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Characterization of a novel polysaccharide with anti-colon cancer activity from *Lactobacillus helveticus* MB2-1

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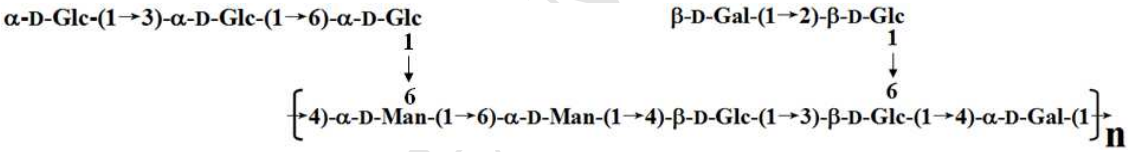
ABSTRACT

The present study aimed at investigating the potential anti-conlon cancer activity of three purified exopolysaccharides fractions (LHEPS-1, LHEPS-2 and LHEPS-3) from the *Lactobacillus helveticus* MB2-1. The experimental evidence showed that LHEPS-1 significantly inhibited cell proliferation of human colon cancer Caco-2 cells in both time- and concentration-dependent manners. In contrast, no significant improvements of the inhibitory effects of LHEPS-2 and LHEPS-3 on Caco-2 cells were observed with increasing sample concentrations or prolonged incubation time. Furthermore, the structure of LHEPS-1 was elucidated using methylated analysis, gas chromatography–mass spectroscopy (GC–MS) and nuclear magnetic resonance spectroscopy (NMR), including one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR). Results indicated that the LHEPS-1 consisted of a decasaccharide repeating unit with the following structure (n ≈ 122):



Our results suggested that the LHEPS-1 produced by *L. helveticus* MB2-1 might be suitable for using as natural anti-conlon cancer drugs and functional foods ingredients.

***Keywords*:** *Lactobacillus helveticus* MB2-1; Exopolysaccharides; Anti-conlon cancer activity; Structural characterization; Methylated analysis; Nuclear magnetic resonance spectroscopy.

1. Introduction

Colon cancer is a major health problem of global concern and the third leading cause of cancer-related mortality throughout the world.[1] Usually, colon cancer is treated by surgical resection and chemotherapy. Both 5-Fluorouracil (5-Fu) and oxaliplatin are the most effective cytotoxic agents used in colon cancer treatment. However, some undesirable effects, including nausea, vomiting and fatigue, declined the quality of patients' lives.[2,3] In addition, inherent and acquired resistances to the drugs limited their applications in colon cancer chemotherapy.[4] One obstacle ahead is the high risk of locoregional relapse and distant metastasis.[5] Therefore, it is important to search for new alternative strategies for the prevention and treatment of colon cancer.

Polysaccharides are important components derived from plants, animals and microorganisms, and have been attracted intensive attentions in the medical and food research fields due to their immunmodulatory and anticancer activities.[6-8] Among microbial polysaccharide, those exopolysaccharides (EPSs) produced by lactic acid bacteria (LAB) are receiving increasing attention because of the food-grade status of these microorganisms coupled with their long historical application.[9,10] EPSs, one of the most important secondary metabolites produced by LAB, have also been correlated with multiple pharmacological activities, such as immunomodulatory, cholesterol lowering, antioxidant and anticancer activities.[11-13] Thus, EPSs derived from LAB might serve as ideal candidate for prevention and therapeutics of colon cancer.

The anticancer activity of polysaccharides including EPSs is affected by many factors such as monosaccharide composition, molecular weight (Mw), structure of the polymeric backbone and side chains, and even number of branching points.[14,15] The moderate size of polysaccharides has been proved to be favorable for anticancer activity.[16] Other structural properties such as the presences of

mannose and glucose residues and existing of branching points in repeating unit are also conducive to increase their anticancer activity.[17-19] Recently, we reported that the production, purification, characterization and antioxidant activities *in vitro* of EPS from *Lactobacillus helveticus* MB2-1. The crude EPS obtained from the whey medium was purified by anion exchange and gel filtration chromatography, affording three fractions of LHEPS-1, LHEPS-2 and LHEPS-3.[20] The Mw of three purified EPS fractions (LHEPS-1, LHEPS-2 and LHEPS-3) were estimated to be 2.08×10^5, 2.04×10^5 and 2.01×10^5 Da, respectively, and they were composed of galactose, glucose and mannose with a molar ratio of 1.33:2.75:1.00, 1.00:1.43:9.34 and 1.17:1.00:2.96, respectively. *In vitro* antioxidant activities assays demonstrated that LHEPS-1, LHEPS-2 and LHEPS-3 had strong scavenging activities against free radicals.[20] In addition, we found that all the LHEPS fractions, especially LHEPS-2 exerted high inhibitory activity *in vitro* on human gastric cancer BGC-823 cells. Furthermore, we elucidated the fine structure of the purified LHEPS-2, which was a →4,6-D-Man*p*-(1→6)-D-Man*p*-(1→4)-D-Gal*p*-(1→ backbone with a single T-D-Glc*p*-(1→4)-D-Man*p*-(1→6)-D-Glc*p*-(1→ side-branching in O-6 of →4,6-D-Man*p*-(1→ residue.[21] These results indicated that EPSs from *L. helveticus* MB2-1 had potential application as natural anticancer drugs or functional food ingredients for gastric cancer therapies. However, little information is presented regarding the anticancer activity of EPS fractions against human colon cancer Caco-2 cells. Thus, in the present study, the *in vitro* anticancer effects were determined using Caco-2 cells for three purified EPS fractions from *L. helveticus* MB2-1. In addition, for a better understanding and evaluation of the structural characteristic and high anticancer activity of EPS fractions, the structure of neutral LHEPS-1 was elucidated by using methylated analysis, gas chromatography–mass spectroscopy (GC–MS), and nuclear magnetic resonance spectroscopy (NMR), including one- and two-dimensional

nuclear magnetic resonance (1D and 2D NMR).

2. Results and discussion

2.1. Assay of inhibitory effects of LHEPS on the growth of human colon cancer Caco-2 cells

It has been reported that some polysaccharides exerted anticancer activity.[22,23] However, only a few studies investigated the anticancer activities of the polysaccharides of LAB.[21,24] In the present study, therefore, the anticancer activity of LHEPS *in vitro* was evaluated. The growth inhibitory effects on colon cancer Caco-2 cells were measured using MTT assay after the cells were treated with increasing concentrations (0, 50, 100, 200, 400 and 600 μg/mL) of the three purified LHEPS fractions and 5-Fu (50 μg /mL) for 24, 48 and 72 h, respectively. As shown in Fig. 1, LHEPS-1 significantly inhibited the growth of Caco-2 cells in both time- and dose-dependent manners within 72 h of incubation ($p < 0.05$). However, the inhibitory effects of LHEPS-2 and LHEPS-3 on Caco-2 cells did not significantly affected ($p > 0.05$) by the different sample concentrations (50–600 μg/mL) at 72 h of incubation, and no significant differences were observed ($p > 0.05$) for the inhibitory effects of LHEPS-2 and LHEPS-3 at the treatments of 24, 48 and 72 h when the sample concentrations were higher than 400 μg/mL. At a concentration of 600 μg/mL and 72 h incubation, the inhibitory effect of LHEPS-1 on Caco-2 cells reached the highest (inhibition rate, 56.34%), although slightly lower than that of 5-Fu (79.98%) at 50 μg/mL. However, the level of LHEPS-1-mediated cytotoxicity in normal colon HCoEpiC cells was not dramatic. Even at the high dose of 600 μg/mL, the cytotoxicity induced by a 72 h incubation of normal colon cells with LHEPS-1 was only 4% (data not shown). Apparently, LHEPS-1 possessed a relative higher anti-colon cancer activity *in vitro* than LHEPS-2 and LHEPS-3 whereas it was no cytotoxic effect on normal colon cells. These results suggested that LHEPS-1 might be a potential natural drug or health food for colon cancer therapeutics. Therefore, the fine structure characterization of LHEPS-1

was further investigated in the present study, which was based on our previous data.[20]

