Biomolecules & Therapeutics, **17(1)**, 62-69 (2009)
www.biomolther.org

ISSN: 1976-9148(print)/2005-4483(online)
DOI: 10.4062/biomolther.2009.17.1.62

Suppressive Actions of Astragali Radix (AR) Ethanol Extract and Isolated Astragaloside I on HCl/ethanol-Induced Gastric Lesions

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(Received November 30, 2008; Revised December 26, 2008; Accepted December 26, 2008)

Abstract − Roots of *Astragalus membranaceus* (*A. membranaceus*) (*Astragali Radix*, AR) has been used as a herbal medicine for gastrointestinal disorders in China, Korea, Japan, and other Asia countries. In this study we investigated the effects of the AR 70% ethanol extract and compounds isolated from AR on gastritis in rats and growth of human gastric cancer cells. AR 70% ethanol extract showed the potent acid-neutralizing capacities and partly cytotoxicity of *Helicobacter pylori* and human AGS gastric cancers cell. Astragaloside I and daucosterol, which were isolated from AR, significantly inhibited HCl/ethanol-induced gastric lesions. In addition, daucosterol increased the mucus content to almost the same as the positive control. The results of this study suggest that astragaloside I and daucosterol may be good candidates for the development of new drugs or neutraceuticals which can be used for the treatment or prevention of gastritis.

Keywords: *Astragalus membranaceus*, Astragali Radix (AR), Astragaloside I, Gastritis, *Helicobacter pylori*

INTRODUCTION

Recently it has been recognized that the infection of the gastric mucosa with *H. pylori* usually causes both an acute and chronic inflammation cell infiltrate, leading to an increase of reactive oxygen species (ROS), which are highly reactive compounds capable of combining with DNA in a number of potentially genotoxic ways, and are accumulated in *H. pylori* gastritis (Drake *et al*., 1998). The gastrointestinal lesions, such as gastric ulcers, and gastric cancers are strongly associated with *H. pylori* infection. One way in which it may be possible to prevent carcinogenesis would be to reduce ROS damage to cellular constituents, especially DNA. In addition, the pathological condition of the recurring gastritis and gastric ulcers causes an imbalance between aggressive factors (i.e., gastric acid, pepsin, and secretion of gastrin) and protective factors (i.e., mucus productivity, mucus secretion, and prostaglandins) (Shay *et al*., 1945). Physiological factors of these gastric diseases include acid-pepsin secretion, parietal cell activity, mucosal barrier, mucus secretion, blood flow, cell regeneration, and the release of endogeneous protective agents, especially prostaglandins and epidermal growth factors (Gyires, 2005).

The genus *Astragalus* includes approximately 2,000 species and is distributed mainly in the northern temperate regions and tropical African mountains (Evans, 2002); five species of this genera have been identified in Korea (Lee, 1989). Astragali Radix (AR), the dry root of Astragalus membranaceus (*A. membranaceus*) (Fisch.) Bge. (Leguminosae), has been used as one of the most important tonic herbs in traditional medicine. Pharmacological studies of AR have demonstrated that AR possesses many biological functions, including hepatoprotective (Ríos *et al*., 1997; Gui *et al*., 2006), neuroprotective effect against ischemic brain injury (Luo *et al*., 2004; Tohda *et al*., 2006), immunological properties (Shao *et al*., 2004; Li *et al*., 2007; Cho *et al*., 2007a), cardiotonic (Zhang *et al*., 2006a; Zhang *et al*., 2006b), antiaging activities (Lei *et al*., 2003), gastroprotective (Navarrete *et al*., 2005), adjuvant (Rajput *et al*., 2007), antitumor effects (Cho *et al*., 2007b; Tin *et al*., 2007), anti-inflammatory (Zhang *et al*., 2003), and the growth of new tissues (Cheng *et al*., 2006; Choi *et al*., 2007).

The present work was carried out to investigate the potential anti-ulcerogenic and anti-gastric effects of AR extract and its several constituents. This result suggests that

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AR may be a good candidate for the development of new drugs or neutraceuticals which can be used for the treatment or prevention of gastritis.

MATERIALS AND METHODS

Materials

Three years-old roots of *A. membranaceus* were harvested at Jungsun, Kangwon, Korea in September, 2004. The plant and root were identified by Prof. S. Kang. (College of Pharmacy, Seoul National University). The voucher specimen (SNU-594) was deposited at the herbarium of the College of Pharmacy, Seoul National University, Seoul, Korea.

