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Enhancement of Heparin and Heparin Disaccharide Absorption by the *Phytolacca americana* Saponins

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We studied the effects of phytolaccosides, saponins from *Phytolacca americana*, on the intestinal absorption of heparin *in vitro* and *in vivo*. The absorption enhancing activity of these compounds (phytolaccosides B, D_2, E, F, G and I) was determined by changes in transepithelial electrical resistance (TEER) and the transport amount of heparin disaccharide, the major repeating unit of heparin, across Caco-2 cell monolayers. With the exception of phytolaccoside G, all of them decreased TEER values and increased the permeability in a dose-dependent and time-dependent manner. *In vitro*, phytolaccosides B, D_2, and E showed significant absorption enhancing activities, while effects by phytolaccoside F and I were mild. *In vivo*, phytolaccoside E increased the activated partial thromboplastin time (APTT) and thrombin time, indicating that phytolaccoside E modulated the transport of heparin in intestinal route. Our results suggest that a series of phytolaccosides from *Phytolacca americana* can be applied as pharmaceutical excipients to improve the permeability of macromolecules and hydrophilic drugs having difficulty in absorption across the intestinal epithelium.

Key words: Phytolaccosides, Heparin, Heparin disaccharide, Caco-2 cells, Absorption enhancer

INTRODUCTION

Most protein/peptide- or carbohydrate-based drugs, including insulin, calcitonin and heparin, do not penetrate intestinal epithelium easily because of their high molecular weight and hydrophilicity. The paracellular pathway is a dominant pathway for the passive transepithelial transport of these hydrophilic compounds in the small intestine, and its permeability depends on the regulation of the intercellular tight junctions (TJ) (Anderson and van Itallie, 1995). The transport of hydrophilic molecules via the paracellular pathway is severely restricted by the presence of the TJ. The barrier function of the TJ is dynamic and appears to be modulated by cellular processes that regulate the movement of hydrophilic molecules across the epithelium. The controlled and reversible opening of the TJ, using absorption enhancers, suggests a way to increase the absorption of hydrophilic drugs across the intestinal epithelium (Ward *et al*., 2000). The use of absorption enhancers to increase the paracellular transport of heparin, one of the least absorbable drugs, has been studied. Earlier approaches include: the use of salts of ethylenediaminetetraacetic acid (EDTA) to obtain the intestinal absorption of heparin and synthetic heparinoids (Winsor and Cronheim, 1961; Tidball and Lipman, 1962); the preparation of heparinic acid complexes with organic acid (Koh and Bharucha, 1972; Sue *et al*., 1976); formulations of heparin complex with hydrophobic compounds (Caramazza *et al.,* 1991; Dal Pozzo *et al*., 1989); the use of modified polysaccharides (Thanou *et al*., 2001); and the application of pharmaceutical preparations such as liposomes (Kim *et al*., 1986; Ueno *et al*., 1982;), emulsions (Engel and Fahrenbach, 1968) and microspheres (Leone-Bay *et al*., 1998). Furthermore, several reports have been published showing that heparin in combination with sodium *N*-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC) affords significant elevations of the in-

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testinal absorption of heparin in rats, primates (Rivera *et al*., 1997) and humans (Baughman *et al*., 1998) and enhances *in vitro* penetration of tissue barriers (Brayden *et al*., 1997).

The naturally occurring saponins, complex molecules composed of sugars conjugated to triterpenes or steroids, have also shown to exert transmucosal permeation enhancing effects. In a previous report, surface active agents, including saponin, increased the permeability of cell membranes (Brayden *et al*., 1997). There are also indications that surface active agents can affect not only the cell membranes but also the TJ. Saponin shows significant promoting effects on corneal and conjuctival *Papp* (apparent permeability coefficient) of thyrotropin-releasing hormone and luteinizing hormone-releasing hormone and its action may be attributable to an increase in the permeability in paracellular pathways (Sasaki *et al*., 2000). *Quillaja* saponin and the chemically modified compound increased the paracellular permeability and induced the dysfunction of the TJ and opening of the paracellular route (Chao *et al*., 1998; Narai *et al*., 1997). It is clear that saponins have utility as intestinal absorption enhancers at low concentrations by affecting the transport mechanisms and permeability in the intestinal epithelium. However, there are few reports on the structural requirements of saponins to have absorption enhancing activity.

