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Anti-adhesive effect of an acidic polysaccharide from *Aloe vera* L. var. *chinensis* (Haw.) Berger on the binding of *Helicobacter pylori* to the MKN-45 cell line

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Abstract

Objectives The emergence of antibiotic-resistant *Helicobacter pylori* strains has necessitated a search for alternative therapies for the treatment of this infection. The aim of this study was to evaluate whether or not polysaccharide fractions from *Aloe vera* are effective in inhibiting the adherence of *H. pylori in vitro*.
Methods Polysaccharide fractions were extracted from *A. vera* and subjected to carbohydrate analysis. The adhesive effect was determined by co-incubation of *H. pylori* and cells with polysaccharides followed by fluorescein isothiocyanate labelling and Gram staining *in vitro*. Inhibition of *H. pylori* growth and cellular viability was tested by agar diffusion and MTT assay.
Key findings APS-F2 contained significant amounts of galacturonic acid, galactose and arabinose. APS-F1 was galacturonic acid-free and consisted of mannose, glucose and galactose. APS-F2 (0.1, 0.5 and 1.0 mg/ml) reduced the count of *H. pylori* attached to MKN45 cells to 88, 76 and 64%, respectively. APS-F1 did not show the same effect. Neither polysaccharide revealed an inhibitory effect on the growth of *H. pylori* or cell viability. In addition, APS-F2 was shown to have a potent anti-adhesive effect against *Escherichia coli*.
Conclusions The results show that the acidic polysaccharide from *A. vera* has a potent anti-adhesive effect against *H. pylori in vitro*. However, there have yet to be any in-vivo studies to demonstrate the clinical relevance of this finding.
Keywords *Aloe vera*; anti-adhesive; *Helicobacter pylori*; polysaccharide

Introduction

Helicobacter pylori, a spiral, Gram-negative, non-invasive microaerophilic bacterium, lives exclusively in the human stomach and plays a major role in the development of chronic gastritis, duodenal and gastric ulceration, adenocarcinoma[1] and gastric MALT lymphoma.[2] It has been defined as a class 1 carcinogen.[3] The first-line eradication therapy for *Helicobacter pylori* in clinical practice is the triple-therapy, a combination of a proton-pump inhibitor combined with clarithromycin and amoxicillin or metronidazole.[4] However, the emergence of resistant strains of the bacterium overshadows the therapeutic effect of this treatment, and makes the search for novel treatments for the condition important.

One such strategy is to use anti-adhesive agents, which target the primary step of bacterial infection: adhesion of the organism to the host. Adhesive inhibitors, usually based on carbohydrate-mediated cell recognition, have a lower risk of reduced efficacy due to the development of resistant strains because these agents do not act by killing or arresting the growth of the pathogen.[5] Carbohydrate-based compounds are regarded as ideal candidates for possible anti-adhesives.

Aloe vera L. var. *chinensis* (Haw.) Berger, a traditional Chinese medicine prescribed for the treatment of gastric illnesses, was found to have extraordinary anti-ulcerogenic effects, inhibiting gastric acid secretion and gastric lesion, protecting from mucosal injury and accelerating the cicatrisation of gastric ulcers in different experimental models.[6,7] However, the effect of *A. vera* on bacterial adhesion to host cells has not been explored.

Phytochemically, polysaccharides have been characterised as the main component in *A. vera*, and they are responsible for many of the biological activities of *A. vera*. Since

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carbohydrate plays an important role in bacterial adhesion to the host and some oligo/polysaccharides have been reported to be effective for the inhibition of bacterial adhesion, the aim of this study was to evaluate the anti-adhesive effect of polysaccharide fractions isolated from *A. vera* on *H. pylori*. The results of this study will not only rationalise the traditional prescription of *A. vera* in the treatment of gastric disorders, but will probably also shed new light on its mechanisms of action.

