BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 19(4), 487-492 (2011)

Effects of *Chenopodium album* Linne on Gastritis and Gastric Cancer Cell Growth

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Abstract

In our previous study, we investigated *Chenopodium album* Linne (CAL) ethanol extract and its fractions on anti-gastritic actions using the HCl/ethanol and indomethacin induced gastric lesion model and *Helicobacter pylori* (*H. pylori*). Based on the results, butanol fraction was most effective among fractions obtained from CAL. This study aims to elucidate the mechanisms of butanol fraction, and betaine as a constituent of the butanol fraction, on gastritis and anti-gastric cancer cell growth. First, we examined antioxidant properties using hydrogen peroxide and superoxide radical, and we found that butanol fraction and betaine may be good antioxidants. Second, cytotoxicity was assessed by measuring cell viability and 4,6-diamidino-2-phenylinodole dihydrochloride (DAPI) staining of human gastric cancer cells (AGS cells). We also examined the relationship between the cytotoxicity and intracellular Ca^{2+} signaling mechanism. The butanol fraction demonstrated cell viability 71.49% at the concentration of 100 μg/ml and increased intracellular Ca^{2+} concentration in a dose dependent manner. Finally, we observed the mucus content as a defensive factor and gastric secretion as an aggressive factor, and found that the mucus content noticeably increased when treated with butanol fraction and betaine and gastric secretion decreased when treated with betaine *in vivo* study. From these results, we suggest that CAL butanol fraction and betaine may have protective effects on gastritis.

Key Words: *Chenopodium album* Linne, Betaine, Anti-gastritis, Gastric cancer cell growth

INTRODUCTION

In our previous study, we investigated anti-gastritis and anti-*Helicobacter pylori* (*H. pylori*) effects of *Chenopodium album* Linne (CAL) ethanol extract and its fractions. CAL has long been used as a folk remedy due to its effectiveness in treating various illnesses such as neuralgia, gastralgia, and hepatocirrohsis (Kim and Jeong, 2010). We have found that butanol fraction was the most effective in inhibiting HCl/ethanol and indomethacin induced gastric lesions among fractions obtained from CAL leaves. The aim of this study is to elucidate the protective mechanisms of butanol fraction and betaine as a constituent (Fig. 1). Betaine is widely distributed in microorganisms, plants, and animals (Craig, 2004) and also called trimethylglycine, glycine betaine, lycin or oxyneurine. In addition, it can promote insulin release by stimulating the Langerhans islets in the pancreas and help treat alcoholic fatty liver. According to the other study, they found that betaine reduces the concentration of cholesterol in the blood by influencing the synthesis of low density lipoprotein cholesterol (LDL). Traditionally, betaine has frequently been used as a treatment for hepatic disorders (Bessieres *et al.*, 1999; Janssens *et al.*, 1999; Lee *et al.*, 2004; Jeong *et al.*, 2010).

Free radicals could cause tissue injuries, carcinogenesis, inflammation, and aging (Oh *et al.*, 2010). Recently, free radicals have been getting attention as a common mechanism of related injuries of gastric mucosa.

Intracellular Ca^{2+} signal appears to be commonly involved in the mechanism of cell death (McConkey and Orrenius, 1996). So, we examined the relationship between the cytotoxic action and intracellular Ca^{2+} signaling mechanisms. For this, we measured the change of intracellular Ca^{2+} concentration using Fura-2 fluorescence technique. Gastric ulcers and gastritis seem to be caused by over-secretion of gastric juice. Also mucin has a gastroprotective activity. We observed the mucus content as a defensive factor and gastric secretion as an aggressive factor using the rats.

