Inhibition of hyperhomocysteinemia in Indomethacin induced peptic ulcer: Impact of pomegranate juice supplementation

Jihan Hussein[1*], Zakaria El-Khayat[1], Abdel Razik Farrag[2], Dalia Medhat[1], Yasmin Abdel Latif[1] and Fatma Oraby[1]

[1]*Medical Biochemistry Department, National Research Center, Doki, Giza, Egypt*
[2]*Pathology Department, National Research Center, Doki, Giza, Egypt*

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ABSTRACT

Peptic ulcer disease (PUD), is the most common ulcer of the gastrointestinal tract. Several investigations have confirmed the role of oxidative stress in developmental peptic ulcers, possibly via the formation of free radicals. Antioxidant therapies may be useful in decreasing the risk of peptic ulcers, several natural products have been reported to poses anti-ulcerogenic activity by virtue of their predominant effects on mucosal defensive factors including apple, pomegranate, bananas, and brindle berry which acts as an antioxidant, inhibiting lipid peroxidation and scavenging free radicals. This study aimed to identify the effect of pomegranate juice on gastric ulcers induced by indomethacin in rats and exhibits its powerful antioxidant properties. Forty male albino rats were used in this study, divided into four groups (control, pomegranate, peptic ulcer and treated groups). Liver and kidney function test, stomach malondialdehyde (MDA) as oxidant marker and superoxidedismutase (SOD) and total antioxidant capacity (TAC) as antioxidant markers were estimated. Tissue IL-1α and TNF-α as markers of inflammation were estimated. Also serum homocysteine was measured by high performance liquid chromatography (HPLC). The data showed that indomethacin in peptic ulcer group significantly increased stomach MDA, IL-1α, TNF-α and serum homocysteine concomitant with a reduction in SOD and TAC. Contrarily, pomegranate supplemantion improved these values in treated group. In conclusion, pomegranate seems to be a highly promising compound in protecting the peptic ulcer rats against oxidative damage and preventing inflammation represented in elevation of homocyteine.

Keywords: Peptic ulcer, Indomethacin, Pomegranate, Antioxidants, Homocysteine.
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INTRODUCTION

The gastrointestinal tract represents an important barrier between the human hosts and microbial populations. One potential consequence of host microbial interactions is the development of mucosal inflammation, which can lead to gastritis and ulcer [1].

Peptic ulcer disease (PUD) is the most common ulcer of the gastrointestinal tract. Peptic ulcers occur worldwide and gastric cancer is the second commonest cause of death from malignant disease [2].

It is defined as mucosal erosions equal to or greater than 0.5 cm almost all ulcers are associated with Helicobacter pylori, a spiral shaped bacterium that lives in the acidic environment of the stomach. They are caused by many

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factors such as drugs, stress or alcohol, due to an imbalance between offensive acid pepsin secretion and defensive mucosal factors like mucin secretion and cell shedding [3].

A long-term use of non-steroidal anti-inflammatory drugs (NSAIDs) causing inflammation of the gastric mucosa, gastrointestinal associated with a range of toxicity, and may finally cause ulceration, bleeding and changes with a very high morbidity and mortality [4].

Indomethacin is an indol derivative, non-steroidal, anti-inflammatory drug with anti-inflammatory, analgesic, and antipyretic effects. Indomethacin became the first choice drug to produce an experimental ulcer model as a result of having a higher ulcerogenic potential than other NSAIDs [5].

A number of drugs including prostaglandins analogs, histamine receptor antagonists, proton pump inhibitors, and cytoprotective agents are available for the treatment of peptic ulcer. By the way various side effects of these products such as, hepatotoxicity and anaphylaxis, are not totally managed yet, so medicinal plant as an alternative treatment always has been the focus of many studies [6].

Several natural products have been reported to poses anti-ulcerogenic activity by virtue of their predominant effects on mucosal defensive factors including apple, bananas, papeeta, and brindle berry [7].

