Original Article

Gastroprotective effect of hydroalcoholic extract of *Ocimum africanum* leaves

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Abstract

Ocimum africanum Lour. (hairy basil, hoary basil or lemon basil) is an aromatic herb in Asian and Thai cuisine that has been used as a carminative, a digestion enhancement and remedy for gastrointestinal disturbance in Thai traditional medicine. This study was performed to evaluate the gastroprotective effect of *O.africanum* leaf extract in acid/alcohol-induced gastric lesion in rats. Pretreatment of the plant extract can prevent the gastric damage in a dose-dependent manner. At 125 mg/kg of body weight, the plant extract attenuated the inflammation with % ulcer index of 5.08±1.41, whereas at doses of 250 and 500 mg/kg of body weight, the gastric lesion was completely prevented in the manner similar to what was observed in omeprazole pretreated animals (% ulcer index of 1.0±61.94, 0.1±10.45 and 0.00±0.00, respectively). The protective effect of the plant extract against acid/alcohol-induced gastric damage relates to its high antioxidative property and the ability to enhance the activities of three antioxidative enzymes, i.e. superoxide dismutase, catalase and glutathione peroxidase. Preliminary phytochemical investigation demonstrated that certain phenolic compounds such as rosmarinic acid, caffeic acid and vanillic acid might take part in the protective effects.

Keywords: gastroprotection, acid/alcohol-induced gastric lesion, *Ocimum africanum*, antioxidation, superoxide dismutase, catalase, glutathione peroxide

1. Introduction

Gastric ulcer is a common and chronic gastrointestinal disease affecting a number of people worldwide (Kangwan, Park, Kim, & Hahm, 2014). The disease etiology involves multifactors as a result of the imbalance between mucosal defensive mechanisms and various endogenous and exogenous offensive factors (Ibrahim *et al.*, 2012; Stewart & Ackroyd, 2011). Although there are many models which can

be applied to induce ulcer in experimental animals, a mixture of acid and alcohol (HCl/EtOH) has been widely used to induce gastric lesions in rats (Ishikawa *et al.*, 2008; Sripanidkuchai, Techataweewan, Tumsan, Pannangrong, & Sripanidkuchai, 2010). Omeprazole, a proton pump inhibitor is commonly used to treat the disease. However, under prolonged treatment, many adverse effects were reported, such as a high risk of neurological damage and other complications (Lam, Schneider, Zhao, & Corley, 2013; Valuck & Ruscin, 2004). Natural products have increasingly received attention due to their diversified activities and low toxicity. Many plant extracts and plant-derived compounds were found to have gastroprotective effects and potential use in place of

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synthetic drugs. The examples of such plant extracts include the leaf extract from *Centella asiatica* (Abdulla, AL-Bayaty, Younis, & Abu-Hassan, 2010; Sripanidkuchai, Techataweewan, Tumsan, Pannangrong, & Sripanidkuchai, 2007), *Cratoxylum formosum* (Sripanidkuchai *et al.*, 2010), *Solanum cernuum* (Miranda *et al.*, 2015), friedelin from *Azima tetracantha* (Antonisamy *et al.*, 2015), crocin from *Crocus sativus* (El-Maraghy, Rizk, & Shahin, 2015) and thymol, a monoterpene in essential oil from several plants (Ribeiro, Diniz, Pinheiro, Albuquerque-Júnior, & Thomazzi, 2016).

