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The Potential Anti-ulcerogenic Effects of Baicalein and/or Empagliflozin in Induced Gastric Ulcer in Rats: Modulating HO-1/SIRT1 / HMGB1 signaling Pathway

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- Gastric ulcer
- Baicalein
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Abstract

Background: Gastric ulcer is one of the most ubiquitous gastrointestinal tract disorders, causing high morbidity. The goal of this study was to explore at the mechanistic effects of Baicalein (BA) and/or Empagliflozin (EMP) treatment on gastric ulcer caused by indomethacin and prednisolone, with an emphasis on the role of the HO-1/SIRT1 / HMGB1 signaling pathway.Material and methods: Fifty adult albino rats were allocated into 5 equal groups: control, ulcer (Prednisolone 10mg/kg for 6 days followed by indomethacin as single oral dose 30 mg/kg), BA (30 mg/kg), EMP (10 mg/kg), and BA + EMP groups. The volume of gastric juice, pH, free and total acidity was estimated. The gene expression of HO-1 and SIRT1 in gastric tissues was assessed by qRT - PCR. Biochemical analysis of gastric tissues homogenates including HMGB-1, PGE2 & nitrites levels was performed. Assay of inflammatory markers and redox status were detected. Additionally, histological, scanning electron microscopic and immunohistochemical analyses were determined.Results: After 4 weeks of treatment, there was remarkable improvement of the histological architecture of rat gastric tissues. Upregulation of HO-1 and SIRT1 gene expression, as well as a decrease in HMGB1 level, resulted in improved inflammatory and oxidative stress biomarkers. Furthermore, immunohistochemical analysis revealed increase in Bcl-2 expression and decreased expression of Bax in the treated groups. Conclusion: Concurrent usage of BA & EMP against gastric ulcer in rats could be related to the interaction of their anti-oxidant, anti-inflammatory, and anti-apoptotic activities via modulation of HO-1/SIRT1 / HMGB1 signaling pathway..

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INTRODUCTION

Gastric ulcer is one of the most common gastrointestinal disorders, causing high morbidity in about 10 % of the world population. Although gastric ulceration pathogenesis is complex multifactorial and contentious, prosperity of upshots disrupts the balance between the destructive factors such as increased gastric acidity, non-steroidal anti-inflammatory drugs (NSAIDs), reactive oxygen species (ROS), inflammatory mediators, and *Helicobacter Pylori*, and the defensive factors in the gastric mucosa such as mucin, prostaglandins (PGs), nitric oxide (NO), bicarbonate, and growth factors [1].

Prednisolone, one of the glucocorticoids, has been extensively used as a therapeutic agent in many inflammatory and immune-mediated diseases. Despite its undeniable efficacy, long term use and/or high dose administration have been related to several adverse effects as gastric mucosal injury [2]. Corticosteroids have been reported to inhibit cyclooxygenase-2 enzymes (COX-2) expression with subsequent suppression of the synthesis of PGs that protect gastric mucosa [3]. Indomethacin (IND) is an analgesic and anti-inflammatory agent that belongs to the non-steroidal anti-inflammatory drug (NSAID) family. However, through various mechanisms such as inhibition of both COX and PGE2 production, as well as further suppression of mucosal cell regeneration, free radical production and activation of gastric cell apoptosis, aggressive ulcerogenic potential is provoked [4]. The mutual use of corticosteroids and NSAIDs may therefore be linked to the progression of gastric ulcers [5].

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme in the oxidation of heme into its products carbon monoxide, free iron and biliverdin. It is an inducible enzyme, expressed in gastric epithelium as a primary adaptive response of the cellular defense mechanism. Recent research suggests that pharmacologically induced HO-1 overexpression has cytoprotective effects and mitigates oxidative stress, therefore protecting the gastric mucosa from gastropathy [6].

Sirtuin 1 (SIRT1) (silent information regulator factor 2-related enzyme 1), a member of class III histone deacetylases, is an imperative stress-responsive element. The connections between protein deacetylation and various pathophysiological processes such as apoptosis, cell survival, antioxidant stress, autophagy, inflammation and metabolism are known to control different targets as a critical factor [7]. SIRT1 has recently been linked to gastric cancer as a potential tumor suppressor that regulates the cell cycle and apoptotic pathway [8]. The specific role of SIRT1 is, however, not fully articulated in ulcerative gastric lesions.

High Mobility Group Box 1 (HMGB1), an evolutionarily conserved DNA-binding protein, is ubiquitously expressed in virtually all mammalian cells [9]. In response to infections and injuries, it is released to the extracellular milieu either by activated immune cells or passively after cell death, acting as an alert triggering inflammatory signals [10]. HMGB1 has been shown to decelerate gastric ulcer healing and to enhance inflammatory cytokines expression [11].

Due to the restricted effectiveness and numerous adverse effects associated with the extended use of the antisecretory agents, treatment

of gastric ulcers using accessible traditional medicines such as proton pump inhibitors, H2 receptor blockers, and antibiotic therapy faces a significant setback [12]. Therefore, there is an urgent need to focus on novel therapeutic alternatives that provide higher effectiveness and fewer side effects for gastric ulcer treatment and prevention.

Baicalein (BA) is a bioactive phenolic flavonoid derived from the roots of *Scutellaria baicalensis* Georgi and is widely used as a Chinese herbal medicine. Besides its wound healing properties, BA has been shown to exert various beneficial effects, such as antioxidant, antipyretic, analgesic, anticancer [13]. While the antioxidant activities of BA in gastrointestinal diseases have been shown in recent studies, the exact molecular mechanisms of gastric ulcers are still not fully clarified.

Empagliflozin (EMP) is a recently FDA-approved agent of oral anti-diabetic drugs, and one of selective sodium glucose co-transporter 2 (SGLT2) inhibitors. Other biological effects of EMP have been identified in experimental animal models, including potent antioxidant, anti-inflammatory, and anti-apoptotic effects, in addition to its antihyperglycaemic properties [14]. Although the beneficial effects of EMP are endorsed by several lines of substantiation, its impact in gastrointestinal disorders has not been studied before now.

Based on the above-mentioned considerations, the goal of the present study is to identify mechanisms underlying the potential therapeutic effects of BA &/or EMP on induced gastric ulcer in rats via HO-1/SIRT1 /HMGB1 axis regulation and further study their downstream effects in gastric tissues.

