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ORIGINAL ARTICLE

High-performance Liquid Chromatography Identification of Gastroprotective and Antioxidant Effects of Purified Fractions A-E from the Stem of *Coscinium fenestratum*

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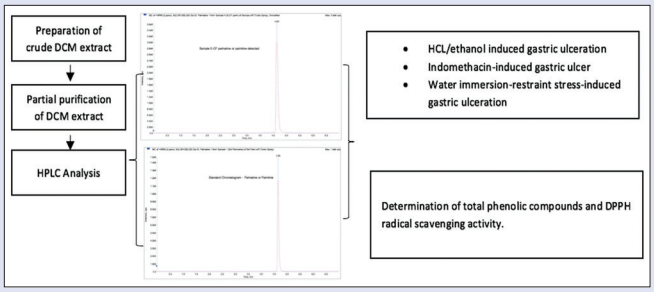
ABSTRACT

Objective: *Coscinium fenestratum* belongs to the family of *Menispermaceae*. It is a woody plant that originated from Indomalaya regions such as southern India, Sri Lanka, Thailand, Vietnam, Cambodia, and West Malaysia. *C. fenestratum* has been reported to possess various pharmacological actions such as antioxidant, antiproliferative, antidiabetic, and antibacterial activities. In addition, this medicinal plant has also been used to treat gastrointestinal disorders, including ulcer. The purified fractions A-E of dichloromethane (DCM) extract obtained from the stem of *C. fenestratum* were investigated for antioxidant activity and its ability to protect the gastric mucosa against hydrochloric acid (HCl)/ethanol, nonsteroidal anti-inflammatory drugs (NSAIDs), and stress-induced ulcer. The bioactive compounds were identified using high-performance liquid chromatography (HPLC) and simple biochemical screening. **Methods:** Air-dried stem extract was soaked in DCM to obtain crude extract which was purified using column chromatography. HPLC and basic biochemical screening were used to identify the bioactive compound present. HCl/ethanol induced, NSAIDs, and stress of animal were used to induce gastric ulcer. The rats were orally administered with purified A-E extract for gastroprotective and antioxidant activity. **Results:** The oral administration of fractions A-E exhibited gastroprotective activity by reducing the ulcerative index induced by HCl/ethanol, NSAIDs, and stress compared to saline. The extract showed high antioxidant effect, especially fraction E. The HPLC and phytochemical analysis of fraction E showed the presence of palmatine and flavonoid. **Conclusion:** The extract showed a very strong gastroprotective and antioxidant activities and the highest activity was observed by fraction E. The activity may be because of the presence of palmatine and flavonoid.

Key words: Antioxidant, flavonoids, gastroprotective, high-performance liquid chromatography, nonsteroidal anti-inflammatory drugs

SUMMARY

- Fraction E from the stem crude extract of *Coscinium fenestratum* plant has been shown to possess a very strong gastroprotective and antioxidant effects. Palamatine a protoberberine class of isoquinoline alkaloids was identified to be the bioactive present in Fraction E.



Abbreviations Used: DCM: Dichloromethane, HCl: Hydrochloric acid, HPLC: High-performance liquid chromatography, NSAIDs: Nonsteroidal anti-inflammatory drugs, EtOH: Ethyl acetate, MeOH: Methanol, NaCl: Sodium chloride, FCR: Folin–ciocalteu reagent, DPPH: 2, 2-diphenyl-1-picrylhydrazyl, TLC: Thin-layer chromatography, IMR: Institute of Medical Research, TPC: Total phenolic compounds, UPM: University Putra Malaysia, R_f: Retardation factor, LD50: Lethal dose.

