**Protective Effect of 4-(3,4-Dihydroxyphenyl)-3-Buten-2-One from *Phellinus linteus* on Naproxen-Induced Gastric Antral Ulcers in Rats**

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The present study investigated the protective effect of naturally purified 4-(3,4-dihydroxyphenyl)-3-buten-2-one (DHP) from *Phellinus linteus* against naproxen-induced gastric antral ulcers in rats. To verify the protective effect of DHP on naproxen-induced gastric antral ulcers, various doses (1, 5, and 10 µg/kg) of DHP were pretreated for 3 days, and then gastric damage was caused by 80 mg/kg naproxen applied for 3 days. DHP prevented naproxen-induced gastric antral ulcers in a dose-dependent manner. In particular, 10 µg/kg DHP showed the best protective effect against naproxen-induced gastric antral ulcers. Moreover, DHP significantly attenuated the naproxen-induced lipid peroxide level in gastric mucosa and increased the activities of radical scavenging enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, in a dose-dependent manner. A histological examination clearly demonstrated that the gastric antral ulcer induced by naproxen nearly disappeared after the pretreatment of DHP. These results suggest that DHP can inhibit naproxen-induced gastric antral ulcers through prevention of lipid peroxidation and activation of radical scavenging enzymes.

**Keywords:** Anti-ulcer drug, DHP, gastric antral ulcer, naproxen, *Phellinus linteus*

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**Introduction**

Various nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for their analgesic, antipyretic, and anti-inflammatory effects. Ulcerations of the gastrointestinal tract are one of the major side effects associated with NSAID therapy. Gastric ulcers, bleeding, and perforation are the major limitations to their use as anti-inflammatory drugs [2, 3, 6, 12, 24]. Naproxen is a noncorticosteroid drug with anti-inflammatory, antipyretic, and pain-relieving properties, which is known to produce erosions, antral ulceration, and petechial bleeding in the mucosa of the stomach as an adverse effect [4, 21, 23]. In particular, generation of oxygen free radicals and lipid peroxides plays a crucial role in the development of naproxen-induced gastric antral ulcers [20, 25]. In this study, we chose naproxen as the ulcer-causing NSAID in rats, because it is used more frequently than other NSAIDs for arthritic patients, and also because the naproxen-induced gastric antral ulcer model is suitable in the human situation, where NSAID-induced gastric ulceration occurs mainly in the gastric antrum [5, 14, 15, 22].

Mushroom extracts and constituents have a long history of traditional use for treating various diseases. *Phellinus linteus*, a medicinal fungus known as “Sang-hwang” in Korea and “Mesimakobu” in Japan, is extremely rare to find in nature. They can be found rarely on wild mulberry trees that have grown for many decades in deep forests. The various medicinal effects of Sang-hwang in treating illnesses have been well known in oriental medicine since ancient times. Sang-hwang mushrooms have been proven to have anticarcinogenic effects by fully restoring the human immune system [9, 11, 17]. Sang-hwang extracts are highly effective in the treatment and preventive treatment of liver cancer [18], lung cancer [7], and prostate cancer [26]. In our study, 4-(3,4-dihydroxyphenyl)-3-buten-2-one (DHP) as a lipid-soluble organic compound and a naturally occurring antioxidant was purified from *Phellinus linteus*. Its
biological and pharmacological effects are still poorly defined. Moreover, there are no reports of the protective effect of DHP against naproxen-induced gastric antral ulcers. Because naproxen-induced gastric antral ulcers are mediated by the generation of free radicals, we hypothesized that DHP can inhibit naproxen-induced gastric antral ulcers, and such protective effect may be directly involving its antioxidant property. Therefore, we examined the protective effect of DHP by measuring the amount of lipid peroxidation and by comparing activities of enzymatic scavengers, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase.