Pomegranate (*Punica granatum* L.) is a well-known table fruit of tropical and subtropical regions of the world. Some botanists place it in the family Lythraceae, of the peculiar type of fruit, called as balausta, most authorities make it the only genus in the family Punicaceae [8].The biological activity of PG has been widely investigated *in vitro* and *in vivo* studies [9].

Homocysteine is an intermediate in the metabolism of methionine. Increased serum concentration of homocysteine is, in most cases, caused by a deficiency of folate or vitamin B12. Hyperhomocysteinemia has been amply documented in patients with vascular disorders, and increased homocysteine levels are a well-defined risk factor of atherosclerosis and thrombosis. Hyperhomocysteinemia has also been documented in cancer patients [10].

The aim of this study was to identify the effect of pomegranate on gastric ulcers induced by indomethacin in experimental rats and exhibits its powerful antioxidant anti-inflammatory properties as well as its role in the reduction of hyperhomocysteinemia during peptic ulcer.

EXPERIMENTAL SECTION

Materials
Chemicals
Homocysteine standard (HPLC grade) was purchased from Sigma-Aldrich Company, St. Louis, MO, USA. All other chemicals were of HPLC grade. Indomethacin as Liometacen ampoules was purchased from The Nile Company for Pharmaceuticals and Chemical Industries. Pomegranate was purchased from local market.

Experimental Animals
Forty male albino rats weighing 180-200 g were obtained from the animal house of National Research Center, Giza, Egypt., and fed a standard commercial diet (control diet) purchased from the Egyptian Company of Oils and Soaps. Water was available adlibitum for acclimatization before starting the experiment; rats were kept under constant environmental conditions at room temperature. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre.

Methods
Induction of peptic ulcer
Acute gastric ulcers were induced by oral administration of indomethacin at a dose of 100 mg/kg body weight once five hours before rats were sacrificed [11].

Pomegranate processing
Pomegranates fruit was crushed, squeezed, and treated enzymatically with pectinase to yield the PJ and byproducts, which included the inner and outer peels and the seeds. Pectinase hydrolyzes α-1,4-galacturonide bonds in pectin

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and thus improves extraction and filtration and prevents the formation of pectin gels. The juice was filtered, pasteurized, concentrated, and stored at -18˚C [12].

Experimental design
Forty male albino rats were classified into four groups (10 rats in each group) as follows:
Group I: healthy rats, received 0.5 ml of vehicle / rat / day / orally.
Group II: healthy rats, received 0.5 ml pomegranate juice (100mg/kg body weight / day orally).
Group III: healthy rats, received 0.5 ml of vehicle / rat/ day orally, then injected with indomethacin.
Group IV: healthy rats, received 0.5 ml pomegranate juice (100mg/kg body weight / day orally), then injected with indomethacin.

After the experimental period (4 weeks), animals were kept fasting for 12 hours before blood sampling, blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes and collected in clean tubes. Stomach was removed quickly; part of it was homogenized and prepared for estimation of other biochemical parameters. Other part of the stomach was kept immersed in 10 % formalin for histopathological and histochemical examinations.

Preparation of tissue homogenate
The frozen tissues were cut into small pieces and homogenized in 5 ml cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 per 500 ml deionized water (pH 7.4) per gram tissue, then centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was removed and used in estimation of biochemical parameters [13].

Biochemical assays
Serum liver enzymes alanine amino-transferase (ALT) and aspartate amino-transferase (AST) were measured according to the method of [14], blood urea and serum creatinine were measured by the method decribed by [15], [16] respectively. Stomach malondialdehyde (MDA) was measured by the method described by [17], superoxide dismutase (SOD) was measured according to the method of [18] and total antioxidant capacity (TAC) was estimated according to the method described by [19]. All kits were purchased from BioMed. Diagnostics. Serum TNF-α and IL-1α were measured by ELISA according to the method described by [20] and [21] respectively.

Determination of serum Homocysteine (SAH)
SAH was estimated by high performance liquid chromatography (HPLC) system, Agilent technologies 1100 series, equipped with a quaternary pump (Quat. pump, G131A model).

