Food Sci Biotechnol (2018) 27(4):1175–1184
https://doi.org/10.1007/s10068-018-0342-0

# Improvement of gastroprotective and anti-ulcer effect of kenaf seed oil-in-water nanoemulsions in rats

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Received: 7 November 2017 / Revised: 8 February 2018 / Accepted: 19 February 2018 / Published online: 1 March 2018


**Abstract** Kenaf seed oil-in-water nanoemulsions (KSON) and kenaf seed oil-in-water macroemulsions were produced to access their gastroprotective effect against indomethacin- and ethanol-induced ulcers in comparison with non-emulsified kenaf seed oil (KSO). Emulsifier mixture (EM) that used to emulsify KSO was also included in the study. Ulcer index, stomach tissue oxidative status, and histopathological changes in indomethacin-induced and ethanol-induced ulcer models were both evaluated. KSON had demonstrated good gastroprotective effect against both ulcer models than non-emulsified KSO and KSOM. In addition, the gastroprotective effect of KSON was comparable to the standard drug, Omeprazole. EM also exhibited gastroprotective effect, especially in indomethacin-induced ulcers. This may be attributed to its high antioxidant activity and cytoprotective effect of sodium caseinate contained in the EM. Results supported that KSON enhanced the bioavailability of native KSO; therefore it offers gastroprotective effect for the prevention of gastric ulceration as a natural alternative to the synthetic drug.

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**Keywords** Kenaf seed oil · Nanoemulsions · Gastroprotective effect · Indomethacin-induced ulcers · Ethanol-induced ulcers

**Abbreviations**

| | |
|---|---|
| KSO | Kenaf seed oil |
| KSOM | Kenaf seed oil-in-water macroemulsions |
| KSON | Kenaf seed oil-in-water nanoemulsions |
| EM | Emulsifier mixtures |
| SC | Sodium caseinate |
| T20 | Tween 20 |
| β-CD | Beta-cyclodextrin |
| NSAID | Non-steroidal anti-inflammatory drug |
| PPIs | Proton-pump inhibitors |
| UI | Ulcer index |
| H&E | Hematoxylin and eosin |
| SOD | Superoxide dismutase |
| GSH | Reduced glutathione |
| MDA | Malondialdehyde |
| ROS | Reactive oxygen species |
| COX | Cyclooxygenase |

## Introduction

Gastric ulcer is the lesions on the mucosal epithelium of the stomach. It is one of the most predominant gastrointestinal diseases that occur in more than 10% of the world's population. The aetiology of gastric ulcer is believed to be related to the imbalance between the gastric aggressive factors such as secretion of hydrochloric and pepsin, and mucosal defensive factors such as blood flow, production of prostaglandins, secretion of mucus and bicarbonate. Regular intake of non-steroidal anti-inflammatory drugs (NSAIDs), for instance, indomethacin, aspirin and

ibuprofen, and *Helicobacter pylori* infection are the major causes of gastric ulcer. An estimated 16,500 patients with arthritis were reported to die as a result of NSAID yearly [28]. In addition, heavy alcohol consumption, stressful conditions, and smoking are frequently associated with gastric ulcer formation [2, 28]. Various synthetic anti-ulcer drugs are available from the market, including histamine H2- receptor antagonists, antacids, sucralfate, misoprostol and more recently, proton-pump inhibitors (PPIs) have been used for the prevention and treatment of gastric and duodenal ulcers. However, each of these drugs provokes mild to severe side effects. For instance, the most common minor side effects of PPIs include skin rash, headache, nausea, constipation, diarrhoea, abdominal pain, and dizziness [26]. Prolonged use of PPIs may cause adverse effects, for instance, thrombocytopenia, hepatotoxicity, gynecomastia, and enteric infections [26]. Therefore, natural products of plant sources for the prevention and treatment of gastric ulcers have become attractive sources due to their perceived lower side effects, easily available and affordable than the high cost of synthetic drugs.

Kenaf seed (*Hibiscus cannabinus* L.) oil has always been suggested as a good source of functional edible oil owing to its high amount of linoleic and oleic fatty acids [13, 22]. Kenaf seed oil (KSO) contains a wide range of naturally occurring bioactive phytochemicals, including phytosterols, vitamin E, polyphenols, saponins, terpenoids and alkaloids [21, 23]. Therefore, KSO has recently been considered as an important medicinal crop. Nevertheless, the functional properties of KSO are hindered by its poor water solubility, which leads to the low bioavailability. KSO has been reported to possess anti-inflammation [21] and anti-ulcer activity [23]; however, the efficacy in both studies was not high, probably due to the poor solubility of KSO in the gastrointestinal tract. Nanoemulsion-based formulations are one of the several encapsulation approaches that not only able to increase the solubility and stability of lipophilic bioactive compounds; they also enhance its bioavailability.

A stable kenaf seed oil-in-water nanoemulsion (KSON) stabilised by using sodium caseinate (SC), Tween 20 (T20), and beta-cyclodextrin (β-CD) complexes has been successfully optimized and developed by using high pressure homogenizer [10, 11]. In the previous study, KSON demonstrated good lipolysis rate (85.25%) and higher bioaccessibility of vitamin E and total phenolic content than non-emulsified KSO in the in vitro study [12]. Furthermore, in the bioavailability study, KSON also revealed higher bioavailability than kenaf seed oil-in-water macroemulsion (KSOM) by 1.4-fold and non-emulsified kenaf seed oil (KSO) by 1.7-fold (unpublished). The aim of this study was to assess and compare the gastroprotective effect of KSO, KSOM, KSON, and the emulsifier mixture (EM) which was used in the preparation of emulsions against indomethacin- and ethanol-induced ulcers.

