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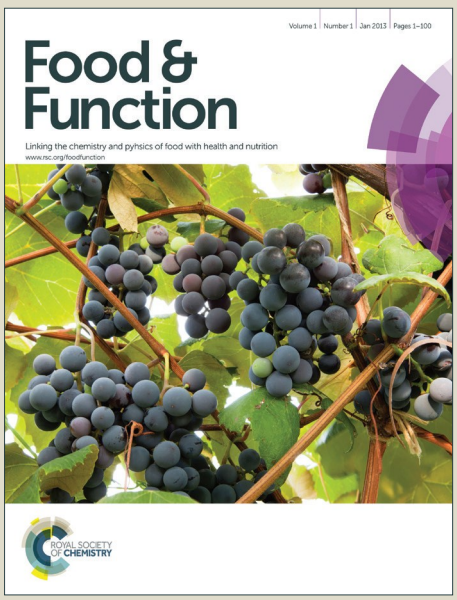

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Protective effects of trigonelline against indomethacin-induced gastric ulcer in rats and potential underlying mechanisms

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ABSTRACT

The present study was undertaken to explore gastroprotective effects of trigonelline (TRG) and to determine the potential mechanisms involved in this action. In order to evaluate the gastroprotective efficiency of TRG, indomethacin-induced ulcer model has been applied. Antioxidants, cytokines, adhesion markers and apoptosis level have been analyzed for the biochemical mechanism involved in TRG activity. TRG (45 mg/kg) pretreated rats significantly inhibited gastric lesions by 81.71 %. Indomethacin administration raises the level of leukotriene B_4 (LTB_4), lipid peroxidation and myeloperoxidase (MPO) with the significant declines of prostaglandin E_2 (PGE_2), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-px). Conversely, TRG (45 mg/kg) pretreated animals showed significant rises in PGE_2, antioxidants level along with substantial reductions in LTB_4, lipid peroxidation and MPO level. Indomethacin-induced rats also exhibits considerable increases of pro-inflammatory cytokines including interleukin- 6 (IL-6), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) level and decreases of anti-inflammatory cytokine such as interleukin-10 (IL-10) and interleukin-4 (IL-4), but these imbalances were normalized through treatment of TRG. Protective activity of TRG against indomethacin-induced gastric ulcer has been ascribed to three important mechanisms: (1) anti-inflammatory; (2) antioxidant; (3) anti-apoptotic pathways.

Keywords: Trigonelline; Indomethacin; Ulcer; Gastroprotection; Antioxidant; Apoptosis

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs), such as indomethacin and ketoprofen, are generally used to alleviate swelling and pain of inflammatory diseases including rheumatoid arthritis and osteoarthritis. Despite their benefits as anti-inflammatory nature, these drugs may cause peptic ulcers.[1,2] The major causes of peptic ulcers include gastric acid, pepsin, bile salts, NSAIDs, *Helicobacter pylori* infection, consumption of alcohol and tobacco. Earlier report stated that the NSAID users have a greater threat of peptic ulcers than those with *Helicobacter pylori* infection.[3] Thus, it is imperative to search for novel compounds that may help to prevent ulceration of the gastrointestinal tract. The NSAID gastropathy is considered a "silent epidemic" and, therefore, has been an area of intense research. Among the commonly used NSAIDs, indomethacin possesses highest ulcerogenic potential to humans.[4,5] Inhibition of cyclooxygenases (COXs) and associated reduced prostaglandin (PG) synthesis were previously believed to be the major reasons for gastric pathogenesis caused by NSAIDs including indomethacin.[6-8] However, accumulated evidence suggests that other COX-independent factors also play equally important roles in the process.[9-11] In order to prevent and treat gastric ulcer, indigenous healers and herbalists traditionally used phytogenic agents. In recent decades, gastroprotection using medicinal plant products as possible therapeutic alternatives has become a subject of active scientific investigations.[12]

Trigonelline (TRG) is a pyridine alkaloid, commonly found in *Trigonella foenum-graecum* L. (fenugreek) seeds and coffee beans.[13,14] TRG as a coffee ingredient is one of the most often consumed alkaloids. Anti-diabetic properties of TRG and its beneficial influence on

lipid profile have been proven.[15,16] TRG attenuates adipocyte differentiation and lipid accumulation in 3T3-L1 cells.[17] Also, this alkaloid has been taken into consideration as a potential neuroprotective agent, especially in Alzheimer's disease (AD). Previous reports exhibit that TRG shows memory improvement in β-amyloid-induced memory impairment in rats and in neurite outgrowth of rats and humans.[14,18] It has also shown an antioxidant property.[19] TRG has antioxidant effectiveness in cell-free systems and human colon cell lines.[20] However, the role of TRG on acute gastric ulcer induced by indomethacin remains unidentified. The objective of this study was to assess the effects of TRG against indomethacin-induced gastric damage in rats and its potential gastroprotective mechanism.

Materials and Methods

Animals

A total of 102 male Sprague-Dawley (SD) rats (200-220 g) were used for this experiment. For the dose selection study 48 animals were used (8 groups with 6 rats each). For the role of different antagonists on TRG produced gastroprotectivity study, 54 animals were used (9 groups with 6 rats each). Animals were kept at precise temperature 23±2 °C, relative humidity 65–80% and exposed to 12 h dark–light circles (lights on at 6:00) and fed with standard pellet diet (Samyang, Daejeon, Republic of Korea) and tap water *ad libitum*. Animals were maintained accordance with guidelines delivered by National Institute of Health for the Care and Use of Laboratory Animals (NIH Publication 80-23, revised in 1996). All the in vivo studies were performed accordance with Ethics Committee norms (permit number CBNU-2014-92) established by the Institutional Animal Care and Use Committee at Chonbuk National University (Jeonju, Republic of Korea).

Drugs and chemicals

Trigonelline (TRG), indomethacin, omeprazole, SC560, celecoxib, N_{ω}-Nitro-L-arginine methyl ester (L-NAME), N-ethylmaleimide (NEM), yohimbine, glibenclamide and ELISA kits for SOD (superoxide dismutase) caspase-3 where purchased from Sigma Chemical Co. (St. Louis, MO, USA). Apoptosis assay kit was acquired from Boehringer Mannheim. ELISA kits for ICAM-1, VCAM-1, E-selectin, P-selectin, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) were obtained from R & D Systems (Minneapolis, MN, USA). ELISA kits for TNF-α, IL-6 and IL-10 were from Bio Legend (San Diego, CA, USA). ELISA kits for hepatocyte growth factor (HGF), vascular epidermal growth factor (VEGF), epidermal growth factor (EGF) and transcription factor kit were obtained from Cayman Chemical (Ann Arbor, USA). Antibodies for COX-1 and β-actin were from Sigma-Aldrich. Antibodies for eNOS, iNOS, IL-10 and TNF-α were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals used were of analytical reagent grade.