Sample extraction
200 µl serum were treated with 16 µl of 1000g/L TCA, mixed well and incubated in ice for 30 min to precipitate protein. After centrifugation for 20 min at 4000 rpm at 4°C, supernatants were filtered through 0.2-µm filter.

HPLC condition
30 µl from the solution were injected in HPLC; separation was achieved on reversed phase column (C18, 25, 0.46 cm i.d. 5 µm). The mobile phase consisted of 40 mmol/L sodium phosphate monobasic monohydrate; 8 mmol/L heptanesulfonic acid and 18% (v/v) methanol adjusted to pH 3.1 by addition of phosphoric acid and filtered through a 0.45-µm membrane filter and was delivered at a flow rate of 1 ml/min at 40°C. UV detection was performed at 260 nm. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentrations in samples were obtained from the standard curve.

Histological and histochemical studies
The sections of the stomach, stained with hematoxylin and eosin (H & E), were assessed for histological changes such as congestion, edema, hemorrhage and necrosis. Another sections stained with periodic acid-Schiff (PAS) staining were assessed for histochemical changes of mucus distribution [22].

Statistical analysis
All data were expressed as mean ± standard error. Data were analyzed using one-way ANOVA using SPSS (Version 16). Duncan's new multiple-range test was used to assess differences between means. Pearson's correlation test was used to assess correlations between means. A significant difference was considered at the level of $P < 0.05$.

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RESULTS AND DISCUSSION

The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is considered to be the major risk factor in gastric ulcers. The mechanisms suggested for the gastric damage caused by NSAIDs are inhibition of prostaglandin synthesis and inhibition of epithelial cell proliferation in the ulcer margin, which is critical for the reepithelization of the ulcer crater [23]. There has been a considerable interest in finding natural antioxidants from plant materials to replace synthetic ones for effective management of therapeutic drug toxicity such as peptic ulcer [24].

In this study, the mean values of serum ALT, AST, urea and creatinine were significantly increased in indomethacin group compared to control although this elevation was still in the normal range for all of them. The effects on these parameters might have been partly a consequence of the intestinal damage (Table 1).

Indomethacin is known to induce the reactive oxygen metabolites in animal models, which may contribute to mucosal injury ,these free radicals also damage the cellular antioxidant enzymes which acting as the first line of cellular defense against oxidative stress. This might lead to aggravated tissue damage during stomach ulceration [25]. In agreement, our study appeared that indomethacin-induced stomach ulceration was accompanied with a severe oxidative stress in gastric tissue. This was apparent from the stimulated lipid oxidation leading to increased accumulation of malondialdhyde (MDA) as well as reduction in the gastric superoxide dismutase (SOD) activity and total antioxidant capacity (TAC) (Table 2).

There have been several conflicting reports about the ulcerogenic mechanism of indomethacin. It has been suggested that indomethacin induces gastric damage via inhibiting the release of protective factors like cyclooxygenase-1 (COX-1), prostaglandin E2 (PGE2), bicarbonate, and mucus; increasing aggressive factors like acid; and increasing oxidant parameters while decreasing antioxidant parameters. Classic antiulcer drugs are known to produce antiulcer effects by activating against indomethacin (increasing PGE2, mucus, and bicarbonate production; inhibiting acid secretion; decreasing oxidant parameters; and increasing antioxidants) [5].

 [26] have shown that indomethacin can induce jejunoileitis, and an increase in mucosal myeloperoxidase (MPO) activity which associated with significantly increased production of serum and tissue levels of TNF-α, IL-1, and nitric oxide. The gradual rise in serum IL-1 and nitric oxide synthase (iNOS)-derived NO levels suggests that TNF-α may up-regulate other cytokines and pro-inflammatory mediators, thereby contributing to intestinal damage, and thus may in part explain the protective effect of pomogranate that possess TNF-α, IL-1α and homocyseine properties, thus in treated group in this study, the mean value of serum inflammatory markers were significantly decreased compared to indomethacin group indicating the reduction of inflammation by pomegranate supplementation (Table 3).

Homocysteine concentrations are raised in the presence of low vitamin B12 and folate, and chronic gastric inflammation could result in malabsorption particularly of vitamin B12 and secondary hyperhomocysteinemia.

