

Co-treatment Effect of *Syzygium cumini* (L.) Skeels on Indomethacin Induced Gastric Ulcer on Mice Model[†]

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Abstract

Syzygium cumini (L.) Skeels or SCC originally from India and Southeast Asia, commonly used as a medicinal and acted as antioxidant and anti-inflammatory plant. Indomethacin, which is a part of nonsteroidal anti-inflammatory drugs (NSAIDs) family, induced gastric damage and perforation through the excess generation of reactive oxygen species (ROS). This research focus on co-treatment administered between SCC and indomethacin in the subsequent 7 days and evaluated on oxidative damage, inflammatory parameter and epidermal growth factor (EGF) receptor. SCC showed a concentration and dose dependent reduction in ulcer index (UI) values leading to the increasing of inhibition percent when compared to indomethacin treated mice, confirmed by photographer which showed maximum efficacy on day 5 of treatment. On day 1 and day 3 of ulceration, malondialdehyde (MDA), oxidized glutathione (GSSG), nitrile contents and tumor necrosis factor-alpha (TNF- α) were increased. Gastric wall mucus and glutathione peroxidase (GPx) were down. After that gastric mucosa were recovered by healing processed and were regenerated the mucosal cap to promote EGF receptor. SCC was increased the up-regulation of COX-1 enzyme resulting in down-regulation to COX-2 expression at day 3 of ulceration. At day 5 and 7, the gastric ulceration were regenerated themselves. These all results indicated that SCC have a great protective effect against indomethacin induced gastric ulcer in *in-vivo* co-treatment model.

Keywords: Co-treatment, *Syzygium cumini* (L.) Skeels, indomethacin, gastric ulcer and mice model

Introduction

The lifetime risk for developing a gastric ulcer is approximately 10 %. It can develop more than 1 time in the lifetime of a man. It is quite common and developed in about half a million people each year in the United States and can be expected to develop in 5 - 10 % of the adult population during the lifetime in Western countries [1-3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly used drugs worldwide due to their analgesics, anti-inflammatory and anti-pyretic therapeutic properties [4,5]. More than 30 million people use NSAIDs every day, and they account for 60 % of the US over the counter analgesic market [4,6]. Indomethacin is a NSAID that also has anti-pyretic, antithrombotic and analgesic effects [7]. Its most common side effect is gastrointestinal tract damage, such as stomach ulcers [8]. Several pharmacological agents, antioxidants and plant extracts have been

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used to protect against gastric damage, and successful results have been obtained [9-11] and *Syzygium cumini* (L.) Skeels or SCC is one of it. SCC, belongs to the Myrtaceae family. Its bark, leaves, fruits and seeds are prepared and administered in different ways according to their properties such as potential antidiabetic action [12], antioxidant [13], antimicrobial [14], anti-inflammatory [15], anthelmintic, antifungal, diuretic and anti-diarrheal [16]. The main compounds of the plant that gives these all properties is the high content of flavonoids [17]. Eighty two % of the essential oils present in the plant is gallic acid, kaempferol, myricetin, ellagic acid, chlorogenic acid, quercetin, rutin, anthocyanins and tannins [16,18].

On the model of indomethacin induced acute gastric ulceration, SCC caused discernible decrease gastric lesion and lipid peroxide content and also restored oxidized glutathione (GSSG), glutathione peroxidase (GPx), nitric oxide (NO) levels and gastric wall mucus. Indomethacin induced inflammation by activated inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF- α) [15]. However, in the long term of indomethacin usage still have less in literature. Therefore, this research was aimed to consider the SCC co-treatment with indomethacin in the subsequent 7 days on oxidative stress and inflammatory parameter. Thus, our experiment will provide some baseline information which may highlight the potential of SCC as a gastric ulceration inhibitor.

Materials and methods

Plants

Syzygium cumini (L.) Skeels was collected from various areas at Nakhon Si Thammarat province, Thailand. To prepare an aqueous leaf extracts, young fresh leaves were rinsed with distilled water, chopped and homogenized into distilled water in a blender for 1 min. The suspension was filtered and lyophilized by freeze dryer at -20 °C for 20 h. The powder of *Syzygium cumini* (L.) Skeels aqueous crude extract (SCC) was kept at -30 °C for until used.

The effect of SCC on indomethacin induced gastric ulcer *in vivo*

Animals

Mice were bred at Mahidol University, Bangkok, Thailand and were procured after obtaining clearance from the respective Animal Committee of the Walailak University, Thailand. Mice were handled following International Animal Ethics Committee Guidelines. Male mice (30 - 40 gBW at 5 weeks old) were kept in controlled environment, at a constant temperature (23 \pm 2 °C) and humidity (60 \pm 10 %), and a 12 h light/dark cycle. Mice were acclimatized for one week before any experimental procedures and were allowed standard rat chow and tap water *ad libitum*.

Co-treatment effect of SCC on indomethacin induced gastric ulcer

Male mice were divided into 7 groups (n = 6) and orally treated as follows:

- Group 1: Untreated control (phosphate buffer solution: PBS),
- Group 2: PBS + indomethacin (10 mg/kgBW),
- Group 3: SCC (100 mg/kgBW) + indomethacin (10 mg/kgBW),
- Group 4: SCC (250 mg/kgBW) + indomethacin (10 mg/kgBW),
- Group 5: SCC (500 mg/kgBW) + indomethacin (10 mg/kgBW),
- Group 6: omeprazole (100 mg/kgBW) + indomethacin (10 mg/kgBW),
- Group 7: vitamin C (100 mg/kgBW) + indomethacin (10 mg/kgBW).

Ten mg/kgBW of indomethacin treated in group 2 to group 7 was suspended in an aqueous solution of sodium bicarbonate (5 %) and was administered after one hour of PBS, SCC, omeprazole and vitamin C pretreatment. The mice of all groups were orally treated as follows at the same time for 7 days.