Ocimum species (Lamiaceae family) are annual or perennial and rapidly grown herbs in tropical Asia (Smitinand, 2014) that have immense pharmaceutical, nutraceutical and cosmeceutical significance. *O.basilicum* L. (sweet basil) and *O. sanctum* L. (holy basil) are well-known worldwide as aromatic herbs used in several cuisines, but in Thailand, *O. africanum* Lour (common name: hairy basil, hoary basil, lemon basil; synonyms: *O.americanum* L., *O.canum* Sims.) is also widely consumed. Hairy basil leaves are eaten as side-dish vegetables, or used as ingredient in Thai curry to increase lactation, while seeds can be used for laxative and weight control purposes as they have high mucilage content. Hairy basil leaves have also been used in traditional medicine as a carminative, a digestion enhancement and remedy for gastrointestinal disturbance (Bunyapraphatsara & Chokechaijaroenporn, 2000; Krisanapan, Somnapan, & Suriyapananon, 1998). *Ocimum* spp. contains various essential oil concentrations and compound types. Phytochemical analysis of essential oil in four *Ocimum* taxa showed that the plant in *americanum* group was dominated by camphor and other compounds including longipinanol, limonene, α-pinene, β-selinene and camphene (Verma *et al.*, 2015). Other compounds found in leaves of *Ocimum* spp. are phenolic compounds such as ursolic acid and rosmarinic acid (Mahajan, Rawal, Verma, Poddar, & Alok, 2013; Pandey, Chandra, Kumar, Dutt, & Sharma, 2016).

Various *Ocimum* spp, such as *O. sanctum*, *O. basilicum* and *O. gratissimum* has been reported to possess many pharmacological activities (Mahajan *et al.*, 2013; Vasudevan, Kashyap, & Sharma, 1999), but there are very few reports on *O. africanum*. The aim of this study was to investigate the protective effects of the hydroalcoholic extract of *O. africanum* leaves using acid/alcohol induced gastric ulceration model and to determine some of the underlying mechanisms of action in order to support the usage of the herb for nutraceutical development.

2. Materials and Methods

2.1 Chemicals

Beta-nicotinamide adenine dinucleotide phosphate (β-NADPH), catalase, copper sulfate, cytochrome c, 1,1-diphenyl-β-picryl hydrazyl radical (DPPH), gluthathione reductase, glutathione peroxide, superoxide dismutase, tannic acid, xanthine, xanthine oxidase were products of Sigma-Aldrich Co. (USA), Bovine serum albumin, eosin, Folin reagent, hematoxylin, hydrogen peroxide were obtained from Merck (Germany). Omeprazole was a product of Guadalajara (Spain). All other chemicals were analytical- grade substances sourced from local distributors.

2.2 Plant extract

Ocimum africanum Lour. was obtained from local markets in Khon Kaen province, Thailand. After identification, the herbarium of the voucher specimen (CRD-021) was deposited at the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University. The leaves were cleaned, dried and pulverized. The leaf powder was macerated in 50% ethanol in a closed container for five days. The liquid part was filtered and centrifuged at 4,000 g for 5 minutes, and then the supernatant was collected and dried using a rotary evaporator and a freeze dryer. The obtained extract at a yield of 3.02% was kept in a tight and light protected container at -20°C until used. The extract was suspended in distilled water before intragastrically administered to experimental animals.

2.3 Total phenolic content and antioxidative activity

Total phenolic content of the extract was determined by modified Folin-Ciocalteu method (Sripanidkulchai & Fangkrathok, 2014; Singleton, Orthofer, & Lamuela-Raventos, 1999). The extract dissolving in 50% ethanol at various concentrations (50 µl) was reacted with Folin-Ciocalteu reagent (80 µl) in the microplate for 4 min at room temperature. The absorbance was measured at 650 nm and the total phenolic content was expressed as tannic acid equivalent (TAE) in terms of mg/g of extract. For antioxidative activity, the DPPH radical scavenging methanol was used (Shimada, Fujikawa, Yahara, & Nakamura, 1992). The extract dissolving in methanol at various concentrations (250 ml) was incubated with 2.8 ml of 1 mM DPPH solution at room temperature for 20 minutes. The absorbance was measured at 515 nm and the DPPH radical scavenging activity was calculated and expressed as 50% effective dose (ED_{50}) and vitamin C was used as a positive control.

