

INHIBITORY EFFECT OF HYALURONIDASE (HAASE) AND HYDROXYL RADICALS (OH) OF *FRAXINUS RHYNCHOPHYLLA*

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ABSTRACT

Hydroxyl radicals (OH) generated in the human body may play an important role in tissue injury at sites of inflammation in oxidative stress-originated diseases. Hyaluronidase (HAase) depolymerizes the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue. The purpose of the present study is to evaluate plant extracts as sources of natural antioxidants and to examine whether *Fraxinus rhynchophylla* having significant hydroxyl radicals (OH) and hyaluronidase (HAase) inhibitory activity. OH of outer bark extracts of *F. rhynchophylla* was evaluated at 4.0 mg/ml was 58.4% and that of endodermis was 48.2% at same concentration. OH scavenging activity of leaf extracts of *F. rhynchophylla* was evaluated at 4.0 mg/ml was only 42.0% at same concentration. The outer bark of *F. rhynchophylla* showed maximum inhibition of OH activity (IC₅₀ = 54.5 ug/ml). The highest HAase inhibition was recorded in the outer bark extract (46.4%) among three tissues. Although the degree of inhibition of HAase by *F. rhynchophylla* was different among leaves, outer cortex, and endodermis at different concentrations, there were not show a statistically significant difference ($p < 0.05$). Strong inhibition of HAase enzymes by extract from *F. rhynchophylla* makes this pharmacopeial plant material an interesting topic for further biological and phytochemical examination.

Keywords: Hydroxyl radicals (OH), *Fraxinus rhynchophylla*, Hyaluronidase (HAase), Pharmacopeial plant.

INTRODUCTION

There are various antioxidant materials that scavenge free radicals in human plasma. It is possible that the radical-scavenging function causes a radiation protective effect in humans [1].

Living cells generate free radicals and other reactive oxygen species (ROS) as by-products of various physiological and biochemical processes. Reactive oxygen species (ROS), which consist of free radicals such as hydroxyl(OH[•]), superoxide (O²⁻), nitric oxide (NO), peroxy (RO²⁻), lipid peroxy (LOO[•]) radicals and non-free radical species such as hydrogen peroxide (H₂O₂), singlet oxygen(O¹), ozone (O₃), lipid peroxide (LOOH), are different forms of activated oxygen [2].

Hyaluronidase (HAase, EC.3.2.1.35) is a family of enzymes that depolymerizes the polysaccharide hyaluronic acid (HA) in the extracellular matrix of connective tissue

by catalyzing the hydrolysis of hyaluronan. The enzyme is found both in organs (testis, spleen, skin, eye, liver, kidney, uterus and placenta) and in body fluids (tears, blood and sperm) [3, 4]. 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 [5].

The genus *Fraxinus* L., the ashes, comprises 43 species occurring in temperate and subtropical regions of the northern hemisphere. The two main distribution areas are North America (20 species) and eastern Asia (20 species) [6]. Three species occur in Europe and western Asia. *Fraxinus rhynchophylla* Hancei (Oleaceae) is a tree plant growing to 25.0 m tall. The species has been known for its bright, cutting tone and sustaining quality. Thus it is

often used as material for acoustic guitar bodies.

F. rhynchophylla has been used a kind of commonly Chinese herbal drug and is officially listed in the Chinese Pharmacopoeia [7]. People usually use it to clear away pathogenic heat and remove the toxin, eliminate pathogenic heat from the blood to treat dysentery, and remove excessive heat from liver to improve visual acuity [8]. Furthermore, it also has been shown to possess expectorant, antitussive and anti-asthmatic effects. The extraction of the stem barks of *F. rhynchophylla* showed significant inhibitory activity on adipocyte differentiation as assessed by measuring fat accumulation [9]. The bark extract is analgesic, anti-inflammatory, antitussive, astringent, diuretic, expectorant and stomachic [10].

The purpose of the present study is to evaluate plant extracts as sources of natural antioxidants for hydroxyl radicals (OH) and to examine whether the herbal medicine (*F. rhynchophylla*) having significant HAase inhibitory activity.

MATERIALS AND METHODS

Sample extract

The plants of *F. rhynchophylla* was divided into three parts: leaves, outer bark, and endodermis. Endodermis was chopped with microtome (Leica Biosystems Nussloch GmbH, Germany). Each sample (100 g) of *F. rhynchophylla* was ground with pestles and liquid nitrogen at -70°C and homogenized prior to beginning extraction experiments. The ground powders were dissolved in 1000 ml ethanol and 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 stirred with a magnetic bar at 65°C for 12 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 dried extract, samples placed in a low temperature vacuum chamber.