Fetal bovine serum (FBS), RPMI Medium 1640, and Hank's balanced salt solution were obtained from GIBCO Co. (Grand Island, NY). Dantrolene sodium, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), trypan blue, probenecid, dimethyl sulfoxide (DMSO), sodium bicarbonate, penicillin-streptomycin, trypsin-EDTA, cimetidine, and ampicillin were obtained from Sigma Chemical Co. (St. Louis, MO). HCl, ethanol, and other solvents were purchased from Duksan Pure Chemical Co. Ltd. (Kyunggi-do, Korea). Silica gel 60, Kieselgel 60, Kieselgel 77, and TLC plate were from MERCK, Ltd. (Darmstadt, Germany). All other reagents and solvents were the pharmaceutical or analytical grade.

Animals

Male Sprague-Dawley rats weighing 180-200 g were purchased from Samyook Animal Laboratories, Kyunggi-do, Korea, and were acclimatized to standard laboratory conditions (24 ± 2°C, 55 ± 5% humidity and 12 h light/dark cycle) for 14 days in the animal facility in Duksung Women's University. The samples dissolved in saline were administered in a volume of 0.5 ml per 100 g (body weight). The control group was given saline only. The experimental procedures for Sprague-Dawley rats were conducted in accordance with the Guidelines of the Care and Use of Laboratory Animals, Duksung Women's University. The animals were allowed free access to food (standard pellet diet) and water ad libitum. All this study was carried out in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 1999-61) issued by the Korea Food and Drug Administration, the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies (Notification No. 2000-63) issued by the Korea Food and Drug Administration, and the Principles of Good Laboratory Practice issued by the Organization for Economic Cooperation and Development.

Preparing extract and isolation constituents from AR

AR (17.8 kg) was chopped into small pieces and refluxed with 70% ethanol for 3 h at 70-80°C. The 70% ethanol extract was evaporated under reduced pressure and then fractionated successively with water and hexane (137 g), ethyl acetate (145 g), and then butanol (340 g). The ethyl acetate (143.8 g) was separated by column chromatography (CC) over silica gel with dichloromethane/ methanol (gradient) to yield 51 subfractions (Fr. E-01-Fr. E-51). Fr. E-45 (20 g) was further purified on a silica gel column (ethyl acetate/methanol/water; 100:1:0.5→100:2:1) to yield 70 subfractions (Fr. E-45-01 - Fr. E-45-70). Subfraction E-45-50 (4.5 g) were chromatographed on an RP-18 column with 80% methanol to afford E-45-50-8 (3.2 g) and repeated silica gel CC (chloroform/methanol/water; 7:0.5:0.5) to afford astragaloside I (1.61 g, Fig. 1) from E-45-50-8-25. Subfraction E-23 (1 g) was purified on an RP-18 column with hexane-ethyl acetate (gradient) to yield subfraction E-23-221, which was crystallized from dichloromethane/ methanol to yield formononetin (150 mg, Fig. 1). The butanol soluble fraction was fractionated by CC over silica gel (dichloromethane/methanol/water; 7:1:0.5→7:2:0.5 - 7:3:1) to yield 39 fractions (Fr. B-01 - Fr. B-39). Fr. B-20 (1.5 g) was rechromatographed on an RP-18 column with 80% methanol to afford astragaloside IV (250 mg, Fig. 1) from subfraction B-20-23. The compounds were identified as daucosterol, formononetin, astragaloside I, and astragaloside IV by NMR and LC/MS and comparison with literature data (Hirotani *et al*., 1994; Kim *et al*., 1996; Lee *et al*., 2007).

Acid-neutralizing capacity

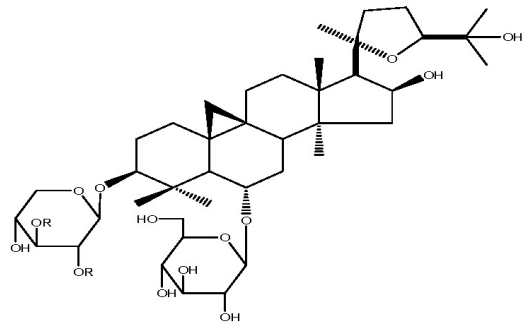
One gram of extracts and constituents was added to 100 ml of 0.1 N HCl and then incubated for 1 h at 37°C with a shaking. Acid-neutralizing capacity was determined by titrating with 0.1 N NaOH using methyl orange as an indicator. Hydrotalcite and cimetidine were used as a positive control.