2- Materials and Methods:

2.1. *Reagents and Chemicals:*

IND was purchased from Kahira pharmaceutical Co., (Cairo, Egypt). Prednisolone was obtained as a product of Sanofi- Aventis pharmaceutical Co, (Cairo, Egypt). Baicalein was obtained from Sigma-Aldrich Chemical Co., (Louis, MO, USA; purity (HPLC) ≥ 97.5%). Empagliflozin was purchased from Boehringer Ingelheim (Ingelheim, Germany) with 99.98% purity for the drug, based on the company analysis certificate. All drugs and chemicals used were of analytical grade.

2.2. Experimental animals:

In the experiment, fifty adult male albino rats, of average 150-180 grams; about 8-12 weeks of age were collected from the animal house of the Faculty of Medicine, Tanta University. All rats were housed in stainless steel cages individually and each rat had a tag number. During acclimatization before starting the experiment, rats were fed commercial rat chow and allowed free access to water for two weeks within a constant temperature of 25 ± 3 with relative humidity of 56 ± 3% and exposed to 12 h light/dark cycle. All the experiments were conducted according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 85-23, revised in 1996). This study was performed in accordance with the Research Ethics committee of Tanta University, Faculty of Medicine.

2.3. Animal grouping and experimental design:

24 hours prior to ulcer induction, the rats were fasted but had free access to water to prohibit exogenous dietary effect. To eliminate variations due to diurnal rhythms of potential regulators of gastric functions, all studies were conducted at the same time of day. The rats were randomly allocated into five experimental groups (10 rats / group) as following:

1- Group I (control group): Rats were given 0.9% sodium chloride solution (1 ml/100 g of body weight) via oral gavage for 4 weeks as a vehicle.

2- Group II (ulcer group): Rats received Prednisolone 10 mg/kg orally by gavage once daily for 6 days followed by a single oral dose of IND 30 mg/kg [15, 16].

3- Group III (BA group): Rats were given Prednisolone and IND as in ulcer group. Then, after 6 hours they received BA 30 mg/kg orally by gavage once daily for 4 weeks [17].

4- Group IV (EMP group): Rats were given Prednisolone and IND as in ulcer group. Then, after 6 hours they received EMP 10 mg/kg orally by gavage once daily for 4 weeks [18].

5- Group V (BA + EMP group): Rats were given Prednisolone and IND as in ulcer group. Then, after 6 hours they received both BA 30 mg/kg and EMP 10 mg/kg orally by gavage once daily for 4 weeks.

All animals were rapidly sacrificed after being anaesthetized with diethyl ether at the end of the experiment. The stomachs were gently mobilized and dissected along the greater curvatures after the anterior abdominal wall was incised. The contents of the stomach were collected in centrifugation tubes and centrifuged for 15 min at 3,000 rpm to remove any solid debris and separate the supernatant for further investigations. The gastric tissues were divided into two parts, one for histological & immunohistochemical examinations, while the other part of the stomach used for the preparation of tissue homogenates and was stored at -80˚C for biochemical and real time gene expression analyses.

2.4. Evaluation of gastric mucosal lesions:

The excised stomachs were macroscopically examined for gross hemorrhagic mucosal lesions. Ulcer index (UI) and curative index (CI) were used to evaluate the degree of ulcer damage according to the previous methods described by Ganguly et al, and Adinortey et al. respectively [19, 20].

2.5. Analysis of the gastric juice:

The volume of the supernatant was measured and analyzed for pH, free and total acid concentrations after centrifugation of the collected gastric juice [21].

2.6. Real time quantitative PCR estimation for gastric HO-1 and SIRT1 relative genes expression:

Briefly, gastric tissue was used for total RNA extraction by gene Jet RNA purification kit in accordance to the kit instructions (Thermo scientific, # k 0731 USA). Total RNA concentrations and quality were determined by NanoDrop spectrophotometer (NanoDrop Technologies, Inc., Wilmington) at the OD260 and OD260/280 ratios respectively, then stored at -80˚C. Next, Extracted RNA was reverse transcribed by a revert Aid H Minus Reverse Transcriptase (Thermo scientific, # Ep0451) into

cDNA, a template for further quantification of relative gene expression of HO-1 and SIRT1 using Step One Plus Real Time PCR system (Applied Biosystem). The primers were designed by software (Primer 5.0) as shown in Table 1. Finally, the expression levels were calculated relative to housekeeping gene (β-actin), using the comparative method formula $2^{-\Delta\Delta CT}$ [22].

Table (1): Primer sequences for real-time RT-PCR:

Gene	Primer Sequence
HO-1 (NCBI GenBank Nucleotide accession # NM_012580.2)	F 5'- GGCTGTGAACTCTGTCTC -3' R 5'- GGCATCTCCTTCCATTCC -3'
SIRT1 (NCBI GenBank Nucleotide accession # NM_001372090.1)	F 5'- ATTTATGCTCGCCTTGCTGTG -3' R 5'- AGAGATGGCTGGAACTGTCC -3'
β-actin (NCBI GenBank Nucleotide accession # NM_001101.4)	F 5'- CTCTTCCAGCCTTCCTTCCT -3' R 5'- AGCACTGTGTTGGCGTACAG -3'

2.7. Biochemical analysis of gastric tissues homogenates:

A portion of gastric tissue was homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). Then, gastric homogenates were then centrifuged for 10 min at 10,000 rpm at 4˚C, and the collected supernatants were kept at -80˚C to be used directly for further experimental procedures. Total proteins concentrations in gastric tissue homogenates were assessed (#Cat 500–0006, Bio-Rad Protein Assay).

2.7.1. Determination of HMGB-1 levels:

The HMGB-1 levels in gastric tissue homogenates were quantitatively assayed using a specific enzyme-linked immunosorbent assay (ELISA) kit (Sunred Biological Technology Co, Shanghai, China, Cat # 201-11-0258). The color intensity established was read at 450 nm and the results were expressed as ng/mg protein.

2.7.2. Assay of inflammatory markers:

The levels of tumor necrosis factor-α (TNF-α) in the gastric tissues were measured by commercially available ELISA kits (MyBiosource, San Diego, USA, Cat# No MBS355371) at optical density of 450 nm according to the manufacturer's recommendations. The results were expressed as pg/mg protein.

Myeloperoxidase (MPO) activity in gastric tissue homogenates was assayed in accordance to the method of Bradley et al. [23]. The activity of MPO was detected spectrophotometrically at 460 nm and expressed as U/mg protein.

