

Effects of *Apium graveolens* Extract on the Oxidative Stress in the Liver of Adjuvant-Induced Arthritic Rats

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ABSTRACT: *Apium graveolens* Linn. (Apiaceae) is an indigenous plant of the North and South Americas, Southern Europe, and Asia and has been widely used as a food or a traditional medicine for treatment of inflammation and arthritis. The purpose of this study was to investigate the antioxidant effects of a methanolic extract of *A. graveolens* (AGE) against liver oxidative stress in an adjuvant-induced arthritic rat model. The AGE (250, 500, and 1,000 mg/kg) was given orally for 24 consecutive days after induction by injecting complete Freund's adjuvant. Liver and spleen weights were recorded. The superoxide anion level, total peroxide (TP), glutathione peroxidase (GPx) activity, superoxide dismutase (SOD) activity, total antioxidant status, and oxidative stress index (OSI) were also measured. AGE treatment significantly decreased the levels of the superoxide anion, TP, and OSI whereas the GPx and SOD activities significantly increased in the liver of the arthritic rats. These results indicated that AGE showed an ameliorative effect against liver oxidative stress in adjuvant-induced arthritic rats by reducing the generation of liver free radicals and increasing the liver antioxidant enzyme activity.

Keywords: Chinese celery, rheumatoid arthritis, adjuvant-induced arthritis, reactive oxygen species, liver oxidative stress

INTRODUCTION

Rheumatoid arthritis (RA), characterized by a chronic and systemic inflammation that results in synovitis and destruction of cartilage and bone (1,2) contributes to the impairment of the quality of life as well as increasing the costs of general medical treatment for patients. T cells, B cells, macrophages, fibroblasts, and proinflammatory cytokines, particularly interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α are involved in the pathogenesis of arthritis that involves articular cartilage hyperplasia (3). In addition to the increase of proinflammatory cytokines, a high level of oxidative stress has been identified as one of the strongest risk factors of joint damage in RA. Overproduction of the cytokines stimulates inflammatory cells such as neutrophils and macrophages to secrete reactive oxygen species (ROS) in the synovial fluid, and this acts as a mediator of tissue injury (4,5). Apart from the destruction of articular cartilage, rheumatoid arthritis is also a key cause of inflammatory responses and immunological alterations in other organs, including vascular tissue, liver, and brain (6-9). Previous studies have shown that liver from adjuvant-induced ar-

thritic rats showed high levels of ROS as well as reduced gluconeogenesis, increased glycolysis, and decreased xenobiotic metabolism (8,10,11) and is also associated with evidence for a critical role of the liver in modulating the immune response in chronic inflammatory disease. Therapeutic agents approved for treatment of RA such as the non-steroidal anti-inflammatory drugs, corticosteroids, disease modifying antirheumatic drugs, and biological agents over long-term administration can induce a series of undesirable side effects including gastrointestinal bleeding, cardiovascular and liver complications (12,13).

Natural sourced compounds have been used as agents to prevent and treat arthritis and liver disease. *Apium graveolens* Linn., is a plant in the family Apiaceae, that has been widely used as food. It is found in the North and South Americas, Southern Europe, Africa, and Asia (14, 15). Many studies have revealed some pharmacological effects of *A. graveolens* extracts that were related to their antioxidant (16-19), hepatoprotective (20,21), and anti-inflammatory activities (22,23). Its major active constituents are phenolic compounds, flavonoids, and volatile oils that can be extracted from its various parts includ-

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ing roots, leaves, and seeds (16-19,22). *A. graveolens*, may be an alternative medicine for the reduction of the symptoms and other organ manifestations of arthritis because it has a natural origin and has been used over a long-term as food and in folk medicine. Thus, the aim of this study was to investigate the antioxidant effects of an *A. graveolens* extract against liver oxidative stress in an adjuvant-induced arthritis of rats.

MATERIALS AND METHODS

Chemical

Complete Freund's adjuvant (CFA) was from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used throughout this study were of analytical grade.