In the present report, we focused on the enhancement of paracellular permeability of heparin and heparin disaccharide by plant saponins (phytolaccosides) with different sugars and side chains. Especially, heparin disaccharide was used as a model compound for the comparison of the ability of saponins as enhancers. It contains unsaturated bond, which can be easily detected by HPLC with UV detector. We demonstrated that some of phytolaccosides have the absorption enhancing effects on hydrophilic heparin and further suggest that these results can be extended to the application of these compounds as pharmaceutical additives to increase the transport of hydrophilic compounds such as carbohydrate- or peptide/protein-based drugs.

MATERIALS AND METHODS

Materials

Heparin (porcine mucosa) was provided by New Zealand Pharmaceuticals. Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Dulbecco's modified Eagles medium (DMEM), Dulbeccos phosphate buffered saline (DPBS), non-essential amino acids, trypsin and EDTA, collagen type I, penicillin-streptomycin (10,000 units/mL and 10 mg/mL in 0.9% sodium chloride, respectively), *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES), Hanks balanced salt solution (HBSS), fluorescein isothiocyanate (FITC)-dextran (MW 4000), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT), activated partial thromboplastin time (APTT), and thrombin time (TT) reagents were purchased from Sigma (St Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA) and other reagents and chemicals were of the best grade available. Phytolaccosides were isolated and characterized from the roots of *Phytolacca americana* as previously reported (Kang and Woo, 1987 and 1991; Woo *et al*., 1978). They were initially dissolved in 5% DMSO and diluted in transport media for *in vitro* experiments.

Cell culture

Caco-2 cells between passage 31 and 49 were cultured routinely in DMEM (pH 7.4) supplemented with 1% non-essential amino acid, 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin (complete media) at 37°C in an atmosphere of 95% air, 5% CO_2 and 90% relative humidity. Cells were harvested by treatment with trypsin-EDTA and resuspended in complete media before reaching the confluence. They were finally seeded at a density of about 2.8×10^5 cells/mL on collagen-coated Transwell® polycarbonate filters (surface area = 1 cm^2, pore size = 3.0 μm) from Costar (Cambridge, MA, USA). Media in the apical and basolateral chambers were changed every other day and cells were allowed to reach the confluence for 21 days before the experiments.

Preparation of heparin disaccharide

Heparin was depolymerized with heparinase I prepared from *Bacteroides stercoris* HJ-15 as previously described (Kim *et al*., 2000). The depolymerization mixture was freeze-dried and size-fractionated on a Bio Gel P-10 column (2.5×110 cm) equilibrated with 0.1 M NaCl. The main fraction of the disaccharide was desalted by using a Bio-Gel P-2 chromatography and freeze-dried. Its homogeneity was examined by HPLC and the structure was characterized by ^{1}H-NMR (Merchant *et al*., 1985).

HPLC analysis of heparin disaccharide *in vitro*

HPLC was performed to analyze the amount of heparin disaccharides (MW 665.4) transported through the cell monolyers. The system was equipped with a 5 μm particle size strong-anion exchange (SAX) analytical column from Phenomenex (Torrells, CA, USA) of dimension 0.46×25 cm using ÄKTA purifier controlled by UNICORN software 3.1 from Amersham Pharmacia (Uppsala, Sweden). In brief, samples were applied to the column equilibrated with water (pH 3.5). The column was washed with the same mobile phase for 8 min corresponding to two column volumes. Then, the linear gradient of 0-2.0 M NaCl (pH 3.5) was performed at a flow rate of 1.0 mL/min. The elution profile was monitored by absorbance at 232 nm. The cumulative amount of transported heparin disaccharide

was calculated according to the standard curve.

