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Chemoprotective activity of 3-indolylmethyl glucosinolate on induced gastric mucosal injury in male rats

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Abstract Mucosal damage in stomach therapy faces a major drawback in modern days due to the unpredictable side effects of the long-term uses of commercially available drugs. The aim of the present investigation study is to evaluate the chemoprotective activities of 3-indolylmethyl glucosinolate against aspirin-induced gastric mucosal damage in male albino rats. Rats were divided into two large groups, control group and ulcerated group. Aspirin (500 mg/kg/body weight for a week), and followed by the beginning of different experimental regimens for a total experimental duration of one month. Results of the present work showed that, the groups were treated with 3-indolylmethyl glucosinolate possessed protective activity by an increase the mucus level, protein granules and a great number of proliferation cells in the stomach. So this study suggests that, 3-indolylmethyl glucosinolate possesses significant gastroprotective and antioxidant activities against aspirin-induced gastric ulcer in male albino rats.
Key Words: Histochemical, Gastroprotective, Aspirin, 3-Indolylmethyl glucosinolate, Rats.

النشاط الكيميائي الوقائي لثلاثي–إندوليل ميثيل الجلوكوزينولات على إصابة الغشاء المخاطي للمعدة المحدثة في ذكور الجرذان

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الملخص يواجه الغشاء المخاطي عند معالجة المعدة عيباً كبيراً في الأيام الحديثة بسبب الآثار الجانبية غير المتوقعة للاستخدامات الطويلة الأجل للأدوية المتوفرة تجارياً. الهدف من هذه الدراسة هو تقييم الأنشطة الوقائية الكيميائية لثلاثي إندوليل ميثيل الجلوكوزينولات ضد تلف الغشاء المخاطي المعوي المحدث بالأسبرين في ذكور الجرذان البيضاء. تم تقسيم الجرذان إلى مجموعتين كبيرتين، المجموعة الضابطة والمجموعة المتقرحة. الأسبرين (500 ملغ / كغ / وزن الجسم أعطي لمدة أسبوع) ، يليه أعطيت النظم التجريبية المختلفة لمدة شهر واحد. أظهرت نتائج العمل الحالي أن المجموعات التي عولجت بـــثلاثي إندوليل ميثيل جلكوزينولات أنها تمتلك نشاط وقائي وذلك بزيادة مستوى المخاط وحبيبات البروتين وعدد كبير من خلايا الانتشارية في المعدة. لذلك تشير هذه الدراسة إلى أن ثلاثي إندوليل ميثيل الجلوكوزينولات يمتلك أنشطة وقائية ومضادة للأكسدة كبيرة ضد قرحة المعدة التي يسببها الأسبرين في ذكور الجرذان البيضاء.
الكلمات المفتاحية: هيستوكيميائي، الوقائي المعدي، الأسبرين، ثالث–إندوليل ميثيل جلكوزينولات، الجرذان.

Introduction:

Gastric mucosal layers are continuously exposed to a wide variety of injurious agents originating either endogenously or exogenously [25]. These include hydrochloric acid, pepsin, *Helicobacter pylori*, alcohol, and non-steroidal anti-inflammatory drugs (NSAID). When these aggressive factors exceed the ability of the mucosa resist to multiple pathologies such as gastritis, gastric ulcers, and even gastric cancer can develop [1]. Peptic ulcer disease (PUD) is one of the oldest diseases known to human kind. The term PUD generally refers to spectrum of disorders that includes gastric ulcer, pyloric channel ulcer, duodenal ulcer and postoperative ulcers at or near the site of surgical anastomosis [2]. Gastric mucosal injury can be treated by allopathic drug therapy. However, there are many examples of recurrence. Therefore, a useful method for prevention of recurrence is needed. Food and other natural resources have been attracting attention as a preventive method, and many studies have been performed [3]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin. Aspirin (ASA) often used as an analgesic to relieve minor aches and pains, as an antipyretic to reduce fever and as an anti-inflammatory medication [4]. Aspirin also has an antiplatelet or anti-clotting effect and is used in long-term at low doses to prevent heart attacks, strokes and blood clot formation in people at high risk for developing blood clots [7].

