Preventive Role of Punica Granatum Peel and Seed Extract in Murine Model of Peptic Ulcer: A Histopathological Perspective

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Abstract

Objectives: To histologically assess prophylactic antiulcer effect of Punica granatum (Anar) peel and seed. To compare stomach histological aspects of Punica granatum peel and seed with standard drug pantoprazole in diclofenac induced murine model of peptic ulcer.

Material and Methods: This experimental animal study was conducted in post graduate medical institute (PGMI) Lahore, after approval from Institutional Review Board (IRB) of Federal post graduate medical institute (FPGMI). Eighty one male rats were segregated into 9 groups having 9 rats each. Control groups; G-1 (healthy control) and G-2 (disease control) were given only distilled water. Pantoprazole and Punica granatum peel (PGPE) and seed (PGSE) extracts were given orally once daily to the treatment groups (3-9) for 15 days as follow:G-3: pantoprazole 60mg/kg/d, G-4:PGPE 100mg/kg/d, G-5 PGSE 500mg/kg/d, G-6: PGPE 50mg/kg/d±PGSE 250mg/kg/d, G-7:Pantoprazole 30mg/kg/d±PGPE 50mg/kg/d, G-8:Pantoprazole 30mg/kg/d±PGSE 250mg/kg/d, G-9:Pantoprazole 30mg/kg/d±PGPE 50mg/kg/d±PGSE 250mg/kg/d. Groups 2-9 were then given 100mg/kg diclofenac orally on day 17 for induction of peptic ulcer. Histopathological analysis of stomach was done at the end of the study.

Results: The gastric mucosa of all the animals in healthy control group was intact and had a continuous epithelium. Disease control showed severe mucosal damage grade 3 involving entire mucosa, grade 2 involving 2/3rd of mucosa and grade 1 superficial erosion injury. The difference between mucosal findings of all groups was highly significant (p-value= 0.000). None of the treatment groups showed grade 3 mucosal damage.

Conclusion: Best results were observed in the group where Pantoprazole 30mg/kg/d ± PGPE 50mg/kg/d ± PGSE 250mg/kg/d combination was used.

Keywords: pomegranate, peptic ulcer disease, pantoprazole, diclofenac

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Introduction

Peptic ulcer, considered as the most common gastrointestinal disease causes severe complications such as bleeding, perforation, and may also lead to death if it is accompanied by other morbidities.[1] Though anti-inflammatory drugs, including nonsteroidal anti-inflammatory drugs (NSAIDs) such as Diclofenac and Piroxicam. These agents are widely used as analgesics and have been reported to cause gastric ulcers, ulcer perforation, gastric and duodenal bleeding, and ulcer death. Prostaglandin analogues like, proton pump inhibitors (PPIs) are used to treat peptic ulcer.[2] The disease management approaches have been markedly changed by the PPIs introduction. PPIs has become a pillar of medical therapy for peptic ulcer-related gastrointestinal bleeding.[3] It has been revealed that reactive oxygen species (ROS),

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especially the hydroxyl radical and superoxide anion, play an important role in the pathogenesis of acute experimental gastric lesions induced by NSAIDs. Cyclo-oxygenase isozymes in the gastric mucosa promote mucus production by the continuous production of prostaglandins and the inhibitions of cyclooxygenase and prostaglandin synthesis by NSAIDs could disrupt the intrinsic ability of the mucosa to prevent the injuries induced by exogenous and endogenous factors.[4]

Medicinal plants and herbal recipes have played substantial role in the management and cure of various diseases. Punica granatum (Pomegranate) peel and seed extracts have anti-inflammatory and antioxidants which have shown their significant protective effect against neuro-inflammation,[5] hepatotoxicity, renal damage,[6] cardio-vascular dysfunction,[7] metabolic syndromes,[8] and auto-immune diseases.[9] Several natural products showed that pomegranate possesses antiulcerogenic activity by their predominant effects on mucosal defensive factors.[10] The prophylactic antiulcer effect of PG peel and seed extracts (PGPE, PGSE) individually and in combination and in comparison, to Pantoprazole, were investigated against diclofenac induced gastric ulcer in murine model. Moreover, no previous research existed to compare the stomach histological aspects with PGPE and PGSE in comparison to the standard drug pantoprazole.