The aim of this study was to investigate the mechanism of anti-gastritis and anti-gastric cancer cell growth of the CAL butanol fraction and betaine by measuring their anti-oxidant activities, cytotoxicity to the gastric cancer cells, intracellular Ca^{2+} concentration, the mucus content, and gastric secretion.

www.biomolther.org

Open Access **http://dx.doi.org/10.4062/biomolther.2011.19.4.487**

pISSN: 1976-9148 eISSN: 2005-4483

Received Aug 24, 2011 **Revised** Oct 1, 2011 **Accepted** Oct 5, 2011

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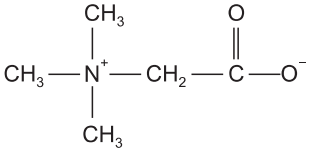

Fig. 1. Chemical structure of betaine.

MATERIALS AND METHODS

Reagents

Dimethyl sulfoxide (DMSO), fura-2AM, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue, probenecid, dantrolene sodium, sodium bicarbonate, dimethyl sulfoxide, 4,6-diamidino-2-phenylinodole dihydrochloride (DAPI), cimetidine were obtained from Sigma (Sigma-Aldrich Inc., MO, USA). Cell culture media and reagents, including Hank's balanced salt solution, RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA were obtained from GIBCO (Invitrogen Inc., NY, USA). Other solvents were purchased from Duksan pure Chemical Co. Ltd. (Kyunggi-do, Korea). All other reagents were of pharmaceutical or analytical grade.

Laboratory equipments

Equipments included: pH meter (IQ Scientific Instruments, Inc.), clean Bench (Johnsam Co.), CO_2 incubator (Forma Scientific), water bath (Vision), inverted microscope (Olympus), autoclave (Duksan Chem. Co.), micropipette (Gilson Co.), centrifuge 5810R (Eppendorf), high speed centrifuge (Sorvall RT-6000), liquid nitrogen Dewars (CHART MVE), fluorescence spectrophotometer (HITACHI), UV-spectrophotometer (Agilent Technologies Inc., CA, USA), UV-spectrophotometric plate reader (ASYS UVM340), etc.

Animals

Male Sprague-Dawley rats, weighing 190-200 g, were purchased from Samtako, Kyunggi-do, Korea, and were acclimatized to standard laboratory conditions (22 ± 2°C, 55 ± 5% humidity and 12 h light/dark cycle) for 14 days in the animal facility in Duksung Women's University. The experimental procedures for rats were conducted in accordance with the Guidelines of the Care and Use of Laboratory Animals, Duksung Women's University (2009-03-009). The animals were allowed free access to food (standard pellet diet) and water ad libitum. The entire study was conducted in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 1999-61) and the Good Laboratory Practice Regulations for Non-clinical Laboratory Studies (Notification No. 2000-63) issued by the Korea Food and Drug Administration.

Cell culture

AGS gastric cancer cells were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured with RPMI-1640 containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 lg/ml) in a 5% CO_2 humidified incubator at 37°C. For subculture, 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.

Antioxidant effects

Hydrogen peroxide scavenging: Hydrogen peroxide scavenging activity is assessed according to the modified method of Ilhami (2006). CAL butanol fraction and betaine were mixed with 0.6 ml of hydrogen peroxide solution (40 mM in phosphate buffer, pH 7.4). After 10 minutes, the absorbance of the mixture was measured at 230 nm. The data is presented as hydrogen peroxide scavenging activity (%) values calculated using the formula $[(A_c-A_s)/ A_c]\times100$. A_c and A_s are the mean absorbance values of the control and the sample respectively.

Superoxide radical scavenging: The superoxide radical scavenging activity was determined according to the method of Nishikimi (1972). 0.1 ml of Tris-HCl buffer (pH 8.5) and 0.2 ml of 100 μM phenazine methosulfate (PMS) were added to 0.5 ml of CAL butanol fraction and betaine solution. The absorbance of the mixture was determined at 560 nm using UV-spectrophotometry (S_0). 0.2 ml of 500 μM nitro bluetetrazolium (NBT) and 0.4 ml of 500 μM (β-nicotinamide adenine dinucleotide) NADH were continuously added to the mixture. The absorbance of the mixture was determined at 560 nm again (S). C_0 and C were obtained from control. The data is assessed as superoxide radical scavenging activity (%) values calculated using the formula $[\{(C-C_0)-(S-S_0)\}/(C-C_0)]\times100$.

