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Anti-gastric Actions of Eugenol and Cinnamic Acid Isolated from Cinnamomi Ramulus

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We investigated the evidence of gastric protection for ulcer and gastritis by Cinnamomi Ramulus (*Cinnamomum cassia* Blume, Geiji, CR) extract and its several constituents. CR ethanolic extract showed the potent antioxidant activity and cytotoxicity of *Helicobacter pylori* (*H. pylori*) and acid-neutralizing capacity. Especially, eugenol exerted a significant antioxidant activity and inhibited the colonization of *H. pylori*. *In vivo* test, eugenol and cinnamic acid significantly inhibited HCl/ethanol-induced gastric lesions and increased the mucus content though they didn't inhibit gastric secretion effectively. Taken together, eugenol and cinnamic acid, which were isolated from CR, exhibited the antioxidant activity *in vitro* and protective effect against gastric damage *in vivo* through stimulation of mucus secretion and so on. It suggested that they are useful as the neutraceuticals for gastritis.

Key words——eugenol; cinnamic acid; gastritis; Cinnamomi Ramulus; *Helicobacter pylori*

INTRODUCTION

Traditional medicine has been extensively used to prevent and cure the human diseases in oriental area. Due to their low toxicity and the side-effect free therapeutical performance, the traditional medicine has been attracted considerable attention as alternative drugs recently. In addition, the pharmaceutical researches for active ingredients of medicinal herbs have become important for development of novel medicines.

Cinnamomi Ramulus (*Cinnamomum cassia* Blume, Geiji, CR) is a well-known traditional medicine for a long time in Korea. The medicinal prescription containing CR on anti-atherosclerosis activity has been used for the treatment of cardiovascular disorders. As a matter of fact, CR is very effective in treating thrombosis in those patients who have difficulties with more conventional antithrombotic drugs. In addition, CR has been used as a remedy for menstrual problems, gastrointestinal disorders, headache, amenorrhea, and postpartum hemorrhage.[1] It was reported that CR has a remarkable central stimulant effect, a transient hypertensive effect, and positive inotropic and chronotropic effects.[1] It has also been known that CR protects the myocardium against ischemia-induced derangement. The inhibitory effect of CR on the growth of cancer cell lines such as HepG2 cell and Hep3B cell has been demonstrated for a potential cancer chemopreventive agent in humans, especially in hepatological cancers.[2] *In vivo*, the feeding CR-extract reduced endothelial damage and the severity of atherosclerosis in cholesterol-fed NZW rabbits. Low density lipoproteins (LDLs) from CR-treated animals contained more vitamin E and were more resistant to oxidation *ex vivo*.[3] CR inhibits the aggregation of human platelets in a dose-dependent manner, and that CR exerts its vasodilator effects *via* an endothelial nitric oxide (NO)-dependent mechanism. CR protects the myocardium against ischemia-induced derangement. An ethanolic extracts of CR contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes, and erythrocytes against oxidative damage. CR acts as an antioxidant and antimutagen and to stimulate phase II drug-metabolizing enzymes; it mediates anti-inflammatory effects and inhibits cyclooxygenase and hydroperoxidase functions; and it induces human promyelocytic leukemia cell differentiation.

Cinnamaldehyde, coumarin, β-sitosterol, and protocatechuic acid were isolated from ethanolic extract of CR.[4] Cinnamaldehyde exerts the anti-spasms, analgesic, removal of fever, antimicrobial, anti-cancer, and strength of stomach, and coumarin has biological functions for the anti-aggregation, vascular dilation, antimicrobial, and anti-inflammation.[5]