However, the major limitations of their clinical application are serious gastrointestinal side effects, especially peptic ulcerations and gastrointestinal bleeding [5]. Some NSAIDs, particularly those of acidic nature, can directly kill epithelial cells. NSAIDs can also reduce mucus and bicarbonate secretion, thereby decreasing the effectiveness of the juxtamucosal pH gradient in protecting the epithelium [6]. Despite the cardiovascular benefits of ASA, a potential gastrointestinal harm has been noted in several

clinical and preclinical studies. The main undesirable side effects of ASA are gastrointestinal ulcers, stomach bleeding, and tinnitus, especially in higher doses [8],[9]. Furthermore, the pharmacological activity of NSAIDs is related to the suppression of prostaglandin biosynthesis popularly known as cyclooxygenase-2 (COX-2). Prostaglandins synthesized by the gastric mucosa are one of the defensive factors known to inhibit the secretion of gastric acid and stimulate the secretion of mucus and bicarbonate [10]. A number of antiulcer drugs like gastric antisecretory drugs-H_2 receptor antagonists, antimuscarinic agents, proton pump inhibitors, mucosal protective agents carbenoxolone sodium, sucralfate and prostaglandin analogues are available which are shown to have side effects and limitations [11]. Selective proton pump drug as omeprazole (OMZ) is an effective agent in the treatment of peptic ulcer disease. The effects of OMZ against gastric mucosal injuries have been thought to depend on its inhibitory action on gastric acid secretion [12]. However, clinical evaluation of this drug has shown relapse in the long run, side effects and drug interactions [13]. This has been rational for the development of innovative drug that reduces the offensive factors and is proved to be safe, clinically effective, having better patient tolerance, relatively less expensive and globally competitive.

However, some plant extracts, are the most attractive sources of new drugs and have been shown to produce promising results in the treatment of gastrointestinal tract ulcers [14]. A number of natural products found in fruits and vegetables are known to possess anti-mutagenic and anti-carcinogenic properties [15]. Cruciferous vegetables are a rich source of many phytochemicals, including indole derivatives, dithiolthiones, and isothiocyanates. 3-Indolylmethyl glucosinolateis a naturally occurring hydrolysis product of glucobrassicin found in vegetables of the *Cruciferae* family such as broccoli, brussels sprouts, and cauliflower. Epidemiological studies suggest high dietary intake of cruciferous vegetables is associated with lower cancer risk, and it is possible that the chemo-preventive properties are in part attributable to 3-indolylmethyl glucosinolate [16].

The aim of study:

The current study was undertaken to evaluate the gastroprotective effects of 3-indolylmethyl glucosinolate (3-IM) (as a new safer chemo-protective and antioxidant activities compound found in cruciferous vegetables) against aspirin induced gastric ulcers in albino male rats and the effects of treatments of OMZ and 3-IM either alone or combined with each other's on the acute phase of inflammation, models induced by aspirin as one of the NSAIDs.

Material and Methods:

Drugs:

3-Indolylmethyl glucosinolate(3-IM) was purchased from Sigma-Aldrich Chemical Company U.S.A. (Cairo, Egypt), aspirin (ASA) tablets (Bayer AG, Germany) and omeprazole (OMZ) tablets (European Egyptian Pharmaceutical Industries, Cairo Egypt) were obtained from pharmacy.

Experimental animals:

Adult male albino rats (*Rattusnorvegicus*) weighing about 140-160 g were used throughout the experiment. The animals were housed in polypropylene cages with sterile, inert husk materials as bedding. The experimental animals were maintained under controlled environment conditions of light and dark cycle (12/12 hrs light/dark, temperature 23 ± 2 ˚C). They were allowed to acclimatize for 10 days and were provided a free access to standard pellet diet and water *ad libitum*. Animals were fasted for 24 hour with free access to drinking water before starting the experiment.