Fig. 1

2.2. Methylation analysis of LHEPS-1

After methylation for four times, the hydroxyl group absorption at (3700–3100 cm^{-1}) in FT-IR disappeared, indicating the completeness of methylation. The individual peaks of the methylated monosaccharide residues and fragmentation patterns were identified by their retention time in GC and by comparison with literature MS patterns.[25-29] The identification and the related linkage patterns are shown in Table 1, and the molar ratio of monosaccharide residues was calculated according to the peak areas and response factor of the total ion chromatogram (TIC) in Agilent GC–MS system. Methylation analysis of the LHEPS-1 showed the presences of ten components, namely 2,4,6-Me_3Glc*p*; 2,3,4-Me_3Man*p*; 3,4,6-Me_3Glc*p*; 2,3,6-Me_3Glc*p*; 2,3,4,6-Me_4-Glc*p*; 2,3,4,6-Me_4-Gal*p*; 2,3,6-Me_3Gal*p*; 2,3,4-Me_3Glc*p*; 2,3-Me_2Man*p* and 2,4-Me_2Glc*p*. Based on the data available in Table 1, LHEPS-1 was composed of (1→3)-linked-D-Glc*p*, (1→6)-linked-D-Man*p*, (1→2)-linked-D-Glc*p*, (1→4)-linked-D-Glc*p*, (1→)-linked-D-Glc*p*, (1→)-linked-D-Gal*p*, (1→4)-linked-D-Gal*p*, (1→6)-linked-D-Glc*p*, (1→4,6)-linked-D-Man*p* and (1→3,6)-linked-D-Glc*p*, in molar ratio of 1.25, 1.21, 1.07, 1.00, 1.31, 1.14, 1.19, 1.26, 1.16 and 1.33 (about 1:1:1:1:1:1:1:1:1:1), respectively. Furthermore, methylation linkage analysis of LHEPS-1 indicated that the residues of branch structure were linked to the oxygen of C-6 positions of (1→4)-linked-D-Man*p* and (1→3)-linked-D-Glc*p*, respectively. In addition, the molar ratios of D-Glc*p*, D-Man*p* and D-Gal*p* residues in fully methylated analysis were respectively 3.10:1.02:1.00, which was in good agreement with the monosaccharide composition of LHEPS-1 obtained from GC analysis.[20]

Table 1

2.3. 1D and 2D NMR analysis of LHEPS-1

In order to get more insight into the structural information of the LHEPS-1 from *L. helveticus* MB2-1, both detailed 1D and 2D NMR studies on the LHEPS-1 were performed. The general representative peaks of sugar residue were ascribed as follows: anomeric proton (H-1) signals of glycosides were assigned to δ 4.6–5.6 ppm, and H-2, H-3, H-4, H-5 and H-6 from glycosidic ring were assigned to δ 3.5–4.5 ppm. The signal for residual D_2O in the sample occurred at the anomeric region in the spectrum at δ 4.7 ppm. All the signals were assigned based on component analysis, linkage analysis and literature values.[20,21,26-28,30] 1D NMR spectra of LHEPS-1 are shown in Fig. 2A and B. Ten obvious chemical shift signals of anomeric protons were found at δ 5.46, 5.33, 5.29, 5.26, 5.24, 5.23, 4.71, 4.70, 4.66 and 4.64 ppm in ^{1}H NMR spectrum (Fig. 2A). The ^{13}C NMR (Fig. 2B) chemical shifts in the area of anomeric carbon atoms also suggested ten kinds of α- or β-linkages existed for the D-Glc*p*, D-Man*p* and D-Gal*p* residues. The C-1 signals of the residues were detectable at δ 105.72, 105.68, 105.38, 105.33, 104.58, 104.46, 104.29, 102.90, 100.73, 100.62 ppm, respectively. It was evident from Fig. 2 that there were three regions of H-1/C-1 signals corresponding to α- anomers (H-1 5.2-5.6 ppm, C-1 100-105 ppm) and beta-anomers (H-1 4.6-4.8 ppm, C-1 105-107 ppm). Combined with the result of monosaccharide analysis, the **A**–**F** residues might reflect six different α-type glycosidic bonds of sugar residues, and **G**–**J** residues might indicate four different β-type glycosidic bonds of sugar residues. Molar proportion of the ten residues was about 1.00:0.86:1.04:0.83:0.84:1.20:1.20:1.18:1.15:1.19, which estimated by the ratio of peak area of the integration of the H-1 signal for **A**-**J** residues. However, the corresponding H chemical shifts of residues **A**–**J** for other positions were packed in the range of δ 3.5–4.5 ppm and even difficult to discriminate.

Fig. 2

The complete assignments of the ^{1}H and ^{13}C chemical shifts of the LHEPS-1 were carried out by means of the 2D HSQC, COSY, TOCSY, HMBC and NOESY experiments based on FTIR analysis (data not shown), methylation analysis and the data from literatures.[18,20,21,25–32] The single-bond correlations between the protons and the corresponding carbons obtained from HSQC spectra (Fig. 2C) of LHEPS-1 in D_2O enabled all the ^{13}C NMR to be assigned. The C-1 signals at δ105.72, 105.68, 105.38, 105.33, 104.58, 104.46, 104.29, 102.90, 100.73, 100.62 ppm could be assigned to the **J**, **I**, **H**, **G**, **B**, **E**, **F**, **A**, **C** and **D**, respectively. These signals cross link to the proton signals at chemical shifts δ 4.64, 4.66, 4.70, 4.71, 5.33, 5.24, 5.23, 5.46, 5.29 and 5.26, respectively. The results from COSY (Fig. 3A), based on stepwise magnetization transfers from the anomeric protons, could help to assign the chemical shifts of other protons. The TOCSY spectrum provided all intraresidue connectivity of all protons of the **A**–**J**, respectively, as shown in Fig. 3C. From the HMBC spectrum, the linkage of residue was obtained. Inspection of the NOESY spectrum showed on the **F** and **I** H-1 track NOE connectivity with **D** and **J** H-6 in agreement with the **F**-(1→6)-**D** and **I**-(1→6)-**J** linkage, respectively. In addition, inter-residue NOE cross-peaks between **D** H-1 and **A** H-6 supported the **D**-(1→6)-**A** linkage, and between **J** H-1 and **B** H-6 supported the **J**-(1→4)-**B** linkage, which indicated that the existing two branching points and the branching position of residue **D** and **J**, respectively (data not shown). Thus a summarized of the information from the 1D and 2D NMR spectra gave a complete assignment of all the linkage patterns which was shown in Table 2. According to the results, it has been considered that residues **A**, **B**, **E**, **F**, **G** and **I** were substituted at C-6, C-4, C-3, C-6, C-4 and C-2 position, respectively. Thus, residues **A**, **B**, **E**, **F**, **G** and **I** belonged to (1→6)-linked-D-Man*p*, (1→4)-linked-D-Gal*p*, (1→3)-linked-D-Glc*p*, (1→6)-linked-D-Glc*p*, (1→4)-linked-D-Glc*p* and

