

## Quercetin Ameliorate Oxidative Stress-Induced Small Intestine Toxicity by Mefenamic Acid

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### ABSTRACT

The objectives of this study were to determine the involvement of oxidative stress and the ameliorative effect of quercetin (QR) on mefenamic acid-induced small intestine toxicity by measuring the level of superoxide radical, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), protein carbonylation (PC), and advanced oxidation protein products (AOPPs). In this experiment, a small intestine was taken from male rats (*Rattus norvegicus*). Samples then homogenized and divided into three groups with; T1 served as control which contains small intestine homogenate only; T2 which contains small intestine homogenate + 500 mg of mefenamic acid; and T3 which contains small intestine homogenate + 500 mg of mefenamic acid + 250 mg/L of quercetin. After treatment, every 15-minute superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs levels were estimated. The results revealed that mefenamic acid increased the level of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs, while QR decreases the level of all parameters. These results indicated that mefenamic acid induce small intestine toxicity through oxidative stress mechanisms as can be seen from the increasing of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs levels. Also, the results indicated that QR could inhibit these process as can be seen from the decreasing of all parameters.

**Keywords:** Small Intestinal Mucosa Damage, Mefenamic Acid, Oxidative Stress, Quercetin.

### INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used of therapeutic agents. Taken singly or in combination with other classes of drug, they relieve symptoms across multiple clinical indications, including short and long term pain states and a range of musculoskeletal disorders<sup>1</sup>. One of the commonly used NSAIDs is mefenamic acid. Mefenamic acid is NSAIDs are included in the group of an anthranilic acid derivative. It is commonly used to treat pains, including menstrual pain, osteoarthritis, headache, dental pain, postoperative and postpartum pain<sup>2</sup>.

Despite the wide usage, mefenamic acid causes a large variety of serious toxicity which includes severe gastrointestinal tract disorders, hepatotoxicity, and nephrotoxicity<sup>3</sup>. According to the previous study, the use of NSAIDs including mefenamic acid is associated with significant damage to the more distal regions of the small intestine. Small intestinal injury includes bleeding, erosion, and ulceration. Serious complications include massive bleeding, perforation, and strictures, leading to death<sup>4</sup>. The precise mechanism by which NSAIDs induce the injury of the small intestine is poorly understood. Previous reports suggest that small intestine injury by NSAIDs was associated with the increasing of intestinal

permeability, the magnitude of which directly correlates to the potency of their ability to inhibit cyclooxygenase-1 (COX-1)<sup>5</sup>. But still, the underlying mechanisms are incompletely understood, and currently, there are no clinically approved therapies available.

It is widely known that oxidative stress is involved in and play an important role in the development of gastrointestinal mucosa injury<sup>6</sup>. Our previous results show that mefenamic acid-induced gastric mucosal damage through oxidative stress and inflammation through the measurement of the level of superoxide radical, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Protein Carbonylation (PC), and Advanced Oxidation Protein Products (AOPPs)<sup>7</sup>. In this present study, we try to a different place of mucosa injury but with the same parameters. In this present study, we try to evaluate the involvement of oxidative stress mechanism in small intestinal mucosa injury by mefenamic acid through the measurement of those four parameters.

In this present study, we also used a quercetin (QR) to inhibit the small intestine toxicity. Our previous study found that QR has a protective effect to gastric mucosa which can be seen from the decreasing of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs levels in gastric mucosa<sup>7</sup>. Since the use of QR is useful to inhibit the gastric

mucosa injury by mefenamic acid, QUR may be able to use to inhibit the small intestine toxicity. Thus our present study aimed to investigate the involvement of oxidative stress mechanism in small intestine toxicity by mefenamic acid, and investigate the protective effect of QUR to inhibit this process.

## MATERIAL AND METHODS

### *Animals and Homogenate Preparation*

Male rats (*Rattus norvegicus*) weighing 200–250 gram with 2-3 months old were obtained from the Abadi Jaya farm at Yogyakarta, Indonesia, in healthy condition. The experiment was approved by the Ethical Committee of the Lambung Mangkurat University, South Kalimantan, Indonesia. Animals were fed under standard conditions and acclimatized with a 12 hours light/dark cycle. The animals were sacrificed by surgical procedure and the small intestinal was removed. Rinsed the mucosa with PBS. Then, the small intestine was homogenized in phosphate buffer saline (pH 7.0). Centrifuged the small intestinal at  $1000 \times g$  for 15 min at 4°C. The supernatant was collected and stored at -80 °C until determination of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs level.