Materials and Methods

This experimental animal study was conducted in post graduate medical institute (PGMI) Lahore, after approval from Institutional Review Board (IRB) No. F-39/NHRC/Admin/IRB/88 daated:09-08-2016 of Federal Post Graduate Medical Institute (FPGMI). It took 17 days for the completion of the experiment. Eighty-one healthy male albino rats weighing130-170 grams were purchased from University of Veterinary and Animal Sciences (UVAS) Lahore, and kept in the polypropylene cages in standard housing and lighting conditions; 60-70% humidity, 25-270C, 12/12-hour light/dark cycle. The albino rats were randomly assigned to 9 groups (G-1 to G-9) having 9 rats each by lottery method. The rats were acclimatized to the new environment for one week before starting the experiment. Pantoprazole tablets (40mg) and Diclofenac tablets (50mg) were bought from Servaid Pharmacy, Lahore, Fresh PG fruits were purchased from local fruit market of Lahore. The identification, verification and extract preparation of the extracts were done in Applied Chemistry and Research Centre at Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Lahore. Fruits were washed thrice with distilled water and peeled manually. One kilo peel and seeds were shade dried and finely ground in the grinder. Separate extraction was carried out for the peel and seeds with 80% methanol in a Soxhlet apparatus for 4hrs, and further concentration was performed at 40°C under controlled reduced pressure using a rotary vacuum evaporator. The extracts were collected in capped bottles and stored 12 at 4°C till further use. After acclimatization the animals in the healthy (G-1) and disease control groups(G-2) were given only 0.5ml distilled water orally daily for 15 days and the remaining seven groups were given prophylactic oral treatment (extracts and pantoprazole tablets dissolved in distilled water once daily for 15 days as per group designation detailed below.

Group 3:(standard group) Pantoprazole 60mg/kg/d
Group 4: PG peel extract (PGPE) 100mg/kg/d
Group 5: PG seed extract (PGSE) 500mg/kg/d
Group 6: PGPE + PGSE (50+250mg/kg/d)
Group 7: Pantoprazole + PGPE (30+50mg/kg/d)
Group 8: Pantoprazole + PGSE (30+250mg/kg/d)
Group 9: Pantoprazole + PGPE + PGSE (30+50+250 mg/ kg/d)

Animals of groups 2-9 were then fasted for 24 hrs (day 16) and given Diclofenac 100mg/kg orally in a single dose on the 17th day. After 4 hours the rats were anesthetized using chloroform and then surgically sacrificed for histological evaluation of the stomach. Stomach tissues were fixed in 10% formalin in properly labeled containers. The tissue was processed using a tissue processor, paraffin blocks were made for the processed tissue and about 5-µm thick sections were cut using a rotary microtome. The sections were then stained with hematoxylin and eosin for examination under light microscope. Slides were examined by the histopathologist in Fatima Jinnah Medical University (FJMU) Lahore, who was blinded to the study. The following stomach histopathological parameters were evaluated.

Grades:

Grade 0 = No ulceration
Grade 1 = Superficial erosion
Grade 2 = Ulceration involving 2/3rd of mucosa
Grade 3 = Ulceration involving entire mucosa
Inflammation: Present/ Absent
Hemorrhage: Present/ Absent

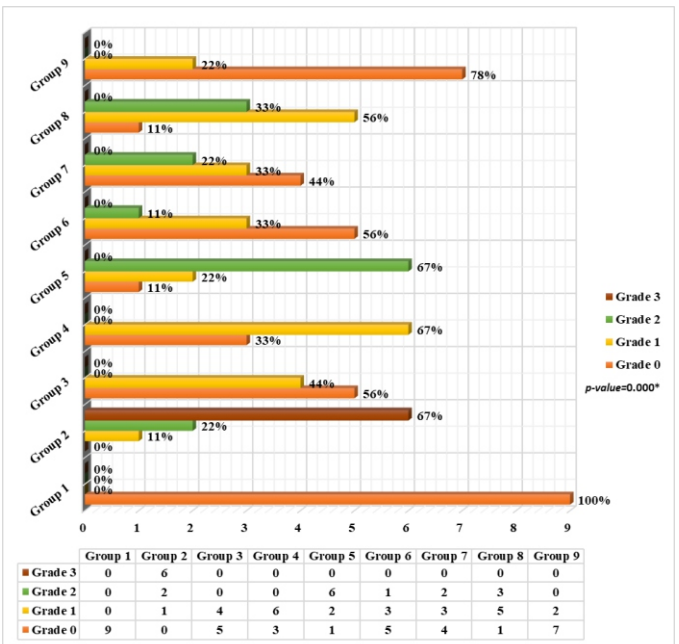
The data was entered and analyzed using SPSS version 23.0. Data for qualitative variables; grades, inflamma-