(1→2)-linked-D-Glc*p*, respectively. Residues **D** and **J** were assigned to (1→4,6)-linked-D-Man*p* and (1→3,6)-linked-D-Glc*p* containing branching point, respectively. In addition, residues **C** and **H** were determined as α-terminal-D-Glc*p* and β-terminal-D-Gal*p*, respectively. On the basis of above mentioned results, the suggested repeat unit of LHEPS-1 was concluded (Fig. 4). In this study, the value of 'n' that represented the repeated structural unit of LHEPS-1 was calculated using the following formula. The value of 'n' = (Mw of LHEPS-1) / [(Mw of monosaccharide × the number of monosaccharide residues in the repeating unit) – (Mw of water molecule × the number of glycosidic bonds in the repeating unit)]. In our study, Mw of LHEPS-1, Mw of monosaccharide, the number of monosaccharide residues in the repeating unit, Mw of water molecule and the number of glycosidic bonds in the repeating unit were 2.08 × 10^5 Da, 180 Da, 10, 18 Da and 11, respectively.[20] Therefore, the value of 'n' that repeat structural unit of LHEPS-1 was approximately equal to 122. In the current study, a neutral EPS fraction **(**LHEPS-1**)** was derived from *L. helveticus* MB2-1, and its structure was identified as heteropolysaccharide.

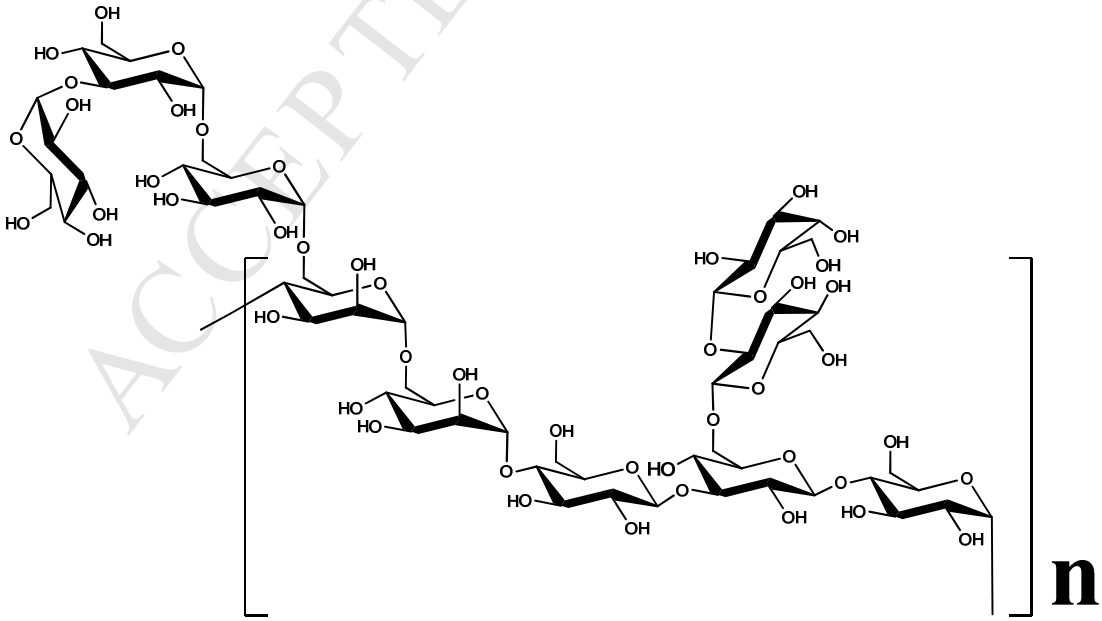


Table 2

Fig. 3

Fig. 4

In general, EPS from *Lactobacillus* spp. are divided into heteropolysaccharide and homopolysaccharide as other bacterial EPS.[33,34] Homopolysaccharides are composed of one kind of monosaccharide (ie. glucose or galactose), whereas several different types of monosaccharides constitute heteropolysaccharides. Heteropolysaccharides have a great variability in structures. EPS backbones of *Lactobacillus* spp. (*L. helveticus*, *L. rhamnosus* and *L. delbrueckii bulgaricus*) have repeated units composed of seven monosaccharides, where glucose, galactose and mannose are the main sugar residues.[30,35] Heteropolysaccharides are often linear or branched with different types of linkages. Their structures are also complex depending on the principal monosaccharide, for example, glucogalactan or galactoglucan and depending on the ratios of each sugar they are named as glucomannogalactan or galactoglucomannan. Obviously, LHEPS-1 was a kind of moderate branching glucomannogalactan.

2.4. Structure-anticancer activity correlation

It has been reported that the anticancer effects of polysaccharides were influenced by their chemical composition and structure of the polymeric backbone.[19] According to our previously study, although the Mw of LHEPS-1 was similar with that of LHEPS-2 and LHEPS-3, the chemical composition and structure of LHEPS-1 was greatly differed from that of LHEPS-2 and LHEPS-3. LHEPS-1 exhibited more average molar ratios between the composed monosaccharide than LHEPS-2 and LHEPS-3.[20] Moreover, differed from LHEPS-2 and LHEPS-3, LHEPS-1 possessed more side chains which probably facilitated the exposure of active groups and/or its spatial structure. In our study, the degrees

of branching of the different LHEPS fractions were calculated using the following formula. The degree of branching = the number of branching point / the number of monosaccharide residues in the repeating unit. In our study, the number of branching points of LHEPS-1, LHEPS-2 and LHEPS-3 were 2, 1 and 1, respectively. The number of monosaccharide residues in the repeating unit of LHEPS-1, LHEPS-2 and LHEPS-3 were 10, 6 and 7, respectively[20, 21]. Thus, the degree of branching was 20% for LHEPS-1 which was higher than LHEPS-2 (17%) and LHEPS-3 (14%), according to the molar ratios of branched to linear sugar residues. Therefore, structural characteristics such as the average molar ratios of monosaccharide composition, moderate Mw, configuration and high degree of branching might attribute LHEPS-1 a high anti-colon cancer activity. However, the mechanism for the inhibitory activity of cancer cell growth of polysaccharide is complex. Ma et al.[3] found a water-soluble polysaccharide named SFPSA could inhibit the proliferation and growth of human colon cancer cells in a time- and dose-dependent manner within 48 h, induce the apoptosis and increase the accumulation in G_2/M phase of colon cancer cells. Furthermore, it was found that SFPSA could decrease Bcl-2 mRNA level, and enhance the mRNA expressions of Bax and p53, as well as the activity of caspase-3. It could induce apoptosis of colon cancer cells through regulation of apoptosis-associated gene expressions and the activation of caspase-3. Therefore, further study was needed to shed light on the mechanism for the inhibitory activity of cancer cell growth of LHEPS-1 from *L. helveticus* MB2-1 .

