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ORIGINAL ARTICLE

Gastric Ulcer Healing Activity against Acidified Ethanol-Induced Gastric Ulcer and Gastroprotective Mechanisms of *Zingiber simaoense* Rhizome Ethanol Extract in Rats

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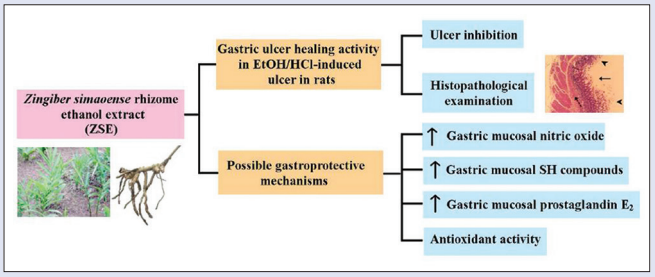
ABSTRACT

Background: *Zingiber simaoense* Y. Y. Qian (*Zingiberaceae*) rhizomes have been widely used to alleviate gastric disorders in Thai traditional medicine. **Objectives:** This study aimed to investigate the gastric ulcer healing activity of *Z. simaoense* rhizome ethanol extract (ZSE) against acidified ethanol-induced gastric ulcer and its possible gastroprotective mechanisms. **Materials and Methods:** The gastric ulcer healing activity of ZSE was evaluated using an acidified ethanol-induced gastric ulcer model in rats. The involvement of endogenous nitric oxide (NO) and sulfhydryl (SH) compounds in ZSE gastroprotection was also examined in addition to the determination of NO, malondialdehyde (MDA), and prostaglandin (PG) E_2 levels in rat gastric tissues as well as the determination of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and total phenolic content in ZSE. **Results:** ZSE at the dose of 240 mg/kg/day significantly accelerated gastric ulcer healing when observed on days 4 and 8 following ulcer induction. Pretreatment with either N^G-nitro-L-arginine methyl ester or N-ethylmaleimide inhibited the gastroprotective activity of ZSE. Moreover, ZSE significantly increased NO and PGE_2 levels and decreased MDA levels in rat gastric tissues. DPPH radical scavenging activity and total phenolic compounds were also presented in ZSE. **Conclusion:** This study demonstrates the gastric ulcer healing activity of ZSE against acidified ethanol-induced gastric ulcer in rats. The possible gastroprotective mechanisms underlying the cytoprotective effect of ZSE might also involve gastric mucosal NO, SH compounds, and PGE_2 as well as its antioxidant activities.

Key words: Gastric healing, gastroprotective, mechanism, rats, rhizome, *Zingiber simaoense*

SUMMARY

- Oral administration of *Zingiber simaoense* rhizome ethanol extract (ZSE) at the dose of 240 mg/kg/day significantly accelerated gastric ulcer healing when observed on days 4 and 8 following acidified ethanol-induced gastric ulcer induction in rats
- Pretreatment with either N^G-nitro-L-arginine methyl ester or N-ethylmaleimide inhibited the gastroprotective activity of ZSE
- Pretreatment with ZSE significantly increased NO levels to near-normal levels, diminished malondialdehyde production caused by EtOH/hydrochloric acid, and prevented the depleting effect of indomethacin on tissue prostaglandin E_2 levels
- On phytochemical screening, ZSE was found to contain phenolic compounds (flavonoids and tannins), and the antioxidant activity of these compounds was confirmed in 1,1-diphenyl-2-picrylhydrazyl assay.



Abbreviations used: COX: Cyclooxygenase; DPPH: 1,1-diphenyl-2-picrylhydrazyl; EtOH/HCl: Acidified ethanol; GAE: Gallic acid equivalent; HCl: Hydrochloric acid; HCO_3^-: Bicarbonate; H_2SO_4: Sulfuric acid; L-NAME: N^G-nitro-L-arginine methyl ester; MDA: Malondialdehyde; NaCl: Sodium chloride; NEM: N-ethylmaleimide; NO: Nitric oxide; NOS: Nitric oxide synthase; NSAIDs: Nonsteroidal anti-inflammatory drugs; PG: Prostaglandin; PPIs: Proton pump inhibitors; ROS: Reactive oxygen species; SH: Sulfhydryl; ZSE: *Zingiber simaoense* rhizome ethanol extract.

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INTRODUCTION

Peptic ulcer is one of the major common chronic digestive problems affecting humans worldwide, including Thailand.[1] Gastric acid, pepsin, alcohol, and drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs), can alter mucosal defensive and repair mechanisms including mucus, bicarbonate (HCO_3^-), prostaglandins (PGs), and epithelial renewal, leading to epithelial cell injury.[2-4] Proton pump inhibitors (PPIs) are the most commonly prescribed drugs for peptic ulcer because of their higher efficacy, although their weak points include side effects and drug interactions. Recent findings have shown an association between PPIs and increased risk of kidney damage, hip fracture, pneumonia, dementia, and gastric cancer.[5,6] In addition, the US Food and Drug Administration has issued a warning about an important adverse interaction between clopidogrel and PPIs.[5] Medicinal plants are attractive sources of new alternative compounds that may have potential

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to be developed into new anti-ulcer agents.[7-10] Many experimental and clinical studies have demonstrated that some herbal medicines for peptic ulcer treatment have comparable or superior efficacy with lower side effects when compared to conventional drugs.[11] The first effective drug developed from a medicinal plant for the treatment of peptic ulcer is carbenoxolone (from *Glycyrrhiza glabra*); however, its use is limited by side effects due to electrolyte disturbance.[12,13]

Zingiber is a genus of the family Zingiberaceae. The gastroprotective activity of *Zingiber* rhizomes has been shown in many studies.[14-17] *Zingiber simaoense* Y. Y. Qian is a widely distributed *Zingiber* that can be found in many regions of Thailand. The rhizome of *Z. simaoense* ("*Khing Krang*" in Thai) is a variety of Thai ginger that has been used in Thai traditional medicine to relieve symptoms in gastric disorders similar to the rhizome of *Zingiber officinale* ("*Khing*" in Thai).[18] Recently, the gastroprotective activity by pretreatment with *Z. simaoense* rhizome ethanol extract (ZSE) in experimental models in rats has been demonstrated.[19] However, the gastric healing activity of ZSE, which would be beneficial to provide a faster gastric ulcer healing rate than that of the natural healing process alone, was still unrevealed. As any ideal anti-ulcer agents should be effective for both the prevention and treatment of peptic ulcer, in the present study, we aimed to investigate the gastric ulcer healing activity of ZSE against acidified ethanol (EtOH/hydrochloric acid [HCl])-induced gastric ulcer in rats. In addition, further investigations of the possible mechanisms of its gastroprotection were also performed.

