Biochimica et Biophysica Acta 1850 (2015) 769–783

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Morin protects gastric mucosa from nonsteroidal anti-inflammatory drug, indomethacin induced inflammatory damage and apoptosis by modulating NF-κB pathway

Krishnendu Sinha, Pritam Sadhukhan, Sukanya Saha, Pabitra Bikash Pal, Parames C. Sil *

Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Kolkata 700054, India

ARTICLE INFO

Article history:
Received 8 July 2014
Received in revised form 24 December 2014
Accepted 12 January 2015
Available online 17 January 2015

Keywords:
NSAID
Morin
Gastropathy
Inflammation
Anti-inflammatory compound
Antioxidant

ABSTRACT

Background: Deregulation in prostaglandin (PG) biosynthesis, severe oxidative stress, inflammation and apoptosis contribute to the pathogenesis of nonsteroidal anti-inflammatory drug (NSAID)-induced gastropathy. Unfortunately, most of the prescribed anti-ulcer drugs generate various side effects. In this scenario, we could consider morin as a safe herbal potential agent against IND-gastropathy and rationalize its action systematically.
Methods: Rats were pretreated with morin for 30 min followed by IND (48 mg kg^{-1}) administration for 4 h. The anti-ulcerogenic nature of morin was assessed by morphological and histological analysis. Its effects on the inflammatory (MPO, cytokines, adhesion molecules), ulcer-healing (COXs, PGE_2), and signaling parameters (NF-κB and apoptotic signaling) were assessed by biochemical, RP-HPLC, immunoblots, IHC, RT-PCR, and ELISA at the time points of their maximal changes due to IND administration.
Results: IND induced NF-κB and apoptotic signaling in rat's gastric mucosa. These increased proinflammatory responses, but reduced the antioxidant enzymes and other protective factors. Morin reversed all the adverse effects to prevent IND-induced gastric ulceration in a PGE_2 independent manner. Also, it did not affect the absorption and/or primary pharmacological activity of IND.
Conclusions: The gastroprotective action of morin is primarily attributed to its potent antioxidant nature that also helps in controlling several IND-induced inflammatory responses.
General significance: For the first time, the study reveals a mechanistic basis of morin mediated protective action against IND-induced gastropathy. As morin is a naturally abundant safe antioxidant, future detailed pharmacokinetic and pharmacodynamic studies are expected to establish it as a gastroprotective agent.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed to recover clinical cases of pain and inflammation in rheumatic disorders and osteoarthritis [1]. These drugs are also used as antineoplastic agents and for the prevention and treatment of ischemic heart disease [2]. However, they also contribute to severe gastrointestinal complications like gastric mucosal bleeding, decreased gastric mucosal blood flow and induced mucosal cell apoptosis [1,3–7]. Among arthritis patients alone, around 16,500 NSAID related deaths occur in the United States annually [8]. Inhibition of the cyclooxygenases (COXs), subsequent reduction in prostaglandin (PG) synthesis along with the NSAID-induced production of reactive oxygen species (ROS) and associated gastric mucosal apoptosis are thought to be the most important reason of gastropathy [1]. Indomethacin (IND), a potent NSAID, was found to bind to a site near complex I and ubiquinone of mitochondrial electron transport chain to generate ROS [9]. ROS can also generate hydroxyl radical (•OH) by inactivating mitochondrial aconitase [10]. This inactivated aconitase produces free iron which subsequently generates more mitochondrial •OH [1,4,6]. The oxidative stress is associated with the uncoupling of mitochondrial respiration, formation of the mitochondrial permeability transition pore, mitochondrial dysfunction and generation of mitochondrial oxidative stress, which is associated with proinflammatory cytokine production and inflammation [1,3]. Inflammation plays a significant role in the pathogenesis of gastric mucosal injury [5,11]. The attraction and subsequent involvement of leukocytes to the specific tissue site are a hallmark incident in the initiation of inflammation and pathogenesis of gastric mucosa [12–15]. It is also becoming increasingly evident that various adhesion molecules mediated leukocyte–endothelial cell (EC) interaction which is an early and significant episode in the NSAID-induced gastropathy [16]. Activated neutrophils can induce injury by physically occluding micro-vessels via the production of many proinflammatory and pro-oxidative enzymes, for instance, myeloperoxidase (MPO) or through production of superoxide and other reactive oxidants [5,9,17]. These dramatically

* Corresponding author at: Division of Molecular Medicine, Bose Institute, P-1/12, CIT Scheme VII M, Kolkata 700054, West Bengal, India. Tel.: +91 33 25693243; fax: +91 33 2355 3886.
E-mail addresses: parames@jcbose.ac.in, parames_95@yahoo.co.in (P.C. Sil).

http://dx.doi.org/10.1016/j.bbagen.2015.01.008
0304-4165/

increase the oxidative burden of gastric mucosa and also damage endothelial lining [11,18–20]. Therefore antioxidant, anti-inflammatory and antiapoptotic therapy would be a rational approach in preventing NSAID-induced gastropathy.

In spite of medicinal advances, many of the currently prescribed anti-ulcer drugs (such as omeprazole, lansoprazole etc.) confer various side effects, and are expensive particularly for the rural population [21–26]. Considering these limitations, the development of affordable and safe anti-ulcer formulations is an important goal in medicinal research. From this perspective, herbal antioxidants may provide the desired anti-ulcer medications.

Morin (2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one) (Fig. 1a) is a kind of flavonoid belonging to the group of flavonoids found in almond (*Psidium guajava*) [27], mill (*Prunus dulcis*), old fustic (*Chlorophora tinctoria*), osage orange (*Maclura pomifera*), fig (*Chlorophora tinctoria*) and other family members of Moraceae [28]. These are used as food and also as herbal medicines [29]. It has been shown to be acting as a potent antioxidant [30] and possessing various biological and biochemical effects including anti-inflammation [31], xanthine oxidase inhibition activity [32] etc. Most importantly morin also shows intestinal anti-inflammatory activity on chronic experimental colitis in the rat [33]. However, the protective role of morin against IND-induced gastric injury has not been investigated yet.

In this study, we investigated the protective effect of morin against IND-induced gastric injury in rats. For detailed study on the mechanism we have used rats and AGS cells as models. Here we report that morin corrects NSAID-induced inflammatory gastric mucosal damage and apoptosis by inhibiting ROS generation, inducible nitric oxide synthase (iNOS) activation, NF-κB activation and neutrophil infiltration. The study for the first time, reveals a mechanistic basis of morin mediated protection against IND-induced inflammatory gastric pathophysiology.

2. Materials and methods

2.1. Materials and reagents

Indomethacin (IND), DPPH (2, 2-diphenyl-1-picrylhydrazyl), collagenase type I, hyaluronidase, Rhodamine123 (Rh123) and 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Sigma (St Louis, MO, USA). Morin ($C_{15}H_{10}O_7$) (CAS number: 654055-01-3), methylthiazolyldiphenyl-tetrazolium bromide (MTT), and bovine serum albumin (BSA) were purchased from Sisco Research Laboratory (Mumbai, India). Formalin and dimethyl sulfoxide (DMSO) were obtained from Merck (Worli, Mumbai). Fetal bovine serum (FBS) was obtained from Gibco, Invitrogen (Carlsbad, CA, USA). Ham's F-12 medium was purchased from HIMEDIA (Mumbai, India). Halt Protease and Phosphatase Inhibitor Cocktail was obtained from Thermo Fisher Scientific Inc., USA. Primary antibodies against phospho-NF-κB p65 (p-NF-κB p65) (#3033), phospho-IKK (p-IKK) (#2859), phospho-IκBα (p-IκBα) (#2859), caspase-3 (#9662), PARP (#9532) and β-actin (#4970) were purchased from Cell Signaling (Cell Signaling Technology Inc., Danvers, MA). Anti-HSP70 (ab79852), anti-PGE_2 (ab2318), anti-IκB (ab32518), and HRP (ab97051) and FITC-tagged secondary (ab6717) antibodies were obtained from Abcam (Cambridge, UK). Anti-γ-actin

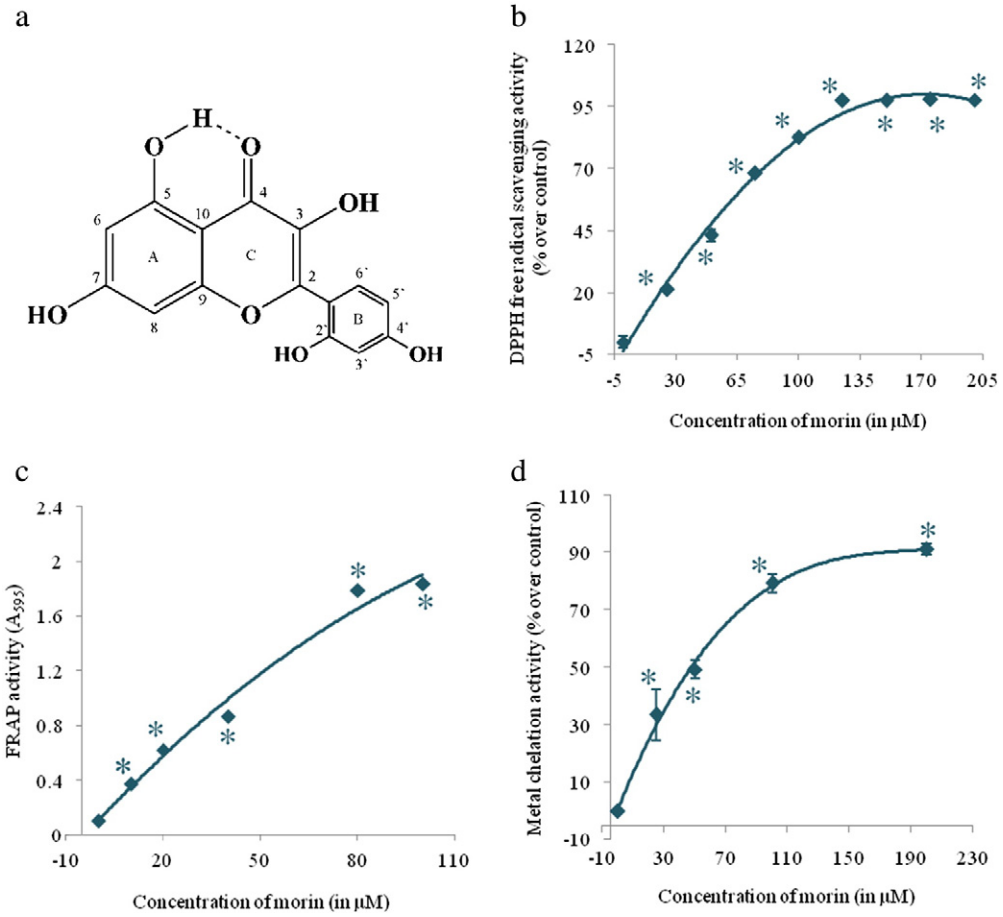

Fig. 1. Structure and biochemical properties of morin in vitro. (a) Structure of morin. (b)Morin showed dose dependent DPPH free radical scavenging activity expressed as % over control. (c). Change of absorbance at 595 nm wave length at various doses of morin in the FRAP activity assay. (d) Metal (iron) chelation activity of morin at various concentrations expressed as % over control. Data are represented as the mean ± SEM of three independent experiments. *P < 0.05 vs. Control.

(BB-AB0025) and anti-GAPDH (BB-AB0060) primary antibody was purchased from BioBharati (Kolkata). All additional chemicals used in the study were of the highest experimental grade available.

2.2. Determination of free radical scavenging activity of morin in vitro

DPPH radical scavenging activity was performed to check radical scavenging activity in vitro according to Blois [34]. Briefly, 1 ml of DPPH solutions (125 μM in methanol) and 1 ml of tested samples at different concentrations (0 to 200 μM with an increase of 25 μM) of morin were mixed in the tubes. The solution was incubated at 37 °C for 30 min in dark. The decrease in absorbance at 517 nm was measured spectrophotometrically against methanol blank. The ability of scavenging DPPH radical was calculated as % of DPPH scavenging over control. Ascorbic acid was used as the positive control.

2.3. Determination of total antioxidant activity (ferric reducing antioxidant power, FRAP) of morin in vitro

The FRAP assay was performed according to the protocol of Benzie and Strain [35] with few modifications. The fresh working FRAP solution was made up of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-striazine) and 20 mM $FeCl_3 \times 6H_2O$ solutions (10:1:1 respectively) and then warmed at 37 °C before using. Different concentrations of morin from 0 to 100 μM (0, 10, 20, 40, 80, 100 μM) were allowed to react with the FRAP solution for 30 min in the dark. The readings of the colored product were taken at 595 nm and the values were plotted against concentration of morin used (the more the antioxidant power the more the absorbance at 595 nm). Ascorbic acid was used as the positive control.

2.4. Determination of ferrous (Fe^{2+}) metal chelation activity of morin

The ferrous chelating power of morin was evaluated by the protocol of Dinis et al. [25] with few modifications. Briefly, morin at different concentrations ranging from 0 to 200 μM (0, 25, 50, 100, 200 μM) was incubated with 20 μM of Fe^{2+} and reaction was initiated by adding 100 μM ferrozine. After 10 min, the absorbance was read at 562 nm. The percentage of inhibition of Fe^{2+}–ferrozine complex formation (competitive inhibition) was calculated and expressed as metal chelation activity (% over control). EDTA was used as a positive control.