2.4 Thin layer chromatographic and high performance liquid chromatographic analyses

The plant extract was analyzed on TLC (thin layer chromatographic) plate (pre-coated aluminum-backed supported silica gel 60 F254, Merck) with a mixture of dichloromethane, acetic acid and formic acid (72:21:7) as a mobile phase. The TLC chromatogram was detected under ultraviolet light (254 and 366 nm), or sprayed with DPPH reagent. The retention time (Rf values) of each band was calculated and compared with the four commercially available standard compounds. To standardize the extract, high performance liquid chromatography (HPLC) analytical

method was used to quantity the content of rosmarinic acid. The HPLC system (Aligent LC12000) consisted of Hypersil ODS C18 column (4.6 x 250 mm, 5 μm) with UV detector of 326 nm. A gradient elution at a flow rate of 1 ml/min with a gradient program was performed by varying the portion of solvent A (0.5% formic acid in acetonitrile) and solution B (water) for 50 min (A:B at 10:90, 0-30 min; 25:75, 30-35 min; 100:0, 35-40 min; 10:90, 40-50 min). Injection volume was 20 ml. By comparing the peak area with the standard curve, the amount of rosmarinic acid in the extract was calculated and expressed as mg/g of crude extract.

2.5 Experimental animals

The experimental protocols were approved in accordance with the national guideline stipulated by the Animal Ethics Committee of Khon Kaen University (AE90/ 2555). Male Wistar rats (weighing 150-200 g) were obtained from the National Animal Center of Mahidol University, Bangkok. The animals were placed in a sterile and climate-controlled room at 25±2°C with a 12-h dark-light cycle and sound less than 85 decibels. The rats were acclimatized for seven days before the experiment with standard food pellet (Chareonpokpan, Bangkok) and *ad libitum* drinking water. An acid/alcohol solution containing hydrochloric acid (1.7%), ethanol (60%) in water (HCl/EtOH) was used to induce the gastric damage in this study. The animals were overnight fasted, then group I animals (control group) were orally given distilled water (0.5 ml/kg of body weight). The animals in group II - VI (ulcer, omeprazole and three *O. africanum* treated) were administered with distilled water (0.5 ml/kg of body weight), omeplazole (20 mg/kg of body weight), and *O. africanum* extract (125, 250, 500 ml/kg of body weight), respectively. One hour later, the animals (group II-VI) were given the acid/alcohol solution (5 ml/kg of body weight) to induce the gastric lesions. Then after six hours of treatment, the animals were killed by euthanasia with intraperitonal injection of pentobarbitol sodium (60 mg/kg of body weight). From each group, seven animals were taken for anatomical / histological studies and another seven animals were taken for biochemical analysis.

2.6 Assessment of gastric lesion

For gross anatomical and histological analyses, the abdomen was opened and the ligation between pyloric sphincter and esophagogastric junction was performed. Then the stomach was rapidly removed and injected with 1 ml of 10% formalin solution (in 0.1 M phosphate buffer, pH 7) and further fixed in 10% formalin solution for 24 hours. After that the stomach was opened along the greater curvature, rinsed with ice-cold 0.9% sodium chloride solution and extended on the paraffin board using needles. The mucosal lesion was determined for the bleeding area using an image analyzer (UTHSCSA image tool, version 3.00) as described by

Sripanidkulchai *et al.* (2006). The severity of gastric mucosa lesion was expressed as % ulcer index $\left(\frac{\text{ulcer area}}{\text{total area}} \times 100 \right)$ and % ulcerative protection $\left(\frac{\text{\% ulcer index of control} - \text{\% ulcer index of treated}}{\text{\% ulcer index of control}} \right)$. After gross anatomical examination, the stomach was processed through paraffin sections at 5 mm thickness by a microtome and stained with hematotoxylin and eosin (Anderson & Gordon, 1996). Then the histological analysis was carried out under a light microscope.

2.7 Biochemical analysis of the gastric mucosa

The stomach obtained from another seven animals in each group was also cut along the greater curvature and rinsed with ice-cold 0.9% sodium chloride solution. The gastric mucosal layer was scraped and homogenized in cold 0.1 M phosphate buffer saline solution (pH 7.4). After centrifugation at 10,000 g for 10 minutes at 4°C, the supernatant was collected for further analysis on protein content (Lowry, Roseburgh, Farr, & Randall, 1951) and antioxidant enzymatic activities. Three enzymes involving in the antioxidative process including superoxide dismutase (SOD), catalase and glutathione peroxide were determined in this study.