MATERIALS AND METHODS

Plant material and extraction

Rhizomes of *Z. simaoense* were collected in March 2014 from Chiang Rai Province, Thailand. Plant identification and authentication were done by a botanist at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The voucher specimen (no. 147) has been deposited at the School of Medicine, Mae Fah Luang University, Thailand. Preparation of ZSE can be described briefly as follows: air-dried (at room temperature) powdered rhizome was macerated in 95% ethanol overnight followed by filtration through a filter paper. The filtrate was concentrated at 55°C under reduced pressure using a vacuum rotary evaporator and then lyophilized to obtain ZSE. The extraction yield was 6.59% (w/w).

Drugs and chemicals

Absolute ethanol, HCl, sodium dodecyl sulphate (SDS), acetic acid, pyridine, and sodium carbonate were purchased from VWR Prolabo BDH chemicals (Leuven, Belgium). Omeprazole, Tween 80, N^G-nitro-L-arginine methyl ester (L-NAME), N-ethylmaleimide (NEM), carbenoxolone, Griess reagent, sodium nitrite, 2-thiobarbituric acid, n-butanol, indomethacin, Bradford's solution, 1,1-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, and Folin–Ciocalteu's reagent were purchased from Sigma-Aldrich (Thailand) Co., Ltd. (Bangkok, Thailand). PGE_2 competitive Biotrak™ enzyme immunoassay kit was purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA).

Gas chromatography-mass spectrometry analysis

Phytochemical analysis of ZSE was conducted using gas chromatography-mass spectrometry (GC-MS) analysis. All conditions and procedures were the same as described by Baiubon *et al.*[19]

Phytochemical screening

Phytochemical screening was performed using standard procedures described by Sofowora[20] and Evans.[21]

Test for alkaloids

About 1.5 g of ZSE was stirred with 15 mL of 2 N HCl and 0.5 g of sodium chloride (NaCl) in a water bath for 10 minutes (min) and then filtered. The filtrate was placed into four test tubes, 0.5 mL/test tube. A few drops of Mayer's reagent, Dragendorff's reagent, Wagner's reagent, and Hager's reagent were added to the test tubes numbered 1–4, respectively. The appearance of cream (with Mayer's reagent), orange (with Dragendorff's reagent), red-brown (with Wagner's reagent), and yellow precipitate (with Hager's reagent) indicated the presence of alkaloids.

Test for tannins

About 1 g of ZSE was boiled with about 20 mL of distilled water in a test tube and then placed into four test tubes (2 mL/test tube). A few drops of 0.1% ferric chloride solution, 1% gelatin solution, 1% gelatin solution with 10% NaCl, and distilled water (negative control) were added to the test tubes numbered 1–4, respectively. The observation of brownish green or blue-black color indicated the presence of tannins.

Test for terpenoids

ZSE (0.5 g) was mixed with 2 mL of chloroform, and then 2 mL of concentrated sulfuric acid (H_2SO_4) was slowly added to form a layer. The appearance of reddish brown color indicated the presence of terpenoids.

Test for anthraquinones

About 0.5 g of ZSE was boiled with 10 mL of 5% H_2SO_4 and then filtered. Chloroform (5 mL) was then added to the filtrate, and the mixture was shaken. The chloroform layer was pipetted into another test tube, and 1 mL of 25% ammonia was added. Appearance of red color was taken as evidence of the presence of anthraquinones.

Test for flavonoids

ZSE (0.2 g) was dissolved in 10 mL of 50% methanol and then filtered. A few fragments of magnesium ribbon were added to the filtrate followed by a few drops of concentrated HCl. A yellow-orange coloration appeared after few minutes, which indicated the presence of flavonone, whereas a reddish color indicated the presence of flavonol.

Test for saponins

ZSE (0.5 g) was added to 5 mL of distilled water in a test tube. The solution was shaken vigorously for about 5 min. Stable persistent bubbles were taken as evidence of the presence of saponins.

Test for glycosides

One milliliter of ZSE (8% in methanol) was mixed with 1 mL of 1% 3, 5-dinitrobenzoic acid in methanol and 1 mL of 1 N potassium hydroxide. The immediate appearance of a violet color indicated the presence of glycoside in the extract.

Experimental animals

Male Sprague-Dawley rats weighing between 200 and 250 g were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, Thailand. They were kept in the animal room maintained under environmentally controlled conditions of 24°C ± 1°C, 50% ± 10% relative humidity, and a 12-hour (h) light/dark cycle for a minimum of 1 week before starting the experiments. They were fed with commercial rodent chow (Perfect Companion Group, Co., Ltd., Samut Prakan, Thailand) and tap water *ad libitum,* but the food and water were withdrawn 48 h[22-24] and 1 h, respectively, before the beginning of each experiment. All experimental procedures followed the International Guiding Principles for Biomedical Research

Involving Animals of the Council for International Organizations of Medical Sciences and were approved by the Animal Ethics Committee of Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (protocol number 20/2558).