Four hour after administering the last dose of the test samples on each sampling day (1, 3, 5 and 7 days), the stomachs of mice were removed. The ulcerated portions of the stomach were sectioned after fixing in 10 % formal saline solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 μ m onto a glass slide, stained with haematoxylin-eosin and the histology examined under a light microscope. The damage scores, biochemical parameter, cyclooxygenase 1 and 2

(COX-1 and COX-2), inducible nitric oxide synthase (iNOS), Tumor necrosis factor-alpha (TNF- α) and epidermal growth factor (EGF) receptor of untreated and treated mice was measured.

The gastric ulcer was evaluated by measurement the length of ulcer in mm. scale. Ulcer index (UI) was calculated from the total of gastric ulcer area (mm) divided with total mice in each group. Inhibition percent (% inhibition) was calculated follow the formula;

$$\% \text{ Inhibition} = \frac{(\text{UI of control group} - \text{UI of treatment group}) \times 100}{\text{UI of control group}} \quad (1)$$

The mucus bound to the epithelial surface ($\mu\text{g/g}$ tissue) was performed according to the method of Corne *et al.* [19].

Determination of oxidative damage

Determination of lipid peroxides

Gastric mucosal was homogenized with 1 % (v/v) orthophosphoric acid and 0.6 % (w/v) thiobarbituric acid, then boiled for 45 min at 100 °C. After cooling, the colored product was extracted by n-butanol, vortexed and centrifuged at 3000 g for 15 min. The absorbance of the upper layer was read spectrophotometrically at 535 and 520 nm. The difference in absorbance was calculated as gastric mucosal lipid peroxides level and expressed as nmol/g tissue malondialdehyde [20].

Determination of oxidized glutathione (GSSG)

The gastric tissue was homogenized and diluted to 10 folds, 10 μl of EDTA and 10 μl of NADPH were added. Mixed and recorded the baseline level of NADPH absorbance. For measurement of standard GSSG solution, 1 ml sample was replaced by 1 ml buffer and standard GSSG at volume containing 5 nmol. The oxidized glutathione levels were present as ng/mg protein [21].

Determination of glutathione peroxidase (GPx)

The activity was expressed as U/g protein following the reaction mixture consisted of 100 μl of buffer, 20 μl of GSH, 100 μl of GR, 100 μl of NADPH, 10 μl of homogenated gastric tissue, 10 μl of NaN_3 and 660 μl of distilled water. This mixture was warmed at 37 °C for 10 min and then 10 μl of t-butyl hydroperoxide was added. The OD at 340 nm was then followed. A blank value was obtained without any addition of substrates was subtracted from the assay values. The activity of glutathione peroxidase was calculated from the change in optical density in 1 min and the molar extinction coefficient for NADPH at 340 nm of 6.22 nM/cm. Protein (mg/g tissue) was determined by Lowry *et al.* [22] method.

Determination of nitric oxide level

Nitric oxide (nmol/g tissue) were examined indirectly as nitrite/nitrate concentration using Griess reaction-dependent method [23]. Gastric mucosal homogenates were deproteinated with absolute ethanol for 48 h at 4 °C, centrifuged at 12000 g for 15 min at 4 °C. An aliquot of the supernatant vanadium trichloride 0.8 % (w/v) in 1 M HCl was added for the reduction of nitrate to nitrite, followed by the rapid addition Griess reagent consisting of 0.1 % (w/v) N-(1-Naphthyl) ethylenediamine dihydrochloride and 2 % (w/v) sulfanilamide in 5 % (v/v) HCl and incubated for 30 min at 37 °C. Mixtures were cooled and read at 540 nm was measured.

Determination of inflammatory parameter

Tumor necrosis factor-alpha (TNF- α) assay

The plasma samples were collected from the mice. Each sample was incubated with TNF- α detection antibody solution while shaking for 1 h. Samples were discarded and washed 4 times with assay buffer and conjugated with avidin-HRP A solution for 30 min while shaking finally, discard and washed to minimize background. One hundred μl of substrate solution F was added and incubated for 15 min in

the dark to form a complex with a stabilized chromogen. The reaction was stop by adding 100 µl of stop solution to get yellow color. Absorbance was read at 450 nm within 30 min and subtracted with the absorbance at 570 nm.

Western blot technique for COX-1, COX-2 and iNOS expression

The gastric mucosa was washed with PBS containing protease inhibitors, minced and homogenized in a PBS buffer containing protease inhibitor. After centrifugation at 14,000 g for 20 min at 4 °C, the supernatant were collected, aliquoted, and kept at -80 °C before use in the western blots. The proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in Tris-buffered saline with Tween 20 (TBST) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.02 % Tween 20) containing 5 % bovine serum albumin or skim milk and incubated overnight at 4 °C with the appropriate primary antibody. The membrane was washed with TBST twice for 7 min and Tris-buffered saline (TBS). Then membrane was incubated with the appropriate peroxidase conjugated secondary antibody. The bands were detected using an enhanced chemiluminescence detection kit and quantified with respect to the bands of a suitable loading control, using the Kodak Gelquant software [24].

EGF receptor assay

The immunostaining of EGF receptor was carried out with minor modifications. In this case, after blocking the endogenous peroxidase and treatment with the protein blockers, the tissue sections were incubated overnight at 4 °C with primary antibody (mouse polyclonal, 1:200). The peroxidase conjugated goat anti-mouse IgG (1:500) was used as the secondary antibody. Excess secondary antibodies were removed by washing three times in PBS and once with deionized H₂O. Tissue slides were counter staining with nuclear staining dye (DRAQ5) and mounted with mounting medium. Photomicrographs were obtained using a Leica confocal fluorescence microscope.

Statistical analysis

All statistical analyses were run in triplicate. Data obtained was expressed as mean ± standard error of mean (SEM). Parameter data of all the biochemical parameters were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. A probability values of $P < 0.05$ were considered statistically significant.