Experimental design:

Forty eight adult male albino rats were randomly divided into two large groups: Control groups administrated distilled water and ulcerated groups administrated aspirin (ASA) at a dose of 500 mg/kg/body weight for a week. Aspirin administration was stopped after one week representing the initial duration for the experiment and was followed by the beginning of different experimental regimens for a total experimental duration of one month.

Control group was further divided into four sub-groups (six male rats each) which are normal control group, 3-indolylmethyl glucosinolate(3-IM) group receiving a dose of 20 mg 3-IM /kg/body weight, omeprazole (OMZ) group receiving a dose of 20 mg OMZ /kg body weight and OMZ + 3-IM group receiving both treatments of OMZ and 3-IM. Ulcerated group (U) was further divided into another four sub-groups (six male rats in each) which are ulcer group (U), U + 3-IM group, U + OMZ group and U + 3-IM + OMZ group at same doses above. All chemicals were given to male rats orally by stomach tube. All rats ware dissected after an experimental period of one month.

Histochemical examination:

Sections were stained with bromophenol blue for the demonstration of sites of total protein content [17]. According to Mowry, [18], sections were stained with combined alcian blue periodic Schiff technique to demonstrate the presence of mucins and clearly distinguish between acid and neutral mucins.

Immunohistochemical studies:

- Immunohistochemistry of Cyclooxygenase-2 (COX-2) examination:

From 10 % formalin fixed paraffin embedded samples, 6-µm thick sections were prepared. The sections were deparaffinized in xylene, hydrated through standard graded ethanol solutions and treated with 0.2 % saponin (CliniLab Cairo, Egypt. Aran B Chemical Company U.K.) at room temperature for 30 minutes. After the sections were treated with methanol containing 3% hydrogen peroxide (H_2O_2) for 15 minutes to eliminate endogenous peroxidase, the sections were reacted with 10 % normal rabbit serum for 10 minutes to block non-specific reactions. As the primary antibody, cyclooxygenase-2 (COX-2) polyclonal antibodies

were diluted 100 times in phosphate-buffered saline (PBS) and reacted with the sections at 4°C for 15 minutes. After the streptavidin–biotin complex method in kits of (CliniLab Cairo, Egypt. Aran B Chemical Company U.K.) biotin-labeled anti-rabbit immunoglobulin G antibody as the secondary antibody was reacted with the sections at room temperature for 15 minutes, and the peroxidase-labeled streptavidin was reacted at room temperature for 10 minutes, followed by color development using diaminobenzidine (DAB) reagent. After counterstaining with Mayer's hematoxylin the sections were observed [19].

- Immunohistochemistry of Proliferating Cell Nuclear Antigen (PCNA) examination:

Tissue samples were fixed in 10 % formalin. The formalin fixed specimens were embedded in paraffin, sectioned 6-µm. The sections were deparaffinized in two changes of xylene, hydrated and autoclaved at 121°C for 15 minutes to increase antigenicity. The basic methodology used was the same as that for COX-2. As the primary antibody, anti-PCNA monoclonal antibody (CliniLab Cairo, Egypt. Aran B Chemical Company U.K.) was diluted 100 times in phosphate-buffered saline (PBS) before use [19].

Method of counting: The count of immunopositive cells in relation to the number of COX-2 and PCNA were carried out by of a cell Imaging Software on fine picture (×400) randomly selected [19],[20].