2.7.3. Assay of lipid peroxidation, and antioxidant enzymes:

Malondialdehyde (MDA), a marker for lipid peroxidation, was detected in gastric tissue homogenate spectrophotometrically by measurement of the color occurring at 532 nm that resulted during MDA reaction with thiobarbituric acid (TBA) using semiautomatic BTS-350 Biosystems spectrophotometer. The values were expressed as nmol/mg protein.

The levels of reduced glutathione (GSH) were estimated by the commercial kits (Biodiagnostic, Egypt) according to the manufacturer's instructions. The results were expressed as mg/g protein.

2.7.4. Determination of nitrites and PGE2 levels:

Nitrite levels were used as an indicator of gastric nitric oxide (NO) production. It was measured in gastric tissue homogenate supernatant based on Griess diazotization reaction [24]. The results obtained were well compared with standard curve obtained with sodium nitrite reagent as reference. PGE2 levels were determined by the standard commercial kit (Cat# No. ABIN365372) according to the manufacturer's instructions.

2.8. Histological assessment of gastric tissues:

Immediately after extraction, specimens from the fundus and body of stomach were immediately fixed in 10% buffered formalin for 24 h, then embedded in paraffin blocks and cut into 5 µm sections. These were placed on glass slides, then stained with haematoxylene and eosin (H&E), coded and examined by a light microscope.

2.9. Immunohistochemical detection of apoptotic markers:

The slides first were heated at 60 °C for 25 min, then de-paraffinized with xylene, graded alcohol, after that boiled in antigen retrieval solution and then incubated for 15 min with primary antibodies (anti -Bax) and (anti -Bcl-2) at dilutions of 1:100, 1:50 respectively (Thermo Fischer scientific ,UK). After that, slides were incubated for 15 min with ultravision hydrogen peroxide block to reduce nonspecific back ground staining [25]. Then apply antibody enhancer and incubate for 10 minutes then apply HRP polymer, incubate 15 minutes then wash and incubate with peroxidase compatible chromagen sensitive. Then counter stain and cover slip using an aqueous mounting medium [26]. To evaluate the expression of Bax and Bcl-2 in cells, ten non-overlapping fields were analyzed for each animal at a magnification of 400×. Measuring of pixel density of Bax and Bcl-2 immunoreactivity was determined, to determine Bax and Bcl-2 expressions, and the mean intensity levels were measured using "Fiji" version of Image J. ANOVA with Tukey's post–hoc test was carried out to compare the difference between groups.

2.10. Scanning electron microscopic examination of the stomach tissues:

Specimens from the stomach were excised and then cut open along its longitudinal axis were fixed in 3% glutaraldehyde / 0.1 M PBS for 1 h at room temperature. Then specimens were rinsed in 0.1 M phosphate buffered saline (PBS), post-fixed in 1% OsO4 for 30 min and dehydrated through a graded series of ethanol (70^100%) for 20 min. Then stomach were critical-point dried, mounted on brass studs using double adhesive tape and sputter coated with 20 nm layer of gold in a JFC-ion sputter. Examination of the specimens was done by JEOL JSM-IT 200 scanning electron microscope, in Electron Microscope Unit of Faculty of Science, Alexandria University- Egypt.

2.11. Statistical analysis

Data were represented as means ± standard deviation (SD). Statistical analysis was performed using SPSS software (Version 23.0, IMB, NY). The differences between the studied groups were done by one-way ANOVA followed by Tukey's post hoc test. Statistically significant differences were considered at $P < 0.05$.

3. Results:

3.1. Effects of BA &/or EMP on gastric mucosal lesions:

Compared to the control group, the treatment of rats with IND + prednisolone produced a significant increase in UI, while treatment with BA & EMP (either alone or in combination) revealed

significant reduction in UI. Moreover, combined treatment with BA & EMP showed higher CI % than the BA treated group and the EMP treated group, Table 2.

Table (2): Effect of BA &/or EMP on gastric mucosal injury.

Parameters	Control Group	Ulcer Group	BA Group	EMP Group	BA + EMP Group
Ulcer Index	0.32 ± 0.18	11.24 ± 2.47*	7.06 ± 0.84 *#	6.58 ± 0.49*#	3.81 ± 1.28 *#$&
Curative Index (%)	___	___	82.7 %	81.3 %	85.6 %

Data are expressed as Mean ± SD.*$p < 0.05$ Compared to control Group, # $p < 0.05$ Compared to Ulcer Group, $ $p < 0.05$ Compared to BA Group, & $p < 0.05$ Compared to EMP Group.

3.2. Effects of BA &/or EMP on gastric juice parameters:

As shown in Figure 1, a significant increase in gastric juice volume, total and free acidity with concomitant significant decreases in gastric pH were observed in the ulcer-induced group receiving combined IND + prednisolone as compared to the control group. However, the three treated groups by BA & EMP (either alone or in combination) revealed significant reduction of gastric juice volume, total and free acidity with significant increase in gastric pH when compared to the ulcer group.

3.3. Effects of BA &/or EMP on HO-1 and SIRT1 relative genes expression:

In the ulcer-induced group, IND + prednisolone administration significantly decreased the expression of HO-1 and SIRT1 compared to the control group (figure 2). On the other hand, in contrast to the ulcer group, treatment with BA & EMP (either alone or in combination) was found to significantly upregulate the expression of both genes. It was noted that, when compared to the BA treated group and the EMP treated group, the BA+EMP group showed significant differences in these parameters.

3.4. Effects of BA &/or EMP on gastric HMGB-1 levels and inflammatory markers (TNF-α and MPO):

As compared to the control group, IND + prednisolone administration in the ulcer induced group revealed significant increase in HMGB-1, TNF-α and MPO levels in gastric tissues. However, in the other treated groups, treatment with BA & EMP (either alone or in combination) significantly attenuated this reduction as opposed to the ulcer group. No significant differences were found between the BA + EMP group with regard to the TNF- α level compared to the BA treated group and the EMP treated group, Figure 3.

3.5. Effects of BA &/or EMP on oxidative stress biomarkers (gastric MDA & GSH):

As shown in Table 3, in the ulcer-induced group, IND + prednisolone administration showed a significant elevation in the level of gastric MDA with concomitant significant decreases in the level of GSH compared to the control group. Compared to the ulcer group, treatment with either BA or EMP (alone or in combination) significantly prohibited the elevation of MDA and significantly increased the level of GSH. It was noted that, compared with the BA treated group and the EMP treated group, the BA+EMP group showed significant differences in these parameters.