Cytotoxicity assay

MTT assay: Cytotoxicity to AGS cells (gastric cancer cell lines) was examined using the modified MTT assay. Cells were seeded at a density of 1×10^4 cells/well in 96-well culture plates (Corning Inc., USA), and were cultured for 24 hours at 37°C in a 5% CO_2 humidified incubator. The samples were added to the plate in the manner of serial dilution and incubated for 24 hours. MTT agent was added at a final concentration of 0.5 mg/ml and the plates were incubated for 4 hours at 37°C. After discarding all media from the plates, 0.1 ml of DMSO was added to all wells. The plates were held for 5 minutes at room temperature with shaking in order to completely dissolve formazan. The absorbance of the MTT formazan was determined at 540 nm using a UV-spectrophotometric plate reader (Choi *et al.*, 2004).

DAPI staining: DAPI staining was performed to observe the change in a cell nucleus. AGS gastric cancer cells were seeded at a density of 2×10^5 cells/ml onto a 60 mm cell culture dish 24 hours before the drug treatment. Then, cells were cultured for 24 hours with 300 and 200 μg/ml samples respectively. After treatment, cells were fixed with 4% *p*-formaldehyde solution for 20 minutes, stained with 4 μg/ml DAPI for 10 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy.

Measurement of intracellular Ca^{2+} concentrations: Intracellular Ca^{2+} concentrations were measured using a modified version of the method described by Kim *et al.* (2003). Aliquots of the AGS cells were washed in PBS. Then, 5 μM Fura-2/AM was added, and the cells were incubated for 1 hour at 37°C. Unloaded Fura-2/AM was removed by centrifugation at 2,000 rpm for 3 minutes. Cells were resuspended at a density of 2×10^6 cells/ml in Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 4°C for 30 minutes before the fluorescence measurement. For each experiment, 0.5 ml aliquot of Fura-2-loaded cells was equilibrated to 37°C in a stirred quartz cuvette.

Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. In the data intracellular free Ca^{2+} concentrations are presented through the 340:380 nm fluorescence ratios.

In vivo experiments

Mucus content by the absolute ethanol induced gastric lesion in rats: Using the method of Kitagawa *et al.*(1986), rats were fasted for 24 hours with free access to water before experiment. The samples were administered orally to the rats. 30 minutes later, the absolute ethanol (1 ml/100 g) was given orally for induction of gastric lesions in rats. One hour later, the animals were killed and the secreted mucus was determined. The glandula 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 hours. The mucus combined with the alcian blue was extracted with 20 ml of 70% ethanol containing 30% dioctyl sodium sulfosuccinate. The optical density of the supernatant was measured at 620 nm in an UV spectrophotometer.

Gastric secretion: After 24 hours of fasting, with free access to water prior to the experiment, the rats were immediately administered with samples intraduodenally (Shay *et al.*, 1945). 4 hours after the pyloric ligation, the animals were killed, and the contents of the stomach were collected and centrifuged at 3,000 rpm for 10 minutes. The total volume of gastric juice and pH were measured, and an acid output (mEq/ml) was determined by titrating the gastric juice with 0.1 N NaOH using phenol red as an indicator.

Statistical analysis

All experiments were performed in triplicate. Data was analyzed using the Student's *t*-test. *p*-values <0.05 were considered statistically significant.