Results:

Histochemical results:

Stomach sections stained with histochemical examination in OMZ and/or 3-IM treated animals ratified no change in their distribution throughout the whole experimental duration. A strong reactivity was displayed by the peptic and oxyntic cells of the control animals in the bromophenol blue stain. Their contents of protein were located in a mildly reactive ground cytoplasm. Their nuclei exhibited a strong reactivity with bromophenol blue (Fig. 1). Sections of normal control rats demonstrated normal distribution of mucin content in the gastric tissue throughout the whole experimental period, which was showed by combined alcian blue periodic Schiff technique (Fig. 2). Histochemical staining of gastric sections from animals treated with aspirin for the identification of the mucin content represented by combined alcian blue periodic Schiff technique (Fig. 2) and total protein content (Fig. 1) manifested a diminution in mucosal cells total content. The stainability of the cytoplasm and nuclei of the oxyntic, peptic and mucous neck cells was greatly reduced and the protein granules in most cells were highly reduced. In such case, a weak feeble stainability with both stains was quite clear in the constituent cells, whereas proteinic content began to regain affinity to stainability. The cytoplasm and nuclei of peptic, parietal mucous neck cells and surface mucous cells had restored a greater part of their reactivity with bromophenol blue or with combined alcian blue periodic Schiff by the 3-IM, OMZ and the combined treatments of 3-IM +OMZ. It is obvious from the stomach sections that 3-IM treatment alone to ASA-ulcerated rats has more pronounced effects on the gastric tissue mucin content than OMZ treatment alone.

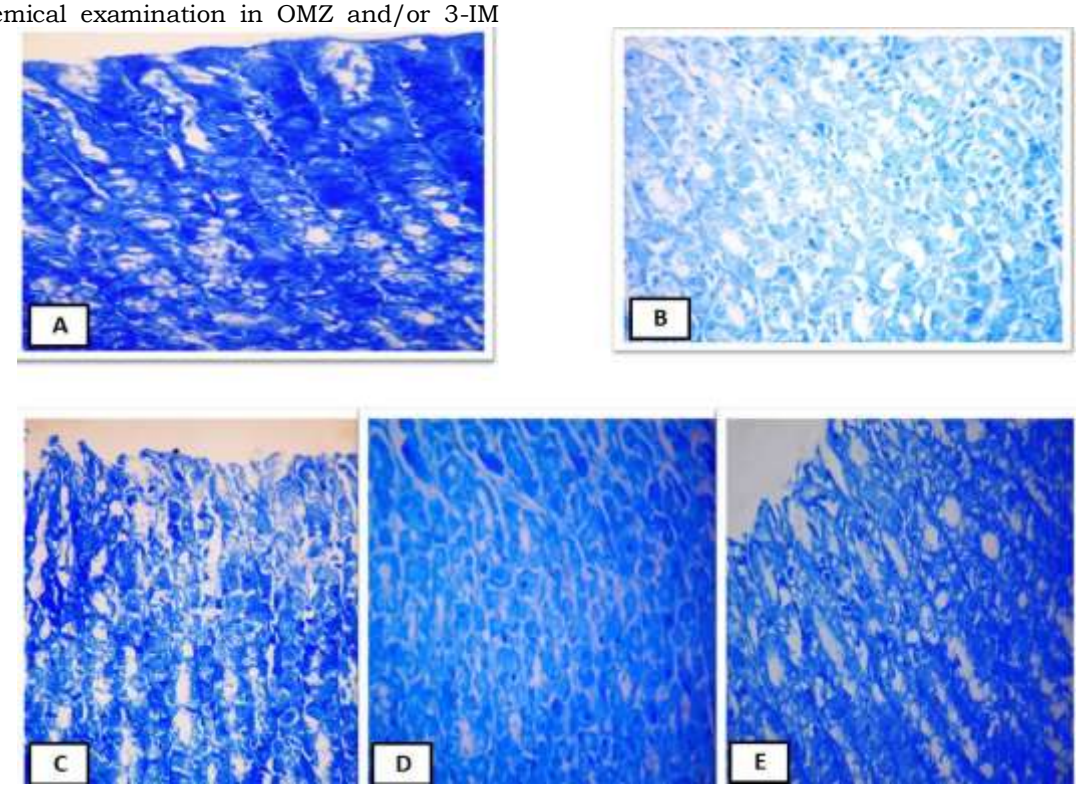

Figure 1. Histochemical changes of bromophenol blue stain in normal control stomach tissue (photomicrograph (A)), ulcerated ASA rats (photomicrograph (B)), OMZ treated tissue (photomicrograph (C)), 3-IM treated tissue (photomicrograph (D)) and OMZ + 3-IMtreated tissue (photomicrograph (E)) (400x magnification).