Apium graveolens extracts

Whole plants of *A. graveolens* were purchased from Lumpang Herb Conservation, Thailand. A voucher specimen (BKF no. 188856) has been deposited at the Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The dried plants were ground into a powder and extracted with 70% methanol for 3 days and filtered. The solvent was evaporated to dryness under reduced pressure (Rotavapor R-300, BÜCHI Labortechnik AG, Flawil, Switzerland) and the residue kept in a freezer.

Laboratory animals and adjuvant-induced arthritis

Fifty adult male Wistar rats (2-month old, 180~220 g) were obtained from the Laboratory Animal Faculty Unit, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. All animals were maintained under standard environmental conditions (12-h light/dark cycle, 25 ±2°C, and 50% humidity). Rats were allowed free access to food and drinking water. The experimental protocol was approved and performed according to the principles of animal care outlined by Faculty of Science, Prince of Songkla University (Ethical no: MOE 0521.11/466). Before studies, the rats were anesthetized with isofurane (Baxter Healthcare Co., LTD., Bangkok, Thailand) and arthritis was induced by a single intra-dermal injection 0.1 mL of complete Freund's adjuvant (CFA) (dry heat-killed *Mycobacterium tuberculosis*) into the subplantar region of the left hind paw (24,25). The rats were randomly divided into 5 groups of 10 rats as follow; Group I: normal control rats, Group II: arthritic control rats, and Group III~V: arthritic rats administered with the *A. graveolens* methanolic extract (AGE) at 250, 500, and 1,000 mg/kg body weight (BW), respectively. All treatments were oral, started on day 5 after induction by CFA injection, until day 28. The BW was measured at days 0, 7,

14, 21, and 28. At the end of the treatment (day 29), liver and spleen were separated under anesthesia (50 mg/kg sodium thiopental, i.p.) for analysis of superoxide anion level, total peroxide (TP), glutathione peroxidase (GPx), superoxide dismutase (SOD) activities, total antioxidant status (TAS), and oxidative stress index (OSI).

Preparation of liver tissue homogenates

Liver tissues were cut into small pieces and washed with ice-cold saline. Liver homogenate tissues were prepared using 0.01 M Tris-HCl buffer pH 7.4 at 4°C and centrifuged at 12,000 g for 45 min. Supernatant fluid from the liver homogenates were used for the assays.

Measurement of %inhibition superoxide anion

The assay for the %inhibition of superoxide anion was conducted according to the xanthine/xanthine oxidase (XO) system that involves the conversion of nitro blue tetrazolium (NBT) to formazan. The reagent mixture (ethylenediaminetetraacetic acid, NBT, xanthine, and XO) was incubated with the homogenized liver samples and the optical density was measured at 560 nm in comparison to a standard curve of 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl. The data were expressed as %inhibition calculated based on the following formula (26):

$$\%inhibition = \frac{\text{optical density (OD) of reagent} - \text{OD of sample}}{\text{OD of reagent}} \times 100$$

Measurement of the TP level

The TP levels of liver were determined by the ferrous oxidation-xylenol orange 2 (FOX2) method as previously described (27). A supernatant from the liver tissue was mixed with FOX2 reagent (ammonium ferrous sulphate buffer) and incubated at room temperature for 30 min. Then, the mixture was centrifuged at 14,000 rpm for 10 min. The absorbance of the supernatant was then determined at 560 nm. The TP level of the homogenized liver samples was determined using a solution of H₂O₂ as standard.

Measurement of the TAS level

The TAS level of the homogenized liver samples was measured as previously described by Erel (28). Supernatant from the homogenized liver tissue was incubated with reagent 1 (acetate buffer 0.4 mol/L pH 5.8) and reagent 2 [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) in acetate buffer 30 mmol/L pH 3.6]. The reaction mixture was incubated for 5 min and then measured at 660 nm. The TAS value of the homogenized liver was expressed as an equivalent of the millimolar concentration of the Trolox solution.