Results and discussion

The effect of SCC on ulcer index (UI) and inhibition percent on co-treatment study

Table 1 was shown an UI values on co-treatment experiment. The mice receiving PBS (g.1) was not show any lesions in the gastric mucosa while the treated mice with indomethacin (g.2-7) produced typical time dependent mucosal lesions in terms of UI values. Mice treated with indomethacin was the highest and showed significant increase in UI values at 66.67±15.44 and 69.69±18.68 at day 1 and day 3, respectively when compared with untreated control group. On day 5 and day 7, the area of indomethacin treated groups were progressive recovery and reduced the UI values to 57.78±27.99 and 48.89±13.11, respectively.

The UI values of SCC treated groups at 100, 250 and 500 mg/kgBW were 60.00±19.02, 69.69±18.68 and 57.78±27.99, respectively at the first day of ulceration. On day 3 of experiment, UI values were 61.27±30.93, 60±7.72 and 47.22±7.18, respectively. The UI values were reduced to 48.83±16.54, 45.24±10.80 and 40.63±6.87, respectively at day 5 and were 42.5±8.53, 38.89±11.55 and 33.79±4.81, respectively at day 7. Treated mice with SCC showed a concentration and dose-dependent reduction in UI values compared to indomethacin treated mice of the corresponding days.

The inhibition percent of gastric ulcers were calculated and the results showed that inhibition percent of gastric ulcer showed no significant difference in the group of indomethacin treated mice on day 1 and day 3. As well known, the mucosal were natural restored and the inhibition percent of gastric ulcer showed significantly increase to 13.34±9.69 and 29.67±11.34 %, respectively in day 5 and day 7.

Treatment with SCC increased inhibition percent as dose dependent manner and at the dose 500 mg/kgBW showed 49.31±1.41 % and showed the greater protective action than in omeprazole and vitamin C treated mice (**Table 1**).

Pathology and histopathology of gastric tissue of SCC prevent gastric ulceration by co-treatment treated

Ulcer healing is a complex process involving various factors. The gastro-toxicity of the NSAIDs including indomethacin in animals can be attributed to their ability to induce the reactive oxygen metabolites [25]. They also delay ulcer healing by reducing the prostaglandin level [26] and preventing the prostaglandin mediated angiogenesis. After haemorrhagic ulcer, re-epithelialization process are occur by forming a mucoid cap and also the growth factors and their receptors such as EGF receptor assist in ulcer healing by potential angiogenesis, and increasing the release of gastric mucin. SCC was arrested ulcer progression by antioxidant action due to powerful in vitro antioxidant property. Photograph of gastric tissue (**Figure 1**) and histopathological examinations (photograph not shown) revealed that administration of indomethacin at 10 mg/kgBW caused marked damage to the gastric mucosa within one day, with elongated haemorrhagic lesions, confined to the glandular portion. Maximum ulcerative damage was observed on the day 3 after administration of indomethacin and the nature of ulceration was evident from natural recovery (autohealing) of the gastric tissues even without any treatment.

In comparison, the mice treated with SCC at 500 mg/kgBW showed significant greater and faster in wool healing within 3 days. However, on the day 7 of ulceration, the natural recovery itself was substantial and the beneficial effect of the test samples was not very important. Overall, SCC (at highest dose) showed maximum efficacy on day 5 of treatment. SCC was similarly effective on gastro-protection and the qualitative histopathological assessment also correlated well with these data.

Determination of malondialdehyde (MDA) in plasma and tissue of co-treatment study

Compared to the normal mice, the content of thiobarbituric acid reactive substances of indomethacin treated mice was elevated to 15.50±0.91 and 19.71±1.38 nM/ml respectively on day 1 and day 3 of ulceration in plasma samples. Treatment of SCC, omeprazole or vitamin C showed immediate effect significantly reducing the MDA from day 1 of ulceration ($P < 0.05$) and the highest MDA levels were found on day 3 of ulceration. At day 3, SCC at concentration of 100 - 500 mg/kgBW reduced MDA levels to 14.33±0.59, 11.88±0.65 and 3.38±0.46 nM/ml respectively. Omeprazole or vitamin C at 100 mg/kgBW was decreased MDA level to 3.83±0.34 and 8.96±0.68 nM/ml compared to indomethacin treated group at day 3 of ulceration. After day 7 of ulceration, MDA level of all groups were significantly reduced when compared to indomethacin treated group and remained almost similar to normal level. The MDA level in plasma of SCC (100-500 mg/kgBW), omeprazole or vitamin C was 7.45±1.69, 4.08±0.77, 3.32±0.55, 3.42±0.39 and 4.04±0.79 nM/ml, respectively at day 7 of ulceration.

The MDA levels in gastric tissues were demonstrated in table 1. The MDA levels of indomethacin treated mice were significantly increased to 5.91±0.30 and 7.19±0.27 nM/mg protein of day 1 and day 3 of ulceration ($P < 0.05$) and were continuously reduced until the last day of ulceration by natural wool healing process.

Determination of gastric wall mucus on indomethacin-induced gastric ulcer of co-treatment study

Treatment with indomethacin at 10 mg/kgBW was rapidly decreased mucin secretion when compared to normal mice at day 1 and was lowest decreased at day 3 of ulceration at 10.38±1.89 µg Alcian blue/g wet stomach. Ulceration by indomethacin might reduce the ability of the mucosal membrane to protect the mucosa from pathological damage and back diffusion of hydrogen ions [25]. However, at day 5 and 7 of ulceration gastric mucosa were recovered by healing processed and was regenerated the mucosal cap to promote epithelial mucus, then the gastric wall mucus increased to 12.87±2.35 and 15.73±0.24 µg Alcian blue/g wet stomach, respectively as shown in **Table 1**.

The gastric wall mucus in SCC at 500 mg/kgBW treated group was also reduced from normal level, but significantly increased compared to indomethacin treated mice ($P < 0.05$). The reduction of gastric

wall mucus at day 3 of this SCC treated group was at $22.06 \pm 0.51 \mu\text{g}$ Alcian blue/g wet stomach. After day 5 and day 7 of ulceration the gastric wall mucus levels were increased to 30.35 ± 0.26 and $32.89 \pm 0.50 \mu\text{g}$ Alcian blue/g wet stomach, respectively. Omeprazole and vitamin C treated group, gastric wall mucus levels were reduced at day 3 and were recovered at day 5 and day 7 of ulceration. An increase in mucus production usually associated with an increase in the mucus layer in the gastric mucosal. Amongst the test samples, SCC restored the mucus level maximum, which was even better than omeprazole treated group.