Dose selection

To determine lowermost effective dose of TRG, gastric ulcers were induced by indomethacin after TRG treatment. In this analysis, forty eight (48) SD rats were used, divided eight (8) groups containing six (n = 6) rats each. Rats were starved for 24 hours and accommodated in cages. They were only allowed free access to drinking water. Drugs were dissolved in 0.5% **CMC (carboxymethyl cellulose)** as vehicle, and administered orally via orogastric-intubations. Experimental gastric ulcer was induced based on previous method described by Antonisamy et al. (2014) [21] using indomethacin as ulcerogen.

Group 1 (normal control) received 0.5 mL of 0.5% CMC.

Group 2 (ulcer control) received 0.5 mL of 0.5% CMC.

Group 3 (positive control) received 0.5 mL of 0.5% CMC of the standard drug omeprazole (40 mg/kg) based on the previous report.[21]

Groups 4, 5, 6, 7 and 8 received 0.5 mL of 0.5% CMC of the TRG at a dosage of 15, 30, 45, 60 and 75 mg/kg respectively.

After 30 min, Group 1 received 0.5 mL of 0.5% CMC. Group 2–8 received indomethacin (20 mg/kg orally). Animals were sacrificed under anesthesia with ketamine/xylazine (0.5 mL of 100 mg/mL ketamine combine with 0.05 mL of 20 mg/mL xylazine) at a dosage of 0.55 mL/ 100 g body weight an six (6) hour after indomethacin administration and ulcer score were macroscopically examine according to previous method.[22]

Role of different antagonists on TRG produced gastroprotectivity

Rats were assigned to nine (9) groups, each comprising of six rats (n = 6). The treatment groups and experimental protocol are detailed below:

Group 1 (normal control) received 0.5 mL of 0.5% CMC.

Group 2 (ulcer control) received 0.5 mL of 0.5% CMC.

Group 3 (TRG treatment) received TRG (45 mg/kg p.o.).

Group 4 (SC560+TRG treatment) received SC560 (5 mg/kg p.o.) and TRG (45 mg/kg p.o).

Group 5 (celecoxib+TRG treatment) received celecoxib (3.5 mg/kg p.o.) and TRG (45 mg/kg p.o).

Group 6 (YO+TRG treatment) received YO (2 mg/kg i.p.) and TRG (45 mg/kg p.o).

Group 7 (L-NAME+TRG treatment) received L-NAME (50 mg/kg i.p.) and TRG (45 mg/kg p.o).

Group 8 (NEM+TRG treatment) received NEM (10 mg/kg s.c.) and TRG (45 mg/kg p.o).

Group 9 (GLIB+TRG treatment) received GLIB (5 mg/kg p.o.) and TRG (45 mg/kg p.o).

All drugs were administered using 0.5% CMC as the vehicle. After 30 min, each group of animals except normal group received 20 mg/kg of indomethacin. Selective COX-1 inhibitor (SC560), COX-2 inhibitor (celecoxib), α_2- receptors antagonist (yohimbine), nonselective nitric oxide synthase (NOS) inhibitor (L-NAME), endogenous sulfhydryl antagonist (NEM), and K^+ATP channels antagonist (glibenclamide) were administered to rats 30 min before TRG treatment and 1 h prior to indomethacin induction. Six hours later, animals were killed under anesthesia with ketamine/xylazine at a dosage of 0.55 mL/ 100 g body weight and stomach was surgically removed, opened along the greater curvature and macroscopically examine lesions according to ulcer score described by previous method.[22] Concisely, ulcers are either circular (assessed on the basis of diameter) or linear (assessed on the basis of length). Deep circular ulcers more than 8 mm = 10; 7–8 mm = 8; 6–7 mm = 7; 5–6 mm = 6; 4–5 mm = 5; 3–4 mm = 4; 2–3 mm = 3; 1–2 mm = 2 and 0–1 mm = 1. The deep linear ulcers more than 10 mm in length = 6 and linear ulcer less than 10 mm in length = 3. The score for each single lesion was then summed up for the determination of ulcer index (mm).

The percentage inhibition was calculated through the method described by Demirbilek et al. (2004)[23]: (UI nontreated – UI treated)/UI nontreated) × 100.

Gastric tissue homogenate preparation

Immediately after animals were killed, gastric mucosa was removed from rats and washed carefully with ice-cold saline. Using a homogenizer small fragment of each stomach was homogenized (10% w/v) in ice cold PBS (0.1 mol/l) containing mammalian protease inhibitor cocktail. The homogenates were centrifuged at 10000 g at 4 $^{\circ}$C for 15 min. The pure supernatant was used to quantify the biochemical markers.

Determination of the effect of TRG on biochemical markers

MPO activity was determined as previously described.[24] The absorbance was read at 650 nm. MPO activity was expressed as mU/100 mg wet tissue.

The SOD activity was evaluated based on manufacturer instructions. The absorbance was read at 560 nm.[25] The results were expressed as U/mg proteins.

Catalase activity was measured using the method described by Aebi (1984).[26] Absorbance was measured at 510 nm. The results were expressed as (U/g tissue).

Lipid peroxidation was determined by estimating the level of thiobarbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA), according to the method of Mihara and Uchiyama.[27] The absorbance was measured at 535 nm. The results were expressed as nmol of MDA/g tissue.

Glutathione peroxidase (GPx) activity was spectrophotometrically determined based on the previous method.[28] The absorbance was measured at 340 nm. The results were expressed as unit/g wet tissue.

PGE_2 and LTB_4 assay was performed according to the manufacturer's instructions. Results were expressed as ng/g wet tissue (for PGE_2) and pg/g wet tissue (for LTB_4).

Measurement of apoptosis and caspase-3 was performed according to the manufacturer instructions. Level was expressed as μM pNA/min/g wet tissue.

For apoptotic measurement, the gastric mucosal cells were collected from the stomachs of freshly dissected rat for the quantitative analysis of apoptosis. Gastric mucosal cells were collected and incubated in the lysis buffer and centrifuged, the supernatant having the cytoplasmic histone-associated DNA fragments were reacted with immobilized anti-histone antibody in the microtitrator wells. After the wells were washed, the retained complex was

reacted with anti-DNA peroxidase and probed with ABTS [2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid)] reagent for spectrophotometric quantification.[29] Apoptosis level was expressed as U/mg protein.

The levels of P-selectin, E-selectin, VCAM-1 and ICAM-1 in serum samples were estimated using ELISA kits according to manufacturer's protocol. The values were expressed as ng/ml (for ICAM-1, VCAM-1 and P-selectin) and pg/ml for E-selectin.

Levels of VEGF, EGF and HGF in the gastric tissue were estimated using commercially available ELISA kits. The values were expressed as ng/g wet tissue.

Pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-1β, IL-6, and anti-inflammatory cytokines including IL-10 and IL-4 levels were evaluated based on manufacturer instructions. The values were expressed as pg/mg proteins.

Gastric mucosal NOS activity was measured by previous methods.[30,31] The deference between absorption at 401 and 421 nm was frequently detected with a dual wavelength recording spectrophotometer at 37 °C. Induced NOS (iNOS) level was calculated by subtraction of constitutive NOS (cNOS) level from total NOS (tNOS) level.

NO content was quantified by measuring nitrite/nitrate concentration using Griess assay.[32] The absorbance was measured at 540 nm. The results were expressed as μmol/g tissue.

Preparation of nuclear fraction and determination of the effect of TRG on transcription factor

Stomach tissues were homogenized ice cold PBS, and centrifuged at 3,000g for 5 min. Discarded the resulting supernatants. Precipitates were washed twice by ice cold PBS and then re-suspended in buffer A (10 mM HEPES buffer, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1mM dithiothreitol (DTT), 50 mM NaF, 30 mM β glycerophosphate, 1 mM

Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10% NP-40), resulting homogenates were centrifuged at 15,000g for 15 min after the incubation of 15 min on ice and strong shocked for 45s. The pellets were washed three times with buffer A, then resuspended in buffer B (20 mM HEPES buffer, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF) and shocked in 4 °C for 30 min, then centrifuged at 15,000g for 15 min. The resulting supernatants were consider as nuclear extracts, and frozen at −80 °C for measurements of NF-*κ*B. NF-*κ*B p65 and NF-*κ*B p50 subunits were detected by ELISA depends on manufacturer instructions. The optical density (OD) was measured at 655 nm. Percentage inhibition was calculated by following formula: (OD of control− OD of treated/ OD of control × 100).

Western blot analysis

Western blot analysis was carried out to detect the expression of various target proteins. Gastric mucosal samples were homogenized in RIPA lysis buffer. The total protein content in supernatant was assayed by BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal amount of proteins (20μg) were loaded to 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and then electro transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membrane was incubated with a 1:1000 dilution of respective primary antibody, followed by a 1:2000 dilution of horseradish peroxides-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare, Pittsburg, PA, USA). ImageJ analysis software was used to quantify the density of each band.

Determination of the effect of TRG on microvascular permeability

In this experiment rats were allocated into five different groups, having six animals each. Prior to experimentations rats were fasted for 24 h and permitted free access to water. The treatment groups and experimental protocol are given below:

Group 1 (normal control) received 0.5 mL of 0.5% CMC.

Group 2 (ulcer control) received 0.5 mL of 0.5% CMC.

Group 3 (TRG treatment) received TRG (45 mg/kg).

Group 4 (SC560+TRG treatment) received SC560+TRG (30 mg/kg+45 mg/kg).

Group 5 (celecoxib+TRG treatment) received celecoxib+TRG (30 mg/kg+45 mg/kg).

All drugs were suspended in 0.5% CMC and treated by oral administration 1 h before ulcer induction using indomethacin. Microvascular permeability was assessed 6 h after indomethacin treatment through measuring the amount of extravasated Evan's blue dye in mucosa based on previous method.[33] Briefly, an each rat received 1mL of 1% (w/v) Evan's blue in sterile saline through intravenous injection 30 min before sacrifice. Under ether anesthesia, rats were killed by bleeding from descending aorta, stomachs were removed, and gastric mucosa was scraped off and immersed in distilled water. The dye was extracted with formamide and quantified spectrophotometrically at 620 nm, and results were expressed as μg/mg proteins.

Statistical analysis

All results were expressed as mean±S.D. (standard deviation). Data were analysed for normal distribution using Kolmogorov-Smirnov test. Normally distributed data were analysed with one way ANOVA using a Tukey's post hoc test; otherwise Kruskal-Wallis test was used. If the Kruskal-Wallis test for analysis of variance was significant, Mann-Whitney U-test was used for comparison between two selected groups. Statistical significance was accepted at *P* value less than 0.05.

Results

Macroscopic reflection indicated that pretreatment of TRG **(Fig. 1C)** or omeprazole **(Fig. 1D)** considerably reduced gastric mucosal injury compared to the indomethacin-induced ulcer control group; where elongated band of hemorrhages have been observed in gastric mucosa **(Fig. 1B)**. However, normal group displays undamaged stomach without any injuries **(Fig. 1A)**.

Figure 2 represents the effective dose determination. In comparison with indomethacin treated group, TRG at 45 mg/kg provided significant gastroprotective effect, inhibiting the gastric ulcer by 81.71%, which does not vary statistically from upper doses such as 60 and 75 mg/kg. Therefore, 45 mg/kg was selected as a lowermost effective dose of TRG.

Levels of mucosal SOD, CAT, GSH-px and PGE_2 were reduced on indomethacin group by 3.36, 2.62, 1.84 and 1.94 fold respectively as compared to normal rats. These levels were reverted by TRG (45 mg/kg) pretreatment by 3.15 (SOD), 2.31 (CAT), 1.86 (GSH-px) and 1.69 (PGE_2) fold respectively. On the other hand, MDA, MPO and LTB_4 levels were significantly increased on indomethacin group as compared with normal group by 2.17, 3.94 and 1.52 fold respectively. However, pretreatment of TRG (45 mg/kg) significantly reduced MDA (2.07 fold), MPO (3.50 fold) and LTB_4 (1.58 fold) levels compared to indomethacin group (Figs. 3A, B and C).

TNF-α (14.78 fold), IFN-γ (3.16 fold), IL-1β (2.25 fold) and IL-6 (7.98 fold) levels were significantly increased and IL-4 (6.05 fold) and IL-10 (1.91 fold) levels were considerably reduced in indomethacin group as compared to normal group. Pretreatment of TRG (45 mg/kg) significantly reduced TNF-α, IFN-γ, IL-1β, and IL-6 levels by 12.47, 2.34, 2.03 and 7.11 fold respectively as compared to indomethacin-induced ulcerated group **(Figs. 4A and B)**.

iNOS (12.62 fold) and TNF-α (4.18 fold) protein expression levels were significantly increased and eNOS (3.88 fold), COX-1 (17.15 fold) and IL-10 (1.56 fold) levels were considerably reduced in indomethacin group as compared to normal group. Pretreatment of TRG (45 mg/kg) significantly reduced iNOS and TNF-α level by 3.46 and 4.59 fold respectively. The levels of eNOS (5.13 fold), COX-1 (16.92 fold) and IL-10 (2.84 fold) were significantly increased in TRG pretreated group compared to indomethacin-induced ulcerated group **(Fig. 5)**.

