Natural Product Sciences
15(2) : 96-100 (2009)

Gastric Ulcer Healing Effects of *Dioscorea japonica*, *Halloysite* and *Ostrea gigas* Mixtures

Jeong-Sook Park and Kun Han*

College of Pharmacy, Chungbuk National University, Cheongju, Heungdukgu Gaeshindong 12, Chungbuk 361-763, Korea

Abstract – A novel gastric ulcer healing formulation, a mixture of Dioscoreae Rhizoma, Halloysitum Rubrum and Ostreae Testa (Dihaos), was examined for gastric ulcer healing effects. The effect of Dihaos was assessed in various gastric ulcer models in rats. Oral administration of Dihaos significantly reduced HCl-ethanol-induced gastric ulcers. Dihaos also significantly reduced gastric and duodenal ulcers induced by cysteamine. Ostreae Testa decreased secretion of gastric juice and increased the pH of gastric juice. Furthermore, the extracts of Dioscoreae Rhizoma affected the cell proliferation of MKN 74 cells. These results suggest that the healing effect of Dihaos on gastrohemorrhagic lesions results from its protective effect against acid secretion and proliferation of mucosal cells in induced gastric ulcers in rats.
Key words – Dioscoreae Rhizoma, Halloysitum Rubrum, Ostreae Testa, Gastric ulcer, Cysteamine

Introduction

Gastric ulcers are caused by an imbalance between offensive factors within the lumen and defensive factors within the gastro-duodenal mucosa (Goel and Bhattacharya, 1991). In this study, we investigated the effect of Ostreae Testa on offensive factors such as acid-pepsin secretion and the effect of Dioscoreae Rhizoma and Halloysitum Rubrum on defensive factors such as mucin secretion, cellular mucus, cell shedding and cell proliferation.

Dioscoreae Rhizoma is traditionally used to strengthen stomach function, improve anorexia, eliminate diarrhea, dilute sputum and moisturize skin (Wu, 2005). Substances that have been identified in Dioscoreae Rhizoma include mucilage, steroidal saponin (dioscin), starch (16%), vitamin C, 3,4-dihydroxy phenyethylamine (dopamine), phytic acid, and polyphenol oxidase glycoprotein.

Halloysitum Rubrum consists of Si (42.93%), Al (36.58%), Fe_2O_3, Mn, Mg, Ca, and H_2O (14.75%). In oriental medicine, it is known to affect the functions of spleen, stomach and large intestine (Wu, 2005). It acts as an astringent, adsorbs poison and has anti-hemorrhagic effects in lesions. Furthermore, it is known to affect cell proliferation (Wu, 2005).

Ostreae Testa is composed of mainly $CaCO_3$ and $CaHPO_4$, in addition to Mg, Al, Si and Fe_2O_3. In oriental medicine, it acts at the liver and kidney and has a soothing effect on accelerated liver function (Kee, 1993). It is used to treat palpitations, anxiety, insomnia and anger in combination with other herbs and also has antisecretion activity on gastric juice.

This study was designed to evaluate the gastric ulcer healing effects of Dihaos mixtures for development as an anti-ulcer agent.

Material and Method

Preparation of extracts – Dioscoreae Rhizoma, Halloysitum Rubrum and Ostreae Testa were purchased from a local herbal store (Cheongju, Korea). The materials were authenticated by Dr. Hwang, a botanical professor at the College of Pharmacy, Chungbuk National University (Cheongju, Korea). Air-dried rhizomes of Dioscoreae Rhizoma were extracted with boiling water for 2hrs 30 min. The extracts were separated by filtration and concentrated on a rotary evaporator (EYELA, Japan) and then freeze-dried (EYELA, Japan) under reduced pressure. Dried, pulverized extracts of Dioscoreae Rhizoma were sieved to a fine powder (75 μm). The powders were packed into airtight sample bottles and stored in the freezer until used. Halloysitum Rubrum and Ostreae Testa were fine powders and were used without any further purification.

Test animals – Six-week-old Sprague-Dawley albino male rats (Samtako, Kyungkido, Korea), weighing 220 ± 10 g, were used in this study. The animals were

*Author for correspondence
Tel: 82-43-261-2820; E-mail: khan@chungbuk.ac.kr

maintained under controlled conditions of temperature (23 ± 1 °C), relative humidity (55 ± 10%), and lighting (12 hrs per day). Standard laboratory chow and tap water were provided *ad libitum*. The rats were allowed three to four days to acclimatize to the experimental environment.