Determination of antioxidant activity

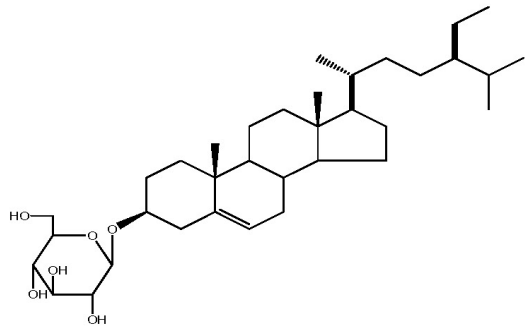
One milliliter of DPPH (150 μM) was added to 4 ml of extracts/constituents (2.5-120 μg/ml), and then the mixture was stirred. After 30 min incubation at room temperature, the absorbance of the samples was read against a blank on 520 nm. Scavenging the DPPH free radical in percent (Inhibition %, *I* %) was calculated as followings;

$$I\ \% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

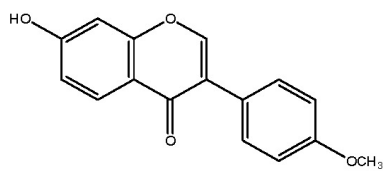
A_{blank} is the absorbance of the mixture without samples



Astragaloside I R = Ac
Astragaloside IV R = H



Daucosterol



Formononetin

Fig. 1. Chemical structure of astragaloside I, astragaloside IV, daucosterol, and formononetin.

and A_{sample} is the absorbance of the mixture with samples. Concentration providing 50% inhibition (IC_{50}) was determined from the graph plotted inhibition percentage against concentration. L-Ascorbic acid was used as a positive control (Tepe and Sokmen, 2007).

Anti-*H. pylori* activity

H. pylori strain was obtained from American Type Culture Collection (ATCC, Rockville, MD). The inhibitory effect of AR ethanol extract and its constituents on the growth of *H. pylori* was investigated. Six hundred microliter of samples was mixed to 5.4 ml of brucella agar medium containing 7% horse serum in the petri dish. *H. pylori* (5×10^5 CFU) was seeded in this media and then incubated for 3 days at 37°C incubator (AnaeroPak Campylo: 85% N_2, 10% CO_2, 5% O_2). Ampicillin was used as a positive control (Kim *et al*., 2003).

Cell culture

Human gastric cancer cell lines, AGS and SNU638, were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Gastric cancer cells were grown at 37°C in a 5% CO_2 humidified incubator in a MEM containing 10% FBS, 200,000 IU/L penicillin, 200 mg/L of streptomycin, and 1 mM sodium pyruvate. After reaching confluence, the cells were subcultured by trypsinization. SNU and AGS cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum (which can inhibit trypsin) and were subdivided using 0.05% trypsin with 0.53 mM EDTA.

Cytotoxicity assay

Cell viability was assessed by the MTT method (van de Loosdrecht *et al*., 1991). Cells were seeded in 24-well plates at the density of 5×10^4 cells/well. After 24 h incubation with samples, 100 μl of MTT (5 μg/L in water) were added to every well and the plates were incubated for a further 4 h. Two hundred microliter of DMSO was added to every well and mixed by pipetting to dissolve the MTT formazans completely. Relative cell viability was obtained by measuring the absorbance by an ELISA reader (Molecular Devices, Menlo Park, CA) on 540 nm.

HCl/ethanol-induced gastric lesion

The rats, which were fasted for 24 h with free access to water prior to the experiment, were orally administered with AR 70% ethanol extract or its constituents (Mizui and Dodeuchi, 1983). After 30 min, 0.5 ml/100 g of a HCl/ethanol (150 mM HCl in 60% ethanol) solution was given orally for the induction of gastric lesions. One hour later, the animals were anesthetized with ether, and their stomachs were removed and fixed with 2% formalin for 10 min. The amount of hemorrhage on the glandular portion was measured by summing the total length (mm) of each lesion and expressed as a lesion index.