Materials and Methods

Materials

Fresh leaves of *A. vera* L. var *chinensis* were bought from local market and identified by Dr Yuping Zhu, School of Life Science, Nanjing University. A voucher specimen was kept in the herbarium of Nanjing University. *H. pylori* 43504 was obtained from American Type Culture Collection (ATCC) and the clinical strain was acquired from Jiangsu People's Hospital in Nanjing, China. *Escherichia coli* strains BL21 (DE3) were kindly provided by Dr Changhong Liu, School of Life Sciences, Nanjing University, Nanjing, China. Fluorescein isothiocyanate (FITC), trypsin, dextran, arabinose, fucose, galactose, glucose, rhamnose, mannose, xylose, carbazole and phenol were purchased from Sigma (St Louis, MO). Sephacryl s-200 gel was bought from GE Healthcare, Sweden. DEAE52 (OH^-) anion-exchange columns were bought from Whatman, UK.

Bacteria and cell line cultivation

The *H. pylori* strains used in this study were from one standard strain, ATCC 43504, and a clinical isolate of *H. pylori*, which was obtained from antral biopsies of an adult patient hospitalised at Jiangsu People's Hospital in Nanjing. Both strains were cagA-positive and the presence of cagA was analysed by the RT-PCR method. Both strains were cultured on Columbia Agar (BioMerieux, France) supplemented with 7% sheep blood and cultured for 3 days at 37°C under microaerophilic conditions, with high humidity, as detailed previously.[8]

Escherichia coli strains BL21 (DE3) were cultured aerobically on Luria-Bertani (LB) agar plates. The colony was inoculated and grown in LB broth without antibiotics at 37°C for 6 h before being harvested by centrifugation (6000*g* for 30 min) and kept at −70°C until required for experimental use.

MKN45 (human gastric cancer) and LOVO (human colonic cancer) cell lines were both grown in Roswell Park Memorial Institute Medium 1640 (RPMI 1640) medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 U/ml streptomycin and 10 mM HEPES. Cells were grown in 10 cm dishes and incubated in 5% CO_2 at 37°C for 24–48 h before the experiments were carried out.

Extraction and isolation of polysaccharide fractions

The crude extract of *A. vera* was prepared according to the method described by Wu *et al*.[9] A sample of 50 g of the crude extract was then twice soaked with 70% ethanol for 24 h and dried at a temperature below 50°C. The crude extract was then dissolved in hot (45°C) distilled water (2000 ml) and stirred for 12 h. The supernatants were collected by centrifugation and deproteinated twice using trichloroacetic acid (TCA, final concentration 20%). The crude polysaccharide (APS) was obtained through precipitation with ethanol (final concentration 80%) and then lyophilised. The APS was then dissolved in water, centrifuged, and applied to a DEAE52 (OH^-) anion-exchange column (6×8 cm). The column was eluted with deionised water and 0.2 M NaCl at a flow rate of 1.5 ml/min. The eluate was monitored for carbohydrate content by phenol–sulfuric acid assay. According to the elution profile, the eluates were collected, concentrated, dialysed and lyophilised to afford two fractions: APS-1 and APS-2. These fractions were further purified on a Sephacryl s-200 column (1.6×60 cm) eluted with deionised water at a flow rate of 0.25 ml/min. This purification protocol yielded two polysaccharides, namely APS-F1 and APS-F2.

Carbohydrate analysis and molecular weight

Total carbohydrate and uronic acid contents were determined by the phenol–sulfuric acid and sulfate–carbazole methods. Glucose and galacturonic acid were used as the standards. Monosaccharide composition of APS-F2 and APS-F1 was determined as described previously,[10] with some modification. Briefly, 10 mg of polysaccharide was hydrolysed in 2 M trifluoroacetic acid (TFA) (2 ml) at 100°C for 8 h in a sealed tube. Subsequently, the hydrolysate was repeatedly co-evaporated with methanol at reduced pressure to remove excess TFA. The residue was dissolved in a minimum volume of water and analysed by TLC on a GF_{254} silica gel plate developed with butanol–pyridine–H_2O (20 : 7 : 5), and detected with aniline-o-phthalatic reagent. The remaining hydrolysate was mixed with hydroxylamine hydrochloride (8 mg) and pyridine (0.4 ml) and kept at 90°C for 30 min before being cooled to room temperature. The product was mixed with 0.4 ml acetic anhydride and reacted at 90°C for 30 min. The reaction mixture was directly analysed by gas-liquid chromatography over a HP-5 column (crosslinked 5% phenyl methyl siloxane, 30 m × 0.25 mm × 0.25 μm) with a temperature gradient programmed to rise from 150 to 220°C at a speed of 2°C/min, and from 220°C to 280°C at 30°C/min. The molecular weight was determined on a sephacryl S-400 column using a set of Dextrans (T-2000, T-500, T-70, T-40 and T-10) as molecular weight references.