Evaluation of gastric ulcer healing activity

The acute gastric ulcer healing model was performed with some modifications.[19,25-27] Rats were divided into two arms of five groups per arm and six rats per group. All were induced to have gastric ulcer by oral gavage of 1 mL EtOH/HCl (absolute EtOH and HCl in a ratio of 60:40, v/v). In the first arm (3-day treatment), at the 4th h after ulcer induction on day 1 and once daily on days 2 and 3, Groups I–V were treated (in an equivalent volume of 5 mL/kg) with 5% Tween 80 (control group), omeprazole 30 mg/kg (reference group), or ZSE 60, 120 and 240 mg/kg (test groups). All rats in the first arm were sacrificed on day 4, 18 h after the administration of the last dose. In the second arm (7-day treatment), all groups were treated similarly to the first arm except that the protocol was continued through day 7 and the rats were sacrificed on day 8. After stomach removal and opening, quantification of gastric lesions in each rat followed by calculation of the mean ulcer index of each group and the percentage of gastric healing was performed.[19,28] The entire stomachs were used for further histopathological evaluation. The stomachs were fixed in 10% neutral buffered formalin, serially cut longitudinally into eight continuous sections, and routinely processed with a semi-automated tissue processor before being embedded in paraffin. Four-micrometer-thick paraffin sections were taken and stained with hematoxylin and eosin prior to evaluation.

Investigations of possible mechanisms of gastroprotection

Involvement of endogenous nitric oxide and sulfhydryl compounds in gastroprotection

This experiment was performed following Arrieta *et al.*[29] and Caldas *et al.*[30] with slight modification. Fifty-four fasted rats were divided into three main groups (18 rats per group); each group was pretreated with intraperitoneal injection of normal saline solution, L-NAME (an inhibitor of nitric oxide synthase [NOS]) 70 mg/kg, or NEM (a sulfhydryl [SH] compound blocker) 10 mg/kg. Thirty minutes later, each main group was divided into three subgroups (six rats per group) and each subgroup orally received 5% Tween 80, carbenoxolone (100 mg/kg), or ZSE (120 mg/kg). One hour after that, all the rats were induced to have gastric ulcer by oral gavage of 1 mL EtOH/HCl and were then sacrificed 1 h later for gastric lesion examination.

Determination of nitric oxide concentration and lipid peroxidation product in gastric tissue

Twenty-four rats were divided into four groups (six rats per group). The normal group did not receive any test drug, whereas the control, reference, and test groups orally received 5% Tween 80, omeprazole 10 mg/kg, and ZSE 120 mg/kg, respectively. Rats in all groups, except the normal group, were induced to have gastric ulcer by oral gavage of 1 mL EtOH/HCl. One hour later, all the rats were sacrificed and their stomachs were removed for further preparation of tissue homogenate. The ground stomach tissue was weighed and homogenized in chilled phosphate buffer (pH 7.4) at a concentration of 10% w/v. The homogenates were centrifuged at 3500 rounds per minute (rpm) for 15 min at 4°C. The clear supernatant was used for the determination of NO concentration and lipid peroxidation product.

Determination of nitric oxide concentration

Determination of the reduction of nitrate into nitrite in the supernatant was performed using a colorimetric assay with Griess reagent (0.1% N-1-naphthylethylenediamide dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid).[31] One hundred microliter (μL) of Griess reagent was added to the supernatant (1:1). The mixture was incubated for 10 min at room temperature and the absorbance was measured at 540 nanometers (nm) by a microplate reader with an ultraviolet-visible spectrometer. The nitrite concentration was determined using a standard sodium nitrite curve (0–100 μg/mL). The results were expressed as μg of nitrate/nitrite per gram of protein.

Determination of lipid peroxidation product

The concentration of lipid peroxidation product in the supernatant was determined by estimating the amount of malondialdehyde (MDA) using the thiobarbituric acid test.[32] Briefly, the supernatant (0.2 mL) was added to a solution containing 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid solution (adjusted to pH 3.5), and 1.5 mL of 0.8% aqueous 2-thiobarbituric acid. The mixture was made up to 4 mL with distilled water and heated at 95°C for 1 h. Upon cooling, 1 mL of distilled water and 5 mL of n-butanol: pyridine (15:l) were added. The mixture was vortexed for 1 min and centrifuged for 15 min at 3,500 rpm. Absorbance of the supernatant was measured at 532 nm. A standard curve was obtained using 1,1,3,3-tetramethoxypropane 0–20 nanomolar (nM). The tissue level of MDA was expressed as nanomoles (nmol) per mg of protein. The protein content in the supernatant was determined using the method of Bradford.[33] The supernatant (40 μL) was added to a 96-well plate. Then, 200 μL of Bradford's solution was added to each well. After incubation at room temperature for 5 min, the absorbance was measured at 595 nm. The protein concentration was determined from a standard bovine serum albumin curve (0–10 mg/mL).

Determination of prostaglandin E_2 levels in gastric tissues

The experiment was performed as described by de-Faria *et al.*[34] with slight modification. The fasted rats were divided into three groups (six rats per group). The normal group did not receive any test drug, whereas the control and the test groups received 5% Tween 80 and ZSE 120 mg/kg orally, respectively. One hour later, all except the normal group were induced to have gastric ulcer by oral gavage of indomethacin (in 0.5% carboxymethylcellulose) 100 mg/kg. All rats were sacrificed 5 h later, and their stomachs were removed. Stomach tissues were homogenized as described above. Tissue homogenates were adjusted for equal protein concentration and then purified. The homogenized tissues at a volume of 0.5 mL were mixed with 0.5 mL of water in ethanol solution (1:4) and 10 μL of glacial acetic acid. The mixture tubes were allowed to stand for 5 min at room temperature and then were centrifuged at 4,725 rpm for 2 min. The clear supernatant was used for the determination of PGE_2 level using the PGE_2 competitive Biotrak™ enzyme immunoassay system with slight modification. Briefly, the tissue supernatant and standard PGE_2 were added to a 96-well plate (precoated with sheep anti-mouse immunoglobulin G) followed by the addition of mouse anti-PGE_2 and PGE_2 conjugated to horseradish peroxidase reagent. The plate was then incubated at room temperature for 1 h on a microplate shaker. After that, all wells were washed, and enzyme substrate 3,3',5,5' tetramethylbenzidine was added and mixed. Finally, 1M H_2SO_4 was added to stop the reaction and the absorbance was measured at 450 nm.

