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Attenuation of Aspirin-Induced Gastric Ulcer in Rats By Linseed, Pumpkin and Lupine Seed Oils

KEYWORDS: peptic ulcer; aspirin; linseed oil; pumpkin oil; lupine oil; rats

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ABSTRACT *This study aimed to investigate, whether the oils of linseed, pumpkin and lupine seeds can heal the gastric ulcer produced by aspirin (ASP) in rats compared to ranitidine drug. F¬¬orty eight male Sprague Dawely rats (150-200 g) were divided into 6 groups (8 rats /group). Group 1 was normal control; the other groups were treated with ASP (400 mg/kg body weight, p.o). In addition to ASP, group 2 (positive +ve, control) received saline, group 3 was treated with ranitidine (50 mg/kg body weight, p.o), and groups from 4 to 6 were treated daily with one of the three tested oils (1.5 ml/kg body weight, p.o) for 7 days. The volume of gastric juice, total acidity of gastric secretion and ulcer index were significantly reduced while the pH of the gastric fluid was significantly increased in the oils treated groups, compared with the +ve control. These results were associated with significant increase in serum total antioxidant capacity (TAC) and a reduction in total oxidant capacity (TOC). Also, The levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), were suppressed after randitine or oils treatment. The gastric prostaglandin E2 (PGE2) and cytochrome P450 reductase (CYP450 red) levels were also increased in ranitidine or each of pumpkin and linseed oils treated groups, whereas gastric cyclooxygenase 2 (COX-2) and total nitric oxide (NO) were significantly reduced in ranitidine and linseed oil treated groups. The best improvement was found in the group treated with ranitidine followed by linseed oil group. These results suggest that the three tested oils attenuated the aspirin-induced gastric lesions via antisecretory, antiulcer, antioxidant and anti-inflammatory mechanisms.*

Introduction

Peptic ulcer is the most common gastrointestinal (GI) tract disorder in clinical practice, which affects approximately 5-10% of the people during their life (**Zapata-Colindres *et al.*, 2006**). The patho-physiology of peptic disease is attributed to the imbalance between aggressive factors like acid, pepsin, and Helicobacter infection, and the local mucosa defenses like bicarbonate secretion, mucus and prostaglandins (PG) (**Jain *et al.*, 2007**). Hemorrhagic injury to the GI mucosa is produced by exogenous compounds as well. Non-steroidal anti-inflammatory drugs (NSAIDs) particularly ASP can cause inflammation and ulceration in the GI tract (**Whittle, 2004 and Takeuchi *et al.*, 2010**). ASP damages the GI mucosa by several mechanisms. Suppression of endogenous PG production by cyclooxygenase (COX) inhibition is considered to be important (**Wang *et al.*, 1989**). On the other hand, oxygen-derived free radicals are directly implicated in the mechanism of ASP-induced acute gastric mucosal injury, and scavenging these free radicals protects against injury (**Muratoglu *et al.*, 2001**). TNF-α is a proinflammatory cytokine and has been shown to be a crucial mediator of NSAID-induced gastric mucosal injury. TNF-α augments neutrophil-derived superoxide generation (**Yoshikawa *et al.*, 1992**), leading to oxygen radical-mediated tissue damage. Pretreatment with TNF-α inhibitors suppresses the gastric mucosal injury caused by ASP and other NSAIDs (**Naito *et al.*, 2001**).

