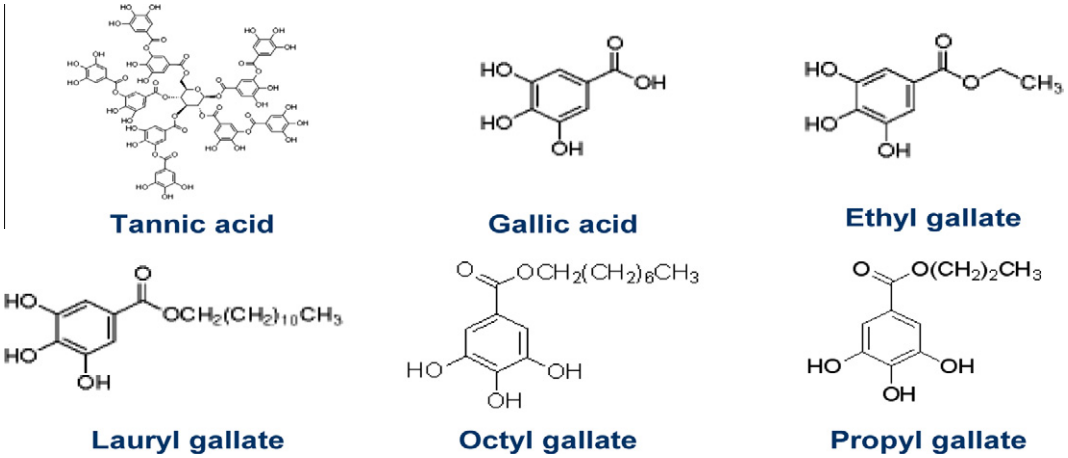

Figure 5. Chemical structures of tannic acid and its derivatives.

(2 μg/ml) were coated to a microtiter plate by using streptavidin (5 μg/ml).[11]

5.6. MTS cytotoxicity assay

Human cervical carcinoma (HeLa) cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.[47] LLC-MK2 cells, originally from the kidney tissue of adult rhesus monkeys, were grown at M199 medium supplemented with 10% fetal bovine serum and penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The cells were seeded at 5×10^4 per ml onto a 96-well plate and incubated overnight before testing. The MTS cytotoxicity assay was performed using CellTiter 96 aqueous nonradioactive cell proliferation kits (Promega, Madison, WI). The assay solution contained the tetrazolium compound 3-(4, 5-dimethylthiazole-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and an electron-coupling reagent, phenazine ethosulfate. The cytotoxicities of individual herbs or tannins were determined by the decrease in cellular reduction of MTS into the colored product. Briefly, after the incubations with herbs or tannins at various concentrations for 3 days, the culture medium was replaced with fresh medium with 100 μl of MTS-phenazine methosulfate/well, incubated at 37 °C for 2 h, and measured with a plate reader at an absorbance of 490 nm. The 50% cytotoxic concentrations (CC_{50}s) were determined as the concentrations of compounds that caused 50% inhibition of cell growth compared to the growth of control cells cultured without compound. The selective index was calculated as the values for the CC_{50} divided by the IC_{50}s determined by the saliva-blocking assay described above.[48]

Acknowledgments

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