ASA: aspirin; 3-IM: 3-Indolylmethyl glucosinolate; OMZ: omeprazole.

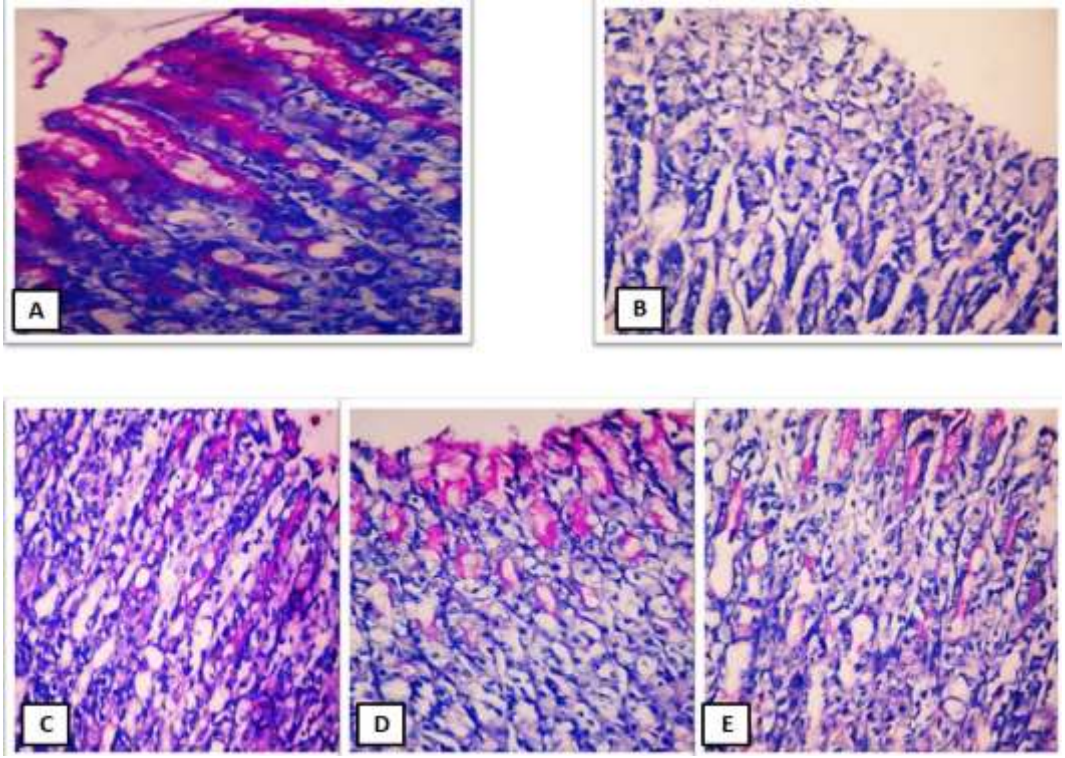

Figure 2. Histochemical changes of combined alcian blue periodic Schiff technique in normal control stomach tissue (photomicrograph (A)), ulcerated ASA rats (photomicrograph (B)), OMZ treated tissue (photomicrograph (C)), 3-IMtreated tissue (photomicrograph (D)) and OMZ + 3-IMtreated tissue (photomicrograph (E)) (400x magnification).

ASA: aspirin; 3-IM: 3-Indolylmethyl glucosinolate; OMZ: omeprazole.

Immunohistochemical results:

The gastric tissues obtained in the ASA model of gastric ulcer were used for immunohistochemical localization of COX-2 antibodies and PCNA antibodies. Immunohistochemical slices (Figs. 3& 4) showed a great number of proliferation cells in the stomach of animals treated with 3-IM, OMZ and 3-IM +OMZ. Thus, the results indicate that this protein participated in the healing of the gastric ulcer treated with 3-IM, OMZ and 3-IM +OMZ. Table 1 shows that the most pronounced expression of COX-2 and PCNA stain was in 3-IM treated ulcerated rats.

