



mechanism of increased oxygen-free radicals increases the protease secretion and capillary blood flow obstruction which will result in gastric injury.<sup>[11,12]</sup> Besides interfering of PGE<sub>2</sub> pathway, NSAIDs also raise the inducible nitric oxide synthase (iNOS) activity and iNOS messenger RNA (mRNA) expression. Consequently, this will cause increase in nitric oxide (NO) production.<sup>[13]</sup> Massive formation of NO is indirectly stimulated through the increase of other pro-inflammatory cytokine production induced by NSAIDs. These pro-inflammatory cytokines can stimulate the iNOS. The damage of gastric mucosal caused by the peroxynitrite, a cytotoxic substance, is due to the reaction of NO and superoxide.<sup>[14]</sup> Moreover, previous studies have revealed that IMN causes an increase in malondialdehyde (MDA), a metabolite of intracellular lipid peroxidase, level compared to the control group which were given quercetin (one of the flavonoid compounds).<sup>[15,16]</sup>

Flavonoids and phenolic compounds can be found in many edible plants, for instance, the leafy vegetables, fruits, and plant-based beverages. Flavonoids are categorized into five classes, the flavone, isoflavone, flavonol, flavanone, and anthocyanin.<sup>[17]</sup> These compounds seem to have many beneficial biological effects including anti-inflammatory, antioxidant, antihistaminic, free radical scavenging abilities, antiviral, antithrombotic, and anti-ischemic effects.<sup>[17]</sup> Some abilities of flavonoids have preferable outcomes on gastritis, gastric ulcer, and even gastric cancer. The known mechanisms of this gastroprotective effects of flavonoids are the increase of endogenous prostaglandin, reduction of histamine secretion, scavenging of oxygen-derived free radicals, stimulation of gastric mucus secretion, and inhibition of “nuclear factor-κB” (NF-κB) and “signal transducer and activator of transcription-1” (STAT-1).<sup>[17,18]</sup> NF-κB and STAT-1 are the main factors of NO production as they play a role of transcription factors for iNOS.<sup>[17]</sup> Flavonoid compounds (including genistein) are observed to decrease lipopolysaccharide-induced NO production on J774 macrophage, decrease iNOS protein expression, and decrease iNOS mRNA levels.<sup>[19]</sup>

Genistein, one of the flavonoids, isoflavone, is generally found in soybean. Genistein has various abilities including estrogen-like activity, anti-inflammatory, antioxidant, and anticancer effects. Genistein has similar structure to estradiol. Genistein binds to the estrogen receptor at the estrogen-responsive tissue. However, genistein may produce an antagonistic effect of estrogen as genistein is a weak estrogen agonist.<sup>[20,21]</sup> The other effects of genistein as mentioned above can be seen in the previous research. Genistein can lead to gastroprotective ability as it seems to reduce gastric acid secretion and suppress TNF-α and cytokine-induced neutrophil chemoattractant (CINC)-1, which are inflammatory cytokines related to gastric ulceration.<sup>[19]</sup>

There are many researches that confirm the gastroprotective effect of genistein, but unfortunately, there have not yet been research on the effect of genistein on NSAIDs-induced gastropathy. This study aims to test the gastroprotective properties of genistein on inflammatory attenuation, oxidative stress reduction, and improvement of histopathology in rats with IMN-induced gastric injury.

## MATERIALS AND METHODS

### Animal, indomethacin, and genistein preparation

The protocol for this study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (Approval No. 17/2557). Eighteen male Sprague-Dawley rats weighing 180–220 g that were purchased from the National Laboratory Animal Center, Mahidol University, Salaya Campus, Nakornpathom, Thailand, were used in this study. All rats were kept in a controlled temperature room at 25°C ± 1°C under standard conditions with a 12-h light/12-h dark cycle. Care for animals in this study complied with the rules and regulations set forth by the Chulalongkorn University Animal Care and Use Committee.

IMN, which is an NSAID, was dissolved in 5% NaHCO<sub>3</sub><sup>-</sup> (Atlantic Laboratories Corp., Ltd., Bangkok, Thailand). Genistein (Cayman Chemical, Ann Arbor, MI, USA) was dissolved in 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich Co. LLC., St. Louis, MO, USA).