3. Conclusion

In the present study, the inhibitory effects of three purified LHEPS fractions (LHEPS-1, LHEPS-2 and LHEPS-3) from the *L. helveticus* MB2-1 to the proliferation potential of human colon cancer Caco-2 cells were studied. The results suggested the potential prospects of LHEPS-1 significantly inhibited cell proliferation in Caco-2 cells in both time- and concentration-dependent manners. Further

study revealed LHEPS-1 was a moderate branching glucomannogalactan that possessed a backbone consisting of the repeating unit →4)-α-D-Man*p*-(1→6)-α-D-Man*p*-α-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→, branching at O-6 of →4)-α-D-Man*p*-(1→ and →3)-β-D-Glc*p*-(1→, respectively, and terminated with D-Glc*p* and D-Gal*p*. This structure differed from the previously reported EPS from other *Lactobacillus* spp. These results suggested the potential prospects of LHEPS-1 as foods and natural anticancer drugs to prevent human colon cancer.

4. Experimental

4.1. Materials

The strain of *L. helveticus* MB2-1 was isolated from traditional Sayram ropy fermented milk which was collected from Xinjiang, China.[36] Human colon cancer Caco-2 cells were obtained from the Cell Bank of Shanghai Institute of Cell Biology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Gibco/Invitrogen (Gibco BRL, Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), 5-Fu, penicillin and streptomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

4.2. Preparation of purified LHEPS fractions

The LHEPS was isolated and purified according to our previously method.[20,21] Briefly, after 24 h of incubation period, the cell was separated by centrifugation at 12,000 × *g* for 15 min at 4 °C. Then trichloroacetic acid (TCA) solution was added to the culture to give a final concentration of 4% (w/v), and the precipitated proteins were removed by centrifugation (12,000 × *g* for 30 min at 4 °C). The supernatant was filtered through a 0.45 μm membrane filter, mixed with three volumes ice cold ethanol,

stirred vigorously and kept at 4 °C for overnight. Crude LHEPS was collected by centrifugation at 15,000 × *g* for 15 min. The LHEPS pellet was dissolved in distilled water and dialyzed (Mw cut-off 8,000– 14,000 Da, Solarbio Co., Ltd, Beijing, China) against distilled water for 48 h at 4 °C and then lyophilized. The freeze-dried sample was fractionated with a DEAE-cellulose column (2.6 × 30 cm), followed by stepwise elution with distilled water, 0.1 and 0.3 M NaCl solutions at a flow rate of 60 mL/h. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method.[36] Three fractions (LHEPS-1, LHEPS-2 and LHEPS-3) eluted with 0, 0.1 and 0.3 M sodium chloride solutions were collected, concentrated, dialyzed and lyophilized, respectively. These three fractions were then purified by a Sephadex G-100 column (2.6 cm × 100 cm) with distilled water at a flow rate of 12 mL/h. Finally, the three purified fractions were collected, concentrated, dialyzed and lyophilized for further study.

4.3. Anticancer activity of three purified LHEPS fractions

To investigate the inhibition effects of three purified LHEPS fractions, human colon cancer Caco-2 cells and human colonic epithelial HCoEpiC cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/L) under 5% CO_2 atmosphere at 37 °C, respectively. The inhibition effects of different LHEPS fractions on Caco-2 and HCoEpiC cells *in vitro* was evaluated using MTT-based colorimetric method according to our previously reported method.[24] Briefly, 100 μL of cells were incubated on a 96-well plate at a concentration of 2×10^5 cells/well. After inoculation under 5% CO_2 at 37 °C for 24 h, the cells were then incubated with DMEM medium containing various concentrations of purified LHEPS fractions (0, 50, 100, 200, 400 and 600 μg/mL) and 5-Fu (50 μg /mL) for 24, 48 and 72 h, respectively. At the end of each treatment, 10 μL (5 mg/mL) of MTT was dissolved in DMEM medium and was added and the cells were inoculated for another 4 h.

The liquid was then removed and 100 μL DMSO was added to the well. After dissolving of the formed crystal formazan, the absorbance was measured by a Synergy™-2 microplate reader (BioTek Instruments, Inc., Burlington, VT) at 570 nm. The inhibitory rate was expressed as follows:

Inhibitory rate (%) = [1 – (A_{sample} – A_{blank}) / ($A_{control}$ – A_{blank})] × 100

where $A_{control}$ and A_{blank} were the absorbance of the system without the addition of samples and cells, respectively.

4.4. Methylation and GC–MS analysis for LHEPS-1

Methylation and GC–MS analysis of LHEPS-1 were performed by using our previously reported method.[37] Complete methylation was confirmed by the disappearance of O–H absorption band (3700–3100 cm^{-1}) in fourier-transform infrared (FT-IR) spectrum. Briefly, the permethylated LHEPS-1 was hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 2 h. Then the methylated sugars were reduced with $NaBH_4$ for 2 h and acetylated with pyridine-acetic anhydride (1:1, v/v) at 100 °C for 1 h. The resulting methylated alditol acetate derivatives were analyzed by GC–MS. GC–MS analysis was performed on a Agilent 5975MSD-6890 GC–MS (Agilent Technologies, CA, USA), equipped with a HP-5 capillary column was used with He as carrier gas. The oven temperature was initially set at 100 °C, programmed at 6 °C/min to 250 °C and held at 250 °C for 5 min.

4.5. 1D and 2D NMR analysis

The structure of LHEPS-1 (50 mg/mL) was identified by NMR. ^{1}H NMR, ^{13}C NMR and 2D NMR spectra were recorded in D_2O as solvent at 323 K with a Bruker AVANCE AV-500 spectrometer (Bruker Group, Fällanden, Switzerland) using the residual solvent signal as internal standard. The chemical shifts (δ) are given in parts per million (ppm), and coupling constants (J) are given in Hz. The 2D ^{1}H–^{1}H correlated spectroscopy (COSY), ^{1}H–^{1}H total correlation spectroscopy (TOCSY), ^{1}H–^{13}C

heteronuclear single quantum coherence (HSQC), $^{1}H–^{13}C$ heteronuclear multiple quantum coherence (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) measurements were used to assign signals and to determine the sequence of sugar residues.

4.6. Statistical analysis

Statistical analysis was performed using SPSS for windows, version 16.0. One-way analysis of variance (ANOVA) and the Fisher's least significant difference (LSD) test were used to determine significant differences for different samples. Differences were considered to be significant when $P < 0.05$. Data were obtained from three independent experiments and each sample was analyzed in triplicate.