## Materials and methods

### Materials

Kenaf (*Hibiscus cannabinus* L.) seeds were purchased from Malaysian Agricultural Research and Development Institute (MARDI) (Selangor, Malaysia). Beta-cyclodextrin (β-CD) was bought from Zibo Qianhui Fine Chemical Co., Ltd. (Shandong, China). Sodium caseinate (SC) was purchased from VIS Food Tech Ingredient Supplies (Kuala Lumpur, Malaysia). Reduced Glutathione (GSH) Assay Kit and Superoxide Dismutase Assay Kit (SOD) were purchased from BioAssay Systems (California, USA). Lipid Peroxidation (MDA) Assay Kit was purchased from Sigma (New York, USA). Omeprazole was bought from Milrin Pharmaceutical Co. (M) Sdn. Bhd (Penang, Malaysia). Indomethacin was bought from Y.S.P. Industries (M) Sdn. Bhd. (Selangor, Malaysia). HPLC-grade solvents (isopropanol, and methanol) were purchased from Fisher Scientific (Leicestershire, UK). All other reagents were of analytical grade.

### Extraction of KSO from kenaf (*Hibiscus cannabinus* L.) seeds

Soxhlet extraction of KSO was performed according to the previously described method [11].

### Preparation of KSOM and KSON and EM

The preparation of KSON was according to the previously optimized formulation and methods [10, 11]. Briefly, sodium caseinate, SC (5.79 wt%), and Tween 20, T20 (2.76 wt%) were dissolved in 45 °C of ultrapure water (40 wt%). Sodium azide (0.02 wt%) was added to inhibit microbial growth. Kenaf seed oil (10 wt%) was added drop-wise into the aqueous phase containing SC and T20 under magnetically stirring at 45 °C and continued to stir for another 10 min after the oil was added into the aqueous phase. Primary emulsions were produced by using a high shear mixer (Ultra-Turrax, IKA, UK) at 8600 rpm for 3 min. Prior to high pressure homogenisation, the predissolved of beta-cyclodextrin (β-CD) (1.45 wt%) in 70 °C ultrapure water (40 wt%) was then added to the primary emulsions. Then, the KSON was produced by passing through the high pressure homogeniser (Nano Debee, BEE International, USA) at 28,000 psi for four cycles. KSOM were prepared according to the composition of KSON. In brief, KSO was added into pre-dissolved emulsifier

mixtures (SC, T20, and β-CD) under magnetically stirring at 45 °C and continued to stir for another 10 min after the oil was added into the aqueous phase. Conventional macroemulsions were produced by using a high shear mixer (Ultra-Turrax, IKA, UK) at 8600 rpm for 3 min. EM was prepared by just mixing the ternary emulsifiers (SC, T20, and β-CD) with water according to the same concentration of KSON and KSOM, but without the addition of KSO.

### Animals

Adult Sprague–Dawley rats of either sex (250–300 g) were procured from the institutional animal house (Universiti Kebangsaan Malaysia, Bangi, Malaysia). All animals were housed in standard cages under standard conditions of temperature at 25 ± 2 °C and humanity (75 ± 5) % with 12 h light/12 h dark cycle throughout the period of study. The animals were fed with standard pellet (Gold Coin, Kuala Lumpur, Malaysia) and allowed to drink water ad libitum. The rats were left to acclimatize for 2 weeks before commencement of the experiment. The study was approved by the Faculty's of UCSI University (Cheras, Malaysia) with project code Proj-FAS-EC-15–42.

### Indomethacin-induced ulcer

Sprague–Dawley rats were randomly divided into six groups of five rats in each group. The experiment was carried according to the described method with slight modification [32]. The rats were fasted for 24 h before the experiment, but given free access to water. A dose of 10 mL/kg b.w of EM, 10 mL/kg b.w of deionised water, 20 mg/kg b.w of omeprazole, 1 mL/kg by body weight (b.w) of KSO, 10 mL/kg b.w of KSOM and 10 mL/kg b.w of KSON with each oral dose of 1000 mg oil/kg b.w were administrated by oral gavage as treatment groups. Omeprazole was prepared by crushing into fine powder, followed by addition of water and stirred under sonication bath until fully dissolved. After 1 h, indomethacin (100 mg/kg b.w) was orally gavaged to all rats to induce gastric ulcers. After 4 h, the rats were sacrificed by overdosing with diethyl ether.

### Gross evaluation of gastric lesions

After scarification, the stomachs were excised, opened along the greater curvature and then rinsed with deionised water. The stomachs were pinned on a dissecting tray, photographed with digital camera together with a ruler and the gastric ulcer area was determined using Image J software [27]. The ulcer index (U.I) and protection percentage were calculated using the following formula [25]:

$$\mathrm{UI} = (\text{ulcerate area/total stomach area}) \times 100. \quad (1)$$

$$\text{Protection}\,(\%) = \big(\text{UI in control} - \text{UI in pre} - \text{treated group/UI in control}\big) \times 100\% \quad (2)$$

### Ethanol-induced ulcer

Sprague–Dawley rats were randomly divided into six groups of five rats in each group. The fasted rats (24 h) were orally received KSO, KSOM and KSON with each oral dose of 1000 mg oil/kg b.w, EM (10 mL/kg b.w), deionised water (10 mL/kg b.w) or omeprazole (20 mg/kg b.w). After 1 h, ulcer was induced with absolute ethanol (5 mL/kg b.w). One hour later, the rats were sacrificed by overdosing with diethyl ether [32]. The gross evaluation of gastric lesions was performed as described above.