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INTRODUCTION

Coscinium fenestratum is a common medicinal plant in the family of *Menispermaceae*.[1] It originated from Indomalaya regions such as southern India, Sri Lanka, Thailand, Vietnam, Cambodia, and West Malaysia.[2] The local names for this plant include kunyit-kunyit babi (Peninsular), abang asuh (Sabah), and perawan (Sarawak). Darvi, Daruharidra, Peetadaru, Peetadru are its synonyms in Sanskrit, Daruhaldi, Jharihaldi, and Jhari-huldi in Hindi, Maramanjal in Tamil, and "Ham" or "Ka-min-kreu" in Thai. However, it is commonly known as tree turmeric, columbo wood, false calumba, and sekunyit.[3] It is critically interested species due to its huge demand in the medicinal plant and has been reported to possess various pharmacological actions such as antioxidant,[4] antiproliferative,[5,6] antidiabetic,[3,7-9] antiplasmodial and antibacterial activities.[1] The traditional folk medicine practitioners claim that *C. fenestratum* has a strong effect on peptic ulcer disease.[10] Unfortunately, the stem of *C. fenestratum* is only

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used to produce natural fabric yellow dye in Malaysia for dyeing in the past. However, at present scientific data, about the pharmacological effect of this species on the gastrointestinal system is very limited.[11] Peptic ulcer is the most predominant of the gastrointestinal diseases in the world. It is an excoriated area of the gastric or duodenal mucosa caused by a lack of equilibrium between the gastric aggressive factors and the mucosal defensive factors[5] such as acid–pepsin secretion, parietal cell, mucosal barrier, mucus secretion, blood flow, cellular regeneration, and endogenous protective agents, such as prostaglandins and epidemic growth factors.[12] Stress, smoking, nutritional deficiencies, excessive ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs), and *Helicobacter pylori* infection augment gastric ulcer incidences.[13] There are many pharmaceutical products available for the treatment of gastric ulcers such as antacid, proton-pump inhibitor, or H2-blockers, but they are not completely effective and produce several adverse reactions.[14] Therefore, numerous efforts have been pursued to find a novel class of antiulcer agent which strengthens defensive mechanisms with less toxic effect.[15] In recent years, there has also been growing interest in alternative therapies and the use of natural plant products.[14] Plant extracts have been shown to produce promising results in the treatment of gastric ulcers.[15] The purpose of this study was to investigate the antioxidant activity and the ability of fractions A-E from the stem of *C. fenestratum* to protect the gastric mucosa against hydrochloric acid (HCl)/ethanol, NSAIDs, and stress-induced gastric ulcer. The bioactive compounds present in the extract were identified using simple biochemical screening and high-performance liquid chromatography (HPLC).

METHODS

Plant material

The stems of *C. Fenestratum* were collected in the jungles of Gombak, Selangor Darul Ehsan, and West Malaysia. The plant was authenticated by Dr. Shamsul Khamis, a plant taxonomist, and a voucher specimen was deposited at the herbarium of the Laboratory of Natural Products, Department of Biosciences, University Putra Malaysia (UPM).

Chemicals and solvents

Dichloromethane (DCM), hexane, ethyl acetate, ethanol (EtOH), methanol (MeOH) and chloroform (R and M Chemicals, England), HCl, ammonium hydroxide, ranitidine, indomethacin (Sigma Chemical Co., UK), Tween 40® (Synth, Brazil), tragacanth gum powder, glycerol, sodium chloride and folin–ciocalteu reagent (FCR) (Merck, Germany) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (Fluka, Switzerland), sodium bicarbonate anhydrous (Bendosen, Norway) and gallic acid (Acrōs, USA), methylcellulose solution, and Dragendorff's reagent were used in this study. The extract was dissolved in saline, tragacanth powder, Tween 40®, and glycerol (vehicle). All substances were prepared immediately before use, and the reagents used were of analytical grade.

