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Research article

# The potential beneficial effects of sildenafil and diosmin in experimentally-induced gastric ulcer in rats

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ABSTRACT

*Objectives:* research in the treatment of gastric ulcer has involved the investigation of protective drugs. These drugs may be used as adjacent therapy with the traditional pharmacologic treatment of peptic ulcer. The present study is designed to investigate the gastro protective effects of diosmin (DIO), sildenafil (SILD) and their combinations with ranitidine (RANT) against indomethacin (INDO)-induced gastric ulcer in rats. Additionally, the potential mechanisms of their effect are addressed.

*Methods:* DIO (100 mg/kg) and SILD (10 mg/kg) were administered by oral route for seven days prior to ulcer induction. Moreover, other rats were treated with RANT (50 mg/kg) not only to compare efficiency of the medications but also, to help clarify potential mechanisms of their effect. Following, after 24 h of fasting, INDO (100 mg/kg) was administered for induction of gastric ulcer. Furthermore, rats in each group were sacrificed 4 h later. Biochemical analysis of DIO, SILD, RANT and their combinations pre-treated host tissues demonstrated reduction in tumor necrosis factor (TNF)-α and malondialdehyde (MDA) contents and concomitant increase in gastric pH, nitric oxide (NO) and reduced glutathione (GSH) contents.

*Result:* It is observed, that SILD and DIO pre-treatment showed non-significant effect on gastric juice PH. However, their combinations with RANT is superior to using RANT alone. In addition, the results revealed, that combinations of (RANT and SILD) and (RANT and DIO) showed the highest increase in gastric tissue NO levels. But, these two combinations achieved the lowest MDA levels relative to the control (INDO) group. Despite, all groups displayed non-significant effect on reduced GSH content, (RANT and SILD) group increased GSH concentration by 39.75% relative to INDO group. In addition, DIO, RANT and (RANT and DIO) pre-treatment have anti-apoptotic activity on gastric mucosa. On the other hand, SILD did not affect caspase-3 immunostaining. These results are confirmed by histopathological findings.

*Conclusion:* The work outcomes provide a new gastro protective agents in clinical gastropathy. So, this study not only provides an efficient way for peptic ulcer protection, but also it may be considered for future studies in ulcer healing and gastric cancer.

## 1. Introduction

Worldwide, peptic ulcer disease and its complications remain the cause of much suffering and significant mortality. A peptic ulcer is a sore on the lining of stomach which named (gastric ulcer) or on the lining of duodenum (duodenal ulcers). It is believed, that peptic ulcers develop due to imbalance between aggressive factors (reactive oxygen species, stress, helicobacter pylori, NSAIDs, gastric acid) and protective factors (mucus-bicarbonate barrier, mucosal blood flow, prostaglandins) leading to interruption in mucosal integrity [1]. Indomethacin, belongs to non-steroidal anti-inflammatory drugs (NSAIDs). Also, it is considered as one of the most commonly prescribed drugs in the world to treat pain and inflammation. Unfortunately, it is observed, that long term use of indomethacin is associated with severe gastropathy by different mechanisms independent on gastric PH [2]. Moreover, these mechanisms include the topical irritant effect on the epithelium, impairment of the barrier properties of the mucosa, suppression of gastric prostaglandin synthesis, reduction of gastric mucosal blood flow and interference with the repair of superficial injury [3, 4]. In addition, other mechanisms include, reduction of bicarbonate and mucus secretion [5], inhibition of both COX isoforms [6], induction of gastric mucosal apoptosis and necrosis [3, 7], enhance leukocyte adherence to the vascular endothelium and

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microcirculatory disturbances [8]. On the other hand, sildenafil (SILD) is a selective and potent inhibitor of cGMP specific phosphodiesterase (PDE5), which catalyzes hydrolysis of cGMP leading to increase Nitric oxide (NO) level [9]. Thus, it can prevent indomethacin (INDO)-induced gastric ulceration, through a reduction of leukocyte-endothelium adhesion, modulation of epithelial barrier function. Further mechanisms include, increase in mucus and bicarbonate secretion, reduction of gastric acid secretion. Also, SILD exerts gastroprotective effect by inhibiting HCl producing cells, inhibition of apoptosis and maintenance of gastric mucosal blood flow [10].

Diosmin (DIO) is a natural citrus flavone with remarkable antioxidant, anti-inflammatory and anti-apoptotic features [11]. These features are responsible for its protection against cardiac, hepatic and renal injuries [12]. However, these papers have not considered the peptic ulcer protection through incorporating the effect of DIO and SILD with ranitidine (RANT). Accordingly, RANT an H2-receptor antagonist which used in gastric acid disorder treatments was incorporated for comparative purposes. Furthermore, RANT was employed not only to compare efficacy of the medications but also, to assist clarify potential mechanisms of their effect.