### Histopathological analysis

After gross evaluation, gastric samples from each group were immediately fixed in 10% buffered formalin solution for overnight. The tissues were processed and dehydrated with alcohol and xylene using spin tissue processor (Model STP-120, Thermo Fisher Scientific, Germany). Prior to sectioning, the tissues were embedded in paraffin wax (Paraplast, Leica, Singapore) under embedding workstation (Microm EC 350, Thermo Fisher Scientific, Germany). The embedded tissue blocks were sectioned (3–5 μm) using rotary microtome (Accu-Cut SRM 200, Netherlands). A group of slides was stained with haematoxylin and eosin (H&E) according to the recommended method with slight modification [8]. Photomicrographs were viewed using an inverted microscope (Carl Zeiss, Germany).

### Preparation of tissue homogenates

After gross evaluation, stomachs were freeze dried under liquid nitrogen and stored at − 80 °C until analysis. The freeze dried stomach samples were thawed, minced and homogenised with 5 mL/g of self-prepared cold lysis buffer (50 mM phosphate buffer, 0.1 mM EDTA, 0.5% Tween 20) under sonication bath. The homogenates were centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatants were used for SOD, GSH and MDA assays.

### Determination of superoxide dismutase (SOD) activity

The SOD activity was measured using assay kit (ESOD-100, BioAssay Systems, USA) by according to the manufacturer's recommendations. The absorbance was read at

440 nm using microplate reader (FLUOstar Omega, BMG Labtech, Germany). The results were expressed as U/g tissue.

### Determination of reduced glutathione (GSH)

The GSH was measured using assay kit (DIGT-250, BioAssay Systems, USA) based on the manufacturer's instructions. The absorbance was measured at 412 nm using microplate reader (FLUOstar Omega, BMG Labtech, Germany). The results were expressed as nmol/g tissue.

### Determination of lipid peroxidation (MDA)

The MDA was determined by using assay kit (MAK085, Sigma, USA). The absorbance was measured at 532 nm using microplate reader (FLUOstar Omega, BMG Labtech, Germany). The results were expressed as nmol/g tissue.

### Statistical analysis

The results were expressed as mean ± standard deviation (SD) and analysed using One-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests to compare the treatments with ulcer control group. All assays were carried out at room temperature and in duplicates. The significance levels were analysed at $p < 0.05$ and $p < 0.001$.

## Results and discussion

### Indomethacin-induced ulcers

Indomethacin is known to induce ulcers by local effect and indirect effect. Local effect is referring to the direct local contact between mucosa and drug, leading to irritation, cytotoxic to tissues and mucosal damage [17]. The indirect effect of indomethacin that induces ulcers is by inhibiting prostaglandin synthesis via cyclooxygenase (COX) pathway. Prostaglandins are present in many mammalian tissues, including the stomach. Prostaglandins are generated by COX-1 and COX-2. COX-1 generates prostaglandin E2 (PGE2) and thromboxane (TXA2) that is responsible for maintaining the integrity of gastric mucosa and platelet aggregation, respectively; whereas COX-2 regulates the generation of prostaglandins that mediate inflammation, pain, and fever [7]. Indomethacin inhibits COX-1 more than COX-2; therefore, it increases inflammation and reduces bicarbonate and mucus secretion, decreases mucosal blood flow, impairs platelet aggregation, and immunocyte function in the stomach [2, 7]. Apart from that, indomethacin can also induces oxidative mucosal damage by increasing the production of reactive oxygen species (ROS) and neutrophil infiltration [29].

### Gross evaluation of gastric lesions

The gastroprotective effect of different pre-treatments on indomethacin-induced gastric damage in rats was macroscopically evaluated (Table 1). Gross observation for ulcer control group presented few long dark haemorrhagic streaks [Fig. 1(A)]. Omeprazole group (standard drug) exhibited the greatest protection (97.52%) on indomethacin-induced ulcer with only some red coloration on certain parts [Fig. 1(B)]. Among the pre-treatment samples, KSON group showed significant ($p < 0.001$) reduction of ulcer area, followed by KSOM group ($p < 0.05$). Macroscopic evaluation of KSON and KSOM showed some red coloration and pinpoint erosion, respectively [Fig. 1(E, F)]. The gastroprotective effect of KSON pre-treated group was significant and comparable to Omeprazole pre-treated group. Pre-treatment with EM was also found to have significant ($p < 0.05$) ulcer reduction with a protection of 76.34%. Surprisingly, rats pre-treated with KSO displayed no gastroprotective effect.