Anti-adhesion assay

Helicobacter pylori strains were collected and resuspended (OD_{600}: 2.0) in 1 ml RPMI and then incubated with 10 μl 0.1% FITC in DMSO for 1 h at room temperature in the dark. The FITC-labelled *H. pylori* were then washed three times in RPMI containing 0.1% Tween 20 to remove excess FITC.

For the adhesion assay,[11] MKN45 cells were seeded in 96-well plates (Corning, NY) in 100 μl of RPMI 1640 culture medium with 10% bovine serum at approximately 5×10^4 cells/ml. After 48 h, cells reached confluence, and the medium was replaced with fresh RPMI containing 2% bovine serum and the combination of either APS-F1 or APS-F2 at a serial concentration of 0, 0.01, 0.1, 0.5 and 1.0 mg/ml. The cultures were incubated for 1 h before being infected with FITC-

labelled *H. pylori* (5×10^6 cfu). One hour later, the cells were washed three times with RPMI to remove unbound bacteria and were then lysed at 37°C in trypsin-EDTA. Bacterial adherence was quantified by fluorescence (FITC excited at 485 nm and detected at 528 nm, TECAN Safire A-5082, Austria).

The effect of polysaccharides on *H. pylori* binding to MKN-45 cells was evaluated by Gram staining and light microscopy.[12] For Gram staining, MKN-45 monolayers were prepared on glass coverslips placed in six-well tissue-culture plates. The MKN45 cells were seeded in the six-well plates to obtain confluence. Then they were treated with APS-F2 at a final concentration of 1 mg/ml for 1 h. Next, 100 μl *H. pylori* suspension (10^7 cfu/ml) was added and incubated for 1 h. Subsequently, the cell monolayers were washed twice with sterile phosphate-buffer saline (PBS, pH 7.4) to remove non-adhered bacteria. The monolayers were fixed with methanol for 30 min at room temperature. After fixing, the MKN45 cells were washed three times with PBS, dried in air and Gram stained. Adherence was examined microscopically.

The anti-adhesion assays were performed with *E. coli* and its host LOVO cells to assess the specificity of anti-adhesion.

Disk diffusion method

The influence of polysaccharides on bacterial growth was investigated using the disk diffusion method,[13] which was performed as recommended by the national committee for clinical laboratory (NCCL) standards. Briefly, the *H. pylori* suspension was spread on the surface of Columbia agar. Filter paper disks (4 mm diameter) with different concentrations (2.0, 1.0, 0.5 and 0.25 mg/ml) of APS-F1 or APS-F2 were placed on the agar surface. After incubation for 48 h at 37°C, the inhibition zones were measured with a ruler or caliper, and the size of the zones was recorded in millimeters. Amoxicillin was used as the positive control. A similar procedure was used for the investigation of the influence of the tested polysaccharides on the growth of *E. coli*.

MTT assay

Cell viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) assay. Briefly, MKN 45 cells were grown for 24 h on 96-well plates before being incubated with APS-F1 or APS-F2 at serial concentrations of 2.0, 1.0, 0.5 and 0.25 mg/ml for 24 h. Then, 10 μl MTT (5 mg/ml) was added to each well for a 4 h period. After the formation of formazan crystals, the culture medium supernatant was removed. The formazan crystals were then dissolved in 100 μl DMSO per well. The results were determined using a microplate spectrophotometer. (570 nm; DYNEX Technologies, Chantilly, VA). The influence of polysaccharides on the viability of LOVO cells was also examined.

Statistical analysis

Data were presented as means with standard error for the individual groups and analysed by one-way analysis of variance (ANOVA). Where differences were observed, Dennett's multiple test was used to determine their statistical significance. The observed differences were considered significant at $P \leq 0.05$ in all analyses.