tion, mucosal hemorrhage was described by using frequency and percentages for each group. Comparison among groups was made by using Chi-square test. P-value of < 0.05 was considered statistically significant and <0.01 highly significant.

Results

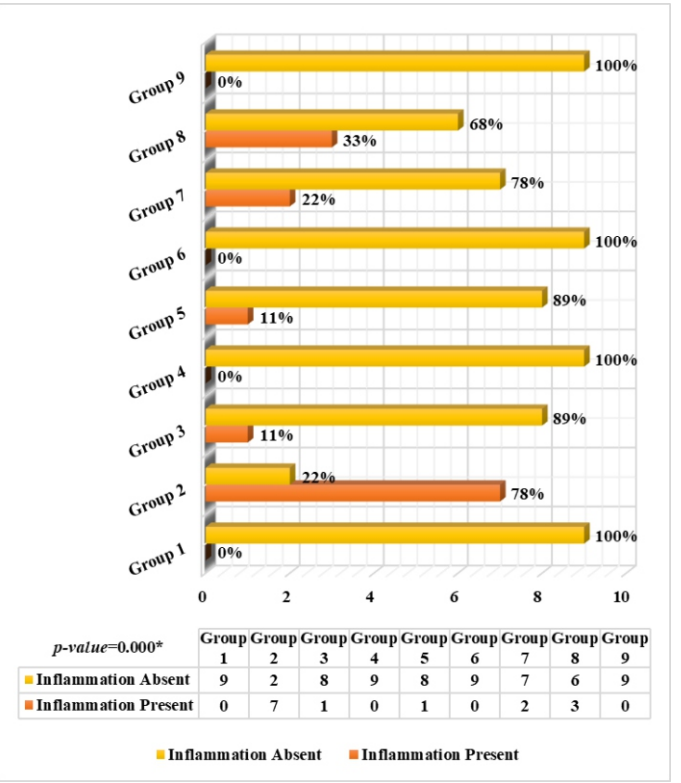
Out of 81, 46 (56.8%) rats had grade 1 to grade 3 changes. Chi square test revealed that there is an association between grades and groups. All the rats of group 1 were normal. In group 9, 7 (77.8%) rats and in group 3 & 6, 5(55.6%) rats were normal.

Out of 81, 14(17.3%) rats had inflammatory changes in their stomach. Chi square test revealed that there is an association between inflammation and groups. No inflammation was present in group 1, 4, 6 and 9. In group 2,7 (77.8%) rats, in group 3,1 (11.1%), in group 7, 2(22.2%) and in group 8 only 3(33.3%) rats had inflammation. Out of 81, 3 (3.7%) rats showed hemorrhage. Chi square test revealed that there is an association between hemorrhage and groups. No hemorrhage was observed in all groups except group 2, 3(33.3%) rats showed hemorrhage.