Measurement of transepithelial electrical resistance (TEER)

Cell monolayers were treated on the apical side with absorption enhancers at various concentrations dissolved in HBSS containing 11 mM D-glucose and 25 mM HEPES, pH 7.4 (transport media) for 20 min and washed with the same media. The effects of phytolaccosides on Caco-2 cell monolayers were measured with a Millicell® electrical resistance system (Millipore Corp., Bedford, MA) before and after the application of phytolaccosides. The resistance due to cell monolayers was determined and the results were presented as the percentage of the initial (t = 0) value in the same monolayers. A change of TEER was examined for 3 h after washing phytolaccosides with transport media. Control values were measured in the range of 500-600 $\Omega \cdot cm^2$.

Assessment of paracellular permeability of heparin disaccharide

To evaluate the effect of phytolaccosides on the permeability of heparin disaccharide, transport studies were performed on Caco-2 cell monolayers with or without these saponins at 37°C. Complete media in the apical sides were replaced with fresh transport media and preincubated for 1 h at 37°C. Phytolaccosides in transport media (0.5 mL) were applied to the monolayers and incubated for 20 min. After removing phytolaccosides, heparin disaccharide (1 mM in transport media) was added to the monolayers. The transport media was taken from the basolateral side of the transwell hourly for 3 h. At each sampling, the inserts were rapidly transferred to another well containing the fresh transport media (1.5 mL). The amount of heparin disaccharide in the collected media was determined by SAX-HPLC as described above. In addition, the flux of FITC-dextran (1 mg/0.5 mL transport media) was assessed across the Caco-2 cell monolayers for testing the integrity of cell monolayers. Transport amount of FITC-dextran was determined with microfluorometer (Spectra Max, Gemini XS) from Molecular Device (Sunnyvale, CA, USA) at 495 nm for excitation and 525 nm for emission. All measurements were determined in triplicate and expressed as means±SD.

Evaluation of cytotoxicity of enhancers

Cytotoxicity of phytolaccosides (0.02, 0.05, and 0.1%) was evaluated by measuring mitochondrial dehydrogenase (MDH) activity based on MTT assay (Liu *et al*., 1999) and lactate dehydrogenase (LDH) activity (Choksakulnimitr *et al*., 1995). The percentage of released LDH was calculated by % $LDH_{release}=LDH_{medium}/(LDH_{medium}+LDH_{detached\ cell}+LDH_{cell}) \times 100$. Triton X-100 was used as a cytotoxicity control.

Heparin administration and plasma preparation

ICR male mice, aged 5 weeks, were used and had been fasted during 12 h before sacrificed. Each heparin sample (100 mg/kg) containing 10, 40, and 80 mg/kg of phytolaccoside E was orally administered to mice for 4 days. After 30 min the last administration, mice were anesthetized with ether and blood samples (900 μL) were collected by cardiac puncture and mixed with 3.8% sodium citrate (100 μL) using the syringe. Plasma was prepared by centrifugation at 2000 × *g* for 15 min at 4°C. All animal studies were carried out in accordance with the procedure outlined in the Guide for the Care and Use of Laboratory Animals of Seoul National University.

Measurement of clotting time and anti-Factor IIa activities in plasma

The anticoagulant activity was typically monitored by measuring APTT on a BBL®FibroSystem® and anti-factor IIa activity was determined by measuring thrombin clotting time (Wu *et al*., 1998).

Histological examination

The effects of phytolaccoside E on morphology and viability in the intestinal and gastric tissues were examined and compared with those of the untreated control group by histological examinations. After the oral administration of phytolaccoside E (80 mg/kg) for 4 days, gastric and intestinal tissues were excised and fixed with 10% formalin and they were treated for histological processing as previously described (Kusakabe *et al*., 1984). Isolated gastric and intestinal tissues were examined by light microscopy after staining paraffin-embedded sample with hematoxylin and eosin.