Materials and Methods

Purification of DHP from *Phellinus linteus*

The 4-(3,4-dihydroxyphenyl)-3-buten-2-one (DHP) was extracted with ethanol for 1 week. The ethanol extract was suspended in hexane and H2O. The hexane extract was subjected to repeated silica gel (0.063–0.200 mm; Merck) column chromatography, using a hexane-ethyl acetate gradient as the eluting solvent, and quantitatively analyzed by high-performance liquid chromatography (YMC-Pack ODS-AM, 250 × 6.0 mm, ID 5 µm; with 70% acetonitrile) (Fig. 1).

Chemicals

Naproxen was purchased from Sigma Chemicals (St. Louis, MO, USA). Naproxen was dissolved in distilled water and subsequently administered by orogastric gavage, with an appropriate feeding needle in a volume of 5 ml/kg. DHP was dissolved in dimethyl sulfoxide (DMSO) immediately before use and administered intragastrically to rats in a volume of 5 ml/kg.

Animals

Male Sprague-Dawley rats (200–250 g, 7 weeks old) were purchased from Daehan Biolink Co., Ltd. Rats were placed singled in cages with wire-net floors in a controlled room (temperature 22–24°C, humidity 70–75%, lighting regimen of 12 h light and 12 h dark), and they were fed a normal laboratory diet. Typically, rats were fasted for 18 h prior to the studies. Following the first dose of naproxen, rats were provided with food for the remainder of the study. Rats were also allowed tap water throughout the study period. The animal experiment was performed in accordance with guidelines established by the Animal Care and Use Committee of Dong-Eui University and approved by the committee.

Experimental Strategy

The optimal condition for induction of naproxen-induced gastric antral ulcers in rats was determined on the basis of our previous study [10]. To investigate the protective effect of DHP derivative against naproxen-induced gastric antral ulcers, the rats were divided into six groups (n = 8 rats per group). The untreated normal rats received distilled water twice daily (at 07.00 h and 17.00 h) for 3 days, in comparable volume by the oral route. The control rats received only 80 mg/kg naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. Each of the remaining four test groups was treated with a vehicle (DMSO, 0 µg/kg DHP) and one of three doses (1, 5, and 10 µg/kg) of DHP twice daily (at 07.00 h and 17.00 h) for 3 days, and then treated with 80 mg/kg naproxen twice daily (at 07.00 h and 17.00 h) for 3 days. All the rats were killed under deep ether anesthesia 4 h after the naproxen treatment. The rat stomachs were promptly excised, weighed, and chilled in ice-cold 0.9% NaCl. After washing with 0.9% NaCl, the mucosa was homogenized in 50 mM potassium phosphate buffer at pH 7.5. Mitochondria and cytosol fractions were prepared according to the method of Hogeboom [8]. The quantitative analysis of protein was measured by Bradford protein assay.

Measurement of Lipid Peroxidation

Lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA) in the gastric mucosa according to the modified method of Ohkawa *et al.* [19]. The stomach homogenate was supplemented with 8.1% sodium dodecyl sulfate, 20% acetic acid (pH 3.5), and 0.8% TBA, and boiled at 95°C for 1 h. After cooling with tap water, the reactants were supplemented with n-butanol and pyridine (15:1 ν/ν), shaken vigorously for 1 min, and centrifuged for 10 min at 3,500 xg. Absorbance was measured at 532 nm. Lipid peroxidation was calculated from the standard curve using the MDA tetrabutylammonium salt. MDA concentrations were expressed as nM/g of tissue.

Measurement of SOD Activity

The activity of SOD in gastric mucosa of rats was determined according to the method of McCord and Fridovich [16]. The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.8, containing 0.1 mM EDTA, in a cuvette thermostated at 25°C. The reaction mixture contained 0.1 mM ferricytochrome c, 0.1 mM xanthine, and sufficient xanthine oxidase to produce a reduction rate of ferricytochrome c at 550 nm of 0.025 absorbance unit per minute. Tissue homogenate was mixed with the reaction mixture (50 mM potassium phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 0.1 mM ferricytochrome c, and 0.1 mM xanthine). Kinetic spectrophotometric analysis was started by

![Chemical structure of purified 4-(3,4-dihydroxyphenyl)-3-buten-2-one (DHP) from *Phellinus linteus*.](Image)
adding xanthine oxidase at 550 nm. Under these conditions, the amount of SOD required to inhibit the reduction of cytochrome c by 50% was defined as 1 unit of activity. The results were expressed as units/mg of protein.