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Table 1. Methylation analysis of LHEPS-1 from *L. helveticus* MB2-1

Retention time (min)	Methylated sugar	Primary mass fragments (*m/z*)	Molar ratio	Deduced linkage
15.459	2,4,6-Me_3Glc*p*	43.1, 71.1, 101.1, 129, 161.1, 205.1, 253.1, 282.1	1.25	→3-D-Glc*p*-(1→
15.812	2,3,4-Me_3Man*p*	43.1, 65, 85, 117, 139, 159.1, 201.1, 231, 261.1, 281.9	1.21	→6-D-Man*p*-(1→
16.985	3,4,6-Me_3Glc*p*	43.1, 87, 129, 161.1, 189.1, 281.1, 326.9	1.07	→2-D-Glc*p*-(1→
17.095	2,3,6-Me_3Glc*p*	43.1, 87, 117, 145, 173, 207, 234.1, 281.1, 401	1.00	→4-D-Glc*p*-(1→
17.222	2,3,4,6-Me_4-Glc*p*	43.1, 71, 101.1, 129, 161.1, 205.1, 249, 355.2	1.31	T-D-Glc*p*-(1→
17.536	2,3,4,6-Me_4-Gal*p*	43.1, 71.1, 101.1, 129, 161.1, 189.1, 233, 281.1, 341.1	1.14	T-D-Gal*p*-(1→
18.048	2,3,6-Me_3Gal*p*	43.1, 71, 101.1, 125, 145.1, 173.1, 205.1, 245.2, 281.1	1.19	→4-D-Gal*p*-(1→
18.996	2,3,4-Me_3Glc*p*	43.1, 87, 129, 159.1, 189.1, 233.1, 261, 355.1	1.26	→6-D-Glc*p*-(1→
19.073	2,3-Me_2Man*p*	43.1, 68, 87.1, 117, 142.1, 161.1, 189.1, 208.9, 233.1, 281.1	1.16	→4,6-D-Man*p*-(1→
20.996	2,4-Me_2Glc*p*	43.1, 85, 115, 145, 187, 217.1, 259.1, 289.1, 361.1	1.33	→3,6-D-Glc*p*-(1→

Table 2. Chemical shifts (ppm) of ^{1}H and ^{13}CNMR signals for the LHEPS-1, recorded in D_2O at 323 K.

Residue		**H-1**	**H-2**	**H-3**	**H-4**	**H-5**	**H-6**
A	→6-α-D-Man*p*-(1→	5.46	4.29	4.12	4.08	3.94	4.20
B	→4-α-D-Gal*p*-(1→	5.33	4.25	4.14	4.11	3.85	3.95
C	α-D-Glc*p*-(1→	5.29	4.19	3.98	3.95	3.86	4.09
D	→4,6-α-D-Man*p*-(1→	5.26	4.19	3.99	3.96	3.95	4.08
E	→3-D-α-Glc*p*-(1→	5.24	4.29	4.02	4.22	3.94	4.09
F	→6-D-α-Glc*p*-(1→	5.23	4.39	4.25	4.15	3.96	4.08
G	→4-D-β-Glc*p*-(1→	4.71	3.51	3.72	3.81	3.64	3.69
H	β-D-Gal*p*-(1→	4.70	3.50	3.71	3.80	3.65	4.04
I	→2-D-β-Glc*p*-(1→	4.66	3.66	4.13	4.11	3.85	4.11
J	→3,6-D-β-Glc*p*-(1→	4.64	3.68	4.07	4.10	3.84	3.89
		C-1	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**
A	→6-α-D-Man*p*-(1→	102.90	80.52	80.63	71.00	71.42	80.83
B	→4-α-D-Gal*p*-(1→	104.58	72.40	80.25	77.25	75.08	63.38
C	α-D-Glc*p*-(1→	100.73	80.75	75.51	75.13	69.22	63.38
D	→4,6-α-D-Man*p*-(1→	100.62	80.83	75.73	75.01	69.18	69.18
E	→3-D-α-Glc*p*-(1→	104.46	71.31	81.00	71.22	73.90	63.44
F	→6-D-α-Glc*p*-(1→	104.29	71.93	71.20	71.32	69.29	69.19
G	→4-D-β-Glc*p*-(1→	105.33	75.45	75.10	73.18	77.10	62.88
H	β-D-Gal*p*-(1→	105.38	75.32	75.27	73.09	77.15	62.90
I	→2-D-β-Glc*p*-(1→	105.68	78.00	77.98	75.41	71.95	63.21
J	→3,6-D-β-Glc*p*-(1→	105.72	78.02	80.98	71.90	69.25	69.15

Figure captions

Figure 1. Inhibitory effect *in vitro* of LHEPS-1, LHEPS-2 and LHEPS-3 against human colon cancer Caco-2 cells at 24 h (**A**), 48 h (**B**) and 72 h (**C**) treatment. Data are means ± SD of triplicate.

Figure 2. The 500-MHz ^{1}H, ^{13}C NMR and HSQC spectra of LHEPS-1 from *L. helveticus* MB2-1 recorded on Bruker Avance DRX-500 spectrometer in D_2O. ^{1}H NMR spectrum (**A**); ^{13}C NMR spectrum (**B**); HSQC spectrum (**C**). The assignments of sugar residues in anomeric region (**D**) and non-anomeric region for HSQC spectrum (**E**, **F** and **G**).

Figure 3. COSY spectrum of LHEPS-1 from *L. helveticus* MB2-1 recorded on Bruker Avance DRX-500 spectrometer in D_2O (**A**). The assignments of sugar residues in non-anomeric region for COSY spectrum (**B**). TOCSY spectrum of LHEPS-1 recorded on Bruker Avance DRX-500 spectrometer (**C**). The assignments of sugar residues in anomeric region for TOCSY spectrum (**D**).

Figure 4. Proposed structure of LHEPS-1 from *L. helveticus* MB2-1.

