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Gastro-Protective, Antioxidant, and Anti-Inflammatory Potential of Three Vegetable Oils Against Indomethacin-Induced Gastric Ulceration in Rats: in Vivo and in Vitro Study

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Abstract

The objective of the current study was to evaluate the antioxidant, anti-inflammatory, and anti-ulcerogenic properties of flaxseed, pumpkin, and lupin seed oils, adopting an indomethacin-induced gastric ulceration rat model. Gastric ulcers were generated in Sprague Dawley rats by a single indomethacin oral dose. The tested oils and famotidine were orally administrated for 15 days before ulcer induction. Flaxseed, pumpkin, and lupin seed oils pretreatment attenuated gastric ulcers, as evidenced by the notable reduction in the total acidity, gastric juice volume, and ulcer index, and the increase in gastric pH and the preventive index in addition to restoring gastric levels of PGE-2, nitric oxide, and COX-2 levels. Furthermore, the tested oils significantly prevented the upsurge in gastric TNF-α and IL-1β and enhanced the serum antioxidant capacity with concomitant reduction in oxidant capacity. Histopathological findings supported these results. The prophylactic effects of the tested oil are possibly ascribed to its varied bioactive substances and the free radicals scavenging activities, which we have demonstrated in the *in vitro* study. Conclusion: Flaxseed, pumpkin, and lupin oils exhibited a potential gastroprotective efficacy against the ulcerative consequences of indomethacin. Their effects may be mediated by suppressing oxidative stress and gastric inflammation and maintaining gastric PGE-2, nitric oxide, and COX-2 levels.

***Keywords*:** Gastric ulcer; Indomethacin; Flaxseed oil; Pumpkin oil; Lupin oil; Rats

1. Introduction

Peptic ulcers are among the most prevalent gastrointestinal illnesses, affecting about 5-10% of the population worldwide [1]. It is a complex, multifactorial ailment that can arise from the compromised balance of gastroprotective elements like the prostaglandin E2 (PGE-2) secretion and the mucosal-bicarbonate barrier and hostile elements such as Helicobacter pylori (H. pylori) infection, gastric acid, pepsin [2], and eventually a rise in the release of the proinflammatory cytokines such as interleukin-1β (IL-1β), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and interleukin-17 (IL-17) in the mucosa. The ulcerative process is further aggravated by the rise in reactive oxygen species generation by neutrophil cells that have invaded the inflammatory region [3]. Chronic non-steroidal anti-inflammatory drug (NSAID) use, stressors, a high-spice diet, and smoking have contributed to an escalating prevalence of stomach ulcers [2]. The use of NSAIDs, including indomethacin, induces topical damage by disrupting the stomach epithelium, which allows hydrogen ions to diffuse while additionally weakening the mucosal surface's hydrophobic properties, allowing gastric acid and pepsin to destroy the epithelium, coupled with the suppression of cyclooxygenase (COX) and the endogenous PGE production, which lowers epithelial mucus, epithelial proliferation, mucosal blood flow, and mucosal resistance to damage [4-6].

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Consequently, treating stomach ulcers should emphasize restoring this protective barrier and reducing gastric acid, inflammation, and oxidative stress to minimize tissue damage.

Natural products found in plants and diets are typically safe gastro-protective medications and have shown promising outcomes when used to treat stomach ulcers [7, 8]. Plants contain active ingredients such as flavonoids, terpenoids, tannins, and polyunsaturated fatty acids (PUFAs), often exhibiting antiulcer properties [5]. Research on animal models revealed that various plants and their active constituents possess substantial anti-ulcerogenic properties through antioxidant, anti-secretory, muco-protective, anti-inflammatory, antimicrobial, and anticancer activities compared with reference drugs [9, 10].

The flax plant (Linum usitatissimum) is grown worldwide for its oil and fiber. Flaxseed has soluble fibers or mucilage (6%), insoluble fibers (18%), proteins (25%), and oil (30 to 40%), with alpha-linolenic acid (ALA) accounting for 50 to 60 percent of all fatty acids in flaxseed oil (FSO) [11]. ALA is a precursor to omega-3 fatty acids that makes flaxseed the primary plant-based source of omega-3 fatty acids [12]. FSO has been shown to improve lipid and glucose profiles, mitigate cardiac, hepatic, and renal dysfunction, eliminate oxidative damage, and boost the antioxidant protection mechanism in patients with metabolic syndrome. It also suppressed the growth of cervical cancer in mice and reduced cell proliferation in various human tumors [13,14]. Pumpkin is in the genus Cucurbita and Cucurbitaceae family. It is a valuable food. Containing plentiful nutrients, including protein, lipid, carbohydrate, fiber, and phytochemical substances such as carotenoids, tocopherols, and β sitosterol [15]. Pumpkin seed oil (PSO) is abundant in different fatty acids and beneficial substances, including lutein, β-carotenes, α-tocopherol, vitamin B, and other minerals. [16]. PSO has been reported to exert effects that are wound-healing, antimicrobial, anti-inflammatory, and antioxidant [17]. Additionally, animal studies have demonstrated that PSO could be valuable for treating hypertension, arthritis, hypercholesterolemia, and diabetes [17-19]. Lupins are members of the family Leguminosae. Lupin seed oil (LSO) is distinguished by increased levels of unsaturated fatty acids and minor beneficial substances like phytosterols and γ-tocopherol that provide anti-mycotoxicological and antioxidant potency for the oil [20-22].

The intended objective of the current study was to evaluate the phytochemical components, the fatty acids profile, free radicals scavenging properties, and the reducing power of flaxseed, pumpkin seed, and lupin oil and to estimate the potential antioxidant and anti-inflammatory, and gastroprotective efficacy of these oils against an indomethacin-induced gastric ulcer rats model compared to famotidine, the conventional anti-ulcer agent.

2. Materials and Methods

2.2. In vitro experiments

2.2.1. Fatty acids evaluation

The detection and quantification of fatty acids in the flaxseed, pumpkin, and lupin seed oils were conducted by adopting the Gas Chromatography Flame Ionization Detection (GC FID) method. Fatty acid methylation was done according to the procedure outlined by Radwan and Ahmed [23]. Then, fatty acid methyl esters (FAMEs) were investigated utilizing a gas chromatograph (Agilent Technologies 7890A GC) outfitted with a flame ionization detector (FID). The separation of FAMEs was done on a Supelco SP2330 column (30 mm long × 0.32 mm i.d. × 0.2 μm film thickness, Cat. No. 24073, Sigma-Aldrich, St. Louis, MO). The splitless injection method and flow rate conditions through the GC column were carried out in compliance with the techniques clarified by Radwan and Ahmed [23]. The fatty acid relative peaks were determined by comparing their retention time with the reference mixture that underwent the same analytical process. The total area was measured and served to calculate the percentage of each area beneath each peak, which corresponds to each fatty acid. The oxidizability (Cox value) of the tested oils was computed by employing the formula: Cox value = (C18: 3 × 21.6) + (C18: 2 × 10.3) + (C18: 1×1) \ 100 [24].

2.2.2. Determination of total phenolic

The total phenolic concentration in flaxseed, pumpkin, and lupin seed oils was evaluated using the Folin-Ciocalteu technique [25]. Twenty μL of each tested oil was added to 100 microlitres of a Folin-Ciocalteu Reagent (0.2 N). The tubes were set in the dark and at the ambient temperature. 80 microlitres of 7.5% sodium carbonate (Na_2CO_3) solution was added to the mixture. The absorbance was determined after an hour against a blank at 765 nm. All measurements were conducted three times. Total phenolics content has been specified as milligrams of gallic acid

equivalents for each gram of oil (mg gallic acid eq/g oil).

2.2.3. Total flavonoids determination

The total flavonoid contents in the tested oils were estimated using the aluminum chloride ($AlCl_3$) colorimetric technique [26]. One mL of each tested oil was mixed with one mL of 2% $AlCl_3$ solution. The reaction mixture's absorbance was measured after a 15-minute incubation period at room temperature at the wavelength of 430 nanometers. Quercetin was utilized as the reference standard. The amount of flavonoid compound present was reported as quercetin equivalent in milligrams for each gram of oil (mg QtE/g oil).

2.2.4. Gas chromatographic determination of some phenolic compounds

Each oil was extracted utilizing methanol, and the produced extract was analyzed using gas chromatography. A capillary column, HP INNOWax Column (30 m × 0.25 mm × 0.25 µm film thickness), and a flame ionization detector (FID) were utilized. The temperatures at the entry and detection points were 250 -320 °C, respectively. Split injection was done with a split ratio of 20 to 1. Nitrogen was the carrier gas. The compressed air and hydrogen pressures were 35 psi and 22 psi. The oven program was: initial temperature at 50 °C, first ramping at eight °C/min for twenty min, maintained for four min, then a second ramping at 12 °C/min for four min [7].