### Experimental protocol

All rats were fasted with water available *ad libitum* for 22–24 h before the experiment. Rats were randomly divided into three experimental groups. Group 1 (control; *n* = 6) was given distilled water 1 mL/rat through intragastric tube at 0 and 4 h. Group 2 (IMN; *n* = 6) was given IMN 150 mg/kg dissolved in 5% sodium bicarbonate (NaHCO<sub>3</sub><sup>-</sup>) 1 mL/rat through intragastric tube at 0 and 4 h as previously described.<sup>[22,23]</sup> Group 3 (genistein; *n* = 6) was given genistein 100 mg/kg dissolved in 0.1% DMSO and IMN 150 mg/kg dissolved in 5% NaHCO<sub>3</sub><sup>-</sup> 1 mL/rat through intragastric tube at 0 and 4 h.

Four hours after the second dose (total elapsed time 8 h), all rats were anesthetized with an intraperitoneal injection of thiopental sodium 50 mg/kg (Jagsopal Pharmaceuticals Limited, Haryana, India). The stomach of each rat was removed and dissected along the greater curvature. Stomach specimens were then washed twice with ice-cold phosphate-buffered saline, frozen in liquid nitrogen, and stored at –80°C until laboratory evaluation for the expression of iNOS using Western blot (Abcam Plc., Cambridge, MA, USA) and for MDA using TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). The remaining stomach specimens were fixed in 10% formalin solution, which was later stained with hematoxylin and eosin (H and E) for histopathologic examination of pathologic change of gastric mucosa, as measured by ulcer index. A blood sample was then collected by cardiac puncture. The blood was allowed to clot at room temperature for 2 h before being centrifuged at 1000 g for 20 min. Serum was separated and stored at –80°C until measurement of TNF-α and PGE<sub>2</sub> levels using enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN, USA).

### Histopathology

Stomach tissue samples were excised and fixed in 10% formalin and later processed by routine technique before paraffin embedding. Sections were cut into 5 μm thickness and stained with H and E stain. One experienced gastrointestinal pathologist (NK) examined all blinded samples using light microscopy with ×40 magnification. All histopathological findings were recorded and graded using gastric erosion and polymorphonuclear (PMN) leukocyte infiltration scores, as follows: Gastric erosion: 0, no erosion; 1, erosion 1/3<sup>rd</sup> of epithelium depth; and 2, erosion 2/3<sup>rd</sup> of epithelium depth, or development of ulcer. For PMN infiltration: 0, no infiltration (normal/no gastric mucosal injury); 1, PMN infiltration 1/3<sup>rd</sup> of epithelium (mild injury); 2, PMN infiltration 2/3<sup>rd</sup> of epithelium (moderate injury); and 3, PMN infiltration in all depths of epithelium (severe injury).<sup>[24]</sup>

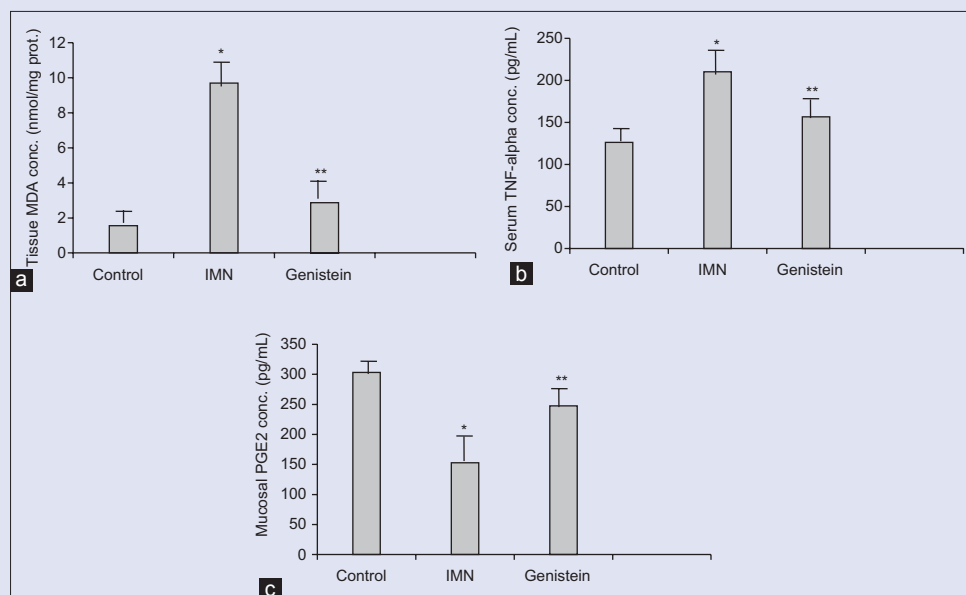
### Statistical analysis

All data are presented as mean ± standard deviation. Means were compared by one-way analysis of variance (one-way ANOVA), followed by Fisher's least significant difference *post hoc* test. All statistical analyses were performed using SPSS for Windows version 22.0 (SPSS, Inc., Chicago, IL, USA). *P* < 0.05 was considered statistically significant.