Preparation of crude dichloromethane extract

The plant material (stem) was air-dried at 25°C for a week. The dried stem was cut into thin pieces and ground into coarse powder form using a miller (Hsiangtai Machinery Industry Co Ltd, Taiwan) in Institute of Bioscience, UPM. Dried ground stem powder (1 kg) was extracted with DCM (4 L) for 48 h at room temperature. Then, the extract was filtered through Whatman filter paper and concentrated using rotary evaporator at 35°C–37°C to yield a solid mass crude DCM extract. The procedure was repeated until an adequate amount of crude DCM extract was obtained.

Partial purification of dichloromethane extracts

DCM extract was purified using column chromatography technique with silica gel as stationary phase and solvents mixture (hexane, ethyl acetate, and MeOH) as mobile phase. Five fractions, namely, A, B, C, D, and E were eluted. Fractions were eluted as a single spot on thin-layer chromatography (TLC) silica-coated aluminum plate (F254) and examined under ultraviolet (UV) light of wavelength 365 nm. Eluents were pooled together based on its TLC pattern and similar measured retardation factor (R_f) value of each spot in each fraction. The pooled fractions were concentrated with rotary evaporator until dry at temperature 35°C. These steps were repeated until appropriate amounts were obtained for pharmacological assay.

Phytochemical screening of fraction E

Phytochemical screening of fraction E was performed for the presences of alkaloids, tannins, saponins, flavonoids, cardiac glycosides, and terpenoids.[16,17]

High-performance liquid chromatography analysis

Targeted HPLC analysis of the purified fraction E extract was performed using Perkin Elmer Flexar FX15 Ultra HPLC system. The column used was Agilent Zorbax XDB-C18–150 mm × 4.6 mm × 5 uM. The mobile phase consisted of buffer A: water with 0.1% formic acid and 5 mM ammonium formate and B: acetonitrile with 0.1% formic acid and 5 m ammonium. Gradient run program was 10% B to 95% B from 0.01 min to 5.0 min, hold for 1 min, and back to 10% B in 0.1 min and re-equilibrated for 1 min. All samples were dissolved accordingly as per instructed (saline which is aqueous, MeOH or 50:50 MeOH: water). Samples were then filtered with 0.22 uM nylon filter before injection and volume of injection 5 ul.

Animals

Albino Wistar rats of both sexes weighting (180–200 g, 11 weeks) were purchased from Institute of Medical Research, West Malaysia. They were housed in standard metal cages in UCSI animal holding unit. All animals were given access to food and water *ad libitum*. They were deprived of food but not of water before the commencement of the experiment because the drugs and test substances were administered orally.

Acute toxicity studies

The acute toxicity study of the fractions A-E from stems of *C. Fenestratum* was performed in rats (n = 10). In this assay, increasing concentration of fractions A-E was orally administered to groups of animals for each concentration after a 12 h fast. Animals receiving the vehicle (saline) served as control. The signs and symptoms associated with the fractions A-E administration (5000 mg/kg, p. o. volume of 10 mL/kg of body weight) were observed at 0, 30, 60, 120, 180, and 240 min after administration and then once a day for 14 days. At the end of the period, the number of survivor was recorded. The acute toxicological effect was estimated by the method described by Souza-Brito,[18] and the death, when occurred, was expressed as LD50 according to Litchfield and Wilcoxon.[19]

Hydrochloric acid/ethanol-induced gastric ulceration

The gastroprotective activity of the fractions A-E of *C. fenestratum* was studied in HCl/ethanol-induced gastric ulcer. The experiments were performed as described by Hara.[20] Albino Wistar rats of both sexes were divided into groups (n = 5), and animals were fasted 24 h before receiving an oral dose of the vehicle, saline (10 mL/kg), ranitidine (30 mg/kg), crude DCM (100 mg/kg), and fractions A-E (100 mg/(kg body wt). After 90 min, all groups were orally treated with 1 ml of a 0.3 M HCl/60% ethanol solution (HCl/ethanol) for gastric ulcer induction. The animals

were sacrificed 1 h after the administration of HCl/ethanol, and the stomachs excised and inflated by saline injection (2 mL). The length (mm) of each lesion was measured, and the lesion index was expressed as the sum of the length of all lesions as described by Szelenyi and Thiemer.[21] The percentage of ulcer inhibition (% I) was determined as follows:

$$\text{Inhibition of ulcer}\,(\%) = \frac{\text{Control mean ulcer index} - \text{test mean ulcer index}}{\text{Control mean ulcer index}} \times 100$$