2.5. Cell culture and treatment

AGS cells were cultured in Ham's F-12 medium supplemented with 10% FBS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} amphotericin B at 37 °C and in 5% CO_2 [36]. The cells were exposed to different doses of IND (400, 800 and 1600 μM). After that the cells were maintained for 24 h and the viability was measured by MTT assay at the end. Briefly, MTT solution (0.5 mg ml^{-1} final concentrations) was added to the wells and incubated for 4 h at 37 °C in CO_2 incubator. After the incubation, 10 volumes of DMSO was added and the MTT reduction (absorbance of formazan dye) was measured at 570 nm (with a background subtraction of 630 nm) [37]. All experiments were carried out under sterile conditions in triplicate for the reproducibility. Here this is to mention that, we have chosen the secondary cell line (AGS) for the mitochondrial activity assay because it is a well-established supportive model (with in vivo experiments) in NSAID-induced gastric damage related studies [36].

2.6. Assay for mitochondrial metabolic function

Mitochondrial metabolic function was studied by observing the ability of mitochondrial dehydrogenases to reduce MTT into formazan dye [1,38]. Approximately 3.2×10^3 AGS cells were placed in each well of a 96-well plate and the wells were divided into six groups: control, IND treated (800 μM), 20 μM morin pretreated + IND treated (800 μM), 40 μM morin pretreated + IND treated (800 μM), 80 μM morin pretreated + IND treated (800 μM) and 160 μM morin pretreated + IND treated (800 μM). All the morin pretreatment was done 2 h before the IND treatment [39]. After incubation of 24 h (counted from IND exposure), MTT solution (0.5 mg ml^{-1} final concentration) was added to the wells and incubated for 4 h at 37 °C in a CO_2 incubator. After that, 10 volumes of DMSO was added and the MTT reduction (absorbance of formazan dye) was measured at 570 nm (with a background subtraction of 630 nm) [37].

2.7. Determination of mitochondrial membrane potential

In order to observe the change in the mitochondrial transmembrane potential ($\Delta\Psi_m$), cell permeant fluorescent cationic dye, Rhodamine123 (Rh123) was used. AGS cells were plated on 6-well plates in appropriate density and divided into three groups: control, ND treated (800 μM), 80 μM morin pretreated IND treated (800 μM). The morin pretreatment was done 2 h before the IND treatment [30]. After 24 h incubation, each group was pelleted by centrifugation at 1000 *g* for 3 min at 4 °C. Then 500 μl of Rh123 working solution (1 μg ml^{-1} Rh123 solution in PBS–BSA) was added to each pellet after optimum flicking. The mixture was then incubated for 10 min at 37 °C. At the end of incubation, tubes were maintained on ice. The tubes were centrifuged at 4 °C, at 1000 *g* for 10 min and resuspended in 500 μl of cold PBS–BSA. Throughout the procedure the samples were actively protected from light. FACS analyses of the samples were then carried out with FACSVerse at an excitation at 488 nm blue laser and collection filter for the green emission (530 nm) (FITC-A default filter) using the FACSuite software. The protocol has been adopted from Ferlini and Scambia [34].

2.8. Animals

Male albino Sprague–Dawley rats, weighing approximately 120–130 g, were acclimatized under laboratory conditions for 2 weeks before the experiments. The animals were maintained in a standard diet and water ad libitum. They were subjected to 12 h light and dark cycles under standard conditions of temperature and humidity. All the experiments with animals were carried out according to the guidelines of the Institutional Animal Ethical Committee (IAEC), Bose Institute, Kolkata.

2.9. Experimental protocol for induction of gastropathy and ulcer score (US) scoring

IND-induced gastric injury was carried as described below, following the established protocol of Biswas et al. [1,3,40]. Briefly, animals were starved for 24 h and then divided into three groups (n = 9 for each group): Control: received vehicle only (saline); IND: received IND treatment only at a dose of 48 mg kg^{-1} body weight (in saline); morin + IND: received 30 min pretreatment of morin (i.p.) at a dose of 50 mg kg^{-1} body weight (in saline), prior to IND treatment; morin: received only morin (i.p.) at a dose of 50 mg kg^{-1} body weight (in saline) [1,41]. They were supplied with water ad libitum. All the animals were sacrificed at 4 h after the IND treatment. Stomachs were collected, cut opened through greater curvature and cleaned properly in PBS. Then photographs were taken for the analysis of US and samples were stored at −80 °C for subsequent studies. The US scoring was done by using ImageJ 1.48s (NIH, USA). Briefly by using 'Threshold' application the damaged area was measured (Thresholding method: Default, Thresholding color: black and color spaces: HSB) and the score was represented as % of damage area over the total stomach area.

Here this is to mention that, the present study was conducted using the 48 mg kg^{-1} (p.o.) dose of IND based on earlier studies that identified this dose as the most effective dose for a robust and reproducible acute gastric ulceration in rats without any mortality [41]. The time course studies on the IND-induced gastric ulcerative damage in rats showed

gradual increase in the US that reached peak value at 4 h (Fig. 2). Hence, the ulcer-preventive properties of morin were evaluated on the 4 h followed by IND administration. Several studies showed that 30 min to 1 h pretreatment (i.p.) with potent gastroprotective agents (like omeprazole, gallic acid, NAC etc.) was optimally effective [41,42]. Initially, a pilot study was done to select the optimum dose of morin dissolved in water. Thirty minute pretreatment at three different doses (10, 30, 50 mg kg^{-1} body weight, i.p.) was selected for this purpose. It was observed that, at a dose of 50 mg kg^{-1}, morin significantly ($P < 0.05$) prevented the pathophysiological changes in gastric mucosa after IND administration (Fig. 2). The observation was based on the morphological changes, US and histopathological changes. Hence, the dose of 50 mg kg^{-1} was selected for this study. In this respect this is to mention that, several high doses (e.g. 400 mg kg^{-1}) of morin had already been used to see its pharmacological activities and were found to be entirely safe [43–46].

2.10. Histomorphometric evaluation of rat gastric mucosal damage

The histomorphometric estimation of gastric mucosal damage was carried out as per Natale et al. with few modifications [47]. Stomachs from the rats of different groups were opened through greater curvature, washed carefully in PBS and were fixed in 10% buffered formalin for 24 h at RT. Fixed tissues were processed for paraffin sectioning. Sections of 6 μm thickness were taken at different depths, in order to detect possible lesions lying within the 2 mm of thickness of the strips. These were stained with hematoxylin and eosin to evaluate the gastric pathophysiological changes under bright field light microscope (Leica Microsystem DN1000; camera: DFC450 C). The length of both total and damaged mucosa was evaluated by means of a micrometric scale. At least three histological sections of gastric mucosa were then examined for each rat, leading to a more accurate histological reading. The length fraction of damaged mucosa over the total mucosal length

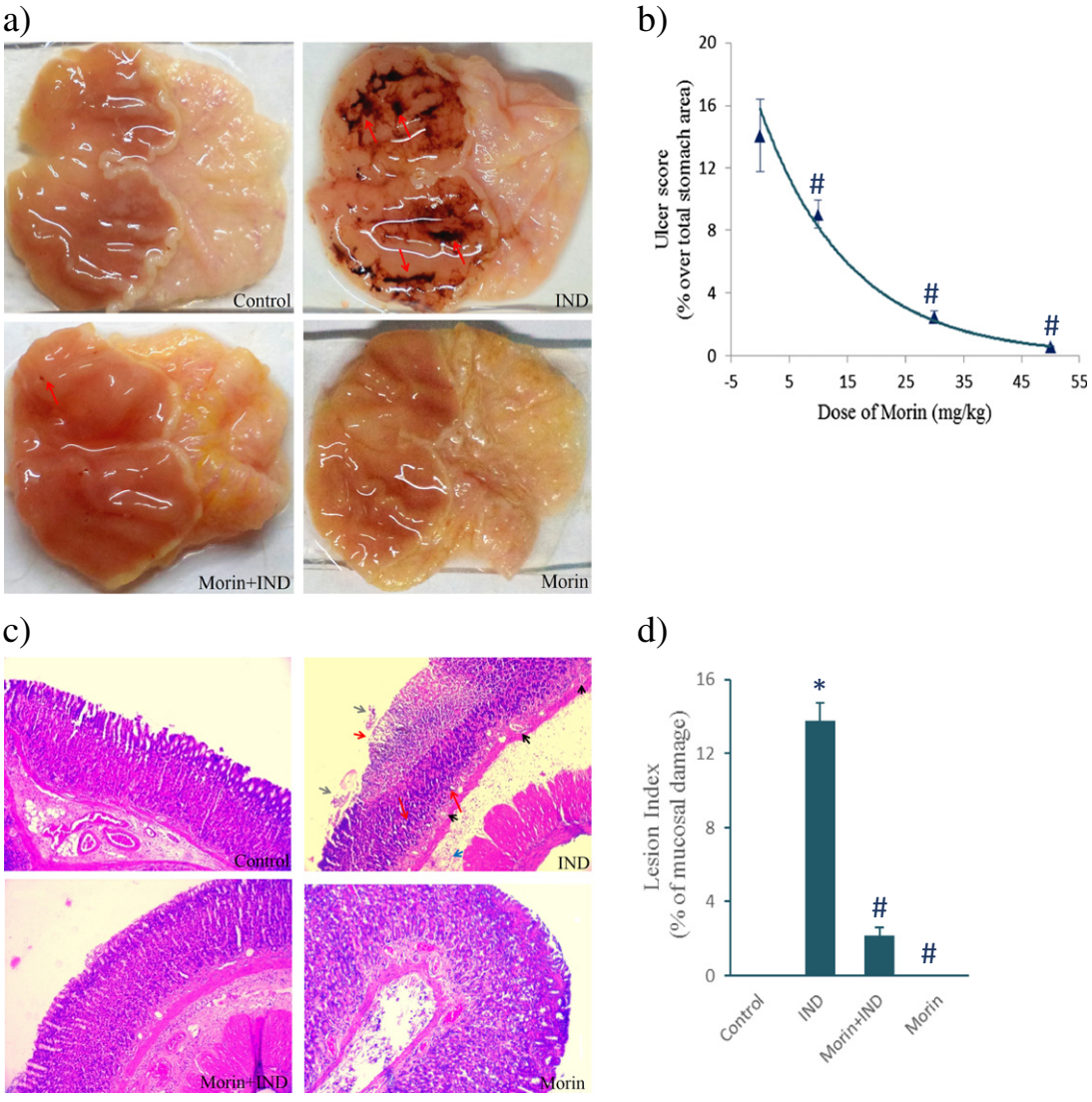

Fig. 2. Effect of morin on gastric mucosa in IND-induced gastric injury. Control: vehicle treatment alone; IND: 48 mg kg bw^{-1} IND treatment alone (for 4 h); morin + IND: treatment with 50 mg kg bw^{-1} morin (30 min pretreatment) and 48 mg kg bw^{-1} IND (for 4 h); Morin: 50 mg kg bw^{-1} morin treatment alone. (a) Open stomach showing injured mucosa. Note that the injury (in the form of reddish black ulcers of different sizes) is highest in the IND (red arrows) and morin almost completely prevented the ulceration. (b) Gastric injury was scored in US in respect to the whole opened stomach area (see the 'Materials and methods' section for the scoring scheme). (c) Sections of gastric mucosa were stained with hematoxylin–eosin 4 h after IND administration. The IND group showed marked changes with outward mucosal damage (red arrow), presence of inflammatory exudates (green arrows), extensive vasocongestion (black arrows) and damaged submucosa (blue arrow). (d) Histomorphometric estimation was scored in LI (see the 'Materials and methods' section for the scoring scheme). These changes were less evident in the morin + IND group and morin group showed no notable changes over the control group. Data are represented as the mean ± SEM ($n = 9$ for each experimental group). *$P < 0.05$ vs. Control; #$P < 0.05$ vs. IND.

was estimated as the lesion index (total damage; LI) and expressed in percentage of damages. The histomorphometric analysis was performed by three independent blind observers (i.e. who were unaware of the model of gastric damage and of the treatment received by animals).