RESULTS

Antioxidant effects

A reduction in reactive oxygen species (ROS) protects against gastritis (Wang *et al.*, 2005). For example, ascorbic acid known as antioxidant plays important roles in preventing the development of gastritis and gastric cancer (Block, 1991 and Block G *et al.*, 1991). Hydrogen peroxide (H_2O_2) is not a

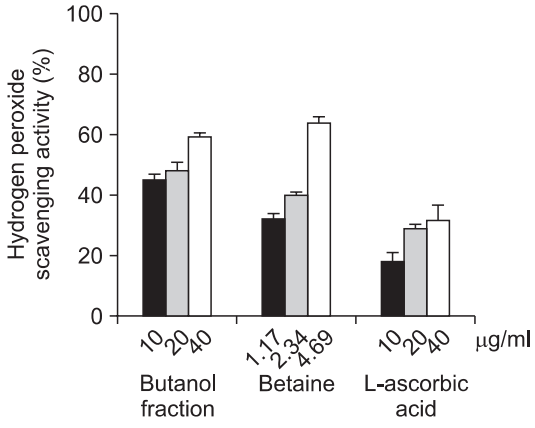

Fig. 2. Hydrogen peroxide scavenging activity of butanol fraction and betaine. The values are means ± S.E.

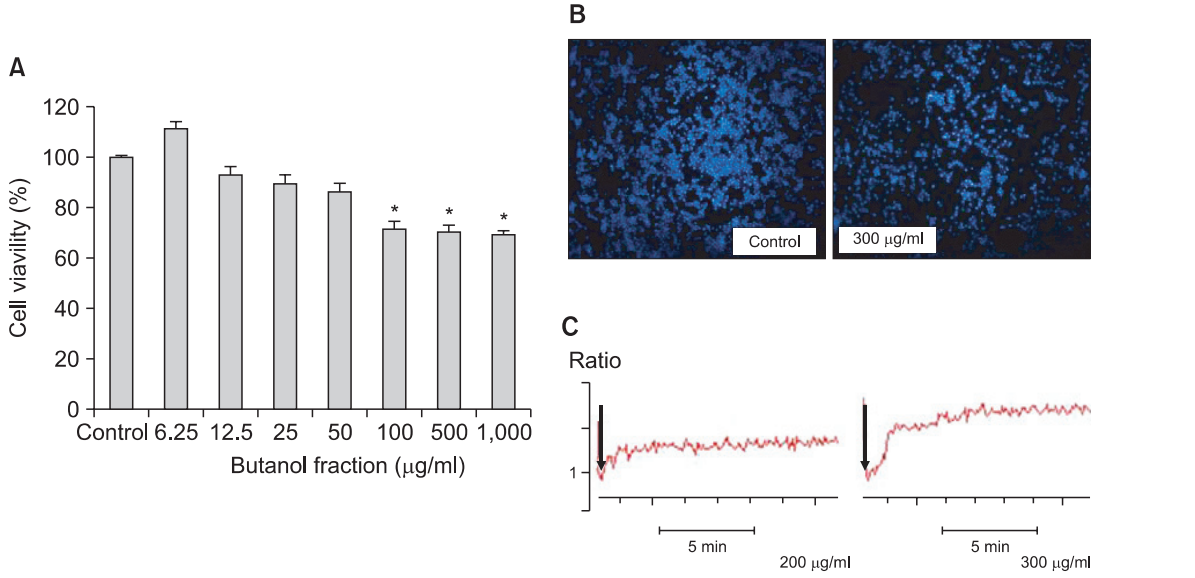

Fig. 3. MTT assay, DAPI staining, Intracellular Ca^{2+} concentration of *Chenopodium album* butanol fractions against AGS cells. (A) Cell viability. The values are means ± S.E. *p<0.05 compared to the control group. (B) The change in a cell nucleus. Morphological change: Control is observed with rounded and bright nuclei. Butanol fraction (300 μg/ml) showed condensed, fragmented nuclei and enriched chromatin. (C) Intracellular Ca^{2+} concentration. Internal Ca^{2+} release mechanism mediates increased intracellular Ca^{2+} concentration. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique. The data represent intracellular Ca^{2+}changes with time. The arrow shows the time point for addition of butanol fraction of CAL.

