Regular Article

Protective Effects of Genipin on Gastrointestinal Disorders

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Genipin, an aglycone of geniposide, is a major component of *gardeniae fructus*, and has been used to treat jaundice, various inflammatory disorders, and liver disease, and has also been used as a natural cross-linking agent. The authors conducted several experiments to evaluate the protective effects of genipin on gastrointestinal disorders, such as, gastritis and gastric ulcers. Genipin showed inhibitory effects against HCl·ethanol-induced acute gastritis and indomethacin-induced gastric ulcers in rats and increased prostaglandin E_2 (PGE_2) in AGS gastric cancer cell. Genipin had significant effects on aggressive factors, acid-neutralization, and gastric secretion, and inhibited H^+/K^+-ATPase (a proton pump), which secretes gastric acid. The results obtained indicate that genipin has significant gastroprotective effects and might be useful for treating and preventing gastric lesions.

Key words genipin; gastrointestinal disorder; gastric secretion; prostaglandin E_2; proton pump

Numbers of patients with gastrointestinal disorders continue to increase. Gastritis is a condition involving inflammation of the gastric mucous membrane[1] and is classified histologically as atrophic and non-atrophic gastritis. Gastritis is classified as acute or chronic gastritis. Aggressive factors, such as, gastric acid and pepsin damage gastrointestinal tract mucosa, and *Helicobacter pylori* (*H. pylori*) inflammation and non-steroidal anti-inflammatory drugs (NSAIDs) are also relevant factors.[2] Currently, antacids, H_2 antagonists, proton pump inhibitors (PPIs), *H. pylori* antimicrobial agents, prokinetics, antidepressants, and antianxiety drugs are widely used to treat gastrointestinal disorders[3] and though these agents are effective, they have many side effects associated with their chemical syntheses. Genipin (Fig. 1) is an aglycone of geniposide and has been used to treat jaundice, various inflammatory diseases, and liver disease,[4] and has antibacterial, anticancer, anti-inflammatory, anti-oxidative, and neuroprotective properties.[5–7] Therefore, in the present study, we evaluated the protective effects of genipin on gastrointestinal disorders, such as, gastritis and gastric ulcers with the objective of developing medicines based on natural compounds with few side effects or interactions with other drugs.

MATERIALS AND METHODS

Genipin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI medium 1640, penicillin-streptomycin, fetal bovine serum (FBS), phosphate buffered saline (PBS) 10×, and trypsin–ethylenediaminetetraacetic acid (EDTA) were from Gibco Inc. (MA, U.S.A.). ATP, indomethacin, cimetidine, phenol red, and Tris–HCl were from Sigma-Aldrich Inc. (MO, U.S.A.). Rebamipide was purchased from DAEHE Chemical. Activated charcoal, carboxymethyl cellulose (CMC), HCl, sodium bicarbonate, sodium hydroxide, methanol, and methyl orange were obtained from Duksan Pure Chemical Co., Ltd. (Kyunggi-do, Korea). All other reagents and solvents used were of analytical grade and obtained commercially. In the present study, the following equipment was used; an inverted microscope (Olympus Co., Tokyo, Japan), a CO_2 incubator (Forma Scientific), micropipettes (Gilson Inc., WI, U.S.A.), a 5810R centrifuge (Eppendorf Co., Hamburg, Germany), a microcentrifuge (Vision Co., Ltd., Daejeon, Korea), a high speed centrifuge (Sorvall RT-6000, MA, U.S.A.), and a UV spectrophotometer (ASYS UVM340, Cambridge, U.K.).