### Histopathological evaluation of gastric lesions

In order to further evaluate gastric mucosa lesions, histological observation stained by H&E was performed and presented in Fig. 1(a–f). Histological examination of indomethacin-induced stomach tissue with ulcer control group showed disruption of mucosa and epithelial layer (blue arrow) with fibrosis (red arrow). Fibrosis with loss of glandular tissue occurred as a result of inflammation and necrosis of epithelial cells and connective tissue. Infiltration of inflammatory cells (circle) was seen underneath the fibrosis tissues in submucosa layer. Besides, haemorrhage was observed in the mucosa (black arrow) and the part of submucosa was expanded by fluid, indicating oedema (#) [Fig. 1(a)]. Pre-treatment with KSO did not seem to have much protection effect on the indomethacin-induced rats. Oedema and leucocyte infiltration were observed but milder fibrosis with no infiltration of inflammatory cells beneath [Fig. 1(d)]. Stomach tissue pre-treated with EM and KSOM also demonstrated erosion but with reduction of oedema and inflammation [Fig. 1(c, e)], compared to ulcer control group and KSO group. KSON pre-treated group has comparable histological changes with Omeprazole pre-treated group, in which both demonstrated normal mucosa architecture with only mild mucus depletion and mild oedema and leucocyte infiltration in submucosa layer [Fig. 1(b, f)].

**Table 1** The effect of different pre-treatment groups on indomethacin- and ethanol-induced gastric lesions in rats

| Ulcer model | Groups | Ulcer index | Protection (%) |
|---|---|---|---|
| Indomethacin-induced | Ulcer control | 1.26 ± 0.83 | – |
| | Omeprazole | 0.03 ± 0.03*** | 97.52 |
| | EM | 0.30 ± 0.23* | 76.34 |
| | KSO | 1.22 ± 0.45 | 3.42 |
| | KSOM | 0.23 ± 0.16* | 81.55 |
| | KSON | 0.07 ± 0.04*** | 94.33 |
| Ethanol-induced | Ulcer control | 39.38 ± 7.94 | – |
| | Omeprazole | 7.82 ± 4.90*** | 80.15 |
| | EM | 23.43 ± 12.04 | 40.50 |
| | KSO | 19.80 ± 17.52* | 49.73 |
| | KSOM | 5.99 ± 2.69*** | 84.78 |
| | KSON | 1.99 ± 1.21*** | 94.95 |

Values are expressed as mean ± S.D. (n = 5) Statistical comparison was determined by One-Way ANOVA followed by Dunnett's multiple comparison tests

Percentage is calculated as compared to ulcer control group

*EM* emulsifier mixture, *KSO*, kenaf seed oil, *KSOM* kenaf seed oil-in-water macroemulsions, *KSON* kenaf seed oil-in-water nanoemulsions

*$p < 0.05$ statistically significant compared to the ulcer control group

***$p < 0.001$ statistically significant compared to the ulcer control group

## Oxidative stress in the gastric tissues after induced by indomethacin

Gastric homogenates from all study groups were analysed for SOD, GSH, and MDA, in order to evaluate the extent of oxidative stress. SOD is the first line of defence in cell against reactive oxygen species (ROS) that cause damage to the cellular membranes. The SOD activity level denotes the intracellular antioxidant capacity of the cell. GSH plays a dominant role in coordinating body's antioxidant network, which is important in maintaining the integrity of gastric mucosa [3]. MDA is a lipid peroxidation product, which is a useful marker for oxidative stress [31]. Table 2 shows the lowest GSH level and the highest MDA level in the ulcer control group in comparison with other pre-treatment groups. This indicated the increased production of ROS, which resulted in oxidative damage to the cell as shown in Fig. 1(a).

Recently, more studies have supported that the gastric mucosa lesions induced by the indomethacin in short term were primarily due to oxidative stress [1, 31]. Oxidative stress refers to the lack of equilibrium between the free radical formation and antioxidant activity, leading to lipid peroxidation and tissue damage. Defence against oxidative stress is largely dependent on the synergism between endogenous and exogenous antioxidants to scavenge the over production of free radicals as a result of indomethacin/ethanol-induced ulcers [16]. The changes of GSH levels in this study is in agreement with the studies reported by authors, who concluded that severe gastric lesions exhibited lower GSH level than moderate gastric lesions [27, 30]. Among the treatment groups, the significantly higher GSH contents in both Omeprazole group and KSON group corresponded with their highest percentage of gastroprotection. These results support the fact that GSH plays an imperative role in maintaining the gastric mucosal integrity. Omeprazole was reported to have antioxidant effect in addition to anti-secretory activity [6]. This explains the high content of GSH in Omeprazole group. EM pre-treated group also demonstrated significantly higher GSH level and lower MDA level than ulcer control group; these results further suggested that indomethacin induced gastric ulcer was mostly due to oxidative stress. EM contains SC, T20, and β-CD, which was used to emulsify KSOM and KSON. In the previous study, SC was found to have very high ABTS free radical scavenging activity (97.6%) and EM which contained a high concentration of SC had 93.5% of ABTS scavenging activity (unpublished). Therefore, the higher GSH level and lower MDA level of EM group, compared to ulcer control group and its significant gastroprotection (76.3%) were likely due to its high scavenging activity.