The activity of superoxide dismutase was determined using xanthine/xanthine oxidase as a superoxide generator as described by McCord & Fridovich (1969) and the reduction of cytochrome c was determined at a wavelength of 550 nm. For catalase activity, the method as described by Goldblith & Proctor (1950) was carried out. The reaction of H_2O_2 with potassium permanganate/sulfuric acid was detected at the absorbance of 515 nm (Aebi, 1984). Glutathione peroxidase activity was determined by coupled enzymatic reaction in which the reduced glutathione reacted with NADPH. The decrease in NADPH was measured at 340 nm (Wendel, 1980). The enzymatic activities of these three enzymes were expressed as unit/mg protein.

2.8 Statistical analysis

Data were expressed on mean±S.E.M. using the SPSS version 13 and analyzed by one-way analysis of variance followed by Fisher's least significant difference test for multiple comparison. The statistical significance was set at $p < 0.05$.

3. Results

3.1 Total phenolic content and antioxidative activity of the extract

As shown in Table 1, *O. africanum* leaf extract contained high phenolic content (476.06±9.08 mg TAE/g) and very high antioxidatvie activity which was 5-fold higher than the standard vitamin C (EC_{50} at 0.92±0.01 and 4.75±0.02 mg/ml, respectively).

Table 1. Percent yield, total phenolic content and antioxidative activity of *O. africanum* leaf extract.

Sample	%yield	total phenolic content* (mg TAE/g)	EC_{50}* (mg/ml)
O. africanum	3.02	476.06±9.08	0.92±0.01 (r^2 = 0.984)
Vitamin C	-	-	4.75±0.02 (r^2 = 0.990)

*Values are expressed as mean ± S.E.M. (n = 3).

3.2 TLC and HPLC analyses of the plant extract

Considering the TLC chromatogram and comparing it with the Rf-values of four available standards, it can be suggested that the plant extract contains many phenolic compounds with at least four known compounds, i.e, rosmarinic acid, caffeic acid, gallic acid and vanillic acid (Figure 1A, B and C). HPLC analysis showed more than 18 peaks of compounds in the extract (Figure 1D and E). By comparing the retention time of standard rosmarinic acid (27.3 min), the extract contained rosmarinic acid at a concentration of 0.73±0.04 mg/g.

3.3 Gross anatomical evaluation of gastric lesions

Oral pretreatment of the standard drug omeprazole and *O.africanum* leaf extract demonstrated the protective effects on HCl/EtOH induced gastric lesions. When compared to the control, the bleeding spots and inflammation were observed in the stomach of the rats receiving HCl/EtOH solution (Figure 2A and B). Omeprazole, a proton pump inhibitor which has been used for curing of gastric ulcer, at 20 mg/kg of body weight can prevent the bleeding and inflammation of the rat stomach (Figure 2C). The significant and dose-dependent inhibition of gastric lesions was detected in the animals pretreated with *O. africanum* leaf extract (Figure 2D, E, F). After pretreatment with the plant extract, the % ulcer index significantly decreased at doses of 250 and 500 mg/kg of body weight (1.06±1.94 and 0.11±0.45) compared to the ulcer group (8.24±6.08). In terms of % ulcerative protection, omeprazole gave complete protection and the plant extract showed a dose-dependent protection of the ulceration, which were 24.58±2.00, 94.53±0.00 and 98.69±0.001, respectively. The protection of the highest dose of the extract is almost the same as that of omeprazole (Figure 3).