2.2.5. Scavenging ability against 1,1-Diphenyl-2-picrylhydrazyl Radical (DPPH) assessment

The scavenging activity towards the DPPH radical of each flaxseed, pumpkin, and lupin oil was assessed by applying the procedure reported by Bendaoud et al. [27]. Twenty µL for every oil was included in 180 µL of 0.1 mM DPPH solution. After shaking the mixture vigorously, it was left to sit at the ambient temperature for 1/2 an hour in the dark. The absorbance was determined against blank at 520 nm. Ascorbic acid (60 mg/L) was used as standard. All measurements were done in triplicate. The results were displayed as Vit. C eq/g oil.

2.2.6. Superoxide anion scavenging evaluation

A riboflavin-light-nitro tetrazolium blue system backed the superoxide anion radical scavenging activity test [28]. Briefly, One mL of each oil was mixed with 0.3 ml riboflavin (50 mM), 0.5 mL of phosphate buffer (50 mM, pH 7.6), 0.25 mL phenazine methosulfate (PMS, 20 mM), and 0.1 mL NBT (0.5 mM). A fluorescent bulb was used to illuminate the mixture to initiate the reaction. After incubation for twenty minutes, the absorbance at 560 nanometers was measured. The standard was ascorbic acid (60 mg/L).

2.2.7. Hydrogen peroxide scavenging activity

40 mM hydrogen peroxide solution was made using 50 mM phosphate buffer (pH 7.4). 0.1 mL of each oil was added to a hydrogen peroxide solution in the test tubes. The volumes were made up to 0.4 mL with the phosphate buffer. Then, the tubes were vortexed. After ten minutes, hydrogen peroxide's absorbance at 230 nm was measured and compared to a blank solution containing a phosphate buffer but no hydrogen peroxide [29].

2.2.8. Reducing power

The reducing power was calculated based on Fe^{+3} to Fe^{+2} conversion in the presence of solvent fractions [30]. The Fe^{+2} can be assessed by measuring Perl's Prussian blue production at 700 nm wavelength. One mL of each oil was mixed with one mL of phosphate buffer (0.2 M, pH 6.6) and one mL of potassium ferricyanide (ten mg per mL). The mixture was incubated for twenty minutes at 50°C, and one mL of trichloroacetic acid (100 mg per L) was added. Then, the mixture was centrifuged for ten minutes at 3000 rpm to separate the solution's upper layer. A volume of one mL from each mixture was mixed with 0.2 mL of 0.1% (w/v) fresh ferric chloride and one mL of distilled water. After ten minutes of reaction, the absorbance was measured at 700 nm.

2.3. In vivo experiments

2.3.1. Animals

Forty-eight adult male Sprague-Dawley albino rats weighing around 200 to 250 g were procured from the Agricultural Research Center, Giza, Egypt. Every animal was kept in a separate cage made of metal and remained at an adjusted temperature (23–25°C) and ambient humidity (50–60%) and on a 12-

hour light-dark cycle. Throughout the trial and a week-long adaption period, the animals received a standard laboratory chaw and unrestricted access to water. All animal housing and handling methods were evaluated and approved by the Research Ethics Committee of Women College, Ain Shams University, code: sci1532309003.

2.3.2. Experimental design

The rats were assigned into six groups, each including eight rats. The first and the second group received a daily oral dose of physiological saline (1.5 mL/ kg body weight/day, p.o) and served as the negative and positive control, respectively. The third group (the famotidine group) was treated with a daily oral dose of famotidine (20 mg/kg body weight) [31]. Groups 4 through 6, FSO, PSO, and LSO groups, got a daily oral dose of flaxseed, pumpkin, or lupin seed oil, respectively (1.5 ml/kg body weight, p.o.) [32]. The previous treatment continued for 14 consecutive days. On day fifteen, after pretreatment of saline, famotidine, or the tested oils and after 24 hours of fasting, all animals were subjected to a single oral administration of indomethacin (100 mg/kg, p.o.) [33], apart from the negative control group, which was given physiological saline. Four hours following indomethacin administration, animals were sacrificed by cervical dislocation under diethyl ether anesthesia. The blood samples were collected from the retino-orbital sinus straight into centrifuge tubes without heparin. Serum aliquots were separated by centrifugation at 3000 r.p.m for 15 minutes and were frozen at -20 °C for subsequent biochemical analysis.

After blood sampling, The abdominal cavity was longitudinally incised to obtain the stomach, the upper and the lower ends of the pylorus and esophagus were closed, and the stomach was removed. The gastric juice was emptied into a glass tubing. Then, the stomachs were opened around the larger curvature and washed with ice-cold saline, and the stomach tissues were examined to assess the formation of ulcers. Following the macroscopical inspection for ulcers, the stomach was divided in half right away. One was fixed in a neutral formalin solution for histological examination, while the other was homogenized in phosphate buffer (0.1 M) followed by a 10-minute, 12,000 rpm, 4°C centrifugation. The supernatants were used for PGE-2, COX-2, total nitric acid (NO), Cyto-P450 reductase, TNF-α and IL-1β determinations.

2.3.3. Mucosal damage evaluation

The macroscopical mucosal lesion examination was performed on the stomach glandular section. The lesions were identified utilizing an illuminated magnifying lens (10×). The ulcer's severity was indicated in scores: 0 = typical coloring, 0.5 = coloring in red, 1.0 = confined ulcer, 1.5 = hemorrhagic patches, 2.0 = profound ulcer, and 3.0 = perforations [34]. The ulcer index was computed by the formula: $UI = (UN + UP + US) \times 10^{-1}$ where UI is the ulcer index; UN is the average ulcer number per animal; US is the average severity scores number; and UP is the animal percentage with ulcers. The following formula was used to evaluate the Preventive Index [1].

$$\text{Preventive Index (\%)} = \frac{\text{UI (Positive control group)} - \text{UI (Treated group)}}{\text{UI (positive control group)}} \times 100$$

2.3.4. Gastric Volume, pH, and total acidity

The stomach contents were collected and centrifuged at 3000 × g for ten minutes. Gastric juice volume was measured (mL), and the pH of the supernatant was examined using a pH meter for each sample [35]. Then, one mL of gastric juice was titrated versus sodium hydroxide (0.1 mol L^{-1} NaOH), utilizing phenolphthalein as an indicator until the endpoint (faint pink) was reached. The NaOH volume utilized was recorded, and total acidity was calculated as described by Dashputre & Naikwade [36].

2.3.5. Biochemical analysis

2.3.5.1. Determination of gastric levels of COX-2, PGE2, total nitric acid, and Cyto-P450 reductase

Gastric levels of COX-2 were assessed using rat ELISA kits (Catalog No: 27187) obtained from Immuno-Biological Laboratories Co., Ltd. (Gunma, 370-0831, JAPAN). Rat ELISA kits were also used to assess the gastric levels of PGE-2 (Catalog No: MBS7606497, MyBioSource, Inc. San Diego, CA 92195-3308, USA) and Cyto-P450 reductase (Catalog No: MBS2022373, MyBioSource, Inc. San Diego, CA 92195-3308, USA). All kits adopted the sandwich ELISA technology following the manufacturers' instructions. The concentration of NO in the tissue was quantified indirectly as the

concentration of nitrate (NO^{3-}) and nitrite (NO^{2-}) by the Griess reaction using a nitric oxide assay kit (Colorimetric) (Abcam, Waltham, MA 02453, USA).

2.3.5.2. Determination of gastric TNF-α and IL-1β

The levels of the gastric inflammatory cytokines, TNF-α, and IL-1β were determined by rats-specific ELISA kits, adopting the instructions supplied by the manufacturer (DuoSet kits, R&D Systems; Minneapolis). The results are represented as pg of cytokine /mL.

2.3.5.3. Determination of serum total antioxidant and oxidant capacity

Serum oxidant and total antioxidant capacities were assessed using standard kits bought from Labor Diagnostika Nord GmbH & Co.KG, Germany, according to the methods of Flohe & Gunzler [37] and Cao et al. [38], respectively.

2.3.6. Histopathological examination

Stomach samples were gathered from experimental groups, fixed in 10% neutral formalin, dried, cleaned, dehydrated, and embedded within paraffin. Five-micron thickness sections of the paraffin blocks were prepared for histopathological analysis by staining with hematoxylin and eosin [39]. Tissue alterations in the stomach were observed and rated as follows: (-) no changes, (+) mild, (++) moderate, and (+++) severe changes. The grading was indicated by percentage as <30% changes (mild), 30% – 50% (moderate), and >50% (severe changes) [40].