Gastric diseases including gastric ulcer, infection of

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H. pylori, gastritis, and gastric cancer affected a large portion of the world population and are induced by several factors, such as stress, smoking, nutritional deficiencies, and ingestion of nonsteroidal anti-inflammatory drugs.[6] Peptic ulcers appear to result from overproduction of gastric acid and or decrease on gastric mucosal protective mechanisms. Consequently, reduction of gastric acid production as well as reinforcement of gastric mucosal protection has been main approaches for peptic ulcer therapy. The recurring gastritis and gastric ulcers are caused by an imbalance between aggressive factors (*i.e.*, gastric acid, pepsin, stimulation of the vagus nerves, secretion of gastrin, and increasing the number of parietal cells) and protective factors (*i.e.*, bicarbonate ion, mucus productivity, mucus secretion, and prostaglandins).[7] 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 endogenous protective agents, especially prostaglandins and epidermal growth factors.[8] One of the greatest concerns is to ascertain whether *H. pylori*-induced gastritis may lead to gastric cancer. According to several epidemiologic studies concerning the association between gastric cancer and *H. pylori*, the antibodies for *H. pylori* was increased and higher in the patients with gastric cancer than in the control group.[9] Numerous approaches for the treatment of gastric ulcers have been focused on the control of acid secretion, *H. pylori* level, and H^+/K^+-ATPase activity for the alleviation of mucosal damage and inflammation.[10]

The present work was carried out to investigate the evidence of gastric protection for ulcer and gastritis by CR extract and its several constituents. We expect that CR 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 The young branches of CR were harvested at Kangwon, Korea in September, 2004, identified by Prof. K. W. Bae, College of Pharmacy, Chungnam University, Korea. The voucher specimen (CNU-771) was deposited at the herbarium of the College of Pharmacy, Chungnam National University, Daejeon, 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, penicilin-streptomycin, trypsin-EDTA, cimetidine, sucralfate 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.

Preparing the Extract and the Isolation of Constituents From CR CR (30.0 kg) was chopped into small pieces and refluxed with 70% EtOH for 3 h at 70–80°C. The 70% EtOH extract was evaporated under reduced pressure and then fractionated successively with H_2O and hexane (410 g), ethyl acetate (EtOAc) (230 g), and then butanol (BuOH) (135 g). The hexane fraction (410 g) was separated by column chromatography (CC) over silica gel with hexane/EtOAc (gradient) to yield 7 subfractions (Fr. H-01–Fr. H-07). Fr. H-02 and Fr. H-03 were chromatographed on a silica gel column with hexane-EtOAc (gradient), and were crystallized to yield cinnamaldehyde (6000 mg, Fig. 1A) and eugenol (800 mg, Fig. 1B), respectively. Fr. H-04 was further purified on a silica gel column (hexane/EtOAc; 20:1→15:1) to yield coumarin (4000 mg, Fig. 1C). Fr. H-07 was purified by HPLC (MeOH/water; 50:50) to yield cinnamic acid (2000 mg, Fig. 1D). The purity of these constituents is over 96% by chromatogram (Supplemental data to Fig. 1).

Animals Male Sprague-Dawley rats, weighing 170–210 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 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. Saline was given to the control group. The experimental procedures for 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.