Gastric secretion

The rats were immediately administered daucosterol and Astragaloside I isolated from AR or cimetidine intraduodenally after the pyloric ligation (Shay *et al*., 1945). Four hours after the pyloric ligation, the animals were sacrificed, and the contents of the stomach were collected and centrifuged at 1,050×g for 10 min. The total volume of gastric juice and pH were measured, and an acid output (mEq/ml) was determined by titrating the gastric juice with 0.05 N NaOH using phenolphthalein as an indicator.

Mucus secretion

The constituents from AR were administrated orally to the rats. After 30 min, an absolute ethanol (1 ml/100 g) was given orally to induce the gastric lesions. One hour later, the animals were sacrificed and the secreted mucus was determined (Kitagawa *et al*., 1986). The glandular portion separated from the excised stomach was opened along the lesser curvature and everted. The stomach was soaked in 0.1% alcian blue 8GX dissolved in 0.16 M sucrose buffered with 0.05 M CH_3COONa (adjusted to pH 5.8 with HCl) for 2 h. The mucus combined with the alcian blue was extracted with 20 ml of 70% ethanol containing 30% dioctyl sodium sulfosuccinate and centrifuged for 10 min at 500×g. The optical density of the supernatant was measured at 620 nm using by UV-spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA).

Statistical analysis

All experiments were performed four times. Data were expressed as mean ± standard error of the mean (SEM), and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. *p*-values less than 0.05 are considered statistically significant.

RESULTS AND DISCUSSION

Acid-neutralizing capacity

AR 70% ethanol extract inhibited approximately 38.80% of NaOH-consumption volume, which was better than that of cimetidine as a positive control (Table I). NaOH-consumption volume by astragaloside IV, daucosterol, and formononetin, which were isolated from AR, was decreased to 5.01, 6.15, and 2.01%, respectively. However, astragaloside I did not show the acid-neutralizing activity. Acid-neutralizing capacity of AR 70% ethanol extract, which is extra-ordinarily superior among the natural extract materials, may be expected to exert the protective influence on stomach damage by secretion of gastric acid, when taken in a long period.

Table I. Acid-neutralizing capacity of AR 70% ethanol extract and its constituents

Material	NaOH consumption volume (% control)	Inhibition (%)
Control	99.33 ± 1.15	−
AR 70% ethanol extract	60.08 ± 5.36*	38.80
Astragaloside I	101.94 ± 4.11	−1.94
Astragaloside IV	94.35 ± 3.04*	5.01
Daucosterol	93.85 ± 5.11*	6.15
Formononetin	97.33 ± 2.52	2.01
Hydrotalcite	31.66 ± 5.77**	68
Cimetidine	87.06 ± 5.65*	12.94

The values are mean ± SEM. Significant difference, *$p < 0.05$, **$p < 0.01$, as compared to the control.

Antioxidant activity

The antioxidant property plays an important role for the inhibition of oxidation processes, which are involved in the mechanisms of several gastric disorders, including ulceration (La Casa *et al*., 2000). IC_{50} of L-ascorbic acid as positive control was < 5 μg/ml. However, AR ethanol extract and its any constituents did not show the effective scavenging activity against DPPH free radicals (Table II), in spite of its potent acid-neutralizing capacity.

It has been widely accepted that a large number of free radicals is generated in the peptic ulcer and gastritis. In addition, the involvement of oxygen derived free radicals are well established in the pathogenesis of ischaemic injury of gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol, and *H. pylori* (Drake *et al*., 1998). The radicals also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism, and DNA damage. From this report, it is likely to speculate that AR ethanol extract and its constituents have low antioxidant action to play a role to anti-ulcer and gastritis.

Anti-*H. pylori* activity

H. pylori is the major cause for bacterial gastrointestinal infections in adults and children. Also, it is believed to be a cause of peptic ulcer (Gerrits *et al*., 2006) and gastric cancer (Matysiak-Budnik and Megraud, 2006). Antibiotic therapy using the combination of two or three drugs has been widely used to eradicate these infections. However, the development of drug-resistance in bacteria needs novel sources of drugs, and therefore plants seem to be a desirable source of new antibacterial compounds. Indeed, the

Table II. DPPH-radical scavenging activity of AR 70% ethanol extract and its constituents

Material	IC_{50} (μg/ml)
AR 70% ethanol extract	> 160
Astragaloside I	> 160
Astragaloside IV	> 160
Daucosterol	> 160
L -Ascorbic acid	< 5

medicinal plants and/or their chemical components have been reported to have a potential benefit in eradicating such problems (Nostro *et al*., 2005). Recently the phenolic phytochemicals, such as cinnamic acids, cinnamaldehydes, coumarins phenolic acids, capsaicin, flavonoids, and tannins from peppers, wine, and many other natural products, showed the high anti-*H. pylori* activity (Bae *et al*., 1999). This result shows that AR 70% ethanol extract and astragaloside I inhibited partly the colonization of *H. pylori* at 50 and 100 μg/ml of concentration (Table III).