Table 1: Liver and kidney function tests

Parameters Groups	ALT U/L	AST U/L	Urea mg/dl	Creatinine mg/dl
Control	11.8 ± 1.2	32.3 ± 1.1	40.8 ± 1.6	0.20 ± 0.02
Pomegranate	11.3 ± 0.8 [b]	32.0 ± 2.2	47.4 ± 0.3 [a]	0.2 ± 0.03
Indomthacine	15.7 ± 0.4 [a]	62.7 ± 1.6 [a]	51.7 ± 2.1 [a]	0.5 ± 0.01 [a]
Treated	13.3 ± 0.8	47.0 ± 1.6 [a, b]	49.1 ± 2.6 [a]	0.3 ± 0.01

Data presented as mean ± SE
Significant p value < 0.05
a : significant difference compared to control group
b : significant difference compared to indomethacin group
n : number of cases = 10

The health benefits of pomegranate juice and extracts is derived from a spectrum of bioactive agents. In addition to the unique family of polyphenols in the pomegranate called punicalagins, pomegranate phytochemicals include anthocyanins, flavanols, and a seed oil which can be converted to conjugated linoleic acid. In addition to alkaloids, such as pelletierine, pseudopelletier pseudopelletierine [27]. In general, epidemiological studies suggest that intake of flavonoids, a group of polyphenolic compounds is beneficial for prevention of cardiovascular [28], inflammatory

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and other diseases [29]. It has been suggested that free radical scavenging and antioxidant activities play an important role in prevention of free radical-related diseases, including aging and ulcer [30]. Meanwhile, anthocyanidines, which differ structurally from other flavonoids except flavan-3-ol, and which do not have a carboxyl group in the C-ring, prevented lipid peroxidation [31].

Table (2): Oxidant/antioxidant parameters in different studied groups

Parameters / Groups	SOD U/g tissue	Total antioxidant mM/g tissue	MDA nmol/g tissue
Control	383.5 ± 2.5	3.7 ± 0.3	68.7 ± 1.6
Pomegranate	379.0 ± 1.7 [b]	3.8 ± 0.2 [b]	62.3 ± 1.7 [b]
Indomthacine	126.1 ± 1.3 [a]	1.6 ± 0.1 [a]	174.0 ± 1.2 [a]
Treated	286.0 ± 1.5 [a, b]	3.6 ± 0.3 [b]	72.3 ± 1.7 [b]

Data presented as mean ± SE
Significant p value < 0.05
a : significant difference compared to control group
b : significant difference compared to indomethacin group
n : number of cases = 10

Table 3: Inflammatory markers in different studied groups

Parameters / Groups	Homocysteine µmol/L	IL-1α Pg/ml	TNF-α Pg/ml
Control	4.5 ± 0.4	19.1 ± 0.2	40.5 ± 0.2
Pomegranate	3.0 ± 0.1 [b]	21.0 ± 0.2 [a, b]	41.2 ± 0.3 [b]
Indomthacine	34.5 ± 1.7 [a]	53.2 ± 0.3 [a]	71.5 ± 0.3 [a]
Treated	13.1 ± 2.9 [a, b]	23.7 ± 1.0 [a, b]	48.4 ± 1.0 [a, b]

Data presented as mean ± SE
Significant p value < 0.05
a : significant difference compared to control group
b : significant difference compared to indomethacin group
n : number of cases = 10

The histopathological Results in this study confirmed these results as follows

Fundus is the chosen part of stomach in the present study and its sections were stained with haematoxylin and eosin. The surface epithelial cells are present at the sides of the gastric pits and in the isthmus of the gastric gland. These cells are columnar with oval basal nuclei. The mucous cells are present in the neck of the gastric glands. They are columnar cells with triangular basal nuclei. Their cytoplasm is foamy in appearance.

The microscopic observation of control tissue revealed that the structure of gastric mucosa had an intact epithelial layer and glandular cells with continuous gastric pits (Figure 1- A).