2.11. Gastric emptying (GE) determination

Gastric emptying (GE) was determined using a modification of the technique previously described by Santos et al. [48]. Briefly, animals were starved for 24 h and then divided into three groups (n = 9 for each group): Control: received vehicle only (saline); IND: received IND treatment only at a dose of 48 mg kg^{-1} body weight (in saline); morin + IND: received 30 min pretreatment of morin (i.p.) at a dose of 50 mg kg^{-1} body weight (in saline), prior to IND treatment; Morin: received only morin (i.p.) at a dose of 50 mg kg^{-1} body weight (in saline). They were supplied with water ad libitum. Gastric emptying was determined in unanesthetized rats with the use of a non-absorbable marker, phenol red. Phenol red was dissolved in the physiological saline at the concentration of 0.5 mg ml^{-1} [49]. This solution (1.5 ml rat^{-1}) was gavage-fed. After 30 min, rats were sacrificed. Then the gastro-esophageal and gastroduodenal junctions were quickly clamped by laparotomy. Then the stomach and the small intestine was removed from the body and divided into stomach, proximal, medial and distal small intestine. Each segment volume was measured by adding 0.1 N NaOH solution (100 ml) to a graduated tube and was homogenized for 30 s. 5 ml of the supernatant was centrifuged for 10 min (1600 *g*). Proteins in 5 ml of homogenate were precipitated with 0.5 ml of 20% trichloroacetic acid (w/v), centrifuged for 20 min (1600 *g*), and 3 ml from the supernatant was added to 4 ml of 0.5 N NaOH solution. The residual dye in the stomach was determined from it by reading the absorbance at 560 nm. The gastric fractional phenol red retention was expressed in percentage, according to the following formula: gastric dye retention = amount of phenol red recovered in stomach/total amount of phenol red recovered from all four segments [48].

2.12. Evaluation of indomethacin absorption in serum via RP-HPLC

Blood samples from each group were collected after 2 h of IND administration for evaluation of its absorption. Blood samples were centrifuged at 500 *g* for 20 min and subsequently at 1000 *g* for 20 min. The resulting plasma was mixed with of acetonitrile (1:1 v/v). Denatured protein precipitates were separated using centrifugation at 12,000 *g* for 10 min. The resultant supernatant was directly injected into the column after filtering. The concentration of indomethacin in plasma was measured using a Shimadzu model LC20-AT pump system equipped with a diode array model SIL-M20A detector and an analytical Phenomenex C_{18} reversed-phase column or analytical Inertsil ODS-3 column (MetaChem Technologies) attached to a model SIL-20A autosampler. The IND was eluted using a programmed gradient solvent system at a flow rate of 1.0 ml min^{-1} and detected at 254 nm along with diode array analysis. The mobile phase was a 10 min isocratic flow of acetonitrile: 50 mM phosphate buffer (pH 4) (40:60 v/v). The data was analyzed by the software LCsolution Version 1.21.

2.13. Quantitative and qualitative measurements of PGE_2 in gastric mucosa by ELISA and immunofluorescence microscopy

Rats were divided into 4 groups: treated with vehicle, IND and/or morin as per the experimental design discussed above. All the animals were sacrificed at 4 h after the IND treatment. Stomachs were collected, weighed and kept in 100% ethanol supplemented with 100 mM indomethacin. Tissues were then cropped into pieces and homogenized and centrifuged at 12,000 *g* for 10 min at 4 °C. Supernatant was used for quantitative measurement of PGE_2 by indirect ELISA following standard protocol of Abcam using Abcam anti-PGE_2 primary antibody. Again to check the expression pattern of PGE_2, immunofluorescence was used using the Abcam anti-PGE_2 primary antibody. Isolated stomach tissues from each group were fixed by immersion in 10% buffered formaldehyde and embedded in paraffin. Tissue sections (6 μm) were deparaffinized with xylene and rehydrated with ethanol water down gradation. The slides were treated with heat mediated antigen retrieval method (in microwave) by boiling samples in 0.1 M citrate buffer (pH 8.8) for 20 min to obtain an adequate signal. The samples were then rinsed with tris buffered saline (TBS). After treatment with blocking buffer, the slides were incubated overnight at 4 °C with an anti-p-PGE_2 antibody (dilution 1:100). The primary antibody was localized using the goat anti-rabbit FITC-linked secondary antibody, followed by mounting in VECTASHIELD Mounting Medium with DAPI (Vector laboratories). All the slides were imagined under Leica Microsystem DN1000 (camera: DFC450 C) microscope.

2.14. MPO activity assay of gastric mucosa

MPO activity with 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was measured following the method of Suzuki et al. [50]. Briefly, 10 μl samples (gastric mucosa tissue homogenate) from each group were combined with 80 μl of 0.75 mM H_2O_2 and 110 μl TMB solution and incubated at 37 °C for 5 min. The reaction was terminated by adding 50 μl of 2 M H_2SO_4 and absorbance was measured at 450 nm to calculate the MPO activity, expressed as μM of H_2O_2 consumed min^{-1} mg^{-1} protein.

2.15. SOD and GST activity assay of gastric mucosa

SOD activity measurement was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with nitro blue tetrazolium (NTB) to form formazan dye [51]. It was then determined at 560 nm by the degree of inhibition of this reaction and is expressed as millimole per minute per milligram tissue (mmol min^{-1} mg $tissue^{-1}$).

Total GST activity was determined as described by Dengiz et al. [51] Briefly, the enzyme activity was assayed spectrophotometrically at 40 nm in a 4-ml cuvette containing 0.1 M PBS (pH 6.5), 30 mM glutathione, 30 mM 1-chloro-2,6-dinitrobenzene, and tissue homogenate. Enzyme activity was expressed as nanomole per minute per milligram protein (nmol min^{-1} mg $tissue^{-1}$).

2.16. Subcellular fractionation of gastric mucosa to separate nuclear, mitochondrial and cytoplasmic fractions

The subcellular fractionation was performed according to Cox and Emili [52] with slight modification. Briefly, after removing the stomach the gastric mucosa was scrapped and collected into appropriate containers. Then the tissue was cleaned in cold PBS rinsed once in 5 volumes ice-cold 250-STMDPS buffer (250 mM sucrose, 50 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 25 μg ml^{-1} Spermine, 25 μg ml^{-1} Spermidine) supplemented with protease and phosphatase inhibitors [Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Fisher Scientific Inc., USA] and homogenized in 5 volumes of ice-cold 250-STMDPS Buffer. The homogenate was centrifuged at 800 *g* for 15 min at 4 °C. For isolation of nuclear protein, 5 volumes of 250-STMDPS buffer was added to the pellet and re-homogenized and centrifuged at 800 *g* for 15 min at 4 °C. The pellet (nuclei) was then suspended in five volumes of protease and phosphatase inhibitor supplemented NET buffer (20 mM HEPES pH7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% glycerol, 1% Triton-X-100, 1 mM DTT, 1 mM PMSF) and incubated for 30 min with occasional vortexing at 4 °C and finally lysed by sonication. Then, it was centrifuged at 14,000 *g* for 25 min at 4 °C and the supernatant was used for Western blotting in respect to NF-κB p65 and Lamin B1.

2.17. Western immunoblotting

For Western blot analysis, an equal amount of protein from whole lysate of each sample was resolved by 10–12% SDS-PAGE (as required) following standard protocol [39] and allowed to react against anti-Hsp70, anti-Iκβ, anti-caspase-3, anti-PARP and anti-β- and γ-actin and GAPDH primary antibodies separately at provider's recommended dilutions. Nuclear fraction was used for the Western blot analysis of p-NF-κB. Primary antibodies were detected against HRP-conjugated secondary antibody using the HRP substrate ECL solution.

2.18. RNA extraction and reverse transcriptase PCR (RT-PCR)

RNA was extracted from the gastric mucosal tissue of all three groups of rats using the TRIzol reagent, following the manufacturer's protocol (Invitrogen, Carlsbad, CA). The amount of RNA was measured spectrophotometrically using nanodrop, HellmaTrayCell Type 105.810 (Hellma Analytical). Two (2) μg of RNA from each sample was converted to cDNA using Thermo Scientific Verso cDNA synthesis kit (Thermo Scientific, USA). Thermal cycling was performed as follows: 95 °C for 300 s (initial denaturation) followed by the set of cycles: 95 °C for 30 s (denaturation), 55 °C for 30 s (primer annealing), and 72 °C for 45 s (primer extension). After 35 cycles the time for DNA extension was 5 min at 72 °C. The PCR amplification products were then held at 4 °C. After that, the PCR amplified products were subjected to electrophoresis on 1.5% agarose gel. The product size and annealing temperatures of the primers used were given in Table 1.

2.19. Immunohistochemical analysis of p-IKK level

Immunohistochemical staining for p-IKK was performed as described elsewhere [53]. Isolated stomach tissues from each group were fixed by immersion in 10% buffered formaldehyde and embedded in paraffin. Tissue sections (6 μm) were deparaffinized with xylene and rehydrated with ethanol water down gradation. The slides were treated with heat mediated antigen retrieval method (in microwave) by boiling samples in 0.1 M citrate buffer (pH 8.8) for 20 min to obtain an adequate signal. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min and the samples were then rinsed with tris buffered saline (TBS). After treatment with blocking buffer, the slides were incubated overnight at 4 °C with an anti-p-IKK antibody (dilution 1:100). The primary antibody was localized using the goat anti-rabbit HRP-linked secondary antibody, followed by reaction with diaminobenzidine (DAB) as the chromogen and counterstaining with hematoxylin and mounted in DPX. All the slides were imagined under Leica Microsystem DN1000 (camera: DFC450 C) microscope and evaluated by using ImageJ 1.48s plugin extension, IHC Profiler (NIH, USA).

2.20. Agarose gel electrophoresis for DNA fragmentation

The DNA fragmentation assay was performed by using electrophoresing genomic DNA samples, isolated from normal as well as experimental kidney, on agarose/EtBr gel by the procedure described by Sellins and Cohen [54,55].

2.21. Statistical analysis

Results are expressed as mean ± SEM. Statistical evaluation of data has been done by means of one-way analysis of variance (ANOVA) and the group means were compared by Tukey test. A p-value less than 0.05 is considered as statistically significant.

3. Results

3.1. Morin possesses excellent free radical scavenging, antioxidant and metal chelation activities

Fig. 1b shows that the morin significantly ($p < 0.05$) scavenges free radical (DPPH free radical) in a concentration dependent manner showing IC_{50} at approximately 59 μM. Morin also has excellent antioxidant activity assessed by FRAP method showing IC_{50} at 38 μM (Fig. 1c). Fig. 1d shows that the morin extensively chelated metal (iron) with IC_{50} of 50 μM.

3.2. Morin inhibits mitochondrial dysfunction in IND-exposed gastric cells

Mitochondrial dehydrogenases are one of the most important machineries of the electron transport chain and are prone to ROS mediated damage [56]. IND dose dependently reduced mitochondrial dehydrogenases activity in AGS cells showing IC_{50} at 800 μM (Fig. 3a). Morin pretreatment significantly recovered ($p < 0.05$) the mitochondrial dehydrogenase activity in a dose dependent manner (Fig. 3b). Mitochondrial dehydrogenase activity was restored optimally in AGS cells treated with both 80 μM morin and 800 μM IND than in those treated with only IND. In all the respective doses morin remains non-toxic as assessed by MTT assay (data not shown).

Table 1
Primer details.

Gene	Primer sequence (5′ to 3′)	Annealing temperature (°C)	Amplicon size (bp)
TNF-α	Fp: CTGAAGTAGTGGCCTGGATTG Rp: GCTGGTAGTTTAGCTCCGTTT	51	424
IL-1β	Fp: CTTCCTAAAGATGGCTGCACTA Rp: ATCCCATACACACGGACAAC	51	307
IL-6	Fp: CAGAGCAATACTGAAACCCTAGT Rp: TTCTGACCACAGTGAGGAATG	51	262
MCP-1	Fp: GTGTCCCAAAGAAGCTGTAGTA Rp: AAGGCATCACATTCCAAATCAC	51	297
ICAM-1	Fp: CACCATGCTTCCTCTGACAT Rp: CACTGCTCGTCCACATAGTATT	51	283
iNOS	Fp: TCAGGAAGAAATGCAGGAGATG Rp: GCTCTTGAGCTGGAAGAAGTAA	58	283
Catalase	Fp: GTACAACTCCCAGAAGCCTAAG Rp: CCAAACAGAAGTCCTAAGCAGA	51	347
COX-1	Fp: GCCATGGCTGCATAGTTAGA Rp: CCCGCCAGATAGTCAACATT	47.3	170
COX-2	Fp: ACTCATATAGCAAACTGCGTAGA Rp: ATTTGTAATCAGTTCTCGATGC	67.5	180
β-Actin	Fp: TCCCTGGAGAAGAGCTATGA Rp: ATAGAGCCACCAATCCACAC	51	332

