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Effects of Alismatis Rhizoma Pharmacopuncture Extracts on the Elastase Activity and DPPH and NO Scavenging Activities

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澤瀉 藥鍼液의 elastase 활성과 DPPH, NO 소거능에 미치는 영향

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Abstract

목적 : 엘라스타제는 폐의 탄성 섬유를 파괴하여 폐기종과 폐고혈압을 유발하는 역할을 한다. 이에 본 연구는 澤瀉 藥鍼液의 엘라스타제 억제효능과 항산화효능을 측정하여 폐기종과 폐고혈압 치료의 가능성을 확인하고자 실시하였다.

방법 : 澤瀉 藥鍼液의 엘라스타제에 대한 억제효능과 di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) 및 nitric oxide (NO) 자유기 소거능을 측정하였다.

결과 : 澤瀉 藥鍼液은 엘라스타제 활성을 통계적으로 유의하게 억제하였고, DPPH 및 NO 자유기 소거능이 있음이 통계적으로 유의하게 확인되었다.

결론 : 본 연구를 통해 澤瀉 藥鍼液의 엘라스타제 억제효능과 항산화 효능이 확인되었다. 본 연구 결과를 근거로 시험관내 실험이외의 추가적인 연구를 통해 폐기종과 폐고혈압 치료제로서 澤瀉 藥鍼液의 활용을 고려해 볼 수 있을 것으로 기대한다.

Key words : Alismatis Rhizoma, *Alisma orientalis*, elastase, DPPH, NO

Ⅰ. Introduction

Alismatis Radix is the root of *Alisma orientalis*, which riches in alisol A, B, C, alisol A monoacetate, alisol B monoacetate, alisol C monoacetate, epialison A, alismol, alismoxide, choline, and other elements[1]. It is used to regulate water circulation and resolve dampness. It is also used to clear deficiency fire from the Kidney[1,2].

Neutrophil elastase (or leukocyte elastase) is a serine protease in the same family as chymotrypsin and has broad substrate specificity. As with other serine proteases it contains a charge relay system composed of the catalytic triad of histidine, aspartate, and serine residues that are dispersed throughout the primary sequence of the polypeptide but that are brought together in the three dimension conformation of the folded protein. The gene encoding neutrophil elastase, ELA2, consists of five exons. It breaks down elastin, an elastic fiber that,

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together with collagen, determines the mechanical properties of connective tissue[3]. This neutrophil elastase (NE) is a potent non-specific serine protease which plays a role as bactericidal agent in the degradation of immune complexes by intraphagosomal processes. It promotes inflammation, pulmonary emphysema, and chronic obstructive pulmonary disease[4-7]. The proposed pathogenesis of emphysema development involves a combination of inflammation, elastase, matrix metalloprotease imbalance, apoptosis, and oxidative stress[8]. It is generally accepted that pulmonary exposure to porcine pancreatic elastase (PPE), an enzyme that acts predominantly on elastin[9], elicits acute lung inflammatory response with neutrophils and macrophages[10-12]. Clinical studies for human pulmonary hypertension (PH) and systolic left ventricular failure are now in progress as well[4-7].

The production of reactive oxygen species (ROS) was induced by NE[13,14]. They reported that NE enhancement of MUC5AC messenger RNA levels was dependent on the production of intracellular oxidants or an alteration in the redox state of the cell. It means that ROS may play a role in elastase mediated inflammation. Nitric oxide plays an pivotal role in elastase mediated diseases as well[15].

In the present study, we investigated the effects of Alismatis Rhizoma pharmacopuncture extracts (ARPE) on elastase activity. Anti-oxdative activities of ARPE were also examined via measuring the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) free radical scavenging and nitric oxide (NO) scavenging activities .

II. Materials and Methods

1. Sample preparation

Alismatis Rhizoma (the root of *Alisma orientalis*) was purchased from Omniherb (Korea). ARPE was prepared as follow. 100 g of Alismatis Rhizoma in 2,000 ml distilled water was heated in a heating extractor for 3 hours. The extract was filtered and concentrated by using the rotary evaporator. The extracts were lyophilized by using freeze dryer (12.7 g). The lyophilized extract was dissolved in water and filtered three times through microfilter paper (Whatman no. 2, 0.45-0.2 μm). It was placed in a disinfected vial and sealed for further study.