Hydroxyl radical assay

The scavenging activity for hydroxyl radicals was measured with fenton reaction. Reaction mixture contained 60 μL of 1.0 mM FeCl_2 , 90 μL of 1mM 1,10-phenanthroline, 2.4 mL of 0.2 M phosphate buffer (pH 7.8), 150 μL of 0.17 M H_2O_2 , and 1.0 mL of extract at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with UV visible spectrometer Shimadzu, UV-1800, Japan.

Hyaluronidase inhibitory assay

The inhibitory effect of HAase by *F. rhynchophylla* 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 *F. rhynchophylla*. The resulting solution was applied to a microwell plate. A negative control (0.1 M acetate buffer) to serve as a 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 (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 absorbance of all wells was measured at a wavelength of 540 nm within 5 min post stopping.

HAase assay was validated by demonstrating that pure tannic acid (0.07 mg/ml Sigma-Aldrich Co., England) as a negative control, a known HAase inhibitor [9], give 76-80% enzyme inhibition [11]. All experiments were done in duplicate.

Statistical analysis

All the analysis were carried out in triplicate and the results were expressed as the mean \pm SD. Correlation co-efficient (R) to determine the relationship between two or more variables among hydroxyl radicals and among HAase were calculated using the SPSS software (Release 21.0). Regression analysis was used to calculate IC_{50} , defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction.

The percent inhibition was calculated as the decolourization percentage of the test sample using the following formula:

$$\text{Inhibition \%} = (\text{IA} - \text{As}) / \text{IA} \times 100.$$

Where IA is the absorbance of the 100% initial and As is the absorbance of the sample. IA and As were the values which were subtracted the average absorbance of the blank wells.

RESULTS AND DISCUSSION

Table 1 was shown the antioxidant activities of the *F. rhynchophylla*. Various concentrations of outer bark extracts were higher than those of leaves and endodermis. The maximum high antioxidant activity found on outer bark extracts. Hydroxyl radical (OH) of outer bark extracts of *F. rhynchophylla* was evaluated at 4.0 mg/ml was 58.4% and that of endodermis was 48.2% at same concentration. OH scavenging activity of leaf extracts of *F. rhynchophylla* was evaluated at 4.0 mg/ml was only 42.0% at same concentration. The outer bark of *F. rhynchophylla* showed maximum inhibition of OH activity ($\text{IC}_{50} = 54.5$ $\mu\text{g/ml}$) (Fig. 1). The values of IC_{50} of endodermis and leaves at the same level were 45.1 $\mu\text{g/ml}$ and 39.4 $\mu\text{g/ml}$, respectively (Fig. 1). The three groups for leaves, outer

bark and endodermis did not show a statistically significant difference ($p < 0.05$).

Table 2 was shown the HAase activity of *F. rhynchophylla* extracts. The highest HAase inhibition was recorded in the outer bark extract (46.4%) among three tissues. HAase inhibition of matured leaves was 31.1% at 4.0 mg/ml and endodermis was 24.2% at same concentration. Although the values of HAase inhibition of outer bark was higher than those of endodermis and leaves, there were not show a statistically significant difference ($p < 0.05$).

When the tannic acid used as a negative control, extract for outer barks of *F. rhynchophylla* was 38.1% inhibitory effects on the activation of HAase and that of outer bark and endodermis were 36.2% and 24.3%, respectively (Fig. 2). The outer bark of *F. rhynchophylla* showed maximum inhibition of HAase activity ($IC_{50} = 65.9 \mu\text{g/ml}$) (Fig. 3).

Traditional use of herbal medicines refers to the long historical use of these medicines. Traditional herbal medicines treatment has been considered to possess the advantage of improving the health-related quality of life of patients [12].

Their use is well established and widely acknowledged to be safe and effective, and may be accepted by national authorities. WHO estimates that 65–80% of the world's population uses traditional medicines as their primary form of health care and about 85% of traditional medicines involve the use of herbal preparations [13]. The hydroxyl radical and the superoxide anion radical

are strong oxidants that are generally categorised as reactive oxygen species (ROS). These species are responsible for degenerative diseases in humans and rancidity and deterioration in food [14]. Antioxidants can have an effect on oxidation processes in several ways [15]. The many traditional herbal species in Korea exhibited strong oxidant activity [10]. Akular and Odhav [16] reported antioxidants from 18 species in South Africa. *Portulaca oleracea* was high radical scavenging activity (96.5%) [16]. In this study, *F. rhynchophylla* was also relatively high (Table 1).