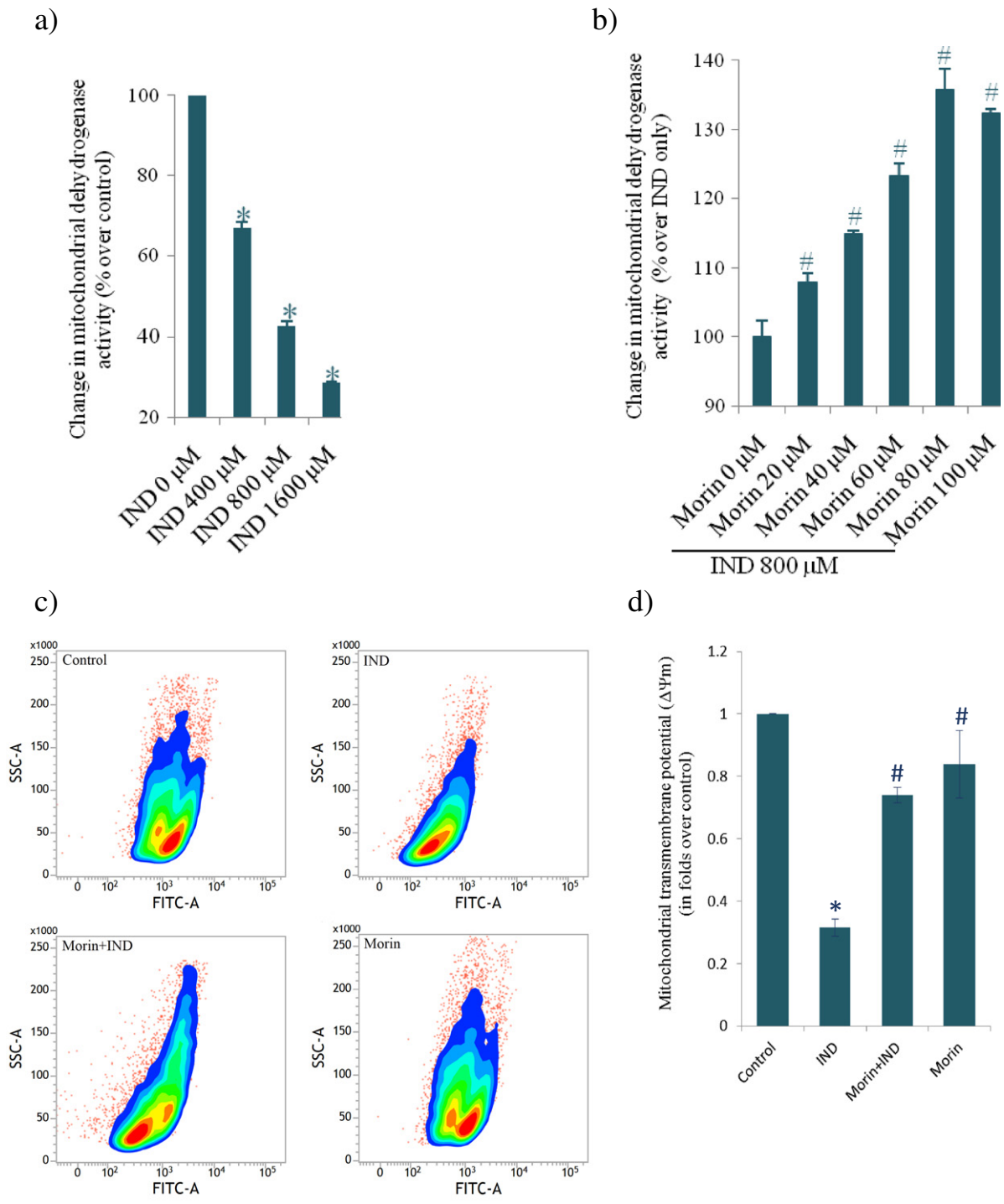

Fig. 3. Effects of morin on mitochondrial dysfunction in IND-exposed AGS cells. Cells were exposed to vehicle alone (Control), 800 μM IND alone (IND), 80 μM morin and 800 μM IND (morin + IND), 80 μM Morin alone (Morin). (a) Effect of IND on the mitochondrial activity of the AGS cells. (b) Effect of morin pretreatment on the mitochondrial dehydrogenase activity of IND exposed AGS cells. (c, d) Contour plots analysis of $\Delta\Psi_m$ along with bar graph showing data expressed as folds of change in Rh123 count over Control. FITC-A filter is the default setting of the FACSuite software used interchangeably with Rh123 spectral range. Data are represented as the mean ± SEM of three individual experiments. *P < 0.05 vs. Control; #P < 0.05 vs. IND.

ROS and related mitochondrial stress and dysfunction play a pivotal role in the IND-mediated gastric inflammation and damage [3]. For this reason, we used the mitochondrial $\Delta\psi_m$ (through Rh123 staining) and dehydrogenase activities (through MTT reducing power) to evaluate the role of morin against IND-induced mitochondrial dysfunction by blocking ROS production. 800 μM IND-exposed AGS cells displayed significant fall ($p < 0.05$) in $\Delta\psi_m$ over control. This fall in $\Delta\psi_m$ was marked ($p < 0.05$) recovered by pretreatment with 80 μM morin (Fig. 3c, d).

3.3. Morin ameliorates IND-induced gastric mucosal damage in a dose dependent manner

IND induced atrophy and degeneration of mucosal lining, ulceration and oozing out of blood in gastric mucosal lining. Blood contains hemoglobin that appeared black due to oxidation in an acidic environment of the stomach [41]. However, morin pre-treatment limited these pathophysiological changes which are visible from Fig. 2a.

Further, US and qualitative histopathological analysis (LI) confirmed the finding. Pretreatment with the morin significantly reduced the US and LI compared to the score in stomachs from rats treated with IND alone (Fig. 2b–d). Histopathological analysis also revealed active gastritis at 4 h post-IND administration. The control group showed intact mucosal epithelium, with the presence of healthy blood vessels in the serosal layer and lack of any inflammatory exudates. In the IND group, superficial mucosal damage, presence of inflammatory exudates and severe vasocongestion were observed. Pretreatment with the morin reduced all these changes and restore the mucosa almost up to the standard conditions (Fig. 2).

3.4. Morin restores catalase level in IND-exposed gastric mucosa

Catalase is an essential endogenous Phase II detoxification antioxidant enzyme that protects virtually all cells and tissues from ROS damage. ROS often negatively regulates this enzyme [57,58]. Therefore, RT-PCR was performed to investigate whether morin could restore catalase levels in IND-treated gastric mucosa after confirming the involvement of ROS (data not shown). Catalase expression was markedly ($p < 0.05$) decreased in the IND-treated rat gastric mucosa with respect to the control rats. Catalase was restored in gastric mucosa treated with both morin and IND than in those treated with only IND (Fig. 4c, d).

3.5. Morin reduces IND-induced delay in gastric emptying and does not hamper IND uptake

Several studies have shown that NSAIDs are associated with altered gastroduodenal motility and it was demonstrated that IND administration considerably delays gastric emptying [48]. Delay in gastric emptying creates abdominal discomfort and gastrointestinal pathophysiology. In our study we found that IND exposure significantly delays gastric emptying while morin has no restoring effect (Fig. 5a). However, it is to mention that morin itself did not affect the gastric emptying.

To see whether morin inhibits IND absorption by any means we have checked the serum level of IND in all the four groups under study. We found that morin has no significant effect on the plasma level of the IND in respect to only IND-treated group ($p < 0.05$) (Fig. 5b). Here it is to mention that we have found no IND in the serum of the vehicle and the only morin-treated group.

3.6. Morin inhibits the infiltration of neutrophil in IND-induced damaged gastric tissue and restores mucosal oxidative status

The process of neutrophil infiltration at the site of tissue injury is a synchronized action controlled by cytokines, chemokines and cell adhesion molecules [3]. MPO activity is being used widely as a biomarker for quantitative assessment of neutrophil infiltration at the site of inflammation because MPO is mainly produced by neutrophils [59]. Thus, the MPO activity was assessed in gastric mucosa obtained from IND-treated rats, which showed a significant increase ($p < 0.05$) compared to those from vehicle-treated control rats. However, morin pre-treatment significantly reduced ($p < 0.05$) the MPO activity in the IND-exposed gastric mucosa compared to the group treated with only IND (Fig. 5c).

Antioxidant enzymes are directly associated with the oxidative status of tissue and protect it under severe oxidative stress conditions, either directly or indirectly [10]. Two of those most important enzymes are SOD and GST. In our study, we have found that, IND exposure extensively decreases their activity while morin pretreatment significantly ($p < 0.05$) restores their activity and thus assists the mucosa to combat IND-induced oxidative stress (Fig. 5d, e).

3.7. Morin reduces a number of proinflammatory cytokines, iNOS and MCP-1 expressions in IND-induced gastric injury

IND administration results in the upregulation of a number of proinflammatory cytokines, proinflammatory enzyme and chemokine expressions in the gastric mucosa. Therefore, RT-PCR was performed to evaluate whether morin could reduce the high expression of these molecules upon IND-induced gastropathy. Gastric mucosa from IND-treated rats showed a marked elevation ($p < 0.05$) in the expression of TNF-α, IL-1β and IL-6 compared to those from vehicle-treated control rats (Fig. 6a, b). Morin significantly ($p < 0.05$) reduced these expressions in the IND-exposed gastric mucosa compared to the group treated with only IND (Fig. 6a, b). As with proinflammatory cytokine expression, the proinflammatory enzyme, iNOS and chemokine, MCP-1 expression was also markedly ($p < 0.05$) elevated in the IND-treated rat gastric mucosa with respect to the control rats (Fig. 6c, d). Morin also considerably ($p < 0.05$) reduced both iNOS and MCP-1 expression from the level seen in IND-treated animals (Fig. 6c, d).

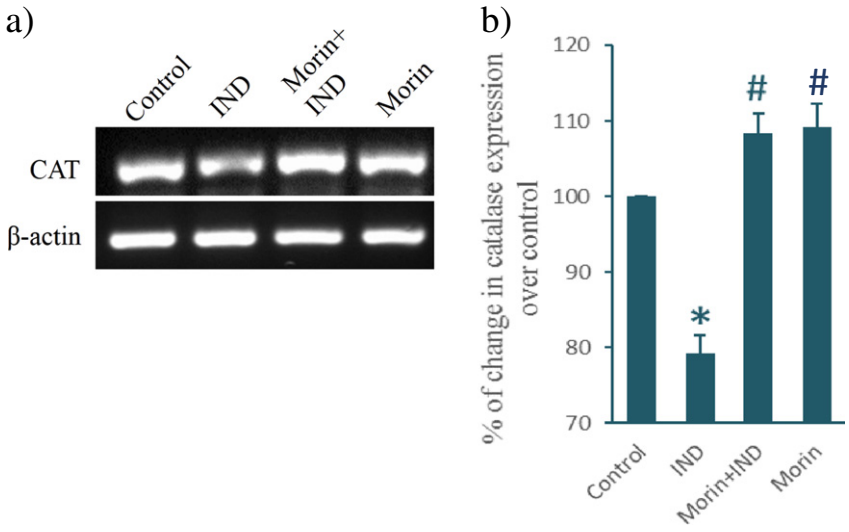

Fig. 4. Effects of morin on the expression of catalase in IND-induced gastric injury. Rats were administered with vehicle alone (Control), 48 mg kg^{-1} IND alone (IND), or 50 mg kg^{-1} morin and 48 mg kg^{-1} IND (morin + IND); Morin: 50 mg kg^{-1} morin treatment alone. (a,b) RT-PCR data and the corresponding densitometric analysis of catalase expression in the gastric mucosa. Note that IND administration decreases catalase expression while morin reverse these changes and there is no notable difference between Control and morin groups. Data in the densitometric analysis are represented as the mean ± SEM of three independent experiments. *P < 0.05 vs. Control; #P < 0.05 vs. IND.