Cytotoxicity

Cytotoxicity was assessed by measuring cell viability of human gastric cancer cells, AGS and SNU-638. AR 70% ethanol extract showed a significant cytotoxicity against AGS gastric cancer cell line (IC_{50}=165.76 μg/ml) (Table IV). AR 70% ethanol extract and its several constituents did not show cytotoxicity against SNU638 gastric cancer cell line.

For the prevention of gastric cancer disease, it is very important to develop the natural herbal medicine to act to the cancer cells directly as well as to associated risk factors, such as *H. pylori* infection and a maintaining ascorbic acid. Recently, the development of new anticancer drug is a key issue for cancer chemotherapy because of the reality that cancer cells, which are resistant to chemotherapy, will eventually cause mortality. Herbal medicines as substitutes for cancer remedies have attracted a great deal of interest, because of their low toxicity and costs. However taken together, it is expected that AR ethanol extract and its constituents have low cytotoxic actions on human gastric cancer cells.

The effects of AR-constituents on HCl/ethanol-induced gastric lesion

The HCl/ethanol-induced gastric lesions are caused by the direct irritation to a gastric mucosal barrier (Seiki *et al*., 1990). The mechanism of ethanol-induced gastric lesions varies, including the depletion of gastric mucus content, damaged mucosal blood flow, and mucosal cell injury. In addition, ethanol-induced gastric mucosal damage is associated with overproduction of free radicals, which lead to an increased lipid peroxidation (Kahraman *et al*., 2003). Ethanol induces both long ulcers and petechial lesions within a relatively short time, which makes this technique suitable for screening methods for investigation of anti-ulcer drugs. The effects of AR-constituents on the HCl/ethanol-induced lesion were summarized in Table V. Lesion index of control group was 66.7 ± 6.77 mm. Astragaloside I (100 mg/kg) and daucosterol (50 mg/kg) significantly decreased approximately 51.1 and 42.6% of lesion index, re-

Table III. Inhibitory effect of AR 70% ethanol extract and its constituents on the colonization of *H. pylori*

Material	Dose (μg/ml)	Colonization
Control		+++[a]
AR 70% ethanol extract	10	+++
	50	++[b]
	100	++
Astragaloside I	10	+++
	50	++
	100	+[c]
Astragaloside IV	10	+++
	50	+++
	100	+++
Daucosterol	10	+++
	50	+++
	100	+++
Formononetin	10	+++
	50	+++
	100	+++
Ampicillin	100	−[d]

[a]+++: colonies(4-5×10^5CFU), [b]++: colonies (3-4×10^5 CFU), [c]+: colonies (1-2×10^5 CFU), [d]−: none.

Table IV. Cytotoxicity of AR 70% ethanol extract and its constituents against SNU638 and AGS cells

Material	SNU638	AGS
	IC_{50} (μg/ml)	
AR 70% ethanol extract	>200	165.76
Astragaloside I	>200	>200
Astragaloside IV	>200	>200
Daucosterol	>200	>200

Table V. Effects of AR-constituents on HCl/ethanol-induced gastric lesion

Material	Dose (mg/kg)	Lesion index (mm)	Inhibition (%)
Control	−	66.7 ± 6.77	−
Astragaloside I	50	51.2 ± 1.65	22.07
	100	32.1 ± 0.99**	51.1
Daucosterol	50	37.7 ± 0.79**	42.6
	100	65.3 ± 0.39	0.6
Formononetin	50	60.7 ± 0.99	7.61
	100	40.8 ± 0.53*	37.9
Sucralfate	375	26.5 ± 0.10**	59.7
Cimetidine	100	45.7 ± 0.30	30.4
Hydrotalcite	100	36.8 ± 0.28**	

The values are mean ± SEM of 6 animals. *p<0.01, **p<0.001, as compared to the control.