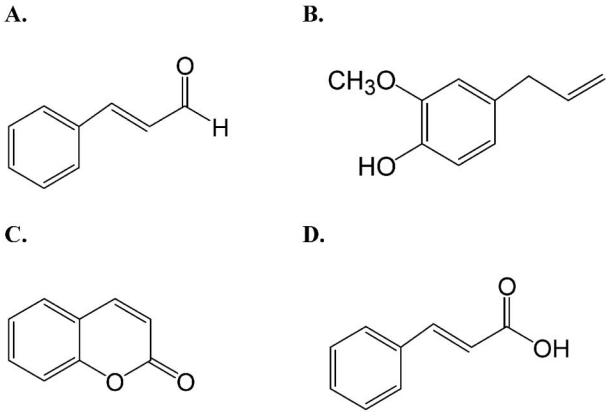
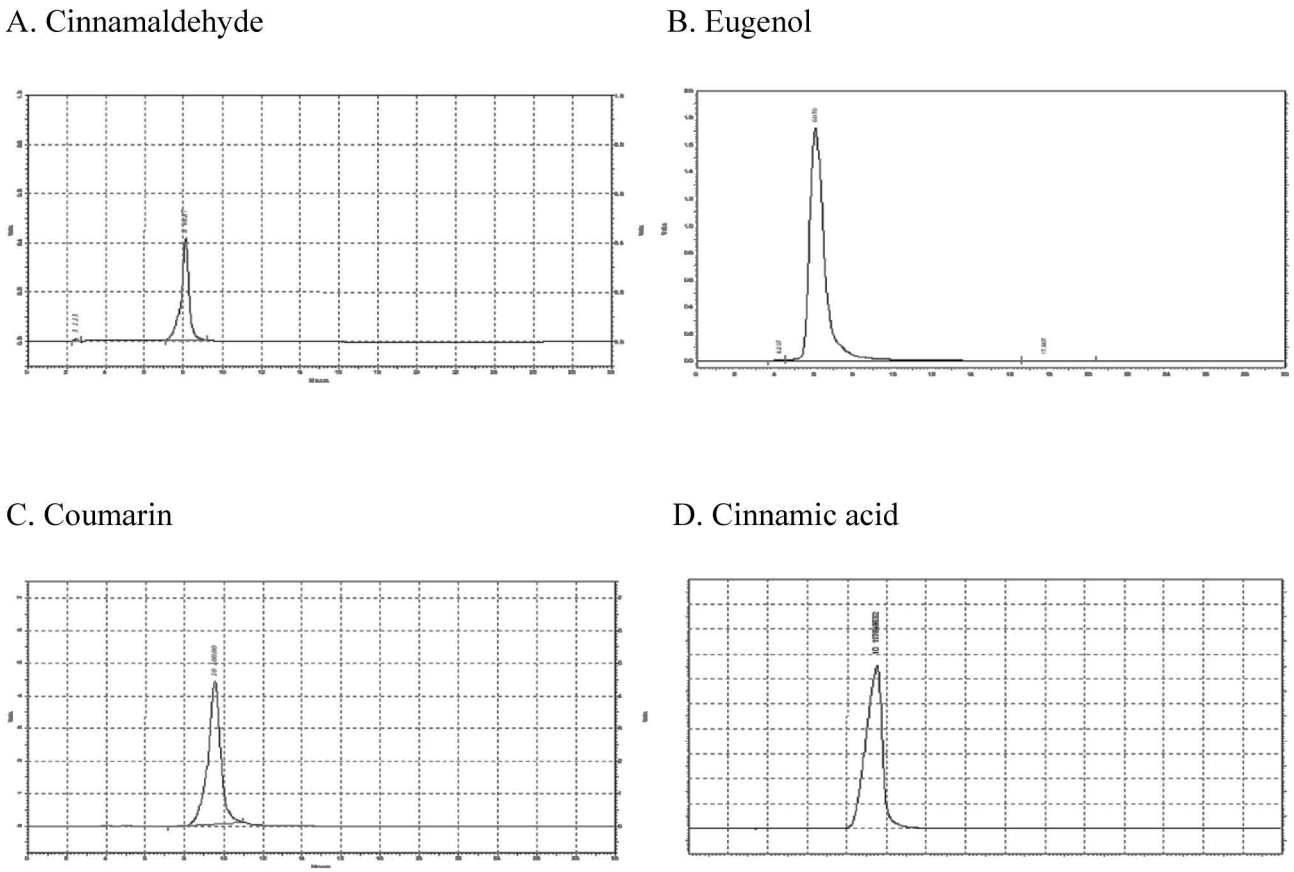


Fig. 1. Chemical Structures of the Compounds Isolated from CR

A, Cinnamaldehyde; B, Eugenol; C, Coumarin; D, Cinnamic acid.