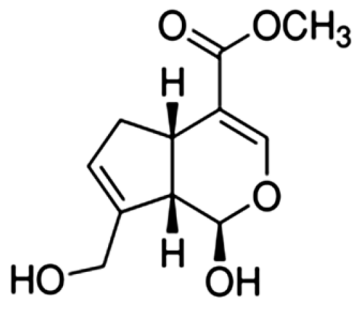

Fig. 1. Structure of Genipin

Animals Male Sprague–Dawley rats weighing 170 to 190 g were purchased from Samtako (Kyunggido, Korea) and were acclimatized to standard laboratory conditions (22±2°C/55±5% relative humidity (RH)/12 h light/dark cycle) for 14 d at the animal facility at Duksung Women's University. Animals were allowed free access to food (standard pellets) and water. All animal experiments were conducted in accordance with recommendations in the National Research Council's Guide (Institutional Animal Care and Use Committee; IACUC) for the Care and Use of Laboratory Animals (Ministry of Food and Drug Safety). The experimental protocol was approved by the Animal Experiments Committee of Duksung Women's University (permit number: 2014-03-004).

Gastric Cancer Cell Lines AGS gastric cancer cells and SNU638 gastric cancer cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured in RPMI-1640 containing 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) in a 5% CO_2 humidified incubator at 37°C. For subculture, AGS and SNU638 cells were rinsed twice with PBS (pH 7.4) to remove all traces of serum (which can inhibit trypsin) and then subdivided using

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1% trypsin–EDTA.

HCl·Ethanol-Induced Acute Gastritis After a 24-h fast but with free access to water prior to the experiment, male rats weighing approximately 180 g were administered a solution of genipin (1 mL/100 g oral administration (*p.o.*)). After 30 min, 1 mL of HCl·EtOH solution (150 mM HCl in 60% EtOH; *p.o.*) was administered. Animals were then allowed to recover for an hour without food and water, before being sacrificed by ether inhalation. Stomachs were excised and inflated by injecting 2 mL of normal saline, and then fixed for 10 min in a 2% formalin solution. Amounts of hemorrhage in the glandular portion were measured by summing the total lengths (mm) of lesions under a dissecting microscope (×10), and expressed as lesion indices.[8]

Indomethacin-Induced Gastric Ulcers Using the method reported by Kasuya *et al.*,[9] rats weighing approximately 200 g were fasted for 24 h but had free access to water prior to the experiment. Genipin was dosed orally and 30 min later, indomethacin (35 mg/kg in 50 mM sodium bicarbonate solution) was injected subcutaneously. The control group received the same amount of 50 mM sodium bicarbonate solution. Animals were sacrificed 7 h after the indomethacin injection. Excised stomachs were placed in a 2% formalin solution for 10 min, incised along the greater curvature, and the glandular portion was then examined for hemorrhage. Lesion area (mm^2) were measured under a dissecting microscope (×10), and the total values were expressed as lesion indices. Cimetidine was used as the positive control.

Acid-Neutralizing Capacity Acid-neutralizing capacity was determined using a method in the Korean pharmacopoeia with slight modification. Genipin (100 μg) was added to 100 μL of 0.1 N HCl and then incubated for one and a half hour at 37°C while shaking. Acid-neutralizing capacity was determined by titrating the remaining acid with 0.1 N NaOH using methyl orange as an indicator. Hydrotalcite and cimetidine were used as positive controls.

Gastric Secretion in Pylorus-Ligated Rats To quantify gastric secretion, we measured pH values and determined total acid output, which are both factors of gastric damage. The rats used weighing approximately 180 to 200 g and were fasted for 24 h, but had free access to water, prior to the experiment. Before pyloric ligation, samples were administered intraduodenally. Four hours after pyloric ligation, rats were sacrificed and stomach contents were collected and centrifuged at 1000×***g*** for 10 min. The total volumes of gastric juice, pH values, and total acid outputs were measured. Acidity was determined by titration *versus* 0.1 N NaOH using phenol red as indicator, and pH was measured using pH meter.[10]

Proton Pump (H^+/K^+-ATPase) Inhibition H^+/K^+-ATPase, the enzyme secreted during the final phase of gastric secretion, was measured as previously described by Naik *et al.*[11] Swine gastric membrane protein determined by Bradford's law was tested with 300 μg protein and pre-incubated at 37°C for 1 h with 0.300 mL of a reaction mixture, containing 20 mM $MgCl_2$, 20 mM ATP, 20 mM KCl, and 20 mM Tris–HCl (pH 7.4) and incubated at 37°C for 30 min. Another 0.3 mL mixture containing 4.5% ammonium molybdate and 30% trichloroacetic acid 0.3 mL was added and centrifuged at 1500×***g*** for 10 min. Supernatants were collected and absorbances were measured at 400 nm.