The aim of this paper is to investigate gastroprotective effect of DIO, SILD and their combinations with RANT against INDO-induced ulcers specifically. Accordingly, seven different groups were resulted. Moreover, the underlying mechanisms of different medications and their combinations were considered to compare different groups. These mechanisms represent gastric PH, MDA, reduced glutathione concentration, TNF-α, NO and caspase-3. The results of these comparisons contribute to provide a new gastro protective agents in clinical gastropathy. So, this study not only provides an efficient way for peptic ulcer protection, but also it may be considered for future studies in ulcer healing and gastric cancer.

## 2. Materials and methods

### 2.1. Materials

Ranitidine hydrochloride (RANT) (Ranitidine; 25 mg/ml) was purchased from MUP Egypt company, (Cairo, Egypt) and indomethacin (INDO) from Cairo Pharmaceutical Ind., (Cairo, Egypt). Furthermore, SILD was used as tablet 20 mg (Respatio) from PHARMA RIGHT company. Additionally, DIO was used as tablet 500 mg (Diovin) from Amriya Pharm. Ind. company. All chemicals were of high analytical grade. Furthermore, all solutions were freshly prepared.

### 2.2. Experimental animals

Adult male *Wistar albino* rats (180–200 g, 8-weeks of age) were obtained from the animal house at the College of Veterinary Medicine, Tanta University (Kafr Elsheik, Egypt). Moreover, all rats were housed in wire mesh cages with a 12-h light:dark cycle at constant temperature (25 $\pm$ 2 °C) and provided ad libitum access to standard rodent chow (El-Nasr, Abuzabal, Egypt). Additionally, all rats were acclimatized in Tanta university pharmacological laboratory for 1 week prior to use in the study which took 7 days. After this period, rats were weighed and randomly allocated into seven experimental groups (n = 8 per group).

Throughout this experimental study, rats in Group I were designated naïve control group; Group II denoted selected INDO-induced ulcer group INDO (100 mg/kg); Group III represented DIO (100 mg/kg) [12] and INDO group; Group IV signified SILD (10 mg/kg) [13] and INDO group; Group V are RANT (50 mg/kg PO) [14] and INDO group; Group VI indicated RANT (50 mg/kg) and DIO (100 mg/kg) and INDO group; Group VII symbolized RANT (50 mg/kg) and SILD (10 mg/kg) and INDO group. It was observed, that low doses (ex: 25–30 mg) of INDO had no effect in the pilot study. So, high dose (100 mg) was used. This may be attributed, to that INDO has poor solubility in water and has good colloidal stability in aqueous solution of Carboxymethyl cellulose (CMC). During the fasting period, rats were allowed to have free access to water only. On the day of ulcer induction, access to water was stopped for 2h before ulcer induction. In addition, all animals were administered treatments orally (P.O.) once a day for 7 days prior to induction of gastric ulcer. Besides, rats in the control and ulcer groups were administered 1 ml CMC solution (0.5%) daily. After 4hr of ulcer induction all rats were euthanized by cervical dislocation. This study is fully consistent with the ARRIVE guidelines. Additionally, it has been carried out in accordance with the National Institutes of Health–Office of Laboratory Animal Welfare policies and laws. The experimental protocol is approved by the local ethical committee of the Faculty of Pharmacy, Tanta University and in accordance with the council for international Organizations of Medical Sciences (CIOMS) guidelines.

#### 2.2.1. Induction of ulceration

All rats were fasted for 24 h before ulcer induction to ensure their stomachs are empty. During the fasting period, rats were allowed to have free access to water only. On the day of ulcer induction, access to water was stopped for 2 h before INDO administration [15]. Rats in Groups II–VII were given 100 mg INDO/kg, P.O. After 4 h, the rats were killed by cervical dislocation. In all cases, the stomach of each rat was excised, opened along the greater curvature and all the gastric juice is collected. Afterwards, the tissue was rinsed with 0.9% saline solution. Then, excised stomach was cut into two halves. One half was immediately fixed in 10% buffered formalin. However, the remainder was cut into pieces. These pieces were weighed and stored at -80 °C for later use in the various assays outlined below.

#### 2.2.2. Gastric pH determination

Gastric pH was determined via the method of Muniappan and Sundararaj [16]. Each obtained stomach was opened along the greater curvature and the gastric content was drained into a centrifuge tube. At that point, it was centrifuged at 1000 rpm for 10 min (4 °C). The clear supernatant was recovered and used for pH measurement using a pH 211 m (Hanna Instruments, Bucharest, Romania).