ICAM-1, VCAM-1, P-selectin and E-selectin levels were considerably elevated following administration of indomethacin to reach 4.50, 2.40, 4.60 and 3.79 fold respectively as compared to normal rats. TRG (45 mg/kg) pretreated group significantly reduced the levels of ICAM-1 (2.59 fold), VCAM-1 (1.97 fold), P-selectin (2.14 fold) and E-selectin (2.30 fold) as compared to indomethacin-induced ulcerated group **(Figs. 6A and B)**.

Indomethacin-induced ulcerated rats shows significant increases of iNOS (11.33 fold) and NO (2.52 fold) levels, as compared with normal rats. Whereas, TRG (45 mg/kg) pretreated animals declines the levels of iNOS and NO by 5.66 and 1.86 fold respectively as compared to indomethacin group **(Figs. 6C and D)**.

Levels of apoptosis (6.24 fold), caspase-3 (10.27 fold), NF-*κ*B p65 (22.33 fold) and NF-*κ*B p50 (14.20 fold) were significantly elevated in indomethacin-induced ulcerated group compared to normal animals. However, pretreatment of TRG (45 mg/kg) significantly reduced apoptosis caspase-3, NF-*κ*B p65 and NF-*κ*B p50 levels by 5.63, 3.52, 2.91 and 3.73 fold respectively **(Figs. 7A and B)**.

Microvascular permeability level was significantly increased in indomethacin group compared to normal group. Pretreatment of TRG (45 mg/kg) reduced the level of microvascular permeability (77.17%). However, administration of SC560 decreased the percentage of

inhibition of microvascular permeability from 77.17% to 3.62%; whereas, treatment of celecoxib did not affect the TRG activity against microvascular permeability **(Fig. 7C)**.

Administration of SC560, L-NAME, and NEM were significantly reduced the ulcer index inhibition percentage elicited by TRG (45 mg/kg) from 81.71% to -6.52%, 3.52%, and -0.87% respectively. But, the treatment of celecoxib, yohimbine and glibenclamide were not affecting the ulcer protective activity of TRG **(Fig. 8)**.

Discussion

Our knowledge of basic understanding about NSAID-induced ulceration has progressed significantly in past 10 years. However, these advancements knowledge have not translated into widespread application in clinical setting. This study will address the gastroprotective activity of TRG against indomethacin-induced gastric ulcer along with underlying mechanisms.

Prostaglandins (PGs) have crucial role in preservation of physiological process including mucosal blood flow, angiogenesis, mucus and bicarbonates secretions.[34] PGs synthesized by cyclooxygenase-I (COX-I) and cyclooxygenase-II (COX-II) isozymes. Since indomethacin as non-specific COX inhibitor, causes gastric ulceration and intensifies forgoing gastric ulcers in humans and rodents through suppression of PGs synthesis.[8] Consistent with these results, our investigational outcomes revealed that exposure of indomethacin significantly reduced gastric mucosal PGE_2 level compared to normal rats. However, pretreatment of TRG (45 mg/kg) significantly increased PGE_2 level compared to indomethacin-induced ulcerated rats. In this study gastroprotective activity of TRG has been reverted by SC560 (COX-I selective inhibitor) and not by celecoxib (COX-II selective inhibitor) indicates the involvement of COX-I synthesized PGs in TRG afford gastroprotection. This observation was consistent with previous reports.[21,35]

Activated neutrophils produce myeloperoxidase (MPO), cytokines, reactive oxygen species (ROS) and reactive nitrogen species (RNS) which have been responsible for oxidative stress in gastric endothelial cells. Since MPO has prominently been produced by neutrophils, infiltration of neutrophils into endothelium identified via quantification of MPO level. Previous experiments elucidate that elevation of MPO activity during indomethacin-induced gastric ulcer.[21,35] Taken together, present data indicate that TRG significantly reduced MPO activity compared to indomethacin-induced ulcerated animals.

Previous experiments explicate that increases of pro-inflammatory cytokines and decline of anti-inflammatory cytokines during gastric ulcer.[21,35] Consistent with these findings, this work revealed that administration of indomethacin significantly increased pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-6) and decreased anti-inflammatory cytokines (IL-10 and IL-4) level; however, pretreatment of TRG significantly revert these markers into normal level. Among the pro-inflammatory cytokines TNF-α possess multiple pathophysiological roles in gastric ulcer including activation of NF-κB, apoptosis, iNOS and neutrophil infiltration. Similarly, IL-6 is another important pro-inflammatory cytokine; activate PMNs into inflammatory site, and triggering oxidative pathway responsible for tissue damage during gastric ulcer.[34,36] Nonetheless, IL-10 is vital anti-inflammatory and immunosuppressive cytokine able to inhibit TNF-α production. Since, TRG considerably inhibits TNF-α and IL-6 along with augmentation of IL-10 elucidated its anti-inflammatory nature.

Neutrophilic PMNs dealings with vascular endothelium are vastly coordinated manners that consist of leukocyte rolling, arrest, firm adhesion, and diapedesis. This interaction occurs under high shear stresses within venules and depends on multiple families of adhesion molecules including ICAM-1, VCAM-1, P-selectin and E-selectin.[37] Adhesion molecules facilitated

transendothelial migration of neutrophils into site of gastric tissue injury. In this study, levels of adhesion molecules were considerably elevated in indomethacin-induced ulcerated animals. However, TRG pretreatment reduce pathological levels of those adhesion molecules into normal. This result was in agreement with previous studies.[34,38]

Lipid peroxidation level in gastric tissue was measured by determining the quantity of MDA; this investigation may convey the level of gastric tissue injury.[39] Our finding shows that significant increases of MDA in indomethacin-induced group, however, pretreatment of TRG significantly prevent MDA production. Superoxide dismutase (SOD) converted superoxide anions (O_2^-) to hydrogen peroxide which in turn is detoxified by glutathione peroxidase (GSH-px) and catalase (CAT). These enzymes constitute an endogenous antioxidant system, and preventing cell damage induced by ROS.[40] In this study, TRG significantly increases the level of SOD, GSH-px and CAT compared with indomethacin-induced ulcerated group suggested its endogenous antioxidant stimulatory potential against indomethacin-induced ulcer.

NF-κB is believed to play a pivotal role in inducible expression of many genes, including TNF-α, IL-1β, IL-6, iNOS and adhesion molecules.[41,42] In this study, indomethacin-induced ulcerated animals show significant increases of both p50 and p65 subunits, nonetheless, pretreatment of TRG considerably decline these pathophysiological markers into normal level. This result was in agreement with previous reports.[43,44]

Nitric oxide (NO) plays an important role in controlling numerous components of mucosal defense, including increased gastric mucus secretion, blood flow, and reduced neutrophil adhesion.[39,40] Previous report shows that considerable increases of iNOS in indomethacin-induced ulcerated rats than normal rats.[35] In agreement with this finding present results revealed that significant increases of iNOS and NO in indomethacin group compared to

normal rats. However, pretreatment of TRG significantly revert the levels of iNOS and NO compared to indomethacin group. Previous study indicated that L-NAME, a nonspecific NOS inhibitor, augmented indomethacin-induced gastric injury in rats,[45] consistent with this finding, present work revealed pretreatment of L-NAME considerably decline the ulcer protective efficiency of TRG and simultaneously increased ulcer index.