Pumpkin (*Cucurbita pepo Linn*.) is one of the well-known edible plants and has substantial medicinal properties. It contains several phyto-constituents belonging to the categories of alkaloids and flavonoids, and palmitic, oleic and linoleic acids (**Yadav *et al.*, 2010**). Previous studies showed a potential anti-inflammatory activity of pumpkin seed oil in the treatment of adjuvant arthritis in rats (**Zámbó, 1998 and Kuhlman *et al.*, 1999).** Linseed (Linum usitatissimum L) oil contains unsaturated fatty acids like oleic acid (12–30%), linoleic acid (8–29%), and linolenic acid (35–67%) (**Kaithwas *et al.*, 2011**). In earlier studies, the linseed oil has been reported to exhibit significant anti-inflammatory, antiarthritic, antiulcer, and antidiabetic properties along with the efficacy against experimental esophagitis in experimental animals (**Kaithwas & Majumdar, 2010**). Lupine (*Lupineus species*) oil is high in polyunsaturated fatty acids and contains a high level of vitamin E. Oil of lupine seeds was composed of 13.5% saturated, 55.4% monounsaturated, and 31.1% polyunsaturated fatty acids **(Erbaş *et al.*, 2005**). **Tsaliki *et al.*, (1999)** reported that the methanol extracts of lupine exhibit a marked antioxidant activity due to the presence of total phenolics and phospholipids at high concentrations.

The present study was undertaken to elucidate whether the seeds oils of pumpkin, lupine and linseed could heal gastric mucosal injury induced by ASP in rats compared to ranitidine, the conventional anti ulcer agent.

Materials and Methods

Drugs and chemicals

Ranitidine was purchased from SEDICO Pharmaceutical Company, Giza, Egypt. Aspirin (acetyl salicylic acid) were purchased from Ameriya Company for Pharmaceutical and Chemical Industries, Cairo, Egypt. Linseed, pumpkin and lupine seeds oils were obtained from Agricultural Research Center, Giza, Egypt. Kits for measurements of TAC and TOC were purchased from Labor Diagnostika Nord GmbH & Co.KG, Germany. Rat PGE_2 ELISA kit was purchased from EIAab, Wuhan, China. ELISA kit for rat CYP_{450} reductase was purchased from Uscn, Wuhan, China, rat NO ELISA kit was purchased from CUSABIO, China and rat IL-1β, TNF-α and COX-2 immunoassay kits were purchased from IBL CO., Japan. All other chemicals were of analytical grade.

Animals

Forty eight adult male Sprague-Dawley albino rats (120-150 g) were obtained from Agricultural Research Center, Giza, Egypt, and housed individually in mesh bottomed

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metallic cages and kept at a controlled temperature (23–25°C) and ambient humidity (50–60%). Light was maintained on a 12-h light-dark cycle (lights on from 0600 to 1800 h). The animals were fed on standard laboratory diet and water ad libitum for one week as an adaptation period and throughout the experimental period.

Experimental design

The animals were deprived from food for 24 hours; afterwards, the ASP (400 mg/kg, orally, dissolved in HCl 0.2N) was given in two doses, with interval of 12 hours between each administration, in a proportion of 1 ml/100 g of body weight (**Main, & Whittle, 1975**). After 24 hours from the last dose of inducement of the lesion, the ASP treated animals were divided into five groups (8 rats /group), the first group, +ve control, was treated with saline, 2nd group, was treated with ranitidine (50 mg/kg body weight) (**Kumar *et al.*, 2012)**, each of the other three groups were treated with one of the tested oils (1.5 ml/kg body weight, *p.o*) **(Eraslan *et al.*, 2013)**. At the end of the experimental period (1 weeks), rats were sacrificed under diethyl ether anesthesia. The blood samples were collected directly from portal vein into non-heparinized centrifuge tubes. Serum aliquots were separated by centrifugation at 3000 r.p.m for 15 minutes and were frozen at -20 °C for subsequent determination of TAC, TOC, IL-1β and TNF-α.

After blood sampling, the lower end of esophagus and pylorus were clamped and the stomach was removed. The gastric juice was drained into a glass tube. The volume of the gastric juice was measured and centrifuged at 2000 rpm for 10 min. From the supernatant, aliquots (1 ml of each) were taken for the determination of pH using pH meter and total acidity according to **Muniappan & Sundararaj (2003**). Then, the stomachs were opened along the greater curvature, rinsed with ice-cold saline, and the stomach tissues were examined by a ×5 magnifier lens to assess the formation of ulcers. The number of ulcers was counted. Ulcer scoring was undertaken according to **Vogel &Vogel (1997)**. The scores were: 0= no ulcer, 1= superficial ulcer, 2= deep ulcer, 3= perforation. Ulcer index was measured by the formula $U_I = U_N + U_S + U_P \times 10^{-1;}$ where U_I is the ulcer index; U_N is the average number of ulcers per animal; U_S is the average number of severity score; and U_P is the percentage of animals with ulcers(**Vogel &Vogel 1997)**. Tissues from stomachs processed for measurement of PGE_2, COX-2, Cytochrome P_{450} reductase and total NO.