#### 2.2.3. Determination of nitric oxide (NO) content

Gastric NO levels were determined via the method of Miranda [17]. Samples of isolated tissues (100 mg) were each homogenized in 10 vol. ice-cold saline solution using a PT 3100 Polytron homogenizer (Kinematica instruments, Lucerne, Switzerland). Upon disruption, absolute ethanol was added (2:1 vol. ratio) to precipitate all proteins. After allowing materials to separate over a 15 min period (at 25 °C), the supernatant was recovered. Moreover, 0.5 ml vanadium chloride (8 mg VCl3/ml) was added to 0.5 ml recovered supernatant. At that time, 0.25 ml of 2% sulfanilamide and 0.25 ml of 0.1% N-(1-naphthyl)-ethylene diamine were added. The mixture is vortexed and incubated at 37 °C for 30 min before its absorbance was measured at 540 nm in a Model UV1601PC spectrophotometer (Shimadzu, Kyoto, Japan). Sodium nitrite was dissolved in distilled water and serial dilutions were prepared. Additionally, 0.5 ml of each dilution was used in place of tissue extract and processed as illustrated above. Then, values of NO in each sample were determined by extrapolation from the prepared standard curve.

#### 2.2.4. Determination of MDA

Sample of 100 mg rat stomach tissue was washed with 0.9% sodium chloride and homogenized in10 volumes of ice cold potassium chloride solution (1.15%) using polytron homogenizer (PT3100). In addition, mixture of 0.5 ml homogenate, 3 ml of TCA (0.5%) and 1 ml TBA (0.6%) were heated in boiling water bath 45 min. After cooling, 4 ml n-butanol

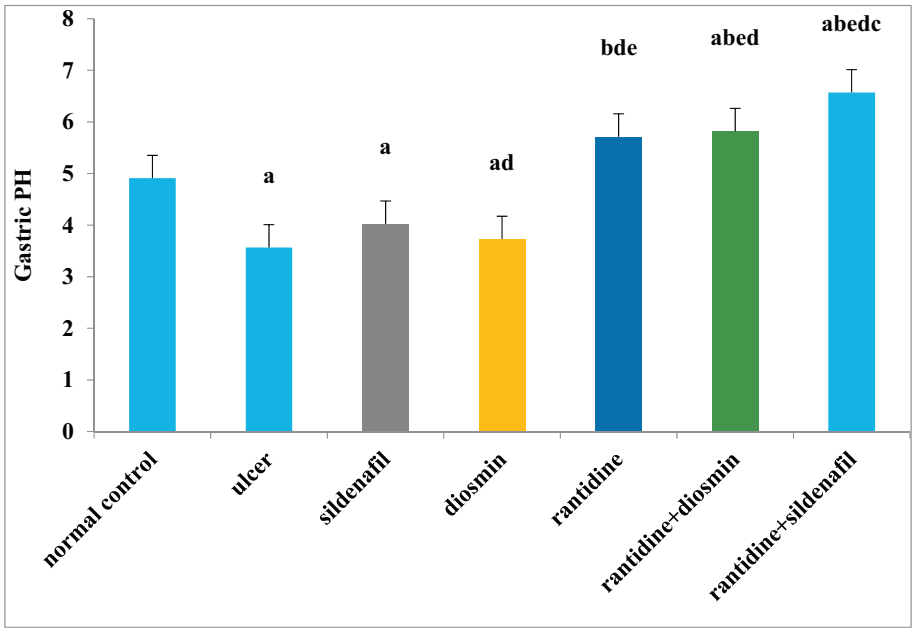


**Figure 1. Effect of different drug pretreatments on gastric pH n = 8 in each group**. a means significant difference as compared to naïve control group at $p < 0.05$. b represents significant difference as compared to ulcer group at $p < 0.05$. c denotes to significant difference as compared to RANT group at $p < 0.05$. d symbolizes significant difference as compared to SILD group at $p < 0.05$. e indicates significant difference as compared to DIO group at $p < 0.05$.

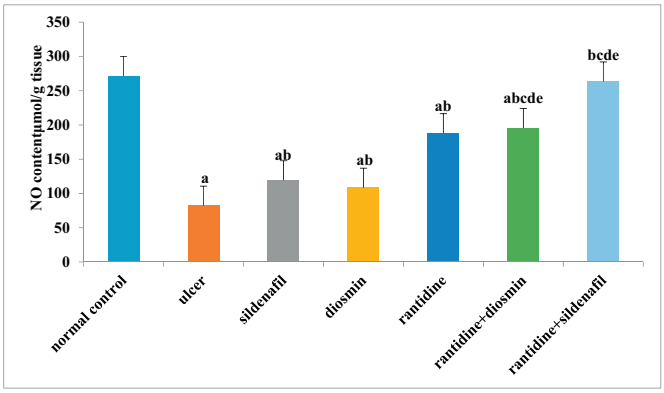


**Figure 2. Effect of different drug pretreatments on gastric nitric oxide (NO) content n = 8 in each group**. a represents significant difference as compared to naïve control group at $p < 0.05$. b means significant difference as compared to ulcer group at $p < 0.05$. c symbolizes to significant difference as compared to RANT group at $p < 0.05$. d indicates significant difference as compared to SILD group at $p < 0.05$. e denotes significant difference as compared to DIO group at $p < 0.05$.