### *Experimental Models*

Homogenate samples were divided into 3 groups (1 control group and 2 treatment groups). Control (T1) group: homogenate only; Treatment 1 (T2) group: homogenate + 500 mg of mefenamic acid; and Treatment 2 (T3) group: homogenate + 500 mg of mefenamic acid + 250 mg/L QUR; Each solution then incubated at 37°C for 60 minutes, and every 15 minutes the SOD activity, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs levels were estimated. In addition, all experimental models and measurement was done in Medical Chemical/Biochemical Laboratory, Faculty of Medicine, Lambung Mangkurat University, Banjarbaru, South Kalimantan, Indonesia.

### *Superoxide radical level analysis*

The superoxide radical level was measured by the method of Misra and Fridovich with slight modifications.<sup>8</sup> Samples were added to 0.800 ml of carbonate buffer (100 mM, pH 10.2) and 100 µl of adrenaline 3 mM resulted in adrenochrome. Superoxide radical levels are calculated based on adrenochrome which formed in solution by measuring the absorbance at 480 nm.

### *H<sub>2</sub>O<sub>2</sub> level analysis*

The H<sub>2</sub>O<sub>2</sub> level was calculated by the FOX2 method with slight modification. Solutions measured spectrophotometrically at  $\lambda = 505$  nm. Standard and test solutions consisted of 1 M H<sub>2</sub>O<sub>2</sub> 200 µL and 200 µL serum, respectively, with the addition of 160 µL PBS pH 7.4, 160 µL FeCl<sub>3</sub> (251.5 mg FeCl<sub>3</sub> dissolved in 250 ml distilled water) and 160 µL o-phenanthroline (120 mg o-phenanthroline dissolved in 100 ml distilled water) for both solutions. The composition of the blank solution was identical to that of the test solution, except for the absence of FeCl<sub>3</sub> in the blank. Subsequent to preparation, all solutions were incubated for 30 minutes at room temperature, then centrifuged at 12,000 rpm for 10 minutes, and the absorbance of the standard (As), test

(Au) and blank (Ab) solutions measured at  $\lambda=505$  nm, using the supernatant of each solution<sup>9-10</sup>.

### *PC level analysis*

PC was calculated by measuring the total protein carbonyl content. The total protein carbonyl content was determined by colorimetric method. The liver homogenate (0.5ml) was pipetted into 1.5 ml centrifuge tube and 0.5 ml of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl was added and allowed to stand at room temperature for 1 hour, with vortexing every 10-15 minutes. Then, 0.5ml of 20% Trichloroacetic acid was added followed by centrifugation. The supernatant was discarded and the pellets were washed 3 times with 1 ml ethanol-ethyl acetate (1:1) to remove free reagent. The obtained precipitated protein was redissolved in 0.6 ml guanidine solution. Carbonyl content was calculated from maximum absorbance (390nm)<sup>11-12</sup>.

### *AOPPs level analysis*

AOPPs measurement was made by spectrophotometric methods as describe by Witko-Sarsat et al., with slight modification. Briefly, AOPPs were measured by spectrophotometry on a microplate reader and were calibrated with chloramine-T solutions that in the presence of potassium iodide at 340 nm. In test wells, 200 ml of plasma diluted 1/5 in phosphate buffer solution were placed on a 96-well microtiter plate and 20 ml of acetic acid was added. In standard wells, 10 ml of 1.16 mol potassium iodide was added to 200 ml of chloramine-T solution (0–100 mmol/l) followed by 20 ml of acetic acid. The absorbance of the reaction mixture is immediately read at 340 nm on the microplate reader against a blank containing 200 ml of phosphate buffer solution, 10 ml of potassium iodide, and 20 ml of acetic acid. The chloramine-T absorbance at 340 nm being linear within the range of 0 to 100 mmol/l. AOPP concentrations were expressed as µmol/l of chloramine-T equivalents<sup>11,13</sup>.

