INHIBITORY EFFECT OF HYALURONIDASE AND DPPH RADICAL SCAVENING ACTIVITY USING EXTRACTION OF *EQUISETUM ARVENS*

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ABSTRACT

Hyaluronidase (HAase, EC.3.2.1.35) is an enzyme that depolymerizes the polysaccharide hyaluronic acid (HA) in the extracellular matrix of connective tissue. The 1, 1- diphenyl 2-picrylhyorazyl (DPPH) is a well-known radical and a trap (scavenger) for other radicals. The purpose of the present study is to evaluate plant extracts as sources of natural antioxidants and to examine whether the herbal medicine (*Equisetum arvense* L.) having significant hyaluronidase inhibitory activity. The inhibitory effect of HAase by *E. arvense* was assayed using a Morgan microplate assay. The antioxidant activity of the *E. arvense* extracts was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical. HAase inhibition of foliage and central stalk was 24.3% at 4.0 mg/ml and that of rhizomatous stem and root was 27.3% at same concentration. DPPH scavenging activity of *E. arvense* was evaluated 96.2% at 4.0 mg/ml foliage and central stalk. DPPH scavenging activity for rhizomatous stem and root was 94.7% at same concentration.

Keywords: *Equisetum arvense* L., Hyaluronidase (HAase), 1, 1- diphenyl 2-picrylhyorazyl (DPPH).

INTRODUCTION

Hyaluronan (HA, also known as hyaluronic acid or hyaluronate) is one of the important matrix components of the ground substance of the subcutaneous tissues and plays important roles in development, growth, and repair of tissues (Pogrel et al., 2003). Hyaluronidase (HAase, EC.3.2.1.35) is an enzyme that depolymerizes the polysaccharide hyaluronic acid (HA) in the extracellular matrix of connective tissue. The enzyme is found both in organs (testis, spleen, skin, eye, liver, kidney, uterus and placenta) and in body fluids (tears, blood and sperm) (Duthie and Chain, 1939; Menzel and Farr, 1998).

The 1, 1- diphenyl 2-picrylhyorazyl (DPPH) is a well-known radical and a trap (scavenger) for other radicals. Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the radical nature of that reaction. All aerobic organisms have antioxidant defense systems to offset harmful effects caused by free radicals. Antioxidants can be found naturally in foods (Kedare and Singh, 2011) and their Antioxidant compounds in food play an important role as a health protecting factor.

The genus Equisetum consists of 30 species. *Equisetum arvense* L. (Equisetaceae), also known as the field horsetail or common horsetail is a herbaceous perennial plant. Horsetail is a member of an ancient and primitive family of plants from the dinosaur period. The species is native throughout the arctic and temperate regions of the northern hemisphere including Korea and Japan. The buds are eaten as a vegetable in Japan and Korea in spring time. All other *Equisetum* species are toxic. The plant is reported to contain a number of flavonoids, alkaloids, minerals, phenolic petrosins, triterpenoids, saponins, phytosterols (Sandhu et al., 2010). The plant contains several substances which can be used medicinally.

The purpose of the present study is to evaluate plant extracts as sources of natural antioxidants for DPPH and to examine whether the herbal medicine (*E. arvense*) having significant hyaluronidase inhibitory activity.

MATERIALS AND METHODS

Preparation of Crude Extracts

We divided the plants of *E. arvense* as two parts: above ground (foliage and central stalk) and below ground (rhizomatous stem and root). Foliage and central stalk or rhizomatous stem and root (100 g) of *E. arvense* were ground with pestles and liquid nitrogen at –70℃ and homogenized prior to beginning extraction experiments. The extraction solvent was ethanol. The sample was treated with ultrasound at room temperature for a given duration. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was further stirred with a magnetic bar at 65°C for 2 hours. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

Hyaluronidase Inhibition Assay

The inhibitory effect of HAase by *E. arvense* was assayed using a Morgan microplate assay. HAase (Type I-S from bovine testis, Sigma-Aldrich Co., England) is dissolved in 0.1 M acetate buffer (pH 3.5) and mixed with extracts of *E. arvense*. The resulting solution was applied to a microplate. A negative control (0.1 M acetate buffer) to serve as a reagent blank was also applied to another wells with enzyme. The plate was put in water bath for 20 minutes at 30°C. 12.5 mM $CaCl_2$ was added to the plate and incubated for 20 minutes at 37°C.