## Gastroprotective effect of treatments on indomethacin-induced ulcer

KSO contains numerous antioxidants and phytochemicals, including vitamin E (543.4 mg/kg of oil) [13], total flavonoid contents (529.4 mg catechin equivalents/kg of oil), saponin (681.4 mg/kg oil), total terpenoid content

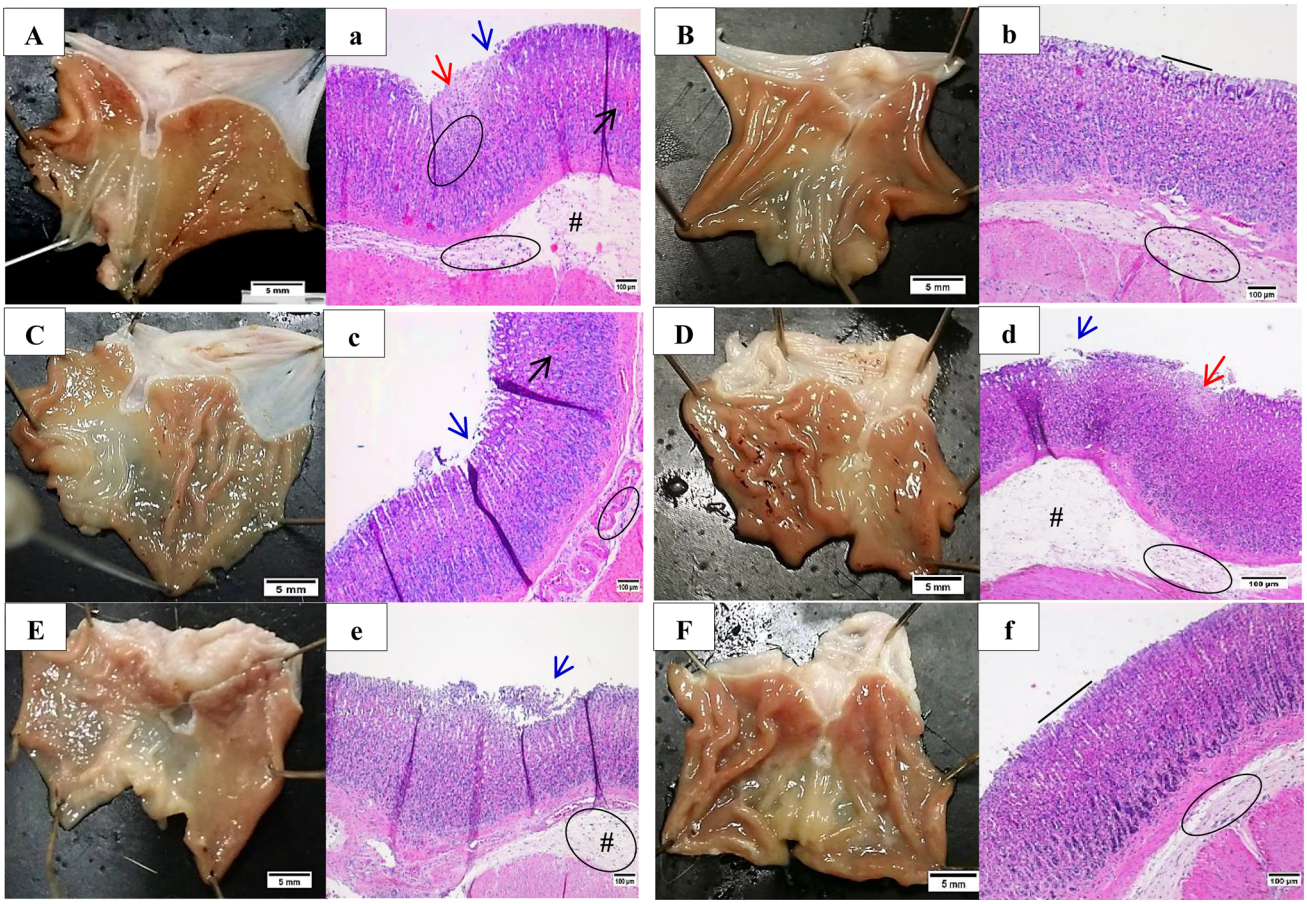


**Fig. 1** Gross and histological examination (×20 magnifications) of the indomethacin-induced gastric lesions in rats. (**A**, **a**) control group; (**B**, **b**) Omeprazole; (**C**, **c**) EM; (**D**, **d**) KSO; (**E**, **e**) KSOM; (**F**, **f**) KSON. Blue arrow: mucosa lesions (erosion); black line: mucus depletion (superficial erosion); red arrow: fibrosis; black arrow: haemorrhage; circle: leukocytes infiltration; #: oedema. *EM* emulsifier mixture, *KSO*, kenaf seed oil, *KSOM* kenaf seed oil-in-water macroemulsions, *KSON* kenaf seed oil-in-water nanoemulsions

**Table 2** The effects different pre-treatment groups on SOD, GSH, and MDA in the gastric tissue homogenates of indomethacin- and ethanol-induced gastric lesions in rats

| Ulcer model | Groups | SOD (U/g tissue) | GSH (nmol/g tissue) | MDA (nmol/g tissue) |
|---|---|---|---|---|
| Indomethacin-induced | Ulcer control | 13.76 ± 0.26 | 49.31 ± 14.88 | 91.77 ± 15.26 |
| | Omeprazole | 14.00 ± 0.07 | 123.20 ± 14.87*** | 79.31 ± 3.05 |
| | EM | 13.80 ± 0.15 | 72.86 ± 8.94* | 83.82 ± 7.89 |
| | KSO | 13.80 ± 0.16 | 75.07 ± 43.19* | 80.63 ± 20.5 |
| | KSOM | 13.85 ± 0.07 | 85.25 ± 19.63* | 78.93 ± 11.58 |
| | KSON | 14.00 ± 0.07 | 102.44 ± 17.26*** | 74.03 ± 7.84 |
| Ethanol-induced | Ulcer control | 13.44 ± 0.20 | 39.55 ± 6.35 | 77.74 ± 4.45 |
| | Omeprazole | 13.52 ± 0.24 | 81.61 ± 11.05*** | 57.05 ± 10.55 |
| | EM | 13.41 ± 0.32 | 59.36 ± 9.81* | 62.08 ± 18.29 |
| | KSO | 13.42 ± 0.36 | 59.85 ± 3.09* | 59.68 ± 8.94 |
| | KSOM | 13.49 ± 0.26 | 59.89 ± 7.58* | 56.07 ± 17.33 |
| | KSON | 13.52 ± 0.04 | 84.31 ± 23.66*** | 52.66 ± 3.83* |