3.4 Histological evaluation of gastric lesions

Under histological observation, when compared to the control group, HCl/EtOH induced gastric lesion, caused damages with severe disruption of the mucosal cells especially at the superficial region (Figure 4A and B). There are several red blood cells and epithelial degradation with edema and infiltration of leukocytes in the lamina propria layer.
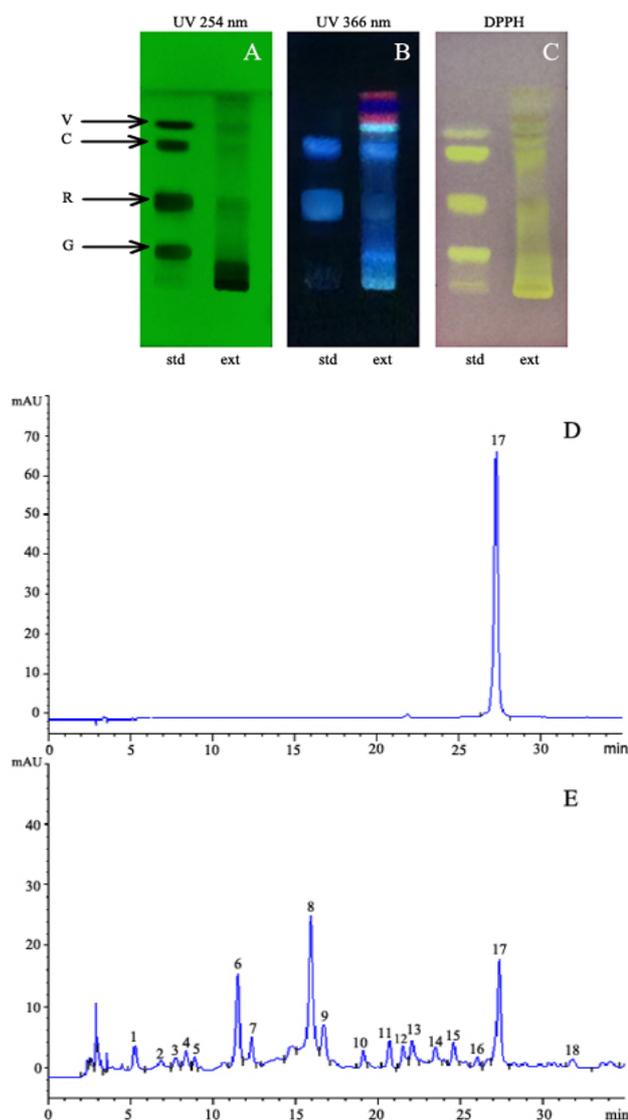