Biochemical analysis

Stomach tissue from each rat was removed, weighed (approximately 1 g), and placed in a test tube containing 5 ml of 0.1 M phosphate buffer. The tissue was homogenized and centrifuged for 10 min at 12,000 rpm at 4°C. PGE_2, COX-2 and CYP_{450} reductase content in supernatant was determined in duplicate by an enzyme immunoassay kit according to **Hamberg & Samuelsson (1973), Hemler & Lands (1976) and Mc-Lean & Day (1974),** respectively. 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 an assay kit, and according to **Griess *et al.* (1982)**. The determination of serum TNF-α and IL-1β was made with an enzyme immunoassay kit from R&D Systems (Minneapolis, MN) according to **Beutler *et al.* (1985) and Grassi *et al.* (1991),** respectively. Serum total antioxidant and oxidant capacities were measured using standard spectrophotometric methods according to **Cao *et al.* (1993)** and **Flohe & Gunzler, (1984)**, respectively.

Statistical analysis

The data are expressed as mean ± standard error of mean (mean ± SEM). The significant differences among groups were determined by one-way analysis of variance using the SPSS package program, version 11. The results were considered significant if the value of p was <0.05, and Duncan's multiple range test was performed if differences were identified between groups **(Bailey, 1994).**

Results

Table (1): Efficacy of linseed, pumpkin and lupine seed oils in aspirin induced gastric ulcer in rats

Groups	Treatment	Ulcer Index	Gastric volume (ml/100g body weight)	Gastric pH	Total acidity (mEq/L)
I	Saline	-	3.05 ± 0.13^{a}	2.96 ± 0.04^{a}	54.1 ± 1.3^{a}
II	Aspirin	10.8 ± 0.1^{a}	4.77 ± 0.17^{b}	2.4 ± 0.18^{b}	93.2 ± 2.2^{b}
III	ASP + Ranitidine	2.6 ± 0.19^{b}	2.48 ± 0.11^{c}	3.2 ± 0.11^{ac}	57.7 ± 1.4^{ac}
IV	ASP + Linseed oil	5.1 ± 0.1^{d}	2.68 ± 0.14^{ac}	2.8 ± 0.05^{a}	61.8 ± 3.3^{c}
V	ASP + Pumpkin seed oil	6.4 ± 0.2^{c}	3.025 ± 0.1^{a}	2.87 ± 0.11^{a}	71.2 ± 2.7^{d}
VI	ASP + Lupine seed oil	6.9 ± 0.01^{c}	3.02 ± 0.12^{a}	2.76 ± 0.07^{a}	72.6 ± 2.2^{d}

The values are expressed as mean ± SEM (n= 8 rats/ group). The same letters means that there is no significant difference between groups. The different letters means that there is a significant difference between groups at p<0.05.

In this study, oral administration of ASP (400 mg/g body weight) induced multiple, elongated, reddish bands of hemorrhagic erosions in rat gastric mucosa. ASP increased ulceration (The ulcer index was 10.8±0.1), gastric volume and total acidity with significant reduction in gastric pH. Ulcer formation by ASP was significantly attenuated by treatment with ranitidine 50 mg/kg or each of the three tested oils. Moreover, it was also observed that treatment with ranitidine and oils significantly reduced gastric volume and total acidity and produced a significant elevation of pH (table 1). Ranitidine showed better protection against ASP induced gastric ulcer followed by linseed oil.