HA (6 mg/ml) which was dissolved in a 0.1 M acetate buffer was added to HAase complex solution and incubated for 40 minutes at 37°C. 0.4 N NaOH and 0.4 M potassium tetraborate were added to terminate the enzymatic reaction for 3 minutes at 100°C. After cooling the mixture until room temperature, 180 µl DMAB solutions (0.04 g/5 ml p-dimethyaminobenzaldehyde, 100% 3.5 ml acetic acid, and 10 N 5.0 ml HCl) were added to each well and incubated for 20 minutes at 37°C. The color change was measured spectrophotometrically at a wavelength of 540 nm.

HAase assay was validated by demonstrating that pure tannic acid (0.07 mg/ml Sigma-Aldrich Co., England) as a positive control, a known HAase inhibitor (Girish and Kemaraju, 2005) and tannic acids give 76-80% enzyme inhibition (Sumantran et al., 2007). All experiments were done in triplicate.

DPPH Free Radical

The antioxidant activity of the *E. arvense* extracts was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhyorazyl (DPPH) free radical according to the method described by Brand-Williams et al. (1995) with slight modifications. 1 ml of 0.1 mM DPPH solution in ethanol was mixed with 1 ml of plant extract solution of various concentrations (0.1, 1.0, 2.0 and 4.0 mg/ml). DPPH was added to the solutions prepared with plant extracts and standard antioxidant substances and stirred. Each mixture was kept in the dark and room temperature for 30 min and absorbance changes are measured

at 517 nm. Corresponding blank sample was prepared and L-Ascorbic acid (1-100 µg/ml) was used as reference standard (positive control). The inhibition % was calculated using the following formula.
Inhibition % = (Ac-As)/Ac×100
Where Ac is the absorbance of the control and As is the absorbance of the sample.

RESULTS

The rates of HAase inhibition of the ethanol extracts were dependent on concentrations (Table 1). Extractions at low concentrations, < 1.0 mg/ml were not shown noticeable HAase inhibition. High concentrations, > 1.0 mg/ml produced significant HAase inhibition. HAase inhibition of foliage and central stalk was 24.3% at 4.0 mg/ml and that of rhizomatous stem and root was 27.3% at same concentration. When the L-ascorbic acid used as a positive control, extracts for foliage and central stalk of *E. arvense* were 30.6 % inhibitory effects on the activation of HAase and that of rhizomatous stem and root was 33.6% (Fig. 1).

Table 2 was shown the antioxidant activities of the *E. arvense.* Although extracts of foliage and central stalk were higher than those of rhizomatous stem and root, there was not shown significant difference. DPPH scavenging activity of *E. arvense* was evaluated at 4.0 mg/ml foliage and central stalk and the highest as found to be 96.2%. DPPH scavenging activity for rhizomatous stem and root was 94.7% at same concentration.

Herbal medicine is a major part of traditional medicine and has been used in medical practice since antiquity to cure human and other animal. The herbal plant is a common element of ayurvedic, homeopathic, and naturopathic medicine (Amit et al., 2013). World health organization (WHO) notes that 74% of the plant derived medicines are used in modern medicine, in a way that their modern application directly correlates with their traditional use as herbal medicines by native cultures (Kumar and Parmar, 2003).