HCl-Ethanol-induced ulcers – The rats were assigned into control and test groups (6 rats/group) according to their body weights. Gastric ulcers were induced with 60% ethanol containing 150 mM HCl (Mizui and Doteuchi, 1983). After 1 hr for ulcer formation, the rats were given cimetidine (20 mg/kg) or Dihaos twice daily by P.O. After 24 hrs, rats were sacrificed by anesthesia and the stomach was removed rapidly. The stomach contents were rinsed with normal saline and the tissue was fixed with 10% formalin solution for 10 minutes. The stomach was incised along the greater curvature and examined for ulcers. The ulcer area was measured by Scion, which was developed by the National Institute of Health (USA) as a PC program for the quantification of gastric lesions (Khan, 2004). The scanned images of ulcers were analyzed by this program. The curative ratio was calculated from the ulcer area using the following equation.

Cysteamine-induced ulcers – Ulcers were induced with a slight modification of the procedure reported by Selye and Szabo (1973). Duodenal ulcers were induced by subcutaneous administration of two doses of cysteamine hydrochloride, 160 mg/kg in solution, at an interval of 4 hrs. Dihaos was administered 1 hr before the first dose of cysteamine hydrochloride and the test solution was re-administered 4 hrs after the second dose of cysteamine hydrochloride. All animals were sacrificed 24 hrs after the first dose of cysteamine. The stomach and duodena were excised and opened along the greater curvature and the anti-mesenteric side, respectively. The ulcer area was measured using Scion.

Estimation of ulcer area by Scion – Stomach samples were placed on the scanner (HP L6000) and covered with a transparent plastic folder (A4 size). The specimens were scanned and the captured images were saved. Five spots of known area (1, 4, 9, 16, and 25 mm^2) drawn on white paper were also scanned for calibration. 125 was subtracted from cysteamine-induced ulcers to correct for the unlesioned area.

***In vitro* evaluation of Ostreae Testa as an antacid** – To evaluate the resistance of Ostreae Testa to pH change, 0.5 or 1.0 g of Ostreae Testa was mixed with 50 ml of 0.1N HCl and stirred constantly. The pH was recorded initially and then after every ten minutes. After each 10-minute pH reading, 2 ml of 1N HCl was added. This step was repeated until a pH of 1.8 was reached. The addition of 2 ml of 1N HCl was an attempt to duplicate continual secretion of HCl by the stomach.

***In vivo* anti-secretion assay of Ostreae Testa** – Ostreae Testa and omeprazole (positive control) suspended in 50% polyethylene glycol (PEG) 400 were administered to rats (250 ± 10 g) by gavage at doses of 100 mg/kg and 20 mg/kg, respectively. The volume administered was 2 ml/kg. The gastric juice was collected 5 hrs after pylorus ligation and centrifuged for 10 min at 3000 rpm and 4 °C; the volume of the supernatant was expressed as ml/250 g body weight. Total acid output was determined by titrating with 0.01N NaOH, using phenolphthalein as an indicator, and was expressed as μEq/h (Shay *et al.*, 1954). Peptic activity was determined using bovine serum albumin as a substrate and was expressed as mM of tyrosine/hr · ml (Debnath *et al.*, 1974).

Cell culture – MKN 74, a human gastric cancer cell line, was purchased from Korean Cell Line Bank (KCLB®, Seoul). Cells were incubated in a 5% CO_2 incubator at 37 °C in RPMI medium supplemented with 10% inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. For testing, cancer cells were incubated in 96-well plates at 5×10^4 cells per well for 24 hrs. The cells were then further cultured in RPMI medium without FBS and containing various concentrations of Ostreae Testa, Halloysitum Rubrum or Dioscoreae Rhizoma extracts.

Determination of cell proliferation – Cell proliferation was measured using the WST-1 method. Assays were performed according to the manufacturer's instruction (Dojindo, Kumamoto, Japan). The cell proliferation reagent WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo phenyl-2H-tetrazolium] (20 μl) was added to each sample 46 hrs after Ostreae Testa, Halloysitum Rubrum or Dioscoreae Rhizoma extract treatment and cells were incubated for an additional 2 hrs. The absorbance of each sample was determined using a microplate reader (Molecular Devices, U.S.A.)

Stomach histology – Rat stomach was fixed in neutralized formalin (10%), dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Sections were cut at 5 μm, stained with Harris' hematoxylin and eosin, and observed under a microscope (Nikon YS 100, Japan).