3.6. Effects of BA &/or EMP on nitrites and PGE2 levels:

Compared to the control group, the treatment of rats with IND + prednisolone produced a

significant reduction in gastric nitrites & PGE2 levels. BA & EMP (either alone or in combination) treatment significantly increased levels of gastric nitrites compared to the ulcer group. With regard to the levels of PGE2, while treatment with EMP alone did not improve the levels of PGE2 with no significant difference compared to the ulcer group, it was observed that the BA and BA + EMP treated groups had significantly improved the levels of PGE2 compared to the ulcer group. In addition, there was no significant difference in the BA + EMP group compared to the BA treated group, Table 3.

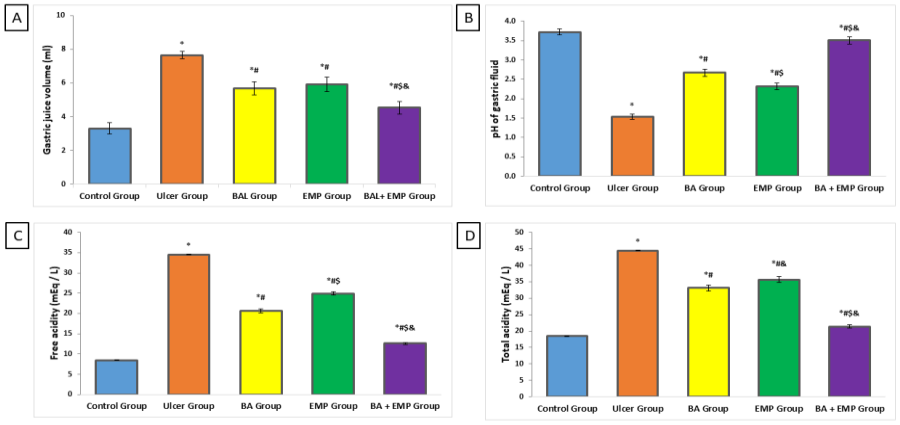

Figure 1: Effect of BA &/or EMP on (A) gastric juice volume, (B) PH, (C) free acidity and (D) total acidity.
Data are expressed as Mean ± SD.*p < 0.05 Compared to control Group, $^{\#}$ p < 0.05 Compared to Ulcer Group, $^{\$}$ p < 0.05 Compared to BA Group, $^{\&}$ p < 0.05 Compared to EMP Group.

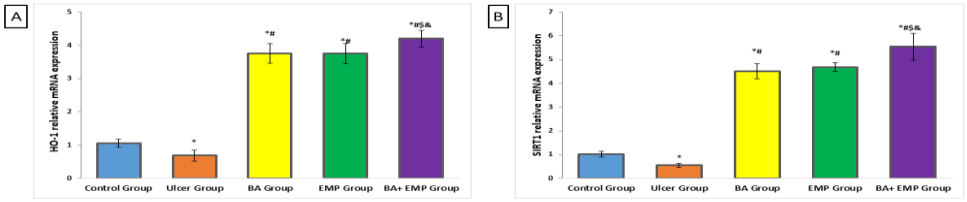

Figure 2: Graphical presentation of the effect of BA &/or EMP on relative expression of (A) HO-1 and (B) SIRT1 in different groups.
Data are expressed as Mean ± SD.*p < 0.05 Compared to control Group, $^{\#}$ p < 0.05 Compared to Ulcer Group, $^{\$}$ p < 0.05 Compared to BA Group, $^{\&}$ p < 0.05 Compared to EMP Group.

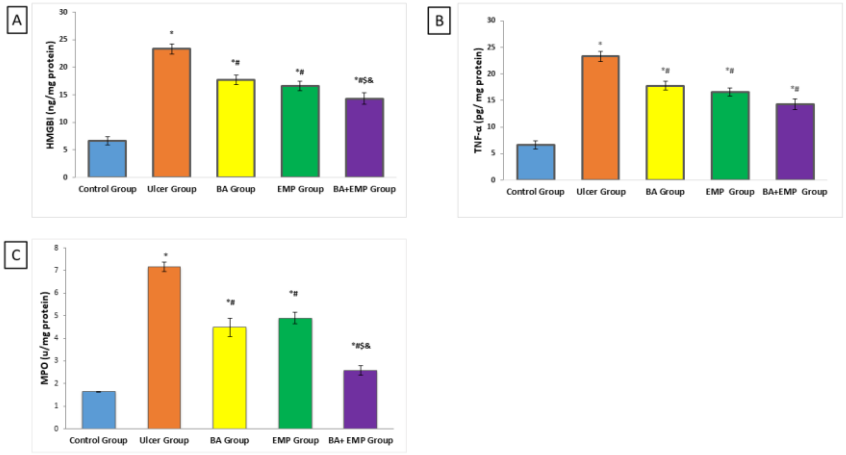

Figure 3: Effect of BA &/or EMP on (A) HMGBI, (B) TNF-α, and (C) MPO levels.
Data are expressed as Mean ± SD.*p < 0.05 Compared to control Group, $^{\#}$ p < 0.05 Compared to Ulcer Group, $^{\$}$ p < 0.05 Compared to BA Group, $^{\&}$ p < 0.05 Compared to EMP Group.

Table (3): Effect of BA &/or EMP on MDA, GSH, Nitrite and PGE2 levels.

Parameters	Control Group	Ulcer Group	BA Group	EMP Group	BA + EMP Group
MDA (nmol/mg protein)	0.92 ± 0.13	4.18 ± 0.23*	2.77 ± 0.12 *#	2.44 ± 0.10*#$	1.26 ± 0.10 *#$&
GSH (mg/g protein)	7.91 ± 0.17	1.65 ± 0.18 *	3.43 ± 0.43 *#	3.63 ± 0.36 *#	5.15 ± 0.24 *#$&
Nitrite (µmol/g wet tissue)	3.42 ±0.04	1.65 ± 0.13*	2.95 ± 0.03*#	2.80 ± 0.13*#$	3.13 ± 0.06*#$&
PGE2 (pg / mg protein)	43.31 ± 1.95	20.98 ± 1.43*	37.68 ± 2.07 *#	20.52 ± 0.9*$	36.31 ± 1.58*#&

Data are expressed as Mean ± SD.*$p < 0.05$ Compared to control Group, # $p < 0.05$ Compared to Ulcer Group, $ $p < 0.05$ Compared to BA Group, & $p < 0.05$ Compared to EMP Group.
Abbreviation: BA: Baicalein; EMP: Empagliflozin; MDA: Malondialdhyde; GSH: Reduced glutathione; PGE2: Prostaglandin E2.