Table 1. Superoxide radical scavenging activity of butanol fraction and betaine

Material	Concentration (μg/ml)	Scavenging activity (%)
Butanol fraction	10	22.13 ± 3.50
	20	23.58 ± 3.60
	40	24.73 ± 4.20
	80	24.30 ± 1.80
	160	27.61 ± 1.89
	320	59.63 ± 15.54
Betaine	1.17	12.82 ± 13.18
	2.34	13.59 ± 14.12
	4.69	14.47 ± 14.52
	9.37	13.05 ± 15.91
	18.74	14.75 ± 18.19
	37.49	37.58 ± 31.17
L-Ascorbic acid	10	24.59 ± 7.54
	20	26.03 ± 2.61
	40	31.36 ± 10.06
	80	39.44 ± 4.77
	160	57.75 ± 4.54
	320	82.26 ± 5.76

The values are means ± S.E.

Table 2. The effect of butanol fraction and betaine on mucus content from absolute ethanol induced gastric lesion in rats

Material	Dose (mg/kg)	Mucin content	Alcian blue (μg/ml)
Control	−	−	292 ± 16
Butanol fraction	300	+++	319 ± 26
Betaine	100	+++	382 ± 49*
Sucralfate	375	+++	192 ± 35*

The values are means ± S.E.
*p<0.01 compared to the control group (n=6).

Table 3. The effect of butanol fraction and betaine on gastric secretion in pylorus-ligated rats

	Dose (mg/kg)	Volume (ml)	pH	Total acid output (mEq/4 hrs)
Control	−	4.2 ± 1.2	1.3 ± 0.8	0.38 ± 0.16
Butanol fraction	300	3.7 ± 0.7	1.1 ± 0.5	0.43 ± 0.14
Betaine	100	3.3 ± 0.7	1.4 ± 0.6	0.32 ± 0.08
Cimetidine	150	1.7 ± 0.5	3.5 ± 0.8	0.22 ± 0.13*

The values are means ± S.E. *p<0.01 compared to the control group (n=6).

free radical, but H_2O_2 can produce the highly toxic hydroxyl radical (· OH) by penetrating through the cell membrane and reacting with metallic ion like a Fe^{2+}. H_2O_2 scavenging activity is assessed by measuring the ability of inhibiting oxidation through reducing involved peroxidase (Finkel *et al.*, 2000). In the hydrogen peroxide scavenging activity test, butanol fraction and bataine showed higher scavenging activity than L-ascorbic acid well-known as antioxidant. Both butanol fraction and betaine showed scavenging activity in a concentration dependent manner (Fig. 2).

Butanol fraction and betaine had little lower superoxide radical scavenging activities than positive control (82.26%), but both butanol fraction and betaine had antioxidant activities of 59.63% and 37.58 %, respectively, at the dose of 320 μg/ml and 37.49 μg/ml (Table 1). Terminating chain reaction of free radicals caused these results.

Cytotoxicity

Cytotoxicity of the butanol fraction was assessed by measuring the cell viability of AGS human gastric cancer cell line. The butanol fraction demonstrated cell viability 71.49% at the concentration of 100 μg/ml. The concentration of 100, 500, and 1,000 μg/ml particularly showed significant cytotoxicity. We visualized the cytotoxicity through the DAPI staining method at the concentration of 300 μg/ml. Control is observed with rounded and bright nuclei, but butanol fraction treated sample showed condensed and fragmented nuclei or enriched chromatin. To elucidate the relationship between cytotoxicity and concentration of intracellular Ca^{2+}, we treated 200 and 300 μg/ml of butanol fraction in AGS cells. Treating the butanol fraction had an increased intracellular Ca^{2+} concentration in a dose dependent manner. In these results, we suggest that increasing the intracellular Ca^{2+} concentration directly affects cytotoxicity such as cell death in AGS gastric cancer cells (Fig. 3).