Measurement of the SOD and GPx activities

SOD activity was determined in order to detect superoxide radicals created from the xanthine/XO system by Cayman's SOD assay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The SOD activity was calculated and compared with the standard curve of SOD. The activity was expressed as units/mg protein. GPx activity was determined by the method of Hussain et al. (29). Briefly, supernatant from the liver tissue was incubated with the reaction mixture containing 48 mM sodium phosphate, 0.38 mM ethylenediaminetetraacetic acid, 0.12 mM β -nicotinamide adenine dinucleotide phosphate (NADP) hydrate, 0.95 mM sodium azide, 3.2 units of glutathione reductase, 1 mM glutathione, 0.02 mM DL-dithiothreitol, and 0.0007% H_2O_2 . The measurement of the absorbance of $NADP^+$ was performed at 340 nm compared to the reagent blank and standard curve of GPx. The values were expressed as units/mg protein. The protein concentration was measured following the method of Lowry et al. (30).

Measurement of the OSI

The percent ratio of the TP level to the TAS level was accepted as the OSI, which is an indicator of the degree of oxidative stress (27). The OSI value was calculated with the formula below:

$$OSI = \frac{TP \text{ (mmol/L)}}{TAS \text{ (mmol Trolox equivalent/L)}} \times 100$$

Statistical analysis

Experimental data were expressed as a mean \pm standard error of the mean (SEM). Significant differences were compared by one-way ANOVA with Tukey's post hoc mean separation. The statistical difference was regarded as $P < 0.05$.

RESULTS

Effect of AGE on body weight, body weight gain, liver weight, % relative liver weight, spleen weight and % relative spleen weight

There was a significant decrease in body weight and body weight gain in the arthritic control rats throughout the duration of the experiment ($P < 0.05$) when compared with the normal control rats. Arthritic rats treated with AGE at 250, 500, and 1,000 mg/kg had a significant increase in body weight and body weight gain when compared with the arthritic control rats (Table 1). In addition, there were significant increases of liver weight and % relative liver weight observed in the arthritic control rats when compared to the control group. However, the liver weight and % relative liver weight in the arthritic rats treated with AGE at dosages of 250, 500, and 1,000 mg/kg/d were significantly decreased when compared to the arthritic control group (Table 1).

Effect of AGE on the %inhibition of superoxide anion

The arthritic control rats demonstrated a significantly decreased %inhibition of superoxide anion ($P < 0.001$) when compared to the unimmunized control rats (Fig. 1). The oral administration of AGE at doses of 500 and 1,000 mg/kg significantly increased the %inhibition of the superoxide anion ($P < 0.001$) in a dose-dependent manner (Fig. 1). These results indicated that AGE decreased the superoxide anion level in the liver of arthritic rats.

Effect of AGE on the TP levels

TP levels in the liver of the arthritic group were significantly raised ($P < 0.001$). The treatment of arthritic rats with AGE at dosages of 250, 500, and 1,000 mg/kg significantly decrease the TP levels in the liver when compared to the arthritic control group (Fig. 2, $P < 0.05$, $P < 0.05$, and $P < 0.001$, respectively).

Table 1. Effects of oral administration of AGE in adjuvant induced-arthritis in rats