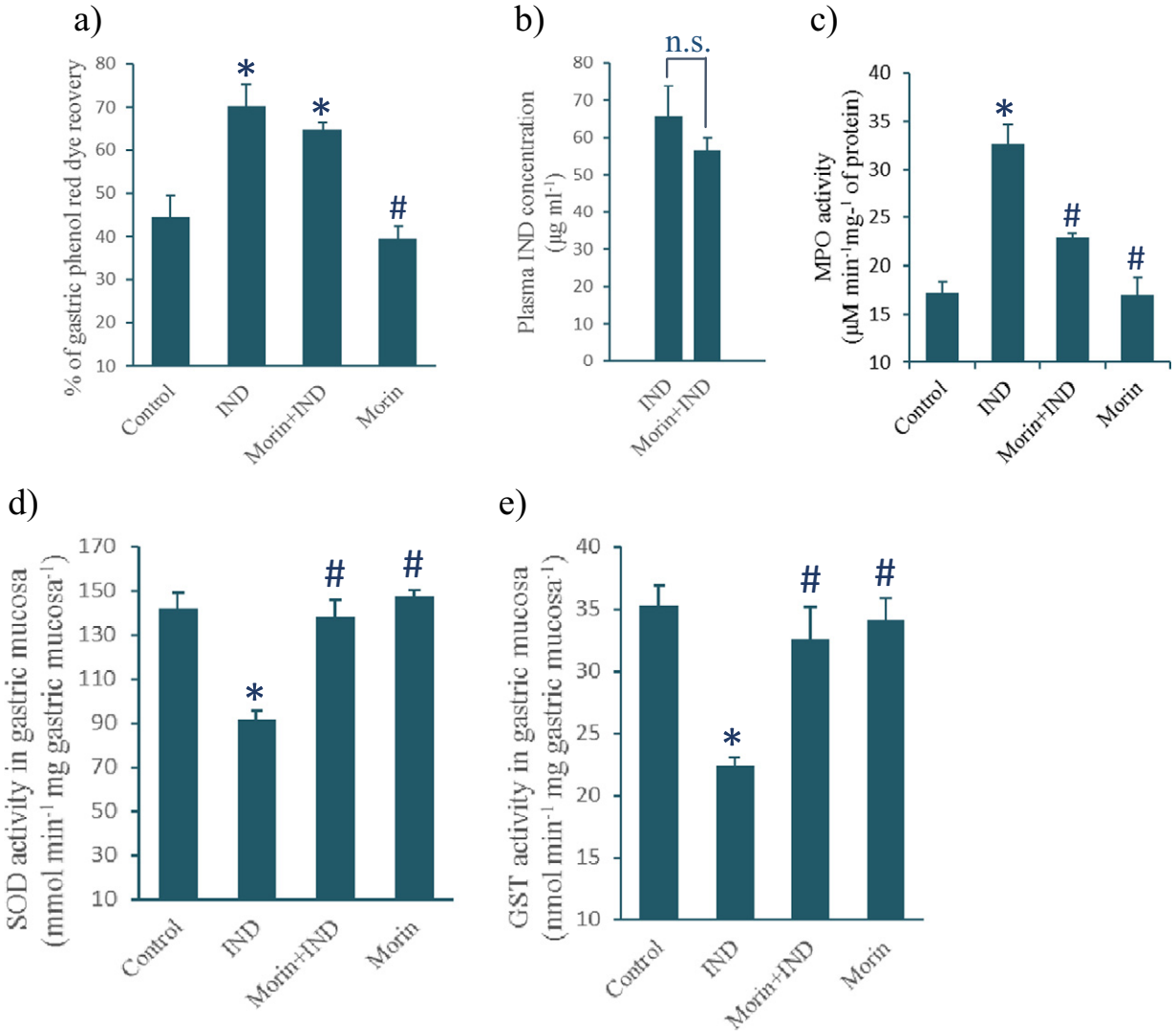

Fig. 5. Effect of IND and morin on GE, plasma level of IND, MPO activity, SOD activity, and GST activity. Control: vehicle treatment alone; IND: 48 mg kg^{-1} IND treatment alone (for 1 h); morin + IND: treatment with 50 mg kg^{-1} morin (30 min pretreatment) and 48 mg kg^{-1} IND (for 1 h); morin: 50 mg kg^{-1} morin treatment alone. (a) There is no notable difference between the Control and morin groups in respect to GE. (b) There is no significant difference between IND and Morin + IND group in respect to serum IND level. (c) MPO activity analysis in gastric mucosa, represented in terms of expressed as μM of H_2O_2 consumed min^{-1} mg^{-1} protein (μM min^{-1} mg^{-1} protein). Note that, IND administration significantly increased MPO activity in the tissue, while 30 min pretreatment of morin significantly decreased the gastric mucosal MPO activity. (d) and (e) respectively representing the SOD and GST activity in gastric mucosa, in terms of expressed as mM min^{-1} mg mucosal $tissue^{-1}$ and nM min^{-1} mg mucosal $tissue^{-1}$ (respectively). Data in the graphical analysis are represented as the mean ± SEM (n = 6–9 for each experimental group). *P < 0.05 vs. Control; #P < 0.05 vs. IND. n.s. indicates 'non-significant' difference.

3.8. Morin reduces ICAM-1 expressions in IND-induced gastric injury

Accumulation of proinflammatory cytokines can trigger the expression of adhesion molecules. In this way, IND administration results in the increased expression of ICAM-1 in gastric mucosa. Hence, to see whether morin could decrease the expression of ICAM-1, with respect to IND exposed rat gastric mucosa, RT-PCR was performed. RT-PCR analysis revealed that the expression of the cell adhesion molecule was significantly ($p < 0.05$) lesser in the gastric mucosa of morin-treated rats than those of only IND-treated ones (Fig. 6c, d).

3.9. IND differentially affects COX-1, COX-2 and PGE_2 irrespective of the morin

IND is a strong non-selective cyclooxygenase inhibitor and this is one of the causes of its adverse effect on gastric mucosa. Hence, RT-PCR and Western blot analyses were performed to evaluate whether morin could alter these molecules upon IND-induced gastropathy. We found that, upon IND exposure transcript level of COX-1 remains almost unaltered while the expression level of COX-2 was significantly elevated ($p < 0.05$) (Fig. 7a–c). Whereas, both ELISA and immunofluorescence study showed decreased level of PGE_2 ($p < 0.05$) in the IND-treated rats with respect to the control or only morin-treated group (Fig. 7d and e). It is notable here that morin itself has no significant effect on the level of COX-1, COX-2 or PGE_2. However, it down-regulates the increased COX-2 level in IND-treated group while it has no significant effect on the IND-induced down-regulation of PGE_2 level.

3.10. Morin prevents IND-induced phosphorylation of IKK in damaged gastric mucosa

IND treatment results in inflammation of the gastric mucosa mediated by activation of IKK [3]. Therefore, we used immunohistochemical staining to assess whether morin could attenuate the activation of IKK. Stomach sections from IND-treated rats displayed significant increased ($p < 0.05$) p-IKK staining in the mucosal layer of the stomach compared

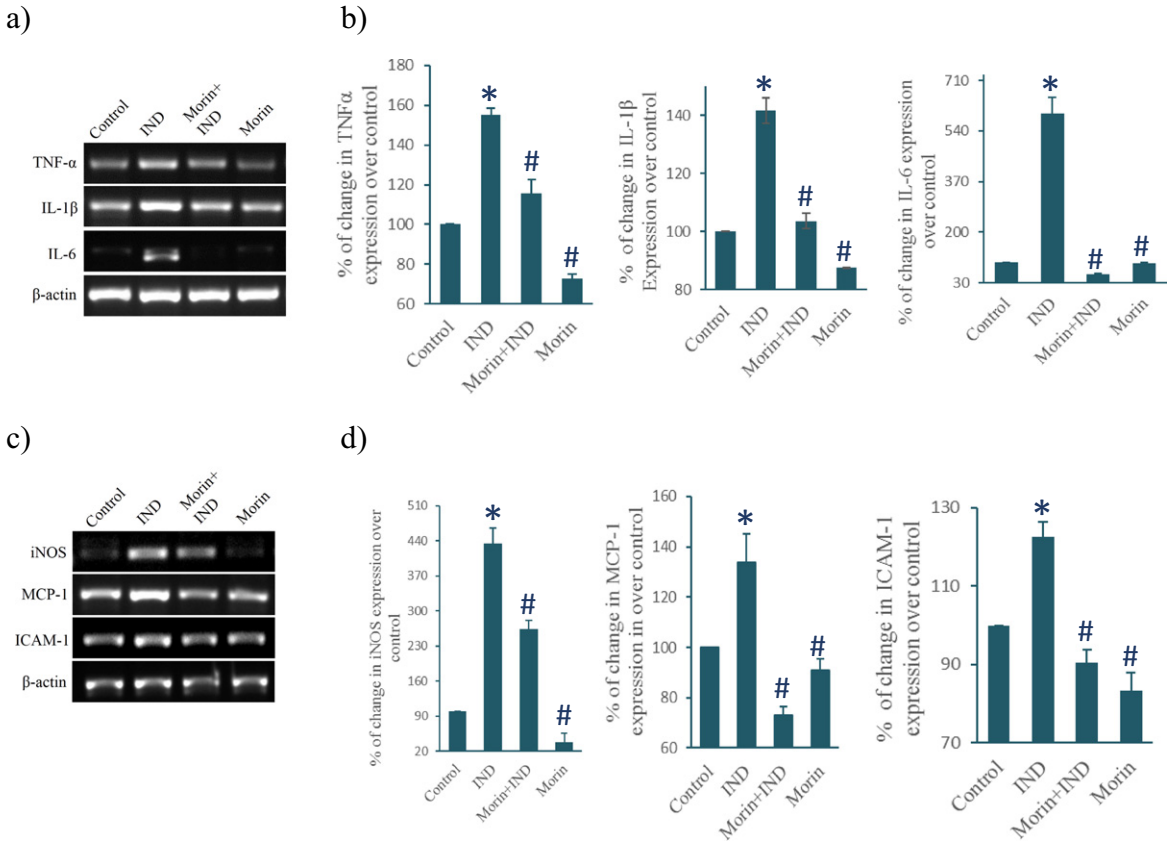

Fig. 6. Effect of morin on proinflammatory cytokines, chemokine, cell adhesion molecule and iNOS expressions in IND-induced gastric injury Control: vehicle treatment alone; IND: 48 mg kg^{-1} IND treatment alone (for 4 h); morin + IND: treatment with 50 mg kg^{-1} morin (30 min pretreatment) and 48 mg kg^{-1} IND (for 4 h); morin: morin: 50 mg kg^{-1} morin treatment alone. (a, b) RT-PCR data showed changes in the expression of proinflammatory cytokines (TNF-α, IL-1β and IL-6) and the respective densitometric analysis of the RT-PCR data. (c, d) RT-PCR data to follow the changes of the expression of iNOS, chemokine (MCP-1) and the cell adhesion molecule (ICAM-1) with respective densitometric analysis. Note that, after 4 h of IND administration, expressions of these proinflammatory molecules, iNOS, MCP-1 and ICAM-1 are highest in the IND groups and 30 min pretreatment of morin significantly decreased the expression of these cytokines. There is no notable difference between Control and morin groups. Data in the densitometric analysis are represented as the means ± SEM of three independent experiments. *P < 0.05 vs. Control; #P < 0.05 vs. IND. (e) Data in the densitometric analysis are represented as the mean ± SEM (n = 9 for each experimental group). *P < 0.05 vs. Control; #P < 0.05 vs. IND.

to those from control rats. Morin pretreatment reduced p-IKK staining ($p < 0.05$) compared to the group treated with IND alone (Fig. 8a, b).

3.11. Morin inhibits IND-induced IκBα degradation and NF-κB activation in gastric injury

Inflammation related genes (e.g. proinflammatory cytokines, chemokines, and cell adhesion molecules) are mostly controlled by the transcription factor NF-κB which in turn is controlled by the inhibitor of NF-κB, the IκBα. Again IκBα activity in turn depends on the modulation of its stability through specific phosphorylation. Upon phosphorylation it got degraded. However, previous reports also confirmed the role of NF-κB in the IND-related gastric inflammation and pathophysiology [42]. Therefore, immunoblotting was carried out to assess the cellular status of IκBα (both the phospho and the native forms) and nuclear localization of p65 in gastric mucosa from IND- and morin-treated rats. As evident from immunoblotting, the amount of p-IκBα increased and IκBα decreased in the gastric mucosa of IND-treated rats over the control group. However, in rats pretreated with the morin as well as IND, or treated with morin alone, the IκBα level was higher than in the IND-treated group (Fig. 8c). This clearly implies that IND-induced the phosphorylation and degradation of IκBα which was prevented by morin. Consistent with the IκBα immunoblotting data, another immunoblot analysis showed that levels of p-NF-κB p65 protein in nuclear protein extracts of IND-treated gastric mucosa were higher than in the control tissue while, morin pre-treatment considerably reduced the p-NF-κB p65 levels than in the nuclear protein extracts of IND-treated group (Fig. 8d).

3.12. Morin restores IND-induced HSP70 to its basal level in IND-exposed gastric mucosa

IND treatment results in stress and activation of HSP70 in the gastric mucosa [41]. Hence, we were interested to see the effect of morin on the HSP70 in the IND-induced gastric mucosal damage and conducted immunoblotting. It was evident that the morin pretreatment reduced the HSP70 up-regulation in the IND-induced gastric mucosa compared to the only IND-treated group (Fig. 9a).

3.13. Morin prevents apoptosis with the down-regulation of extracellular death signal activated apoptotic pathway in IND-exposed gastric mucosa

Increased TNF-α expression is related to NSAID-induced gastropathy and it is well established that many times TNF-α activated extracellular death signal leads to apoptosis following extrinsic pathways [10]. Immunoblot analysis showed an increase in activated caspase-3 and PARP cleavage in IND-treated gastric mucosa while the morin decreased expression of IND-induced apoptotic markers (Fig. 9a). Besides, IND caused the formation of apoptotic DNA laddering in the gastric mucosa, and morin prevents the laddering formation in the IND-treated gastric injury (Fig. 9b).