Prostaglandin E_2 Measurement Prostaglandin E_2 (PGE_2), a protective factor against gastric lesions, concentrations were measured using a commercially available PGE_2 assay kit. Indomethacin was used as a negative control and rebamipide as a positive control.[12] AGS cells (1×10^4 per well) were plated in 96-well culture plates, incubated for an hour, treated with 50 μL of primary antibody solution, and shaken for 1 h. Conjugate (50 μL) was then added, and cells were shaken for two hours, washed four times with wash buffer, treated with 200 μL of substrate solution, and placed in a darkroom for 30 min. Finally, 100 μL of stop solution was added, and absorbance was read within 30 min at 450 nm. To measure the PGE_2 level, we created a standard curve by Bradford assay. Concentration of PGE_2 was calculated corresponding to the mean absorbance from the standard curve (PGE_2 assay kits were from R&D systems, MI, U.S.A.).

Statistics The results were expressed as the mean±standard deviation (S.D.) and were analyzed using the Student's *t*-test. Statistical significance was accepted for p values <0.05.

RESULTS

HCl·Ethanol-Induced Acute Gastritis Using the HCl–ethanol model of gastritis (Table 1), the index of gastric damage was 99.5±19.9 mm in the control group, 32.8±3.6 mm (inhibition, 67.0%) in the 50 mg/kg genipin group, and 8.3±4.8 mm (inhibition, 91.7%) in the 100 mg/kg genipin group. The hydrotalcite (100 mg/kg) positive control had a gastric damage index of 65.0±12.7 mm (inhibition, 34.7%), while cimetidine had a gastric damage index of 52.2±8.3 mm (inhibition, 47.5%). Thus, genipin significantly inhibited HCl–ethanol induced acute gastritis.

Indomethacin-Induced Gastric Ulcers Gastritis damage was observed after indomethacin induction in our animal model (Table 2). Ulcers of mean area 36.0±9.4 mm^2 were observed in the control group, while areas of 24.4±8.0 and 12.0±5.4 mm^2 were observed in groups treated with 50 or

Table 1. Effect of Genipin on HCl·Ethanol-Induced Gastritis in Male Rats

Treatment (n=6)	Dose (mg/kg)	Lesion index (mm)	Inhibition (%)
Control	—	99.5±19.9	—
Genipin	50	32.8±3.6**	67.0
	100	8.3±4.8***	91.7
Hydrotalcite	100	65.0±12.7*	34.7
Cimetidine	150	52.2±8.3**	47.5

Values represent the mean±S.D.s. Significantly different, *$p<0.05$, **$p<0.01$, ***$p<0.001$ *versus* the control.

Table 2. Effect of Genipin on Indomethacin-Induced Gastric Ulcer in Male Rats

Treatment (n=6)	Dose (mg/kg)	Lesion index (mm^2)	Inhibition (%)
Control	—	36.0±9.4	—
Genipin	50	24.4±8.0	32.2
	100	12.0±5.4**	66.7
Cimetidine	200	17.0±5.7*	52.8

Values represent the mean±S.D.s. Significantly different, *$p<0.05$, **$p<0.001$ *versus* the control.

Table 3. Acid-Neutralizing Capacity of Genipin

Material	Dose (μg/μL)	NaOH consumption volume (μL)	Inhibition (%)
Control	—	113.0±2.6	—
Genipin	1	90.7±0.6*	19.8
Hydrotalcite	1	63.0±1.0*	44.2

Values represent the mean±S.D.s. Significantly different, *$p<0.001$ *versus* the control.