3.7. Histological light microscopic results:

Sections of rat stomach of control group showed the regular arrangement of the wall of the stomach which was formed of four layers, were from inner to outer, mucosa containing gastric glands, submucosa (Figure 4A). The gastric mucosa showed surface mucous cells having basal oval nuclei extending down into gastric pits. Mucous neck cells having open-face nuclei and parietal cells having round central nuclei are also found (Figure 5A).

Administration of a combination of IND + prednisolone induced shedding of superficial epithelial lining with marked distortion of mucosal lining gastric glands associated with distorted normal architecture and dilated gastric glands, intact muscularis mucosa and apparent increased thickness of submucosa under the mucosal lesions (Figure 4B). The presence of intra-luminal cells in gastric pits and the apical part of gastric glands, some parietal cells showed vacuolation of their cytoplasm with pyknotic nuclei, others appear karyolitic (Figure 5B).

Administration of BA alone resulted in preserved normal structure of the mucosa, gastric glands and muscularis mucosa. Submucosa show apparent normal thickness (Figure 4C). preserved structure of most of surface epithelial lining, some gastric pits contain intra-luminal cell (Figure 5C), mucous cells having basal oval nuclei, mucous neck cells having open-face nuclei and parietal cells having round central nuclei (Figure 5C). Section of rat stomach in EMP treated group showed preserved normal structure of most of gastric glands except for some areas show cystic dilatations, apparent normal thickness of submucosa (Figure 4D), with preserved normal structure of mucous cells, mucous neck cells and some parietal cells show vacuolation of their cytoplasm (Figure 5 D). Section of rat stomach of BA + EMP treated group showed restoration of normal structure of mucosa, with apparent normal thickness of submucosa (Figure 4E), with preserved normal structure of mucous cells, mucous neck cells and parietal cells (Figure 5E).

3.8. Immunohistochemical expression of apoptotic marker (Bax) & antiapoptotic marker (Bcl-2):

Immunoreactivity of Bax (Figure 6) revealed that (IND + prednisolone) induced ulcer group displayed a highly significant expression of Bax compared to control group. By comparison,

treatment with BA &/or EMP revealed a highly significant modulation of Bax expression compared to ulcer group. On the other hand, immunoreactivity of Bcl-2 (Figure 7) revealed that (IND + prednisolone) induced ulcer group displayed a highly significant reduction of expression of Bcl-2 compared to control group. By comparison, treatment with BA or EMP revealed a significant higher expression of Bcl-2 when compared to ulcer group and the combination of BA + EMP displayed a highly significant increase in expression of Bcl-2 when compared to ulcer group.

3.9. Effect of BA &/or EMP on the scanning electron microscopic picture of rat stomach:

Scanning electron micrograph of rat stomach of control group revealed normal surface gastric epithelium lining, gastric pits together with clear lumen of gastric glands (Figure 8A). Scanning electron micrographs of rat stomach of ulcer group showed wide area of gastric ulceration associated with erosions of interior of gastric glands, abnormal surface epithelium surrounding the gastric ulcer area, together with severe narrowing of gastric pits in some regions (Figure 8 B&C). Scanning electron micrographs of rat stomach of BA treated group showed preserved normal structure of most of gastric pits and gastric glands, gastric epithelial surface show thickening in some regions (Figure 8D). Scanning electron micrographs of rat stomach of EMP treated group showed preserved normal structure of most of gastric pits and gastric glands, most of surface epithelium show normal structure, some regions show damaged gastric epithelium (Figure 8E). Scanning electron micrographs of rat stomach of BA + EMP treated group revealed restoration of normal structure of most of gastric pits and gastric glands with normal surrounding epithelial lining (Figure 8F).

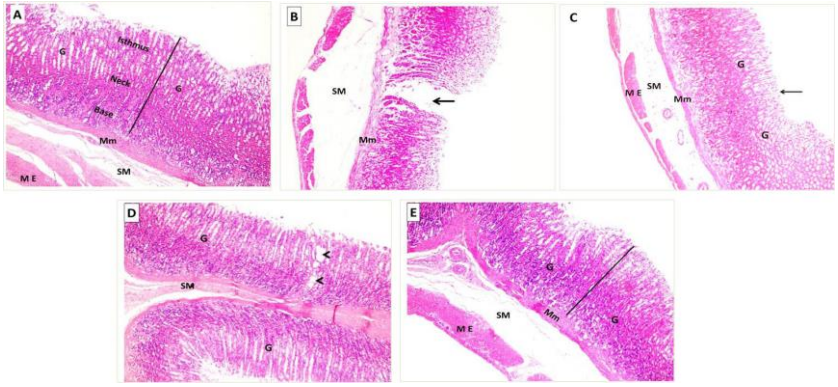

Figure 4: Histopathological findings (H&E X 100):

A) Photomicrographs of section of rat stomach of the control group showing the mucosa (black line) containing gastric glands (G). Each gland has luminal pits (arrow), an isthmus (Isthmus), a neck region (Neck), and a deep basal part (Base). Muscularis mucosa (Mm), submucosa (SM) and muscularis externa (ME) can be seen; **B)** section of rat stomach of ulcer group showing shedding of superficial epithelial lining with marked distortion of mucosal gastric glands (arrow), associated with distorted normal architecture with intact muscularis mucosa (Mm) and apparent increased thickness of submucosa (SM) under the mucosal lesions; **C)** section of rat stomach of BA treated group showing preserved normal structure of most of the mucosal surface epithelium (arrow) gastric glands (G), muscularis mucosa (Mm). Submucosa (SM) shows apparent normal thickness; **D)** section of rat stomach of EMP treated group showing preserved normal structure of most of gastric glands except for some areas show cystic dilatations (arrow heads), apparent normal thickness of submucosa; **E)** section of rat stomach of BA + EMP treated group showing preserved normal structure of mucosa (Black line) and its glands (G), with apparent normal thickness of the underlying submucosa (SM).