2.4. Statistical analysis

The data are clarified as mean ± standard error of the mean (mean±SEM). Using the SPSS package program, version 13, The groups' significant differences were verified by employing a one-way analysis of variance and then Tukey–Kramer multiple comparisons tests. A p-value of less than 0.05 indicated that the differences were significant.

3. Results

3.1. Evaluation of the fatty acid composition

Fig. (1A, B, and C) demonstrate the fatty acid composition chromatographic profile of FSO, PSO, and LSO. The main FAs in FSO were ALA (C18: 3 n-3), oleic acid (C18: 1 n-9), linoleic acid (C18: 2 n-6), palmitic acid (C16: 0), and stearic acid (C18: 0) with percentages of 57.4±4.67, 13.98±1.1, 12.03±1.87, 3.89±0.27 and 2.58±0.197 (g/100 g of total fatty acids), respectively. The % of saturated fatty acids (SFA) was 12.33%, unsaturated fatty acids (UFA) was 86.44%, and that of polyunsaturated fatty acids (PUFA) was 69.43%.

The ratios of UFA/SFA and PUFA/UFA were 7.01 and 80.32, respectively. The Cox value was 13.77± 0:005 (Table 1). In contrast, linoleic, oleic, palmitic, stearic, and palmitoleic (C16:1) acids were the main FAs of PSO, with percentages of 56.08±4.87, 22.15±1.86, 12.68±1.1, 3.21±0.23, and 2.08±0.012.

The % of SFA was 17.34, the % of USFA was 82.75, and the % of PUFA was 56.2. USFA/SFA and PUFA/UFA ratios were 4.77 and 67.91. The Cox value was 6.023 (Table 1). The FAs composition in LSO was as follows: oleic acid (44.48±2.1), linoleic acid (33.95±1.97), palmitic acid (7.98±0.54), α-linolenic acid (6.54±0.43), and myristic acid (C14: 0) (2.25±0.16). SFA, USFA, and PUFA percentages were 13.99, 85.22, and 40.49, respectively. The USFA/SFA and PUFA/UFA ratios were 6.09 and 47.51, respectively. The Cox value was 5.35 (Table 1).

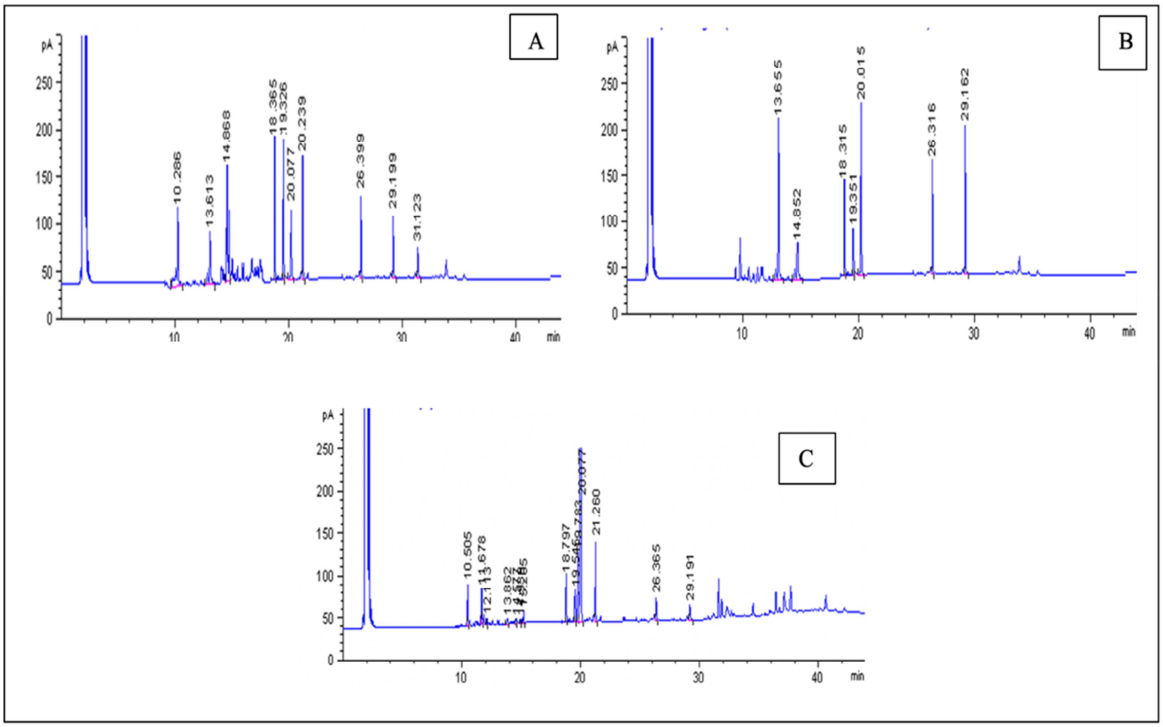

Fig. 1. GC chromatograms of the essential oils of (A) flaxseed oil, (B) pumpkin oil, and (C) lupin oil

Table (1)
Fatty acids composition of flaxseed, pumpkin, lupin seed oils

Fatty acids	Content (g/100 g of total fatty acids)		
	Flaxseed oil	Pumpkin oil	Lupin oil
C14:0 (Myristic acid)	1.65±0.12	Nd	2.25±0.16
C16:0 (Palmitic acid)	3.89±0.27	12.68±1.1	7.98±0.54
C16:1 (Palmitoleic acid)	2.26±0.19	2.08±0.012	0.25±.011
C18:0 (Stearic acid)	2.58±0.197	3.21±0.23	1.08±0.095
C18:1 (Oleic acid)	13.98±1.1	22.15±1.86	44.48±2.1
C18:2 (Linoleic acid)	12.03±1.87	56.08±4.87	33.95±1.97
C18:3 (Linolenic acid)	57.4±4.67	0.12±0.01	6.54±0.43
C20:0 (Arachidic acid)	2.95±0.21	1.45±0.11	1.26±0.093
C20:1 (Eicosenoic acid)	0.77±0.06	0.87±0.06	Nd
C22:0 (Docosanoic acid)	1.01±0.092	Nd	0.88±0.051
C24:0 (Tetracosanoic acid)	0.25±0.01	Nd	0.54±0.038
	Fatty acid groups and relations		
SFA	12.33	17.34	13.99
UFA	86.44	82.75	85.22
USF/SFA	7.01	4.77	6.09
PUFA	69.43	56.2	40.49
MUFA	17.01	25.1	44.73
PUFA/UFA (%)	80.32	67.91	47.51
ω-6 FA	12.03	56.08	33.95
ω-3 FA	57.4	0.12	6.54
Cox value	13.77	6.023	5.35

Values are shown as the average of three measurements ± standard error.

ND: Not detected

3.2. Evaluation of the phenolic compounds

Data in Table (2) show that FSO, PSO, and LSO have a total phenolic content of 64.32±5.7, 51.3±0.59, and 49.6±4.87 mg gallic acid eq/g oil, respectively. Meanwhile, the total flavonoid contents were 33±0.98, 32±1.01, and 29±1.2 mg quercetin eq/g oil in the three oils, respectively. The results shown in Table (2) further show that a considerable amount of phenolic compounds are found in the tested oils. FSO contains catechin (3.98±0.28), chlorogenic acid (0.94±0.074), coumaric acid (2.89±0.14), naringenin (5.97±0.387), and salicylic acid (0.85±0.074). Likewise, PSO contains catechin (0.513±0.043), coumaric acid (0.22±0.016), vanillin (12.9±0.96), and salicylic acid (3.4±0.245). Additionally, LSO includes catechin (1.03±0.097), chlorogenic acid (0.215±0.019), naringenin (0.335 ± 0.0247), vanillin (1.84 ±0.12) and salicylic acid (4.94±0.38).id (0.94 ± 0.074), coumaric acid (2.89±0.14), naringenin (5.97±0.387), and salicylic acid (0.85±0.074).