Table (2): Efficacy of linseed, pumpkin and lupine seed oils in aspirin induced gastric ulcer in rats

Groups	Treatment	TAC (mmol/L)	TOC (mmol/L)	IL-1β (pg/ml)	TNF-α (pg/ml)
I	Saline	1.67 ± 0.01^{a}	0.26 ± 0.005^{a}	12.5 ± 0.27^{a}	3.02 ± 0.018^{a}
II	Aspirin	0.88 ± 0.01^{b}	1.19 ± 0.026^{b}	51.18 ± 0.82^{b}	12.94 ± 0.44^{b}
III	ASP + Ranitidine	1.31 ± 0.03^{c}	0.45 ± 0.015^{c}	23.9 ± 0.69^{c}	5.7 ± 0.14^{c}
IV	ASP + Linseed oil	1.09 ± 0.13^{e}	0.73 ± 0.025^{d}	34.36 ± 0.27^{e}	9.43 ± 0.2^{d}
V	ASP + Pumpkin seed oil	1.01 ± 0.02^{d}	0.79 ± 0.053^{d}	46.5 ± 0.28^{d}	9.9 ± 0.07^{d}
VI	ASP + Lupine seed oil	1.02 ± 0.02^{d}	0.87 ± 0.02^{e}	47.4 ± 0.27^{d}	10.76 ± 0.16^{e}

The values are expressed as mean ± SEM (n= 8 rats/ group). The same letters means that there is no significant difference between groups. The different letters means that there is a significant difference between groups at p<0.05.

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Data present in table (2) demonstrated that ASP caused a significant elevation in serum TOC level, and a significant reduction in TAC whereas, treatment with ranitidine 50 mg/kg or the three tested oils (groups from 3 to 6) obviously reduced the elevated TOC level and increased the level of TAC. As shown in table (2), ASP significantly increased the release of IL-1β and TNF-α, however, this rise was considerably lower in rats treated with ranitidine or the tested oils.

Table (3): Efficacy of linseed, pumpkin and lupine seed oils in aspirin induced gastric ulcer in rats

Groups	Treatment	COX-2 ng/mg	PGE-2 pg/mg	Cyto P_{450} reductase ng/mg	TNO (pg/mg)
I	Saline	4.82±0.09[a]	503.8±3.7[a]	1.87±0.02[a]	35.5±1.16[a]
II	Aspirin	16.07±1.4[b]	308.8±7.06[b]	0.66±0.016[b]	68.13±2.3[b]
III	ASP + Ranitidine	6.73±0.15[c]	451.8±7.2[c]	1.4±0.03[c]	45.43±1.6[c]
IV	ASP + Linseed oil	9.97±0.31[d]	410.9±3.4[d]	1.012±0.03[d]	55.7±1.42[d]
V	ASP + Pumpkin seed oil	16.8±0.13[b]	311.43±2.69[b]	0.67±0.004[b]	64.4±1.36[b]
VI	ASP + Lupine seed oil	16.5±0.31[b]	307.6±3.8[b]	0.66±0.006[b]	65.27±1.27[b]

The values are expressed as mean ± SEM (n= 8rats/ group). The same letters means that there is no significant difference between groups. The different letters means that there is a significant difference between groups at $p<0.05$.

The results presented in table (3) showed that COX-2 and TNO levels were significantly increased in the gastric tissue after treatment with ASP. Treatment with ranitidine or linseed oil reversed the elevation of COX-2 while treatment with ranitidine or all tested oils reversed the elevation of TNO. However, the PGE_2 and CYP_{450} reductase levels were markedly reduced in rats treated with ASP (approximately 78% less PGE_2 than rats in the control group). The PGE_2 generation and CYP_{450} reductase level in gastric mucosa of rats treated with ranitidine or each of pumpkin or linseed oils were increased.

Discussion

Previous studies revealed that under experimental conditions and in humans, the ulceration of the gastric mucosa can be induced after a few oral doses of a NSAID or by low dose of ASP (**Armstrong & Blower, 19**87 **Kontureck *et al.*, 2006**). In accordance with previous results, the present study confirmed that oral administration of ASP (400 mg/kg body weight) induced multiple, elongated, reddish bands of hemorrhagic erosions in rat gastric mucosa. ASP increased ulceration, gastric volume and total acidity with significant reduction in gastric pH.