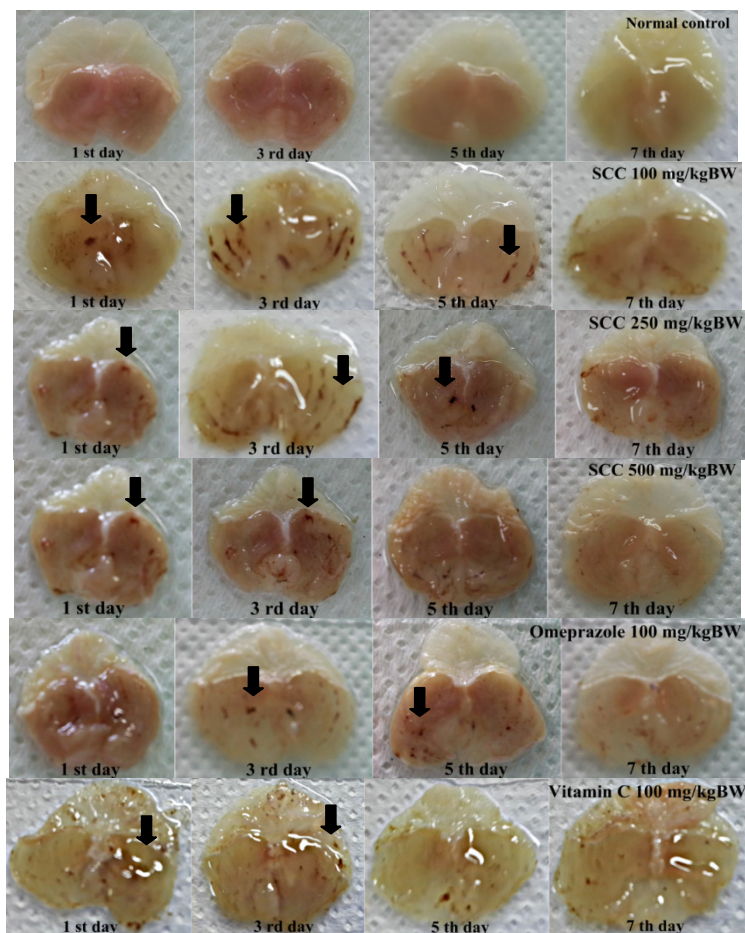


Figure 1 Photograph of protective effect of SCC on gastric tissue after induced with 10 mg/kgBW of indomethacin on day 1, 3, 5 and 7. Black arrow represented the ulcer area in gastric tissue.

Antioxidant effect of SCC in indomethacin induce gastric ulcer of co-treatment study
Effect on oxidized glutathione (GSSG)

GSSG level of indomethacin treated mice was higher increased than the other group at the same day of ulceration. The maximum GSSG level of indomethacin treated mice was found on day 3 and the level of GSSG were decreased after day 5 and 7 of ulceration. Treatment with SCC at all concentrations, omeprazole or vitamin C were significantly increased the GSSG level from day 1 to day 3 of ulceration and the highest level of GSSG are occurred on day 3. However, GSSG levels of SCC treated mice were

significantly reduced when compared to indomethacin group. SCC at 500 mg/kgBW was shown the great reduction on GSSG level after day 7 of ulceration.

Effect on glutathione peroxidase (GPx)

The activity of GPx enzyme was rapidly reduced in all treated mice from day 1 to day 3 of ulceration. After day 5 the recovery processes of gastro endothelial was adapted defense response to mucosa injury. Therefore, the GPx activity was increased in all treated group after day 5. In comparison to indomethacin treated mice, treatment with SCC at all concentration, omeprazole or vitamin C were increased the activities of GPx at the same day of ulceration. On day 7 of experiment, SCC at a concentration of 500 mg/kgBW was maintained the GPx enzyme activity to the basal level compared to normal mice.

Effect on nitrile contents

Nitrile contents of indomethacin treated group were significantly increased from day 1 to day 5 of ulceration compared to normal control. The highest level of nitrile was found on day 5 of ulceration at 3.16±0.42 nM/mg protein. All treated compounds (SCC, omeprazole or vitamin C) were significantly reduced the nitrile contents when compared to indomethacin treated mice at the same day of ulceration. The highest effective of treated compound is SCC at a concentration of 500 mg/kgBW, which was reduced the nitrile contents to 0.47±0.09 nM/mg protein at the last day of ulceration.

Table 1 Ulcer index, inhibition percent, malondialdehyde (MDA), mucus, oxidized glutathione (GSSG), glutathione peroxidase (GPx), nitrite contents and TNF-α on SCC co-treatment study in mice.