Indomethacin-induced gastric ulcers

The experiment was performed according to the method of Ishikawa.[22] In this model, gastric lesions were induced with 1.0 ml of 200 mg/kg indomethacin suspended in 0.1% methylcellulose solution containing 0.2 M HCl and were orally administered to the rats after 24 h fast. Fractions A-E (100 mg/kg), ranitidine (30 mg/kg), and saline (10 mL/kg) were administered orally 30 min before the induction of gastric lesions. The animals ($n = 5$) were killed 1 h after treatment with ulcerogenic agent. The stomachs were removed; gastric damage and inhibition percentage were calculated as described above.

Water immersion-restraint stress-induced gastric ulceration

The experiment was performed according to the method of Nagura[23] modified by Basile.[24] Rats were divided into groups of five animals. After 24 h of starvation, the animals received oral administration of fractions A-E (100 mg/kg), ranitidine (30 mg/kg), and saline (10 mL/kg). One hour after treatment, gastric ulceration was induced by immobilizing the animals in a closed cylindrical cage immersed vertically in water (28 ± 2)°C to the level of xiphoid in the presence of intense light. The animals were sacrificed 24 h after treatment and the stomach removed and examined for ulcer as described previously.

Determination of total phenolic compounds and 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

The amount of total phenolic compounds (TPC) present in the fraction E was determined with FCR using the method of Spanos and Wrolstad.[25] The experiment was done by measuring 2.5 ml of 10% FCR and 2 ml of Na_2CO_3 (2%w/v) was added to 0.5 ml of each sample (3 replicates) of fraction E solution (1 mg/ml). The resulting mixture was incubated at 450°C with shaking for 15 min. The absorbance of the samples was measured at 765 nm using UV/visible light. Results were expressed as milligrams of gallic acid (0–0.5 mg/ml) dissolved in distilled water. The method of Kikuzak and Nakatani[26] was used for the determination of scavenging activity of DPPH-free radical. One milliliter of 0.135 mM DPPH prepare in MeOH was mixed with 1.0 ml of fraction E ranging from 0.2 to 0.8 mg/ml. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance was measured spectrophotometrically at 517 nm. The scavenging ability of the plant extract was calculated using this equation,

$$\text{DPPH scavenging activity}\,(\%) = \left(\frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})}\right) \times 100$$

Where Abs control is the absorbance of DPPH + MeOH; Abs sample is the absorbance of DPPH radical + sample (i. e., fraction E or standard).

Statistical analysis

Statistical significance was accessed using one-way ANOVA followed by Dunnett test to compare the data. All values are presented as means ± standard error mean and probability (P) < 0.05 was considered statistically significant. All values are presented as means ± standard error mean.

RESULTS

Oral toxicity studies

The oral toxicity of fractions A-E was evaluated with concentration fivefold higher than maximum tested gastroprotective concentration (5000 mg/kg, p. o.). At this concentration, no signs and symptoms of acute toxicity were observed in all treated rats. No significant difference was observed in the weight of heart, liver, kidney, or lungs when they were compared with those of control group (saline). None of the treated rats died during 14 days of observation after the administration of fractions A-E (data not shown). The results initially obtained indicated the absence of acute toxic effect of fractions A-E which motivated us to continue the assays.