Traditionally the plant is used by local people and Ayurvedic physicians mainly for its burn healing properties. Horsetail is known for many medicinal usages such as anti-inflammatory, antinociceptive, antioxidant and antimicrobial (Amit et al., 2013). Recently, T cell proliferation was inhibited dose dependently by the *Equisetum* extract without induction of apoptosis or necrosis (**Gründemann et al., 2014).** Oh *et al. (2004)* observed that regular doses of *E. arvense* produced hepatoprotective effect. It is supposed that *E. arvense* is contained many specific drug quantity. *E. arvense* contains about 10 % inorganic constituents with two-third silicic acid (or silicates respectively) of with 10 % are water-soluble (Amit et al., 2013). Flavonoids (0.2 -0.9 %) are present with mostly caempferol and quercetin glycosides and their malonyl esters. Field horsetail contains more than 10% inorganic substances (two-thirds of which are silicic acid and potassium salts). Also, the drug is rich in sterols: β-sitosterol, campasterol, isofucosterol (D`Agostino et al., 1984), ascorbic acid, phenolic acids (cinnamic acids, caffeic acid, di-*E*-caffeoyl-*meso*-tartaric acid and 5-*O*-caffeoylshikimic acids), polienic acids, rare dicarboxylic acids (equisetolic acid), flavonoids (Wichtl, 1994) and styrylpyrones (Veti et al., 1995).

Table 1. Percent inhibition of hyaluronidase by *Equisetum arvense*

Concentration (mg/ml)	Inhibition by foliage and central stalk	Inhibition by rhizomatous stem and root
0.1	1.34±0.32	1.33±0.17
0.5	5.12±0.54	6.25±0.49
1.0	9.95±0.86	11.36±1.51
2.0	15.36±1.74	18.68±3.05
4.0	24.26±3.57	27.31±2.83

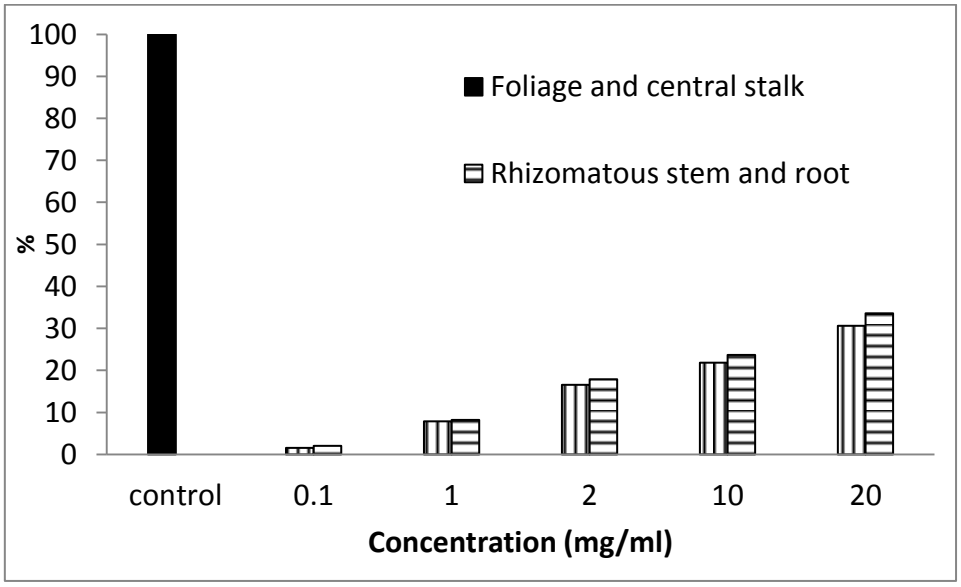

Fig. 1. The rate (100%) of hyaluronidase inhibitory of tannic acid (positive control) and relative inhibitory rate of *E. arvense*.

Table 2. Free radical scavenging effects of ethanol and water extracts of *Equisetum arvense* at different concentrations

Concentration (mg/ml)	Inhibition by foliage and central stalk	Inhibition by rhizomatous stem and root
0.1	71.37±6.42	70.55±5.69
0.5	77.55±3.54	76.79±1.86
1.0	83.28±3.28	82.65±4.47
2.0	92.16±1.62	90.37±1.12
4.0	96.22±1.45	94.66±1.47

CONCLUSIONS

We have shown that 4.0 mg/ml weight of ethanol horsetail extract has inhibitory effect of HAase and antioxidants for DPPH. Although extracts of foliage and central stalk were higher than those of rhizomatous stem and root, there was not shown significant difference.

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