Results and Discussion

HCl-Ethanol-induced ulcer healing effect – In preliminary studies, Halloysitum Rubrum combined with Ostreae Testa did not exhibit significant ulcer-healing

effects against ethanol-induced gastric ulcers. In contrast, Dioscoreae Rhizoma combined with Halloysitum Rubrum or Ostreae Testa showed significant ulcer-healing effects against ethanol-induced gastric ulcers. The ulcer-healing effect of Dioscoreae Rhizoma combined with Ostreae Testa was greater than that of Dioscoreae Rhizoma combined with Halloysitum Rubrum. In this study, we evaluated the ulcer-healing effects of mixtures of Dioscoreae Rhizoma, Halloysitum Rubrum and Ostreae Testa (Dihaos). Dihaos showed significant ulcer-healing effects against gastric ulcers induced by HCl-ethanol and the curative ratio was 78.73% when given twice daily for one day (Table 1). The HCl-ethanol-induced gastric ulcer model was used to evaluate both offensive and protective factors in gastric ulcers.

Cysteamine-induced ulcer healing effect – As reported by Selye and Szabo (1978), cysteamine induced duodenal and gastric ulcers simultaneously. The combination of Dioscoreae Rhizoma, Halloysitum Rubrum and Ostreae Testa showed significant gastric and duodenal ulcer-healing effects and the curative ratio was 82.05% and 42.08%, respectively, when given twice daily for one day (Table 2). Fig. 1 and 2 show the shape of the stomach and the duodenum after administration of Dihaos mixtures.

***In vitro* evaluation of Ostreae Testa as an antacid** – Fig. 3 shows the pH change of Ostreae Testa suspension

Table 1. The healing effects of oral administration of Dihaos mixtures on HCl-Ethanol-induced gastric ulcers in rats.

Administration	Dose/kg	Ulcer Area (mm^2)	Curative ratio (%)
Control	Purified water	51.20 ± 10.44	
Dihaos mixtures	each 100 mg	10.89 ± 2.45	78.73%*
Cimetidine	20 mg	27.97 ± 4.57	45.37%

Data are presented as mean ± S.D. (n = 6).
*, significant difference vs. contol ($p < 0.05$, ANOVA test)

Table 2. The healing effects of oral administration of Dihaos mixtures on cysteamine-induced gastric and duodenal ulcers in rats.

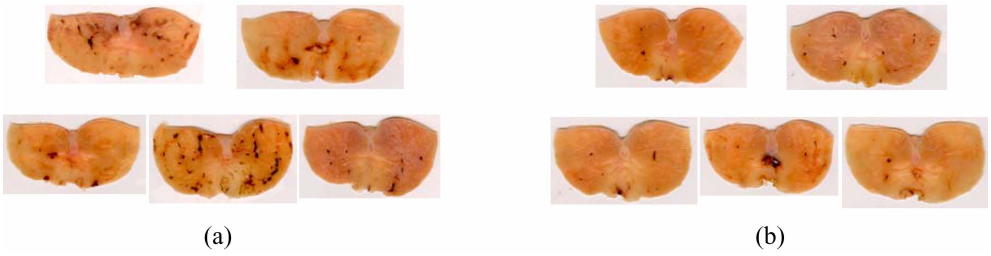
Administration	Dose/kg	Ulcer Area (mm^2)		Curative ratio (%)	
		gastric	duodenal	gastric	duodenal
Control	Purified water	54.15 ± 10.94	27.47 ± 11.71		
Dihaos mixtures	each 100 mg	9.72 ± 4.13	15.91 ± 3.92	82.05%*	42.08%

Data are presented as mean ± S.D. (n = 6).
*, significant difference vs. control ($p < 0.05$, ANOVA test)

Table 3. Effects of Ostreae Testa on gastric acid secretion and pepsin activity in pylorus-ligated rats.

Administration	Dose/kg	Gastric acid volume (ml)	pH	Total acidity (μEq/hr)	Pepsin activity (mM/hr · ml)
Control	Purified water	1.78 ± 0.30	3.42 ± 0.43	31.78 ± 6.94	11.61 ± 1.77
Ostreae Testa	100 mg	1.33 ± 0.15	5.39 ± 0.24*	14.09 ± 2.20*	12.49 ± 2.19
Omeprazole	20 mg	1.52 ± 0.16	5.44 ± 0.27*	14.72 ± 2.33*	12.58 ± 0.51

Data are presented as mean ± S.D.(n = 6).
*, significant difference vs. contol ($p < 0.05$, ANOVA test)



(a) (b)

Fig. 1. The healing effects of oral administration of Dihaos mixtures on Cysteamine-induced gastric ulcers in rats. (a) Control, (b) Dihaos-treated.