Results

Purification and structure characteristic of polysaccharide fractions

The elution profile of the crude polysaccharide (APS) on the DEAE52 (OH^-) anion-exchange column is shown in Figure 1. The fractions eluted from deionised water were pooled to give APS-1, and eluates from NaCl afforded APS-2. These two fractions were further purified by Sephacryl s-200 gel-filtration and yielded APS-F1 and APS-F2.

UV detection revealed that both of the fractions contain no proteins or nucleic acid (data not shown). The yields of the two purified polysaccharides were 0.49 and 0.32% for APS-F1 and APS-F2, respectively (Table 1). The

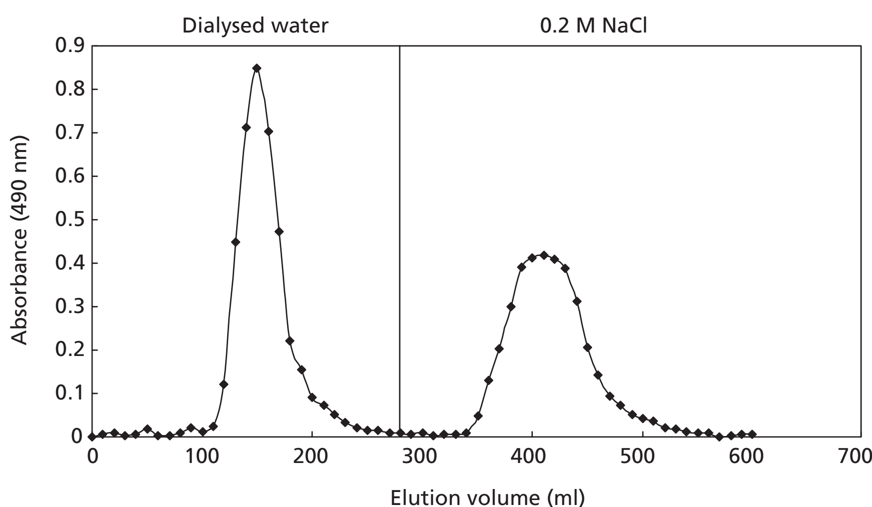

Figure 1 Elution profile of APS by DEAE52 (OH^-) anion-exchange chromatography. A DEAE52 (OH^-) anion-exchange column (6 × 8 cm) was eluted at a flow rate of 1.5 ml/min. The line represents the absorbance at 490 nm from the phenol–sulfuric acid assay.

Table 1 Characteristics of polysaccharide fractions isolated from *Aloe vera* L. var. *chinensis* (Haw.) Berger

	APS-F1	APS-F2
Protein (μg/mg)	0.0	0.0
Yield (%)[a]	0.49	0.32
Molecular weight (kDa)	186.7	29.39
Sugar composition (mol%)[b]		
Rhamnose	0.0	9.0
Arabinose	0.0	15.3
Xylose	0.0	1.4
Mannose	67.9	7.1
Galactose	4.5	37.9
Glucose	27.6	3.7
Galacturonic acid	0.0	25.6

[a]Calculated as weight percent of applied material. [b]Mole percent of total carbohydrate content.

carbazole–sulfuric acid method combined with TLC analysis showed that APS-F1 was galacturonic-acid-free, while APS-F2 was an acidic polysaccharide containing 25.6% galacturonic acid (Table 1). The monosaccharide analysis of the fractions obtained after TFA-hydrolysis of the polymers and GLC analysis indicated that APS-F1 contains a neutral monosaccharide composition of mannose (67.9%), glucose (27.6%) and galactose (4.5%) with a molar ratio of 15.09 : 6.13 : 1.00. APS-F2 contains the monosaccharides of galactose (37.9%), arabinose (15.3%), glucose (3.7%), mannose (7.1%), rhamnose (9.0%) and xylose (1.4%) with molar ratios of 27.07 : 10.93 : 2.64 : 5.07 : 6.43 : 1.00.