Parameter	Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Ulcer index	1	-	66.67±15.44 ^a	60.00±19.02 ^a	58.88±7.22 ^a	48.12±15.58 ^a	57.42±7.17 ^a	62.34±7.52 ^a
	3	-	69.69±18.68 ^a	61.27±30.93 ^a	60.00±7.72 ^a	47.22±7.18 ^{a,c}	62.91±8.11 ^a	64.47±5.54 ^a
	5	-	57.78±27.99 ^a	48.83±16.54 ^a	45.24±10.80 ^a	40.63±6.84 ^a	41.52±4.14 ^a	49.68±8.41 ^a
	7	-	46.89±13.11 ^a	42.50±8.53 ^a	38.81±11.55 ^a	30.79±2.81 ^{a,b}	38.58±3.69 ^a	48.86±10.05 ^a
	7	-	46.89±13.11 ^a	42.50±8.53 ^a	38.81±11.55 ^a	30.79±2.81 ^{a,b}	38.58±3.69 ^a	48.86±10.05 ^a
Ulcer inhibition (%)	1	-	0	10.01±8.44 ^{a,b}	12.13±3.13 ^{a,b}	27.82±2.50 ^{a,b,c}	13.39±7.75 ^{a,b}	6.49±7.59 ^{a,b}
	3	-	0	8.10±6.87 ^{a,b}	10.04±2.85 ^{a,b}	29.17±2.44 ^{a,b,c}	5.64±6.02 ^{a,b}	3.29±1.46 ^{a,b}
	5	-	13.34±9.69 ^a	26.76±7.01 ^a	32.14±8.62 ^{a,b}	39.06±3.33 ^{a,b}	37.72±7.15 ^{a,b}	25.48±7.50 ^a
	7	-	29.67±11.34 ^a	36.25±3.87 ^a	41.79±6.04 ^a	53.82±8.24 ^{a,b,c}	42.13±5.47 ^a	26.71±7.07 ^a
	7	-	29.67±11.34 ^a	36.25±3.87 ^a	41.79±6.04 ^a	53.82±8.24 ^{a,b,c}	42.13±5.47 ^a	26.71±7.07 ^a
Mucus (µgAlcian blue/g wet stomach)	1	37.38±2.13 ^{b,c}	19.89±0.52 ^{a,c}	22.11±0.05 ^{a,b,c}	25.72±0.62 ^{a,b,c}	27.44±0.50 ^{a,b}	27.45±0.04 ^{a,b}	22.78±0.67 ^{a,b,c}
	3	34.62±0.24 ^{b,c}	10.38±1.89 ^{a,c}	16.08±0.11 ^{a,b,c}	18.22±0.05 ^{a,b,c}	22.06±0.51 ^{a,b,c}	24.76±0.20 ^{a,b}	21.04±0.26 ^{a,b,c}
	5	35.95±1.77 ^{b,c}	12.87±2.35 ^{a,c}	24.97±0.35 ^{a,b}	28.54±0.05 ^{a,b,c}	30.35±0.26 ^{a,b,c}	25.12±0.26 ^{a,b}	25.08±0.51 ^{a,b}
	7	33.89±0.32 ^{b,c}	15.73±0.24 ^{a,c}	28.54±0.26 ^{a,b,c}	31.05±1.74 ^{a,b,c}	32.89±0.05 ^{a,b,c}	31.23±0.22 ^{a,b}	31.08±2.82 ^b
	7	33.89±0.32 ^{b,c}	15.73±0.24 ^{a,c}	28.54±0.26 ^{a,b,c}	31.05±1.74 ^{a,b,c}	32.89±0.05 ^{a,b,c}	31.23±0.22 ^{a,b}	31.08±2.82 ^b
Plasma MDA (nM/mL)	1	2.64±0.04 ^{b,c}	15.50±0.91 ^{a,c}	13.84±0.39 ^{a,b,c}	6.14±0.44 ^{a,b,c}	2.68±0.33 ^{b,c}	3.52±0.39 ^{a,b}	6.12±0.26 ^{a,b,c}
	3	2.65±0.50 ^{b,c}	19.71±1.38 ^{a,c}	14.33±0.59 ^{a,b,c}	11.88±0.65 ^{a,b,c}	3.38±0.45 ^{a,b}	3.83±0.34 ^{a,b}	8.96±0.68 ^{a,b,c}
	5	2.66±0.24 ^{b,c}	18.47±0.96 ^{a,c}	10.89±0.59 ^{a,b,c}	8.12±0.62 ^{a,b,c}	3.43±0.35 ^{a,b}	3.56±0.27 ^{a,b}	7.88±1.55 ^{a,b,c}
	7	2.62±0.02 ^{b,c}	9.46±0.65 ^{a,c}	7.45±1.69 ^{a,c}	4.08±0.77 ^{a,b}	3.32±0.55 ^{a,b}	3.42±0.39 ^{a,b}	4.04±0.79 ^{a,b}
	7	2.62±0.02 ^{b,c}	9.46±0.65 ^{a,c}	7.45±1.69 ^{a,c}	4.08±0.77 ^{a,b}	3.32±0.55 ^{a,b}	3.42±0.39 ^{a,b}	4.04±0.79 ^{a,b}
Tissue MDA (nM/mg tissue)	1	0.20±0.02 ^{b,c}	5.91±0.30 ^{a,c}	2.55±0.12 ^{a,b,c}	1.11±0.26 ^{a,b,c}	0.24±0.02 ^{b,c}	0.44±0.08 ^{a,b}	0.52±0.21 ^{a,b}
	3	0.20±0.08 ^{b,c}	7.19±0.27 ^{a,c}	3.96±0.24 ^{a,b,c}	2.09±0.65 ^{a,b,c}	0.72±0.65 ^{a,b}	1.18±0.08 ^{a,b}	1.28±0.59 ^{a,b}
	5	0.20±0.04 ^{b,c}	2.21±0.06 ^{a,c}	1.68±0.83 ^{a,c}	1.43±0.02 ^{a,b,c}	0.25±0.14 ^{b,c}	0.69±0.18 ^{a,b}	1.03±0.02 ^{a,b,c}
	7	0.20±0.02 ^b	1.76±0.02 ^{a,c}	0.88±0.24 ^{a,b,c}	0.39±0.02 ^{a,b,c}	0.20±0.04 ^b	0.24±0.02 ^b	0.26±0.02 ^{a,b}
	7	0.20±0.02 ^b	1.76±0.02 ^{a,c}	0.88±0.24 ^{a,b,c}	0.39±0.02 ^{a,b,c}	0.20±0.04 ^b	0.24±0.02 ^b	0.26±0.02 ^{a,b}