## RESULTS

### Changes in malondialdehyde level

Level of gastric MDA was significantly higher in the IMN group than in the control group (9.70 ± 0.40 vs. 1.56 ± 0.14 nmol/mg protein, *P* = 0.000). There was a significant decrease in gastric MDA level in the genistein group, as compared with the IMN group (2.87 ± 0.37 vs.



**Figure 1:** Effects of genistein on tissue malondialdehyde, serum tumor necrosis factor- $\alpha$ , and prostaglandin  $E_2$  in rat with indomethacin-induced gastropathy. All data are expressed as mean  $\pm$  standard deviation and all data have  $P < 0.01$ . \* $P < 0.01$  versus control group; \*\* $P < 0.01$  versus indomethacin group. (a) Mean of tissue malondialdehyde; (b) Mean of serum tumor necrosis factor- $\alpha$ ; (c) Mean of prostaglandin  $E_2$

9.70  $\pm$  0.4 nmol/mg protein,  $P = 0.000$ ). Mean serum MDA levels for all groups are shown in Figure 1a and Table 1.

### Changes in tumor necrosis factor-alpha level

Average concentrations of serum TNF- $\alpha$  were markedly increased in the IMN group, when compared with the control group (210.28  $\pm$  0.98 vs. 126.4  $\pm$  0.13 pg/mL,  $P = 0.000$ ). There was a significant decrease in gastric TNF- $\alpha$  level in the genistein group, as compared with the IMN group (156.59  $\pm$  0.10 vs. 210.28  $\pm$  0.98 pg/mL,  $P = 0.000$ ). Mean serum TNF- $\alpha$  levels for all groups are shown in Figure 1b and Table 1.

### Changes in prostaglandin $E_2$ level

Level of serum PGE<sub>2</sub> was significantly decreased in the IMN group, as compared with the control group (152.83  $\pm$  0.10 vs. 303.33  $\pm$  2.16 pg/mL,  $P = 0.000$ ). There was a marked increase in serum PGE<sub>2</sub> level in the genistein group, as compared with the IMN group (247.65  $\pm$  0.01 vs. 152.83  $\pm$  0.10 pg/mL,  $P = 0.000$ ). Mean serum PGE<sub>2</sub> levels for all groups are shown in Figure 1c and Table 1.

### Changes in inducible nitric oxide synthase level

Compared with the control group, iNOS band intensity in the IMN group increased. Expression of iNOS band intensity declined in the genistein group, when compared with the IMN group. Band intensity representing iNOS and  $\beta$ -actin is shown in Figure 2.

### Histopathological changes

Histologic appearance of the stomach in control group rats [Figure 2] was normal. In the IMN group, four rats developed gastric erosion (score 2) and two rats developed gastric ulcer. PMN infiltration scores in the IMN group were as follows: Four rats had a score of 1 and two rats had a score of 0. In the genistein group, stomach histopathology improved both gastric erosion and PMN infiltration, when compared to the IMN group. No gastric ulcer was found in the genistein group, as shown in Figure 3 and Table 2.

**Table 1:** Summary of parameters in all the three experimental groups

Experimental group	Parameters		
	MDA (nmol/mg protein)	TNF- $\alpha$ (pg/mL)	PGE <sub>2</sub> (pg/mL)
Control (n=6)	1.56 $\pm$ 0.14	126.4 $\pm$ 0.13	303.33 $\pm$ 2.16
IMN (n=6)	9.70 $\pm$ 0.40*	210.28 $\pm$ 0.98*	152.83 $\pm$ 0.10*
Genistein (n=6)	2.87 $\pm$ 0.37**	156.59 $\pm$ 0.10**	247.65 $\pm$ 0.01**

\* $P < 0.01$  versus control group, \*\* $P < 0.01$  versus Indomethacin group.