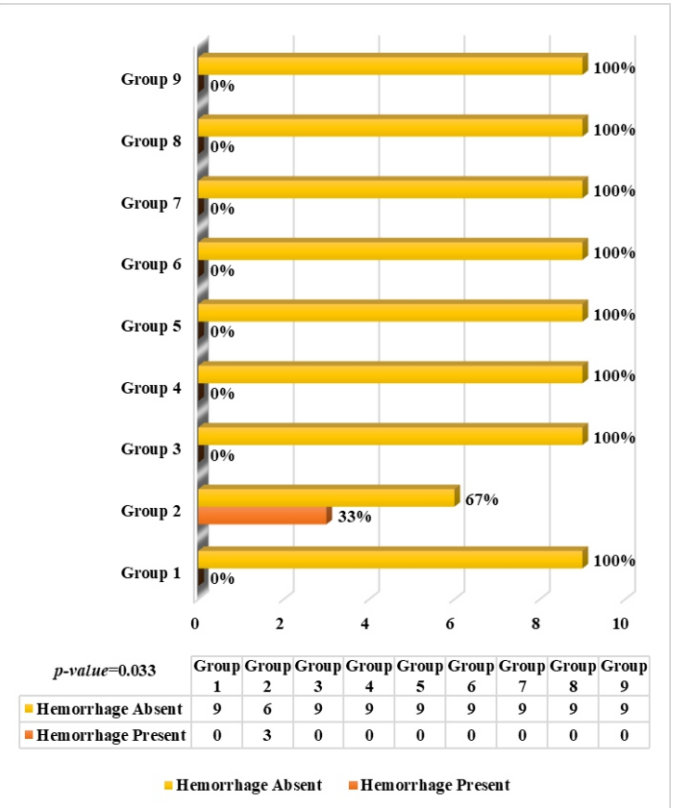
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Grade 3	0	6	0	0	0	0	0	0	0
Grade 2	0	2	0	0	6	1	2	3	0
Grade 1	0	1	4	6	2	3	3	5	2
Grade 0	9	0	5	3	1	5	4	1	7

Figure 1: *Graphical representation of histological grading of stomach.*



p-value=0.000*	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Inflammation Absent	9	2	8	9	8	9	7	6	9
Inflammation Present	0	7	1	0	1	0	2	3	0

Figure 2: *Comparison of Inflammation among groups.*



p-value=0.033	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8	Group 9
Hemorrhage Absent	9	6	9	9	9	9	9	9	9
Hemorrhage Present	0	3	0	0	0	0	0	0	0

Figure 3: *Comparison of Haemorrhage among groups*

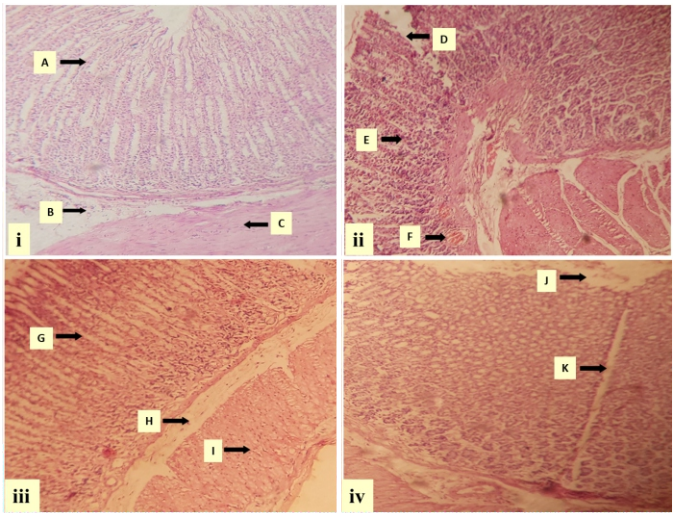


Figure 4: *i: Group 1 showing normal stomach histology; intact epithelium, no inflammation and hemorrhage (10X). A) Mucosa, B) Sub-mucosa, C) Muscularis mucosa. ii: Group 2 showing grade 3 mucosal damage, neutrophilic infiltrates and hemorrhage (10X). D) Ulcer involving the entire length of mucosa, E) Neutro-philic infiltrates, F) Hemorrhage. Iii: Group 3 showing normal stomach histology; intact epithelium, no infla-mmation and hemorrhage (10X). G) Mucosa, H) Sub-mucosa, I) Muscularis mucosa. Group 4 showing grade 1 mucosal damage (10X). J) Erosion, K) Artefact*