Hydrochloric acid/ethanol-induced gastric ulceration

The effects of the oral administration of fractions A-E of *C. fenestratum* on the gastric induced by different damaging agents (HCl/ethanol, NSAID, and stress) were first investigated in rats, and the results are shown in Table 1. The oral administration of HCl/ethanol solution to control group clearly produced the expected characteristic zone necrotizing mucosal lesions. The HCl/ethanol solution induced ulcer lesions after a relatively short time. Pretreatment with fractions A-E given orally at concentration 100 mg/kg and the positive control (ranitidine, 30 mg/kg) induced a significant protective effect in a variable degree. Ranitidine and fractions D and E (100 mg/kg) significantly inhibited ulcer formation by 68.7%, 84%, and 95.4%, respectively. The obtained results suggest that fractions D and E extract of *C. fenestratum* presents a significant gastroprotective effect

Table 1: Effect of ranitidine and fractions A-E of stem dichloromethane extract of stem obtained from stem of *Coscinium fenestratum* on hydrochloric acid/ethanol, nonsteroidal anti-inflammatory drugs (indomethacin), and water immersion-restraint stress-induced gastric ulcers in rats

Model	Treatments (p.o)	Dose (mg/kg)	Ulcer index (mm)	Inhibition (%)
HCl/ethanol model	Control	-	26.2±2.9	-
	Ranitidine	30	8.2±1.9***	68.7
	Fraction A	100	21.6±1.5**	17.6
	Fraction B	100	19.8±2.6***	24.4
	Fraction C	100	24.8±1.9 (NS)	5.3
	Fraction D	100	4.0±1.6***	84.7
	Fraction E	100	1.2±0.8***	95.4
NSAIDs	Control	-	19.6±0.5	-
	Ranitidine	30	4.2±0.2***	78.8
	Fraction A	100	22.1±0.3***	−12.5
	Fraction B	100	15.2±0.4***	22.4
	Fraction C	100	8.2±0.3***	58.3
	Fraction D	100	7.3±0.2***	62.8
	Fraction E	100	3.6±0.1***	81.6
Water immersion-restraint stress	Control	-	12.2±0.8	-
	Ranitidine	30	3.2±0.4***	73.8
	Fraction A	100	12.8±0.8 (NS)	−4.9
	Fraction B	100	8.6±0.9***	29.5
	Fraction C	100	15.6±0.5***	−27.9
	Fraction D	100	4.2±0.4***	65.6
	Fraction E	100	2.2±0.4***	82.0

The results are reported as means±SEM (n=5). Statistical significance one-way ANOVA followed by Dunnett test (**P<0.05, ***P<0.001, NS). In comparison to negative control. HCl: Hydrochloric acid; NSAIDs: Nonsteroidal anti-inflammatory drugs; SEM: Standard error mean; NS: Not significant; ANOVA: Analysis of variance

in this ulcer-induced model. The animals treated with saline (negative control) and fractions A, B, and C 100 mg/kg showed extensive lesion area (26.2 ± 2.9, 21.6 ± 1.5, 19.8 ± 2.6, and 24.8 ± 1.9 mm, respectively).

Indomethacin-induced gastric ulcers

The effect of fractions A-E on gastric lesions induced by indomethacin is also shown in Table 1.

Significant and concentration-dependent inhibition of the gastroprotection was observed from the pretreatment with fractions A-E of *C. fenestratum*. Ranitidine (30 mg/kg) used as positive control in this model and dose of 100 mg/kg of fractions D and E exerted a protective effect by 78.8%, 62%, and 81.6% of ulcer lesion index inhibition, respectively. The animals treated with saline (negative control) and fractions A and B 100 mg/kg showed extensive lesion area (19.6 ± 0.5, 22.1 ± 0.3, and 15.2 ± 0.4 mm, respectively).

Water immersion-restraint stress-induced gastric ulceration

Our data showed that pretreatment with fractions D and E at 100 mg/kg significantly protected the gastric mucosa against water immersion-restraint stress-induced gastric ulcers by 65.6% and 82%, respectively, against 73.8% inhibition obtained with ranitidine (30 mg/kg), the positive control used in this model [Table 1]. These results suggested the ability of fractions D and E to inhibit the gastric prostaglandin depletion and/or decrease of the acid secretion in the mucosa. The animals treated with saline (negative control) and fractions A, B, and C 100 mg/kg showed extensive lesion area (12.2 ± 0.8, 12.2 ± 0.8, 8.6 ± 0.9, and 15.6 ± 0.5 mm, respectively).