Determination of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity

The experiment was performed using the method of Carrasco *et al.*[35] with slight modification. Each 0.5 mL of ZSE solution (in 95% ethanol) at various concentrations was mixed with 1 mL of 40 μM DPPH solution. The mixture was kept in the dark for 30 min, and then the absorbance was measured at 517 nm. Deionized water was used as the blank, while gallic acid was used as a positive control. The percentage of DPPH radial scavenging activity was calculated as $([A_0 - A_1]/A_0) \times 100$, where A_0 is the absorbance of the blank and A_1 is the absorbance of ZSE or gallic acid. The determinations were carried out in triplicate.

Determination of total phenolic content

Determination of the content of phenolic compounds in ZSE was performed using the Folin–Ciocalteu method.[36] Briefly, 0.2 mL of ZSE solution (100 mg/mL of 95% ethanol) and 1 mL of 10% Folin–Ciocalteu reagent were pipetted into a microcentrifuge tube, and then 0.8 mL of 7.5% sodium carbonate solution was added. The mixture was incubated at room temperature for 60 min, and the absorbance was measured at 765 nm. The total phenolic content of ZSE was calculated from the gallic acid calibration curve and expressed in terms of g gallic acid equivalents (GAE) per g of ZSE.

Statistical analysis

Statistical comparison among the groups was conducted using one-way analysis of variance followed by Tukey's honestly significant difference test for parametric data and the Kruskal–Wallis test followed by Dunn's test for nonparametric data. Statistical significance was set at $P < 0.05$. Data were presented as mean ± standard error of the mean.

RESULTS

Zingiber simaoense rhizome ethanol extract gas chromatography-mass spectrometry analysis

The chemical fingerprint of ZSE obtained by GC-MS showed the presence of 33 components [Figure 1], only 22 of which could be identified. The major components were α-Eudesmol (30.38%), γ-Eudesmol (8.67%), and Elemol (7.86%) [Table 1].

Phytochemical screening

Qualitative phytochemical screening revealed the presence of alkaloids, tannins, terpenoids, and flavonoids, and the absence of anthraquinones, saponins, and glycosides in ZSE.

Gastric ulcer healing activity

An oral administration of EtOH/HCl produced gastric lesions in all rats [Table 2]. In the 3-day treatment arm, omeprazole (30 mg/kg/day) significantly reduced the ulcer index observed on day 4 when compared to that of the control group (5% Tween 80). Similarly, the treatment with ZSE at all doses (60, 120, and 240 mg/kg/day) also led to significant reductions in ulcer indexes observed on day 4 (38.55%, 68.04%, and 79.08% healing, respectively) when compared to that of the control group. The effect of ZSE appeared to be dose dependent. However, in the 7-day treatment arm, only omeprazole and ZSE at the highest dose (240 mg/kg/day) significantly reduced the ulcer indexes when compared to that of the control group observed on day 8.

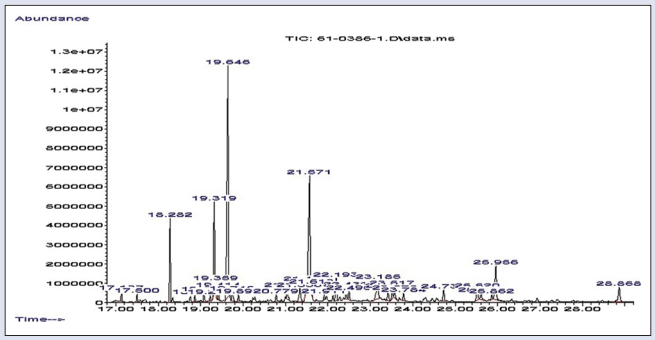

Figure 1: The gas chromatography-mass spectrometry chromatogram of *Zingiber simaoense* rhizome ethanol extract

Table 1: *Zingiber simaoense* rhizome ethanol extract chemical constituents identified using gas chromatography-mass spectrometry

Peak	Retention time (min)	Compound	Percentage of total
1	17.137	α-Humulene	0.92
2	17.500	γ-Gurjunene	0.65
3	18.282	Elemol	7.86
4	18.864	Aristol-1 (10)-ene	0.54
6	19.224	Cadinene	0.45
7	19.319	γ-Eudesmol	8.67
8	19.359	β-Gurjunene	0.79
9	19.414	Agarospirol	1.04
10	19.646	α-Eudesmol	30.38
11	19.746	γ-Selinene	1.22
13	20.779	Cycloheptane, 4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl-	0.74
16	21.486	2-Naphthalenemethanol, 1,2,3,4,4a, 5,6,7-octahydro-α,α,4a, 8-tetramethyl-, (2R-cis)-	0.76
17	21.571	2-Naphthalenemethanol, 1,2,3,4,4a, 5,6,8a-octahydro-.alpha.,.alpha.,4a, 8-tetramethyl-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	15.94
18	21.613	6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydronaphthalene-2,3-diol	0.43
20	22.193	7-Methyl-4-(1-methylethylidene) bicyclo[5.3.1]undec-1-en-8-ol	2.56
23	23.185	n-Hexadecanoic acid	3.19
24	23.418	Isolongifolene, 9,10-dehydro-	1.23
25	23.517	Hexadecanoic acid, ethyl ester	1.09
27	23.784	Isolongifolene, 9,10-dehydro-	0.86
29	25.520	9,12-Octadecadienoic acid (Z, Z)-	1.55
30	25.611	9,17-Octadecadienal, (Z)-	0.88
31	25.862	9,12-Octadecadienoic acid, ethyl ester	0.51

Histopathological evaluation of ulcer healing

On day 4, the stomach mucosa of the control group showed open ulceration with destruction of gastric muscularis mucosa due to inflammatory infiltration [Figure 2]. Treatment with omeprazole and ZSE (240 mg/kg/day) showed ulcer healing with complete epithelialization; the inflammatory process was limited when compared to that of the control group. On day 8, natural healing with complete epithelialization without gland formation was found in the control group, whereas near normalization of both epithelialization and gland formation was found in the omeprazole and ZSE groups. In addition, gastric mucus was also found in the omeprazole and ZSE groups.