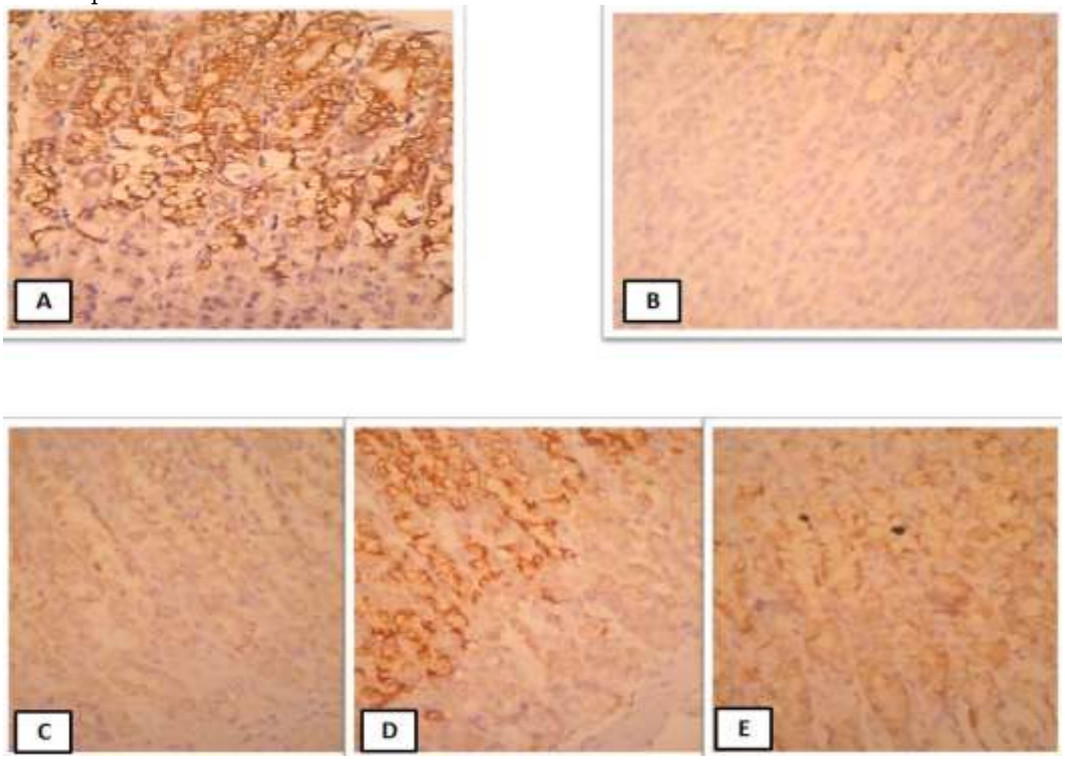

Figure 3. Immunoreactivity of COX-2 in normal control stomach tissue (photomicrograph (A)), ulcerated ASA rats (photomicrograph (B)), OMZ-treated tissue (photomicrograph (C)), 3-IMtreated tissue (photomicrograph (D)) and OMZ + 3-IMtreated tissue (photomicrograph (E)) (400x magnification).
ASA: aspirin; 3-IM:3-Indolylmethyl glucosinolate; OMZ: omeprazole; COX-2:cyclo-oxygenase-2.