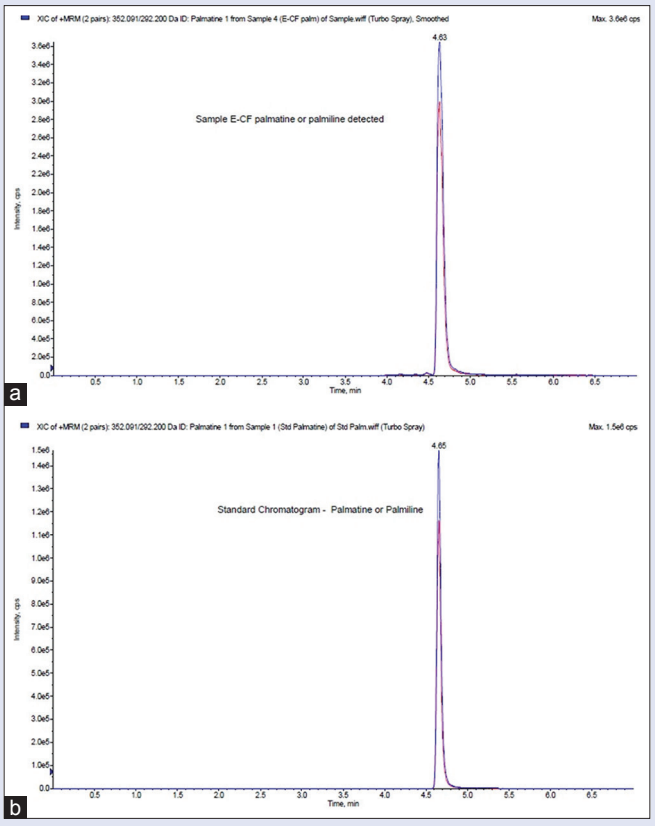

Figure 1: (a) Chromatogram of fraction E. (b) Chromatogram of palmatine fraction E and standard palmatine were dissolved in methanol and filtered with 0.22 uM nylon filter before injection. The injection volume was 5 uL

High-performance liquid chromatography analysis

The chromatograms from HPLC analysis clearly showed the presence of palmatine (palmiline) in the sample of fraction E [Figure 1a and b]. HPLC analysis detected palmatine in fraction E. Palmatine belongs to the protoberberine class of isoquinoline alkaloids. It is a close structural analog of berberine and has been used in the treatment of jaundice, dysentery, hypertension, inflammation, and liver-related diseases.[6] Palmatine has been reported to have an antioxidant effect.[7]

Phytochemical screening, total phenolic compounds, and 2, 2-diphenyl-1-picrylhydrazyl

The phytochemical screening of fractions D and E revealed the presence of flavonoids. The TPC present in fractions D and E was 190 and 256.67 mg GAE/g dry weight, and the radical scavenging activity of the fractions D and E was 16.80 and 21.71%, respectively.