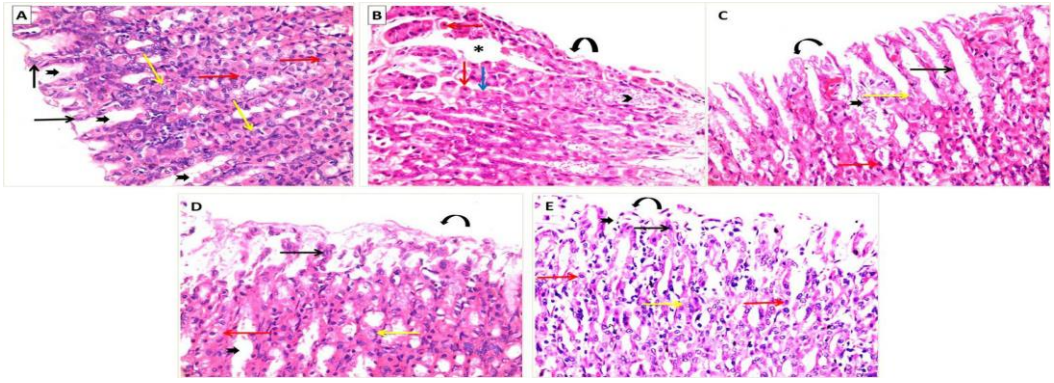

Figure 5: Histopathological findings (H&E X 400):

A) photomicrographs of section of apical parts of rat gastric mucosa of the control group showing surface mucous cells with basal oval nuclei (black arrows) extending down into gastric pits (bifid arrows). Mucous neck cells with open-face nuclei (yellow arrows). Also the parietal cells with rounded vesicular central nuclei (red arrows) can be noticed in apical parts; **B)** rat gastric mucosa of ulcer group showing shedding of superficial epithelial lining (curved arrow) with distortion of gastric glands (*), gastric pits show abundant intra-luminal cell (arrow head), some parietal cells show vacuolation of their cytoplasm with pyknotic nuclei (red arrows) others appear karyolitic (blue arrow); **C& D)** rat gastric mucosa of BA treated and EMP treated groups respectively showing preserved most of the superficial epithelial lining (curved arrows), preserved normal structure of mucous cells (black arrows), mucous neck cells (yellow arrows) and parietal cells (red arrows) , BA group some gastric pits show intra-luminal cell (bifid arrow) , EMP group some parietal cells show vacuolation of their cytoplasm (red arrow) with clear gastric pits (bifid arrow); **E)** rat gastric mucosa of BA + EMP treated group showing restoration of normal structure of gastric pits (bifid arrow) with preserved superficial epithelial lining (curved arrow) , mucous cells (black arrow), mucous neck cells (yellow arrow) and parietal cells (red arrows).

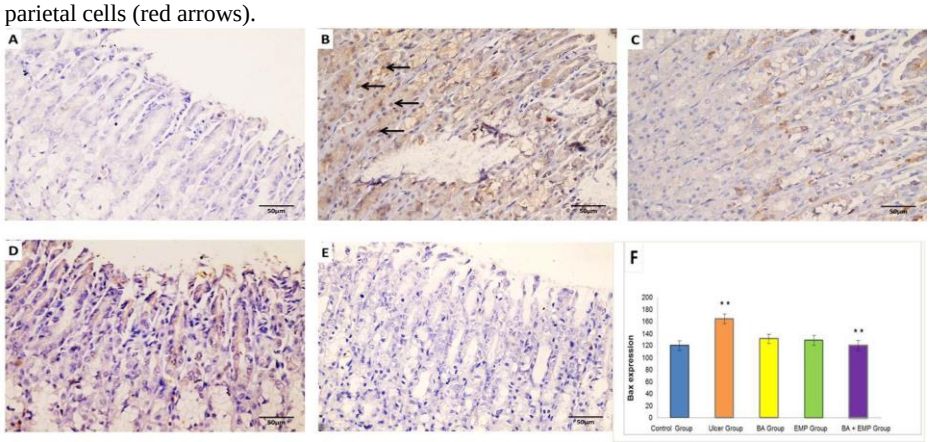

Figure 6: Immunohistochemical analysis of Bax in the gastric mucosa:

Immunoreactivity of Bax photomicrographs **(A, B, C, D, E)** showing the expression of Bax (arrows) in control, ulcer, BA, Emp, and BA + Emp groups respectively. Bar graph **(F)** showing the levels of Bax expression in the gastric mucosa, comparing the mean optical density. ANOVA with Tukey's post–hoc test was carried out to compare the difference between groups. Data are presented as mean ± SD. *P<0.05 is considered significant, **P<0.001 is highly significant. Magnifications; (**A, B, C, D, E** x400, scale bar = 50µm).

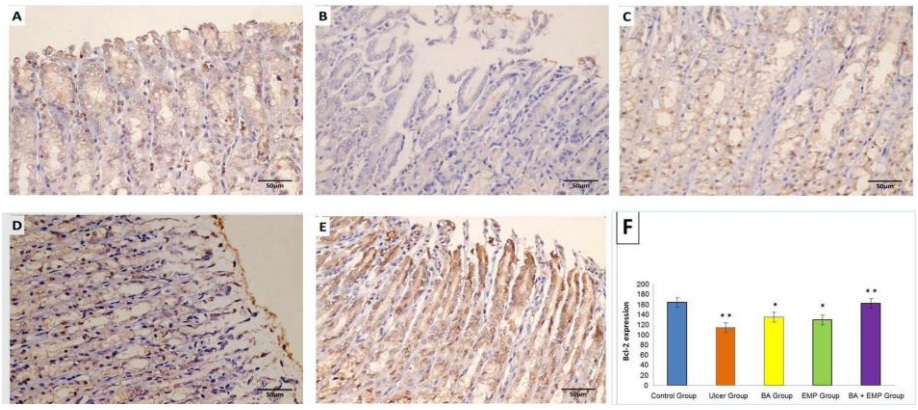

Figure 7: Immunohistochemical analysis of Bcl-2 in the gastric mucosa:

Immunoreactivity of Bcl-2 in the gastric mucosa photomicrographs **(A, B, C, D, E)** showing the expression of Bcl-2 in control, ulcer, BA, Emp, and BA + Emp groups respectively. Bar graph **(F)** showing the levels of Bcl-2 expression in the gastric mucosa. ANOVA with Tukey's post–hoc test was carried out to compare the difference between groups. Data are presented as mean ± SD. *P<0.05 is considered significant, **P<0.001 is highly significant. Magnifications; (**A, B, C, D, E** x400, scale bar = 50µm).