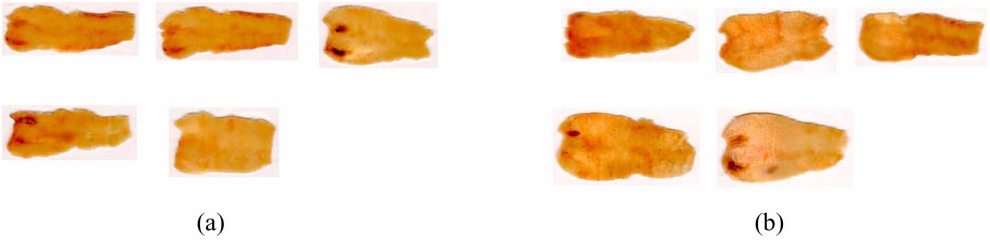


Fig. 2. The healing effects of oral administration of Dihaos mixtures on Cysteamine-induced duodenal ulcers in rats. (a) Control, (b) Dihaos-treated.

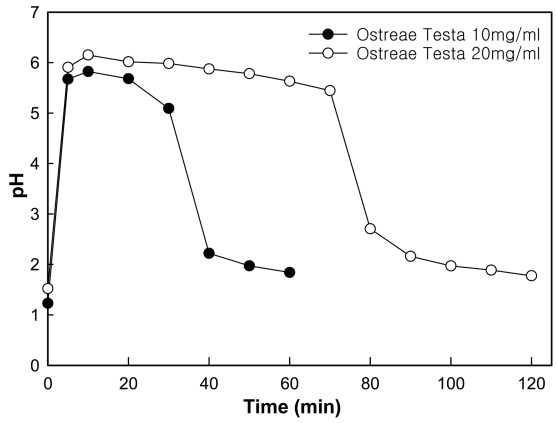


Fig. 3. Profile of pH change in Ostreae Testa suspension.

upon addition of 2 ml of HCl every 10 min. The pH of each Ostreae Testa 1.0 g/50 ml and 0.5 g/50 ml suspension was 6.2 and 5.8 at 10 min. The pH of Ostreae Testa 1.0 g/50 ml and 0.5 g/50 ml was maintained above pH 5 for 70 min and 30 min, respectively.

Effect of Ostreae Testa on acid-pepsin secretion – The effect of Ostreae Testa on various offensive factors was studied regarding the volume, total acid and pepsin activity in the gastric juice of pylorus-ligated rats. Ostreae Testa (100 mg/kg) decreased the volume and acid concentration of gastric juice, while the pH was increased. The pH of control, test and Omeprazole-treated gastric juices were 3.42 ± 0.43, 5.39 ± 0.24 and 5.44 ± 0.27, respectively. The total acidity determined from the titration of control, test and Omeprazole-treated gastric juices with 0.01N NaOH was 31.78 ± 6.94 μEq/hr, 14.09 ± 2.20 μEq/hr and 14.72 ± 2.33 μEq/hr, respectively. On the other hand, pepsin activity was unchanged.

Ostreae Testa showed efficacy as an antacid in *in vitro* experiments but also decreased the total acidity in the *in vivo* pylorus-ligation test. These results indicated that Ostreae Testa exerted its activity through local and systemic action in the stomach. This protection may be an acid-neutralizing action or antisecretory activity via antagonizing muscarinic or H_2 receptors.

Lee *et al.* (1997) reported that Halloysitum Rubrum decreased the volume and acidity of gastric juices.

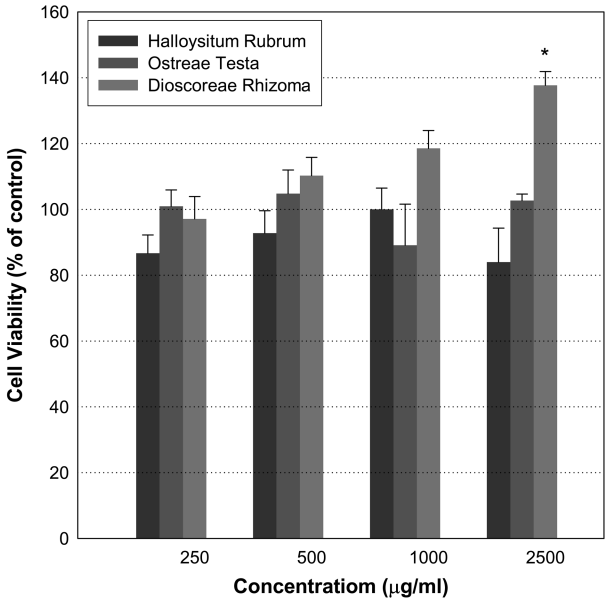


Fig. 4. Effects of Halloysitum Rubrum, Ostreae Testa and Dioscoreae Rhizoma on proliferation of MKN-74 cells. The cells were incubated with *Halloysitum Rubrum*, Ostreae Testa or Dioscoreae Rhizoma extracts for 48 hrs. Data are presented as mean ± S.D. (N = 6).