**Measurement of Catalase Activity**

The activity of catalase in gastric mucosa of rats was determined according to the method of Aebi [1]. The standard assay was performed in 3 ml of 50 mM potassium phosphate buffer at pH 7.0 (1.9 ml), containing 10 mM H$_2$O$_2$ (1 ml) and tissue homogenate (100 µl). Under these conditions, the amount of catalase required to decompose 1.0 µmol of H$_2$O$_2$ per minute at pH 7.0 at 25°C was defined as 1 unit of activity. Absorbance was measured at 240 nm for 2 min, and the results were expressed as units/mg of protein.

**Measurement of Glutathione Peroxidase Activity**

The activity of glutathione peroxidase in the gastric mucosa of rats was determined by a modified method of Lawrence and Burk [13]. The reaction mixture consisted of glutathione peroxidase assay buffer (50 mM potassium phosphate buffer, pH 8.0, and 0.5 mM EDTA) and NADPH assay reagent (5 mM NADPH, 42 mM reduced glutathione, and 10 units/ml glutathione reductase). A sample of supernatant fluid with homogenate solution and 50 mM potassium phosphate buffer at pH 7.5 was prepared by centrifuging it at 1,000 x g for 10 min at 4°C. The cuvette was subsequently filled with 900 µl of glutathione peroxidase assay buffer, 30 µl of NADPH assay reagent, and 50 µl of sample, and mixed by inversion. The reaction started when 10 µl of 30 mM tert-butyl hydroperoxide or 80% cumene hydroperoxide was added. Absorbance was recorded by the following program; wavelength, 340 nm; Initial delay, 15 sec; Interval, 10 sec; and number of readings, 6. The activity of enzyme was the sum of data using 30 mM tert-butyl hydroperoxide and 80% cumene hydroperoxide. The level of glutathione was expressed in terms of µM/min/mg of protein.

**Histopathology**

Stomach tissues were fixed in 10% neutral formalin and embedded in paraffin, and 4-µm-thick sections were prepared and stained with hematoxylin and eosin by standard procedures.

**Statistical Analysis**

All values are represented as means ± SEM. Data were analyzed by ANOVA according to the general linear model procedure. The means were compared by Tukey’s studentized range (HSD) test to detect significant differences at $p < 0.05$.

**Results**

**DHP Prevents Naproxen-Induced Gastric Antral Ulcers in Rats**

To identify the protective effect of DHP against gastric antral ulcers, a vehicle (DMSO, 0 µg/kg DHP) and three doses (1, 5, and 10 µg/kg) of DHP were pretreated twice daily (at 07.00 h and 17.00 h) for 3 days, and then gastric antral ulcers were caused by 80 mg/kg naproxen treatment twice daily (at 07.00 h and 17.00 h) for 3 days. DHP significantly attenuated the gastric antral ulcer area in the mucosa of the stomach in a dose-dependent manner, compared with the control group. Values are expressed as means ± SEM. *$p < 0.05$, significantly different from the untreated normal rats. **$p < 0.01$, significantly different from the control rats.

**DHP Inhibits Naproxen-Induced Gastric Antral Ulcers through Elimination of Lipid Peroxidation and Activation of Radical Scavenging Enzymes**

According to the previous reports, generation of oxygen free radicals and lipid peroxides plays a crucial role in the development of naproxen-induced gastric antral ulcers. Therefore, the protective effect of DHP against naproxen-
induced gastric antral ulcers was evaluated by measuring the level of lipid peroxide and activities of radical scavenging enzymes in the stomach of all test groups.