The molecular weights of the polysaccharides, determined by gel-filtration chromatography using dextran molecular weight standards (1–2000 kDa), were approximately 186.7 kDa (APS-F1) and 29.39 kDa (APS-F2) (Table 1). This finding suggests that APS-F1 may be composed of approximately 1037 sugar units and APS-F2 of about 163 (calculated from an average molecular weight of 180 Da per sugar unit).

Anti-adhesive effects of polysaccharides

As shown in Table 2, APS-F2 significantly reduces the adhesion of *H. pylori* to MKN45 cells in a dose-dependent manner. With the addition of APS-F2 at concentrations of 0.1, 0.5 and 1.0 mg/ml, FITC-labelled *H. pylori* on the cells significantly decreased, to 88% ($P \leq 0.05$), 76% ($P \leq 0.01$) and 64% ($P \leq 0.001$) of the control value, respectively. However APS-F1 did not exhibit the effect even at the highest concentration of 1.0 mg/ml.

For the specific binding studies, another Gram-negative bacterium, *E. coli*, was used as a model and the results (Table 2) showed that APS-F2 (0.5 and 1.0 mg/ml) significantly inhibits the attachment of *E. coli* to LOVO cells, with the bacteria attached to the cells reduced to 73.3 and 68.0% ($P \leq 0.001$) of the control value. APS-F1 did not have this effect.

The anti-adhesive activity of the polysaccharides was observed visually by Gram staining. As shown in Figure 2a and b, in the absence of APS-F2, a large amount of *H. pylori* attached on the surface of MKN45 cells. After treatment with APS-F2 (1.0 mg/ml), the number of attached *H. pylori*

Table 2 Effect of polysaccharide fractions isolated from *Aloe vera* L. var. *chinensis* (Haw.) Berger on the adhesion of FITC-labelled *H. pylori* to MKN45 cell and FITC-labelled *E. coli* to LOVO cell

Inhibitor concentration (mg/ml)	% *H. pylori* attachment to MKN45 cells[a]					% *E. coli* attachment to LOVO cells[a]				
	0	0.01	0.1	0.5	1.0	0	0.01	0.1	0.5	1.0
APS-F1	100.0 ± 1.1	97.4 ± 2.8	98.1 ± 2.9	96.3 ± 2.1	101.3 ± 4.5	100 ± 5.0	103.0 ± 3.5	100.9 ± 2.3	100.2 ± 3.7	99.0 ± 4.7
APS-F2	100.0 ± 6.5	88.0 ± 5.2	80.0 ± 2.8*	76.0 ± 4.1**	64.0 ± 3.4***	100 ± 1.8	102.4 ± 3.4	102.6 ± 2.4	73.3 ± 3.2***	68.0 ± 2.0***

[a]The values represent means with standard error as a percentage of control values. Cells plus bacteria was used as a control, representing 100% adhesion. Significantly different from the control group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