| Groups | Control | Arthritis control | AGE (mg/kg) | | |
|-------------------------------|-------------------|--------------------|---------------------------------|--------------------------------|---------------------------------|
| | | | 250 | 500 | 1,000 |
| Initial body weight (g) | 186.50 \pm 2.38 | 187.40 \pm 1.54 | 182.78 \pm 1.97 | 190.20 \pm 2.50 | 183.00 \pm 1.92 |
| Body weight after 14 days (g) | 278.50 \pm 3.80 | 252.80 \pm 3.27 | 298.44 \pm 4.63 | 271.40 \pm 3.57 | 259.90 \pm 10.29 |
| Final body weight (g) | 342.10 \pm 5.34 | 293.10 \pm 4.69* | 333.78 \pm 10.22 [#] | 336.90 \pm 6.93 [#] | 325.30 \pm 10.68 [#] |
| Body weight gain (g) | 155.60 \pm 5.26 | 105.70 \pm 4.54* | 151.00 \pm 10.40 [#] | 146.70 \pm 5.59 [#] | 142.30 \pm 10.38 [#] |
| Liver weight (g) | 12.67 \pm 0.35 | 13.65 \pm 0.85* | 12.21 \pm 0.54 [#] | 12.36 \pm 0.74 [#] | 12.10 \pm 0.60 [#] |
| Spleen weight (g) | 0.82 \pm 0.04 | 0.81 \pm 0.03 | 0.83 \pm 0.05 | 0.81 \pm 0.03 | 0.83 \pm 0.06 |
| % relative liver weight | 3.71 \pm 0.01 | 4.70 \pm 0.01* | 3.67 \pm 0.16 [#] | 3.69 \pm 0.24 [#] | 3.70 \pm 0.13 [#] |
| % relative spleen weight | 0.24 \pm 0.01 | 0.28 \pm 0.01 | 0.25 \pm 0.01 | 0.24 \pm 0.24 | 0.26 \pm 0.02 |

Data are presented as a mean \pm SEM (n=10).

Significantly different from the *control group and the [#]arthritic control group ($P < 0.05$), respectively.

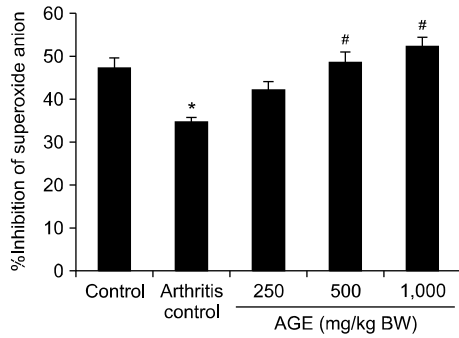


Fig. 1. Effects of methanolic extract of *A. graveolens* (AGE) (250, 500, and 1,000 mg/kg) on the %inhibition of superoxide anions in the liver of adjuvant-induced arthritic rats. The experiment was performed in triplicate for 10 rats in each group. Values are mean \pm SEM. Significantly different from the *control group and the #arthritics control group at $P<0.001$, respectively.

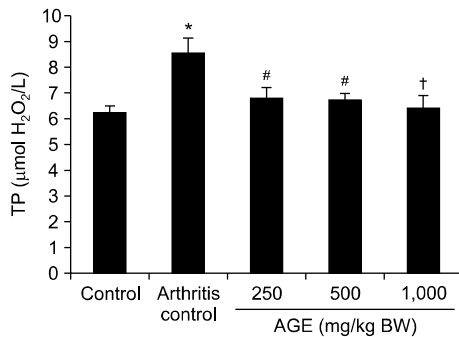


Fig. 2. Effects of methanolic extract of *A. graveolens* (AGE) (250, 500, and 1,000 mg/kg) on the total peroxide (TP) level in the liver of adjuvant-induced arthritic rats. Values are mean \pm SEM obtained from 10 rats. Significantly different from the *control group ($P<0.001$) and the arthritis control group at # $P<0.05$ and † $P<0.001$, respectively.