Involvement of endogenous nitric oxide and sulfhydryl compounds in gastroprotection

As shown in Figure 3, EtOH/HCl-induced gastric ulcers were present in all groups that received 5% Tween 80 following pretreatment with normal saline, L-NAME, or NEM. Among the three groups that received 5% Tween 80, the ulcer indexes of rats pretreated with L-NAME and NEM were significantly higher than that of the control group pretreated with normal saline. In the normal saline pretreated group, both carbenoxolone (100 mg/kg) and ZSE (120 mg/kg) significantly reduced the ulcer indexes when compared to that of rats that received 5% Tween 80, with gastric ulcer inhibition of 86.62% and 82.24%, respectively. However, in the L-NAME pretreated groups, the ulcer indexes of carbenoxolone (105.08±6.00) and the ZSE groups (101.17±7.76)showed no significant differences when compared to that of the 5% Tween 80 group (125.33±3.88), with inhibition decreased to 15.88% (carbenoxolone group) and 19.01% (ZSE group). Similarly, in the NEM pretreated groups, the ulcer indexes of carbenoxolone (122.50±4.31) and the ZSE groups (126.25±8.49) showed no significant differences when compared to that of the 5% Tween 80 group (141.50±6.28), with inhibition decreased to 13.43% (carbenoxolone group) and 10.78% (ZSE group). Results of gastric lesion examination of rats with EtOH/HCl-induced gastric ulcers in all groups [Figure 4] were in line with those shown in Figure 3. In the normal saline pretreated groups, the stomachs of rats receiving carbenoxolone and ZSE showed less severe gastric mucosal injury than those of rats receiving 5% Tween 80 (control group). However, in the L-NAME and NEM pretreated groups, the gastroprotective effects of both carbenoxolone and ZSE decreased.

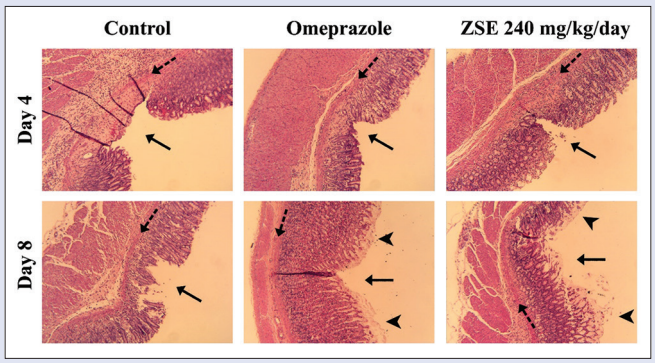

Figure 2: Histopathological examination of gastric mucosa of rats with acidified ethanol-induced gastric ulcers in the control, omeprazole, and *Zingiber simaoense* rhizome ethanol extract-treated groups observed on day 4 and day 8. The solid arrows indicate the ulcer areas, the dashed arrows indicate muscularis mucosa, and the arrowheads indicate gastric mucus (H and E, ×20)

Nitric oxide level in gastric tissues

The effect of ZSE on NO levels in gastric tissues of rats administered EtOH/HCl is presented in Table 3. It was found that NO levels in stomach tissues of the control group were significantly lower than those of the normal group. Pretreatment with omeprazole (10 mg/kg) and ZSE (120 mg/kg) significantly increased the levels of NO (to near-normal levels) when compared to those of the control group.

Table 2: Ulcer healing activity of *Zingiber simaoense* rhizome ethanol extract against acidified ethanol-induced gastric ulcer

Group	Dose (mg/kg/day)	Ulcer index		Healing (%)	
		Day 4	Day 8	Day 4	Day 8
Control	-	50.58±7.24	10.50±2.19	-	-
Omeprazole	30	13.83±1.58*	2.75±1.46*	72.65	73.81
ZSE	60	31.08±3.00*	6.25±0.97	38.55	40.48
	120	16.17±3.38*	6.58±1.51	68.04	37.30
	240	10.58±2.76*	3.58±1.31*	79.08	65.87

Data were expressed as mean±SEM (n=6). One-way ANOVA followed by Tukey's HSD test was used to analyze day 4 data, and the Kruskal-Wallis test followed by Dunn's test was used to analyze day 8 data. *Significantly different from the control group (P<0.05). SEM: Standard error of mean; HSD: Honestly significant difference; ANOVA: Analysis of variance; ZSE: *Zingiber simaoense* rhizome ethanol extract

Table 3: Effect of *Zingiber simaoense* rhizome ethanol extract on nitric oxide levels in stomach tissues of rats with acidified ethanol-induced gastric ulcer

Group	Dose (mg/kg)	The tissue levels of NO (μg/g of protein)
Normal	-	0.19±0.02*
Control	-	0.10±0.01
Omeprazole	10	0.16±0.01*
ZSE	120	0.15±0.01*

Data were expressed as mean±SEM (n=6). One-way ANOVA followed by Tukey's HSD test was used. *Significantly different from the control group (P<0.05). SEM: Standard error of mean; HSD: Honestly significant difference; ANOVA: Analysis of variance; ZSE: *Zingiber simaoense* rhizome ethanol extract; NO: Nitric oxide

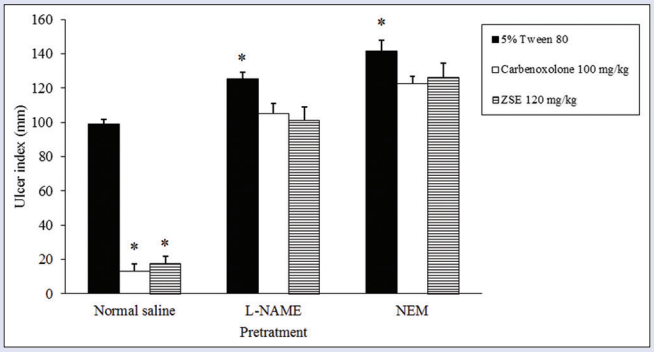

Figure 3: Gastroprotective effect of *Zingiber simaoense* rhizome ethanol extract (p.o.) on acidified ethanol-induced gastric ulcer in rats pretreated (i.p.) with normal saline, N^G-nitro-L-arginine methyl ester (L-NAME) or N-ethylmaleimide (NEM). Data were expressed as mean±standard error of mean (n=6). One-way analysis of variance followed by Tukey's honestly significant difference test was used. *Significantly different from the control group (pretreated with normal saline and received 5% Tween 80) (P<0.05)