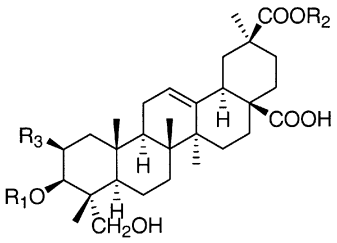
Statistical analysis

Results were expressed as mean±SD. For the statistical analysis of the data, we performed a one-way analysis of variance (ANOVA) for repeated measurements of the same variable. We then used Duncans multiple range *t*-test to determine which means were significantly different from the mean of the control. We considered difference significant at $p<0.01$ and $p<0.05$.

RESULTS

Measurement of transepithelial electrical resistance

Because TEER across Caco-2 cell monolayers is an indicator of TJ integrity, the relative effects of phytolaccosides on TEER values reflect their relative abilities to modulate TJ. The effects of six different phytolaccosides (Fig. 1) on TEER are shown as a function of time (Fig. 2). Phytolaccoside G had little effect on TEER at 0.1%, while phy-



Phytolaccosides	R_1	R_2	R_3
B	xyl	CH_3	OH
D_2	xyl(1→2)glc	CH_3	H
E	glc(1→4)xyl	CH_3	OH
F	rha(1→2)glc(1→2)xyl	CH_3	H
G	xyl	H	OH
I	rha(1→2)glc(1→2)xyl	H	H

Fig. 1. Structure of phytolaccosides. xyl, β-D-xylopyranosyl; glc, β-D-glucopyranosyl; rha, α-L-rhamnopyranosyl

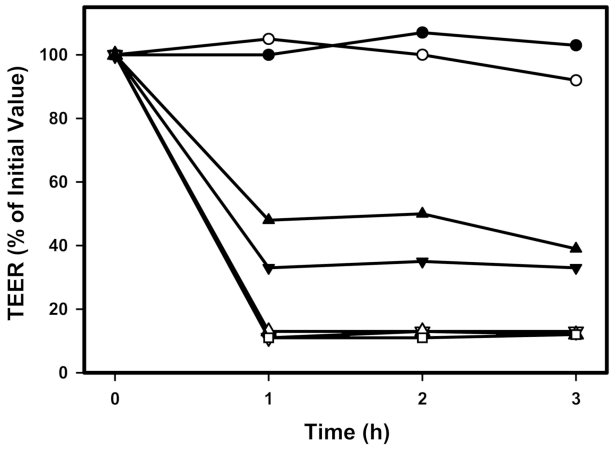


Fig. 2. Effects of phytolaccosides on TEER across Caco-2 cell monolayers. Phytolaccosides were applied to the apical side of the monolayers at 0.1% concentrations for 20 min. TEER values were measured according to Materials and Methods. The symbols correspond to the control (●), phytolaccosides B (▽), D_2 (△), E (□), F (▼), G (○), and I (▲), respectively.

tolaccosides F and I decreased TEER values by 50~70% at the same concentration. In contrast, phytolaccosides B, D_2 and E markedly decreased TEER values, indicating that they had very strong effects on modulating TJ. In the presence of lower concentrations of phytolaccosides B and D_2 (0.02%), the TEER values are 40 and 49% of the control, respectively (data not shown).

Assessment of paracellular permeability of heparin disaccharide by phytolaccosides

At 0.1% concentration, phytolaccosides B, D_2 and E almost completely transported heparin disaccharide

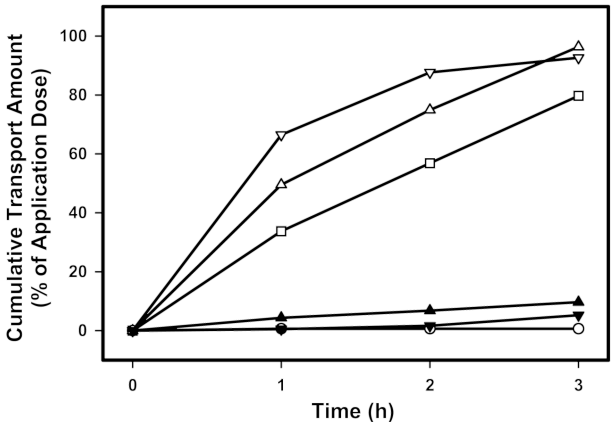


Fig. 3. Measurement of transport amount of heparin disaccharide. Phytolaccosides were applied to the apical side of the monolayers at 0.1% concentrations and the amount transported across Caco-2 cell monolayers was analyzed by SAX-HPLC as described. The symbols correspond to phytolaccosides B (▽), D_2 (△), E (□), F (▼), G (○), and I (▲), respectively.