The major anti-inflammatory and analgesic mechanism of action of NSAIDs is the inhibition of COX enzymes (COX-1 and COX-2). COX is the rate-limiting enzyme to regulate the synthesis of PGs by conversion of arachidonic acid (AA) to PGH_2, the common precursor of bioactive PGs. Two distinct COX isoforms were reported. COX-1 is responsible for constitutive PG formation, whereas COX-2 is usually induced in response to stress (**Cryer & Feldman, 1998**). Because of differences in cellular localization and tissue expression, COX-1 and COX-2 produce a distinct set of PGs that, depending on their type and tissue localization, operate as normal physiologic regulators or as proinflammatory molecules. Therefore, NSAIDs inhibit not only those PGs involved in the inflammatory response but also those responsible for maintaining homeostasis (**Cryer & Feldman, 1998 and Hu *et al.*, 2008**). In the present study, ASP caused increased level of gastric COX-2. This is consistent with many previous studies which reported that ASP can rapidly up-regulate COX-2 expression in the stomach **(D'Argenio *et al.*, 2008 and Hu *et al.*, 2008)**.

In the current study, ASP markedly decreased PGE_2 production. Endogenous PGE_2 derived from COX-2 is closely related to the recovery of gastric mucosal injury (**Brzozowski *et al.*, 2000**), and plays an important role for the maintenance of gastric mucosal integrity by preventing exogenous injury to the stomach and accelerating gastric mucosal healing (**Hatazawa *et al.*, 2007**).

There is substantial evidence to support the claim that reactive oxygen species (ROS) are involved in gastric injury induced by ASP exposure. The results of the present study confirmed that administration of ASP led to enhanced ROS activity, as indicated by elevated level of serum TOC and reduced level of TAC. Such increased activity of ROS often leads to mucosal damage with the subsequent destruction of epithelial basement membrane (**Ames *et al.*, 1993**).

The present study has shown that induction of ulcers increases serum levels of TNF-α and IL-1β. They are members of a group of cytokines that involved in systemic inflammation, act synergistically and stimulate acute phase reaction. TNF-α and IL-1β are produced mainly by macrophages, but a broad variety of other cell types is also involved in their production. They are inducers of endothelial adhesion molecules, which are essential for the adhesion of leukocytes to the endothelial surface prior to their migration into the tissues (**Dinarello, 2000**). They also augment neutrophil–derived superoxide generation (**Yoshikawa *et al.*, 1992**), leading to oxygen radical-mediated tissue damage. Stimulation of intestinal epithelial cells with TNF-α has also induced apoptosis (**Ramachandran *et al.*, 2000**). Previous studies showed a significant increase in proinflammatory cytokines in response to ASP (**Odishama *et al.*, 2005 & 2007**).

In this study, the increased nitric oxide (NO) level in the stomach tissue after ASP administration suggested that ASP-induced peptic ulcer may be contributed by high NO production. This finding is in agreement with an earlier study (**Kontureck *et al.*, 2006**). NO is a free radical produced from L-arginine by a family of isoenzymes called nitric oxide synthase (NOS). Both constitutive and inducible isoforms of NOS are present in the GI tract (**Hoffman *et al.*, 1997**). Low level of NO produced by the constitutive isoform of NOS play a beneficial or homeostatic role in the gastrointestinal tract (**Potoka *et al.*, 2002**). On the other hand, sustained release of NO as a result of inducible isoforms of NOS (iNOS) upregulation may lead to cellular injury and gut barrier failure. The biological effects of NO are achieved through chemical interactions with different targets including oxygen, superoxide and other ROS, transition metals and thiols. It may react with (-SH) groups of amino acids and proteins and form relatively stable nitroso-thiols (**Ignarro, 1990**).