***In Vitro* Assay** Antioxidant activity, acid-neutralizing capacity and anti-*H. pylori* activity of CR 70 % ethanol extract and its constituents were monitored according to the previously study.[11] Briefly, antioxidant activity was determined by the scavenging of DPPH free radical, NO-radical[12] and superoxide anion radical.[13] The growth inhibition of *H. pylori* was investigated by colony counts. Ampicillin was used as a positive control. Acid-neutralizing capacity was determined by titrating with 0.1 N NaOH using methyl orange as an indicator. Hydrotalcite was used as a positive control.

***In Vivo* Assay** The rats administered with eugenol and cinnamic acid were examined by HCl/ethanol-induced gastric lesion,[14] gastric secretion[7] and mucus secretion[15] according to the previously study[11]. Cimetidine was used as a positive control



Supplemental Data to Fig. 1. Chromatographic Profiling of C. Ramulusconstituents

These compounds were analyzed by HPLC using Nova pack C18 (2.1×150 mm) column. These peaks were detected at 254 nm (UV). The condition was as in the following: A. Mobile phase was ACN:H_2O (45:55) and flow rate was 0.5 ml/min. B. Mobile phase was ACN:H_2O (65:35) and flow rate 0.5 ml/min. C. Mobile phase was ACN:H_2O (65:35) and flow rate 0.5 ml/min. D. Mobile phase was ACN:H_2O (65:35) and flow rate 0.4 ml/min.

drug. In mucus secretion assay, sucralfate was used as a positive control. Mucus contents were expressed as microgram of alcian blue determined by standard plots.

RESULTS AND DISCUSSION

In Vitro Study

Antioxidant Activity of CR Extract To investigate the scavenging free radicals by CR ethanolic extract and its constituents, the dose-response relationship was studied (Table 1). CR ethanolic extract and its constituents had the hydrogen-donating activity to DPPH radicals in concentration-dependent manner. IC_{50} value of the reference antioxidant ascorbic acid was found experimentally to be $<1\ \mu g/ml$. CR ethanolic extract and eugenol showed a significant free radical scavenging activity with $IC_{50}<9\ \mu g/ml$. However, cinnamaldehyde, cinnamic acid, and coumarin had low antioxidant activity with an $IC_{50}>300\ \mu g/ml$, respectively. The quenching activity for nitric oxide radicals was consistent to DPPH radical scavenging activity results. IC_{50} value of CR ethanolic extract for NO radicals was investigated to be $52.88\ \mu g/ml$, and eugenol exerted a potent NO radical quenching action with $IC_{50}\leq 9\ \mu g/ml$. In addition, coumarin showed $IC_{50}=27.25\ \mu g/ml$ of NO radical scavenging. Otherwise, the superoxide radical scavenging activity of CR ethanolic extract and its constituents was weak as compared with DPPH and NO radical scavenging.

Gastric cell and the stomach-tissue injury in acute and chronic inflammation are due to the toxicity of reactive oxygen species (ROS) generated in stomach.[16] It has been known that several kinds of free radicals are generated in the peptic ulcer and gastritis, but its mechanism is unclear. ROS play an important role in the progress of the injury in the digestive system,[17] and are involved in the pathogenesis of ischemic injury of gastrointestinal mucosa and in other models of mucosal damage induced by non-steroidal anti-inflammatory drugs, ethanol, and *H. pylori*.[18] The intake of ascorbic acid reduces the gastric cancer risk in a dose-dependent way, and levels of the ascorbic acid are lower than in the normal population in *H. pylori*-infected patients. The retardation of ascorbic acid for gastric cancer risk might be due to antioxidative function quenching harmful ROS in stomach. ROS also promote mucosal damage by causing degradation of the epithelial basement membrane components, complete alteration of the cell metabolism, and DNA damage. Taken together, it is speculated that CR extract and its constituents had the protective effects as a radical scavenger.