VEGF as a growth factor elicits endothelial proliferation, migration and ulcer healing via stimulation of angiogenesis.[21] Similarly, HGF supports angiogenesis process by multiple mechanisms including COX activation and increases EGF expression that is essential for acceleration of ulcer healing by stimulating cell migration and proliferation in epithelial cell monolayers, repairing tissue, and diminishing gastric acid secretion.[46] Current study displayed that indomethacin administration significantly declined the mucosal VEGF, HGF, and EGF levels compare to normal rats. However, pretreatment of TRG considerably augmented growth factors level. These outcomes are in agreement with previous studies.[21,35]

In indomethacin-induced ulcer, apoptosis is another vital pathophysiological pathway. In present study, TRG showed substantial decline of caspase-3 and apoptosis in indomethacin-induced ulcer. Previous results explore that apoptosis was stimulated by lipid peroxidation, TNF-α and inhibited by PGE_2.[40] Present study shows that TRG significantly increased the production of PGE_2 and reduced lipid peroxidation and TNF-α level explicate the possibility that decline of apoptosis and caspase-3 in TRG-pretreated group is due to the augmentation of PGE_2 and inhibition of lipid peroxidation and TNF-α.

Intestinal permeability is thought to be central and essential mechanism of translating the biochemical/ cellular events of NSAIDs to tissue reaction in small bowel.[47] Indeed, elevated microvascular permeability was observed on indomethacin-induced animals. However, this

condition has reduced by TRG treatment. However, treatment of SC560 significantly affects the activity of TRG against vascular permeability. However, treatment of celecoxib did not alter TRG activity. These observations are in agreement with previous reports.[35,48]

Nonprotein endogenous NP-SH compounds binds with free radicals generated by ulcerogens including indomethacin and finally detoxify them. NP-SH compounds are also able to control mucus production, and recycling of antioxidants.[49, 50] Our results expressed that significant inhibition in protective effects of TRG after NEM treatment in comparison with the TRG treated animals, indicating the gastroprotective effects of TRG are at least partly mediated by NP-SH compounds. In stomach physiologic functions such as gastric blood flow regulation, acid secretion, and stomach contractility have been mediated by the opening of K^+ATP channels, a class of ligand-gated proteins. [39] In this study, the gastroprotective mechanism of TRG was K^+ATP -channel independent, since its protective activity was not affected by pretreatment with glibenclamide, a potent antagonist of these channels. Presynaptic α_2-receptors regulate different activities in the gastrointestinal tract including the regulation of gastric acid secretion.[51] Pretreatment of yohimbine (α_2-receptor antagonist) unable to block the protective effect of TRG against indomethacin-induced ulcer, indicating α_2-receptors did not involve in gastroprotective effect of TRG.

Conclusions

To sum up, this is the first report to determine the defensive effects of TRG against indomethacin-induced gastric ulcer model in rats. Overall evidences were depicted in this study that TRG vividly overcome the oxidative stress, cytokines imbalance, inflammation and apoptosis through augmenting the activities of antioxidant enzymes, preventing the production of inflammatory markers, inhibition of microvascular permeability and anti-apoptotic activities.

From these total observations, we concluded that TRG has a solid preventive potential against gastric ulcer induced by indomethacin.

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Figure legends

Figure 1 Macroscopic appearance of the gastric mucosa. (A) Normal group, (B) Indomethacin-induced ulcer group, (C) TRG (45 mg/kg) pretreated group, and (D) OMP (40 mg/kg) pretreated group. Indomethacin-induced sever injuries to the gastric mucosa that appear as elongated bands of hemorrhage (yellow arrow). **Note:** OMP (omeprazole); TRG (trigonelline).

Figure 2 Effect of TRG (15, 30, 45, 60 and 75 mg/kg) on indomethacin-induced ulcer index in rats. Values are mean ± SD ($n = 6$). $^{\dagger}P < 0.05$ compares IND with all the groups; $^{ns}P < 0.05$ compare **TRG 45 mg/kg with TRG 60 and 75 mg/kg. Note:** OMP (omeprazole); TRG (trigonelline); IND (indomethacin).

Figure 3 (A) Effect of TRG (45 mg/kg) on gastric SOD, CAT and GSH-Px level, (B) MDA and MPO level, (C) PGE_2, and LTB_4 level in indomethacin-induced ulcerated rats. Values are mean ± SD ($n = 6$). $^{\dagger}P < 0.05$ compares IND with all the groups. **Note:** TRG (trigonelline); IND (indomethacin).

Figure 4 (A) Effect of TRG (45 mg/kg) on gastric TNF-α and IFN-γ level, (B) IL-6 and IL-1β level, (C) IL-10 and IL-4 level in indomethacin-induced ulcerated rats. Values are mean ± SD ($n = 6$). $^{\dagger}P < 0.05$ compares IND with all the groups. **Note:** TRG (trigonelline); IND (indomethacin).

Figure 5 Effect of TRG (45 mg/kg) on protein expression level of eNOS, iNOS, COX-1, IL-10 and TNF-α in gastric mucosa. Levels of protein of interest were normalized to the level of β-actin. Data were expressed as mean ± SD ($^{\dagger}P < 0.05$ when compared to the IND group). **Note:** TRG (trigonelline); IND (indomethacin).

Figure 6 (A) Effect of TRG (45 mg/kg) on gastric ICAM-1 and VCAM-1 level, (B) E-selectin and P-selectin level, (C) VEGF, HGF and EGF level, D) cNOS, iNOS, tNOS and NO level in indomethacin-induced ulcerated rats. Values are mean ± SD (n = 6). $^{\dagger}P < 0.05$ compares IND with all the groups. **Note:** TRG (trigonelline); IND (indomethacin).

Figure 7 (A) Effect of TRG (45 mg/kg) on caspase-3 and apoptosis level, (B) p50 and p65 level, (C) gastric microvascular permeability level in indomethacin-induced ulcerated rats. Values are mean ± SD (n = 6). $^{\dagger}P < 0.05$ compare IND with all the groups; $^{*}P < 0.05$ compare TRG (45 mg/kg)+IND with SC560+TRG (45 mg/kg)+IND and celecoxib+TRG (45 mg/kg)+IND. **Note:** TRG (trigonelline); IND (indomethacin).