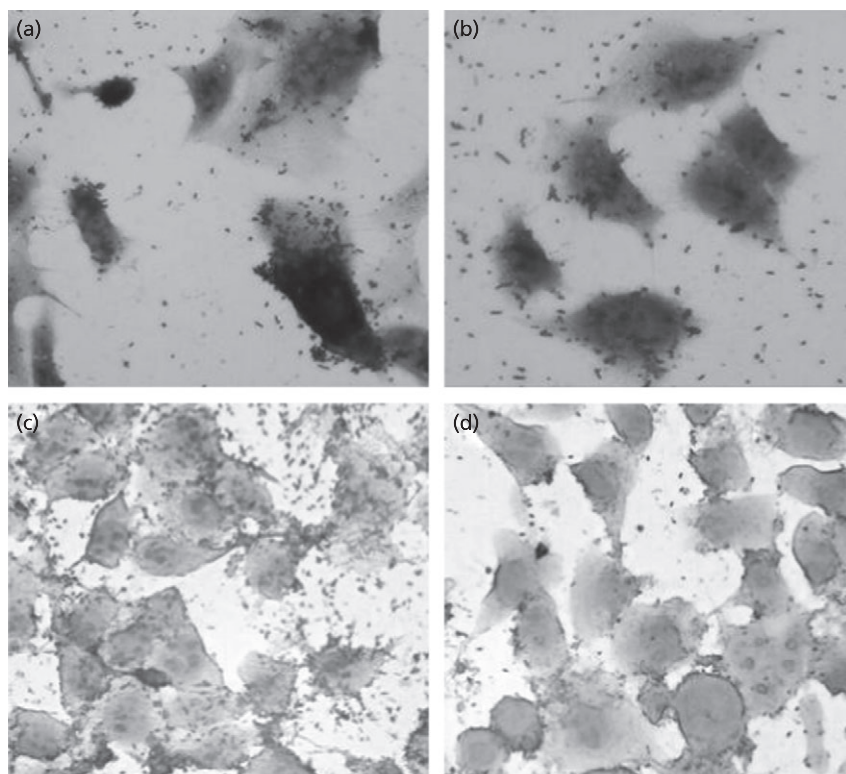

Figure 2 Adhesion of *H. pylori* or *E. coli* to cells in the absence or presence of APS-F2 at 1.0 mg/ml observed using light microscopy after Gram staining. MKN45 cells were infected by 1×10^6 cfu *H. pylori* for 1 h with (b) or without (a) pretreatment of APS-F2; LOVO cells were incubated with 1×10^6 cfu *E. coli* for 1 h with (d) or without (c) pretreatment of APS-F2. The pictures were captured with a 40× optical microscope after Gram staining.

apparently decreased, indicating that APS-F2 greatly reduces the adhesion of *H. pylori* to cells. A similar anti-adhesive effect of APS-F2 was observed in the *E. coli* model (Figure 2c and d). In the presence of APS-F2 (1.0 mg/ml), the quantity of attached *E. coli* on the LOVO cells apparently reduced compared with samples without APS-F2.

Effect of polysaccharides on *H. pylori* growth

The disk diffusion assay showed that while the positive control, amoxicillin (0.5 mg), had a significant effect (inhibition zone of 25 mm), no sign of growth inhibition (there were no inhibition zones) was observed for APS-F1 and APS-F2. This result indicates that these polysaccharides have no inhibitory effect on the growth of the tested *H. pylori* strains at the concentrations used. Similarly, no inhibition of *E. coli* growth was found in the presence of APS-F1 or APS-F2.

Cell viability evaluation

The MTT assay showed that after treatment with APS-F1 and APS-F2, at all tested concentrations, both MKN 45 and LOVO cells did not display detectable changes in their viability compared with the control group, showing that neither APS-F1 nor APS-F2 affects cellular viability at the concentrations examined in this assay (data not shown).

Discussion

Anti-adhesives have drawn great attention in recent years as a new strategy to combat bacterial infection. Studies about the application of botanicals as a source of anti-adhesives have been much-reported. Polysaccharides from *Artemisia capillaries*,[14] *Panax ginseng*,[15] blackcurrant seeds,[16] green tea,[17] procyanidins from cranberries,[18] as well as EPS 7630, an extract from *Pelargonium sidoides* roots,[19] were found to inhibit the adherence of *H. pylori* to gastric epithelial cells. In the current study, polysaccharide fractions were isolated from *A. vera* and their anti-adhesive effects against *H. pylori* were determined.

There have only been a few reports regarding the characterisation of chemical constituents of the polysaccharides from *A. vera* L. var. *chinensis* (Haw.) Berg. Three polysaccharide fractions from *A. vera* L. var. *chinensis* (Haw.) Berg have been isolated, with molecular weights of 10 000, 1300 and 470 kDa, and high mannose content, of 91.5, 87.9 and 53.7%, respectively.[20] Wu *et al*. found that a polysaccharide isolated from fresh leaves of *A. vera* L. var. *chinensis* (Haw.) Berg was composed of mannose and glucose (78.2 and 21.8%) and had a molecular weight around 210 kDa.[9] The APS-F1 and APS-F2 obtained in our study apparently differ from these reported *Aloe* polysaccharides in molecular weight and monosaccharide composition. Although APS-F1 has a high content of mannose and a high molecular weight of 186.7 kDa, which is very similar to these previous reports, it also has a galactose content of 4.5%, which characterises APS-F1 as a glucogalactomannan. APS-F2 possesses 25.6% galacturonic acid and has the much lower molecular weight (29.39 kDa), monosaccharide content and ratios.