Table 1. Free Radical Scavenging Activities of CR Ethanol-extract and Its Constituents

Material	IC_{50} ($\mu g/ml$)		
	DPPH	NO	Superoxide radical
CR 70% ethanol extract	<9	52.88	>300
Cinnamaldehyde	>300	>300	>300
Eugenol	<9	<9	>300
Cinnamic acid	>300	>300	>300
Coumarin	>300	27.25	>300
Ascorbic acid	<1	<1	<1

Anti-*H. pylori* Activity of CR Extract *Helicobacter pylori* is the major microorganism for bacterial gastrointestinal infections, peptic ulcer, and gastric cancer. The acute or chronic inflammation and the denaturation of epithelial cells at mucous membrane cause the loss of gastric mucus and gastric cell death in *H. pylori*-infected patients. The inflammation and toxic substances by *H. pylori* cause the damage of gastric epithelial cells. CR ethanolic extract and its constituents were used to run for *in vitro* experiments on anti-bacterial activity against *H. pylori*. CR ethanol-extract completely inhibited the colonization of *H. pylori* at $100\ \mu g/ml$ concentration, and this effect was equivalent to that of ampicillin ($10\ \mu g/ml$), as shown in Table 2. Among CR constituents, coumarin, eugenol, and cinnamaldehyde had relatively a potent anti-*H. pylori* activity.

Antibiotic therapy through the combination of several drugs has been widely used to eradicate *H. pylori* infections. However, the drug-resistant bacteria need the novel anti-bacterial drugs, and the plants seem to be the ideal sources of novel antibacterial compounds. It is worthy of notice that CR ethanol-extract and its constituents killed the *H. pylori* cells *in vitro* ($<100\ \mu g/ml$) (Table 2). Although the relatively high concentration of CR ethanol-extract and its constituents was needed for the suppression of *H. pylori* growth, the CR ethanol-extract and its constituents are considered to be more effective in use, because the CR extract has extra protective functions for stomach as well as antibacterial effect. The acute gastritis by *H. pylori*-infection is associated with the development of hypochlorhydria, which is a pheno-

Table 2. Inhibitory Activities of CR Ethanol-extract and Its Constituents on the Colonization of *H. pylori*

Material	Dose (μg/ml)	Colonization
Control	—	###
CR 70% ethanol extract	10	##
	50	+
	100	—
Cinnamaldehyde	1.32	##
	6.6	+
	13.2	—
Cinnamic acid	1.48	##
	7.4	##
	14.8	##
Eugenol	1.64	##
	8.2	—
	16.4	—
Coumarin	1.46	##
	7.3	—
	14.6	—
Ampicillin	1	##
	10	—

###: colonies ($4-5\times10^5$ CFU); ##: colonies ($2-4\times10^5$ CFU); +: colonies ($0-2\times10^5$ CFU); —: none.

menon suspected to be caused by an infectious agent.

Additionally, *H. pylori*-infection increases the free radical production in the inflammatory region of stomach. A delicate balance between the free radicals and antioxidants like superoxide dismutase (SOD) and ascorbic acid is the important for physiological functions of the gastric cells. In the deficient antioxidant defenses, the abundantly produced free radicals can initiate lipid peroxidation and DNA damage, which leads to the cellular destruction, chromosomal aberration, and finally cancer. In fact, *H. pylori* were found in 70–90% of patients with gastric adenocarcinoma.[19] Furthermore, the epidemiologic features of gastric adenocarcinoma and *H. pylori*-infection are similar, so that *H. pylori*-infection now is believed to be a risk factor for gastric adenocarcinoma.[20] Therefore, in patients with mucosal *H. pylori*-infection, the eradication of this microorganism is necessary to cure both infection and ulcer disease.[21] Taken together, the anti-bacterial activity of CR extract may suppress several *H. pylori*-caused patho-physiological responses, such as the decrease of ascorbic acid secretion, gastric ulcer, the decrease of resistance for gastric carcinogens, and the induction of mucosal hyperproliferation.