Values are expressed as mean ± S.D. (n = 5). Statistical comparison was determined by One-Way ANOVA followed by Dunnett's multiple comparison tests

*EM* emulsifier mixture, *KSO*, kenaf seed oil, *KSOM* kenaf seed oil-in-water macroemulsions, *KSON* kenaf seed oil-in-water nanoemulsions

*$p < 0.05$ statistically significant compared to the ulcer control group

***$p < 0.001$ statistically significant compared to the ulcer control group

(1487.6 mg linalool/kg oil) [23]. Terpenoids and saponins have been reported to possess antiulcerogenic activity [4]. Many studies showed that vitamin E had effectively inhibited gastric lesions effectively primary via antioxidant and anti-inflammatory mechanisms [18, 30]. In addition, it has been reported that flavonoids exhibited anti-secretory and cytoprotective properties apart from antioxidant activities in gastric ulcer induced by different models [33]. Catechin has been reported to inhibit the oxidative damage of the intestinal mucosa in the in vitro and in vivo studies, suggesting the protective effect of catechin on gastrointestinal ulcers [9]. Results from this study proved that nano-sized particles with higher absorption and bioavailability provided better gastroprotective effect against indomethacin-induced ulcer, compared to macro-size particles and non-emulsified KSO. Endogenous GSH supports the recycling of vitamin E [16], hence higher bioavailability of exogenous vitamin E from KSON provide continuous defence against oxidative stress together with GSH. Therefore, apart from providing anti-inflammatory and anti-secretory activity to against ulcer formation, the presence of vitamin E and flavonoids may help to maintain the gastric GSH level by itself acting to scavenge the free radicals.

KSO group showed significantly higher GSH level than ulcer control group due to KSO contains antioxidants that worked synergistically with GSH to reduce oxidative stress (Table 2). However, KSO group exhibited quite severe mucosal damage without gastroprotective effect on indomethacin-induced ulcers. In the previous study, KSO at the dosage of 500 mg/kg b.w showed mild inhibition effect (47.95%) on indomethacin-induced ulcers [23]. However, in this study, KSO at the dosage of 1000 mg/kg b.w did not show inhibition effect. From the bioavailability study, KSO at the same dosage with anti-ulcer study had demonstrated poor absorption rate (unpublished). It could be due to the slow gastric emptying of KSO, especially at the higher dosage, leading to the longer gastric resistance time. Indomethacin was orally gavaged into the stomach of the rat after 1 h of sample pre-treatment; the majority of KSO may still remain in the stomach during the first hour of digestion. Therefore, the hydrophobic drug indomethacin may be dissolved in KSO, which slowed down the drug absorption rate. Consequently, the prolonged direct contact of indomethacin with mucosa has led to irritation, cytotoxic and mucosal damage. Many researchers are aware of the gastrointestinal toxicity caused by the direct contact of indomethacin with gastric. Hence, many researchers were attempting to develop nanoencapsulated indomethacin and the results showed significant reduction of local irritation and gastric lesions by reducing the direct contact of the drug with mucosa [17].

## Ethanol-induced ulcers

### *Gross evaluation of gastric lesions*

Ethanol-induced rats from ulcer control group showed complete ulceration with extensive and visible haemorrhagic lesions in the gastric mucosa [Fig. 2(A)]. Pre-treatment with KSON had markedly ($p < 0.001$) reduced the ulcer index and demonstrated the highest protection, followed by KSOM ($p < 0.001$), and Omeprazole group ($p < 0.001$), compared to ulcer control group (Table 1). KSO pre-treated group showed significantly ($p < 0.05$) but mild inhibition of gastric ulcer formation. Pre-treated with EM group also presented some gastroprotective effect against ethanol-induced ulcers, but no significant difference with the control group. The results of this study indicated that KSON and KSOM pre-treated groups at the concentration of 1000 mg/kg provided better gastroprotective effect against ethanol-induced ulcer than standard drug, omeprazole.

### *Histopathological evaluation of gastric lesions*

Histological appearance of the ethanol-induced ulcer control group displayed extensive destruction of mucosa and epithelial layer (blue arrow) with haemorrhage (black arrow), loss of glandular epithelial cells (rectangle), extensive oedema (#) and leukocyte infiltration (circle) in the submucosa layer [Fig. 1(a)]. Pre-treated with EM and KSO showed a reduction of mucosa and epithelial layer damage, as well as attenuation of submucosa oedema and necrosis [Fig. 1(c, d)], compared to ulcer control group. KSOM pre-treated group demonstrated the lesser destruction of surface epithelium with submucosal oedema and leucocyte infiltration [Fig. 1(e)], compared to control group. Interestingly, the stomach tissues after pre-treatment with KSON exhibited almost normal mucosa and intact epithelial layer with minor mucus depletion. No oedema but mild leukocyte infiltration at submucosa layer was observed [Fig. 1(f)]. Omeprazole pre-treated group showed more disruption of surface epithelium with oedema and leucocyte infiltration than KSOM and KSON [Fig. 1(b)].