2. Reagents

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3. Elastase activity inhibition

The elastase activity was evaluated by using a modification of a previously reported method of Kraunsoe et al[16]. In order to evaluate the inhibition of elastase activity, the amount of released p-nitroaniline, which was hydrolyzed from the substrate, N-succinyl-Ala-Ala-Ala-p-nitroanilide, by elastase, was read with a maximum absorbance at 410 nm[17]. In brief, 2 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide was prepared in a 0.1 M Tris-Cl buffer (pH 8.0), and this solution was added to the stock sample. Each sample solution was diluted to final concentrations of 0.01, 0.1, and 1 mg/ml. The solutions were mixed thoroughly

by tapping before an elastase (0.1360 unit/ml) stock solution was added. Solution was incubated for 10 min at 37°C, and the absorbance was measured at 410 nm (Synergy2, BioTek Inc., USA). The percent scavenging capability was calculated according to the following equation:

Elastase inhibitory activity (%)
= [(OD410 of control) - (OD410 of sample) / (OD410 of control)] × 100

4. DPPH free radical scavenging activity

The scavenging effect of sample on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was assayed according to the procedure described by Shimada et al[18]. The DPPH radical shows a deep violet color due to its unpaired electron, and radical scavenging capacity can be followed spectrophotometrically by the loss of absorbance at 540 nm[17]. In brief, sample was added to 1 ml of freshly prepared ethanolic solution containing a final DPPH radical concentration of 0.2 mM. After it stood for 30 min in the dark, the absorbance of the mixture was measured at 540 nm against an ethanol control with a ELISA reader (Synergy2, BioTek Inc., USA). The percent scavenging capability was calculated according to the following equation:

DPPH free radical scavenging activity (%)
= [(OD540 of control) - (OD540 of sample) / (OD540 of control)] × 100

5. Nitrite scavenging activity

Nitrite scavenging activity (NSA) of sample was determined by using Griess reagent[19]. First, 1 ml of sample was mixed with 1 ml of 1 mM sodium nitrite. The mixture was added to 8 ml of 0.2 M citrate buffer (pH 1.2, 3.0, and 6.0) and incubated for 1 h at 37°C. Then, 1 ml aliquot was removed and added to 2 ml of 2% acetic acid and 0.4 ml of Griess reagent (1% sulfanilic acid and 1% naphthylamine in a methanol solution containing 30% acetic acid). After vigorous vortex mixing, the mixture was placed at room temperature for 15 min and the absorbance was measured at 520 nm. The NSA (%) was calculated by the following equation.

NSA (%)=[1 - (A - C) / B] × 100

Where, A is the absorbance of treated sample, C is the absorbance of sample, and B is the absorbance of 1 mM $NaNO_2$.

6. Statistical analysis

The results were expressed as means ± standard error of the mean (SEM). Significances of changes were determined using the one-way ANOVA with a Dunnett's post hoc test. Values of $p < 0.05$ were considered significant.

III. Results

1. Inhibition of the elastase activity

The inhibitory effect of ARPE on elastase activity was determined according to the method described previously. ARPE showed the elastase inhibitory effect in a dose dependent manner. ARPE was found to inhibit elastase activity significantly at all concentrations of ARPE 10 mg/ml, 1 mg/ml, and ARPE 0.1 mg/ml treated groups (21.5 ± 2.4%, 38.0 ± 2.8%, and 51.3 ± 0.7% compared with 100% elastase activity of control group respectively, Fig. 1).

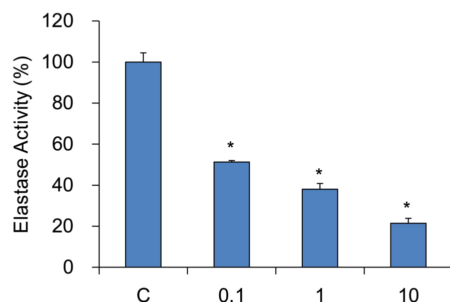

Fig. 1. Effect of ARPE on inhibition of elastase activity.
C: control, distilled water treated group. 0.1, 1, and 10: Alismatis Rhizoma pharmacopuncture extracts treated groups (0.1, 1, and 10 mg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from control, $p < 0.05$.

2. DPPH free radical scavenging capability

It has been reported that reactive free radicals was induced by elastase in neutrophil and that they play a role in inflammation. Assays of the free radical scavenging capacity were carried out by the DPPH method. The free radical scavenging capacity of sample was measured at each concentration (0, 4, 20, 100, and 500 mg/ml). A dose dependent free radical scavenging capability was observed in sample treated groups. ARPE 500 mg/ml treated groups had the highest scavenging capability, of 84.1 ± 11.0%, while 100, 20, and 4 mg/ml treated groups had 49.8 ± 1.2%, 18.6 ± 2.7%, and 7.3 ± 3.2%, respectively(Fig. 2).

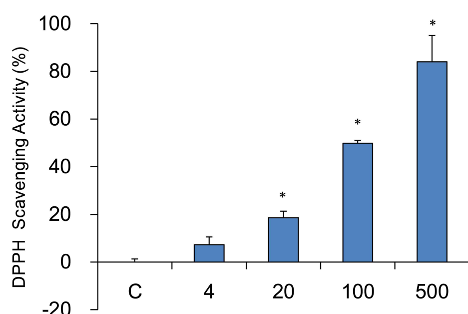

Fig. 2. DPPH free radical scavenging capability.
C: control, distilled water treated group. 4, 20, 100, and 500: Alismatis Rhizoma pharmacopuncture extracts treated groups (4, 20, 100, and 500 mg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from control, $p < 0.05$.