spectively, which were better than or equivalent to that of cimetidine (100 mg/kg), sucralfate (375 mg/kg), and hydrotalcite (100 mg/kg), as positive controls. In case of daucosterol, the inhibitory effect against HCl/ethanol-induced gastric lesions was not concentration-dependency. Based on cytotoxicity of many flavonoids and saponins at high dose to the tissues, its reason is likely to cytotoxicity of high dose-daucosterol. Furthermore, the new drug, effective at low dose, is critical for anti-gastritis treatment. Therefore, the study for lower dose of daucosterol will be performed in the future. Except the potent acid-neutralizing activity, AR 70% ethanol extract and its constituents did not show the effective free radical quenching activity and cytotoxicity on *H. pylori* and human cancer cell lines *in vitro*. However, *in vivo* astragaloside I, which is one of the major components in AR, exerted the significant inhibition on HCl/ethanol-induced lesion, and this anti-gastric activity could be explained by the acid-neutralizing capacity of AR.

The effects of astragaloside I and daucosterol on gastric secretion

The effects of astragaloside I and daucosterol, which were constituents of AR, on gastric secretion, pH, and acid output in pylorus-ligated rats were examined in Table VI. We measured the gastric-juice parameters, such as gastric volume and pH, after submitting the rats to pylorus ligature with or without the astragaloside I and daucosterol intraduodenally. Doses of astragaloside I and daucosterol were selected based on suppression of HCl-ethanol-induced gastric lesion (Table V). Cimetidine was used for a positive control. Astragaloside I (100 mg/kg) and daucosterol (50 mg/kg) showed no significant change in gastric volume and acid output, but increased the gastric pH significantly. Despite of no change in gastric volume and acid output, the increase of gastric pH is presumed to play important role to anti-gastric effect, including the reduction of lesion index *in vivo*.

Table VI. Effect of astragaloside I and daucosterol on gastric secretion in pylorus-ligated rats

Material	Dose (mg/kg)	Volume (ml)	pH	Total acid output (mEq/4 h)
Control	—	5.4 ± 2.53	0.7 ± 0.04	0.47 ± 0.02
Astragaloside I	100	5.7 ± 1.79	1.3 ± 0.04	0.41 ± 0.01
Daucosterol	50	7.2 ± 2.40	1.4 ± 0.04	0.42 ± 0.02
Cimetidine	100	2.8 ± 1.35*	2.7 ± 0.02**	0.09 ± 0.01**

Total gastric juice volume and pH were measured 4 h after the pyloric ligation. The values are mean ± SEM of 6 animals. *$p < 0.01$, **$p < 0.001$, as compared to the control.

Table VII. Effects of astragaloside I and daucosterol on mucus contents from ethanol-induced gastric lesion in rats

Material	Dose (mg/kg)	Mucus content (μg as alcian blue)
Control	—	122.8 ± 7.91
Astragaloside I	100	120.2 ± 7.36
Daucosterol	50	162.9 ± 6.86*
Sucrafate	375	125.6 ± 8.23

The values are mean ± SEM of 6 animals. *$p < 0.01$, as compared to the control.

The effects of astragaloside I and daucosterol on mucus secretion

Daucosterol (50 mg/kg) significantly increased the mucus content to 162.9 ± 6.86 μg, which was better than that of sucralfate as a positive control (Table VII). In the mucus secretion model, even though ethanol induces to reduce the amount of mucus secretion in the rats, daucosterol enhanced the mucus secretion. The amount of mucus secretion by sucralfate was similar to control group. The reason might be by a protective action for stomach damage through sucralfate-coating than by the increase of mucus secretion in response to ethanol irritation. Therefore, the gastro-protective activity of daucosterol was expected to be caused by the stimulation of mucus secretion.

In present study, AR ethanol extract showed the potent acid-neutralizing capacities, the partial inhibition of *H. pylori* and AGS human gastric cancer cell line. Astragaloside I and daucosterol, which were isolated from AR, exhibited protective effect against ethanol-induced gastric mucosal lesion through one or more possible mechanisms including stimulation of mucus secretion. Therefore, AR ethanol extract, Astragaloside I, and daucosterol are expected to have potential protective effect against gastritis.

ACKNOWLEDGMENTS

This study was supported by a grant from the Food & Drug Administration, Republic of Korea and the Korea Research Foundation Grant funded by the Korean Government (MOEHRD)(KRF-2008-005-J00601).

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