Acid-neutralizing Capacity (ANC) of CR Extract

Table 3. Acid Neutralizing Capacity of CR Ethanol-extract and Its Constituents

Material	NaOH consumption volume (μl)	Inhibition (%)
Control	120.0±1.00	—
CR 70% ethanol extract	105.7±1.15*	11.9
Cinnamaldehyde	120.0±1.00	0
Eugenol	113.3±1.53	5.6
Cinnamic acid	113.3±1.53	5.6
Coumarin	114.0±1.73	5.0
Hydrotalcite	10.0±0.77**	91.7

The values are mean±S.E.M. of 6 animals. Significant difference, *$p<0.05$, **$p<0.001$, compared to the control.

The CR ethanol-extract inhibited 11.9% of NaOH-consumption volume as compared to control, whereas cinnamaldehyde, eugenol and coumarin were the low inhibitory action (Table 3). ANC of CR extract is expected to exert the protective influence on stomach damage by secretion of gastric acid, when CR extract is taken in a long period. Generally the ant-acids are effective in accelerating healing of duodenal and gastric ulcers, and the ulcer healing action by ant-acids is due to the neutralization of gastric luminal acid.[22]

In present study, the ANC of CR extract and its constituents imply the increase of pH in gastric content *in vitro*. This result suggests that the CR extract may contribute for gastric protection, and the acid-neutralizing activity works in a variety of conditions, such as the stomach upset, heartburn, the non-ulcer dyspepsia, and the verified gastric and duodenal ulcers. Although CR ethanol-extract and its constituents had less acid-neutralizing capacity than hydrotalcite, it is expected it might be helped for the stomach protection as nutraceuticals.

In Vivo Study

Effects of Eugenol and Cinnamic Acid on HCl/Ethanol-induced Gastric Lesions The HCl/ethanol-induced gastric mucosal damage is associated with the overproduction of free radicals, which lead to an increase of lipid peroxidation.[23] Ethanol induces both wide ulcers and petechial lesions within a relatively short time, which makes this technique suitable for screening methods for investigation of anti-ulcer drugs.[24] The mechanism of ethanol-induced gastric lesions varies in the depletion of gastric mucus content, damaged mucosal blood flow, and mucosal cell injury. This action may be related to an antacid

effects or cytoprotective properties for the gastric mucosa in ethanol-induced gastric lesion in mice. Also, it is suggested to be due to the activation of cellular protection, the reduction of mucosal prostaglandins, and the reduction of gastric vascular permeability.[25]

The effects of eugenol and cinnamic acid on the HCl/ethanol-induced lesion were investigated in this study. Intragastric administration of HCl/ethanol (60% in 150 mM HCl) made gastric tissues thinner and fainter, and multiple band-like lesions in the gastric mucosa, being 87.0±13.51 mm of the lesion index (Table 4), whereas any gastric lesions were not observed in the normal mice (data not shown).

The oral administration of eugenol and cinnamic acid diminished HCl-induced gastric lesions in mice at significant difference. And it is anticipated that this action might be due to the inhibition on cytotoxicity of HCl as attack factor or the stimulation of gastric mucus as defense factor. The severity of these lesions was dose-dependently reduced by *p.o.* administration of eugenol and cinnamic acid. Especially eugenol (100 mg/kg) inhibited approximately 65.8% of HCl/ethanol-induced gastric lesions, and was superior to cimetidine (200 mg/kg), a positive control (approximately 47.5% inhibition). Additionally the pretreatment of mice with cinnamic acid reduced approximately 11.5 and 42.8% of gastric lesions at 50 and 100 mg/kg dose, respectively.

Table 4. Effect of CR Constituents on HCl/Ethanol-induced Gastric Lesion

Material	Dose (mg/kg)	Lesion index (mm)	Inhibition rate (%)
Control		87.0±13.51	—
Eugenol	50	56.7±9.29*	43.9
	100	29.8±11.59***	65.8
Cinnamic acid	50	77.0±10.52	11.5
	100	49.8±11.10*	42.8
Cimetidine	200	45.7±0.30**	47.5

The values are mean±S.E.M. of 6 animals. Significant difference, *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to the control.