## Oxidative stress in the gastric tissues after induced by absolute ethanol

Gastric homogenates from ulcer control group showed markedly decreased of GSH content, although the reduction of SOD was not significant (Table 2). All of the pre-treated groups had significantly restored the depleted GSH level, compared to ulcer control group. The strongest significant ($p < 0.001$) was found in Omeprazole group and KSON group. Although all pre-treated samples showed a

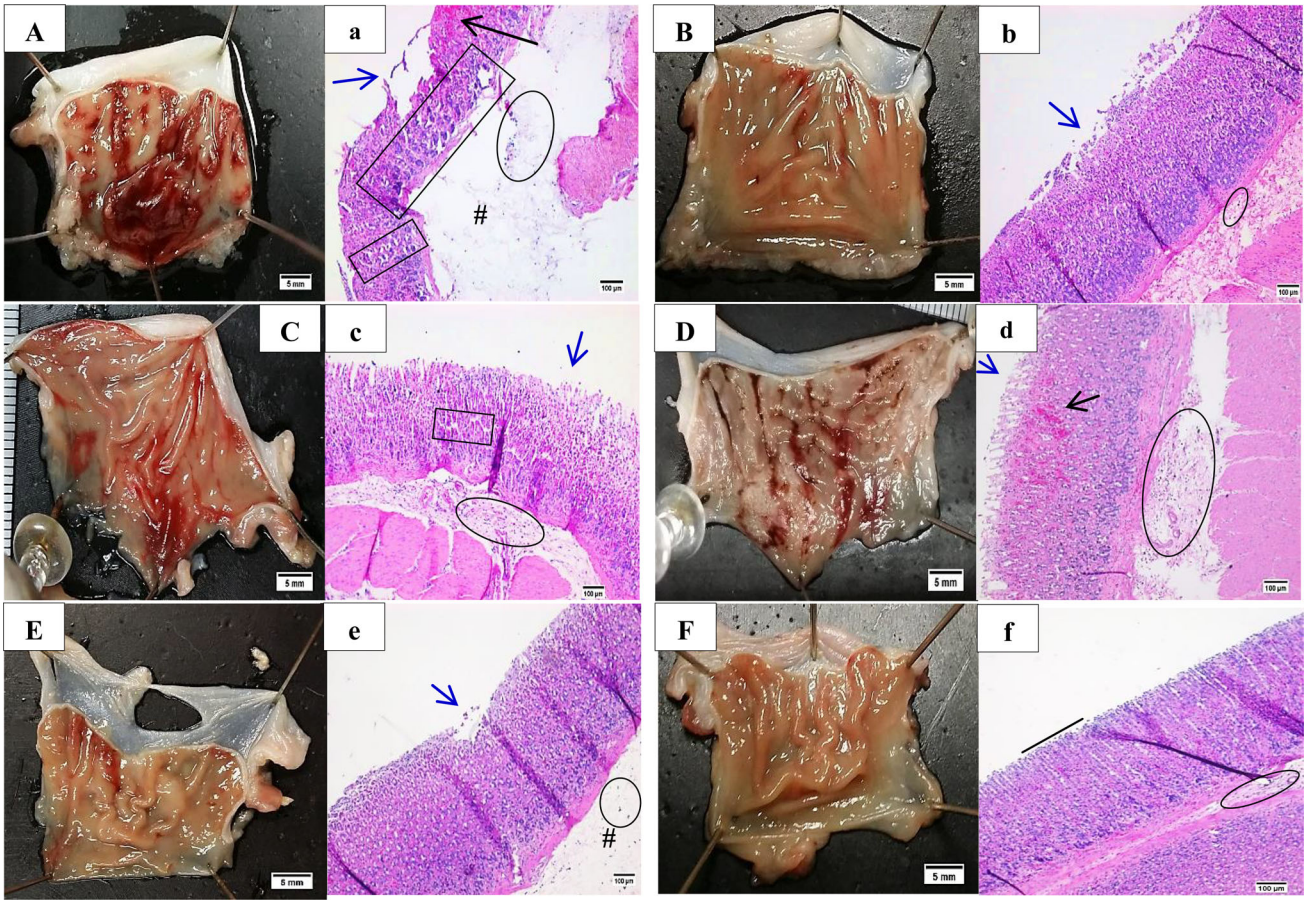


**Fig. 2** Gross and histological examination (20 × magnifications) of the ethanol-induced gastric lesions in rats. (**A**, **a**) control group; (**B**, **b**) Omeprazole; (**C**, **c**) EM; (**D**, **d**) KSO; (**E**, **e**) KSOM; (**F**, **f**) KSON. Blue arrow: mucosa lesions (erosion); black line: mucus depletion (superficial erosion); black arrow: haemorrhage; circle: leukocytes infiltration; rectangle: loss of glandular epithelial cells (necrosis) #: oedema. *EM* emulsifier mixture, *KSO*, kenaf seed oil, *KSOM* kenaf seed oil-in-water macroemulsions, *KSON* kenaf seed oil-in-water nanoemulsions

reduction of MDA, compared to control group; however, only KSON demonstrated significant ($p < 0.05$) reduction of MDA level. These results indicated the efficacy of KSON to enhance intracellular antioxidant by decreasing lipid peroxidation; thereby presented the highest gastroprotective effect to prevent ulcer induced by ethanol.