Figure 8 Effect of SC560 (COX-I specific inhibitor), celecoxib (COX-II specific inhibitor), YO (α_2-receptors antagonist), L-NAME (NOS inhibitor), NEM (endogenous sulfhydryl antagonist) and GLIB (K^+ATP channels antagonist) on ulcer protective effects of TRG (45 mg/kg) against indomethacin-induced ulcer. Values are mean ± SD (n = 6). $^{\dagger}P < 0.05$ compare IND with all the groups; $^{*}P < 0.05$ compare TRG (45 mg/kg)+IND with SC560+TRG (45 mg/kg)+IND or celecoxib+TRG (45 mg/kg)+IND or YO+TRG (45 mg/kg)+IND or L-NAME+TRG (45 mg/kg)+IND or NEM+TRG (45 mg/kg)+IND or GLIB+TRG (45 mg/kg)+IND. **Note:** TRG (trigonelline); IND (indomethacin); L-NAME (N_{ω} -Nitro-L-arginine methyl ester); NEM (N-ethylmaleimide); YO (yohimbine); GLIB (glibenclamide).

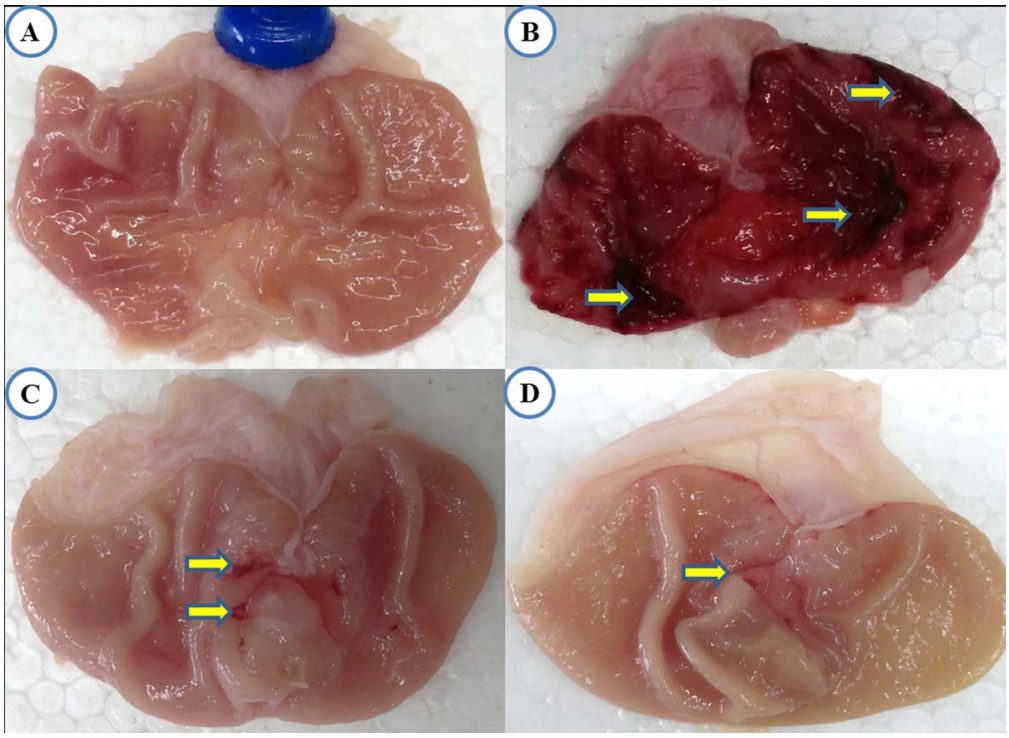

Fig. 1.
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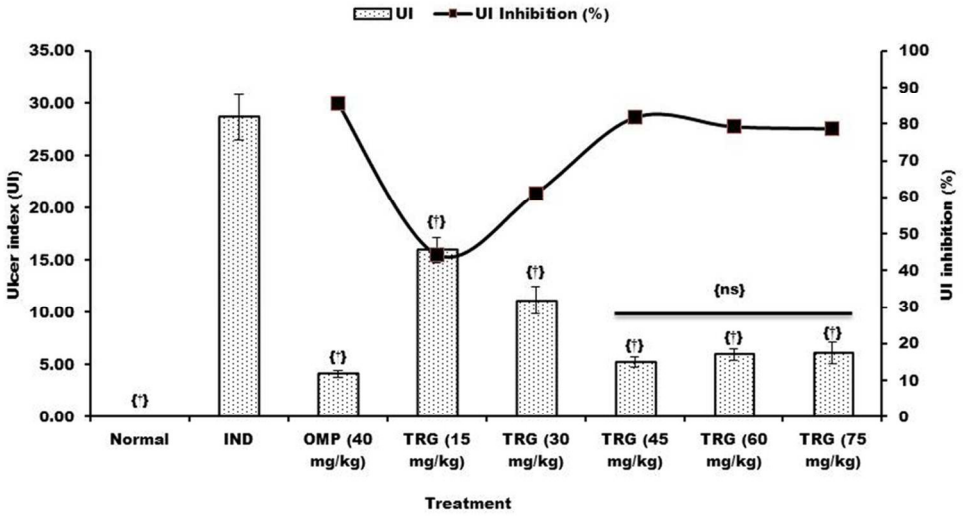

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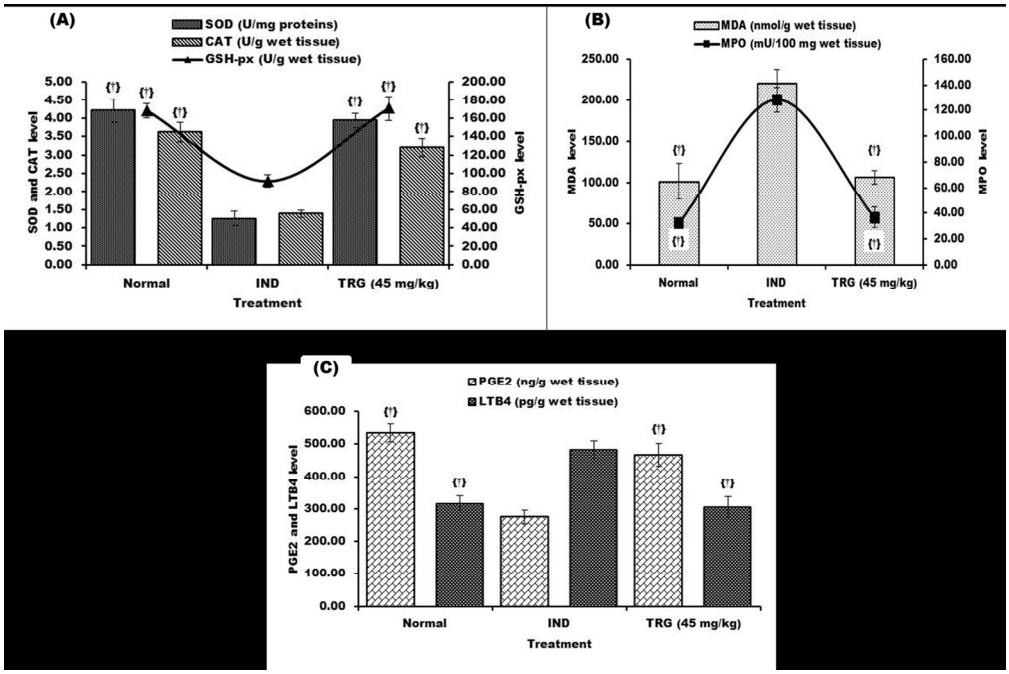