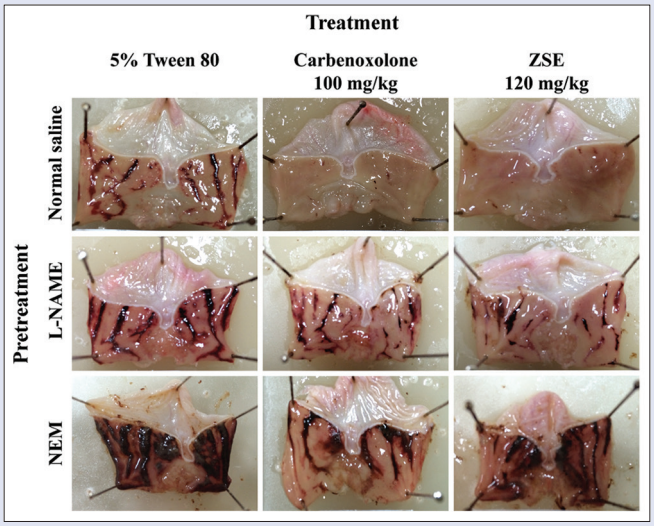

Figure 4: Gross gastric lesions in rats with acidified ethanol-induced gastric ulcers pretreated (i.p.) with normal saline, N^G-nitro-L-arginine methyl ester (L-NAME), or N-ethylmaleimide (NEM) followed by oral gavage of 5% Tween 80, carbenoxolone, or *Zingiber simaoense* rhizome ethanol extract 1 h before gastric ulcer induction

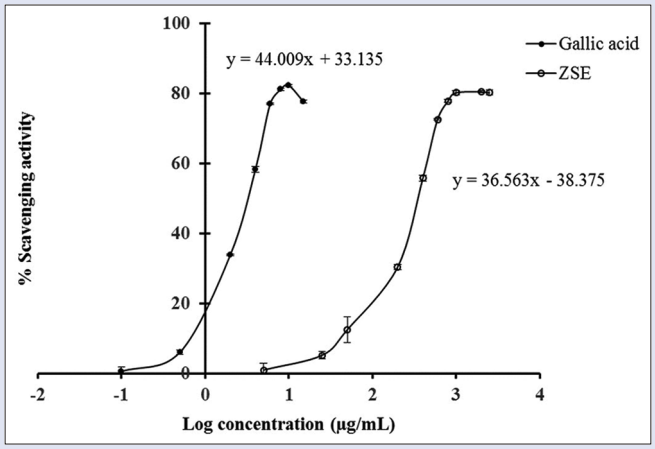

Figure 5: The log concentrations and % 1,1-diphenyl-2-picrylhydrazyl radial scavenging activities of *Zingiber simaoense* rhizome ethanol extract and gallic acid

Lipid peroxidation product in gastric tissues

The effect of ZSE on MDA levels in the gastric tissues of rats administered EtOH/HCl is presented in Table 4. The lipid peroxidation results showed the MDA levels in gastric tissues of the control group were significantly higher than those of the normal group. Pretreatment with omeprazole (10 mg/kg) and ZSE (120 mg/kg) significantly diminished the production of MDA caused by EtOH/HCl. Moreover, MDA levels in the omeprazole and ZSE groups were not significantly different from those of the normal group.

Prostaglandin E_2 levels in gastric tissues

The effect of ZSE on PGE_2 synthesis in gastric mucosal homogenates is presented in Table 5. Indomethacin significantly depleted the tissue levels of PGE_2 in the ulcer control group compared to those of the normal group. Pretreatment with ZSE (120 mg/kg) significantly prevented the depleting effect of indomethacin on tissue PGE_2 levels compared to those of the control group. However, the tissue levels of PGE_2 in the ZSE group were significantly lower than those of the normal group.

Antioxidant activity: 1,1-diphenyl-2-picrylhydrazyl assay

Figure 5 illustrates the relationship between the DPPH radical scavenging activity and log-concentrations of ZSE and gallic acid. The DPPH radical scavenging activities of ZSE and gallic acid seem to be concentration dependent. The maximum effects of ZSE and gallic acid were nearly equal, although the potency of ZSE was less than that of gallic acid. The IC_{50} values of DPPH radical scavenging activities of gallic acid and ZSE were 2.42±0.01 and 261.29±3.33 µg/mL, respectively.

Antioxidant activity: Total phenolic content

The total phenolic content of ZSE in terms of g GAEs/g dry weight of ZSE was 1.66±0.03 g GAE/g.

DISCUSSION

Peptic ulcer, mainly gastric ulcer, is the most common cause of upper gastrointestinal bleeding, that can lead to death, in Thailand.[37] The development of new drugs from medicinal plants for the prevention and treatment of peptic ulcer would offer an alternative way to patients for whom the use of conventional drugs is limited. The present study identifies some more possible mechanisms of gastroprotection of ZSE and demonstrates the gastric healing activity of ZSE against the common model of gastric ulcer induction, the EtOH/HCl-induced gastric ulcer in rats.