3. Nitrite scavenging capability at pH 1.2

Nitrite scavenging capability changes at various pH environments. Accordingly, nitrate scavenging activities at pH 1.2, 3.0, and 6.0 were measured in this study.

The nitrate scavenging capacity of sample was measured at each concentration (0, 0.4, 2, 10, and 50 mg/ml). A dose dependent nitrate scavenging capability was observed in sample treated groups. Three tested concentraions (2, 10, and 50 mg/ml) showed the statistical significances. ARPE 50 mg/ml treated groups had the highest scavenging capability, of 102.8 ± 0.1%, while 10, 2, and 0.4 mg/ml treated groups had 57.8 ± 0.1%, 14.8 ± 0.1%, and 3.3 ± 0.0%, respectively(Fig. 3).

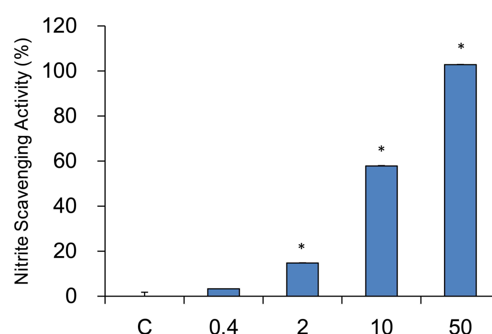

Fig. 3. Nitrite radical scavenging capability at pH 1.2.
C: control, distilled water treated group. 0.4, 2, 10, and 50: Alismatis Rhizoma pharmacopuncture extracts treated groups (0.4, 2, 10, and 50 mg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from control, $p < 0.05$.

4. Nitrite scavenging capability at pH 3.0

The nitrate scavenging capacity of sample was measured at each concentration (0, 0.4, 2, 10, and 50 mg/ml). A dose dependent nitrate scavenging capability was observed in sample treated groups. All tested concentraions showed the statistical significances. ARPE 10 mg/ml treated groups had the highest scavenging capability, of 92.3 ± 0.0%, while 50, 2, and 0.4 mg/ml treated groups had 51.4 ± 0.2%, 20.9 ± 0.1%, and 11.5 ± 0.3%, respectively(Fig. 4).

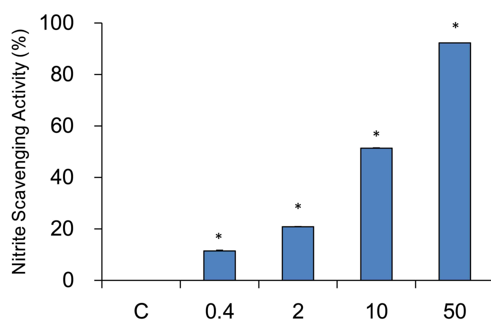

Fig. 4. Nitrite radical scavenging capability at pH 3.0. C: control, distilled water treated group. 0.4, 2, 10, and 50: Alismatis Rhizoma pharmacopuncture extracts treated groups (0.4, 2, 10, and 50 mg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from control, $p < 0.05$.

5. Nitrite scavenging capability at pH 6.0

The nitrate scavenging capacity of sample was measured at each concentration (0, 0.4, 2, 10, and 50 mg/ml). No nitrate scavenging capabilities were observed at all concentrations (Fig. 5). Considering data from Fig. 3 to 5, nitrite scavenging capability was varied with increasing pH, suggesting it is pH dependent. Accordingly, low pH environments (1.2 – 3.0) could be the best environment for nitrite scavenging activity of ARPE. However, pH 6.0 did not show the nitrite scavenging capability at all.

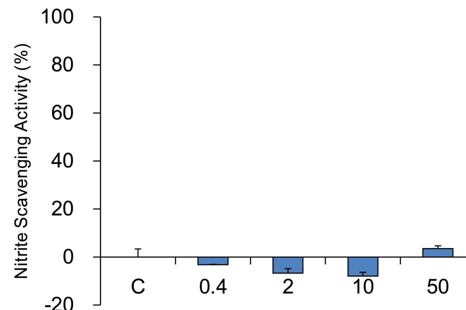

Fig. 5. Nitrite radical scavenging capability at pH 6.0. C: control, distilled water treated group. 0.4, 2, 10, and 50: Alismatis Rhizoma pharmacopuncture extracts treated groups (0.4, 2, 10, and 50 mg/ml). Data are expressed as the mean ± SEM of three experiments. *: significantly different from control, $p < 0.05$.