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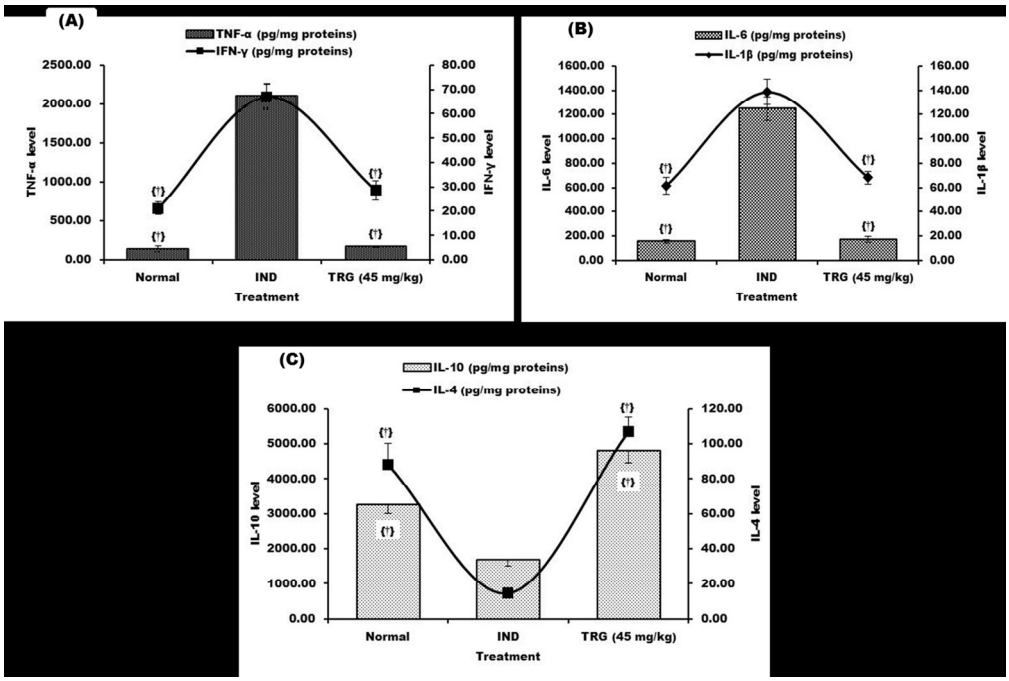

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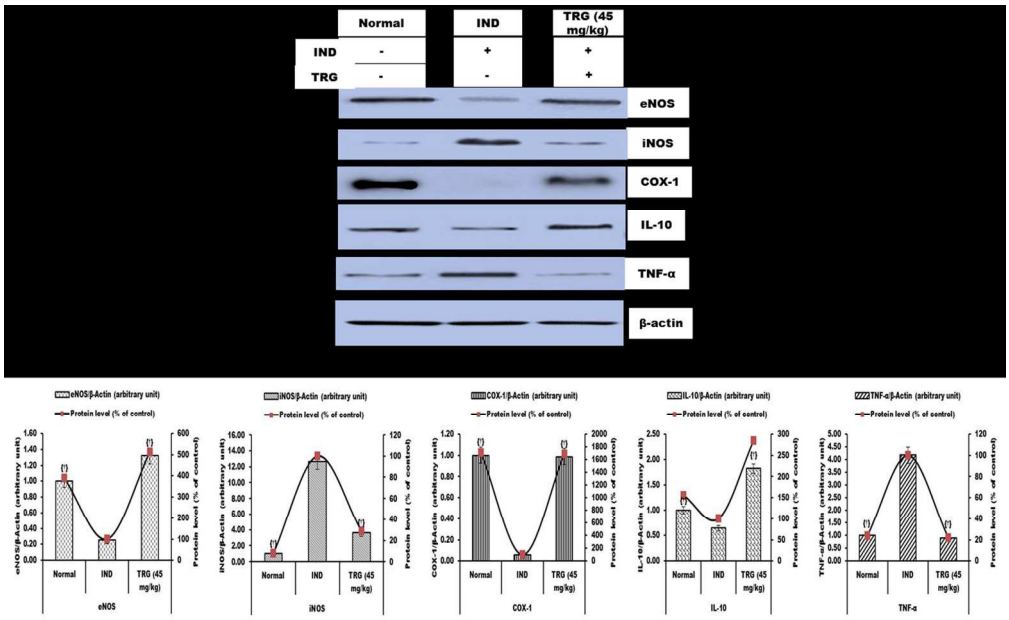

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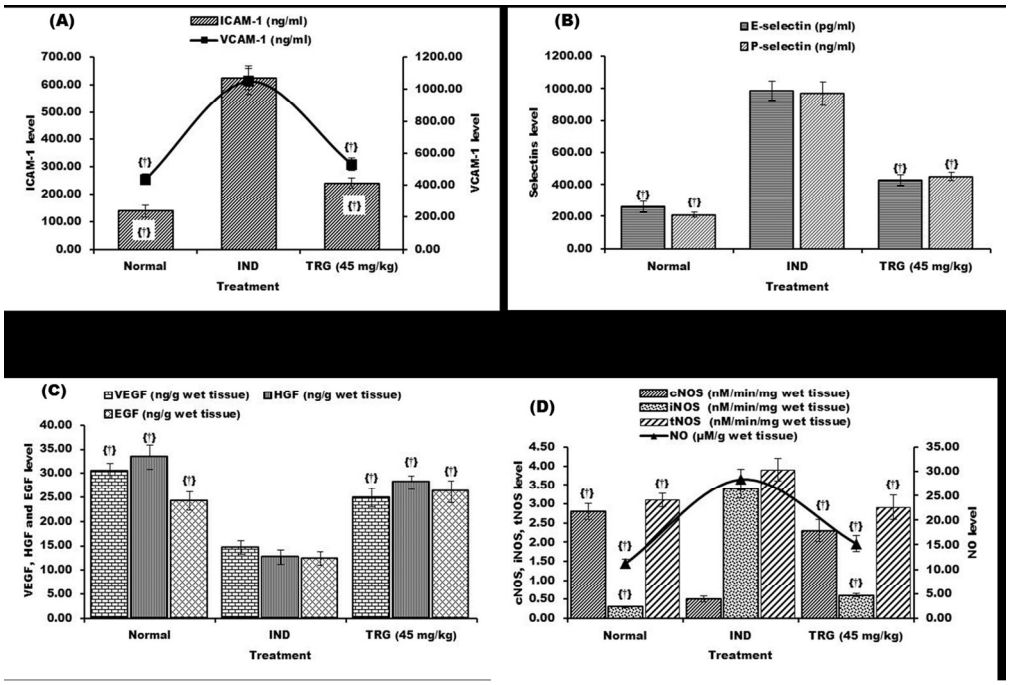