Hwang *et al.* [8] screened the HAase inhibitory activity of 500 methanolic extracts of 500 species from medicinal plants in Korea. They found that *Styrax japonica*, *Deutzia coreana*, and *Osmanthus insularis* were 57.28%, 53.50%, and 53.19%, respectively. *F. rhynchophylla* were 38.1% inhibitory effects on the activation of HAase (Fig. 2). These results show that these plants have some phytochemical constituents which may be active against the HAase enzyme.

The bark of *F. rhynchophylla* contains aesculin, this has anti-inflammatory, anticoagulant and analgesic actions [17]. The plant is used in the traditional medicine of Korea. The bark is analgesic, anti-inflammatory, antitussive, astringent, diuretic, expectorant and stomachic [18]. Although, *F. rhynchophylla* is classified as a medicine species, many studies will be required for the purification of active chemical groups from the crude extracts and to ascertain the mechanisms of action of these crude extracts [19].

Fig 1. Relative antioxidant values of the *Fraxinus rhynchophylla* extracts for control group (H_2O_2)

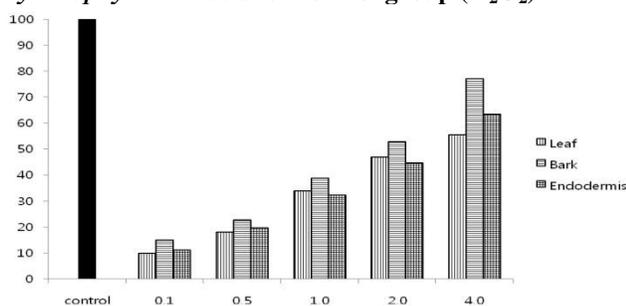


Fig 2. The rate of HAase inhibitory of nordihydroguaiaretic acid (negative control) and relative inhibitory rate of *Fraxinus rhynchophylla*

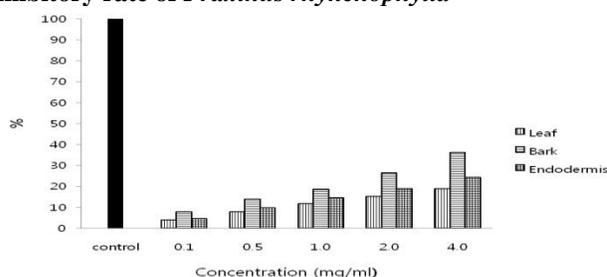


Fig 3. 50% inhibition { IC_{50} (mg/ml)} values on OH and HAase by *Fraxinus rhynchophylla* at 4.0 mg/ml concentration

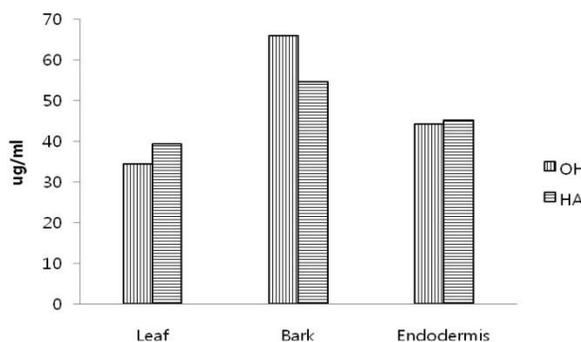


Table 1. Hydroxyl radical scavenging activity of *Fraxinus rhynchophylla* at different concentrations

Concentration (mg/ml)	Leaf	Outer bark	Endodermis
0.1	7.47±4.81	11.34±2.50	8.43±3.16
0.5	13.62±7.66	17.23±3.80	14.96±3.58
1.0	25.79±5.16	29.46±5.02	24.46±3.45
2.0	35.69±5.37	40.03±2.46	33.99±5.42
4.0	42.04±4.44	58.41±4.28	48.17±6.51
F-test	0.215, $p < 0.05$		

Data represent the mean ± SD from three replicates.

Table 2. Percent inhibition of Hyaluronidase by *Fraxinus rhynchophylla* at different concentrations

Concentration (mg/ml)	Leaf	Outer bark	Endodermis
0.1	5.22±1.77	10.13±2.44	5.99±3.11
0.5	10.03±3.14	17.99±1.57	12.52±2.04
1.0	15.01±4.39	23.89±1.60	18.70±2.28
2.0	19.50±1.92	33.92±5.29	24.33±1.75
4.0	24.20±3.02	46.38±6.79	31.14±3.54
F-test	1.512, $p < 0.05$		

Data represent the mean ± SD from three replicates.

CONCLUSION

Outer bark and endodermis of *F. rhynchophylla* have some phytochemical constituents which may be active against the HAase enzyme. We have

shown that 4.0 mg/ml weight of ethanol *F. rhynchophylla* extract has antioxidants for hydroxyl radicals (OH).

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