GC-MS analysis is one of the common chromatographic techniques recommended for quality control of herbal medicines and also medicinal plants harvested at different times, seasons, and areas.[38] Chemical fingerprints obtained by this method can reliably be used to identify the plant. In the present study, although the chemical fingerprint of ZSE and the major identifying components in ZSE were similar to those found in the study by Baiubon *et al.*,[19] some variations in the percentages of total were still detected because *Z. simaoense* rhizomes used in this study were collected at different time periods (in March 2014) from that study

Table 4: Effect of *Zingiber simaoense* rhizome ethanol extract on lipid peroxidation product in acidified ethanol-induced gastric ulcer

Group	Dose (mg/kg)	Tissue levels of MDA (nmol/mg of protein)
Normal	-	1.18±0.07*
Control	-	2.03±0.19
Omeprazole	10	1.17±0.09*
ZSE	120	1.03±0.14*

Data were expressed as mean±SEM (n=6). Kruskal-Wallis test followed by Dunn's test was used. *Significantly different from the control group (P<0.05). SEM: Standard error of mean; ZSE: *Zingiber simaoense* rhizome ethanol extract; MDA: Malondialdehyde

Table 5: Effect of *Zingiber simaoense* rhizome ethanol extract on prostaglandin E_2 levels in indomethacin-induced gastric ulcer

Group	Dose (mg/kg)	Tissue levels of PGE_2 (ng/well)
Normal	-	12.86±0.24
Control	-	7.09±0.95#
ZSE	120	10.53±0.47*,#

Data were expressed as mean±SEM (n=6). One-way ANOVA followed by Tukey's HSD test was used. *Significantly different from the control group (P<0.05); #Significantly different from the normal group (P<0.05). SEM: Standard error of mean; HSD: Honestly significant difference; ANOVA: Analysis of variance; ZSE: *Zingiber simaoense* rhizome ethanol extract; PG: Prostaglandin

(in March 2011). These results could explain the reason of using higher doses (60, 120, and 240 mg/kg) of ZSE in the present study than those in that study (7.5, 15, and 30 mg/kg).

The underlying mechanisms of gastric cytoprotection include increases of mucus and HCO_3^- and PG secretions; increases of SH compounds, blood flow, and free radical scavenging activity; and stimulation of gastric cellular growth and repair.[39] The mucus–HCO_3^-–phospholipid barrier, which covers and protects the entire gastric mucosa against harmful agents, is the first line of mucosal defense.[40] Endogenous SH compounds are key agents in the prevention of gastric ulcer induced by gastric acid, pepsin, and other noxious agents, including ethanol. These agents adhere to the mucus layer, forming a barrier to protect gastric mucosa. By forming disulfide bridges to merge mucus subunits, they prevent gastric mucus from being changed from a water-insoluble gel to a water-soluble form, which can be easily removed by ulcerogenic agents. They also act as recycling antioxidants that can bind to and neutralize harmful free radicals.[41,42] In addition, it has been shown that the gastroprotective effect of PG might be mediated by endogenous SH compounds.[43] Many animal studies have reported that the absence of SH compounds, using SH blocker (NEM), aggravates gastric damage from ethanol.[29,34,44,45] For these reasons, we investigated the involvement of endogenous SH compounds in gastroprotection of ZSE by pretreating animals with NEM in EtOH/HCl-induced gastric ulcer model. It was found that the ability of ZSE, as well as a gastroprotective agent carbenoxolone, to prevent gastric ulcer, measured in terms of percentage of gastric ulcer inhibition and gastric mucosal injury prevention, decreased when the production of SH compounds was blocked by pretreatment with NEM before the administration of ZSE. These results suggest that the gastroprotective mechanism of ZSE may involve the action of endogenous SH compounds.

NO, especially NO derived from endothelial NOS, plays a role in maintaining gastric mucosal defense. It maintains gastric mucosal integrity by increased blood flow in gastric mucosa, modulation of mucus production, inhibition of leukocyte recruitment to the mucosa, and acceleration of gastric ulcer healing.[46] Ethanol reduces gastric mucosal NO production leading to a decrease of gastric mucosal blood flow with the consequence of mucosal tissue hypoxia. In addition, pretreatment with NOS inhibitor can increase the severity of gastric ulcer induced by ethanol,[47] whereas exogenous NO supplements can abolish gastric ulcer lesions induced by ethanol.[48] Therefore, to investigate the role of endogenous NO in gastroprotection, we used L-NAME to assess the involvement of NO in the gastroprotection of ZSE in EtOH/HCl-induced gastric ulcer. It was found that pretreatment with L-NAME (a NOS inhibitor) could increase the aggravation of ulcer induced by EtOH/HCl, thus confirming the role of NO as one of the endogenous substances involved in gastroprotection. In addition, L-NAME pretreatment caused a decrease in the gastroprotective effect of ZSE and carbenoxolone. These findings support that the gastroprotective mechanism of ZSE may also include the action of endogenous NO.

The present study determining NO levels in stomach tissues of rats induced to have gastric ulcer by EtOH/HCl also confirmed the involvement of NO in the gastroprotection of ZSE. NO levels in stomach tissues of the control group were found to be lower than those in the normal group, confirming a study of Masuda *et al.*[47] which reported that ethanol could reduce gastric mucosal NO production. Pretreatment with ZSE also maintained endogenous NO levels in gastric mucosal tissues at near-normal levels, indicating that NO may have a role as one of the antigastric ulcer mechanisms of ZSE.

PG is an important endogenous defensive factor that stimulates and modulates almost all of the mucosal defense mechanisms including the stimulation of mucus, HCO_3^-, and phospholipid secretion; the increase of mucosal blood flow; the inhibition of acid secretion; and the acceleration of gastric epithelial restitution and mucosal healing.[40] NSAIDs are widely prescribed for the management of pain, fever, and inflammation because they have a role in the inhibition of the cyclooxygenase (COX) pathway that is involved in the generation of inflammation and pain.[49] Inhibition of the COX pathway by NSAIDs reduces the synthesis of PGs, leading to gastrointestinal injury by reducing mucus, HCO_3^-, and mucosal blood flow; impairing platelet aggregation; and increasing leukocyte adherence. In addition, the acidic property of NSAIDs also causes local irritation of gastric mucosa, leading to peptic ulcer disease.[50,51] Because of that, in the investigation of the involvement of PGE_2 in the gastroprotective effect of ZSE in this study, indomethacin, a nonselective COX inhibitor, was used to induce gastric ulcer. Pretreatment with ZSE partially prevented the indomethacin-induced depletion of PGE_2 levels in gastric tissue homogenate. This finding is consistent with that of a study that demonstrated the ability of ZSE in protecting against gastric ulcer induced by indomethacin and increase in gastric mucus levels.[19] The present study suggests that the mechanism of anti-ulcer activity of ZSE may be partly modulated by PGE_2.