Parameter	Day	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
GSSG (ng/mgProtein)	1	0.87±0.01 ^{b,c}	1.07±0.04 ^a	1.01±0.04 ^{a,b,c}	0.95±0.01 ^{a,b,c}	0.89±0.20 ^{a,b,c}	1.05±0.03 ^a	0.93±0.01 ^{a,b,c}
	3	0.88±0.01 ^{b,c}	1.16±0.02 ^{a,c}	1.07±0.02 ^{a,b}	1.04±0.11 ^{a,b}	0.92±0.15 ^{a,b,c}	1.08±0.05 ^{a,b}	1.16±0.22 ^{a,c}
	5	0.92±0.02 ^{b,c}	1.14±0.02 ^{a,c}	1.03±0.04 ^{a,b,c}	1.02±0.06 ^{a,b,c}	0.91±0.05 ^{a,b}	0.90±0.04 ^b	1.14±0.32 ^{a,c}
	7	0.88±0.03 ^{b,c}	1.09±0.01 ^a	0.96±0.03 ^{a,b}	0.92±0.02 ^{a,b}	0.89±0.13 ^b	0.99±0.14 ^b	1.09±0.41 ^a
GPx (U/gProtein)	1	2.87±0.09 ^{b,c}	0.51±0.05 ^{a,c}	1.15±0.04 ^{a,b}	1.46±0.10 ^{a,b}	2.92±0.19 ^{b,c}	1.21±0.26 ^{a,b}	1.07±0.21 ^{a,b}
	3	2.78±0.19 ^{b,c}	0.41±0.06 ^{a,c}	0.69±0.09 ^{a,b,c}	1.32±0.04 ^{a,b,c}	2.40±0.07 ^{a,b,c}	0.89±0.05 ^{a,b}	0.82±0.03 ^{a,b,c}
	5	2.82±0.32 ^{b,c}	0.70±0.33 ^{a,c}	0.78±0.09 ^{a,c}	1.36±0.52 ^{a,b,c}	2.53±0.06 ^{b,c}	0.92±0.02 ^{a,b}	0.87±0.07 ^{a,c}
	7	2.82±0.43 ^{b,c}	0.75±0.28 ^{a,c}	0.81±0.05 ^{a,c}	1.51±0.37 ^{a,b,c}	2.59±0.07 ^{b,c}	1.08±0.03 ^{a,b}	0.93±0.32 ^{a,b,c}
Nitrile Contents (nM/mgProtein)	1	0.40±0.07 ^{b,c}	1.34±0.31 ^{a,c}	1.06±0.26 ^{a,c}	0.83±0.10 ^{a,b}	0.72±0.05 ^{a,b,c}	0.86±0.13 ^{a,b}	0.92±0.22 ^{a,b}
	3	0.35±0.08 ^{b,c}	2.48±0.06 ^{a,c}	1.43±0.35 ^{a,b}	1.01±0.06 ^{a,b,c}	0.91±0.12 ^{a,b,c}	1.51±0.49 ^{a,b}	1.70±0.30 ^{a,b}
	5	0.30±0.07 ^{b,c}	3.16±0.42 ^a	2.05±0.23 ^{a,b}	1.53±0.11 ^{a,b,c}	1.21±0.40 ^{a,b,c}	2.46±0.85 ^a	2.29±0.46 ^{a,b}
	7	0.26±0.09 ^{b,c}	1.99±0.33 ^{a,c}	0.66±0.05 ^{a,b,c}	0.60±0.06 ^{a,b,c}	0.47±0.09 ^{a,b,c}	1.26±0.17 ^{a,b}	1.78±0.29 ^{a,c}
Plasma TNF-α (pg/mL)	1	1.75±0.08 ^b	1.98±0.01 ^{a,c}	1.80±0.01 ^{b,c}	1.77±0.01 ^b	1.58±0.01 ^{a,b,c}	1.70±0.01 ^b	1.89±0.01 ^{a,b,c}
	3	1.77±0.06 ^b	2.43±0.07 ^{a,c}	1.88±0.02 ^{a,b}	1.82±0.03 ^b	1.76±0.01 ^{b,c}	1.84±0.04 ^b	1.92±0.03 ^{a,b,c}
	5	1.77±0.06 ^b	2.15±0.10 ^{a,c}	1.85±0.04 ^{a,b}	1.76±0.04 ^b	1.68±0.05 ^b	1.78±0.05 ^b	1.89±0.02 ^{a,b,c}
	7	1.76±0.09 ^b	2.00±0.01 ^{a,c}	1.81±0.01 ^{a,b,c}	1.77±0.01 ^{b,c}	1.62±0.01 ^{a,b,c}	1.69±0.01 ^b	1.90±0.01 ^{a,b,c}

^a*P* < 0.05 compared with normal mice group (Group 1), ^b*P* < 0.05 when compared with indomethacin treated group (Group 2) and ^c*P* < 0.05 when compared with omeprazole treated group (Group 6) at the same day of ulceration.

NSAIDs induced gastric damage is the major side effect associated with usage of this drug. Although the mechanism of NSAID induced gastropathy is generally believed to be related to the ability of these agents to inhibit gastric prostaglandin generation [27], there is a great deal of evidence to suggest that neutrophils are important in the pathogenesis of the gastric damage induced by NSAIDs [28]. Activated neutrophils are a major source of ROS production. On the other hand, organisms have enzymatic and non-enzymatic defenses, including GSH and GPx against the ROS-induced lipid peroxidation [29]. In this experiment results showed that indomethacin markedly increased MDA, an index of lipid peroxidation, accompanied by an increase of GSSG and decreased of GPx, all of which are endogenous antioxidants. These results support an imperative role for oxidative stress in the pathogenesis of indomethacin induced gastric ulcer. Nevertheless, treatment with SCC results in significant increase in the activities of GPx, as well as a decrease of GSSG and MDA formation compared to indomethacin treated mice at the same day, reflecting its antioxidant potential.

The mechanism which underlie SCC effect of oxidative stress in indomethacin induced gastric ulcer could be attributed to the direct antioxidant and free radical scavenging activity of SCC or indirectly due to augmentation of intracellular GSSG and GPx in mice gastric tissues, all of which can scavenge superoxide, hydrogen peroxide, hydroxyl and lipid peroxy radicals, and attenuate damages to the tissue. However, after day 5 of ulceration, ulcer-autohealing is processing even in indomethacin treated group and gastric tissue are recovery, thus treatment with SCC and SCF can enhancing this healing process as well as within 7 days of ulceration.