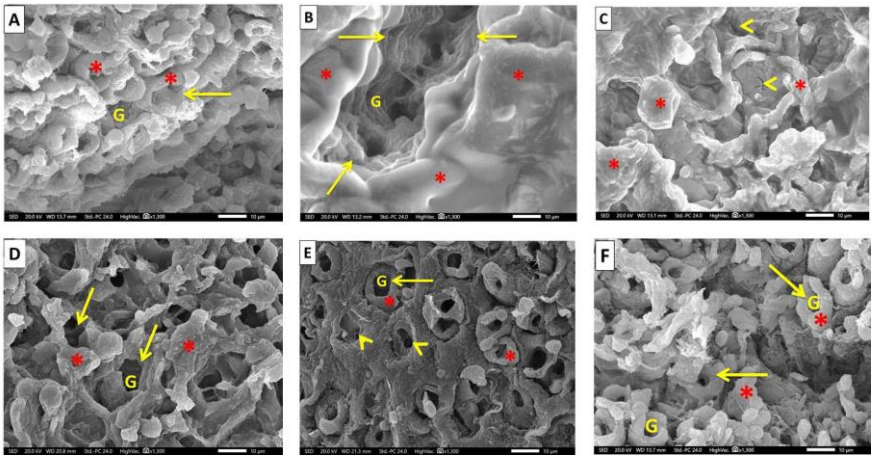

Figure 8: Scanning electron micrograph of gastric mucosa (SEM × 1300):
A) Control group showing normal gastric epithelium (*),gastric glands (G) and gastric pits (arrow)(SEM × 1300); **B &C)** Ulcer group showing wide area of gastric ulceration (arrows) associated with erosions of interior of gastric glands (G), abnormal surface epithelial cells (*) surrounding the gastric ulcer area, together with severe narrowing of gastric pits in some regions (arrow heads); **D)** BA treated group showing, preserved normal structure of some gastric pits (arrows) and gastric glands (G), gastric epithelial surface (*) show thickening in most regions; **E)** EMP treated group showing, preserved normal structure of some gastric pits (arrow) and gastric glands (G) , most of surface epithelium (*) show normal structure ,other regions show damaged gastric epithelium (arrow heads); **F)** BA + EMP treated group showing restoration of normal structure of most of gastric pits (arrows) and gastric glands (G) with normal surrounding epithelial lining (*).

4. Discussion:

The gastroprotective impacts of BA and EMP in IND and prednisolone induced gastric ulcer model in rats, as well as their putative underlying mechanisms, were investigated in this study through the assessment of various biochemical, histological, and immunohistochemical findings in rats stomach tissue. In combination with prednisolone, the choice of indomethacin-induced gastric ulcer model is to imitate the pharmacological treatment most commonly used in most patients, such as those with rheumatoid arthritis, who are more prone to experience gastrointestinal erosions and ulceration [27].

Our data showed, in line with previous studies, that the ulcerogenic potential of combined IND & prednisolone is well established and revealed by the increased gastric content volume and acidity as well as the decreased gastric juice pH [5]. On the other hand, our findings indicated that BA (as an individual compound) has gastroprotective properties shown by significant reduction in the gastric juice volume, free and total acidity and significant increase in the pH of gastric juice. Similarly, it has been documented that anti-secretory effects shown by BA through blocking histaminergic pathways. In addition, BA partially inhibited the activity of in vitro H^+, K^+ -ATPase [17]. Although the effects of EMP on gastric acidity has not been recorded before, a super-additive effect in gastroprotection was created by the combination of BA and EMP, reflecting EMP's potential role in modulating gastric acidity.

HO-1 is one of the most prominently identified gastrointestinal cytoprotective enzymes, which regulates oxidative stress and inflammation through production of biliverdin and bilirubin, as well as the release of CO [28]. Moreover, SIRT1, as downstream effector of HO-1, is known to suppress the inflammatory response via hindering HMGB1 transcription and extracellular secretion by sustaining HMGB1 in a deacetylated state [29]. Here in, we found that regulation of HO-1

/SIRT1/HMGB1 pathway can be considered as an effective approach for the treatment of gastric ulcer as well as other inflammatory diseases.

In this study, we noticed marked decrease in HO-1& SIRT1 gene expression levels with concomitant increase of HMGB1 level in induced gastric ulcer group. Other studies that stated that IND failed to control HO-1 expression but caused gastric mucosal damage via necrosis and apoptosis have confirmed our findings [28, 30]. On the other hand, treated groups with BA and EMP (either as individual drug or combined) reversed these findings and were able to upregulate HO-1 & SIRT-1 expression and decrease HMGB1 levels. However, the effect of combined BA and EMP was more remarkable than that of each drug alone. This comes in agreement with previous studies [31-32]. Furthermore, Jigheh et al., [33] reported that EMP improved both renal oxidative stress and inflammation in diabetic rats through increasing renal HO-1 level, a distinguished anti-oxidant enzyme downstream to the Nrf2 signaling pathway and attenuating the expression of HMGB1.These findings indicated the crucial role of HO-1/SIRT1/HMGB1 signaling in protection against gastric mucosal injury.

It is widely known that inflammation plays a crucial role in the development and etiology of gastric ulcer damage, which comes in agreement with previous report [34]. The current data showed that both gastric TNF-α and MPO levels in the induced gastric ulcer group were significantly increased. The IND-related inhibition of PGE2, an effective suppressor of TNF-α production by inflammatory cells, could explain the elevated TNF-α level. Additionally, IND activates NF-κB with subsequent up regulation of pro-inflammatory cytokines gene expression such as TNF-α. Furthermore, MPO is the principal marker of neutrophil infiltration into gastric tissue that induces inflammation and apoptosis induction, and is considered a preliminary event in the pathogenesis of IND-induced gastric mucosal injury. In contrast, the groups treated with BA &/or EMP revealed significant decrease in TNF-α and MPO levels denoting their anti-inflammatory role in gastroprotection. This may be explained by overexpression of HO-1 and endogenously generated CO which play a key role in inhibiting leukocyte adhesion and proinflammatory cytokines production such as TNF-α & IL-6 [35]. In accordance to earlier studies [33,36], our data revealed that improved redox status along with reduced HMGB1 level may contribute to the reduced inflammatory mediators and increased the resistance to gastric injury by BA &/or EMP treatment.