In the rats treated with pomegranate, histological examination indicated the normal structure of gastric mucosa almost similar to control rat (Figure 1 - B).

In case of stomach of rats treated with indomethacin, histopathological investigation showed disrupted epithelial layer and the glandular cells with discontinuous gastric pits. The erosion of the epithelial layer and evident oedema and infiltration by inflammatory cell were notice (Figure 1 - C).

Examination of stomach of rats pre-administered the extract of pomegranate before indomethacin showed the absence of ulcer crater, proper rearrangement of submucosal layers along with normal glands; complete clearing of inflammatory exudates and reepithelization (Figure 1 - D).

Chronic administration of non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin, during the course of anti-inflammatory therapy, is often associated with the development of adverse gastrointestinal disorders such as gastric erosions, gastric or duodenal ulceration and other severe complications such as gastrointestinal haemorrhage or perforation that often limited their wide spread clinical use [32]. Indomethacin is known to induce gastric ulcer by inhibition of prostaglandins which are cytoprotective to gastric mucosa [33], particularly due to the inhibition of cyclooxygenase pathway of arachidonic acid metabolism resulting in excessive production of leukotrienes and other products of 5-lipoxygenase pathway [34]. In the stomach, prostaglandins play a vital protective role, stimulating the

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secretion of bicarbonate and mucus, maintaining mucosal blood flow, and regulating mucosal cell turnover and repair [35]. Thus, the suppression of prostaglandins synthesis by NSAIDs results in increased susceptibility to mucosal injury and gastroduodenal ulceration. Several studies have indicated that gastroduodenal protection by prostaglandins is due to increasing the mucosal resistance as well as the decrease in aggressive factors, mainly acid and pepsin [36]. The observed antiulcerogenic property of propolis may be due to increased synthesis of mucous and/or prostaglandins or could possibly be due to its 5-lipoxygenase inhibitory effect.
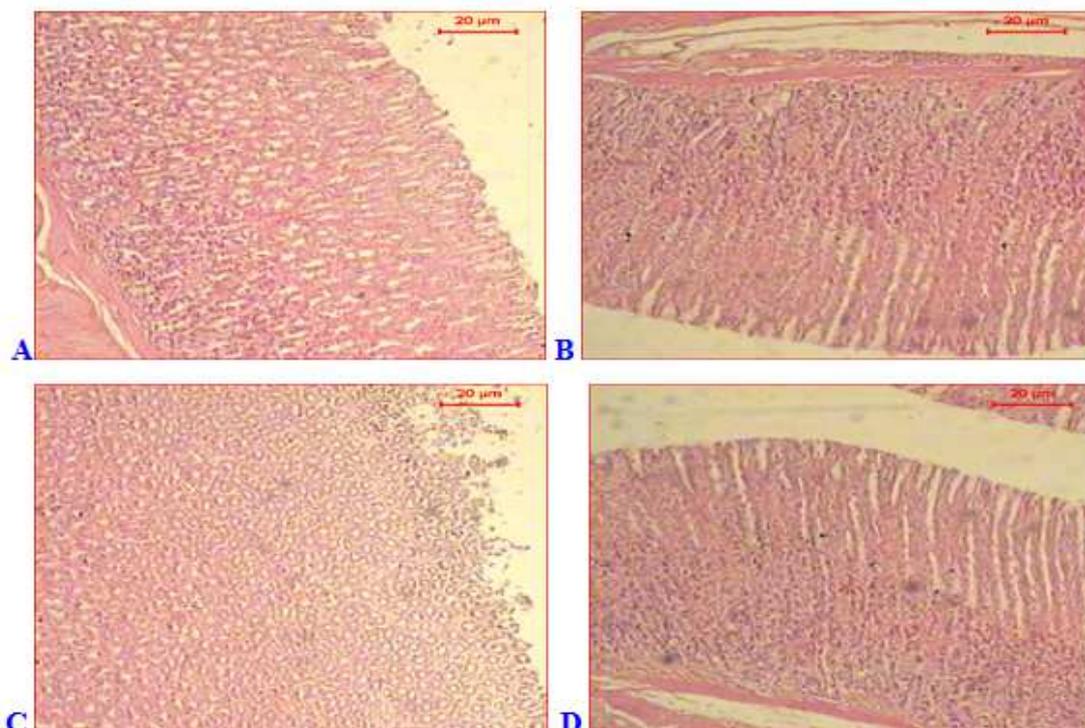