Table 4. Effect of Genipin on Gastric Secretion in Pylorus-Ligated Male Rats

Treatment (n=6)	Dose (mg/kg)	Volume (mL)	pH	Total acid output (mEq/4 h)
Control	—	7.1±2.1	1.2±0.2	1.1±0.4
Genipin	50	5.6±2.0	1.2±1.1	0.8±0.2
	100	3.6±1.8*	1.7±0.4	0.3±0.2**
Cimetidine	150	3.2±1.7***	2.6±1.3*	0.3±0.1***

Values represent the mean±S.D.s. Significantly different, *$p<0.05$, **$p<0.01$, ***$p<0.001$ *versus* the control.

Table 5. H^+/K^+-ATPase Inhibiting Activity of Genipin

Material	Dose (μM)	Inhibition (%)
Control	0	—
Genipin	50	36.3±10.1*
	100	35.4±9.5*
Pantoprazole	50	33.7±5.4*
	100	36.2±7.3*

Values represent the mean±S.D.s. Significantly different, *$p<0.05$ *versus* the control.

Table 6. PGE_2 Synthesis of Genipin

Material	Dose (μM)	PGE_2 (pg/mL)
Control	0	166.0±12.1
Genipin	50	213.4±18.9**
	100	354.4±25.8**
Indomethacin	50	29.6±10.7
	100	14.3±4.5
Rebamipide	50	185.1±21.6*
	100	447.1±38.7***

Values represent the mean±S.D.s. Significantly different, *$p<0.05$, **$p<0.01$, ***$p<0.001$ *versus* the control.

100 mg/kg genipin, respectively. The control drug, 200 mg/kg cimetidine, produced ulcers of area 17.0±5.7 mm^2 (inhibition, 52.8%), and thus, genipin at 50 or 100 mg/kg inhibition inhibited ulcers by 32.2 and 66.7%, respectively, compared the control.

Acid-Neutralizing Capacity When titrated with 0.1 NaOH, genipin inhibited 19.8% as compared to control. Acid-neutralizing capacity of genipin is expected to exert the protective influence against the excessive secretion of gastric acid (Table 3).

Gastric Secretion in Pylorus-Ligated Rats Gastric-juice parameters such as gastric volume and pH were measured after submitting the rats to pylorus ligature with or without the genipin intraduodenally (Table 4). In pylorus-ligated rats, gastric acid volume in the control group was 7.1±2.1 mL, the pH was 1.2±0.2, and total acid output (TAO) was 1.1±0.4 mEq/in 4 h. In rats treated with 50 mg/kg genipin, the gastric acid volume 5.6±2.0 mL, pH was 1.2±1.1, and TAO was 0.8±0.2 mEq/4 h. In rats treated with 100 mg/kg genipin, gastric acid volume was 3.6±1.8 mL, pH was 1.7±0.4, and TAO was 0.3±0.2 mEq/4 h. In the cimetidine group (the positive control), volume, pH, and TAO were 3.2±1.7 mL, 2.6±1.3, and 0.3±0.1 mEq/4 h, respectively. Thus, genipin was found to inhibit aggressive factors but less effectively than cimetidine.

Proton Pump (H^+/K^+-ATPase) Inhibition Orally induced PPIs are absorbed in the intestine, reach cell walls in blood flow through the mucosa of gastric glands, combine with the cystein residue of H^+/K^+-ATPase, which is activated by gastric acid activity, and inhibit acid secretion by controlling the enzyme (Table 5). Pantoprazole (the positive control) at 50 or 100 μM inhibited H^+/K^+-ATPase by 33.7 and 36.2%, respectively, while 50 and 100 mg/kg genipin inhibited it 36.3 and 35.4%, respectively.

Prostaglandin E_2 Measurements Quantitative analysis of PGE_2 was performed in genipin-treated AGS cells using PGE_2 assay kit (Table 6). Treatment with 50 or 100 μM of rebamipide led to the productions of 185.1 and 447.1 pg/mL of PGE_2 in AGS cells, respectively, showing dose-dependent protection of gastric mucosa. Indomethacin at 50 or 100 μM inhibited the synthesis of arachidonic acid to PGE_2, the concentration of which decreased to 29.6 and 14.3 pg/mL, respectively. For genipin at 50 or 100 μM, PGE_2 were 213.4 and 354.4 pg/mL, respectively. These results showed genipin at 50 μM was more effective than rebamipide.