loaded in the apical side to the basolateral side after 3 h. Phytolaccosides F and I increased the transport amount by 9.7 and 5.2%, respectively. But phytolaccoside G behaved similar to the control (0.32%/3 h) and did not influence the paracellular permeability of heparin disaccharide (Fig. 3). As a control experiment, the flux of FITC-dextran (MW 4,000) across the monolayers was less than 0.05%/h. This result indicates that the cell monolayers kept a high integrity.

Cytotoxicity

Fig. 4 shows cytotoxic effects of phytolaccosides on Caco-2 cells. MDH activities were above 80% at 0.02% for all of the phytolaccosides and essentially no difference was observed for these compounds. However, when cells were treated with phytolaccosides B and E at 0.05 and 0.1%, MDH activities were drastically decreased and LDH release was increased in a dose-dependent manner. Phytolaccosides F and I did not show severe cytotoxicity in these assays. Remarkably, phytolaccoside D_2 changed neither LDH release nor MDH activity, compared with other phytolaccosides.

Effect of phytolaccoside E on intestinal absorption of heparin *in vivo*

Fig. 5 shows that the application of phytolaccoside E at two doses of 40 and 80 mg/kg with heparin (100 mg/kg) increased the clotting time, while the APTT value did not change at 10 mg/kg of phytolaccoside E (Fig. 5A). The increase in clotting time, observed in the presence of 40 and 80 mg/kg of phytolaccoside E demonstrated that heparin could be absorbed. Thrombin time was also increased at both 40 and 80 mg/kg of phytolaccoside E (Fig. 5B).

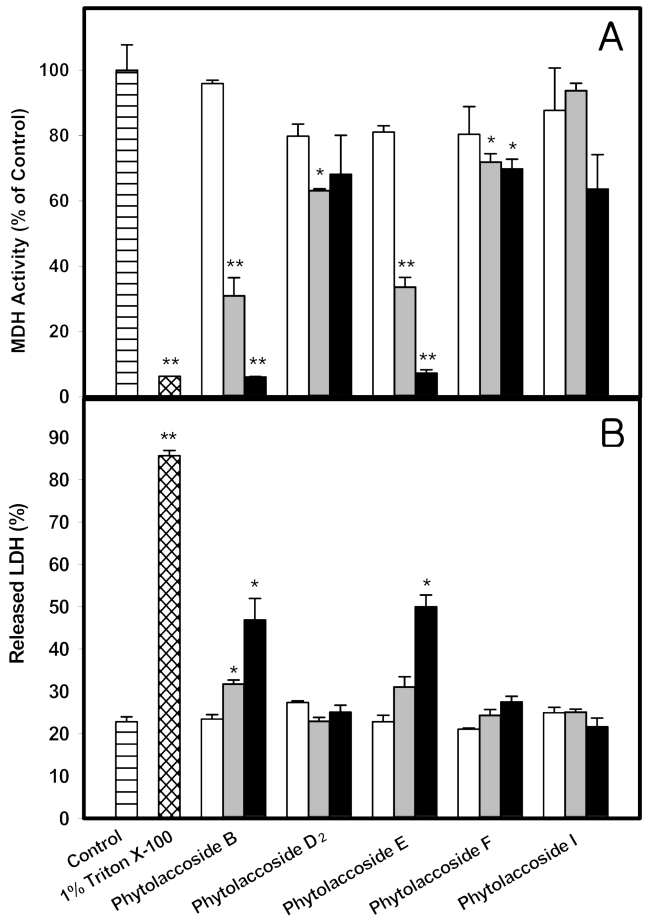


Fig. 4. Cytotoxicity of phytolaccosides on Caco-2 cell monolayers. Effects of phytolaccosides on MDH activity (A) and released LDH (B) were determined. Caco-2 cell monolayers were treated with phytolaccosides at various concentrations. ▤: control, ▨: 1% Triton X-100, □: 0.02% phytolaccosides, ▣: 0.05% phytolaccosides, ■: 0.10% phytolaccosides. Data represent the means±S.D. **p*<0.05, ***p*<0.01