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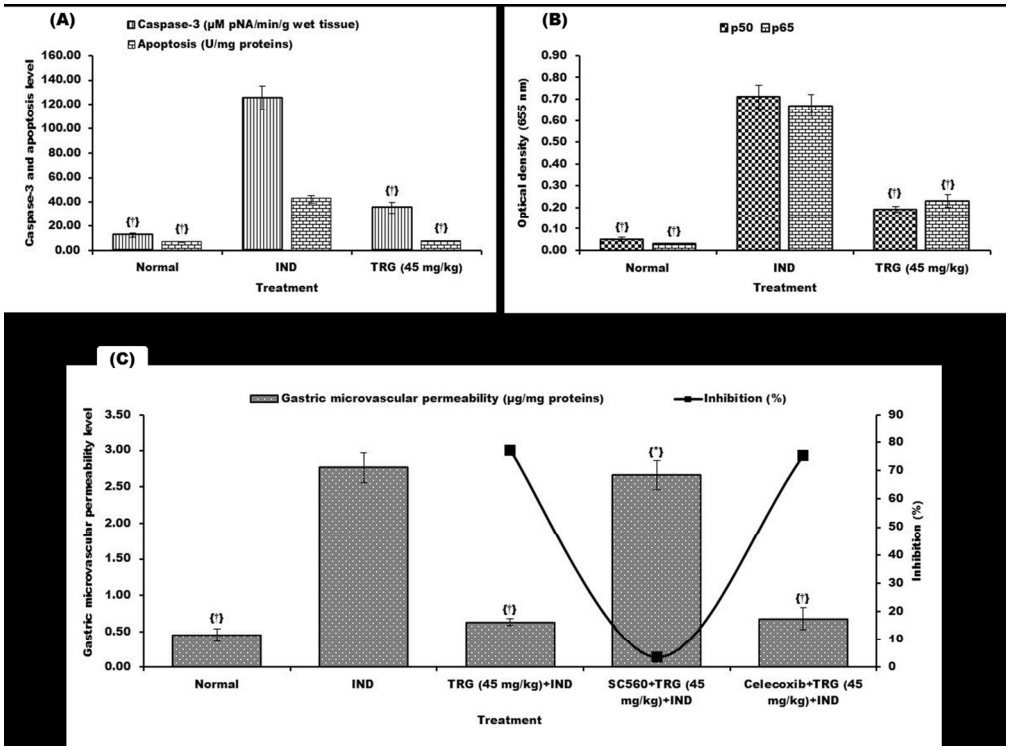

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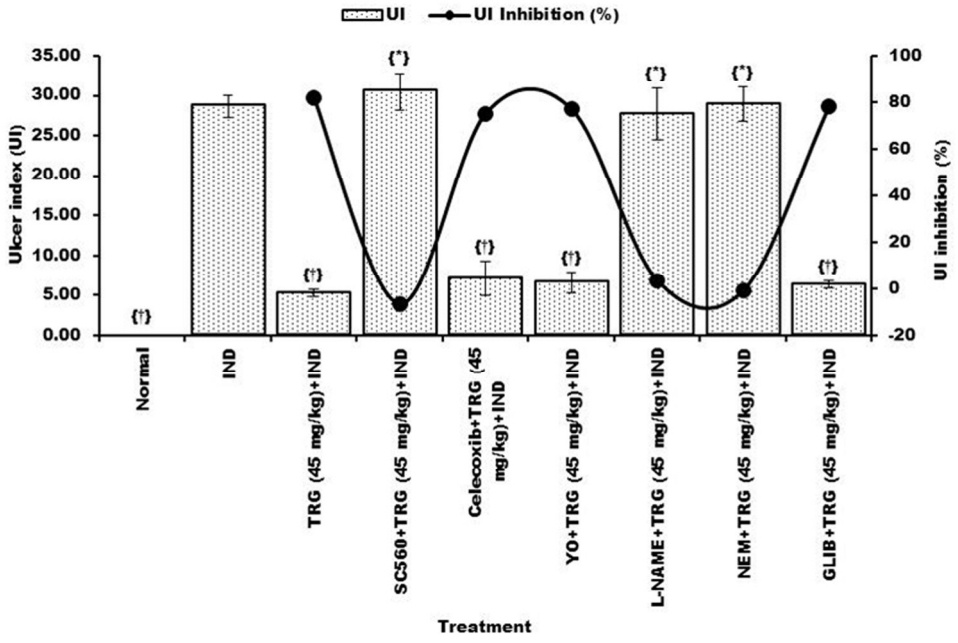

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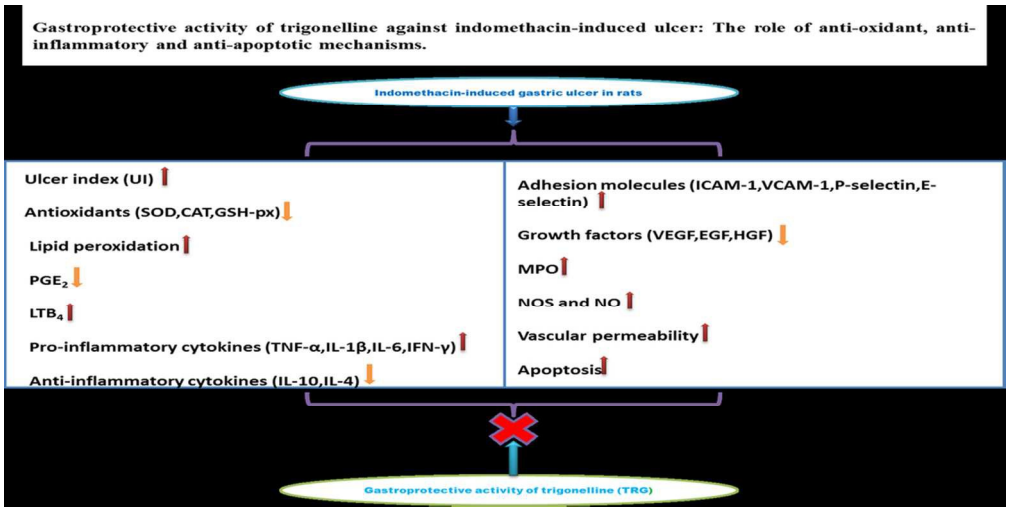

Graphical abstract.
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