DISCUSSION

HCl–ethanol accelerates necrosis and apoptosis of gastric mucosa by damaging its defense system, and is regarded as a standard means of assessing the pathogenesis of acute gastritis. In the present study, genipin at dose of 50 and 100 mg/kg inhibited gastric damage 67.0 and 91.7%, respectively, *versus* control group. Indomethacin, a nonsteroidal anti-inflammatory drug, reduces mucosal blood flow, and as a result, gastric lesions occur in the slime layer due to HCO_3^- reduction and inhibition of PG secretion within the gastrointestinal tract. Previously, a 2 mL/kg subcutaneous injection of a mixture of 30 mg/kg indomethacin and 50 mM sodium bicarbonate solution was observed to produce clear gastric ulcer lesions. In indomethacin-induced gastric ulcer, genipin was more effective than cimetidine, the positive control, which had protective effects. Gastric acid may act as an aggressive factor on damaged gastric mucous membranes. Thus, we evaluated the effects of genipin on acid neutralization and gastric secretion. Gastric mucous membranes tend to be damaged when balance between aggressive and defensive factors is lost, which can lead to gastric ulcers and damage under to muscle layers. Acid-neutralizing drugs serve to control acidity within the stomach and relieve pain rapidly in cases of gastritis and gastric ulcer. Genipin significantly increased acid-neutralizing effects. The result of gastric secretion test suggests that genipin shows the reduction effects of gastric acid volume and TAO. Pylorus-ligation stimulates gastric secretion. And excessive

gastric secretion is acting as an aggressive factor. Therefore decrease of gastric secretion reflects gastroprotective effect. In an inhibition experiment of proton pump (H^+/K^+-ATPase), the gastric acid secreting enzyme involved at the final phase of gastric acid secretion.[13–15] When genipin inhibited the proton pump (H^+/K^+-ATPase), it appeared to inhibit gastric acid secretion. Since the discovery of omeprazole in 1989, numerous drugs, such as, pantoprazole, rabeprazole, esomeprazole, lansoprazole, tenatoprazole, and ilaprazole, have been developed and are now used worldwide to treat peptic ulcers, reflux esophagitis, and other diseases.[16] Genipin protects the stomach by inhibiting H^+/K^+-ATPase, the enzyme involved in gastric acid secretion, whereas PGE_2 preserves[17] and protects gastric mucosa by reducing aggressive factors and increasing the production and secretion of mucus[18] and by increasing the viscosity of mucus and inhibiting the movements of gastric acid and pepsin through the slime layer. Genipin protects the stomach by increasing the production of PGE_2. Antacids, H_2 antagonists, PPIs are widely used in the treatments for gastric disease. These medications are proven the efficacy but side effects have also been consistently reported due to the fact that they are synthesized pharmaceuticals. The side effects of antacids include constipation, diarrhea, reduced concentration of serum phosphate and metabolic alkalosis, while the side effects of the H_2 antagonist cimetidine include gynecomastia, impotence, reduced sexual desire and drug interaction caused by inhibition of CYP3A4. Also, PPIs which are most frequently prescribed drugs worldwide for their effectiveness increase the risk of fracture and cause rise of blood pressure due to decrease a blood vessel relaxation according to the Science Daily report on November in 2013. This study aims to search material from the natural substances of safety and effectiveness empirically which can improve gastric disease treatment.

These findings suggest that genipin, a natural component, can be developed to treat or prevent the adverse effects of cancer chemotherapeutics for acute or chronic gastritis, gastric ulcers, or functional dyspepsia have on the gastrointestinal tract. We recommend toxicity testing of genipin be conducted to support its development as a supplement or medication.

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Conflict of Interest The authors declare no conflict of interest.

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