The antioxidant properties of natural compounds have been found to play a role in gastric mucosa protection through radical scavenging mechanisms.[52] Phenolic compounds are key compounds that possess this scavenging property due to their hydrogen-donating ability.[53,54] In the present study, phytochemical screening of ZSE presented flavonoids and tannins. These compounds are polyphenols.[54] The total phenolic content of ZSE was also measured in this study using the Folin–Ciocalteu assay. The Folin–Ciocalteu reagent gains electrons from phenolic compounds, which results in blue complexes.[55] ZSE was found to contain phenolic compounds at 1.66±0.03 g GAE/g. The DPPH assay, one of the common assays for antioxidants in natural products, was then used to confirm the antioxidant property of these phenolic compounds. This assay estimates the reduction of DPPH free radicals.[56] ZSE showed DPPH radical scavenging activity, suggesting that flavonoids and tannins in ZSE may responsible, in part, for its antioxidant activity.

Reactive oxygen species (ROS) are formed during normal metabolic processes and are removed by antioxidant enzymes. As the accumulation of ROS and the inability of the antioxidant system to scavenge free radicals causes an increase in lipid peroxidation where molecules with unpaired electrons attack the unsaturated fatty acids of cell membranes, resulting in gastric tissue injury,[46,57] the antioxidant role of ZSE involved in lowering lipid peroxidation in gastric tissues was also investigated. Ethanol-induced gastric damage is one of the experiments that causes excessive generation of ROS and lipid peroxidation.[58] The main product of lipid peroxidation is MDA. For this reason, MDA level is a commonly used marker to measure lipid peroxidation in tissue.[32] In this study, the MDA levels in gastric tissues of rats in the control group increased significantly more in response to EtOH/HCl than those of rats in the normal group. Pretreatment with ZSE was found to normalize gastric MDA levels, thus confirming the possibility of its antioxidant effect.

The antigastric ulcer effect of ZSE in several animal models and a cytoprotective mechanism through the increase of gastric mucus have been found.[19] Taken together with the findings in the present study, we conclude that the antigastric ulcer mechanisms underlying the cytoprotective effect of ZSE, in addition to the increase of gastric mucus, might also involve with gastric mucosal NO, SH compounds, and PGE_2 and its antioxidant activities.

Gastric ulcer healing involves the process of mucosal integrity restoration by repairing mucosal defects through the proliferation and migration of epithelial cells, leading to re-epithelialization of the ulcer crater and the reconstruction and differentiation of glands.[40,59] In this study, to investigate the gastric ulcer healing effect of ZSE, the EtOH/HCl-induced gastric ulceration model was used. This model induces peptic ulcers that

resemble acute peptic ulcers in humans and is also widely used for testing the antigastric ulcer activity of potential agents that possess cytoprotective and/or antioxidant activities.[60,61] Ethanol causes necrotic lesions, whereas HCl causes severe damage of the gastric mucosa.[62] These conditions resemble the conditions that humans can be exposed. In the present study, the ulcer index of the control group at day 8 was less than that at day 4 due to the natural healing effect. This result is consistent with prior evidence that the process of complete gastric surface epithelium healing normally takes 3–7 days and that the complete replacement process of glandular cells requires months.[40] However, ZSE at the highest dose in this study (240 mg/kg/day), in addition to the effect at day 4, also accelerated ulcer healing at day 8 when compared with natural healing in the control group. The advantage of ZSE over the natural healing process was confirmed by a histopathological study. It was found that although the control group had complete gastric epithelialization, gland formation and gastric mucus were still absent, whereas the ZSE group (240 mg/kg/day) showed almost normal gastric epithelialization, gland formation, and the presence of gastric mucus. These results of ZSE were similar to those found in rats treated with omeprazole, the PPI with gastric ulcer healing activity. In the EtOH/HCl-induced gastric ulceration model, the gastroprotective factors (e.g., antioxidant enzymes, gastric mucus, and mucosal NO)[63-65] and the healing promoters (e.g., epidermal growth factor and vascular endothelial growth factor)[40,65] that control epithelial cell proliferation and differentiation decreased after ulcer induction. In addition, the gastric healing processes may involve NO, PGs, SH compounds, and antioxidant activity at the gastric mucosa. NO helps to dilate blood vessels, leading to an increase of gastric blood flow[66,67] and stimulation of angiogenesis during the healing processes associated with cell proliferation and re-epithelialization of gastric mucosa.[68] PGs can accelerate ulcer healing via several mechanisms, including their vasodilatory properties which are similar to that of NO;[69,70] reduction of gastric acid secretion;[69] stimulation of mucus and HCO_3^- secretions;[70] and stimulation of the release of vascular endothelial growth factor[71,72] which is an important mediator involved in angiogenesis and ulcer healing.[4,40] SH compounds bind free radicals,[41] leading to the removal of harmful stimuli and enhancement of the healing processes.[73] In addition, SH also stimulates the release of gastric mucin glycoproteins via sulfur donation for the sulfation of acid mucopolysaccharides of gastric mucin.[69] Moreover, scavenging of ROS by antioxidant compounds can also stimulate gastric healing.[70] Therefore, the gastric mucosal NO and PGE_2 levels and SH compounds as well as antioxidant activities might be involved in the mechanisms of action mediating the gastric healing effect of ZSE.

CONCLUSION

The present study demonstrates that ZSE at the dose of 240 mg/kg/day can accelerate gastric ulcer healing in rats on days 4 and 8 following ulcer induction with EtOH/HCl. In addition, the additional gastroprotective mechanisms underlying the cytoprotective effect of this extract might also involve gastric mucosal NO, SH compounds, and PGE_2 and its antioxidant activities. These findings provide convincing evidence to support its traditional use in the treatment of gastric disorders and its potential for further development as an alternative drug for peptic ulcer. However, further studies to determine the most active fraction with the gastroprotective activity of ZSE should be performed.

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Conflicts of interest

There are no conflicts of interest.

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