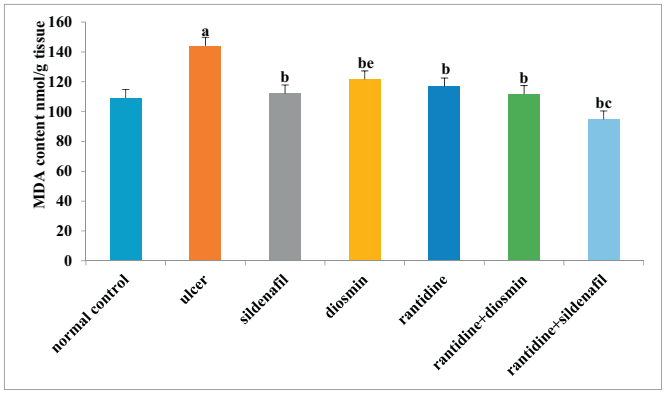


**Figure 3. Effect of different drug pretreatments on gastric MDA content n = 8 in each group**. a symbolizes significant difference as compared to naïve control group at $p < 0.05$. b denotes significant difference as compared to ulcer group at $p < 0.05$. c represents to significant difference as compared to RANT group at $p < 0.05$. d indicates significant difference as compared to SILD group at $p < 0.05$. e means significant difference as compared to DIO group at $p < 0.05$.

was added and mixed vigorously. Next, n-butanol layer was separated and absorbance of pink colored product was measured at 535 nm using double-beam spectrophotometer [18].

### 2.2.5. Determination of reduced glutathione

Reduced glutathione (GSH) in the gastric tissues was determined using Ellman's reagent. In brief, 100 mg gastric tissue was homogenized in 1 ml ice-cold 0.1 M phosphate buffer (pH 7.4). Then, 4% sulfosalicylate solution was added to precipitate proteins. Afterward, materials were centrifuged at 3000 rpm for 10 min at 4 °C. To 0.5 ml of the resultant supernatant, 4.5 ml bis-(3-carboxy-4-nitrophenyl) disulfide (DTNB) reagent was added and the absorbance was measured at 412 nm in the spectrophotometer. Based on the extinction coefficient for the fluorophore generated, the amount of reduced GSH in the sample is calculated [19].

### 2.2.6. Determination of TNF-α

Sample of 100 mg stomach tissue was homogenized in 10 volumes of ice cold phosphate buffered saline (PBS, pH 7). Furthermore, centrifugation at 3000 rpm for 10 min was made for samples. Then, supernatant was collected and analyzed using rat tumor necrosis factor α (TNF-α ELISA kit) Sunred company. Standard curves were prepared using TNF-α provided kit.

### 2.2.7. Histological and immunohistochemical analysis

The excised stomachs that had been fixed in 10% buffered formalin solution for 24 h were subsequently processed in ascending grades of alcohol and, finally, in xylene. The tissues were then embedded in paraffin, serially-sectioned to ~4-μm thickness, mounted on slides and stained with hematoxylin and eosin (H&E; Sigma).

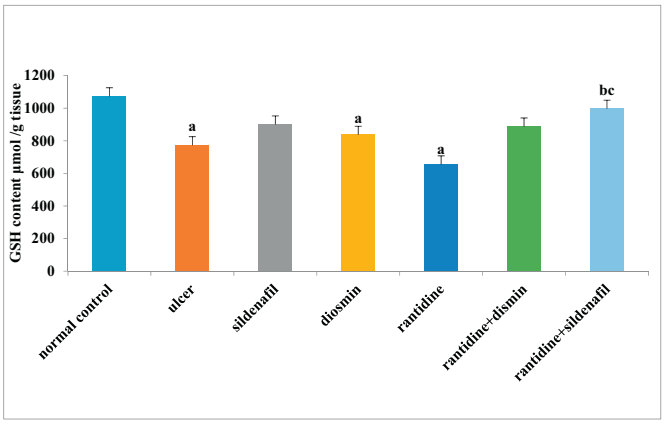


**Figure 4. Effect of different drug pretreatments on GSH content n = 8 in each group**. a indicates significant difference as compared to naïve control group at $p < 0.05$. b symbolizes significant difference as compared to ulcer group at $p < 0.05$. c refers to significant difference as compared to RANT group at $p < 0.05$. d represents significant difference as compared to SILD group at $p < 0.05$. e means significant difference as compared to DIO group at $p < 0.05$.

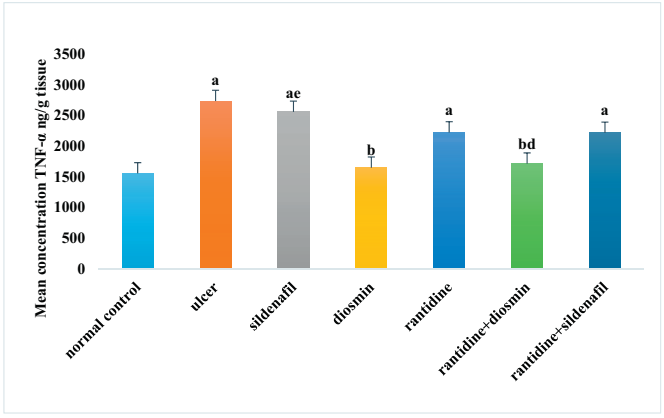


**Figure 5. Effect of different drug pretreatments on gastric TNF-α content n = 8 in each group**. a represents significant difference as compared to naïve control group at $p < 0.05$. b means significant difference as compared to ulcer group at $p < 0.05$. c indicates to significant difference as compared to RANT group at $p < 0.05$. d symbolizes significant difference as compared to SILD group at $p < 0.05$. e refers to significant difference as compared to DIO group at $p < 0.05$.