### *Data analysis*

The results were expressed as mean±SE for two replicates. The data was analyzed between each parameter level and incubation time. For analyzing the data, Microsoft Excel 2010 was used and was examined by simple linear correlation. Furthermore, correlation chart that was generated for each treatment compared to one another.

## RESULTS

We initially measured the radical superoxide formation in each treatment. Compare to the T1 group, the radical superoxide formation is higher than the T2 group in all time of incubation (figure 1). But if we compared to the T3 group, The T1 and T2 group seems to be higher in radical superoxide formation in all time of incubation (figure 1). Another parameter that we analyzed in this study is H<sub>2</sub>O<sub>2</sub>. The result presented in figure 2. From figure 2, it can be seen that the level of H<sub>2</sub>O<sub>2</sub> has a positive correlation with time of incubation in all group of treatments. T1 seems to have a lower H<sub>2</sub>O<sub>2</sub> level than the T2 group in all time of incubation. T3 seems can

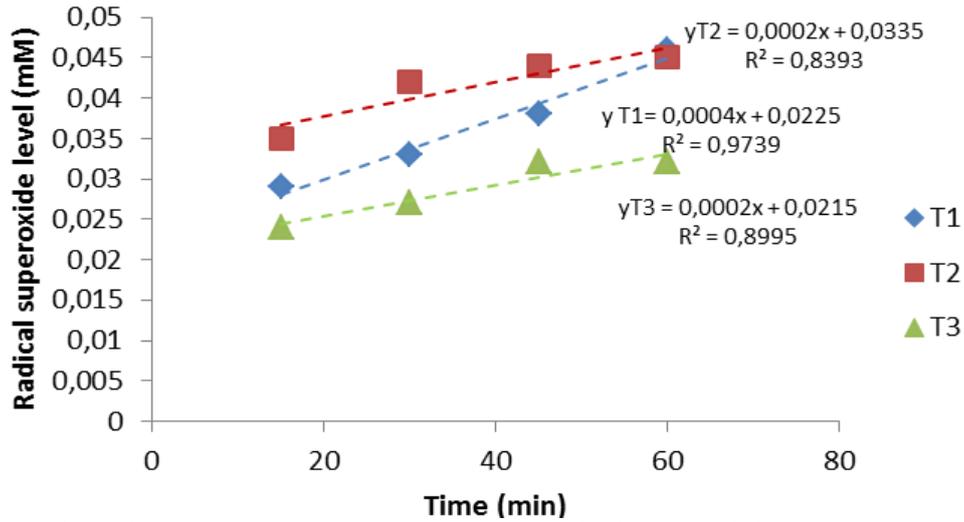


Figure 1: The correlation between time and the rate formation of radical superoxide between group of treatment. T1: homogenate only; T2: homogenate+mefenamic acid; T3: homogenate+mefenamic acid+quercetin.

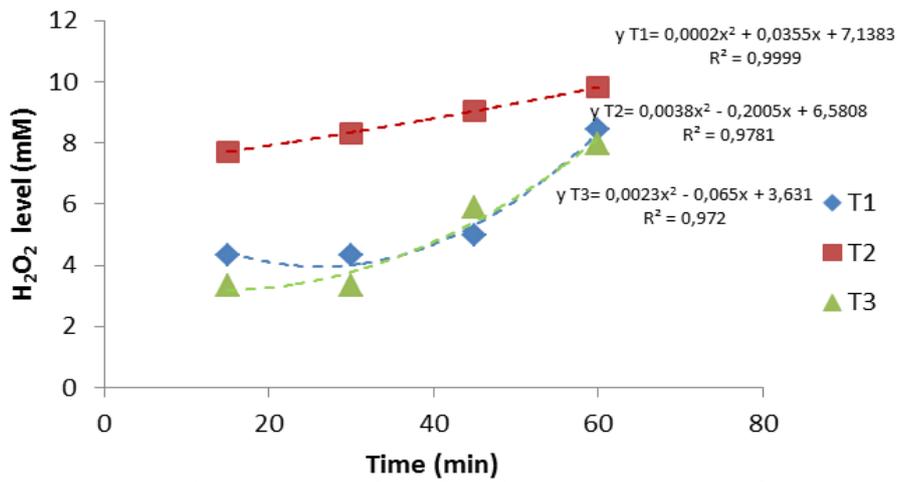


Figure 2: The correlation between time and the rate formation of H<sub>2</sub>O<sub>2</sub> between group of treatment. T1: homogenate only; T2: homogenate+mefenamic acid; T3: homogenate+mefenamic acid+quercetin.