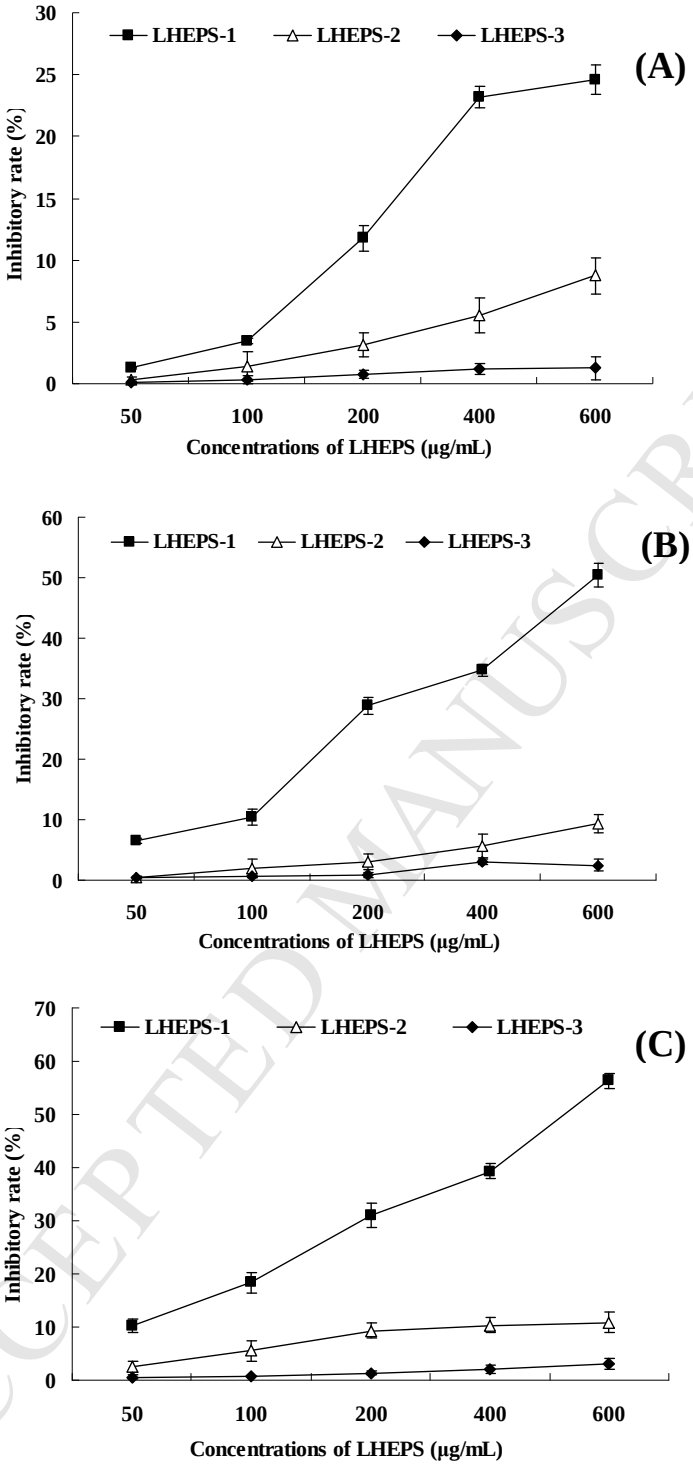
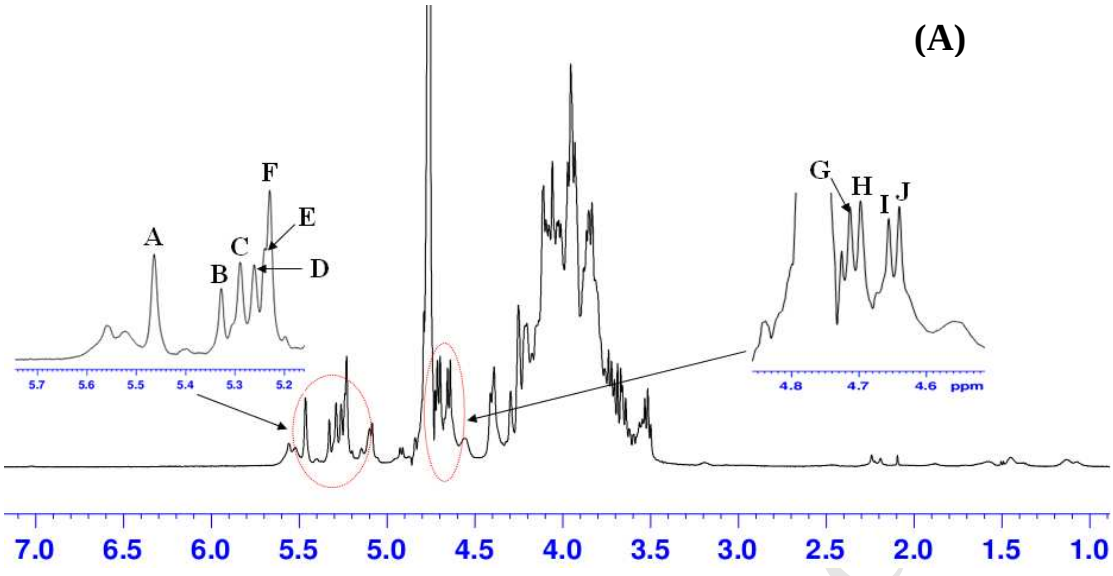
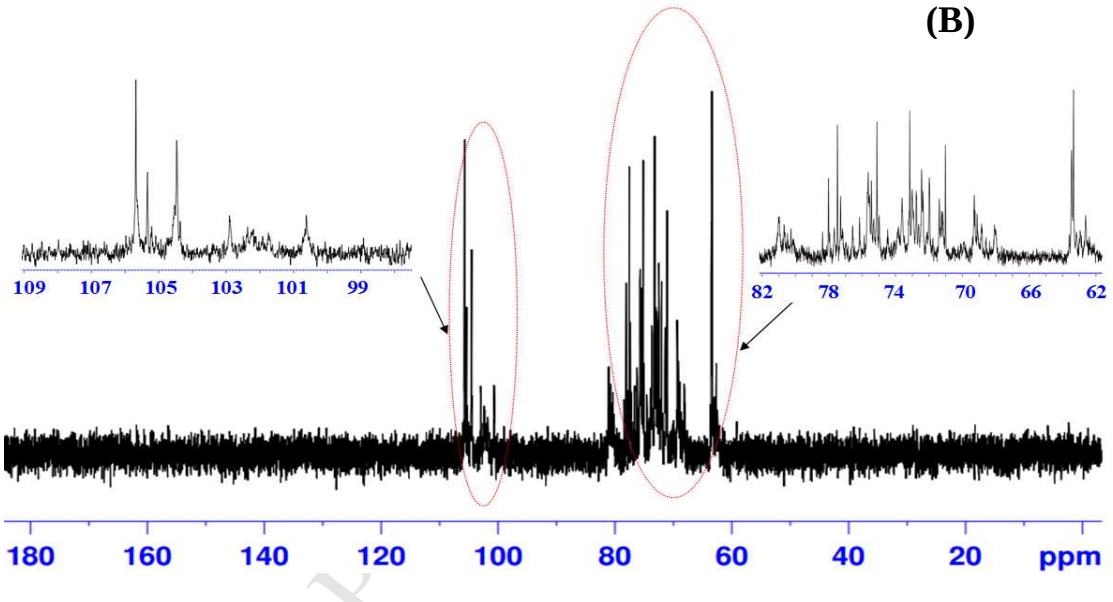
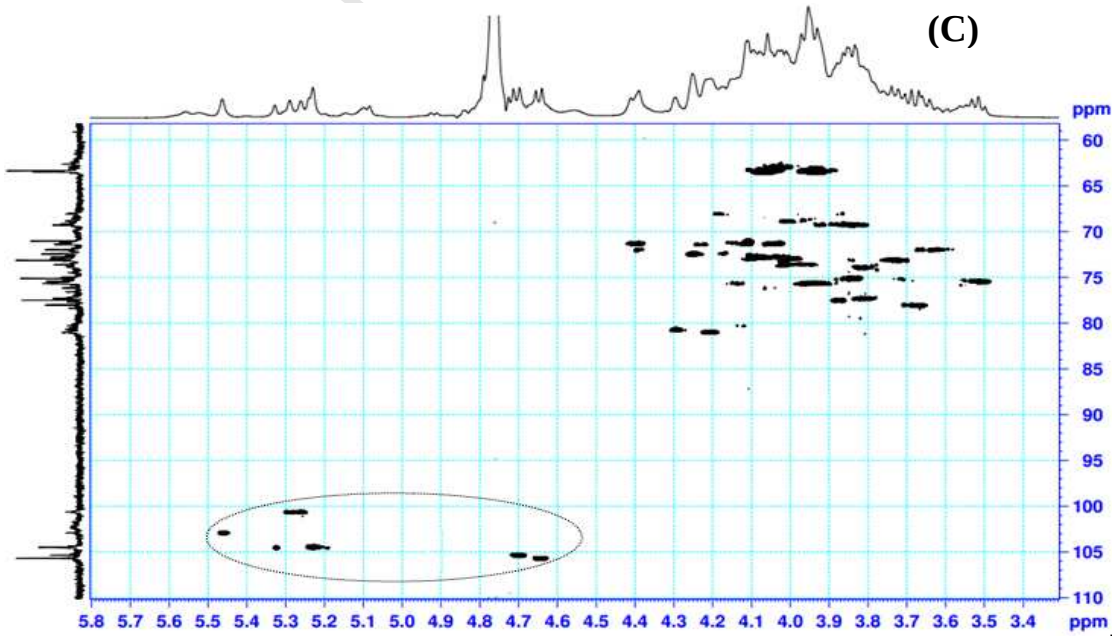