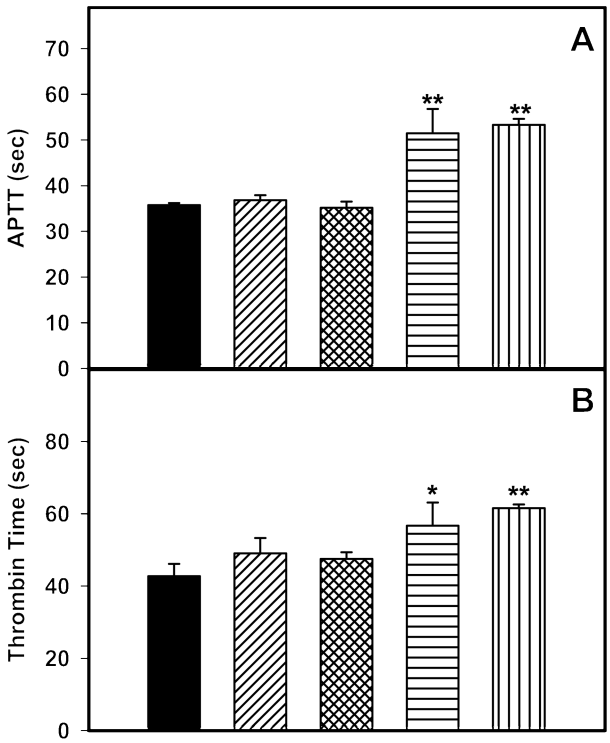


Fig. 5. Effects of phytolaccoside E on heparin absorption *in vivo*. After treatment with phytolaccoside E (40 and 80 mg/kg) for 4 days as described in MATERIALS and METHODS, APTT (A) and thrombin time (B) in plasma were measured using BBL®Fibrosystem®. ■ : control, ▨: heparin (100 mg/kg), ▨: PE (10 mg/kg) + heparin (100 mg/kg), ▤: PE (40 mg/kg) + heparin (100 mg/kg), ▥: PE (80 mg/kg) + heparin (100 mg/kg). Data represent the means±S.D. **p* <0.05, ***p*<0.01.

Histological examination

The cytotoxic effect of phytolaccoside E on isolated gastric and intestinal tissues was studied by histological observation and the result is shown in Fig. 6. The morphology of gastric and intestinal tissues was not noticeably affected by the oral administration of heparin with phytolaccoside E showing a cytotoxic effect *in vitro*.

DISCUSSION

Many studies have attempted to increase the paracellular permeability by applying a variety of absorption enhancers. In this study we demonstrated that *Phytolacca* saponins have absorption enhancing activities *via* modulation of the TJ and increase the paracellular permeability of heparin. As shown in Figs. 2 and 3, there were differences in effects of phytolaccosides on TEER values and paracellular permeability. Phytolaccoside G had no absorption enhancing activity, and phytolaccoside F and I showed a small modulating effect on TJ. In contrast, phytolaccosides B, D_2 and E dramatically decreased TEER values and increased the paracellular permeability. In considering the structural features, phytolaccosides B, D_2 and E have methyl groups on R_2 position (Fig. 1), which are associated with their hydrophobicity. Phytolaccoside G showing the smallest enhancement of permeability does not have methyl group and contains hydroxyl group on R_3. Phytolaccosides F and I showed a little enhancing activities and their activities were placed in the middle of the two groups of enhancers. Of these, phytolaccoside E, which could be obtained in significant amounts, was used for *in vivo* absorption studies. Although it showed cytotoxicity *in vitro*, no specific damages in tissues could be found (Fig. 6).