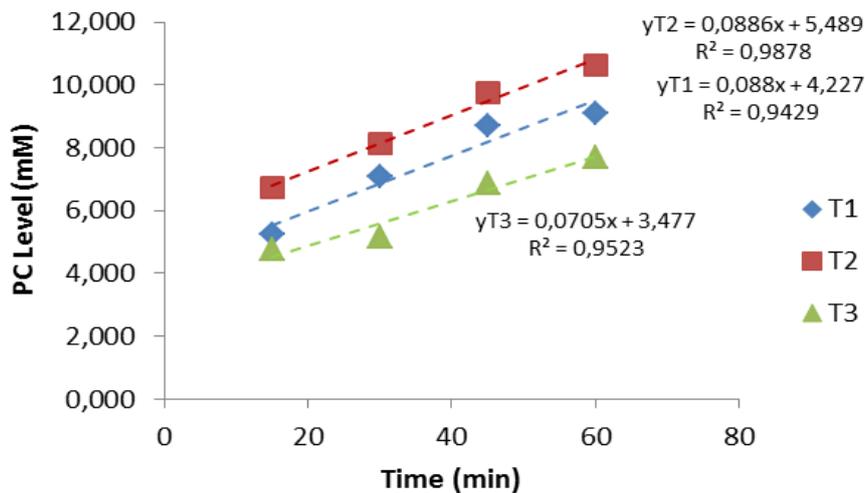


Figure 3: The correlation between time and the rate formation of PC between group of treatment. T1: homogenate only; T2: homogenate+mefenamic acid; T3: homogenate+mefenamic acid+quercetin.

decrease the level of  $H_2O_2$  till below the T1 group in 15th and 30th minute of incubation.

The next parameter that we analyzed in this present study is PC level. The result shows in figure 3. From figure 3, it can be seen that the lowest rate of PC level is in the T3 group and the highest is in T2 group. T2 can increase PC level in all time of incubation period compared to the T1 and T3 group. However, if we compared the rate of PC level between T1 and T3 group, it seems T3 can decrease the rate of PC level in all time of incubation period. The last parameter that we investigated in this present study is AOPPs. The result is shown in figure 4. AOPPs level shows a positive correlation with time of incubation in all group of treatments. The AOPPs level in T2 group is higher than T1 and T3 group in all time of incubation. The AOPPs level in T3 group is lower than T1 and T2 group in all time of incubation.

## DISCUSSION

Mefenamic acid, an anthranilic acid derivative, is an NSAIDs which is widely used to relief mild to moderate pain. Mefenamic acid is classified as class II on the basis of biopharmaceutical classification system. Mefenamic acid is widely used in Indonesia and available as a solid dosage form for oral administration<sup>14</sup>. Like other NSAIDs, mefenamic acid can cause serious gastrointestinal adverse effects (bleeding, ulceration) due to its mechanism of action and act as an irritant of the gastrointestinal mucosa<sup>15</sup>. Previous studies have reported that NSAIDs may induce gastrointestinal injury by acid-independent mechanisms such as by increasing oxidative stress parameters such as mucosal myeloperoxidase levels, together with an increase in mucosal malondialdehyde and reduced glutathione concentration<sup>16</sup>. In this present study, we using this approach to demonstrated the small intestine toxicity due to mefenamic acid, but we used a different parameter, such as radical superoxide,  $H_2O_2$ , PC, and AOPPs levels.

The result of this present study indicated that mefenamic acid could increase the generation of superoxide radical and  $H_2O_2$  in the small intestine. This result is in line with our previous result study that mefenamic acid increases the formation of radical superoxide and  $H_2O_2$  but in gastric mucosa homogenate. The mechanism that we propose in this study may follow the results of our previous study<sup>7</sup>.