Mucus content and gastric secretion in rats

In our previous study, an experiment observing the HCl/ethanol-induced gastric lesion (Mizui and Dodeuchi, 1983) was performed, and we found that butanol fraction decreased the lesion index by approximately 47.4% (Kim and Jeong, 2010). To find the protective mechanism on gastric diseases including gastritis or gastric ulcer, the secreted mucus was determined (Table 2) in this study. After the administration of absolute ethanol, mucus contents of butanol fraction (300 mg/kg) and betatin (100 mg/kg) were 319 ± 26 and 382 ± 49 μg/ml, respectively, compaired with the control (292 ± 16 μg/ml). We observed noticeably increased mucus content as a defensive factor when treated with butanol fraction and betaine. Sucralfat, however, decreases the mucus content. It appears that sucralfate inhibits gastric injuries by coating activities.

As shown in Table 3, the total acid ouput of butanol fraction (300 mg/kg) and betatin (100 mg/kg) were 0.43 ± 0.14 and 0.32 ± 0.08 mEq/4 hr, respectively. Butanol fraction did not reduce volume and total acid output or increase pH level. But betatin reduced volume and total acid output and elevated the pH.

DISCUSSION

Antioxidant action plays an important role for the inhibition of oxidation process, which is involved in the mechanism of

several gastric disorders including ulceration (La Casa *et al.*, 2000). Free radicals are almost scavenged naturally. When the free radicals are produced excessively, however, immune-related factors including lipid, protein, DNA, enzymes, and T-cell are damaged (Sternas *et al.*, 1999). And it is reported that free radicals could cause tissue injuries, carcinogenesis, inflammation, and aging (Oh *et al.*, 2010). Recently, the inflammatory reaction and existence of free radicals have been getting attention as a common mechanism of related injuries of gastric mucosa.

Gastric secretion is caused by various intrinsic and extrinsic factors, along with the free radicals, that affect gastritis or gastric ulcers directly. Three important factors are acetylcholine, gastrin, and histamine. Histamine 2 receptor antagonists, including cimetidine or ranitidine, particularly inhibit the gastric secretion (Waisberg, 2005). When gastric acid is secreted from the stomach wall, intracellular roll of cAMP and Ca^{2+} is important (Dethloff, 1998).

In this study, we chose CAL, as it known for its protective function of stomach and liver as a folk remedy. In the hydrogen peroxide scavenging activity test, both butanol fraction and betaine showed higher scavenging activities than L-ascorbic acid, which well-known as an antioxidant. In the superoxide radical scavenging activities, also, both butanol fraction and betaine had antioxidant activities. From results, we found that butanol fraction and betaine may be good materials for antioxidants though two experiments measuring the antioxidant activities.

And then, we investigated cytotoxicity on butanol fraction in gastric cancer cells. First, we performed the MTT assay using AGS human gastric cancer cells, and visualized it through the DAPI staining method. And to elucidate the relationship between cytotoxicity and concentration of intracellular Ca^{2+}, we measured the intracellular Ca^{2+} influx. With this result, we suggest that increasing the intracellular Ca^{2+} concentration directly affects cytotoxicity such as cell death or modification of cell shape in gastric cancer cells.

Also, to study the mechanism on gastric diseases in connection with the previous study, the mucus content and gastric secretion were determined *in vivo* study. Gastric ulcer seems to be caused from over-secretion and an imbalance of defensive and aggressive factor (McQuaid and Isenberg, 1992). Butanol fraction showed the augmentation of mucus content as a defensive factor and antioxidant activity. Betaine reduced aggressive factors (gastric volume and total acid output), elevated pH, increased mucus content and showed antioxidant activity.

The findings of this study support the conclusion that CAL butanol fraction protect against gastric disease through antioxidant activities, inhibition of gastric cancer cell growth, and increase of mucus content. Betaine protect against gastric disease through antioxidant activities, increase of mucus content and reduction total acidity. Therefore, CAL butanol fraction and betaine are expected to have protective effect against gastric disease.

ACKNOWLEDGMENTS

This research was supported by Duksung Women's University Research Grants 2010.

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