Saponins are naturally occurring amphiphilic compounds composed of hydrophilic sugars conjugated to hydrophobic triterpenes or steroids. In this aspect, some differences in paracellular permeability of phytolaccosides can be due to the balance of hydrophobic/hydrophilic properties in their structures. Although a detailed mechanism for the TEER decrease caused by saponins is still obscure, it is

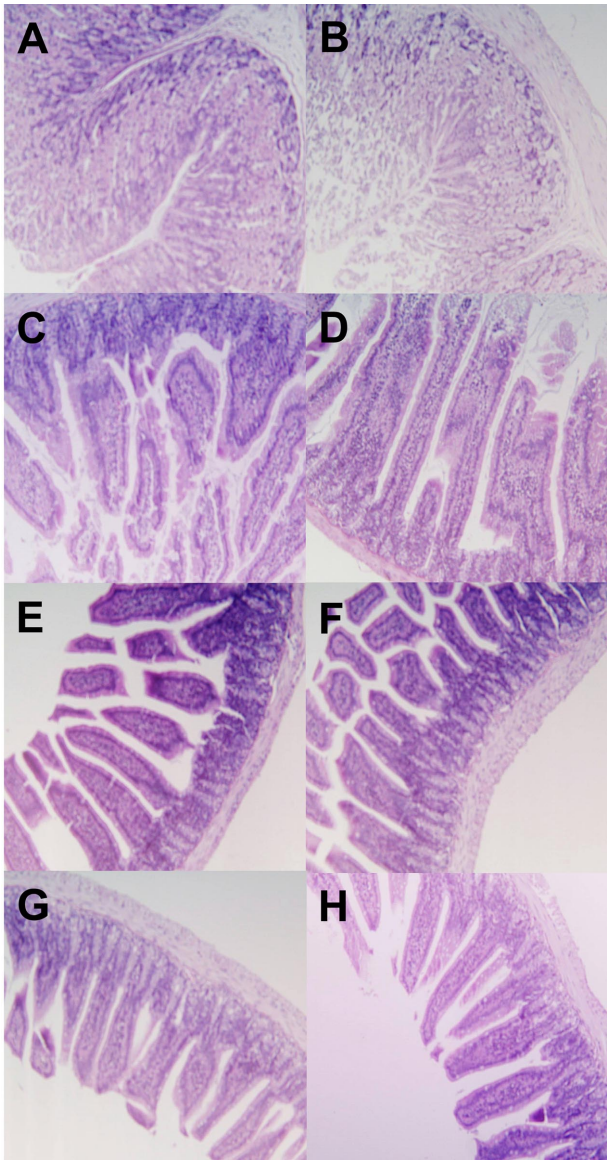


Fig. 6. Photographs of gastric and intestinal tissue sections of mice. After treatment with phytolaccoside E (80 mg/kg) for 4 days, tissues were excised and treated for histological processings. Light micrographs were taken at high magnifications (×200). A: stomach (saline), B: stomach (80 mg/kg of phytolaccoside E); C: duodenum (saline), D: duodenum (80 mg/kg of phytolaccoside E); E: jejunum (saline), F: jejunum (80 mg/kg of phytolaccoside E); G: ileum (saline), H: ileum (80 mg/kg of phytolaccoside E).

presumed that subtle changes in the cell membrane caused by the cytoskeletal changes, result in the TEER decrease. According to the previous reports, saponins from various sources have a dose-dependent effect on intracellular morphology of epithelial cells (Onning *et al*., 1996; Sung *et al*., 1995). Although the separation between cytotoxicity and efficacy of absorption enhancers including saponins is difficult, the ability of many enhancers to increase paracellular permeability is not a direct result of their toxic actions (Fig. 4) and the oral administration of phytolaccoside E exhibits no significant cytotoxicity in the small intestine and stomach (Fig. 6).

In conclusion, a series of plant saponins, phytolaccosides from *Phytolacca americana* have the absorption enhancing activity and increase the intestinal absorption of heparin by modulation of TJs *in vivo* as well as *in vitro*. Although the mechanism of action is still unclear, phytolaccosides may have potential utility as additive agents to regulate TJ structure and function to promote the intestinal absorption of hydrophilic compounds and macromolecules.

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