NO and ROS exert multiple modulating effects on inflammation and play key roles in the regulation of immune responses. In general, the neuronal and endothelial nitric oxide synthase (nNOS and eNOS) isoforms produce low amounts of NO [30]. In contrast, the inducible form of NOS (iNOS) produces NO in higher quantities. Ulcerogenic dose of indomethacin was a 12-fold increase in gastric epithelial expression of iNOS activity compared with controls [31], and this increase was positively correlated with damage to the epithelium. Therefore, these results were shown the high amount of NO production after

day 1 to day 5 of ulceration, thus iNOS generated NO is involved in gastric damage induced by indomethacin. However, the role of NO in granulocyte infiltration induced by indomethacin can be suggest that small amounts of NO released by cNOS (constitutive NOS) are sufficient to down-regulate indomethacin induced gastric granulocyte infiltration. iNOS generated NO is implicated in indomethacin induced gastric damage due to the course of an inflammatory response. Based on the results of our study, SCC was confirmed to have protective effects on indomethacin induced gastric ulceration in mice. The effect of SCC can be attributed to its reduction of oxidative damage, and its inhibitory effects on neutrophil infiltration as well as its anti-inflammatory effects in mice gastric tissue.

Plasma TNF- α of SCC in indomethacin induced gastric ulcer of co-treatment study

Plasma TNF- α in co-treatment study of normal mice was averaged from day 1 - 7 of ulceration is about 1.784 ± 0.01 pg/ml. Treatment with indomethacin was significantly increased from day 1 to day 3 of ulceration compared to normal mice and the peak of plasma TNF- α was 2.428 ± 0.07 pg/ml at the day 3. Treatment of SCC at a concentration of 500 mg/kgBW, plasma TNF- α was less increased when compared to normal group. In comparison with indomethacin treated mice at the same day of ulceration, SCC, omeprazole or vitamin C reduced the plasma TNF- α level from day 1 of experiment and were maintain the TNF- α level as normal mice until day 7 of ulceration (**Table 1**).

A proinflammatory role of TNF- α has been demonstrated to influence the early stage of indomethacin induced small intestinal toxicity [32] and affect experimental NSAID induced gastropathy [33]. TNF- α is activate nuclear transcription factor- κ B (NF- κ B), which lead to activation of transcription of various inflammatory genes [34], including iNOS. Increased plasma level of TNF- α has been reported to induce leukocyte adherence after indomethacin administration [35]. Therefore, TNF- α , as well as the TNF- α dependent expression of adhesion molecules, can be considered a major player in the initiation, progression and persistence of indomethacin induced gastric mucosal inflammation and injury [24]. Similar to our results, TNF- α was increased from 1 - 3 days of ulceration after treated mice with indomethacin but after day 5 of ulceration, the suppression of TNF- α was facilitated ulcer healing. Our finding are confirm that SCC reduced the magnitude of the inflammatory process, triggered modulation of production of inflammatory mediators, including TNF- α [36] and were played a clear beneficial role in gastric defense mechanisms, favoring mucosal barrier efficient, through mucus secretion and reducing the inflammatory response.

The expression of COX-1, COX-2 and iNOS of SCC in indomethacin induced gastric ulcer of co-treatment study

Standard NSAIDs inhibit the enzymatic activity of both the constitutive (COX-1) and inducible (COX-2) isoforms without much selectivity. Our western blot studies of COX-1 were demonstrated in Figure 2A. COX-1 was revealed reduced expression on the day of ulcer induction as well as at peak ulceration (day 3). Treatment with SCC at 500 mg/kgBW for 3 days were increased the expression of the enzymes markedly, while omeprazole showed insignificant effect. However, the effects of SCC was better with COX-1 enzyme restoring its expression to normalcy after day 5 of ulceration. Overall, SCC was more efficient than omeprazole.

In tune with the above results, the COX-2 was significantly increased at day 3 of ulceration, the results were demonstrated in western blot and the ratio of COX-2 compared with β -actin internal control (Figure 2B). When considering both COX-1 and COX-2 expression results together, it is possible to speculate that nonselective COX inhibitors conventional NSAIDs such as indomethacin produce gastric hypermotility, followed by microvascular disturbances and neutrophil activation, and resulting in gastric damage. The gastric hypermotility and subsequent vascular disturbances are associated with a PG deficiency caused by COX-1-inhibition [37]. Therefore, the inhibition of COX-1 up-regulates the COX-2 expression by indomethacin administration. SCC was used as a pretreatment compound 1 h before indomethacin treated. SCC was increased the up-regulation of COX-1 enzyme resulting in down-regulation to COX-2 expression when compared to indomethacin administration alone at day 3 of ulceration. Anyway, at day 5 and 7 the gastric ulceration were regenerated themselves, its make the

constitutive COX-1 enzyme to restored and work as housekeeping role in the stomach and up-regulated COX-2 expression at the same day of ulceration.

The accumulating information indicates that the mucosal tissue injury is accompanied by up-regulation in NOS, epithelial cell apoptosis and the induction of proinflammatory cytokines interleukin-1 β (IL-1 β) and TNF- α that trigger NF-k β activation [38]. From previous results, an inflammation in gastric mucosa by indomethacin is accompanied by increased production of TNF- α , which augments neutrophil-derived superoxide generation resulting to enhancing of NO level in plasma and increased the expression of iNOS in gastric tissue upon indomethacin administration at the peak of ulceration (day 3). However, NO is an antioxidant that regulates acid and gastric mucus release and mucosal blood flow and prevents peroxidation of membrane lipids [39]. Thus at day 5 of ulceration the high expression of iNOS was related with the adaptation processes of gastric tissue formation. SCC was strongly worked as anti-inflammatory compounds by down-regulated iNOS expression from the first day of ulceration compared to indomethacin treated group.