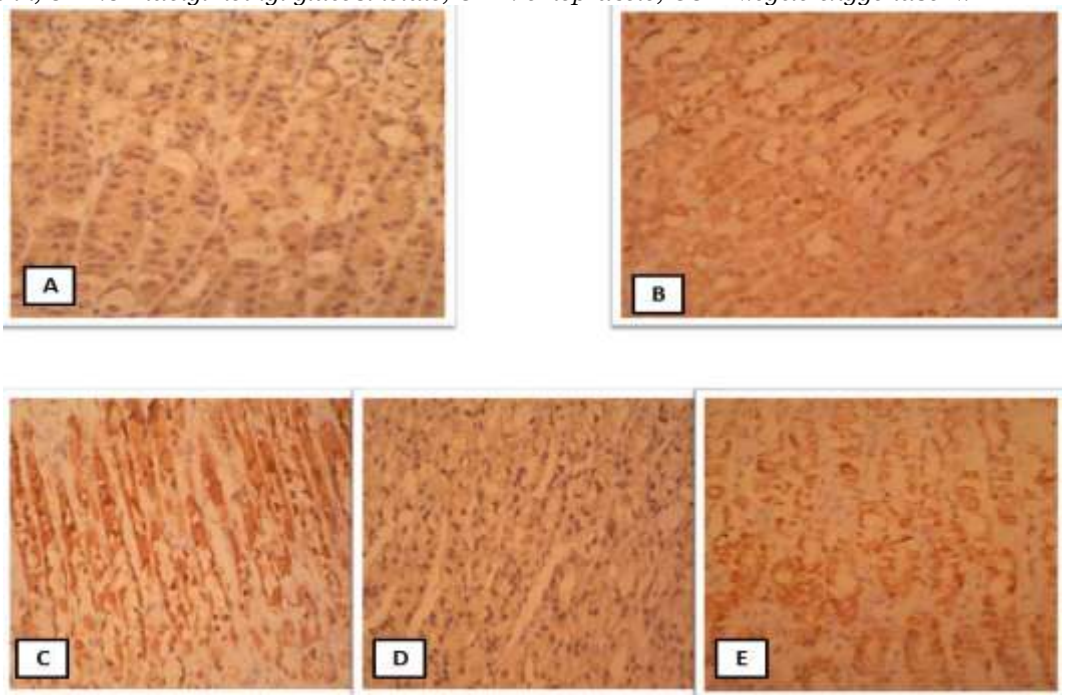

Figure 4. Immunoreactivity of PCNA in normal control stomach tissue (photomicrograph (A)), ulcerated ASA rats (photomicrograph (B)), OMZ-treated tissue (photomicrograph (C)), 3-IMtreated tissue (photomicrograph (D)) and OMZ + 3-IMtreated tissue (photomicrograph (E)) (400x magnification).
ASA: aspirin; 3-IM: 3-Indolylmethyl glucosinolate; OMZ: omeprazole;PCNA: proliferating cell nuclear antigen.

Discussion:

Related to many previous studies were done by, Goel et al. [21], declared that the, gastric ulcers are caused due to imbalances between offensive and defensive factors of the gastric mucosa and the antiulcerogenic activity of many plant products are reported due to an increase in mucosal defensive factors rather than decrease in the offensive factors and, Tan et al. [22], reported that the, the free radicals are produced basically during cellular metabolism and some functional activities and have essential roles in cell signaling, apoptosis and gene expression. On other hand, excessive free radical attack can damage DNA, proteins and lipids, resulting very important diseases. Antioxidants can decrease the oxidative damage by reacting with free radicals or by inhibiting their activity. Moreover, Antioxidants could help to protect cells from damage caused by oxidative stress and enhanced the body's defense systems against degenerative diseases. Administration of antioxidants inhibits ASA-induced tissue injury in rat [23]. In addition, Potrich et al. [24] proposed that, reactive oxygen species (ROS) are involved in the development of gastric ulcers induced by non-steroidal anti-inflammatory drugs. Gastric ulcers are open areas in mucous lining of the stomach [25]. Moreover, aspirin administration causes inhibition of prostaglandins [24]. Prostaglandins are important cytoprotective agents in the gastrointestinal tract because they increase mucosal blood flow. Inhibition of prostaglandin synthesis by aspirin causes damage to the cell membrane of mucosal, parietal and endothelial cells [26]. Besides, aspirin induces gastric damage followed by a multistage pathogenetic event in which ROS, vascular permeability, luminal contents, neutrophils, gastric motility and microcirculation all play a role in the development of inflammation and ulcers [27]. Gastric mucosal lipid peroxidation, mediated by oxygen free radicals, is an important cause of destruction and damage to mucosal cells and gastric mucosal integrity [28]. Also, Bharti et al. [29] confirmed that, the exposure of gastric mucosa to ASA has been shown to affect cellular integrity and such changes are associated with oxidative stress and mitochondrial damage.