DISCUSSION

Hydrochloric acid/ethanol-induced gastric ulceration

Ethanol induces solubilization of mucus constituents in the stomach with a concomitant decrease in the transmucosal potential difference and increases the Na+ and K+ flux into the lumen, while enhancing pepsin secretion, the loss of H + ions, and the histamine content in the lumen.[27] The ingestion of ethanol is also known to evoke acute tissue edema, subepithelial hemorrhages, cellular exfoliation, and time-dependent infiltration by inflammatory cells that may contribute to the induction of mucosal injury through the generation of reactive oxygen species.[28] Ethanol is also able to induce direct oxidative damage against gastric mucosal tissues. It increases superoxide anion and hydroxyl radical production and lipid peroxidation in the gastric mucosa.[12] HCl/ethanol, solution-induced gastric ulcers also promote stasis in gastric blood flow, which contributes to the development of hemorrhagic and necrotic aspects of tissue injury.[29] HCl/ethanol solution induced gastric ulcer by promoting disturbances of mucosal microcirculation, ischemia and appearance of free radicals, releasing endothelin, degranulation of mast cells, inhibition of PGs, and decrease of gastric mucus production.[30] Moreover, it stimulates gastric secretion, gastrin, and histamine release, while enhancing pepsin secretion.[15]

Indomethacin-induced gastric ulcers

In the stomach, prostaglandins have a vital protective role. They stimulate the secretion of bicarbonate and mucus, maintain mucosal blood flow, and regulate mucosal cell turnover and repair.[31,32] The suppression of prostaglandin synthesis by NSAIDs, such as indomethacin, results in increased susceptibility to mucosal injury and gastroduodenal ulceration.[33,34] It is well known that indomethacin induces gastric ulcer by inhibition of prostaglandins, which are cytoprotective to gastric mucosa.[35,36] Fractions D and E effectively reduced the gastric lesions produced by both, indomethacin and HCl/ethanol. These data suggested the involvement of fractions D and E with enhancement of gastric mucosal defensive factors.

Water immersion-restraint stress-induced gastric ulceration

Water immersion-restraint stress-induced gastric ulcers have been used experimentally for evaluation of antiulcer activity in animals by the enhancement of acid secretion and reduction in mucous production mediated by histamine release.[37,38] Bagchi *et al.*[39] hypothesized that free radicals may play a major role in stress involved in gastrointestinal

injury. Stress ulcer is the vagal overactivity that increases gastric acid secretion.[40] Stress significantly increases the indices of oxidative tissue damage such as tissue lipid, protein peroxidation, membrane microviscosity, and DNA fragmentation.[41] Stress is also found to inactivate mucosal prostaglandin synthetase by accumulating H_2O_2, an inhibitor of the prostaglandin synthesis, and this propitiates the generation of reactive oxygen species.[38,42,43]

High-performance liquid chromatography analysis

The chromatograms from HPLC analysis clearly showed the presence of palmatine in the sample of fraction E [Figure 1a and b].[4-10]

Phytochemical screening, total phenolic compounds, and 2, 2-diphenyl-1-picrylhydrazyl

The scavenging activity of compound D and E may be related to the presence of flavonoids in the extract. Several studies showed good correlation between the total phenols and antioxidant activity.[44] Flavonoids have been reported to show significant biological activities such as antiulcer gastric.[45] Flavonoids are able to scavenge free radicals directly mainly due to their antioxidant activity.[12] Berenguer *et al.*[46] testified that flavonoids increased antioxidant enzyme activities to prevent oxidant injury and cell death. Flavonoids inhibit lipid peroxidation and protect the gastric mucosa from oxidative damage,[47] whereas it stimulates PGE2 biosynthesis in gastric mucosal cells.[47] Flavonoids also increase capillary resistance, protect blood vessels, and improve microcirculation.[48,49] Flavonoids show antisecretory effect by reducing acid production in response to histamine.[47] Hence, from this experiment, it seems that the antiulcer effect of fraction E may be attributed to its flavonoids content which has been widely reported to possess gastroprotective activity.

CONCLUSION

Fractions D and E of *C. fenestratum* have shown a strong gastroprotective and antioxidant activity. Fractions A, B, and C showed some gastroprotective and antioxidant activity but way less than fractions D and E. HPLC and phytochemical analysis of fraction E confirmed the presence of palmatine and flavonoids, which may be responsible for the activities observed. Palmatine has potential be development for peptic ulcer treatment and the effect maybe because of its antioxidant effect.

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Conflicts of interest

There are no conflicts of interest.

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