Fig. 1


(A)
A
B
C
D
E
F
G
H
I
J
5.7 5.6 5.5 5.4 5.3 5.2
4.8 4.7 4.6 ppm
7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0


(B)
109 107 105 103 101 99
82 78 74 70 66 62
180 160 140 120 100 80 60 40 20 ppm


(C)
ppm
60 65 70 75 80 85 90 95 100 105 110
5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 ppm

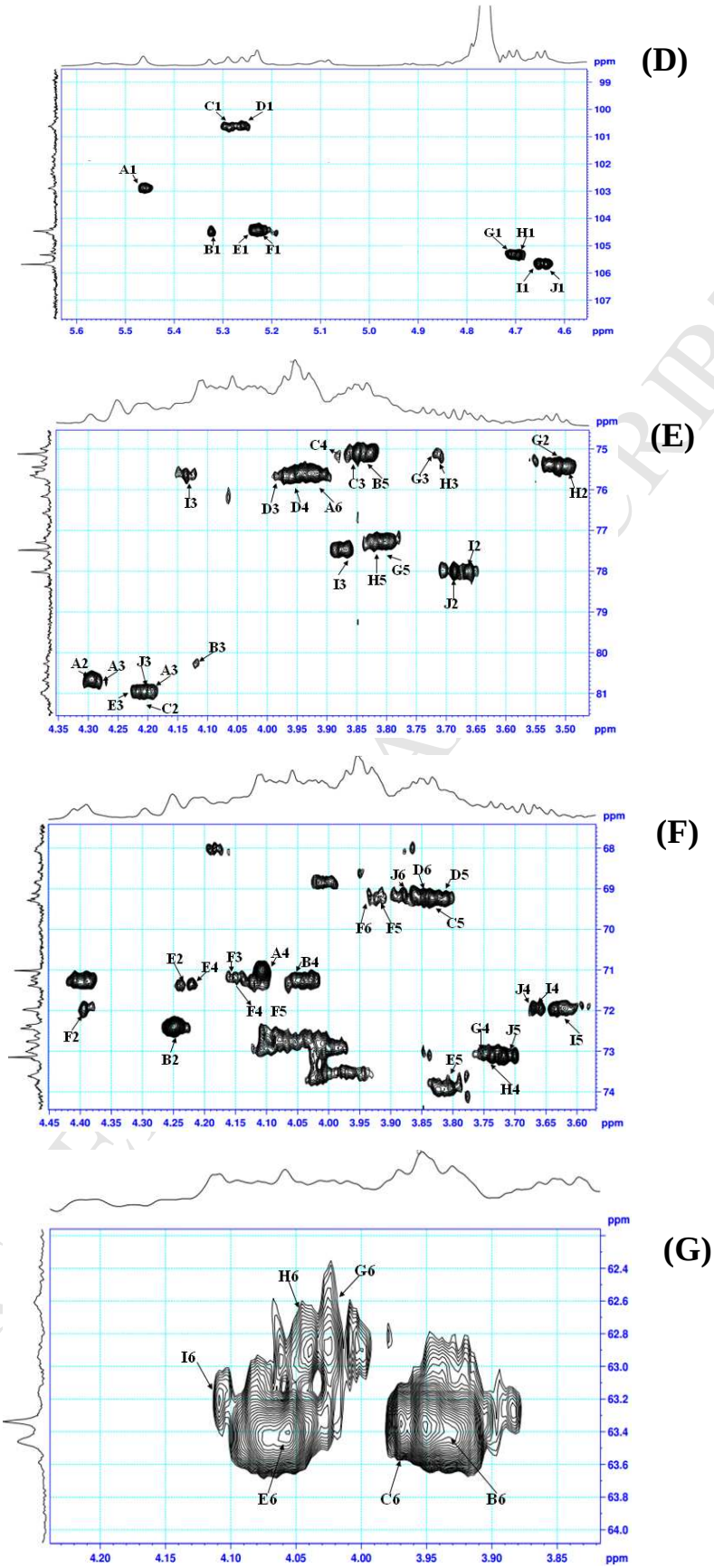
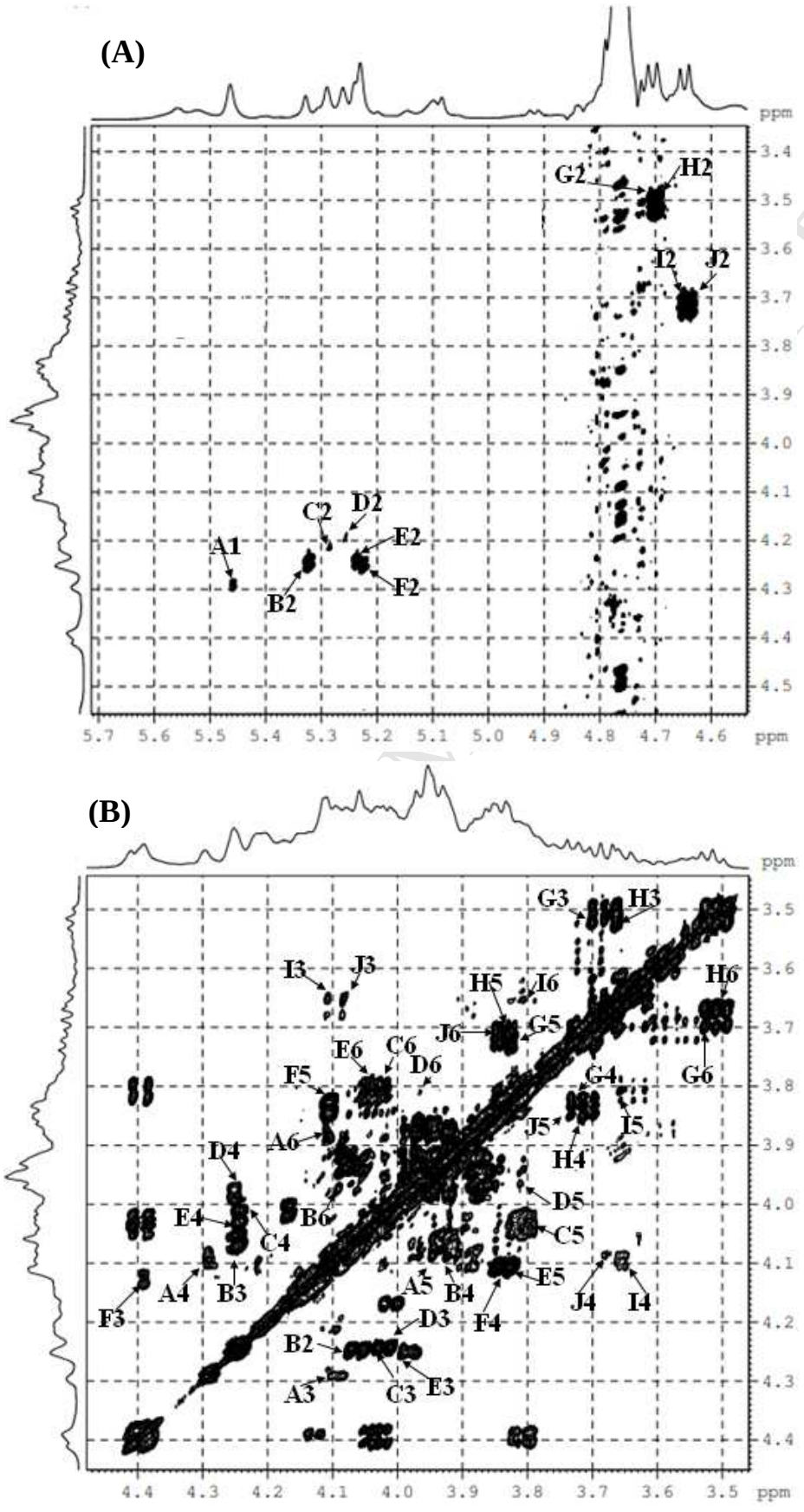