In addition, current findings have shown a redox status imbalance demonstrated by elevated gastric MDA levels with decreased GSH antioxidant levels in induced gastric ulcer group, which is in agreement with previous studies [37, 38]. This could be attributed to an increase in reactive oxygen species (ROS) caused by inhibited mitochondrial oxidative phosphorylation, which contributes to IND cytochrome c release, as well as prednisolone-induced mitochondrial dysfunction and cytochrome P450 isoforms. In addition, ROS suppresses antioxidant enzyme activity, which results in imbalance between oxidants / antioxidants. Meanwhile, in this research, treatment with BA &/or EMP significantly improved redox status and showed protection against antioxidant defense depletion,

referring to their antioxidant and radical scavenging properties that agree with previous studies. In human pancreatic ductal adenocarcinoma cell lines, BA has been demonstrated to partially suppress lipid peroxidation by preventing GSH depletion [39]. Furthermore, taking into account the close association between HO-1, HMGB1 and oxidative stress levels as established in earlier research [33], our results suggested that by targeting the HO-1 / HMGB1 signaling pathway, EMP could be an auspicious agent mitigating oxidative stress in gastric ulcers.

It is well evident that eicosanoids such as PGE2 and NO are vital gastrointestinal mucosal defense mediators. PGE2 is an important cytoprotective endothelial vasodilator and NO that together enhance the healing of ulcer by promoting the release of mucus and bicarbonate and impeding the secretion of gastric acid [40, 41]. Our findings showed that the induced gastric ulcer group showed decreased levels of both gastric PGE2 and NO, leading, as verified by histopathological analysis, to a decrease in mucosal synthesis and mucosal barrier content. Although the current study revealed that EMP as an individual compound failed to attenuate PGE2 level, the combination of BA & EMP revealed significant increase in its level, indicating that PGE2 synthesis could not be attributed to modulating gastropathy through EMP. Furthermore, the present study revealed that both BA & EMP treated group exhibited increase in NO level relative to each drug as individual. Lopez et al., [42] reported that EMP improved myocardial remodeling after myocardial infarction through activation of both endothelial nitric oxide synthase (eNOS) as well as neuronal nitric oxide synthase (nNOS) enzyme and ameliorating inducible nitric oxide synthase (iNOS) activity, leading to an increase of available cardiac NO levels. Furthermore, Ribeiro et al., [17] demonstrated that the gastroprotective activity of BA may be due to the stimulation of NO and PG synthesis.

The present work found significant histological alterations in rat gastric mucosa of the gastric ulcer group, which induced shedding of superficial epithelial lining with marked distortion of mucosal gastric glands associated with distorted normal architecture, apparent increased thickness of submucosa under the mucosal lesions. These results were in accordance to previous studies [43]. However, these changes were significantly reduced by BA &/or EMP administration, suggesting their protective effects. These findings were documented by Ribeiro et al. [17] who explained the ability of BA to maintain a balance between aggressive factors and the natural cytoprotective mechanism of gastric mucosa, by forming natural substances important in cytoprotection. Additionally, the current study confirmed these findings through examining gastric tissue of studied groups by scanning electron microscope, which revealed gastric ulceration and abnormal surface epithelium surrounding the gastric ulcer region, together with erosions of gastric glands in ulcer group. These changes were partially resolved in BA and EMP treated groups with residual damage of some areas and thickening of surface epithelium in other regions. Zaki and Mohamed [44] explained these findings that the epithelial cells which surround the gastric ulcer are the body reaction to repair the damage by covering the ulcer. These changes were

significantly ameliorated by administration of BA in combination with EMP.

Furthermore, frequent independent studies have indicated that apoptosis is another key player in IND-induced gastric ulceration pathogenesis. In the current research, oxidative stress and inflammation that are significantly elevated are involved in activating apoptosis by inducing both extrinsic and mitochondrial apoptotic pathways [45]. This was substantiated by immunohistochemical research, which demonstrated a considerable decrease in the expression of the anti-apoptotic factor (Bcl-2), whereas the expression of the pro-apoptotic factor (Bax) was dramatically enhanced in the induced gastric ulcer group. Treatment with BA &/or EMP, however, showed the opposite effect, thus signifying their anti-apoptotic potential to protect against injury to the gastric mucosa. These findings are endorsed by previous studies that have verified the anti-apoptotic effect of both medicinal products in different diseases [46, 47]. In addition, apoptosis suppression can be explained by overexpression of HO-1/SIRT1 accompanied by decreased levels of HMGB1, thus attenuating inflammation and oxidative stress, as shown in our findings.

Noteworthy, this is the first research to elucidate the protective role of EMP in the gastric ulcer induced model, as well as its interaction with BA. Further research is needed to better characterize the other protective effects of BA and EMP on gastric ulcers, as well as to assess the potential harmful effects of their long-term therapeutic use.

In conclusion, taking into consideration the findings obtained and the related data available in the literature, this study shows that the potent gastroprotective effect of the mutual use of BA and EMP against indomethacin & prednisolone-induced gastric ulcers is greater than that of either of them alone. Both drugs exhibited several protective mechanisms through increased PGE2 content, acid inhibition, anti-oxidant, anti-inflammatory, and anti-apoptotic activities. These effects could be mediated possibly by activation of HO-1/SIRT1 pathway with subsequent decrement of HMGB-1 levels in gastric tissue. It is to be noted that, this combination could launch promising avenues and alternative concept for the treatment of gastric ulcers. The prospective usage of this combination in other gastrointestinal disorders remains a field of potential research to elucidate their novel ameliorative mechanisms.

Abbreviations:

Abbreviations: ***BA:*** Baicalein; ***EMP:*** Empagliflozin; ***NSAIDs:*** Non-steroidal anti-inflammatory drugs; ***IND:*** Indomethacin; ***ROS:*** Reactive oxygen species; ***PGs***: Prostaglandins; ***NO:*** Nitric oxide; ***HO-1:*** Heme oxygenase-1; ***SIRT1:*** Silent information regulator factor 2-related enzyme 1; ***HMGB1:*** High Mobility Group Box 1; ***SGLT2:*** Selective sodium glucose co-transporter 2; ***TNF-α:*** Tumor Necrosis Factor α; ***MPO:*** Myeloperoxidase; ***MDA:*** Malondialdehyde; ***GSH:*** Reduced glutathione.

5. Conflicts of interest:

The authors declare no potential conflict of interest concerning this work.

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