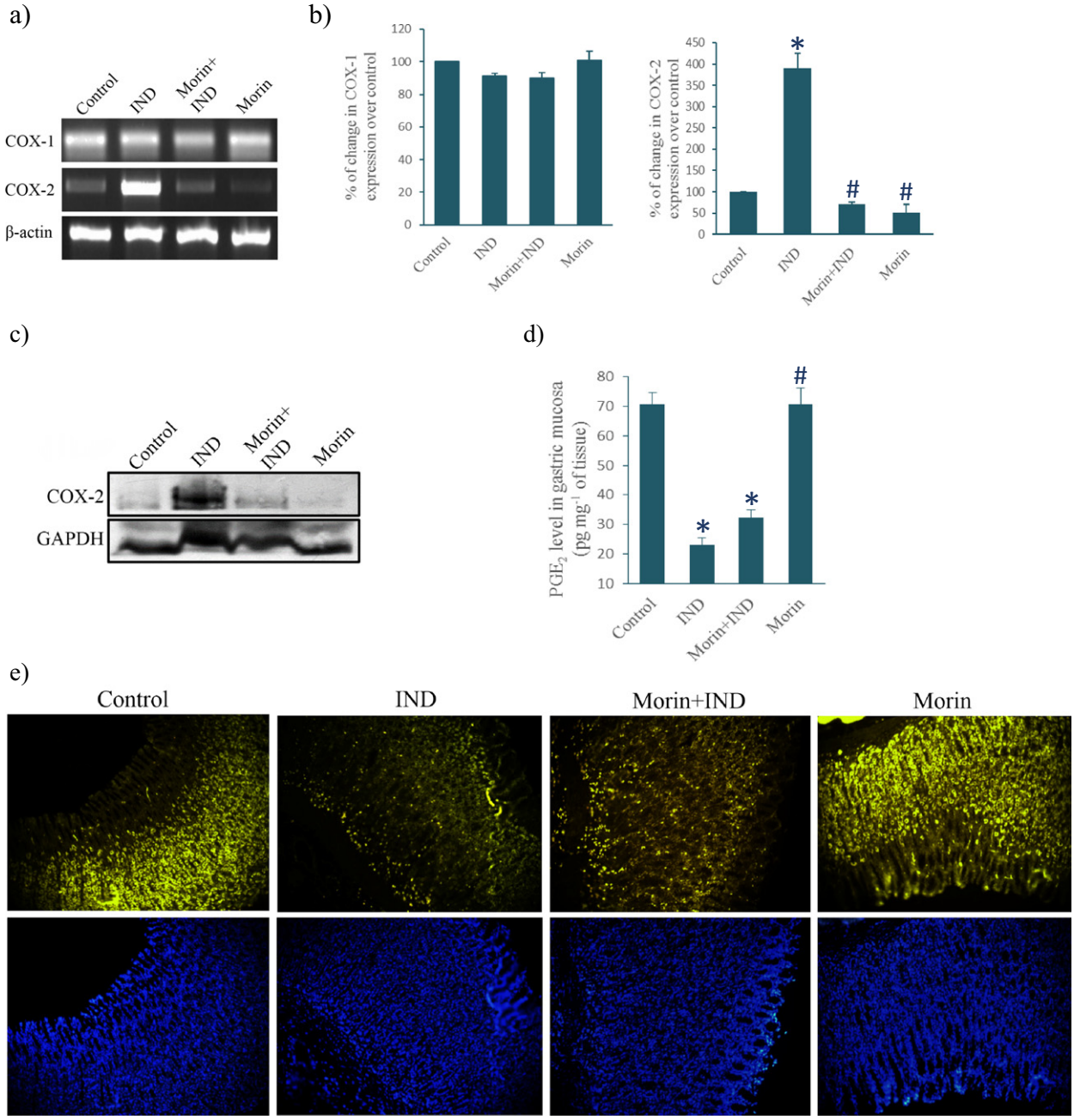

Fig. 7. Effect of IND and morin on COX-1, COX-2 and PGE_2 level in IND-induced gastric injury Control: vehicle treatment alone; IND: 48 mg kg^{-1} IND treatment alone (for 4 h); Morin + IND: treatment with 50 mg kg^{-1} morin (30 min pretreatment) and 48 mg kg^{-1} IND (for 4 h); Morin: Morin: 50 mg kg^{-1} morin treatment alone. (a, b) RT-PCR data showed changes in the expression of COX-1 and COX-2 as well as the respective densitometric analysis of the RT-PCR data. (c) Western blot showing expression change of COX-2 over different groups. (d) ELISA data showing the changes the mucosal tissue level of PGE_2. (e) Immunofluorescence micrograph showing qualitative spatial expression of PGE_2. Here it is clearly visible that the expression of the PGE_2 is mostly restricted to the deep mucosal, submucosal and muscularis region of the gastric mucosa. Note that, after 4 h of IND administration, expression level of COX-2 was increased, that of PGE_2 was decreased and the expression level of COX-1 remained unaltered. There is no notable difference between Control and morin groups. Data in the densitometric analysis are represented as the mean ± SEM of three independent experiments. *$P < 0.05$ vs. Control; #$P < 0.05$ vs. IND.

4. Discussion

In this study, we have established that (1) the administration of morin reduces IND-induced gastric injury as assessed by morphological and histopathological measures; (2) morin reduces the IND-induced inflammation by preventing the up-regulation of iNOS, proinflammatory cytokines (TNF-α, IL-1β, IL-6), chemokine (MCP-1) and cell adhesion molecule (ICAM-1) which results in decreased infiltration of neutrophils into the gastric lesion as evident from decreased tissue MPO activity; (3) administration of morin reduces IND-induced oxidative stress by enhancing the activity and/or production of antioxidant enzymes SOD2, GST and catalase, in gastric mucosal tissue; (4) administration of morin inhibits IKK and thus NF-κB activation in IND-treated gastric mucosa; (6) morin prevents the IND-induced mitochondrial dysfunction in AGS; (7) morin reduces IND-induced cellular stress as evidenced by reduced level of the stress induced molecular chaperone, the HSP70; (8) morin prevents IND-induced apoptosis in gastric mucosa by down-regulating caspase-3 activation and PARP cleavage; and (9) morin exerts its protective action irrespective of any regulation on PGE_2.

The structure of morin (Fig. 1a) shows the presence of a hydroxyl group at position 3, a resorcinol moiety, and a carbonyl group at position 4. The hydroxylation pattern on B-ring, shows both ortho and para

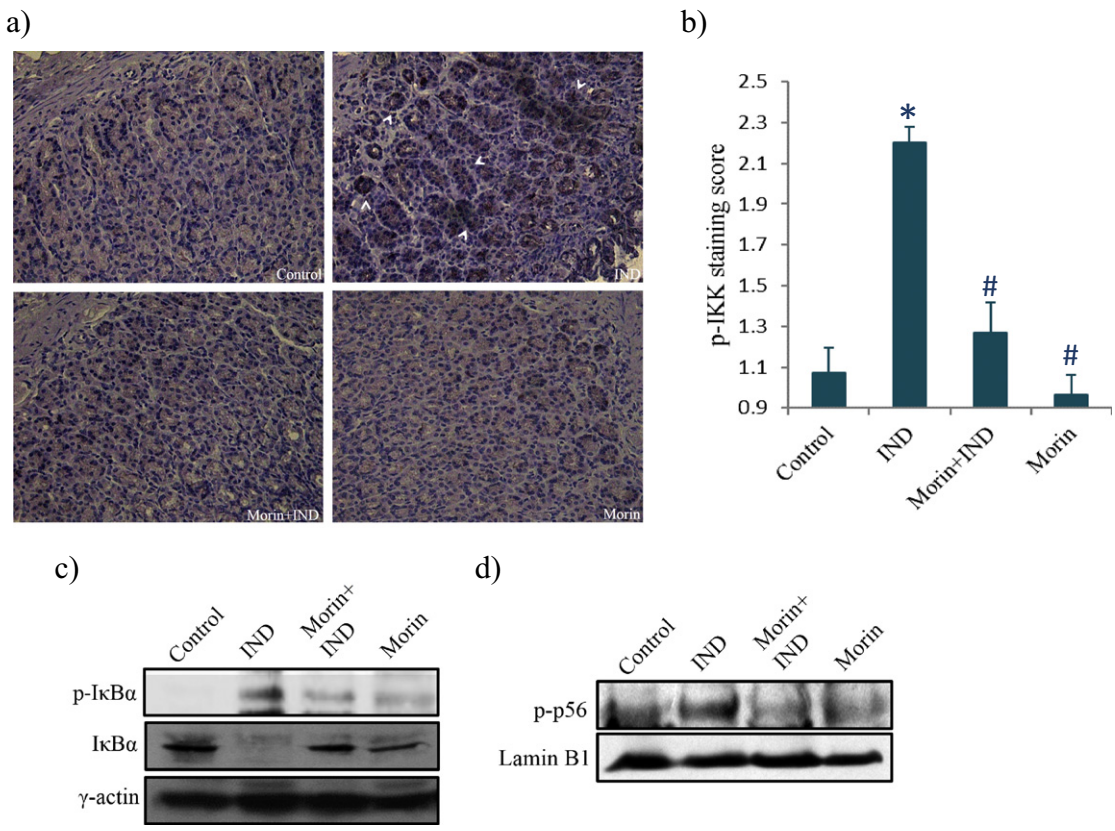

Fig. 8. Effect of morin on NF-κB activation in IND-induced gastric injury. Control: vehicle treatment alone; IND: 48 mg kg^{-1} IND treatment alone (for 4 h); morin + IND: treatment with 50 mg kg^{-1} morin (30 min pretreatment) and 48 mg kg^{-1} IND for 4 h; morin: morin: 50 mg kg^{-1} morin treatment alone. (a) Immunohistochemical detection of the level of p-IKK in stomach sections. (b) Score of p-IKK staining (see the 'Materials and methods' for the scoring method). Note that, after 4 h of IND administration, p-IKK level increased significantly in IND group (soiled black stained regions, arrows) compared to Control while 30 min pretreatment of morin lowers the p-IKK level (Morin + IND group) than the IND group. Data are represented as the means ± SEM (n = 9 for each experimental group). *P < 0.05 vs. Control; #P < 0.05 vs. IND. (c) Immunoblot analysis of p-IκBα and IκBα from whole lysate of the gastric mucosa of all the four groups. (d) Immunoblot analysis of p-NF-kB p65 expression in nuclear fraction from gastric mucosa of all the four groups. Note that, IND administration decreases cellular IκBα level (thus degradation) and increases nuclear localization of p-NF-κB in the IND-treated groups while morin reverses these changes. There is no notable difference between Control and morin groups. Data in the densitometric analysis are represented as the mean ± SEM of three independent experiments. *P < 0.05 vs. Control; #P < 0.05 vs. IND.

hydroxyl group with respect to the flavonoid moiety, suggesting its higher effectiveness, even over quercetin [45]. Morin possesses substantial intestinal antiapoptotic and anti-inflammatory potential [33,60]. It inhibits the release of the proinflammatory mediator, IL-6 and TNF-α release in human mast cells [61]. Although the anti-inflammatory and anti-apoptotic activities of morin were known, no information was

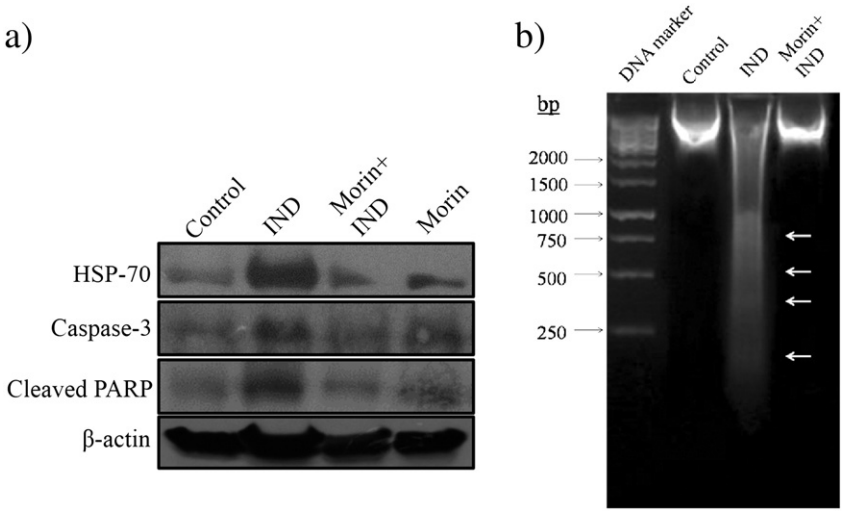

Fig. 9. Effects of morin on IND-induced stress and apoptosis in gastric mucosa. Control: vehicle treatment alone; IND: 48 mg kg^{-1} IND treatment alone (for 4 h); morin + IND: treatment with 50 mg kg^{-1} morin (30 min pretreatment) and 48 mg kg^{-1} IND (for 4 h); morin: morin: 50 mg kg^{-1} morin treatment alone. (a) Immunoblot analysis of HSP70, caspase-3 and cleaved PARP from whole lysate of the gastric mucosa of all the four groups. Note that IND administration increased HSP70, activated caspase-3 along with cleaved PARP and pretreatment of morin decreased these expressions. There is no notable difference between Control and morin groups. (b) Apoptotic DNA fragmentation analysis from all the three groups (white arrows pointing the ladder). Note that IND treatment induced apoptotic DNA fragmentation of the genomic DNA and thus forming laddering effects on agarose gel while morin prevents the this laddering.

available on its gastroprotective nature against NSAIDs. Experimental results of the study showed the free radical scavenging, antioxidant and ferrous (Fe^{2+}) chelating properties of morin that are highly relevant to its pharmacological activities related to this study. ROS are important mediators of IND-induced gastric damage [41]. Interaction between IND and complex I of mitochondrial electron transport chain results in the leakage of electrons, which sequentially leads to the formation of noxious superoxide anion radical ($O_2^{\bullet -}$) [41,62]. An important mechanism of ROS induced toxicity is the direct oxidation and inactivation of iron–sulfur (Fe–S) proteins, such as aconitases, Complex I NADH dehydrogenase etc., which results in the simultaneous release of Fe^{2+} and H_2O_2 [56]. Fe^{2+} and H_2O_2 are ingredients of the Haber–Weiss and Fenton reactions which result in the generation of the harmful hydroxyl radical (•OH) [10]. Hence, it can be predicted that free radical scavengers, antioxidants and Fe^{2+} chelators may stand against IND-induced gastric damage. Here, morin protects the gastric mucosa against IND-mediated insult by using all the three mechanisms. Intracellular catalase reduces the damage caused by the toxic effects of free radicals and thus helps in protecting the ROS mediated changes to its minimal while ROS negatively regulates catalase through cross-talking with the NF-κB pathways [57,58,63]. IND distinctly attenuates catalase in the rat gastric mucosa [64]. In this study, IND considerably increased ROS production (data not shown) and reduced the activity and/or level of antioxidant enzymes SOD2, GST and catalase in the gastric tissue, but pretreatment with morin reduced the ROS production (data not shown) and restored the activity and/or level of the antioxidant enzymes. These clearly suggest that the morin restored balance to the antioxidant system in IND-treated gastric tissue. Morin has a similar effect in vivo: oral intake of morin increased the levels of catalase in the livers of rats treated with ammonium chloride [65].