Table (2)
Phenolic compounds in the tested oils

Compounds	Flaxseed oil	Pumpkin oil	Lupin oil
Total phenolics (mg Gallic acid eq/g oil)	64.32±5.7	51.3±0.59	49.6±4.87
Flavonoid (mg Quercetin eq/g oil)	33±0.98	32±1.01	29±1.2
Catechin (mg/100 g)	3.98±0.28	0.513±0.043	1.03±0.097
Chlorogenic acid (mg/100 g)	0.94±0.074	ND	0.215±0.019
Coumaric acid (mg/100 g)	2.89±0.14	0.22±0.016	ND
Narengenin (mg/100 g)	5.97±0.387	ND	0.335±0.0247
Vanillin (mg/100 g)	ND	12.9±0.96	1.84±0.12
Salicylic acid(mg/100 g)	0.85±0.074	3.4±0.245	4.94±0.38

Values are shown as the average of three measurements ± standard error.
ND: Not detected

3.3. Evaluation of the free radicals scavenging activities and reducing power

Table (3) illustrates that the free radical scavenging activities against DPPH for FSO, PSO, and LSO were 74.32±5.7, 61.3±0.59, and 52. ±4.87 Vit. C eq/g oil, respectively. Likewise, the superoxide radical scavenging activity % was 56.4±1.5, 42.9±2.1, and 49.5±2.2, respectively, in the three oils. While the hydrogen peroxide radical scavenging activity % was 32.95±1.9, 13.56±1.15, and 17.95±0.89 in the three studied oils, respectively. In addition, the data presented in Table (3) show that the reducing power of FSO, PSO, and LSO was 3302±98, 2215±101, and 1958±120 µg/g oil, respectively.

Table (3)
Free radicals scavenging activity and the reducing power of the tested seed oils

Parameters	Flaxseed oil	Pumpkin oil	Lupin oil
DPPH (Vit. C eq/g oil)	74.3±5.7	61.3±0.59	52.6±4.87
Superoxide radical scavenging activity %	56.4±1.5	42.9±2.1	49.5±2.2
Hydrogen peroxide radical scavenging activity %	32.9±1.9	13.5±1.15	17.95±0.89
Reducing power µg/g	3302±98	2215±101	1958±120

Values are shown as the average of three measurements ± standard error.

3.4. Macroscopic investigation of the gastric mucosa

As illustrated in Fig. (2), the macroscopic view of normal rats' gastric mucosa showed a typical surface with no visible lesions. Rats subjected to indomethacin administration exhibited substantial injury to gastric mucosa with excessive bleeding and a wide range of gastric ulceration, manifested by a considerably high ulcer number and ulcer index. However, animals pretreated with famotidine (20 mg/kg body weight, p.o.) have very limited to no damage of gastric mucosa and showed a significant reduction ($p<0.001$) in the number of ulcers and ulcer index, providing a 73% preventive index compared to the gastric ulcer control (positive control). Likewise, rats pretreated with FSO, PSO, or LSO displayed mild disruption of gastric mucosa and a substantial ($p<0.01$) decrease in the number of ulcers by 78.1, 75, and 78.1% and the ulcer index by 72.11, 70.6, and 71.87%, and showing preventive index of 72.11, 70.6, 72.01%, respectively, as compared to the positive control (Table 4).

Table (4)
Macroscopic Examination of the Gastric Mucosa

	Number of ulcers	Ulcer Index	Preventive Index (%)
Negative control	-	-	-
Positive control	32±2.8	20.8±1.78	-
Famotidine	$6±0.54^{b\$}$	$5.6±0.43^{b\$}$	73%
FSO	$7±0.69^{b\$}$	$5.8±0.51^{b\$}$	72.11%
PSO	$8±0.99^{b\$c*}$	$6.1±0.62^{b\$}$	70.6%
LSO	$7±0.97^{b\$}$	$5.85±0.43^{b\$}$	72.01%

The data have been displayed as mean values ± SEM (n = 8). Letters a, b, and c illustrate a significant difference between the negative control, positive control, and famotidine groups, respectively. Statistical significance levels at $p<0.05$, $p<0.01$, and $p<0.001$ were denoted by symbols: *, #, and $, respectively. (ANOVA and a Tukey–Kramer multiple comparisons tests).

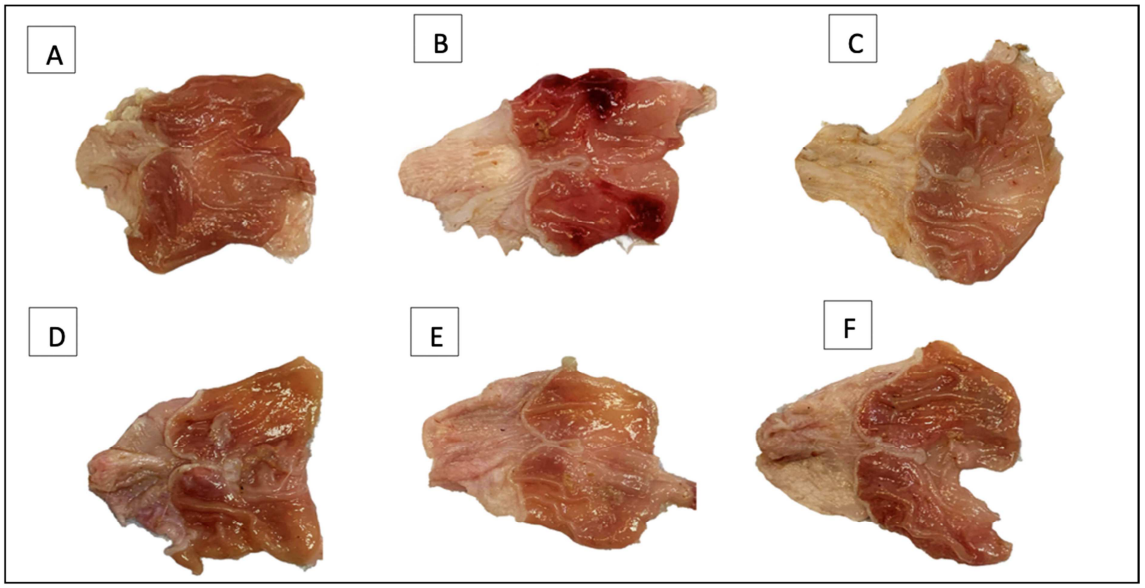

Fig. 2. A Photograph of rat's stomachs demonstrating the protective properties of FSO, PSO, and LSO on indomethacin-induced gastric ulceration in rats; (A) Control showed typical intact gastric mucosa, (B) positive control showed extensive dark red hemorrhage patches on the mucosa, (C) normal appearance with a slight degree of hyperemia and typical mucosal thickening; (D, E, and F) FSO, PSO, and LSO groups, respectively, displayed a normal appearance with a few minor lesions of congestion or hemorrhage

3.5. Gastric Juice volume, total acidity, and pH

Fig. (3) demonstrates that indomethacin administration significantly ($p<0.001$) increased gastric juice volume by 56.39% and total acidity by 70.9% compared to the negative controls. Famotidine, FSO, PSO, and LSO pretreatments anticipate this increase and

significantly ($p<0.001$) prevented this increase in gastric juice volume by 34.5%, 33.7%, 31.86%, and 32.9% and total acidity by 38.4%, 34.3%.25.69%, and 23.88%, respectively, compared to the positive control. Gastric juice pH was significantly ($p<0.001$) decreased by 29.05% in the rats treated with indomethacin as compared to negative control, an effect that was prevented by the pretreatments with famotidine and the three tested oils (33.3%, 33.3%, 27.1%, and 31.4%, respectively).