In this investigation study, ASA-ulcerated rats showed a decrease in the mucin content represented by combined alcian blue periodic schiff technique and sites of protein content demonstrated by bromophenol blue stain, which began to be attenuated by the 3-IM, or the combined treatments of 3-IM and OMZ. These findings strongly support the hypothesis that, ASA stimulate HCl secretion and cause weakness of mucous gel layer which acts as barrier by decreasing mucin production and increasing the secretion of bicarbonate from gastric mucosa. Aspirin causes mucosal damage by interfering with prostaglandin synthesis, increasing acid secretion and blocking the diffusion of H^+ ions [25]. An improvement in mucus production guides the healing process by protecting the ulcer crater against the endogenous aggressors, such as stomach secretions and oxidants, as well as

against exogenous damaging agents, such as NSAIDs. The ulcer prevention or healing by 3-IM was associated with an increase in the mucus layer in the gastric mucosa [30,31]. The PAS (Periodic acid Schiff staining Technique) staining method confirmed the role of 3-IM enhancing the mucus level and protecting the inflammatory cytokine-mediated oxidative damage to gastric mucosa. Thus, the antiulcerogenic activity of 3-IM may involve its beneficial effect on both offensive and defensive gastric mucosal factors [32]. Immuno histochemical stain of COX-2 and PCNA in this study showing that, 3-IM treated ulcerated rats produced an increase in the COX-2 and PCNA production in the stomach tissue verifying the healing process of 3-IM. These results were in agreement with [31, 33] who explained that, COX-2 produces prostaglandins that exert anti-inflammatory actions and play an important role in the healing of gastric ulcers [34]. On the basis of the results made by Hatazawa et al. [35], they brought about that endogenous prostaglandin subtype 2 derived from COX-2, which plays an important part in the spontaneous healing of gastric ulcers and the up regulation of COX-2 appears to be a defensive and anti-inflammatory response aimed at enhancing mucosal defense.

3-IM prevents loss of membrane permeability and dysfunction of cellular proteins, leading to survival of the functionally active cells [36,37]. 3-IM could have a unique capacity to block this oxidative damage similar to that shown by H_2O_2 scavenger, catalase, indicating its potent antioxidant role to protect DNA from the attack of ROS. The effects of 3-IM on PCNA was mimicked, it can be proposed that its beneficial influence on mucosal repair depends on acid-independent mechanisms, which are likely related with its antioxidant properties. This view is supported by Jainu and Mohan [38] who demonstrated that both the 3-IM and omeprazole enhanced the expression of growth factors, including transforming growth factor-alpha, in the gastric mucosa of rats treated with aspirin. Moreover, 3-IM partly counteracted the ASA-induced delay in rat gastric ulcer healing, without affecting mucosal PCNA expression [39]. The results of histochemical and immunohistochemical investigation revealed that 3-IM showed significant inhibiting of lesions in gastric mucosa induced by aspirin. 3-IM treatment was found to preserve the functional cytoarchitecture of the entire gastric mucosa. These findings confirm the gastroprotective nature of 3-IM.

Conclusion:

In conclusion, it was found demonstrated that, 3-IM could significantly gastroprotective the stomach tissue against ASA-induced gastric ulcer and a great number of proliferation cells (COX-2 and PCNA) in the stomach. This study provides evidence that, 3-IM possesses as an effective gastroprotective, anti-inflammatory and antioxidant activities against aspirin-induced gastric ulcer in male albino rats.

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