Cytochrome P_{450} (CYP) enzymes, are heme containing proteins, play a pivotal role in the metabolism of many

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drugs. The reduced level of CYP_{450} reductase observed in this study may be related to the higher level of NO. NO, produced by iNOS, plays important roles in the suppression of CYP_{450} through modification of their heme moiety and/ or their cysteinyl residue(s) (**Takemura *et al.*, 1999**). Inhibition of CYP enzymes usually increases the plasma concentrations of their substrate drugs and can thus alter the safety and efficacy of these drugs. The metabolism of NSAIDs is known to be catalyzed by CYP enzymes. Previous studies demonstrate that the inhibition of CYP enzymes leads to increased concentrations of NSAIDs (**Rodrigues, 2005**).

The present study demonstrated that the three tested oils may have a preventive action against ASP-induced gastric mucosal injury. An anti-ulcer activity, coupled with the restoration of altered biochemical parameters in the gastric tissue and serum, was observed in ASP-induced gastric ulcerated rats treated with the three tested oils. These beneficial effects of the tested oils may be attributed to the presence of high amount of polyunsaturated fatty acids, including linoleic and α-linolenic acid (ALA), along with considerable amounts of tocopherols, polyphenols, flavonoids, tannins and carotenes in these oils (**Jariene *et al.*, 2007 and Kaithwas & Majumdar, 2010**).

ALA (18:3, n-3) is a precursor for eicosapentaenoic acid (EPA, 20:5, n-3), which competes with AA for cyclooxygenase and lipoxygenase pathway. EPA by acting as a substrate for cyclooxygenase and lipoxygenase pathway produces PGE_3 (less potent vasodilator than PGE_2) (**Hawkes *et al.*, 1991**) and leukotriene B5 (LTB_5) (100 times less potent chemotactic than leukotriene B_4 (LTB_4) (**James *et al.*, 2000**). Thus, less vasodilatory PGE3 and chemotactic LTB_5 response of lipid mediators derived from EPA accounts for the inhibition of fluid and protein exudation along with diminished leucocytes migration (**Kaithwas & Majumdar, 2013**).The anti-inflammatory activity of various plant lipids has been reported, and the results revealed that the lipids containing ALA, for example, linseed oil, and soyabean oil had a significant anti-inflammatory activity (**Singh & Majumdar, 1997**).

Polyphenolic compounds have been reported to have a beneficial role in gastric ulcers, as it has been suggested that phenols stimulate PG formation (**Alanko *et al.*, 1999**). Polyphenols clearly improves the status of oxidative stress biomarkers. The biological mechanisms of these possible effects have been attributed to their antioxidant properties through several possible mechanisms, such as their ability to scavenge free radicals, break radical chain reactions, directly reducing peroxides, and stimulating the antioxidative defense enzyme activities (**Williamson & Manach, 2005**).

Flavonoids have been reported to act in the gastrointestinal tract, having antispasmodic (**Lima *et al.*, 2005**), antisecretory, antidiarrheal (**Di Carlo *et al.*, 1993**), antiulcer, and antioxidant properties (**La Casa *et al.*, 2000**). Flavonoids are among the cytoprotective materials for which anti-ulcerogenic efficacy has been extensively confirmed (**Di Carlo *et al.*, 1993 and 1999**). They protect the gastric mucosa against a variety of ulcerogenic agents via several mechanisms of action, mainly free-radical scavenging and antioxidant properties, increased mucus production, antisecretory action, and inhibition of the *Helicobacter pylori* growth (**Galati *et al.*, 2001**). Tannins prevent ulcer development due to their protein precipitating and vasoconstricting effects (**Aguwa & Nwako, 1988**). Their astringent action can help to precipitate microproteins on the ulcer site, thereby, forming an impervious layer over the lining, which hinders induced gastric ulcer in rats, as evidenced by the gut secretions, and protects the underlying mucosa from reduction in the ulcer scores (**Al-Rehaily *et al.*, 2002**).

In conclusions, our results showed that the anti-ulcer activity of the three tested oils was perhaps a result of the interplay between their anti-secretory, anti-inflammatory, anti oxidant properties. These findings suggest the potential for use of these oils as adjuvant in the treatment of gastric ulcer.

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