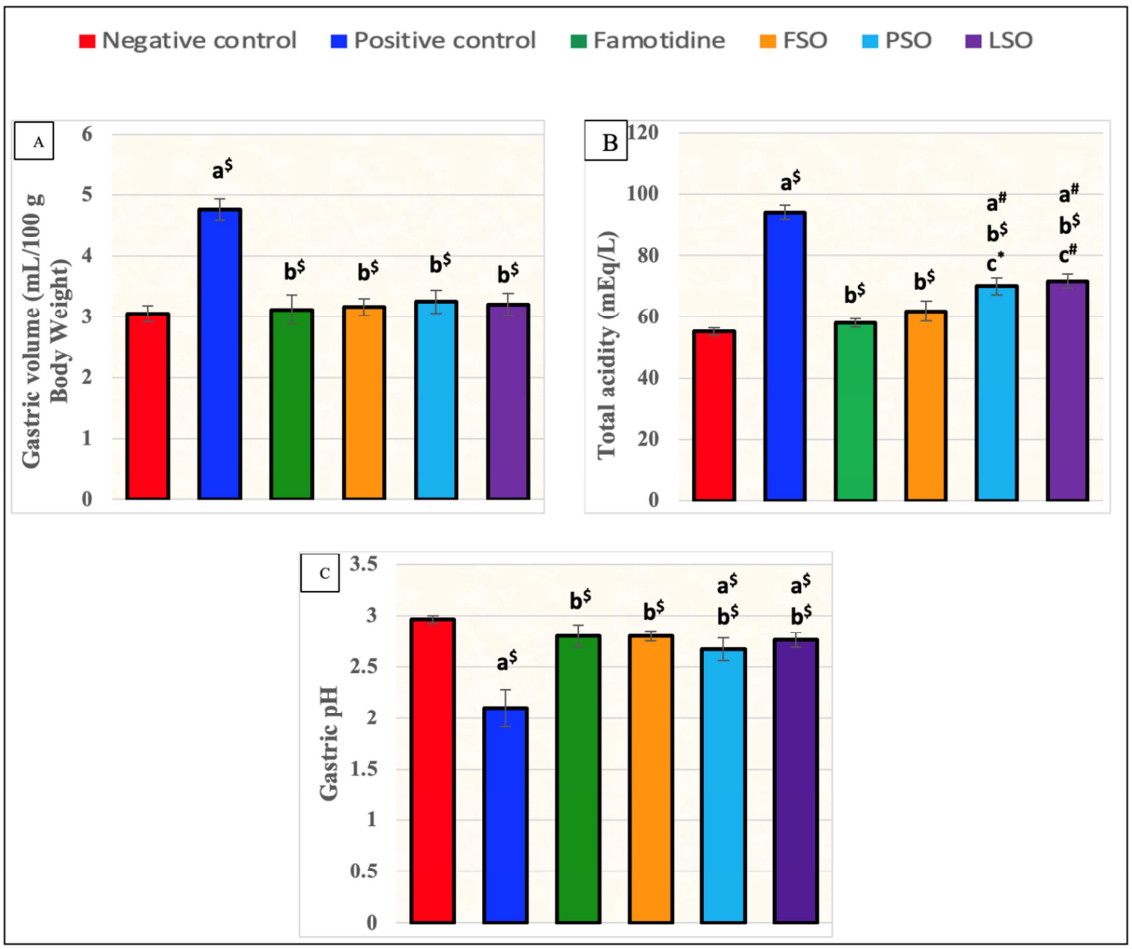

Fig. 3. Gastric volume (A), total acidity (B), and pH (C) of rats in the negative control, positive control, famotidine, FSO, PSO, and LSO groups. The data have been displayed as mean values ± SEM (n = 8). The significant differences between the famotidine, positive control, and negative control groups are displayed in letters a, b, and c, respectively. Statistical significance levels at $p<0.05$, $p<0.01$, and $p<0.001$ were denoted by symbols: *, #, and $, respectively. (ANOVA and a Tukey–Kramer multiple comparisons tests).

3.6. Gastric levels of PGE-2, total nitric acid, COX-2, and Cyto P450 reductase

Results displayed in Fig. (4 A, B, and C) demonstrate that indomethacin administration significantly ($p<0.001$) declined the gastric contents of PGE-2 (38.8%), nitric oxide (30.9%), and Cyto p450 (66.8%) and significantly($p<0.001$) elevated the levels of COX-2 (2.33-fold) in the gastric tissue of the positive control rats as compared to the negative control. Such alterations were markedly attenuated by the pretreatments with famotidine or the tested oils. As shown in Fig. (4), there was a substantial increase in gastric PGE-2 (46.3%, 44.3%, 33.58% and 32.12%, $p<0.001$), nitric acid (30.7% $p<0.01$, 26.37% $p<0.01$,19.6% $p<0.05$, and 22.8% $p<0..05$), and

CytoP450 (1.28-fold, 1.05-fold, 66%, and 54%, p<0.001) in the famotidine, FSO, PSO, and LSO groups as compared to their corresponding levels in the negative control group. Contrariwise, Fig. (4 D) clarified that the gastric tissue levels of COX-2 were significantly (p<0.001) lower by 58.1, 46.48, 32.79 and 28.4% in rats from famotidine, FSO, PSO, and LSO groups, respectively, than the corresponding level in the positive control.

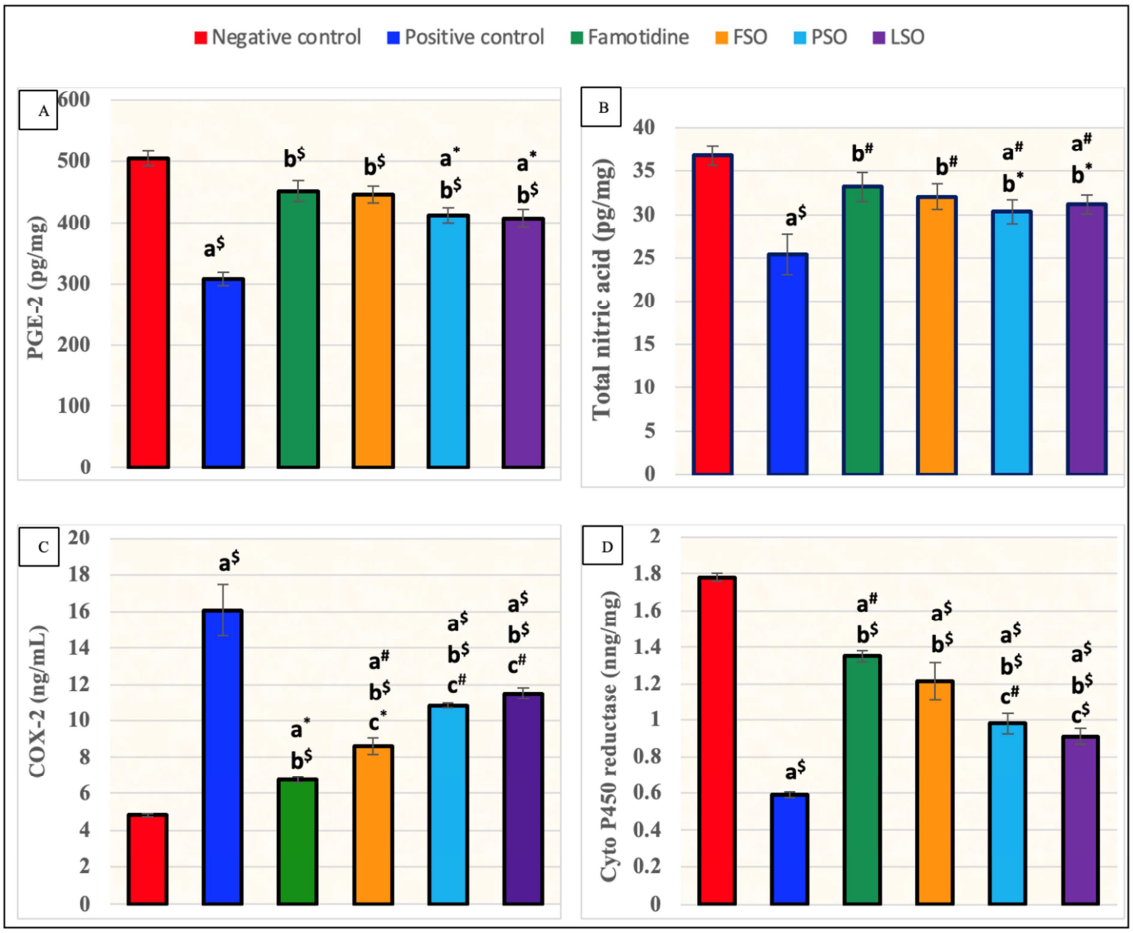

Fig. 4. Gastric levels of (A) PGE-2, (B) total nitric acid, COX-2, and Cyto P450 reductase of rats in the negative control, positive control, famotidine, FSO, PSO, and LSO groups. The data have been displayed as mean values ± SEM (n = 8). The significant differences between the famotidine, positive control, and negative control groups are displayed in letters a, b, and c, respectively. Statistical significance levels at p<0.05, p<0.01, and p<0.001 were denoted by symbols: *, #, and $, respectively. (ANOVA and a Tukey–Kramer multiple comparisons tests).

3.7. Serum total antioxidant capacity and total oxidant capacity

Data in Fig. (5 A and B) reveals that indomethacin administration provoked oxidative stress, evidenced by a marked (p<0.001) elevation in the total oxidant capacity (6.3-fold) and a marked decline (p<0.001) in the total antioxidant capacity 47.3% compared to the negative control rats. Famotidine pretreatment attenuated oxidative stress induced by indomethacin administration as it significantly (p<0.001) reduced the total oxidant by 70.5% and increased the total antioxidant capacity by 47.7%. Similarly, the pretreatment with the tested oils, FSO, PSO, and LSO, mitigated the indomethacin-induced oxidative stress evidenced by significant (p<0.001) reduction in TOC by 88.8%, 63.6%, and 58.4%, coupled with significant increase in TAC by 35.2% (p<0.001), 14.7% (p<0.05),

and 15.9% ($p<0.05$), respectively, in comparison with positive the control.