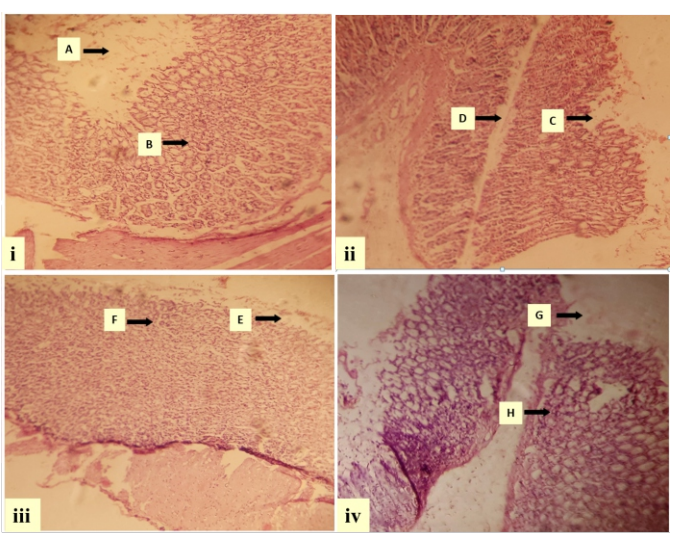


Figure 5: *Group 5 given PGSE 500mg/kg/d showing grade 2 mucosal damage and neutrophilic infiltration. A)ulceration of 1/3rd of mucosa, B) Neutrophilic infil-trates. Group 6 given combination of PGPE 50mg/kg/d & PGSE 250mg/kg/d showing grade 1 mucosal damage (10X). C) Erosion, D) Artefact. Group 7 given combi-nation of Pantoprazole 30mg/kg/d & PGPE 50mg/kg/d showing grade 1 mucosal damage and neutrophilic infiltration (10X). E) Erosion, F) Neutrophilic infiltra-tion. Group 8 given combination of Pantoprazole 30mg/kg/d & PGSE 250mg/kg/d showing grade 2 mucosal damage and inflammation (10X). G) Ulcer involving 1/3rd of mucosa, H) Neutrophilic infiltration.*

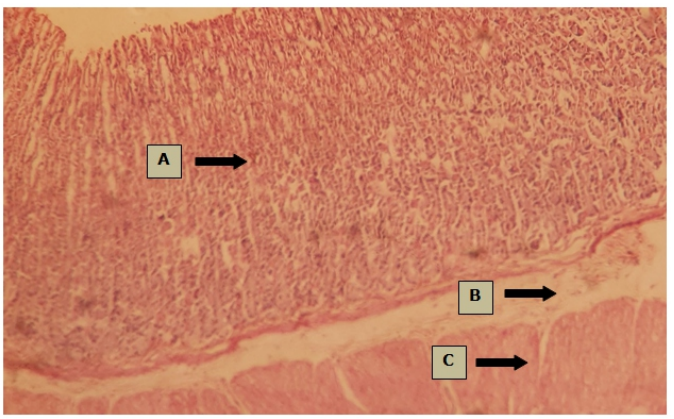


Figure 6: *Group 9 given combination of Pantoprazole 30mg/kg/d, PGPE 50mg/kg/d & PGSE 250mg/kg/d showing normal stomach histology (10X). A) Mucosa. B) Sub-mucosa, C) Muscularis mucosa*

Discussion

Peptic ulcer can be delineated as the presence of a deep destruction of the stomach lining or mucosa and/or duodenum, reaching beyond the muscularis mucosa, specifically to the muscle layer owing to the environmental gastric acid synthesis.[11] Furthermore, nonsteroidal anti-inflammatory drugs (NSAIDs) like diclofenac, have widely been used as therapeutic agents for many of the chronic illnesses but have been reported as one of the major causes of drug-induced gastrointestinal (GI) including peptic ulcer (PU) or bleeding.[12] Other risk factors for PUD are cigarette smoking, alcohol intake, advancing age and stress. Occasionally it may be idiopathic.[13] Punica granatum (Pomegranate) is a miraculous fruit being used in alternative medicine for treatment of various diseases since ancient time. Pomegranate fruit, peels and seeds have shown anti-ulcer effects in murine models due to their strong gastroprotective and antioxidant activity attributed to presence of phenolic compounds, flavonoids, tannins and anthocyanins. These are the main group of antioxidant phytochemicals known for their free radical scavenging activities.[14] The prophylactic antiulcer effect of PG peel and seed extracts (PGPE, PGSE) individually and in combination in comparison to Pantoprazole, a standard