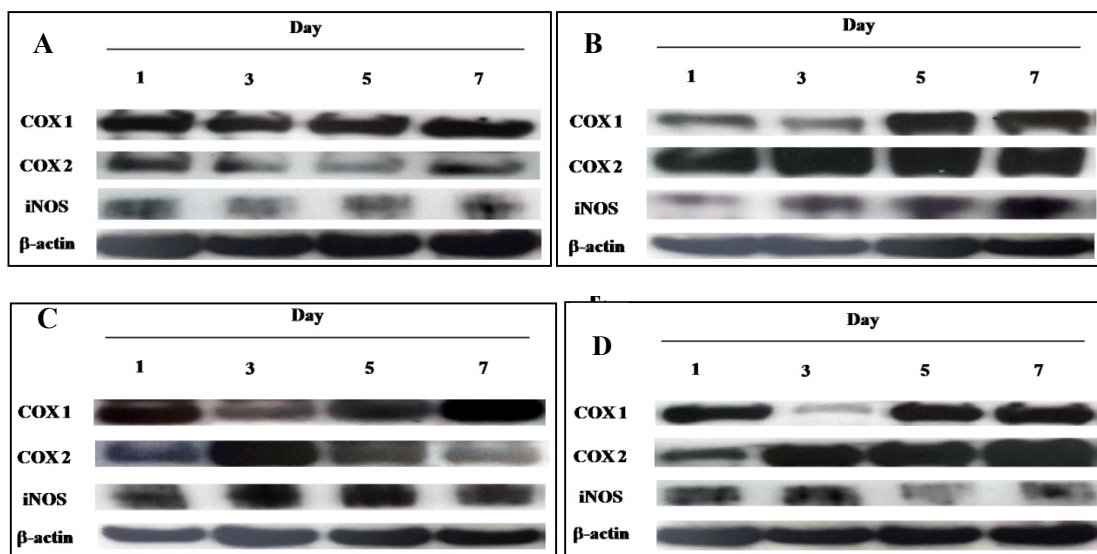


Figure 2 COX-1, COX-2 and iNOS expressed in co-treatment study at day 1, 3, 5 and 7. A: normal group, B: Indomethacin treated group, C: SCC at 500 mg/kgBW treated group and D: omeprazole at 100 mg/kgBW treated group.

The immune staining of EGF of SCC in indomethacin induced gastric ulcer of co-treatment group

EGF receptor accelerates gastroduodenal ulcer healing by promoting intestinal epithelial restitution, an important early process in the re-epithelialization of ulcers [40]. Our studies clearly indicate that activation of EGF receptor is an important early event in gastric mucosal regeneration following acute injury [41]. As shown in Figure 3, EGF receptor was strongly expressed at day 5 and day 7 of ulceration in SCC treated group.

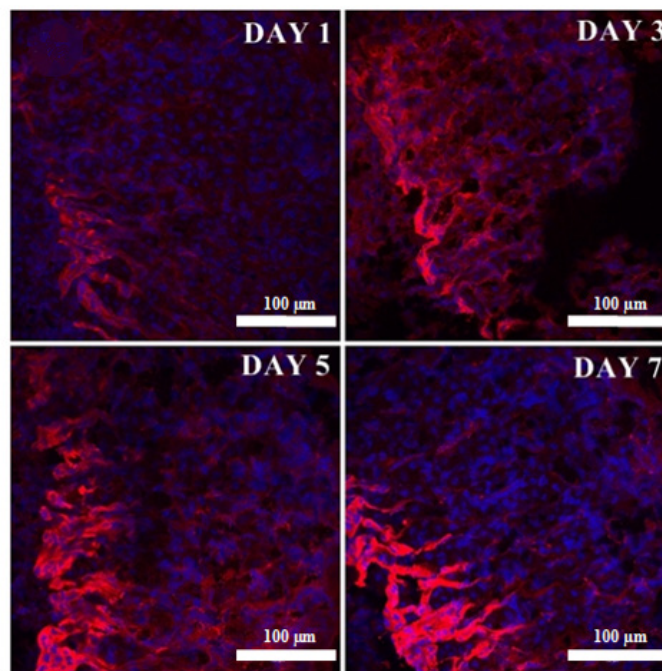


Figure 3 Immunohistograph of EGF receptor expressions in gastric tissues of SCC at 500 mg/kgBW in co-treatment study at day 1, 3, 5 and 7. Red color is showing EGF receptor expression and blue color are nuclei of gastric cells. White bars represent the scale of 100 μm .

Conclusions

Treated with indomethacin revealed mucosal haemorrhagic lesion and inhibited mucus content on mice. Redox imbalance was reflected by increased mucosal GSSG and NO content and decreased GPx activity along with elevated lipid peroxides. SCC caused discernible decreases in indomethacin induced gastric lesion and lipid peroxide content. In addition of co-treatment mice model, GSSG, GPx, NO levels and gastric wall mucus were restored. Indomethacin induced inflammatory by activated iNOS and TNF- α pro-inflammatory cytokines to release large amount of ROS/RNS. Co-treatment with SCC ameliorated TNF- α level and iNOS expression led to decrease the amount ROS/RNS. In a term of drug usage, indomethacin was used as non-selective inhibitor of COX enzyme which inhibited the expression of COX-1 and up-regulated COX-2 expression which may involvement of iNOS activation in this study. In co-treatment mice model, the investigation was continue from day 1 to day 7 of ulceration. The ulceration was peak at day 3 and after that a genetically programmed repair process (ulcer healing) is developed, resulting in the stage of scar formation even in indomethacin treated group. The major of early re-epithelialization of gastric ulcer was depended on an activated of the EGF receptor. Our results on the increased in number of EGF receptor expressing cells at day 5 and day 7 of SCC treatment over that of the first day of ulceration, EGF receptor was expressed in the ulcer margin, indicating the importance of EGF receptor in ulcer healing. Thus, SCC might contribute to their healing activity via increasing the levels of mucin and activated the growth factor receptor as an angiogenesis activity.

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