In Table 1, naproxen in control and vehicle (DMSO, 0 µg/kg DHP) groups significantly increased the level of MDA, as an index of lipid peroxidation, in comparison with the untreated normal group (*p < 0.05). In contrast, DHP reduced the level of MDA in a dose-dependent manner in comparison with the control group. In particular, 10 µg/kg DHP showed a significant decrease in the level of MDA (**p < 0.01), compared with control group.

In Table 2, naproxen in the control and vehicle (DMSO, 0 µg/kg DHP) groups significantly reduced the activities of SOD, catalase, and glutathione peroxidase in comparison with the untreated normal group (*p < 0.05). On the other hand, DHP increased the activities of these enzymes in a dose-dependent manner in comparison with the control group. In particular, 10 µg/kg DHP showed a marked (**p < 0.01) increase of activities of radical scavenging enzymes, compared with the control group.

In conclusion, these results clearly suggest that DHP effectively prevents naproxen-induced gastric antral ulcers through elimination of lipid peroxidation and activation of radical scavenging enzymes, such as SOD, catalase, and glutathione peroxidase.

Discussion

Various NSAIDs induce severe ulcerations as one of the most common side effects and these ulcerative lesions are
the major limitation to their use as anti-inflammatory drugs [2, 4, 5, 12, 23, 24]. These compounds comprise polar lipids that have a high affinity for the lipophilic areas of cell membranes, where their polar groups trigger membrane disruption, with loss of structural phospholipids and membrane proteins [15]. However, most NSAID-induced gastric damage occur mainly in the corpus region of the stomach and tend to be mostly in the form of erosions rather than ulcers. This is unlike the situation in humans, where NSAID-induced gastric ulceration occurs mainly in the gastric antrum [14, 15, 22]. Therefore, we used a simple, reproducible, and relevant naproxen-induced gastric antral ulcer model that is suitable for the human situation. According to our previous study, 80 mg/kg naproxen applied for 3 days clearly caused gastric antral ulcer and showed a low mortality rate in rats [10]. This result was found to be optimal for anti-ulcer drug studies and used in the evaluation of DHP effect against naproxen-induced gastric antral ulcer.

DHP prevents naproxen-induced gastric antral ulcers in rats. DHP (1, 5, and 10 µg/kg) significantly attenuated the gastric damage area and the lipid peroxide level in a dose-dependent manner, compared with naproxen alone. In particular, 10 µg/kg DHP showed the best effect in reducing the gastric damage area and the lipid peroxide level.

The radical scavenging enzymes, such as SOD, catalase, and glutathione peroxidase play a key role in the defense against oxidative damage of the gastric mucosa after naproxen administration [20]. In this study, the activities of these enzymes were significantly reduced by naproxen, which indicated that inhibition of these enzymatic activities was, at least in part, responsible for the oxidative damage of gastric mucosa after naproxen administration, whereas DHP (1, 5, and 10 µg/kg) markedly increased the activities of these enzymes in a dose-dependent manner. Specifically, 10 µg/kg DHP completely protected the gastric mucosa against the loss in the enzyme, resulting in a drastic increase of activities of radical scavenging enzymes up to more than the level of untreated normal rats. Macroscopically, 10 µg/kg DHP distinctly reduced the depth and severity of the naproxen-induced gastric antral ulcer.

These results clearly reveal that DHP effectively prevents naproxen-induced gastric antral ulcers through elimination of the lipid peroxides and activation of radical scavenging enzymes, such as SOD, catalase, and glutathione peroxidase. Therefore, DHP is one of the powerful remedies of gastric antral ulcers and its use may offer an attractive and effective strategy for curing gastric antral ulcers in humans.

Acknowledgments

This work was supported by the Blue-Bio Industry Regional Innovation Center (RIC08-06-07) at Dong-Eui University as a RIC program under the Ministry of Trade, Industry and Energy and Busan City.

References