Immunohistochemical detection of caspase-3 is based on the Peroxidase/Antiperoxidase (APA) technique by using anti-Caspase-3 (Abcam, USA) as primary antibodies, together with goat anti-rabbit immunoglobulin (Biocare Medical, USA) as a secondary antibody. For detection of caspase-3 antibody, sections were subjected to antigen retrieval by boiling in Tris buffered saline solution (0.05 M, pH 7.6)/5 min. After that, cool down at room temperature for 20 min was performed. Then, rinsing with phosphate buffered saline (PBS) for 1 min was completed. Endogenous peroxidase was inactivated by immersing sections in 3% hydrogen peroxide for 10 min followed by washing in PBS/10 min. Blocking is done by incubation with normal goat serum. Sections were incubated overnight with the primary antibodies in a humidity chamber at 4 °C, then washed in PBS/10 min. Sections were incubated with the secondary antibody/60 min at room temperature (RT), washed in PBS/10 min, incubated with peroxidase/antiperoxidase solution/10 min at RT and rinsing with PBS. To develop color reaction, one drop of 3-30-diaminobenzidine-tetra-hydrochloride (DAB) chromogen was added to 2 ml of DAB substrate, mixed and applied on tissues for 5–15 min. Finally, sections were counterstained with Mayer's hematoxylin.

Additionally, Leica DMLB microscopes were used in this study and histological photos were taken by using Leica EC3 digital camera by a blinded pathologist.

## 3. Statistical analysis

Results were expressed as the mean ± SD. Graphpad prism 5.0 Demo (Graphpad software, San Diego, CA) was used for statistical analysis of different groups. Comparison of data groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey, multiple comparison tests. Significant difference is accepted when $P < 0.05$.

## 4. Results

This work introduced a comparative study of the gastro protective effects of DIO, SILD and their combinations with RANT against INDO-induced gastric ulcer in rats. These combinations resulted in seven different groups. Accordingly, a comparison of the different groups was presented in this section. The outcomes of this work give a clear idea about the behavior of DIO, SILD and their combinations with RANT. Moreover, these results were confirmed by histopathological findings.

### *4.1. Effect of different drug pretreatments on gastric pH*

In Group II, INDO significantly decreased gastric juice pH by 27.49% compared to Group I. Also, it was observed, that Group VII, Group VI and Group V showed significant increase in gastric pH values as compared to Group II by 84.55%,63.48% and 60.39%, respectively. On the other hand, SILD and DIO pretreated groups showed non-significant effect in gastric PH from Group II as revealed in Figure 1.

### *4.2. Effect of different drug pretreatments on gastric nitric oxide (NO) content*

In Group II, INDO significantly decreased gastric NO content by 69.68% as compared to Group I. Moreover, SILD and DIO pretreatment significantly increased NO level by 44.92%, 31.89%, respectively as compared to INDO group. Also, Group V, Group VI and Group VII revealed significant increase in NO content by 128.48%, 133.17%, 220.25% respectively, as compared to Group II as shown in Figure 2.

### *4.3. Effect of different drug pretreatments on gastric MDA content*

INDO treatment significantly increased gastric MDA content by 32.16% as compared to Group I. Additionally, Group IV, Group III and Group V showed significant decrease in gastric MDA contents by 22.18%, 15.64% and 18.91%, respectively, relative to that seen with rats received INDO alone. Furthermore, it was observed, that Group VI and Group VII showed significant decrease in gastric MDA contents by 22.48% and 34.27%, respectively, compared to Group II. Consequently, Group VI and Group VII have the highest values compared to other groups as presented in Figure 3.

### *4.4. Effect of different drug pretreatments on reduced GSH content*

INDO significantly decreased GSH levels by 31.84% compared to Group I values. Moreover, pre-treatment with (RANT and SILD) significantly increased GSH by 39.75% relative to that in rats received INDO alone. In addition, other groups showed non-significant effect in GSH values as revealed in Figure 4.