Cell proliferation effect of Dioscoreae Rhizoma extracts on MKN 74 cells – The cell proliferation effects of Ostreae Testa, Halloysitum Rubrum or Dioscoreae Rhizoma extracts were examined in MKN 74 cells. Treatment of MKN 74 cells with various concentrations of Dioscoreae Rhizoma extracts caused a dose-dependent increase of MKN 74 cell growth (Fig. 4). At 2,500 μg/ml, the extracts increased cell proliferation by about 40%. In contrast, cell proliferation was not observed with Ostreae Testa and Halloysitum Rubrum.

Cell proliferation is the major defense mechanism of the gastric mucosa and is required for maintaining mucosal integrity, repairing mucosal injury, and healing of mucosal ulcerations (Tarnawski, 1997). Dioscoreae Rhizoma is thought to contain allantoin and allantoic acid, which prevent inflammation and ulcers in the human body

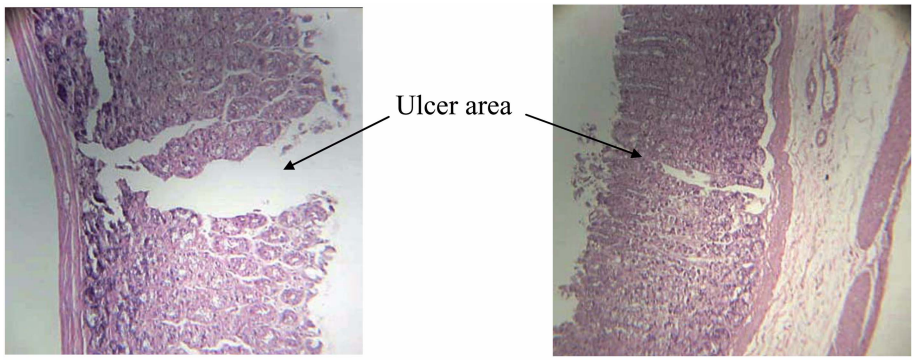


Fig. 5. Representative photomicrographs of ethanol-induced gastric ulcers in control rat (a) and in Dihaos-treated rats (b). Compared to control, Dihaos mixtures promoted restoration of epithelial cells and connective tissue. Magnification × 200.
*, significant difference vs. contol ($p<0.05$, ANOVA test)

(Sagara *et al.,* 1988). We also identified allantoin and allantoic acid in Dioscoreae Rhizoma extracts by HPLC. Allantoin is taken internally to promote cell proliferation. It protects tissues in the stomach, accelerates the healing process throughout the stomach and bowels, and promotes increased tissue repair throughout the entire gastrointestinal tract.

Histopathology of stomach – Histopathologic alterations were assessed in the stomach of rat treated with ethanol (control). Mucosal layers were damaged and connective tissues were thinned. The stomach mucosal layers of rat treated with Dihaos were more closely arranged and the connective tissue was thickened (Fig. 5).

Conclusion

Gastric ulcers are caused by an imbalance between offensive factors within the lumen and defensive factors within the gastro-duodenal mucosa. In an attempt to regain the balance between these factors, combined folk medicines that have different therapeutic effects were tested. Dihaos mixtures showed significant ulcer healing effects against gastric ulcers induced by HCl-ethanol and the curative ratio was 78.73% when Dihaos was given twice daily for one day. Dihaos also showed significant ulcer healing effects against gastric and duodenal ulcer by cysteamine, and the curative ratios were 82.05% and 42.08%, respectively. Ostreae Testa showed efficacy as an antacid in *in vitro* experiments but also decreased the total acidity in the *in vivo* pylorus-ligation test. Treatment of MKN 74 cells with Dioscoreae Rhizoma extracts increased the proliferation of MKN 74 cells. These gastric ulcer therapeutic effects of Dihaos mixtures might be due to the combined cell proliferative effects of Dioscoreae Rhizoma and the anti-secretion effect of Ostreae Testa. However, more studies are needed to identify the mechanism of Halloysitum Rubrum action related to the repair of gastric ulcers.

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Received March 2, 2009
Revised June 16, 2009
Accepted June 18, 2009