(D)
C1
D1
A1
B1
E1
F1
G1
H1
I1
J1
(E)
C4
C3
B5
G3
H3
G2
H2
I3
D3
D4
A6
I2
H5
G5
J2
B3
A2
A3
J3
A3
E3
C2
(F)
J6
D6
D5
F6
F5
C5
E2
E4
F3
A4
B4
F4
F5
J4
I4
I5
F2
B2
G4
J5
E5
H4
(G)
H6
G6
I6
E6
C6
B6

Fig. 2


(A)
G2
H2
I2
J2
C2
D2
E2
A1
F2
B2
ppm
5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 ppm
3.4 3.5 3.6 3.7 3.8 3.9 4.0 4.1 4.2 4.3 4.4 4.5
(B)
G3
H3
I3
J3
H5
I6
H6
J6
G5
E6
C6
D6
F5
G4
G6
J5
I5
A6
H4
D4
D5
B6
E4
C5
C4
E5
A5
B4
J4
I4
A4
B3
F3
D3
F4
B2
A3
C3
E3
ppm
4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 ppm

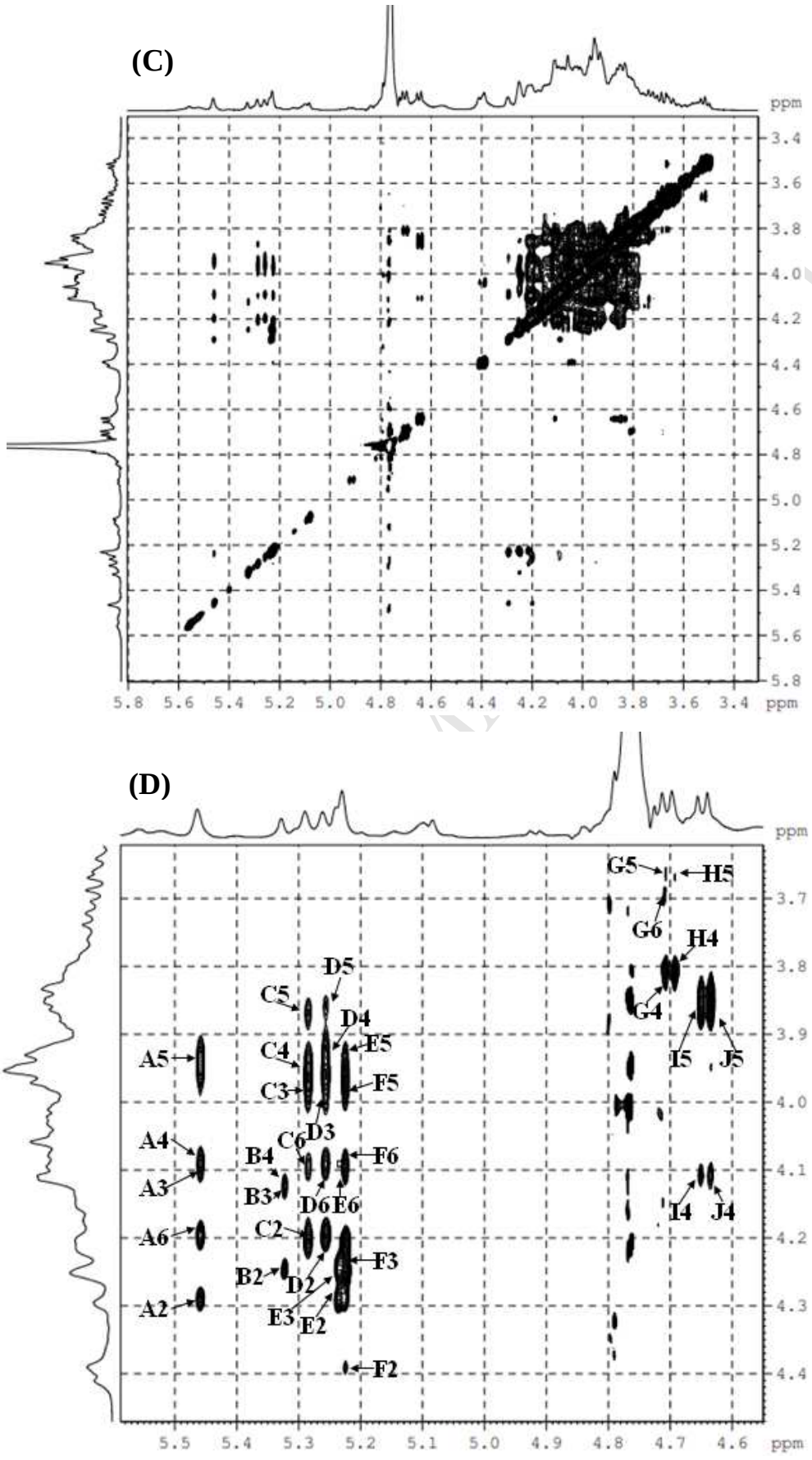

(C)
ppm
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 ppm
(D)
ppm
G5
H5
G6
H4
D5
C5
D4
G4
E5
A5
C4
I5
J5
C3
F5
D3
A4
C6
B4
F6
A3
B3
D6
E6
I4
J4
A6
C2
F3
B2
D2
A2
E3
E2
F2
3.7
3.8
3.9
4.0
4.1
4.2
4.3
4.4
5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 ppm

Fig. 3

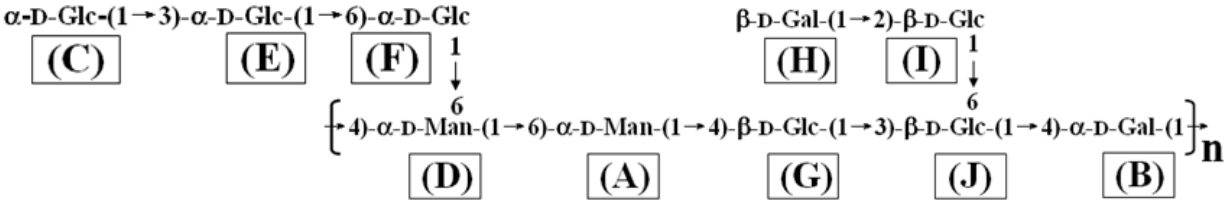


Fig. 4

Highlights:

- LHEPS-1 could significantly inhibit the growth of human colon cancer Caco-2 cells;
- Structural feature of LHEPS-1 was elucidated by methylation, GC-MS and 1D/2D NMR;
- LHEPS-1 was a kind of moderate branching glucomannogalactan;
- A possible structure of LHEPS-1 was proposed;
- Anti-colon cancer activity was correlated with structure of LHEPS-1.