The acidic polysaccharide APS-F2 reduced *H. pylori* adhesion to MKN45 cells by nearly 36% at a concentration of 1.0 mg/ml. This active concentration is comparable to that of sialyl-3′-lactose,[15] which has been considered to be the most active oligosaccharide. Given its apparent molecular weight of 29.39 kDa, the effective concentration of APS-F2 occurred in the region of 4–34 μM. This result is consistent with previous findings, which have reported that acidic polysaccharides derived from plants, such as the acidic polysaccharides from *Artemisia capillarys*,[14] *Panax ginseng*,[15] blackcurrant seeds[16] and green tea ,[17] have the ability to block pathogenic adherence. Notably in these acidic polysaccharides, the high uronic acid content seems to be associated with their anti-adhesive activity and it has been reported that the effectiveness of the anti-adhesive activity is in proportion to the uronic acid content.[21] This observation is supported by our findings: in our study, the acidic polysaccharide APS-F2 possessed a high uronic acid content of as much as 25.6% and has a remarkable inhibitory activity against *H. pylori*'s attachment to host cells. However, the neutral polysaccharide APS-F1, without uronic acid content, shows negative results, also consistent with previous studies.[21,22]

To the best of our knowledge, carbohydrates on the surface of host cells serve as receptors of bacterial adhesin and mediate adhesions between *H. pylori* and the cells. Some carbohydrates mediating this interaction have been identified, such as the Le^b antigen, which recognises the blood group antigen-binding adhesin (BabA), and the sialyl-dimeric-$Lewis^x$ glycosphingolid, for the recognition of sialic acid-binding adhesin (SabA).[23,24] For this reason, exogenous carbohydrate with a homologic structure may competitively bind to the bacterial adhesin and thereby interfere with the bacterial adhesion. Among these homologic structures, negatively charged groups seem to be an important feature and some potential anti-adhesive agents with a negatively charged group in their structure have been reported, such as sialyllactose,[25] sulphated exopolysaccharides,[26] and acidic polysaccharides rich in uronic acid. In this regard, our results for APS-F2 strongly support this point. APS-F1, without uronic acid, did not show the anti-adhesive effect, which may be due to it being unable to act as an acceptor analogue to competitively bind to *H. pylori*.

As a practical therapy for treating pathogenic infection, it will probably be necessary to use multiple agents specifically inhibiting each type of adhesin of the infecting pathogen, or alternatively a single agent that exhibits a broad spectrum of anti-adhesive activity.[5] This is because most pathogens possess genes encoding for more than one type of adhesin and these multiple adhesins, together with their multiple carbohydrate receptors, may be involved in the process of bacterial adhesion. In order to evaluate the specificity of adhesive activity of these polysaccharides, another Gram-negative bacterium *E. coli* adhesive model was used. Similarly to the result of the *H. pylori* experiment, APS-F2 inhibits the adhesion of *E. coli* to LOVO cells, suggesting that the anti-adhesive activity of APS-F2 is not *H. pylori*-specific and so it may be an efficacious anti-adhesive agent against many Gram-negative bacteria, something that would be beneficial in clinical use.

In addition, in-vitro experiments revealed that neither polysaccharide affects the growth of the *H. pylori* strains tested, which shows that the reduction of *H. pylori* adhesion to the cell is truly caused by the inhibition of *H. pylori* attachment, instead of any possible bactericidal effects. Nevertheless, because there is a strain-specific susceptibility to the compounds of *H. pylori*, more strains are required. At the same time, neither polysaccharide exhibits an effect on cell viability at the tested concentrations, which excludes the possibility of any interference in the anti-adhesion assay.

Conclusions

The present study demonstrates, for the first time, that the acidic polysaccharide extract APS-F2 of *A. vera* has a potent inhibitory effect on *H. pylori* adherence to gastric cells *in vitro*. This may be related to its structural characteristic of a high content of the negatively charged group, uronic acid. The adhesive effect may not be *H. pylori*-specific. However, further investigation is required to evaluate whether it is equally effective *in vivo*.

Declarations

Conflict of interest

The Authors declare that they have no conflicts of interest to disclose.

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