Data are presented as mean $\pm$ SD;  $P < 0.05$  indicates statistical significance, all data have  $P < 0.01$ . SD: Standard deviation; IMN: Indomethacin; MDA: Malondialdehyde; PGE<sub>2</sub>: Prostaglandin  $E_2$ ; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$

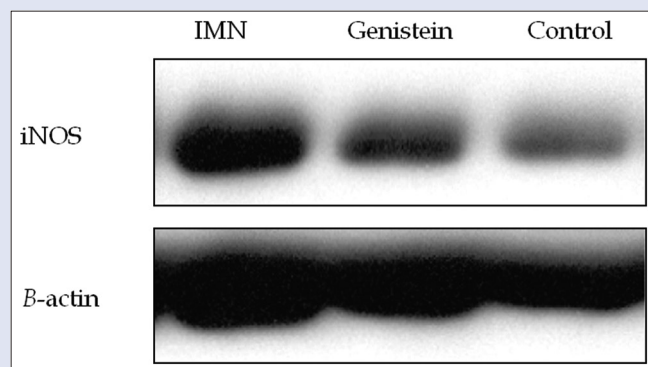
**Table 2:** Summary of the gastric erosion and polymorphonuclear leukocyte infiltration scores

Experimental groups	Gastric erosion			Ulcer	PMN infiltration			
	0	1	2		0	1	2	3
Control (n=6)	6	0	0	-	6	0	0	0
IMN (n=6)	0	0	4	2	2	4	0	0
Genistein (n=6)	1	4	1	-	4	2	0	0

Gastric erosion scoring - 0: no erosion; 1: erosion 1/3<sup>rd</sup> of epithelium depth; 2: erosion 2/3<sup>rd</sup> of epithelium depth or development of ulcer; Gastric inflammation level was estimated and scored by the pathologist following the updated Sydney system. PMN infiltration scoring - 0: no infiltration (normal/no gastric mucosal injury); 1: PMN infiltration 1/3<sup>rd</sup> of epithelium (mild injury); 2: PMN infiltration 2/3<sup>rd</sup> of epithelium (moderate injury); 3: PMN infiltration in all depths of epithelium (severe injury). IMN: Indomethacin; PMN: Polymorphonuclear leukocyte

## DISCUSSION

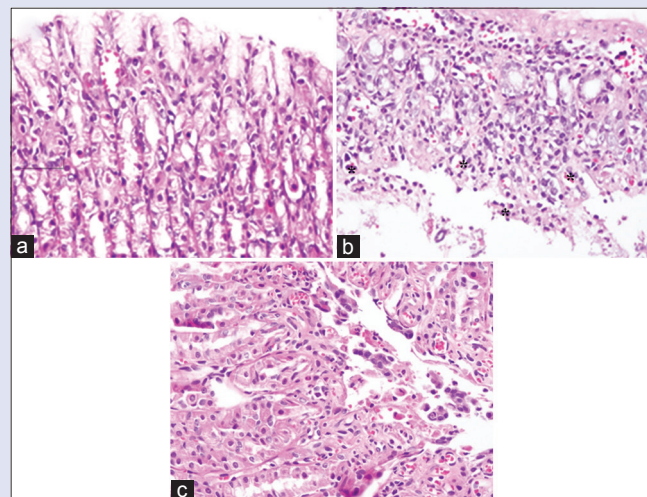
In this research, we study the effects of genistein on IMN-induced gastropathy in rats. It can be seen that the anti-inflammatory effect of genistein helps prevent the formation of gastric ulcer induced by IMN. Recent studies have shown that the early and significant pathogenesis



**Figure 2:** Western blot analysis of inducible nitric oxide synthase expression. Results show that the inducible nitric oxide synthase band intensity was not different between control and genistein groups. Compared with control and genistein groups, inducible nitric oxide synthase band intensity in indomethacin group increased