The ROS activates the transcription factor NF-κB under different circumstances [66,67] while antioxidants suppress this hike [3]. IND also activates proinflammatory cytokines and chemokines. Their accumulation is likely to trigger the expression of adhesion molecules [3]. The in vivo and in vitro administration of morin suppresses NF-κB and NF-κB regulated proinflammatory gene expression [68,69]. It has also been suggested that inactivation of NF-κB occurs through the inhibition of oxidative stress by morin [68]. In our experimental data, IND significantly increased the expression of iNOS, TNF-α, IL-1β, IL-6, ICAM-1, MCP-1, promoted the neutrophil infiltration and the nuclear translocation of p-NF-κBp65 in the gastric mucosa. We, therefore, suggest that morin indirectly suppresses the NF-κB activation induced by ROS and directly by inactivating IKK that resulted in the transcriptional inhibition of an array of inflammatory genes during IND-induced gastric injury in rats.

The activation of HSP70 has been associated with IND-induced severe stress in gastric tissue [36]. Our experimental data showed that morin decreased the IND-related cellular stress induced increase in HSP70 in gastric mucosal tissue. We, therefore, propose that morin decreased IND-induced stress conditions. IND also exerts differential effects in respect to the COX-1 and COX-2 controls. COX-1 level remain unchanged whereas COX-2 level increased upon the IND exposure. But ultimately the PGE_2 level got deceased. In this scenario, we propose that, upon stress inducible cyclooxygenase, the COX-2 got up-regulated to protect the mucosa. Whereas the constitutive COX-1 level remains the same, they got competitively inhibited by the non-specific cyclooxygenase inhibitor IND at their functional level. Hence, the PGE_2 level was decreased. Here morin down-regulated the increased level of COX-2 while it remains neutral for COX-1 and the ultimate effector, PGE_2. This suggests that morin works in a PGE_2 independent pathway to protect the gastric mucosa against IND-induced stress. Moreover it does not inhibit IND absorption in the blood as seen from the RP-HPLC analysis of plasma IND level in both the presence and absence of morin. The activation of apoptotic pathway has been coupled with

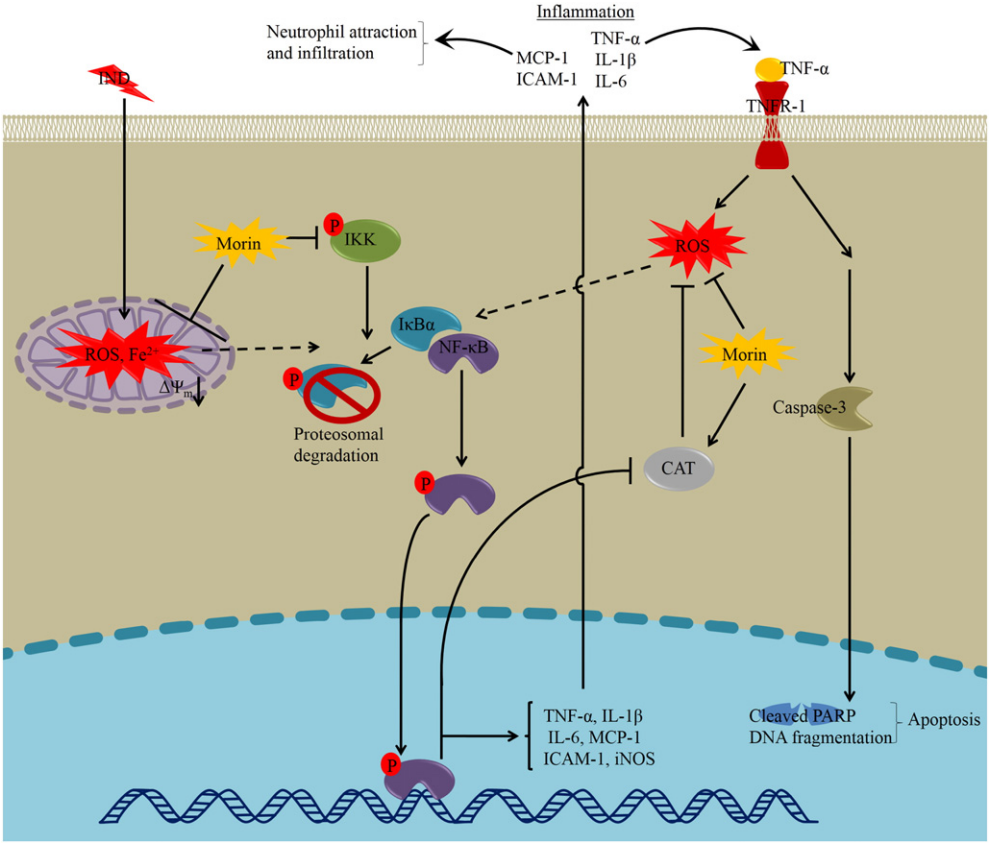

Fig. 10. Graphical abstract representing molecular mechanisms of gastroprotective action of morin against IND-induced gastropathy in rats. ('Solid arrows' indicating stimulatory interaction; 'blunt arrow' indicates inhibitory interaction; 'broken arrows' indicate plausible mechanisms).

IND-induced gastropathy [70]. In our experiments, morin inhibits caspase-3 and PARP cleavage. Thus, we suggest that morin inhibits IND-induced apoptosis through the regulation of these molecules in rat gastric tissue.

Considering all the findings of our study and relevant information available in the literature, we have proposed a scheme to confer a logical explanation regarding therapeutic intervention for IND-induced gastric mucosal injury by morin (Fig. 10). IND disturbs the oxidative balance in gastric tissue leading to oxidative stress and ROS production which in turn inactivates mitochondrial Fe–S proteins accompanied by release of Fe^{2+} that by Fenton reaction, amplifies the stress in several folds. Subsequently the ROS indirectly activates NF-κB. A concomitant proinflammatory response gets initiated leading to neutrophil infiltration and subsequent gastric damage. Activated NF-κB also inhibits catalase production and induces iNOS, and both eventually increase the redox burden. On the other hand, increased TNF-α and IL-1β activate NF-κB, produce ROS, thus create a positive feedback loop. TNF-α and IL-1β also trigger apoptotic signaling pathway, ultimately leading to severe gastric mucosal apoptosis and injury. However, morin effectively inhibits ROS production, scavenges free radicals and chelates noxious Fe^{2+}. Besides, morin pretreatment effectively inhibits IND-mediated NF-κB activation by inhibiting IKK activation. Thus, it reduces proinflammatory cytokine production, related apoptosis and gastric damage. Morin also prevents down-regulation of catalase and thus helps to restore the cellular redox homeostasis. In conclusion, our study provides evidence that morin has significant potential as a therapeutic intervention for IND-induced gastric mucosal injury. It is the first report showing its excellent pharmacological effects against IND-induced gastropathy in rats. Future detailed pharmacokinetic and pharmacodynamic studies on morin might establish it as a safe gastroprotective drug against IND-induced gastropathy.

Transparency Document

The Transparency Document associated with this article can be found, in the online version.

Acknowledgement

Krishnendu Sinha acknowledges the Indian Council of Medical Research (ICMR) for providing financial assistance in the form of fellowship [No.3/1/3/JRF-2011/HRD]. Also the authors are extremely grateful to Ms. Sayantani Chowdhury and Ms. Sharmistha Banerjee for their excellent experimental assistance.

References

[1] C. Pal, S. Bindu, S. Dey, A. Alam, M. Goyal, M.S. Iqbal, P. Maity, S.S. Adhikari, U. Bandyopadhyay, Gallic acid prevents nonsteroidal anti-inflammatory drug-induced gastropathy in rat by blocking oxidative stress and apoptosis, Free Radic. Biol. Med. 49 (2010) 258–267.
[2] P.A. Gladding, M.W. Webster, H.B. Farrell, I.S. Zeng, R. Park, N. Ruijne, The antiplatelet effect of six non-steroidal anti-inflammatory drugs and their pharmacodynamic interaction with aspirin in healthy volunteers, Am. J. Cardiol. 101 (2008) 1060–1063.
[3] S. Bindu, S. Mazumder, S. Dey, C. Pal, M. Goyal, A. Alam, M.S. Iqbal, S. Sarkar, A. Azhar Siddiqui, C. Banerjee, U. Bandyopadhyay, Nonsteroidal anti-inflammatory drug induces proinflammatory damage in gastric mucosa through NF-kappaB activation and neutrophil infiltration: anti-inflammatory role of heme oxygenase-1 against nonsteroidal anti-inflammatory drug, Free Radic. Biol. Med. 65 (2013) 456–467.
[4] A. Lanas, M.A. Perez-Aisa, F. Feu, J. Ponce, E. Saperas, S. Santolaria, L. Rodrigo, J. Balanzo, E. Bajador, P. Almela, J.M. Navarro, F. Carballo, M. Castro, E. Quintero, A nationwide study of mortality associated with hospital admission due to severe gastrointestinal events and those associated with nonsteroidal antiinflammatory drug use, Am. J. Gastroenterol. 100 (2005) 1685–1693.
[5] C. Musumba, D.M. Pritchard, M. Pirmohamed, Review article: cellular and molecular mechanisms of NSAID-induced peptic ulcers, Aliment. Pharmacol. Ther. 30 (2009) 517–531.
[6] J.L. Wallace, How do NSAIDs cause ulcer disease? Baillieres Best Pract. Res. Clin. Gastroenterol. 14 (2000) 147–159.
[7] S.K. Yadav, B. Adhikary, S. Chand, B. Maity, S.K. Bandyopadhyay, S. Chattopadhyay, Molecular mechanism of indomethacin-induced gastropathy, Free Radic. Biol. Med. 52 (2012) 1175–1187.
[8] J.C. Becker, W. Domschke, T. Pohle, Current approaches to prevent NSAID-induced gastropathy—COX selectivity and beyond, Br. J. Clin. Pharmacol. 58 (2004) 587–600.
[9] M. Chatterjee, R. Saluja, S. Kanneganti, S. Chinta, M. Dikshit, Biochemical and molecular evaluation of neutrophil NOS in spontaneously hypertensive rats, Cell. Mol. Biol. (Noisy-le-grand) 53 (2007) 84–93.
[10] K. Sinha, J. Das, P.B. Pal, P.C. Sil, Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis, Arch. Toxicol. 87 (2013) 1157–1180.
[11] A. Uc, X. Zhu, B.A. Wagner, G.R. Buettner, D.J. Berg, Heme oxygenase-1 is protective against nonsteroidal anti-inflammatory drug-induced gastric ulcers, J. Pediatr. Gastroenterol. Nutr. 54 (2012) 471–476.
[12] C. La Casa, I. Villegas, C. Alarcon de la Lastra, V. Motilva, M.J. Martin Calero, Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions, J. Ethnopharmacol. 71 (2000) 45–53.
[13] A.D. Luster, Chemokines—chemotactic cytokines that mediate inflammation, N. Engl. J. Med. 338 (1998) 436–445.
[14] Z. Morise, D.N. Granger, J.W. Fuseler, D.C. Anderson, M.B. Grisham, Indomethacin induced gastropathy in CD18, intercellular adhesion molecule 1, or P-selectin deficient mice, Gut 45 (1999) 523–528.
[15] K. Nishida, Y. Ohta, T. Kobayashi, I. Ishiguro, Involvement of the xanthine–xanthine oxidase system and neutrophils in the development of acute gastric mucosal lesions in rats with water immersion restraint stress, Digestion 58 (1997) 340–351.
[16] L. Osborn, Leukocyte adhesion to endothelium in inflammation, Cell 62 (1990) 3–6.
[17] C.C. Winterbourn, Biological reactivity and biomarkers of the neutrophil oxidant, hypochlorous acid, Toxicology 181–182 (2002) 223–227.
[18] S. Demir, M. Yilmaz, M. Koseoglu, N. Akalin, D. Aslan, A. Aydin, Role of free radicals in peptic ulcer and gastritis, Turk. J. Gastroenterol. 14 (2003) 39–43.
[19] B. Kristal, R. Shurtz-Swirski, J. Chezar, J. Manaster, R. Levy, G. Shapiro, I. Weissman, S.M. Shasha, S. Sela, Participation of peripheral polymorphonuclear leukocytes in the oxidative stress and inflammation in patients with essential hypertension, Am. J. Hypertens. 11 (1998) 921–928.
[20] D. Salvemini, G. de Nucci, R.J. Gryglewski, J.R. Vane, Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 6328–6332.
[21] I. Chattopadhyay, U. Bandyopadhyay, K. Biswas, P. Maity, R.K. Banerjee, Indomethacin inactivates gastric peroxidase to induce reactive-oxygen-mediated gastric mucosal injury and curcumin protects it by preventing peroxidase inactivation and scavenging reactive oxygen, Free Radic. Biol. Med. 40 (2006) 1397–1408.
[22] E.C. Klinkenberg-Knol, F. Nelis, J. Dent, P. Snel, B. Mitchell, P. Prichard, D. Lloyd, N. Havu, M.H. Frame, J. Roman, A. Walan, Long-term omeprazole treatment in resistant gastroesophageal reflux disease: efficacy, safety, and influence on gastric mucosa, Gastroenterology 118 (2000) 661–669.
[23] G.N. Tytgat, Shortcomings of the first-generation proton pump inhibitors, Eur. J. Gastroenterol. Hepatol. 13 (Suppl. 1) (2001) S29–S33.
[24] J.H. Wandall, Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b-245, Gut 33 (1992) 617–621.
[25] N. Kakei, M. Ichinose, S. Tsukada, M. Tatematsu, N. Tezuka, N. Yahagi, M. Matsushima, K. Miki, K. Kurokawa, K. Takahashi, H. Fukamachi, Omeprazole, a proton pump inhibitor, reduces the secretion, synthesis and gene expression of pepsinogen in the rat stomach, Biochem. Biophys. Res. Commun. 195 (1993) 997–1004.
[26] S.E. Miederer, Will anti-ulcer drugs soon differ only in their side effects? Fortschr. Med. 104 (1986) 918–920.
[27] S.S. Wijeratne, M.M. Abou-Zaid, F. Shahidi, Antioxidant polyphenols in almond and its coproducts, J. Agric. Food Chem. 54 (2006) 312–318.
[28] B.B. Aggarwal, S. Shishodia, Molecular targets of dietary agents for prevention and therapy of cancer, Biochem. Pharmacol. 71 (2006) 1397–1421.
[29] M.-X. Xie, M. Long, Y. Liu, C. Qin, Y.-D. Wang, Characterization of the interaction between human serum albumin and morin, Biochim. Biophys. Acta Gen. Subj. 1760 (2006) 1184–1191.
[30] Y. Hanasaki, S. Ogawa, S. Fukui, The correlation between active oxygens scavenging and antioxidative effects of flavonoids, Free Radic. Biol. Med. 16 (1994) 845–850.
[31] S.H. Fang, Y.C. Hou, W.C. Chang, S.L. Hsiu, P.D. Chao, B.L. Chiang, Morin sulfates/glucuronides exert anti-inflammatory activity on activated macrophages and decreased the incidence of septic shock, Life Sci. 74 (2003) 743–756.
[32] Z. Yu, W.P. Fong, C.H.K. Cheng, The dual actions of morin (3,5,7,2′,4′-pentahydroxyflavone) as a hypouricemic agent: uricosuric effect and xanthine oxidase inhibitory activity, J. Pharmacol. Exp. Ther. 316 (2006) 169–175.
[33] J. Galvez, G. Coelho, M.E. Crespo, T. Cruz, M.E. Rodriguez-Cabezas, A. Concha, M. Gonzalez, A. Zarzuelo, Intestinal anti-inflammatory activity of morin on chronic experimental colitis in the rat, Aliment. Pharmacol. Ther. 15 (2001) 2027–2039.
[34] M.S. Blois, Antioxidant determinations by the use of a stable free radical, Nature 181 (1958) 1199–1200.
[35] I.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay, Anal. Biochem. 239 (1996) 70–76.
[36] S. Suemasu, K. Tanaka, T. Namba, T. Ishihara, T. Katsu, M. Fujimoto, H. Adachi, G. Sobue, K. Takeuchi, A. Nakai, T. Mizushima, A role for HSP70 in protecting against indomethacin-induced gastric lesions, J. Biol. Chem. 284 (2009) 19705–19715.
[37] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[38] J. Liu, Z. Yu, S. Guo, S.R. Lee, C. Xing, C. Zhang, Y. Gao, D.G. Nicholls, E.H. Lo, X. Wang, Effects of neuroglobin overexpression on mitochondrial function and oxidative stress following hypoxia/reoxygenation in cultured neurons, J. Neurosci. Res. 87 (2009) 164–170.