Figure (1): Micrographs of stomach of A) control rat shows the structure of gastric mucosa had an intact epithelial layer and glandular cells with continuous gastric pits B) rat treated with Pomegranate shows the normal structure of gastric mucosa C): rat treated with indomethacin shows a disrupted epithelial layer and glandular cells with discontinuous gastric pits. Notice the erosion of the epithelial layer and evident oedema and infiltration by inflammatory cell D): rat administered the extract of pomegranate and indomethacin showing absence of ulcer crater, proper rearrangement of submucosal layers along with normal glands; complete clearing of inflammatory exudates and reepithelization (H & E, Scale bar, 20μm)

Histochemical examination
Histochemical examination of sections of stomach of control rats stained according to the Periodic Acid Schiff`s technique (PAS) to highlight the mucous in the gastric mucosa showed that these materials are mainly localized in the epithelium lining the stomach mucosa. Deep stain is detected in the apical regions of these cells so that a dense coat magenta color is extending along the luminal surface of the stomach epithelium. The surface epithelial cells display intense stainability while the mucous neck cells display strong stainability. The surface epithelial cells are more intense in colouration than the mucous neck cells. The other cells of the gastric mucosa acquires pale stainability (Figure 2 - A) . On the other hand, histochemical examination of the mucosa of rats received the extract of pomegranate showed normal distribution (Figure 2 - B).

In rats received indomethacin, heterogeneous staining was encountered where the degenerated surface epithelial cells and mucous neck cells were almost devoid of stainable material while the healthy cells was densely stained (Figure 2 - C).

Pomegranate induced an increase in mucus production which was most demonstrative in rats that were treated with indomethacin. In these rats the mucus appeared as a thick continuous layer covering the mucosal surface (Figure 2 - D).

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Mucus serves as the first line of the defence against ulcerogens. The mucus is secreted by mucus neck cells and covers the entire gastrointestinal mucosa thereby preventing physical damage and back diffusion of hydrogen ions [37]. Gastric mucus consists of a viscous, elastic adherent and transparent gel formed by water and glycoproteins.

The protective effects of mucous barrier depend not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface. The ability of the gastric mucosa to resist injury caused by endogenous secretions (acid, pepsin and bile) and by ingested irritants such as alcohol, aspirin and NSAIDs can be attributed to a number of factors that have been generally referred as mucosal defense [33].