Another parameters that we used to prove the involvement of oxidative stress in small intestine toxicity by mefenamic acid were PC and AOPPs. It is widely accepted that PC and AOPPs is a protein modification response to an oxidative stress condition.<sup>11,13</sup> The result is also in line with our previous report who found the increasing of both PC and AOPPs in gastric mucosa homogenate by mefenamic acid<sup>7</sup>.

Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species<sup>17</sup>. Protein carbonylation may occur due to direct oxidation of amino acid side chains (e.g. proline and arginine to  $\gamma$ -glutamyl semialdehyde, lysine to amino adipic semialdehyde, and threonine to amino ketobutyrate)<sup>18-19</sup>. In addition, PC derivatives can also result from an indirect reaction of primary amino groups of lysine with reduced sugar or their oxidation product and by a Michael addition reaction of lysine, cysteine and histidine residues with  $\alpha$ ,  $\beta$  unsaturated aldehydes formed during peroxidation of polyunsaturated fatty acids<sup>20</sup>.

AOPPs have been first described in the plasma of uremic patients at high levels as a marker of oxidant-mediated protein damage<sup>21</sup>. AOPPs, formed as a result of irreparable oxidative damage to the proteins, are defined as a novel reliable markers of irreversible oxidative damage<sup>22</sup>. AOPPs is tyrosine containing cross-linked protein products, a definition that is important as it excludes protein aggregates that are formed by disulfide bonds or amino acid modification as a result of oxidative stress<sup>11,23-24</sup>.

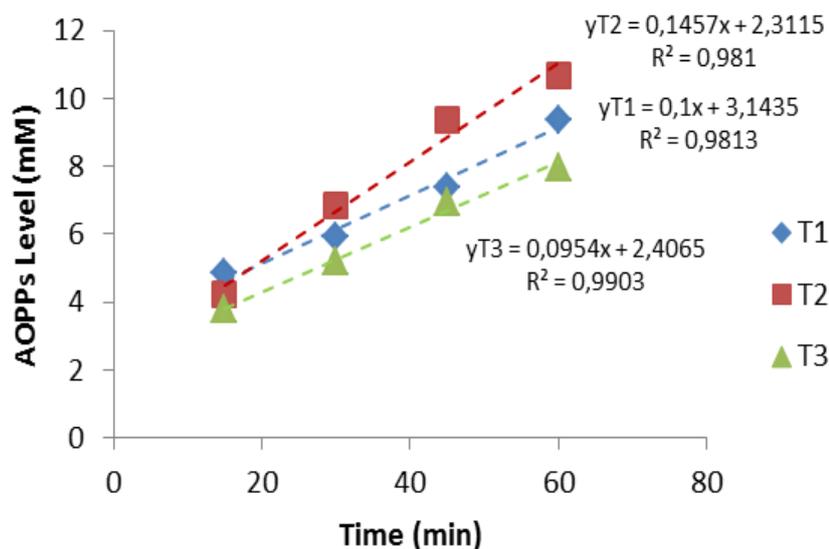


Figure 4: The correlation between time and the rate formation of AOPPs between group of treatment. T1: homogenate only; T2: homogenate+mefenamic acid; T3: homogenate+mefenamic acid+quercetin.

In this present study, we try to use QUR to inhibit the small intestine toxicity by mefenamic acid. The results indicated that QUR inhibit the mefenamic acid-induced small intestine toxicity. QUR can decrease the level of all parameters almost at all time of incubation. It is also in line with our previous result study in mefenamic acid-induced gastric mucosa damage<sup>7</sup>. QUR is a polyphenolic flavonoid compound, behaves as a powerful antioxidant and free radical scavenger and is able to interact with several key enzymes<sup>25</sup>. QUR is a potent scavenger of ROS and RNS. The antioxidant capacity of QUR has been ascribed to the presence of two pharmacophores within the molecule that have the optimal configuration for free radical scavenging, that is, the catechol group in the B-ring and the OH group at position 3<sup>26</sup>.

In conclusion, the present study demonstrated that mefenamic acid-induced small intestine toxicity through oxidative stress mechanisms as can be seen from the increasing level of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs. Also, the present study demonstrated that QUR could inhibit the mefenamic acid-induced small intestine toxicity which can be seen from the decreasing of superoxide radical, H<sub>2</sub>O<sub>2</sub>, PC, and AOPPs levels in small intestine toxicity.

#### CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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