## Gastroprotective effect of treatments on ethanol-induced ulcer

Ethanol can solubilize the protective mucus and rapidly penetrates the gastric mucosa, causing cell and plasma damage. As a result, it increases membrane permeability to sodium and water [19]; therefore, extensive oedema of submucosa layer was observed in ulcer control group [Fig. 2(a)]. Without protection by mucus, the mucosa is exposed to the hydrolytic and proteolytic actions of hydrochloric acid and pepsin, leading to gastric lesions [2]. Ethanol also causes necrotic lesions in the gastric mucosa, leading to the reduction of defensive factors, including bicarbonate secretion, mucus production, blood flow and secretion of prostaglandin E2 ($PEG_2$) [7]. In addition, ethanol also increases cellular oxidative stress by a generation of free radicals and lipid peroxidation in the gastric mucosa [29]. It was evidenced by the higher level of MDA in ulcer control group (Table 2). Gastric mucus plays an important protective role against gastrointestinal damage. Mucus can also capable of acting as an antioxidant to inhibit ROS. Therefore, secretion of mucus is an essential defensive factor against ethanol-induced ulcers [24].

From the gross and histopathological evaluation, it is presumed that the extensive injured of gastric mucosa in ulcer control group was mainly due to direct toxic and corrosive effect of ethanol, which was orally gavaged to the rats for just 1 h before sacrificing the rats. This was supported by the lower levels of lipid peroxides in gastric tissue induced by ethanol than indomethacin (Table 2). EM pre-treated group demonstrated lower protection (40.50%) in ethanol-induced ulcer than indomethacin-induced ulcer (76.34%), which further suggested that indomethacin-

induced ulcer mainly caused by oxidative stress while absolute ethanol-induced ulcers mainly caused by the corrosive and direct toxic effect. Recently, Bessette et al. [5] found out that β-casein peptide showed a protective effect against induced intestinal dysfunction in rats. In addition, Fernández-Tomé et al. [15] reported that $\alpha_{S1}$-casein peptide increased intestinal mucus production in rats. SC consists of four principal caseins ($\alpha_{S1}$-, $\alpha_{S2}$-, β-, k-caseins) in proportions 4: 1: 3.5: 1.5, respectively [14]. Therefore, it can be deduced that SC containing these caseins has caused the increased release of mucus for gastric protection. Therefore, it is speculated that EM provided its gastroprotective effect to ethanol-induced ulcer mostly by the ability of SC contained in EM that increased mucus secretion rather than by its high antioxidant capacity. KSO pre-treated group in ethanol-induced ulcer showed higher protection effect than indomethacin ulcer. This is probably due to the slow gastric emptying of KSO. Ethanol has a considerably low miscibility with KSO as the solubility of oil in ethanol requires high ethanol volume or high solvent temperature. Since the density of absolute ethanol is lesser than KSO, hence KSO is located beneath ethanol in the stomach. The lower layer of KSO gets more contacts with the gastric surface; hence it coats on the gastric surface, which acts as a barrier against the corrosive ethanol on the coated area. Therefore, the low bioavailability and slow gastric emptying of KSO had provided a mild gastroprotective effect in the ethanol-induced model rather than indomethacin induced-model.

The gastric mucosa of ulcer control group showed severe destruction by absolute ethanol, leading to the increased leukocytes (particularly neutrophils) infiltration [Fig. 2(a)]. Neutrophils infiltration is the initial defence reaction caused by tissue damage and can release ROS that is highly cytotoxic to tissue. Therefore, the reduction of neutrophil infiltration was showed to provide protection and healing of gastric ulcers in rats [20]. The ability for KSO, KSOM, and KSON to reduce oedema and leukocytes infiltration is suggested to be attributed to the cytoprotection, anti-inflammatory and antioxidant properties by the phytochemicals contained in KSO. KSON revealed the most significant reduction of oedema and leukocytes infiltration [Fig. 2(f)]. It further proved that nano-sized particles with improved bioavailability exerted better efficacy in protecting and preventing gastric ulcer than macro-sized and non-emulsified KSO.

In conclusion, KSOM and KSON demonstrated anti-ulcer activity in both indomethacin-induced and ethanol-induced ulcer models, which is attributed to the presence of numerous phytochemicals in KSO. KSON had proved to increase its bioavailability and efficacy as an anti-ulcer agent. In addition, this study showed that the choice of emulsifiers as carrier material in an oral delivery system is highly important. EM had demonstrated gastroprotective effect which worked synergistically with KSO in the form of nanoemulsions to further enhance its efficacy in the prevention of gastric ulcers. The overall results revealed that this nanoemulsions formulation could be a promising strategy for improving the bioavailability of KSO, which exhibited a great potential application in nutraceutical fields.

**Acknowledgements** Financial support of this work by COMSTECH-TWAS Joints Research Grants Programme for Young Scientists in OIC Countries (No: 14-391 RG/MSN/AS C) is gratefully acknowledged.

**Compliance with ethical standards**

**Conflict of interest** The authors have declared no conflict of interest.

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