A review of antiulcer drugs of plant origin shows that triterpenes (because their ability to strengthen defensive factors such as stimulation of mucous synthesis or maintenance of the prostaglandins content of gastric mucosa at high levels) are potentially the compounds with antiulcer activity [38].
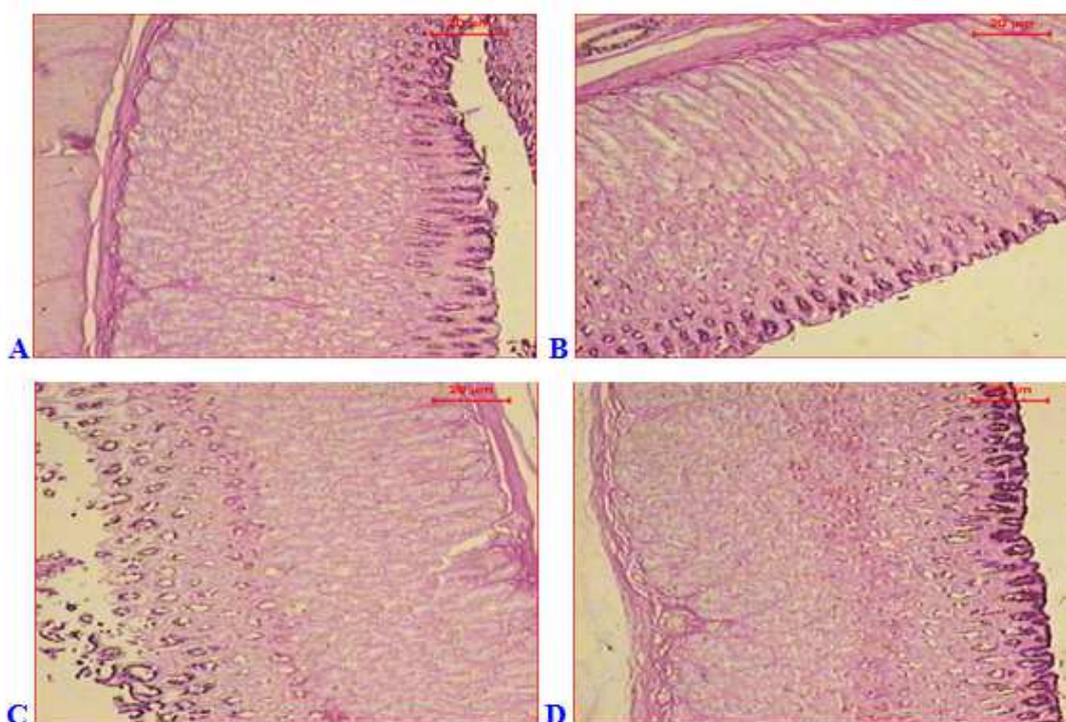

Figure (2): Micrograph of sections of stomach showing the PAS positive materials in the gastric mucosa of A) control rat, these materials displayed a dark pink stainability in the surface epithelial cells and in the mucous neck cells. Notice that the surface epithelial cells give more intense stainability than the mucous neck cells. The other cells of the mucosa acquire pale stainability, B) rat given the extract of pomegranate shows the normal distribution of the PAS positive materials in the gastric mucosa, C): rat received indomethacin shows the polysaccharides in the fundic mucosa. The surface epithelial and mucous neck cells, that represent the positive PAS materials, acquire a pale or disappearance of the stainability, D): rat received the extract of pomegranate before indomethacin administration shows an increase in the distribution of polysaccharides in the mucosa (PAS, Scale bar 20 μm)

CONCLUSION

From these results, we concluded that pomegranate juice supplementation has an important role in prevention of ulceration induced by NSAIDs. In addition to inhibition of hyperhomocysteinemia, the risk factor of many diseases.

REFERENCES

[1]E Colombo; E Sangiovanni; M Dell'Agli. A Review on the Anti-Inflammatory Activity of Pomegranate in the Gastrointestinal Tract, Evidence-Based Complementary and Alternative Medicine, **2013**, 11.
[2] RL Zhang; WD Luo; TN Bi; SK Zhou. *Molecules*, **2012**, 17(7):8419- 8429.

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[3] M Shakeerabanu; K Sujatha; C Praveen-Rajneesh; A Manimaran. *Advances in Biological Research*, **2011**, 5 (1): 64-70.

[4] S Somasundaram; G Sigthorsson; RJ Simpson; J Watts; M Jacob; IA Tavares. *Alimentary Pharmacology & Therapeutics*, **2000**, 14(5):639-650.

[5] H Suleyman; A Albayrak; M Bilici; E Cadirci; Z Halici. **2010**, *Inflammation*, 33(4):224-34.

[6] LK Kohn; CL Queiroga; MC Martini; LE Barata; PS Porto; L Souza. *Pharmaceutical Biology*, **2012**, 50 (10): 1269-1275.

[7] M Umashanker; S Shruti. *International Journal of Research in Pharmacy and Chemistry, IJRPC* **2011**, 1(4) p. 1152-1159. 2231-2781.

[8] PR Bhandari. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, **2012**, 2(3): 171-184.

[9] SD Johanningsmeier; GK Harris. *Annual Review of Food Science Technology*, **2011**, vol. 2, pp. 181–201.