Effect of AGE on TAS level, GPx, and SOD activities

The TAS level was not significantly affected in any of the experimental groups when compared to the normal control group (Fig. 3). However, there was a significant inhibition of the liver GPx and SOD activities in the arthritic control group when compared to the unimmunized

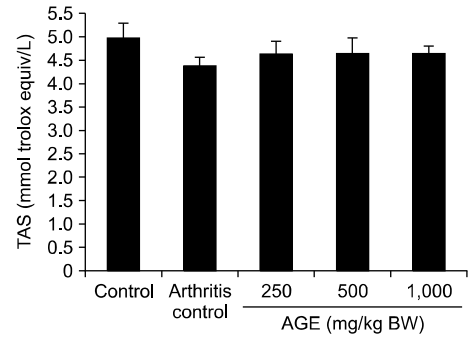


Fig. 3. Effects of methanolic extract of *A. graveolens* (AGE) (250, 500, and 1,000 mg/kg) on the total antioxidant status (TAS) levels in liver of adjuvant-induced arthritic rats. Values are mean \pm SEM obtained from 10 rats.

control group (Fig. 4A and 4B, $P<0.001$ and $P<0.05$, respectively). Treatment with AGE at 250, 500, and 1,000 mg/kg to the arthritis rat significantly increased the liver GPx activity in a dose-dependent manner when compared to the arthritic control group (Fig. 4A). In addition, the activity of liver SOD was significantly increased in all groups of AGE treatment in arthritis rats (Fig. 4B).

Effects of AGE on the OSI

The OSI was significantly increased in the liver of the arthritic control group when compared to the normal control group ($P<0.001$). Administration of AGE in particular at the highest dose of 1,000 mg/kg significantly decreased the OSI in the arthritic rats when compared to the arthritic control group (Fig. 5, $P<0.05$).

DISCUSSION

In this study, we have focused on investigating the beneficial effects of AGE on oxidative stress in the liver of adjuvant-induced arthritis. Recent evidence has indicated that there was a close correlation between the RA and oxidative stress in both humans and animals (31-33).

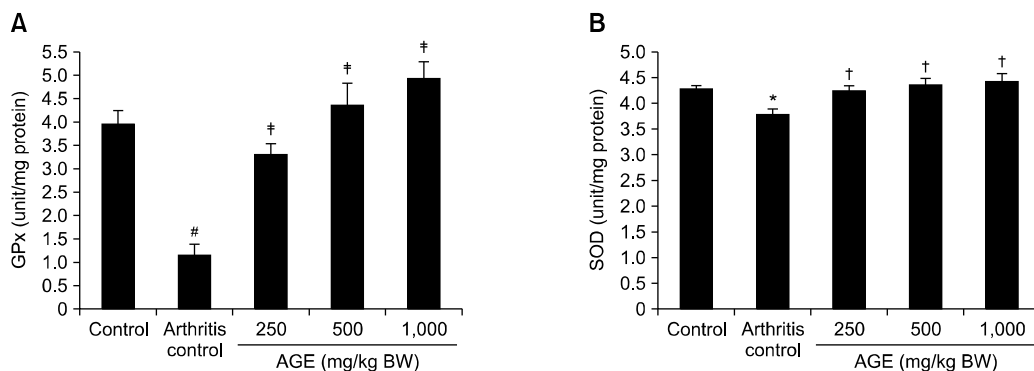


Fig. 4. Effects of methanolic extract of *A. graveolens* (AGE) (250, 500, and 1,000 mg/kg) on the (A) glutathione peroxidase (GPx) and (B) superoxide dismutase (SOD) activities in the liver of arthritic rats. Values are means \pm SEM obtained from 10 rats. Significantly different from the control group at * $P<0.05$ and # $P<0.001$, respectively, and the arthritis control group at † $P<0.001$ and ‡ $P<0.001$, respectively.

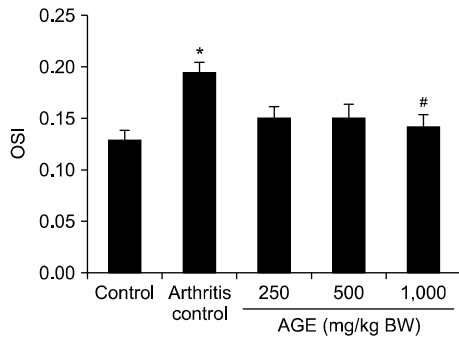


Fig. 5. Effects of methanolic extract of *A. graveolens* (AGE) (250, 500, and 1,000 mg/kg) on the oxidative stress index (OSI). Values are a mean ± SEM. Significantly different from the *control group at $P < 0.001$ and the #arthritic control group at $P < 0.05$.