### *4.5. Effect of different drug pretreatments on gastric TNF-α content*

Group II showed significant increase in gastric TNF-α content by 76% compared to Group I. Besides, pre-treatment with DIO and (RANT and DIO) significantly decreased TNF-α levels by 39.77% and 37.37%,

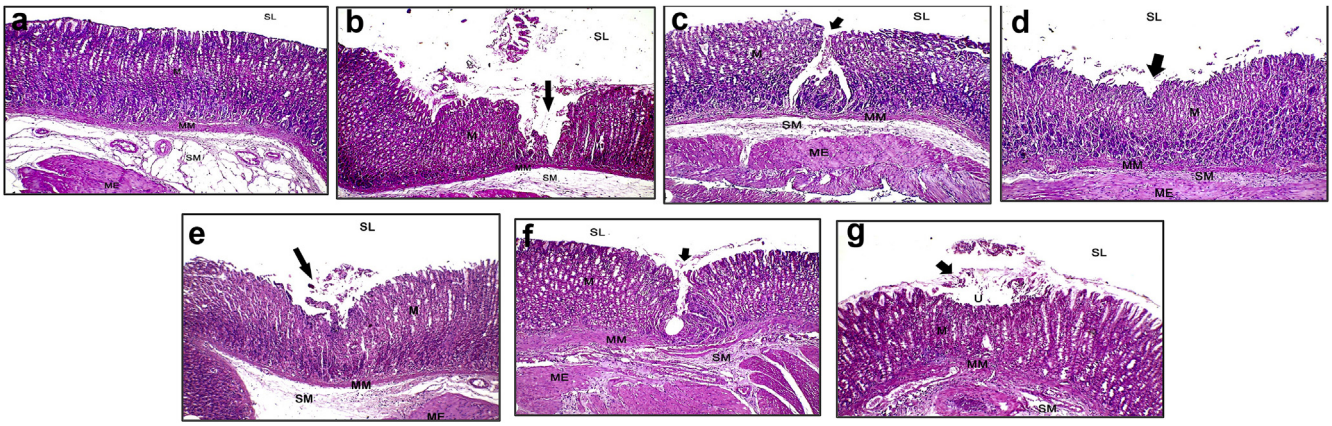


**Figure 6. a. Stomach, rat: vehicle control group**. Showing normal histological architectures. SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **b. Stomach, rat: Indomethacin treated group**. Showing sever erosion of gastric mucosa (arrow) which forming non-perforated ulcer. SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **c. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine**. Showing sever erosion of gastric mucosa (arrow) which forming non-perforated small ulcer. SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **d. Stomach, rat: Indomethacin induced ulcer group treated with Sildenafil**. Showing slight erosion of gastric mucosa (arrow). SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **e. Stomach, rat: Indomethacin induced ulcer group treated with Diosmin**. Showing mild erosion and sloughing of gastric mucosa (arrow). SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **f. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine plus Diosmin**. Showing mild erosion of gastric mucosa (arrow) which forming non-perforated small ulcer. SL, stomach lumen; M, mucosa; MM, mascularis mucosa; SM, submucosa; ME, mucosa externa. HE stain. X 4. **g. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine plus Sildenafil**. Showing mild erosion of gastric mucosa (arrow) which forming non perforated superficial ulcer. SL, stomach lumen; U; ulcer; M, mucosa; MM, mascularis mucosa; SM, submucosa. HE stain. X 4.

respectively, relative to that seen with rats received INDO alone. Moreover, it was noted, that Group IV, Group V and Group VII showed no effect on TNF-α concentration on gastric tissue as shown in Figure 5.

### 4.6. Effect of different drug pretreatments gastric tissue histology

Figure 6a revealed histopathological examination of gastric sections from naïve control group, showing normal histological architectures. After treatment with INDO, sever erosions of gastric mucosa were found (non-perforated ulcer) as shown in Figure 6b. Furthermore, it was noticed, that Group III, Group IV and Group VII showed mild erosions in gastric mucosa relative to Group I as presented in Figure 6c-g.

### 4.7. Effect of different drug pretreatments on caspase-3

Group I gastric tissue showed negative immunostaining for caspase-3 antibody. However, Group II revealed strong positive immunostaining. Additionally, it was observed, that Group V, Group III, Group VI and Group VII showed negative caspase-3 antibody immunostaining. In addition, SILD pretreatment had no effect on the strong expression of