Figure 1. TLC and HPLC chromatograms of the plant extract. TLC plates were detected under ultraviolet exposure at 254 nm (A), 366 nm (B), and sprayed with DPPH reagent (C), and HPLC chromatograms at 326 nm of standard rosmarinic acid (D) and the extract (E). (in A-C: ext = *O. africanum* extract; std = mixture of four commercially available standards, G = gallic acid, R = rosmarinic acid, C = caffeic acid and V = vanillic acid, in D-E: 1-18 = number of detected peaks, peak 17 = rosmarinic acid).
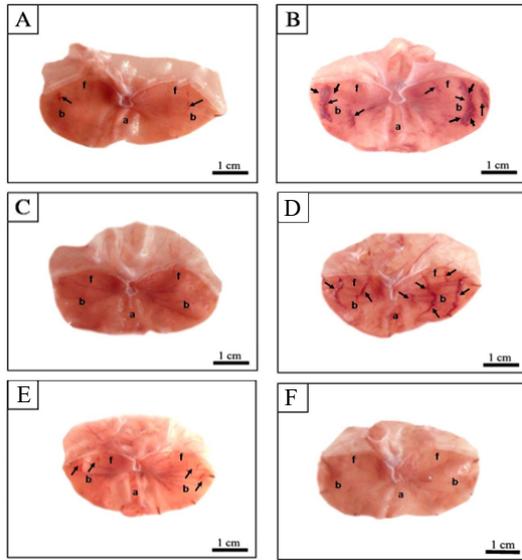
Figure 2   Gross anatomical characteristics of HCl/EtOH induced ulceration of rat stomach after pretreatment with *O.africanum* leaf extract (A= control, B = ulcer, C = omeprazone, D, E, F = *O.africanum* leaf extract at 150, 250, 500 mg/kg of body weight. Arrows indicated the bleeding spots, a = antrum, b = body, f = fundus.
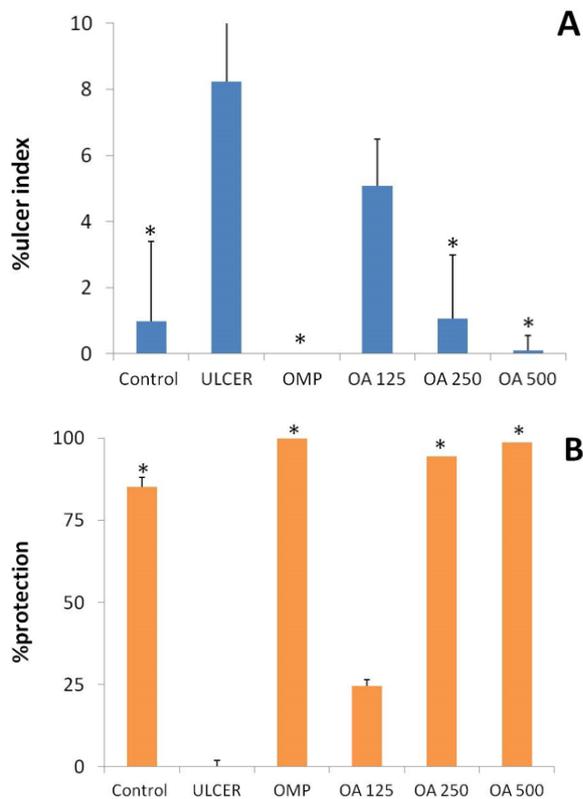

Figure 3.   Percent-ulcer index (A) and % ulcerative protection (B) after pretreatment with *O. africanum* leaf extract of rat stomach receiving HCl/EtOH induction (Data are mean ± S.E.M. n = 7 and * = significant differences from ulcer group at p <0.05).

Omeprazole showed complete gastroprotective effects on the mucosa of stomach as indicated by the absence of edema and white blood cell infiltration (Figure 4C). *O.africanum* leaf extract at low dose (125 mg/kg of body weight) still showed some mucosal damage (Figure 4D), whereas the higher doses of the extract showed no injury, no edema, no leukocyte infiltration and retained the normal morphology of the stomach (Figure 4E and F).

3.5 Effects of the extract on gastric mucosal antioxidant enzymes

Following HCl/EtOH induction, the activities of two antioxidant enzymes including superoxide dismutase and catalase significantly decreased. Pretreatment with omeprazole and the plant extract protected the gastric mucosa against the decrease of these enzymes, resulting in significant increases in both superoxide dismutase and catalase activities (Figure 5A and B). For glutathione peroxidase, the induction by HCl/EtOH slightly decreased the enzyme activity, whereas omeprazole and low dose of the extract (150 mg/kg of body weight) marginally increased the enzyme activity. Moreover,
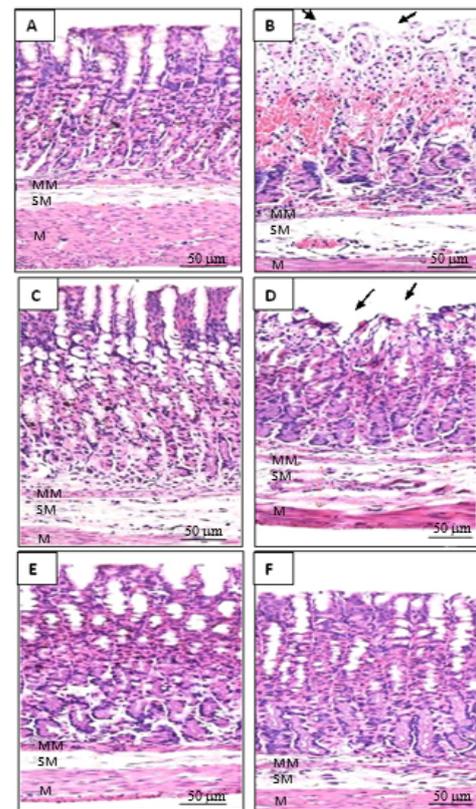