Increased lipid peroxidation, oxidative stress and a decrease in the enzymatic antioxidants including GPx and SOD have been found in RA patients (32,33). Our previous finding showed that high levels of ROS and oxidative stress contributed to the destruction of cartilage in the adjuvant-induced rats (34). Moreover, other studies also reported the destruction of other organs including the liver and brain (8,9). Hepatic involvement has been reported in cases of RA associated with an abnormal liver function test, a mild chronic inflammatory infiltrate of the portal tract, small foci of necrosis and fatty liver (35, 36). Our results show that the oxidative stress was increased in the liver of adjuvant-induced arthritic rats compared to normal rats. This could be concluded from the higher levels of TP, superoxide anions, and the OSI found in liver homogenates of arthritic rats and also there was a decrease in the enzymatic antioxidants including GPx and SOD activities. Our results were consistent with the study of Comar and collaborators who reported a higher level of ROS content in the liver of arthritic rats that seemed to be the effect of both a stimulated pro-oxidant system and an inadequate antioxidant defense mechanism (8). Considering the involvement of ROS in RA, the hepatic biochemical and histological alterations were associated with the changes of the oxidative state in the liver cells. Reducing the oxidative stress would be a useful strategy to prevent and treat the liver organ complications found in arthritis. In the present study, the effect of AGE on the oxidative stress parameters was investigated in the liver of arthritic rats and showed that oral administration of 500 and 1,000 mg/kg AGE to rats with adjuvant induced arthritis also caused an effective reduction in the levels of the superoxide anion, TP, and the OSI in the liver of arthritic rats. In addition, AGE (250, 500, and 1,000 mg/kg) significantly increased the GPx and SOD activities in long-term treat-

ment. However, the current study demonstrated that AGE did not significantly change the TAS level in liver. Besides, our previous report found that *A. graveolens* increased plasma TAS level in arthritis rats (34). We suggested that *A. graveolens* decreased oxidative stress in liver by inhibiting free radicals and increased antioxidant enzyme activity, but did not affect non-antioxidant enzymes. From this study, the information about the long term administration of AGE might provide advantages in terms of the antioxidant status relating to the ameliorative effect against liver destruction in arthritis. It is obvious that *A. graveolens* possesses anti-oxidative properties due to its effect on oxidative stress inhibition and increasing the antioxidant capacity against liver damaged in RA. According to our results, administration of *A. graveolens* extract provided consistent results in various experimental models (21,37). *A. graveolens* administration has also been shown to have a hepatoprotective effect in paracetamol- and carbon tetrachloride-induced liver toxicity via its antioxidant effects and reduced oxidative stress (21,37). Additionally, *A. graveolens* extract mainly contains both phenolic (furanocoumarins, caffeic acid, and ferulic acid, etc.) and flavonoid (apiin, apigenin, luteolin, and chrysoeriol 7-glucosides, etc.) contents (16-19, 22) which are consistent to our previous report (34). Phenolic and flavonoid compounds possess antioxidant properties and have potent radical scavenging activity, which could react with free radicals and eventually terminate the radical chain reactions (16,17,19). Therefore, it is possible that the ability of phenolic and flavonoid in AGE might associate with the reduction of oxidative stress in the liver of arthritic rats.

In conclusion, the results of this study show that the liver of rats with adjuvant-induced arthritis exhibits a decrease in enzymatic antioxidants and also have increased production of ROS that appear to contribute to oxidative stress in the liver. AGE administration reduced oxidative stress by causing a decrease in the superoxide anion and TP, and increase in the activities of GPx and SOD. It can be suggested that *A. graveolens* represents a potential beneficial agent for reduction of liver destruction in arthritis.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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