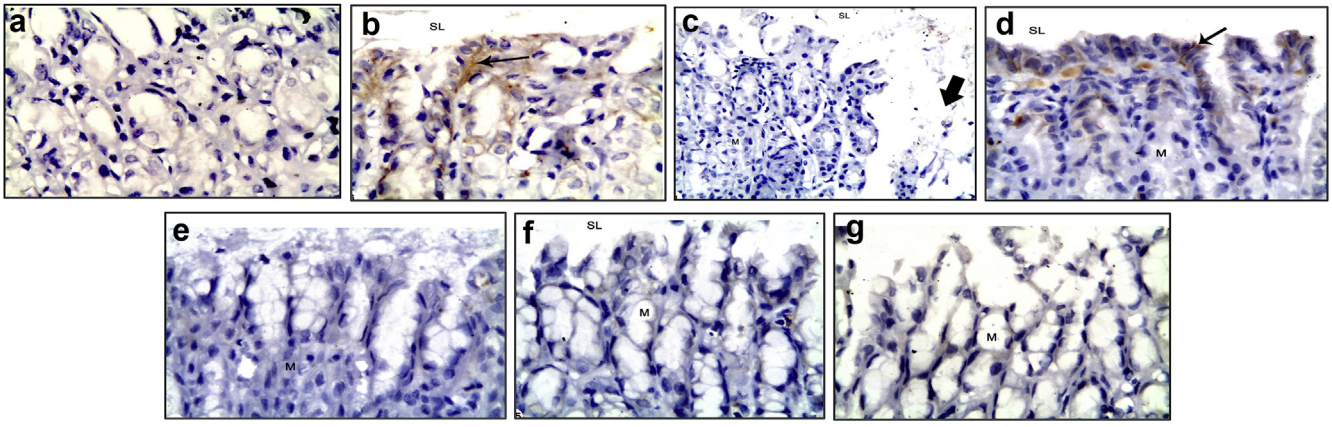


**Figure 7. a. Stomach, rat: vehicle control group**. Showing negative immunostaining for Caspase-3 antibody. IHC, Hematoxylin counterstain. X 40. **b. Stomach, rat: Indomethacin treated group**. Showing strong positive immune signals for Caspase-3 antibody (arrow) in gastric mucosa. IHC, Hematoxylin counterstain. X 40. **c. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine**. Showing negative immunostaining for Caspase-3 antibody; arrow indicating the peptic ulcer which contains necrotic gastric mucosa. SL, stomach lumen; M, mucosa. IHC, Hematoxylin counterstain. X 40. **d. Stomach, rat: Indomethacin induced ulcer group treated with Sildenafil**. Showing positive immunostaining for Caspase-3 antibody (arrow) at the edges of the peptic ulcer. SL, stomach lumen; M, mucosa. IHC, Hematoxylin counterstain. X 40. **e. Stomach, rat: Indomethacin induced ulcer group treated with Diosmin**. Showing negative immunostaining for Caspase- 3 antibody. M, mucosa. IHC, Hematoxylin counterstain. X 40. **f. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine plus Diosmin**. Showing negative immunostaining for Caspase-3 antibody in the mucosa around the peptic ulcer. SL, stomach lumen; M, mucosa. IHC, Hematoxylin counterstain. X 40. **g. Stomach, rat: Indomethacin induced ulcer group treated with Rantidine plus Sildenafil**. Showing negative immunostaining for Caspase -3 antibody in stomach mucosa. M, mucosa. IHC, Hematoxylin counterstain. X 40.

caspase-3 at the two sides of peptic ulcer as a result of INDO administration as presented in Figure 7a-g.

## 5. Discussion

Peptic ulcer occurs due to imbalance between aggressive factors and protective factors that are responsible for the endogenous defense mechanism [20]. Moreover, NSAIDs are known to be aggressive agents that cause damage in gastric mucosa [21]. Consequently, some recent studies have focused on the protective effect of SILD on INDO induced gastric ulcer. SILD citrate is used in the treatment of functional impotence. It increases the effect of the guanosine cyclic 3′, 5′- monophosphate (cGMP), which displays an inhibitory effect on the smooth muscle cells of the arterioles supplying the human corpus cavernosum. Furthermore, SILD effect is due to blockade of the phosphodiesterase-type 5, which inactivates the intracellular cGMP stimulated by nitric oxide. Also, many papers have demonstrated, that DIO (diosmetin 7-O-rutinoside), a natural citrus flavone, displays remarkable antioxidant, anti-inflammatory, and anti-apoptotic activities against cardiac, hepatic and renal injuries. However, these papers did not consider gastro protective effect of DIO on INDO-induced gastric damage. Furthermore, these researches did not consider the gastric protection through incorporating SILD, RANT and DIO. Therefore, based on this complex incorporation, gastro protective effect of 7 different groups were considered in this paper.

Based on histopathological examination of gastric sections, naïve control group showed intact mucosa and no formation of the gastric ulcers as revealed in Figure 6a. Additionally, INDO administration resulted in production of gastric lesions in 100% of the animals as shown in Figure 6b. This result can be attributed to, INDO produce gastric damage mainly via inhibition of cyclooxygenase (COX), depletion of endogenous prostaglandins (PGs) [22], decrease NO level, increase lipid peroxidation [23], increase gastric PH, decrease mucin and bicarbonate production [24].

It is important to note, that rats administered INDO produced a significant decrease in gastric PH from in naïve control group. Furthermore, pretreatment with (RANT and SILD), (RANT and DIO) and RANT resulted in a significant increase in gastric PH from in INDO group. This result thanks to, the RANT effect on gastric juice PH that may be explained as Histamine H2-receptor antagonists which acts directly on the gastric mucosa to decrease acid secretion and inhibit ulcer formation [25]. However, it is observed, that pretreatment with SILD or DIO alone were non-significant as represented in Figure 1.