Figure 4.   Histological characteristics of HCl/EtOH induced ulceration of rat stomach after pretreatment with *O. africanum* extract (A= control, B = ulcer, C = omeprazone, D, E, F = *O. africanum* extract at 150, 250, 500 mg/kg of body weight) MM = muscularis mucosae, SM = submucosal layer, M = muscular layer and arrows indicated the lesion sites.
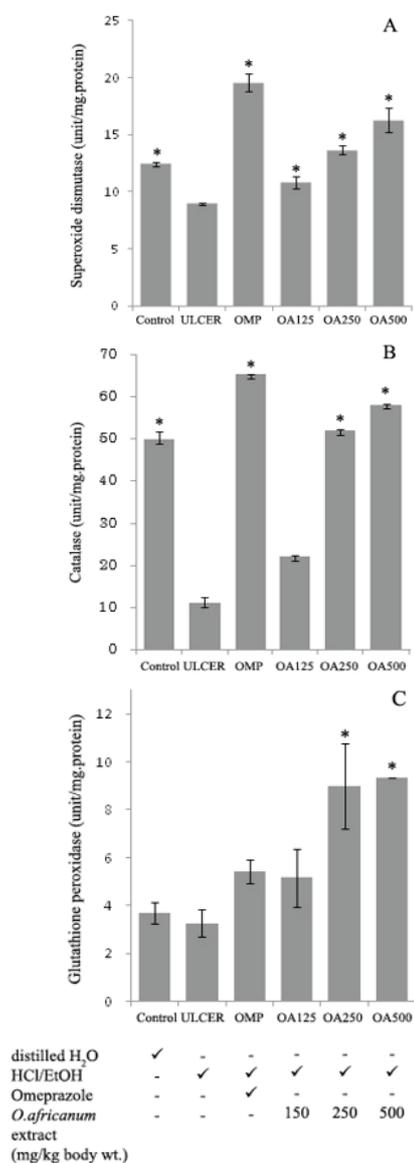
Figure 5. Effect of *O. africanum* leaf extract on the activities of superoxide dismutase (A), catalase (B) and glutathione peroxidase (C) of rat gastric mucosa.
    * = significant differences from ulcer group at $p < 0.05$.

pretreatment with high doses of the plant extract (250 and 500 mg/kg of body weight) significantly enhanced the enzymatic activity of glutathione peroxide (Figure 5C).