[10] B Melichar; H Kalabova; L Krcmova; M Kasparova; E Malirova; K Melicharova; M Pecka; R Hyspler; D Solichova. *Anticancer Research*, **2009**, 29: 4813-4820.

[11] I Puscas; C Puscas; M Coltau; R Pasca; J Torres; M Márquez. *Arzneimittelforschung*, **1997**, 47: 568-572.

[12] M Aviram; L Dornfeld; M Rosenblat; N Volkova; MKaplan; RColeman; T Hayek; D Presser; Bianca Fuhrman *Am. J. Clin. Nutr.,* **2000**,71,1062–76.

[13] F Manna; HH Ahmed; SF Estefan; HA Sharaf; EF Eskander. *Pharmazie*, **2005**, 60, 689-695.

[14] S Reitman; S Frankel. *Am. J. Clin. Pathol.,* **1957**, 28 (1), 56-63.

[15] C Patton; S Crouch. *J. Anal. Chem.*, **1977**, 49:464-469.

[16] DS Young. Effect of disease on clinical laboratory test, **2001**, 4th Ed. AACC.

[17] M Uchiyama, M Mihara. *Anal. Biochem.*, **1978**, 86, 271-278.

[18] M Nishikimi; NA Roa; K Yogi. *Biochem. Bioph. Res. Common.*, **1972**, 46, 849-854.

[19] D Koracevic; G Koracevic; V Djordjevic; S Andrejevic; V Cosic. *J. Clin. Pathol.*, **2001**, 54, 356–361.

[20] P Aukrust; NB Liabakk; F Muller; E Lien; T Espevik; SS Froland. *J. Infect. Dis.*, **1994**, 169: 420-424.

[21] Dinarello, CA. *Blood*, **1991**, 77:1627 -1652.

[22] CFA Culling. Handbook of Histopathological and Histochemical Techniques. 3rd ed. London: Butterworth and Company, **1974**, 126-159.

[23] S Levi; RA Goodlad; CY Lee. *Lancet*, **1990**, 336 (8719), :840-843.

[24] DE Pratt. Natural antioxidants from plant material. In: Huang, I.M.T., Ho, C.T., Lee, C.Y., editors. Phenolic compounds in food and their effects on health. New York: American Chemical Society, **1992**, p.54-72.

[25] IZA Abdallah; HAH Khattab; GH Heeba. *Life Science Journal*, **2011**, 8(3): 433-445].

[26] J Nandi; B Saud; JM Zinkievich; ZJ Yang; RA Levine. *Molecular and Cellular Biochemistry*, **2010**, 336: 17-24.

[27] KR Kirthikar; BD Basu. *Punica granatum.* In: Indian Medicinal Plants, vol. 5. Sri Satguru Publications, **2000**, pp. 1508–1513.

[28] JM Gaziamo; JE Manson; LG Branch; GA Colditz; WC Willett; JE Buring. *Annals of Epidemology*, **1995**, 5, 255–260.

[29] NC Cook; S Sammam. *Journal of Nutrition and Biochemistry*, **1996**, 7, 66–76.

[30] D Harmam. Annals of the New York Academy of Sciences, **2001**, 928, 1–21.

[31] T Tsuda; K Shiga; K Ohshima; S Kawakishi; T Osawa. *Biochemical Pharmacology*, **1996**, 52, 1033–1039.

[32] I Villegas; C La Casa; CA De La Lastra; V Motilva; JM Herrerías; MJ Martín. *Life Sciences*, **2004**, 74: 873-884.

[33] JL Wallace. *The American Journal of Medicine*, **2001**, 110: 19S-23S.

[34] KD Rainsford. *Agents and Actions*, **1987**, 21: 316-319.

[35] J Hayllar; I Bjarnason. *The Lancet*, **1995**, 346: 521-522.

[36] A Aly. *Scandinavian Journal of Gastroenterology*, **1987**, 137: 43-49.

[37] SE Williams; LA Turnberg. *Gastroenterology*, **1980**, 79: 299-304.

[38] DA Lewis; PJ Hanson. *Progress in Medicinal Chemistry*, **1991**, 28: 201-231.