From this result, eugenol and cinnamic acid showed anti-ulcer activity, and especially against HCl/ethanol induced ulcers in rats dose-dependently. Therefore this result implies that CR has effective anti-ulcer activity against HCl/ethanol-induced stomach lesions.

Effects of Eugenol and Cinnamic Acid on Gastric Secretion We measured the gastric-juice parameters, such as gastric volume and pH, after submitting the rats to pylorus ligature with or without the eugenol and cinnamic acid intraduodenally. Doses of eugenol and cinnamic acid were selected based on suppression of HCl-ethanol-induced gastric lesion (Table 4). The effects of eugenol and cinnamic acid on gastric secretion, pH, and acid output in pylorus-ligated rats were showen in Table 5. The amount of gastric secretion by eugenol was approximately 3.6 ml, which were less than that of control (4.2 ml), whereas the amount of gastric secretion by cinnamic acid was approximately 5.2 ml. Eugenol and cinnamic acid increased the hydrogen ion concentration to 1.2 and 1.0 in gastric juice, respectively, as compared to control (pH 1.38). Eugenol showed the decline of total acid output to 0.37 mEq/4 h, as compare to control (0.38 mEq/4 h) without significantly difference.

Taken together, though eugenol and cinnamic acid did not inhibit effectively gastric secretion, it was expected to suppress the attack factor through the reduction of gastric secretion, and to play important role to anti-gastric effect, including the reduction of lesion index *in vivo*.

Effects of Eugenol and Cinnamic Acid on Mucus Secretion Induced Absolute Ethanol In the mucus secretion model, even though ethanol induces to

Table 5. Effect of CR Constituents on Gastric Secretion in Pylorus-ligated Rats

Material	Dose (mg/kg)	Volume (ml)	pH	Total acid output (mEq/4 h)
Control		4.2±1.2	1.38±0.8	0.38±0.16
Eugenol	100	3.6±1.9*	1.20±0.2	0.37±0.23
Cinnamic acid	100	5.2±1.2*	1.00±0.2	0.50±0.22*
Cimetidine	150	1.7±0.5***	3.50±0.8**	0.22±0.13*

Total gastric juice volume and pH were measured at 4 h after the pylorus-ligation. The values are mean±S.E.M. of 6 animals. Significant difference, *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to the control.

Table 6. Effect of CR Constituents on Mucus Contents from Absolute Ethanol-induced Gastric Lesion in Rats

Material	Dose (mg/kg)	Mucus content (μg as alcian blue)
Control		173.9±7.68
Eugenol	100	183.5±8.14*
Cinnamic acid	100	213.4±2.52**
Sucralfate	375	160.4±6.72*

Control means the absolute ethanol-induced rats orally. The values are mean±S.E.M. of 6 animals. Significant difference, *$p<0.05$, **$p<0.01$, compared to the control.

reduce the amount of mucus secretion in the rats, eugenol and cinnamic acid enhanced the mucus secretion. Eugenol and cinnamic acid (100 mg/kg) significantly increased the mucus content to approximately 183.5 and 213.4 μg, respectively, which was superior to that of control (173.9 μg) by the induction of absolute ethanol (Table 6). Eugenol and cinnamic acid might protect stomach from the attack-factor by increase of mucus secretion, as well as by the suppression of HCl/ethanol-induced gastric lesions. The amount of mucus secretion by sucralfate, an effective medicine for anti-ulcer, was lower than that of control group. The reason might be expected to be a protective action of sucralfate for stomach damage through sucralfate-coating, not to be the increase of mucus secretion in response to ethanol irritation.

Taken together, CR ethanol-extract showed the antioxidant activity, acid-neutralizing capacities, and the partial inhibition of *H. pylori*. Eugenol and cinnamic acid, which were isolated from CR, exhibited the potent inhibitory activity against HCl/ethanol-induced gastric lesion and the reduction of gastric secretion. Therefore, CR ethanol-extract, eugenol, and cinnamic acid are expected to have potential protective effect against gastritis.

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