The current results also showed, that rats administered INDO produced a significant increase in gastric MDA content relative to naïve control group. In addition, rats pretreated with RANT, DIO or SILD alone or their combinations produced significant decrease in MDA content compared to INDO group as represented in Figure 3. These results can be credited to, increasing c AMP level that inhibits lipid peroxidation by decrease oxygen free radicals' production [26]. Accordingly, SILD may inhibit lipid peroxidation by increasing the synthesis of cGMP and cAMP.

Moreover, it is observed, that INDO significantly reduced gastric mucosal NO level compared to naïve control group as introduced in Figure 2. These findings are in accordance with AYDINLI [9, 27] who reported a decrease in NO biosynthesis. That can be attributed to, NOS activity decrement from gastric tissue damage. Furthermore, NO mediates gastro-protective effect by inhibition leukocyte adhesion to vascular endothelium [28], maintenance of gastric blood flow, increase cGMP content [29]. Also, NO increases mucus and bicarbonate secretion, decreases gastric acid secretion and promotes ulcer healing [30, 31]. Consequently, many studies considered NO as playing a key role in SILD gastro protection against INDO-induced damage.

Based on the data here, it appears that co-administration of (RANT and SILD) produced a significant increase in gastric GSH relative to INDO group as depicted in Figure 4. This obtained result comes on the line with [13, 29] where RANT (50 mg/kg, p.o.) and SILD (10 mg/kg, p.o.) (pretreated group) suppresses lipid peroxides level as compared to INDO group. Conversely, obtained results showed, that RANT, DIO and SILD pretreatment individually is non-significant.

It was also demonstrated, that INDO produced a significant increase in TNF-α [32] in gastric tissues which is one of the aggressive factors in ulcerogenesis [13, 33]. This result may be accredited to that TNF-α is a potent stimulator of inducible NO expression [34]. Results here showed, that DIO alone or in combination with RANT significantly decreased TNF-α relative to INDO group as shown in Figure 5. This outcome corresponds to the results of a previous study that confirmed an inhibitory effect of DIO on TNF-α levels on metabolic syndrome in rats [35]. On the other hand, it was noticed, that SILD showed inability to decrease upregulation of TNF-α which result from INDO induced gastric damage. Moreover, this result supported a previous study on pulmonary vasoconstriction [36, 37].

It is clear, that INDO produced a significant activation in caspase-3 gastric tissue as shown in Figure 6b. This result is in agreement with the obtained results in [38] that illustrated vanillin effect on indomethacin-induced gastric ulcer. The attained result, can be attributed to, INDO ability to uncouple oxidative phosphorylation, dissipate the mitochondrial transmembrane potential (MTP), and to induce mitochondrial permeability transition pore (PTP), which liberates cytochrome c. This enzyme generates reactive oxygen species (ROS) [39, 40]. Consequently, triggers caspase cascade and cellular lipid peroxidation, resulting in cellular apoptosis [41]. Furthermore, our results demonstrated, that (RANT + DIO) pretreatment significantly inhibit caspase-3 immunosignals as revealed in Figure (7c, 7e). This finding comes in agreement with a previous study where in, DIO antiapoptotic effect by inhibition of caspase-3 is indicated on ulcerative colitis [42]. Additionally, RANT gastroprotective effect by inhibition of caspase-3 was demonstrated in neuronal cell death induced by oxygen-glucose deprivation [43].

## 6. Conclusion

In this paper, the gastro protective effect of DIO, SILD and their combinations with RANT against INDO-induced gastric ulcer in rats was addressed. Additionally, the potential mechanisms of these medications and their combination effect were considered. It is observed, that SILD and DIO pre-treatment showed non-significant effect on gastric juice PH. However, their combinations with RANT was superior to using RANT alone. In addition, the results revealed, that combinations of (RANT and SILD) and (RANT and DIO) attained the lowest MDA levels relative to the ulcer group. But, these two combinations provided the highest increase in gastric tissue NO levels. Despite, all groups displayed non-significant effect on reduced GSH content, (RANT and SILD) group increased GSH concentration by 39.75% relative to INDO group. In addition, DIO, RANT, (RANT and DIO) and (RANT and S ILD) pre-treatment had antiapoptotic activity on gastric mucosa. On the other hand, SILD did not affect caspase-3 immunostaining. Consequently, it can be said, that SILD gastroprotective effect against INDO induced gastric damage was achieved through increasing NO level in gastric tissue besides inhibition of lipid peroxidation. Furthermore, DIO gastroprotective effect was attained by increasing NO level in gastric tissue as well as inhibition lipid peroxidation. Additionally, DIO decreased TNF-α and inhibited caspase-3 in gastric tissue. In light of this investigation, combinations of SILD and DIO with RANT were more successful and might be considered to be drugs of choice in clinical gastropathy in the future.

## Declarations

### *Author contribution statement*

D.Y. Khira: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

A.E. El-Sisi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. Elsayed and S. Sokar: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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*Additional information*

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