of NSAIDs-induced gastropathy presents with leukocyte adhesion to the vascular endothelium which results in tissue inflammation.<sup>[25]</sup> As mentioned above, NSAIDs decrease the PGE<sub>2</sub> level which causes an elevation of TNF- $\alpha$ . TNF- $\alpha$ , a pro-inflammatory cytokine, plays an important role in the expression of iNOS or Type II NOS. iNOS is the main enzyme in NO production. NO is another significant mediator of inflammation. It leads to tissue injury.<sup>[26]</sup> Furthermore, TNF- $\alpha$  stimulates the ICAM-1 expression on the endothelium. ICAM-1 directly relates to an increase of neutrophil adhesion.<sup>[10]</sup> ICAM-1 also induces transendothelial migration of leukocytes to the inflamed tissue which also results in more tissue damage.<sup>[27]</sup> In addition, TNF- $\alpha$  stimulates NF- $\kappa$ B. NF- $\kappa$ B causes gastric injury through various pathways. First, NF- $\kappa$ B increases inflammatory cytokine synthesis such as IL-1, IL-6, and IL-8 and increases these inflammatory cytokine receptors.<sup>[19]</sup> Second, NF- $\kappa$ B regulates adhesion molecule such as ICAM-1 expression.<sup>[28]</sup> Neutrophil accumulation and adhesion stimulated by various inflammatory cytokines will lead to tissue damage.<sup>[29]</sup> Therefore, substance that reduces the adhesion molecule will attenuate the inflammatory process. From a previous study, it was found that administration of monoclonal antibody against endothelial adhesion molecules can help prevent the IMN-induced neutrophil adhesion and consequently decrease the severity of IMN-induced gastric injury.<sup>[25]</sup>

Recent studies have revealed that genistein has anti-inflammatory effect which results in positive outcome in gastric injury. Genistein inhibits tyrosine-specific protein kinase activity of epidermal growth factor receptor which results in the suppression of NF- $\kappa$ B activity, inhibition of TNF- $\alpha$  production in monocytic macrophage cell lines *in vitro*, and also inhibition of CINC-1 or IL-8.<sup>[17,30]</sup> Genistein has direct gastroprotective effect as it suppresses gastrin secretion. The decrease in gastrin leads to decrease in gastric acid secretion. There has been an investigation in the other research on anti-inflammatory effect of genistein. The doses of 50 and 100 mg/kg/day genistein were given to water immersion restraint-stressed Wistar rats for 2 weeks.<sup>[19]</sup> These rats receiving genistein had less ratio of mucosal hemorrhagic erosion area to the whole stomach body area; decreased pro-inflammatory cytokines, including decreased TNF- $\alpha$  and CINC-1 level in gastric tissue; decreased myeloperoxidase activity; increased superoxide dismutase activity; and decreased TBARS level (an index of lipid peroxidation that is expressed as nmol of MDA).<sup>[19]</sup> Another mechanism of genistein is it reduces iNOS resulting in decreased NO. It has been studied that low-dose genistein attenuates immunohistochemical staining for iNOS leading to reduction of peroxynitrite synthesis and improves mucosal architecture.<sup>[31]</sup>



**Figure 3:** H and E stained stomach section ( $\times 40$ ). (a) Control group showing normal stomach histopathology; (b) Indomethacin group showing gastric ulcer formation and infiltration of inflammatory cells; (c) Genistein group showing a significantly reduced degree of gastric ulcer formation and inflammation. \*Indicate ulcer and infiltration of inflammatory cells

In this research, administration of 150 mg/kg body weight of IMN shows significantly higher level of tissue MDA (a metabolite of intracellular lipid peroxidase), serum TNF- $\alpha$  (a pro-inflammatory cytokine), and tissue iNOS (an inflammatory modulator) compared with the control group. In addition, the level of all these three substances significantly decreased in genistein pretreated group compared with IMN group. The result shows that IMN group causes a decrease in mucosal PGE<sub>2</sub> (a mucoprotective factor of the stomach) compared with the control group. On the other hand, in genistein group, the level of PGE<sub>2</sub> raises significantly as compared to the IMN group. Moreover, the IMN group causes more gastric erosions and ulcer formation with increase in PMN infiltration around the gastric lesions, while the group given genistein as a prophylaxis reveals improved gastric histopathology including the gastric erosion severity and PMN infiltration score compared to the IMN group.

## CONCLUSIONS

NSAIDs cause an increase in inflammatory cytokines and leukocyte adhesions leading to gastric mucosal damage in the form of erosion or ulcer. Genistein, an isoflavone with its gastroprotective abilities, can attenuate IMN-induced gastric injury by reducing inflammation and oxidative stress and improving the gastric histopathology. Therefore, according to the results collected in this study, genistein should be considered as a preventive method for NSAIDs-induced gastropathy.

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## Conflicts of interest

There are no conflicts of interest.

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