[39] K. Sinha, P.B. Pal, P.C. Sil, Cadmium (Cd(2+)) exposure differentially elicits both cell proliferation and cell death related responses in SK-RC-45, Toxicol. In Vitro 28 (2014) 307–318.
[40] K. Biswas, U. Bandyopadhyay, I. Chattopadhyay, A. Varadaraj, E. Ali, R.K. Banerjee, A novel antioxidant and antiapoptotic role of omeprazole to block gastric ulcer through scavenging of hydroxyl radical, J. Biol. Chem. 278 (2003) 10993–11001.
[41] C. Pal, S. Bindu, S. Dey, A. Alam, M. Goyal, M.S. Iqbal, S. Sarkar, R. Kumar, K.K. Halder, M.C. Debnath, S. Adhikari, U. Bandyopadhyay, Tryptamine-gallic acid hybrid prevents non-steroidal anti-inflammatory drug-induced gastropathy: correction of mitochondrial dysfunction and inhibition of apoptosis in gastric mucosal cells, J. Biol. Chem. 287 (2012) 3495–3509.
[42] S.K. Yadav, B. Adhikary, S.K. Bandyopadhyay, S. Chattopadhyay, Inhibition of TNF-alpha, and NF-kappaB and JNK pathways accounts for the prophylactic action of the natural phenolic, allylpyrocatechol against indomethacin gastropathy, Biochim. Biophys. Acta 1830 (2013) 3776–3786.
[43] K.S. Al-Numair, G. Chandramohan, M.A. Alsaif, Pretreatment with morin, a flavonoid, ameliorates adenosine triphosphatases and glycoproteins in isoproterenol-induced myocardial infarction in rats, J. Nat. Med. 66 (2012) 95–101.
[44] E.W. Campion, R.J. Glynn, L.O. DeLabry, Asymptomatic hyperuricemia. Risks and consequences in the Normative Aging Study, Am. J. Med. 82 (1987) 421–426.
[45] J. Venu Gopal, Morin Hydrate: Botanical origin, pharmacological activity and its applications: a mini-review, Pharmacogn. J. 5 (2013) 123–126.
[46] V. Sreedharan, K.K. Venkatachalam, N. Namasivayam, Effect of morin on tissue lipid peroxidation and antioxidant status in 1, 2-dimethylhydrazine induced experimental colon carcinogenesis, Investig. New Drugs 27 (2009) 21–30.
[47] G. Natale, G. Lazzeri, C. Blandizzi, G. Gherardi, P. Lenzi, A. Pellegrini, M. Del Tacca, Seriate histomorphometry of whole rat stomach: an accurate and reliable method for quantitative analysis of mucosal damage, Toxicol. Appl. Pharmacol. 174 (2001) 17–26.
[48] C.L. Santos, B.A. Medeiros, R.C. Palheta-Junior, G.M. Macedo, M.A. Nobre-e-Souza, L.E. Troncon, A.A. Santos, M.H. Souza, Cyclooxygenase-2 inhibition increases gastric tone and delays gastric emptying in rats, Neurogastroenterol. Motil. 19 (2007) 225–232.
[49] S. Mizuguchi, T. Ohno, Y. Hattori, T. Ae, T. Minamino, T. Satoh, K. Arai, T. Saeki, I. Hayashi, Y. Sugimoto, S. Narumiya, K. Saigenji, M. Majima, Roles of prostaglandin E2-EP1 receptor signaling in regulation of gastric motor activity and emptying, Am. J. Physiol. Gastrointest. Liver Physiol. 299 (2010) G1078–G1086.
[50] K. Suzuki, H. Ota, S. Sasagawa, T. Sakatani, T. Fujikura, Assay method for myeloperoxidase in human polymorphonuclear leukocytes, Anal. Biochem. 132 (1983) 345–352.
[51] G.O. Dengiz, F. Odabasoglu, Z. Halici, E. Cadirci, H. Suleyman, Gastroprotective and antioxidant effects of montelukast on indomethacin-induced gastric ulcer in rats, J. Pharmacol. Sci. 105 (2007) 94–102.
[52] B. Cox, A. Emili, Tissue subcellular fractionation and protein extraction for use in mass-spectrometry-based proteomics, Nat. Protoc. 1 (2006) 1872–1878.
[53] M.J. Sung, D.H. Kim, Y.J. Jung, K.P. Kang, A.S. Lee, S. Lee, W. Kim, M. Davaatseren, J.T. Hwang, H.J. Kim, M.S. Kim, D.Y. Kwon, S.K. Park, Genistein protects the kidney from cisplatin-induced injury, Kidney Int. 74 (2008) 1538–1547.
[54] P.B. Pal, K. Sinha, P.C. Sil, Mangiferin attenuates diabetic nephropathy by inhibiting oxidative stress mediated signaling cascade, TNFalpha related and mitochondrial dependent apoptotic pathways in streptozotocin-induced diabetic rats, PLoS ONE 9 (2014) e107220.
[55] K.S. Sellins, J.J. Cohen, Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes, J. Immunol. 139 (1987) 3199–3206.
[56] S. Orrenius, V. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 143–183.
[57] J. Das, J. Ghosh, P. Manna, P.C. Sil, Taurine protects acetaminophen-induced oxidative damage in mice kidney through APAP urinary excretion and CYP2E1 inactivation, Toxicology 269 (2010) 24–34.
[58] J. Ghosh, J. Das, P. Manna, P.C. Sil, Arjunolic acid, a triterpenoid saponin, prevents acetaminophen (APAP)-induced liver and hepatocyte injury via the inhibition of APAP bioactivation and JNK-mediated mitochondrial protection, Free Radic. Biol. Med. 48 (2010) 535–553.
[59] K.M. Mullane, R. Kraemer, B. Smith, Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium, J. Pharmacol. Methods 14 (1985) 157–167.
[60] T. Lawrence, C. Fong, The resolution of inflammation: anti-inflammatory roles for NF-kappaB, Int. J. Biochem. Cell Biol. 42 (2010) 519–523.
[61] D. Kempuraj, B. Madhappan, S. Christodoulou, W. Boucher, J. Cao, N. Papadopoulou, C.L. Cetrulo, T.C. Theoharides, Flavonols inhibit proinflammatory mediator release, intracellular calcium ion levels and protein kinase C theta phosphorylation in human mast cells, Br. J. Pharmacol. 145 (2005) 934–944.
[62] N. Demaurex, L. Scorrano, Reactive oxygen species are NOXious for neurons, Nat. Neurosci. 12 (2009) 819–820.
[63] M.J. Morgan, Z.G. Liu, Crosstalk of reactive oxygen species and NF-kappaB signaling, Cell Res. 21 (2011) 103–115.
[64] J.H. Kim, B.W. Kim, H.J. Kwon, S.W. Nam, Curative effect of selenium against indomethacin-induced gastric ulcers in rats, J. Microbiol. Biotechnol. 21 (2011) 400–404.
[65] S. Subash, P. Subramanian, Morin a flavonoid exerts antioxidant potential in chronic hyperammonemic rats: a biochemical and histopathological study, Mol. Cell. Biochem. 327 (2009) 153–161.
[66] S. Lee, S.O. Moon, W. Kim, M.J. Sung, D.H. Kim, K.P. Kang, Y.B. Jang, J.E. Lee, K.Y. Jang, S.Y. Lee, S.K. Park, Protective role of L-2-oxothiazolidine-4-carboxylic acid in cisplatin-induced renal injury, Nephrol. Dial. Transplant. 21 (2006) 2085–2095.
[67] C. Nathan, A. Cunningham-Bussel, Beyond oxidative stress: an immunologist's guide to reactive oxygen species, Nat. Rev. Immunol. 13 (2013) 349–361.
[68] J.M. Kim, E.K. Lee, G. Park, M.K. Kim, T. Yokozawa, B.P. Yu, H.Y. Chung, Morin modulates the oxidative stress-induced NF-kappaB pathway through its anti-oxidant activity, Free Radic. Res. 44 (2010) 454–461.
[69] V. Sivaramakrishnan, S. Niranjali Devaraj, Morin regulates the expression of NF-kappaB-p65, COX-2 and matrix metalloproteinases in diethylnitrosamine induced rat hepatocellular carcinoma, Chem. Biol. Interact. 180 (2009) 353–359.
[70] P. Maity, S. Bindu, V. Choubey, A. Alam, K. Mitra, M. Goyal, S. Dey, M. Guha, C. Pal, U. Bandyopadhyay, Lansoprazole protects and heals gastric mucosa from non-steroidal anti-inflammatory drug (NSAID)-induced gastropathy by inhibiting mitochondrial as well as Fas-mediated death pathways with concurrent induction of mucosal cell renewal, J. Biol. Chem. 283 (2008) 14391–14401.