3.8. Gastric levels of TNF-α and IL-1β

Fig. (5 C and D) demonstrate that indomethacin administration elicited an intense inflammatory response as manifested by the substantial elevation ($p<0.001$) in gastric levels of TNF-α (3.37-fold) and IL-1β (3.79-fold) as compared with the negative control. Such an inflammatory response was significantly attenuated and even normalized in the groups of rats pretreated with famotidine and FSO, PSO, and LSO as they reduced gastric levels of TNF-α by 56.9, 55.3,40.15, and 37.5%, and the levels of IL-1β by 62.7, 58.7, 33.9, and 29.7%, respectively, as compared to the positive control.

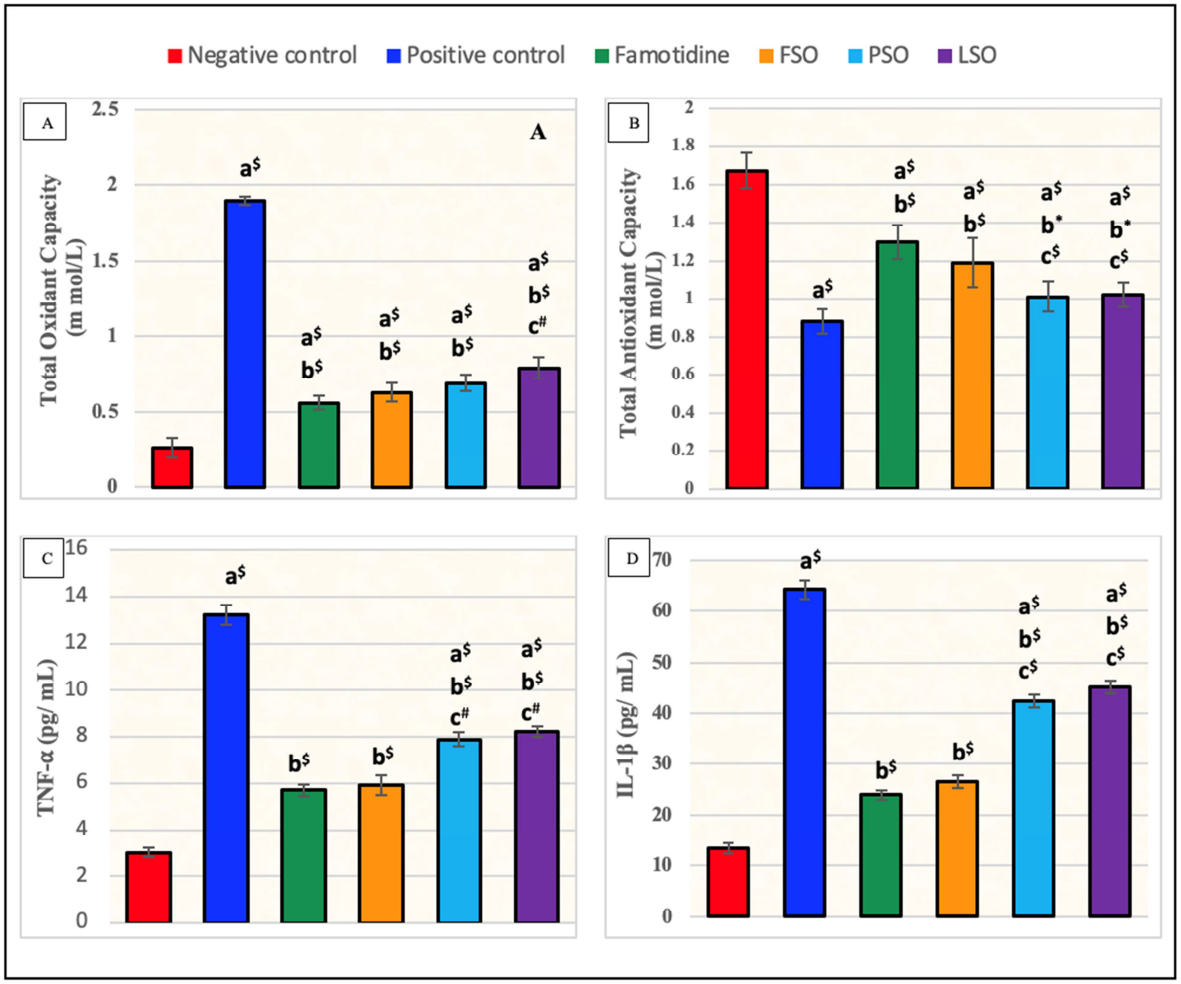

Fig 5. Serum total oxidant capacity (A), serum total antioxidant capacity (B), gastric TNF-α (C), and gastric IL-1 β (D) of rats in the negative control, positive control, famotidine, FSO, PSO, and LSO groups. The data have been displayed as mean values ± SEM (n = 8). The significant differences between the famotidine, positive control, and negative control groups are displayed in letters a, b, and c, respectively. Statistical significance levels at $p<0.05$, $p<0.01$, and $p<0.001$ were denoted by symbols: *, #, and $, respectively. (ANOVA and a Tukey–Kramer multiple comparisons tests).

3.9. Histopathological finding

Light microscopic inspection of stomach sections of the negative control rats depicted the typical histological structure of gastric mucosa and submucosa (Fig. 6 A1 and A2). In contrast, the stomach sections of the positive control group rats showed several histopathological abnormalities with an invasion of mononuclear inflammatory cells in the mucosa (Fig. 6 B1), mucosal necrosis and mucosal hemorrhage (Fig. 6 B2), and extensive invasion of

inflammatory mononuclear cells, diffuse submucosal edema and hemorrhage (Fig. 6 B3). Otherwise, the stomach tissues of the rats in the famotidine group showed an improvement in the histological appearance; the examined sections displayed mild mononuclear inflammatory cell infiltration in both mucosa and submucosa with moderate submucosal edema (Fig. 6 C). Furthermore, a notable remission of the histopathological damage with restoration of the typical histology of stomach tissue was noted in the FSO, PSO, and LSO groups. Stomach sections from these groups exhibited amelioration in the abovementioned lesions as mucosal and submucosal inflammatory cell invasion and edema were mild (Fig 6 D, E, and F, respectively). Table (5) demonstrates the histopathological lesion scores in the stomach tissues of the rats in the different experimental groups.