Ⅳ. Discussion and Conclusion

Alismatis Radix, the root of *Alisma orientalis*, is used to regulate water circulation and resolve dampness. It treats edema, dysruia syndrome, urinary disorders, vertigo, and other disorders characterized by accumulation of water and dampness[1,2]. It is also used to clear the fire charaterized as a deficient fire from the Kidney. It teats spermatorrhea, nocturnal emissions, and premature ejaculation, which are symptoms commonly associated with the rising fire from Kidney yin deficiency[1,2].

Elastase breaks down elastin, an elastic fiber that, together with collagen, determines the mechanical properties of connective tissue. The neutrophil form breaks down the outer membrane protein A (OmpA) of E. coli and other Gram-negative bacteria, and also breaks down Shigella virulence factors. This is accomplished through the cleavage of peptide bonds in the target proteins. The specific peptide bonds cleaved are those on the carboxy side of

small, hydrophobic amino acids such as glycine, alanine, and valine. Actually, elastase is the only enzyme that is capable of degrading elastin, an insoluble elastic fibrous protein in animal connective tissues. It is capable of hydrolyzing nearly all proteins, including supporting and structural proteins of the connective tissue such as collagen and elastin[20]. Elastin is the main component of the elastic fibers of the connective tissue and tendons. The elastic fibers in the skin, together with the collagenous fibers, form a network under the epidermis[21]. Elastase also plays a critical role in inflammatory processes. The enzyme has drawn much attention, primarily because of its reactivity and non-specificity. It is able to attack all major connective tissue matrix proteins, including elastin, collagen, proteoglycans, and keratins[22]. However, recent observations indicate that the role of neutrophil elastase (NE) in inflammation is more complex than the simple degradation of extra-cellular matrix components. This NE is a potent non specific serine protease which plays a role as bactericidal agent and in the degradation of immune complexes by intraphagosomal processes. It promotes inflammation when the granule contents are secreted in the extracellular environment. In certain pathological circumstances, an imbalance between NE and its major plasmatic inhibitor alpha 1-PI (formerly, alpha 1-antitrypsin) leads to abnormal tissue destruction and disease development. Genetic or acquired alpha 1-PI deficiency is thought to be involved in the pathogenesis of pulmonary emphysema. A variety of degenerative and degradative disorders are also associated to uncontrolled proteolysis by NE (rheumatoid arthritis, glomerulonephritis, adult respiratory distress symptom, psoriasis, cancer). Recent studies suggest that NE not only plays a key role in lung destruction (emphysema) but can also modulate proliferative changes (fibrosis) in inflammatory processes. Thus, NE could be considered to have potential multiple roles in the pathogenesis of both emphysema and lung fibrosis[23]. Numerous inhibitors of NE have been reported. Various molecules are currently undergoing clinical trials for emphysema and other pulmonary diseases[24]. The defects of elastic matrix aggravate hypertension which is associated with alteration in the great arteries, arteries, and arterioles. Clinical studies for human pulmonary hypertension (PH) and systolic left ventricular failure are now in progress. An elastase inhibitor is currently being investigated in phase I clinical trials in patients with PH owing to chronic obstructive pulmonary disease[4-7].

In this study, inhibitory effect of ARPE on elastase activity was determined according to the method. ARPE showed the elastase inhibitory effect in a dose dependent manner. ARPE was found to inhibit elastase activity highly at a concentration of 10 mg/ml.

The production of Reactive oxygen species (ROS) was induced by NE[13,14]. They reported that NE enhancement of MUC5AC messenger RNA levels was dependent on the production of intracellular oxidants or an alteration in the redox state of the cell. It means that ROS may play a role in elastase mediated inflammation. Accordingly, anti-oxdative activities of ARPE were also examined. DPPH free radical scavenging capability of ARPE was measured at each concentration (0, 4, 20, 100, and 500 mg/ml). A dose dependent free radical scavenging capability was observed.

Nitric oxide plays an pivotal role in elastase

mediated diseases[15,25]. So, nitrite scavenging activities were also examined. However, nitrite scavenging capability changes at various pH environments. Accordingly, nitrate scavenging activities at pH 1.2, 3.0, and 6.0 were measured in this study. Considering data at pH 1.2, 3.0, and 6.0, nitrite scavenging capability was varied with increasing pH, suggesting it is pH dependent. The low pH environments (1.2 – 3.0) could be the best environment for nitrite scavenging activity of ARPE. However, pH 6.0 did not show the nitrite scavenging capability at all.

In conclusion, ARPE showed the inhibiting effects on the elastase, and free radical scavenging capability of DPPH and nitrite. These results suggest that ARPE may have potential effects for pulmonary emphysema and pulmonary hypertension. Further studies might be needed to unravel exactly under the clinical trial and mechanisms.

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