antiulcer drug (PPI) in the murine diclofenac induced gastric ulcer model were investigated. Moreover, no previous research existed to compare the stomach histological aspects with PGPE and PGSE in comparison to the standard drug pantoprazole against diclofenac. The gastric mucosa of all the animals in healthy control group was intact and had a continuous epithelium. Disease control showed severe mucosal damage grade 3 involving entire mucosa, grade-2 involving 2/3rd of mucosa and grade 1 superficial erosion injury. These results further confirmed ulcerogenic potential of diclofenac which is a result of several mechanisms: local irritant activity due to its acidic nature, prostaglandin synthesis inhibition, NO synthesis inhibition[15] and increased oxidative stress due to formation of free radicals and ROS; O2-, OH and H2O2 which damage lipids, proteins and DNA. Additionally, lipid peroxidation resulting from free radicals, damages the cell and decreases the integrity of mucosal epithelial cells, producing epithelial disruption.[16]

The difference between mucosal findings of all groups was highly significant (P-value= 0.000). None of the treatment groups showed grade 3 mucosal damage. Best results were seen in group 9 (Pantoprazole 30mg/kg/d + PGPE 50mg/kg/d + PGSE 250mg/kg/d) having 77.8% rats with normal mucosa and only 22.2% showed grade 1 mucosal damage (gastric epithelium was not completely intact). These results were even better than the standard group in which 44.4% animals showed grade 1 damage.

This showed a unique, multipronged, preventive antiulcer activity of pantoprazole and PG peel and seed extracts against diclofenac seen by decreasing HCl production, increasing NO levels and mucus secretion,[17] precipitating proteins and forming a mucosal protective layer[18], overcoming the oxidative stress by scavenging ROS and increasing endogenous antioxidants; GSH, CAT and SOD.[19] This effect may be due to the presence of phytochemicals, especially ellagic acid, ellagitannins, punicalagin and flavonoids; anthocyanins and proanthocyanidin.[20]

Inflammation was not seen in gastric histology of all the animals of healthy control group whereas, neutrophilic infiltration was present in one third of the rats of gastric mucosa of disease control group. Diclofenac triggers neutrophil adherence to gastric microvascular endothelium, damaging the gastric mucosa releasing pro-inflammatory cytokines; tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) resulting in acute inflammation presented as mucosal neutrophilic infiltration.[21]

There was significant difference in the presence of inflammation between disease and treatment groups (P-value=0.000). In groups 3 (Pantoprazole 60mg/kg/d), 7(Pantoprazole 30mg/kg/d + PGPE 50mg/kg/d) and 8(Pantoprazole 30mg/kg/d + PGSE 250mg/kg/d) 11.1%, 22.2% and 33.3% rats showed inflammation respectively. Whereas, neutrophil infiltration was absent in all the animals of group 4 (PGPE 100mg/kg/d), 6 (PGPE 50mg/kg/d + PGSE 250mg/kg/d) and 9 (Pantoprazole 30mg/kg/d+PGPE 50mg/kg/d+PGSE 250mg/kg/d) which demonstrates the strong anti-inflammatory effect of PG peel and seeds due to the presence of polyphenols like ellagic acid, gallic acid and ellagitannins mitigating inflammation by suppressing the pro-inflammatory cytokines (TNF-α and IL-1β).[22] Another research showed a potent anti-inflammatory activity of Punica granatum peel extract due to ellagic acid in Carrageenan-induced paw edema model, proving its ability to reduce acute inflammation.[23] Only the diclofenac treated disease control group showed hemorrhage in one third of rats primarily by interfering with the house keeping effects of prostaglandins resulting in decreased mucosal protection[24] and secondarily due to microvascular injury caused by neutrophil and oxygen free radical.[25]

Conclusion

The obtained findings revealed that pretreatment with a combination of pantoprazole, PGPE and PGSE significantly strengthened gastric mucosa, ameliorated the gastric mucosal damage and inflammation caused by diclofenac. Another noteworthy aspect was that hemorrhage was prevented in all groups including those given the drug and extracts individually, which showed that hemorrhage can be prevented by blocking any step in the formation of peptic ulcer whether acid secretion, mucosal damage or inflammation.

Conflict of interest: None

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Authors Contribution

SSA: Conceptualization of Project
SJ: Data Collection
MIP: Literature Search
OS: Statistical Analysis
MR: Drafting, Revision
AA: Writing of Manuscript