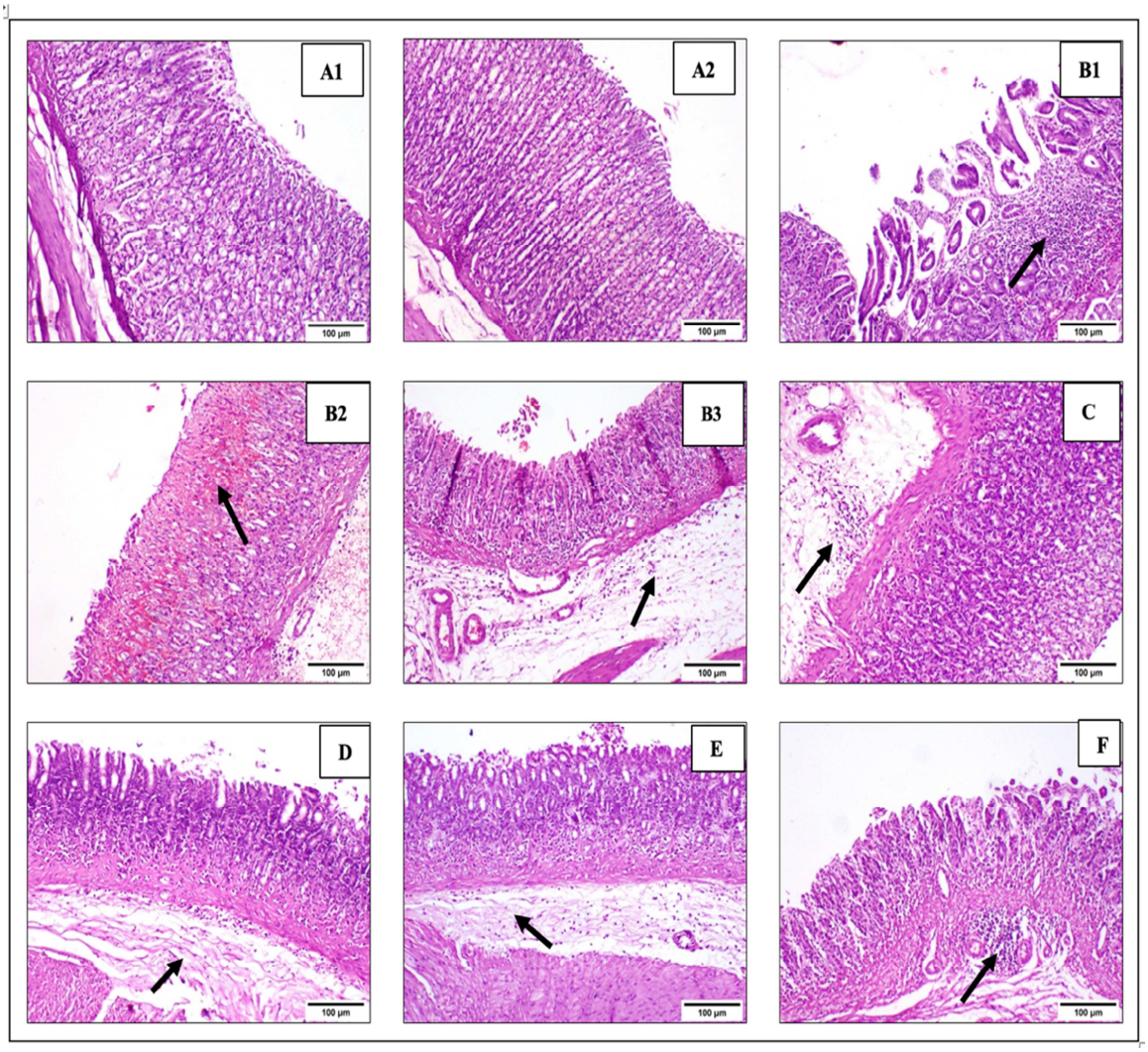

Fig. 6. Representative photomicrographs of rat stomach tissue sections: (A1 and A2) negative control, showing the normal histological structure of mucosa and submucosa. (B1) positive control, showing mucosa infiltration with mononuclear inflammatory cells (arrow). (B2) positive control, showing diffuse submucosal edema (arrow). (B3) positive control, showing diffuse mucosal hemorrhage (arrow). (C), famotidine group, showing few submucosal inflammatory cell infiltration (arrow). (D) The FSO group showed mild submucosal edema (arrow). (E) The PSO group showed few mononuclear inflammatory cell infiltration in the submucosa (arrow). (F) LSO group, showing focal submucosal inflammatory cell infiltration (arrow). (H & E, scale bar, 100 µm).

Table (5):
Scoring of histopathological changes in the stomach of different treated groups

Lesions	Groups					
	Negative control	Positive control	Famotidine	FSO	PSO	LSO
Infiltration of mucosa with mononuclear inflammatory cell	-	+++	+	+	+	+
Mucosal necrosis	-	++	+	-	-	-
Mucosal hemorrhage	-	+++	+	-	-	-
Submucosal mononuclear inflammatory cells infiltration	-	+++	+	+	+	+
Edema of submucosal	-	+++	++	+	+	+
Submucosal hemorrhage	-	+++	+	-	-	-

The scoring system was designed as follows: score (-) = absence of the lesion in all rats of the group (n= 5), score (+)= (<30%), score (++)= (<30% – 50%), score (+++)= (>50%)

4. Discussion

This study's noteworthy finding was that flaxseed oil, pumpkin oil, and lupin seed oil pre-treatments mitigated indomethacin-induced peptic ulcer in rats by diminishing the oxidative stress and inflammatory responses, restoring the levels of PGE-2 and nitric acid and reducing the level of COX-2 in the stomach.

In line with earlier findings, the current investigation verified that the oral administration of indomethacin (100 mg/kg body weight) induced gastric ulceration. This was emphasized by macroscopical and histopathological findings, which revealed gastric mucosal injuries such as a congested appearance and hemorrhagic erosions with edema and severe mononuclear cell infiltration in rat gastric mucosa and submucosa [5, 33]. Moreover, indomethacin-treated rats showed a significant reduction in gastric pH level with inflation in gastric juice volume and ulcer index compared to the normal control group. The elevated hydrogen ion concentration is a hostile element facilitating gastric damage via decreasing pH in gastric juice [41]. It was reported that indomethacin, like NSAIDs, could diffuse into both synthetic and biological membranes, interact with membrane phospholipid molecules, and considerably modify the membrane's hydrophobicity, such as fluidity, bending stiffness and permeability, which can alter the 'gatekeeping functions' of cells, leading to an inevitable gastric mucosal damage such as erosions, ulcers, and bleeding, consequent to membrane pore formation [42]. Extreme stomach mucosa disruption could cause increased gastric content, total acidity, and consequent lowering of gastric pH [6].

Additionally, the here-in observed decline in the gastric PGE-2 and nitric acid and the upsurge in COX-2 could be involved in the gastric ulceration induced by indomethacin. Epithelial cells of the gastric mucosa can be penetrated by indomethacin that inhibits the two cyclooxygenase (COX) isoforms, COX-1 and COX-2; both enzymes are essential in the prostaglandin synthesis from arachidonic acid [31, 43]. COX-1 and COX-2 produce distinct patterns of prostaglandins, and they have diverse tissue localization, distribution, and regulation [31]. Most tissues, including the stomach, express COX-1 constitutively, where it has a housekeeping function [43]. In contrast, the expression of COX-2 appears to result from the response to damage in various tissues; thus, it is referred to as an inducible isoform [44]. Several earlier studies have demonstrated COX-2 induction in inflammatory cells in areas of inflammation [45, 46]. COX-2 mRNA and protein expression were also encountered to be induced at the acute stage of gastric ulceration, with COX-2 expression levels decreasing in correlation with ulcer size during the recuperation stage and dropping to indiscernible amounts with ulcer healing [47].

Similarly, COX-2 mRNA expression was displayed in the rat gastric mucosa damaged by ischemia-reperfusion [48]. Prostaglandin production suppression of COX-1 and COX-2 decreases the mucosal blood flow, bicarbonate, and mucus secretion, provokes vascular damage, and lowers cell turnover [43]. Consistently, indomethacin was reported to obstruct blood microvessels and diminish gastric mucosal blood flow [49]. The reduction in the blood flow of gastric mucosa facilitates neutrophil and leukocyte recruitment in the tissue and directly connects to indomethacin-induced gastropathy. Thus, the gastric ulceration observed in this study could be attributed to the suppression of prostaglandin synthesis due to the blocking of cyclooxygenase by indomethacin. In addition to the observed decline in gastric PEG-2, we found that indomethacin lowered gastric NO levels. Lowering gastric NO can further restrain gastric blood flow, curb PGE-2 synthesis, and force the adhesion of the neutrophil in the stomach mucosa's microvascular endothelium, thereby harming gastric mucosal integrity [50]. Consistent with our findings, a previous study revealed that gastric ulcer caused by a single oral

dose (400 mg/kg bw) of aspirin significantly decreased gastric total nitrite and nitrate levels, suggesting gastric protection from the production of NO was decreased [51]. According to Abdulla et al. [52], maintaining normative nitric oxide levels is among the primary defences against indomethacin's detrimental effects on the stomach mucosa.

Compelling data highlights the implication of inflammatory response perturbations as one of the characteristics of gastric ulcers. In parallel with former reports, the present investigation revealed a heightened state of gastric inflammation in the indomethacin-ulcerated rats as demonstrated by discernibly elevated levels of gastric TNF-α and IL-1β and the severe mononuclear inflammatory cell infiltration in gastric mucosa and submucosa [53-55]. Serum TNF-α and IL-1β levels were markedly elevated after a single dosage of aspirin treatment (200 mg/kg bw, *p.o.*) [53]. Similarly, Li et al. [54] and El-Hussieny et al. [55] reported increased gastric tissue pro-inflammatory cytokines due to ethanol administration. As discussed above, reducing gastric PGE-2 and nitric oxide as a result of indomethacin administration tissue could reduce blood flow to the gastric mucosa and submucosa, promoting neutrophil infiltration in the gastric tissue. These cells secret cytokines such as TNF-α, IL-1β, IL-6, and NF- κB. These molecules play a crucial part by moving to the gastric epithelium cells' nucleus and stimulating the expression of several target genes, such as pro-inflammatory cytokines that can amplify the damage in the gastric mucosa [56]. TNF-α is one of the central pro-inflammatory cytokines that stimulates inflammation and is an efficacious NF-κB activator, so this cytokine fosters the destruction of gastric tissue. Moreover, TNF-α is involved in recruiting neutrophils and other leucocytes by stimulating the production of adhesion molecules in these cells. Furthermore, TNF-α is associated with the build-up of and generation of superoxide molecules as a subsequent of the accumulation and activation of neutrophils in the gastric tissue that causes disruptions in the microcirculation and thus the production of free radicals [49, 56, 57]. Consequently, the control of pro-inflammatory cytokines directly correlates to the decreased ulcerogenic effects of NSAIDs.