4. Discussion

The present study demonstrated the gastroprotective potential of *O. africanum* leaf extract on HCl/EtOH-induced gastric lesion of rats and provided an insight on some underlying mechanisms for the protection via the antioxidative effects of the plant extract. Since multiple factors involved in the development of gastric ulcer and among several inducers, acid/alcohol induction of gastric lesion was widely used as a studied model for gastroprotective effects of natural products (Itoh & Guth, 1985; Miranda *et al.*, 2015; Sripanidkulchai *et al.*, 2010). With the HCl/EtOH induction, we detected the gastric damages as characterized by the disturbance in the gastric mucosa, causing the bleeding and exfoliation of epithelial cells and generation of reactive oxygen species as previously reported (Shimoyama *et al.*, 2013; Terano, Hiraishi, Ota, Shiga, & Sugimoto, 1989). Our results revealed that all doses of the extract prevented the gastric lesions, decreased inflammation, edema and leukocyte infiltrations as earlier reported on the other plants having gastroprotective effects such as *C. asiatica* (Abdulla *et al.*, 2010; Sripanidkulchai *et al.*, 2007), *Aloe vera* (Sripanidkulchai *et al.*, 2006), *Solanum cernuum* (Miranda *et al.*, 2015) and *Monolluma quadrangula* (Ibrahim *et al.*, 2016). We also demonstrated the dose-dependent protective effects of the plant extract. At 125 mg/ kg dose of the extract, the bleeding area decreased and at 500 mg/kg dose of the extract the gastric lesion was completely gone as observed on the omeprazole-treated group, indicating the strong potential of the plant extract to prevent gastric lesion. The used doses are in a range of the previous studies. Generally, the plant extracts were tested at doses between 25-800 mg/kg per oral route (Schmeda-Hirschmann & Yesilada, 2005). Previous studies on ethanolic extract of *O. sanctum* leaves showed a strong gastropotective effects at 100 mg/kg in aspirin, alcohol and pyloric ligation-induced gastric lesion in Sprague-Dawley rats (Dharmani *et al.*, 2004). The gastroprotection of the plant extract is also in line with the increase in three antioxidant enzyme activities of the gastric mucosa. The free radicals generated from acid/alcohol exposures can be scavengered by *O. africanum* extract which showed the high antioxidative activity under the DPPH assay. The association of antioxidative effects and gastroprotective effects had been previously demonstrated in several studies such as crocin from the stigma of *Crocus sativus* (El-Maraghy *et al.*, 2015), friedelin from *Azima tetracantha* (Antonisamy *et al.*, 2015), extract from *Solanum cernuum* (Miranda *et al.*, 2015) and extract of *Cratoxylum formosum* (Sripanidkulchai *et al.*, 2010).

The prevention of gastric lesions according to antioxidative effects of the extract was also supported by the increase of the antioxidant enzyme activity as observed that the activities of superoxide dismutase, catalase and glutathione peroxide increased in the dose-dependent manners, suggesting that *O. africanum* extract can prevent the gastric lesion via its antioxidant property and induction of antioxidant enzyme activities. However, the mechanisms of how the extract increased these enzymatic activities need further studies. Moreover, besides antioxidative mechanism of action the gastroprotective effect of the plant extract may confer via its anti-inflammatory effect as observed that three were no edema and no leukeocyte infiltration in the gastric of treated animals. In terms of the plant chemical constituents, several compounds were reported in essential oil of various species of *Ocimum*. Phytochemical study of *O. basilicum* and *O. sanctum* showed that they contained many terpenoids,

eugenol, geraniol, limalool and eucalyptol as their major constituents (Vasudevan *et al.*, 1999). Although the number of reports on *O. africanum* is very limited, geraniol/neral and estragol are found to be the major compounds of *O. africanum* and its accessions (Carovic-Stanko *et al.*, 2011). Other compounds such as camphor, limonene, α-pinene, β-selinene and camphene were also found in *O.africanum* (Verma *et al.*, 2015). Based on the HPLC and GC/MS analyses, rosmarinic acid and ursolic acid were found in *O. africanum* (Silva *et al.*, 2008). In Thailand, these three *Ocimum spp.*, *O. basilicum*, *O. sanctum* and *O. africanum* are widely used as aromatic herbs and with their easily cultivated nature; they are likely to have hybrid species. The chemical constituents of *O. africanum* might be found in other *Ocimum spp* as well. Our preliminary investigation by TLC and HPLC analyses revealed that *O. africanum* leaf extract contained more than 18 compounds including several phenolic compounds such as rosmarinic acid, caffeic acid, gallic acid and vanillic acid, these compounds may be in part involved in the gastro-pretective effects of *O. africanum* leaf extract. With an attempt to standardize the extract, rosmarinic acid content was successfully determined and it can be used as one of the chemical marker of this plant. Previous report on the ethanolic extract of *Rosmarinus officinalis* also showed high content of rosmarinic acid in association with its gastroprotective effect (Amaral *et al.*, 2013). However, more studies on the mechanism of action and the other compounds should be further done. In conclusion, the extract of *O.africanum* leaf can prevent gastric ulceration via its antioxidative and anti-inflammatory effect and the consumption of this plant as an aromatic herbs in Asian cuisine reflects its gastroprotection benefit.

Acknowledgements

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