Oxidative stress, sparked by reactive oxygen species (ROS) generation, is presumably implicated in the pathogenesis of gastric ulcers [56, 58]. Thus, the elevated production of ROS and depletion of antioxidants observed in our study could account, at least partly, for the corresponding gastric mucosal injury in the indomethacin-treated rats. Consistent with our results, gastric lipid peroxidation was increased in rats with aspirin- or alcohol-induced gastric mucosal injury [55, 59, 60]. The pro-oxidant effects of indomethacin are well known; free radicals such as ROS can be generated through neutrophil infiltration in gastric mucosa [61]. Furthermore, indomethacin can prevent mitochondrial oxidative phosphorylation in the gastric mucosa epithelial cells. As a result, the cytochrome c releases from the mitochondrial intermembranous space into the cytosol, eventually releasing inner ROS in the cells. ROS's multitude of deleterious consequences increases the gastric epithelial cells' permeability and produces mucosal damage in the stomach [56]. Therefore, enhancing the antioxidant capacity and the reduction in the mediators of inflammatory cytokines could be considered a contributing factor in preventing peptic ulcers induced by indomethacin administration.

The present study demonstrated that the three tested oils have excellent protection against the potent ulcerative effect of indomethacin. We observed a prominent reduction in gastric mucosa damage, such as mucosal hemorrhage, inflammatory cell infiltration, and mucosal edema. Furthermore, we noticed a substantial reduction in the number of ulcers and ulcer index while restoring gastric pH to normal levels with simultaneous decreases in gastric secretion and total acidity in indomethacin ulcerated rats pre-treated with the tested oils. These anti-ulcer activities were coupled with the restoration of the altered biochemical parameters. The tested oils significantly stimulated PGE-2 production, increased gastric NO levels, and decreased the inducible COX-2 levels. Additionally, the present study demonstrates that the plant oils pre-treatment had the potential to recuperate the endogenous antioxidant capacity, hamper total oxidant capacity, and curtail the TNF-α and IL-1β upsurge, indicating that these oils could effectively reduce indomethacin-induced oxidative stress and inflammation.

Corroborating with the gastric healing activity demonstrated in vivo study, our in vitro investigation revealed that FSO, PSO, and LSO have antioxidant activity as they exhibited a powerful scavenging action against DPPH radical and superoxide anion and hydrogen peroxide scavenging activity. The observed antioxidant capacities of the tested oils were corroborated by our in vivo findings wherein the tested oils pre-treatment and, more prominently, FSO pre-treatment markedly amended the oxidative aberrations in the ulcerated rats. Additionally, phytochemical analysis in the current study revealed that these oils have high levels of total polyphenols and flavonoids. Furthermore, examination of the phytochemical profile revealed the presence of many phenolic substances, the most important of which are catechins, chlorogenic acid, coumaric acid,

naringenin, vanillin, and salicylic acid. Thus, the observed antioxidant, anti-inflammatory, and anti-ulcer properties of the tested oils in this study could be partly ascribed to the presence of such phenolic compounds.

The health-promoting potential of dietary polyphenols could arise from their anti-inflammatory and immunomodulatory characteristics and their ability to scavenge free radicals like superoxide anion and hydroxyl radicals [9]. By reducing oxidative stress, polyphenols can alter gene expression that promotes inflammation, thereby suppressing downstream cytokine production (e.g., TNF-α and IL-1β) and boosting the tissue levels of antioxidants [10]. Phenolic substances have also been shown to have anti-ulcerogenic properties. They function through anti-secretory and cytoprotective effects as they promote prostaglandin production [62, 63]. Furthermore, several investigations have demonstrated that flavonoids preserved the glycoprotein moiety of the gastric mucosa, decreased mast cell histamine release and lipid peroxidation, and increased nitric oxide activity [62-64]. Catechin, a flavonoid that is present in considerable amounts in the studied oils, has been shown to have a gastro-protective effect against indomethacin-induced gastric ulceration in rats as it promoted the healing of chronic gastric ulcers due to its capacity to increase gastric mucus and its antioxidant properties [65]. Alongside catechin, chlorogenic acid was also detected in oils under the study and has been shown to have a gastro-protective effect on ethanol/HCl and NSAID-induced gastric mucosal damage, which is related to the inhibition of neutrophil influx, antioxidant properties, and inhibition of inflammatory cytokines release [66]. Furthermore, *p*-coumaric acid, also found in the examined oils, has been reported to have gastric healing activity as it increased prostaglandin levels and mucus synthesis in human gastric cells [8].

Additionally, GC analysis in this study revealed that FSO and LSO are rich in n-3 polyunsaturated fatty acid (n-3 PUFA), particularly α-linolenic acid, which makes up 54–65% of the total fatty acids, along with oleic and linoleic acids that have also been found in FSO and LSO. Likewise, linoleic and oleic acids are present in significant amounts in PSO. These fatty acids could be responsible for modulating indomethacin-induced gastric ulcers. A high intake of α-linolenic acid has been linked with lower inflammatory mediators and cytokine production in intensifying inflammatory responses [67]. α-Linolenic acid and its metabolites (EPA and DHA) were reported to inactivate the nucleotide-binding domain and leucine-rich repeat-containing protein-3 (NLRP3) inflammasome, which is accumulated in response to danger signals or infection, then triggers the production of inflammatory cytokines and caspase-1 [68]. Furthermore, n-3 PUFA has been reported to quash the nuclear translocation of nuclear factor-κB, the initial step of inflammasome activation, and suppress pro-inflammatory cytokines [68]. Moreover, the n-3 PUFAs have also been reported to stabilize the membrane by altering membrane structure, disrupting the organization of the membrane lipid raft, and suppressing apoptotic-related cell signaling [69]. Therefore, we suggested that some PUFAs in the oils under study, especially those of the n-3 group, can modulate the inflammatory response and that n-3 PUFAs could act as gatekeepers against the cytotoxic effects of NSAIDs. Several reports have consistently demonstrated the beneficial effects of dietary n-3 PUFAs against gastric damage induced by NSAIDs, ethanol, and water immersion-restraint stress [70, 71].

In conclusion, the findings of the current investigation suggest that flaxseed, pumpkin, and lupin oil pre-treatments exhibited potent antioxidant, anti-inflammatory, and prophylactic potentials against acute gastric ulcers induced by indomethacin. This could be possibly attributed to wealthy phytoconstituents such as total polyphenols, flavonoids, and PUFA, which might act synergistically and in complementary ways to ensure gastroprotection. These findings suggest the potential for using these oils as adjuvant in treating gastric ulcers.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Ethics approval

All methods in the current research were approved by the Ethics Committee in Women's College, Ain Shams University, Egypt (code: sci1532309003).

Consent for publication

Not applicable.

Authors' contributions

MMM: Conceptualization, Investigation, Methodology, Data curation, Formal analysis,

Writing-original draft, Validation, Writing-review. OAF: Conceptualization, Investigation, Methodology. All authors read and approved the final manuscript.

List of abbreviations

1,1-Diphenyl-2-picrylhydrazyl	DPPH
Alpha-linolenic acid	ALA
Cyclooxygenase	COX
Cytochrome P450 reductase	Cyto P450 reductase
Docosahexaenoic acid	DHA
Eicosapentaenoic acid	EPA
Flaxseed oil	FSO
Helicobacter pylori	H. pylori
Interferon-gamma	IFN-γ
Interleukin-17	IL-17
Interleukin-1β	IL-1β
Lupin seed oil	LSO
Mono-unsaturated fatty acids	MUFA
n-3 poly unsaturated fatty acids	n-3 PUFAs
Non-steroidal anti-inflammatory drug	NSAID
Polyunsaturated fatty acids	PUFAs
Prostaglandin-2	PEG-2
Pumpkin seed oil	PSO
Reactive oxygen species	ROS
Saturated fatty acids	SFA
The nucleotide-binding domain and leucine-rich repeat-containing protein-3	NLRP3
Total antioxidant capacity	TAC
Total oxidant capacity	TOC
Tumor necrosis factor-alpha	TNF-α
Unsaturated fatty acids	UFA

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