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ANTIOXIDANT ACTIVITY AND LIPOXYGENASE INHIBITION OF *HYDROCOTYLE NEPALENSIS*

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ABSTRACT

Objective: The objectives of this study were to determine antioxidant activities and lipoxygenase inhibition of ethanol extracts of *Hydrocotyle nepalensis* (Araliaceae) leaves and stems. **Materials and Methods:** The hydroxyl radical (-OH) can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation), and amino acids. The 1, 1- diphenyl 2-picrylhyorazyl (DPPH) is a well-known radical and a trap (scavenger) for other radicals. **Results:** The antioxidant activity of the *H. nepalensis* extracts was measured on the basis of the scavenging activity of the –OH and DPPH free radical. Hydroxyl radical scavenging activity of *H. nepalensis* was 78.8%. DPPH scavenging activity of leaves of *H. nepalensis* was evaluated at 8.0 mg/ml was 96.8%. Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes capable of oxygenating polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. The inhibitory effect of 15-LOX by *H. nepalensis* was assayed using a Morgan microplate assay. The extract of *H. nepalensis* was 67.6% inhibitory effects on the activation of LOX. The IC_{50} values for OH activity, DPPH activity, and LOX inhibition were 14.61 μg/ml, 5.26 μg/ml, and 31.27 μg/ml, respectively. **Conclusion:** These results show that *H. nepalensis* has some phytochemical constituents which may be active against the free radicals (OH and DPPH) and lipoxygenase enzyme.

KEYWORDS: Antioxidant activities, hydroxyl radical, 1, 1- diphenyl 2-picrylhyorazyl (DPPH), *Hydrocotyle nepalensis*, lipoxygenase.

INTRODUCTION

All living organisms utilize oxygen to metabolize and use the dietary nutrients in order to produce energy for survival. Oxygen thus is a vital component for living. While oxygen is one of the most essential components for living, it is also a double edged sword.[1] Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called free radicals. This may lead to damage, disease and severe disorders. Cell damage caused by free radicals appears to be a major contributor to aging and diseases such as cancer, heart disease, decline in brain function, and decline in immune system etc. Antioxidant molecules prevent or inhibit many harmful reactions in human body. Antioxidant scavenge free radicals are very important in inhibiting oxidative mechanisms that lead to degenerative some diseases. The 1, 1- diphenyl 2-picrylhyorazyl (DPPH) is a well-known radical and a trap (scavenger) for other radicals.[2] Antioxidants can be found naturally in plants and their compounds in food play an important role as a health protecting factor.[3]

The hydroxyl radical ($OH^{\bullet}$) is the most potent yet short-lived of the reactive oxygen species (ROS) radicals. The hydroxyl radical constitutes the chemically most reactive species of `activated oxygen' formed by successive monovalent reduction of dioxygen (O_2) in cell metabolism, and is primarily responsible for the cytotoxic effects of oxygen in plants, animals and micro-organisms, living in an oxygenic atmosphere.[4]

Lipoxygenase (LOX) is an enzyme that catalyzes the oxidation of polyunsaturated fatty acids to form a peroxide of the acid. Originally, mammalian cells were thought to contain four type of LOXs (5-, 8-, 12-, and 15-lipoxygenases) corresponding to their prominent regiospecificities toward arachidone acid. The structural determinants of the positional specification of 12- and 15-lipoxygenases are particularly well investigated.[5] 15-lipoxygenases (also termed arachidonate 15-lipoxygenase, 15-lipoxygenase-1, 15-LO-1, 15-LOX-1) is a polyunsaturated fatty acid-metabolizing enzyme that in humans is encoded by the ALOX15 gene located on chromosome 17p13.3.[6] 15-Lipoxygenases have been found in plants as well as in animal tissue. Commercially obtainable 15-1ipoxygenase is isolated from soybeans as the type 1 isoenzyme.

Hydrocotyle nepalensis Lam. (Araliaceae) is a small species native to southeastern Asia. The species is a low-growing perennial plant that only reaches a height of 10 cm, but spreads to form clumps 30 cm or more wide. The plant is gathered from the wild for local use as a food and medicine. The plant is decocted and used in the treatment of abscesses, boils, bruises, cirrhosis, colds, coughs, hepatitis, hepatoma, influenza, itch, jaundice, sinusitis and sore throat.[7] It is a Chinese herbal drug for hepatoma.

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

MATERIALS AND METHODS

Sample extract

The plants of *H. nepalensis* divided into two parts: leaves and roots. Each sample (100 g) of *H. nepalensis* was ground with pestles and liquid nitrogen at -70℃ and homogenized prior to beginning extraction experiments. The samples were blended with 50% ethanol, and then an aliquot of the mixture (100µl, 200 mg sample / ml 50% ethanol) was further mixed with 100 mM Tris-HCl buffer (400µl, pH 7.4). The mixture was further stirred with a magnetic bar at 65℃ for 2 hours. 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 shaken vigorously for one hour at room temperature and left in the dark at room temperature for 20 min. 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.

Hydroxyl radical assay

The scavenging activity for hydroxyl radicals was measured with fenton reaction. Reaction mixture contained 60µL 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), 150µL 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).

DPPH free radical

The antioxidant activity of the *H. nepalensis* 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.[2] with slight modifications. 1 ml of 0.1 mM DPPH solution in ethanol was mixed with 1 ml of the previous plant extracts 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. A solution of DPPH was prepared by dissolving 5 mg DPPH in 2 ml of ethanol, and the solution was kept in the dark at 4°C. A stock solution of the compounds was prepared at 1 mg/ml in DMSO. The stock solution was diluted to varying concentrations in 96-well microplates. Then, 5μl of ethanol DPPH solution (final concentration 300 μm) was added to each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminum foil and placed into the dark. After 30 min, the optical density (OD) of the solution was read using the UVmini-1240 Reader (Shimadzu, Kyoto, Japan) at the wavelength 517 nm. Absorbance changes are measured at 517 nm. Corresponding blank sample was prepared and L-Ascorbic acid (1.0 μg/ml) was used as reference standard (positive control). The inhibition % was calculated using the following formula.

Percentage inhibition was calculated using the following formula: Hydroxyl radical scavenging effect (%) = {(OD of sample-OD of blank)/OD of control} ×100

IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. To determine the IC_{50} value of the active component, the technique using 96-well microplates was employed.[8] A dose response curve was plotted to determine the IC_{50} values.

Lipoxygenase activity

Lipoxygenase (LOX) inhibitor Screening Assay Kit (Abnova, CA, USA) was used and measured the hydroperoxides produced in the lipoxygenation using a purified LOX. Stock solutions of the tested samples 15-lipoxygenase standard (Abnova, CA, USA) were prepared by dissolving the extracts in ethanol or methanol. The reaction was initiated by the addition of aliquots (90μl) soybean LOX solutions (prepared in potassium phosphate buffer, pH 9.0) in a sufficient concentration to give an early measurable initial rate of reaction to 10μl of arachidonic acid in phosphate buffer. The enzymatic reaction was performed in presence or absence of inhibitor and their kinetics were compared. Quertin was used as positive control.

Nordihydroguaiaretic acid (NDGA) and Rutin used as negative control. Lo inhibition activity was determined using a spectrophotometric method at 490 nm.

The concentration that gave 50% inhibition (IC_{50}) was calculated from the outline of the inhibition percentages as a function of the inhibitor concentration. Aqueous extracts ($IC_{50} \geq 100\mu$g/ml) were not taken in this study.

Statistical analysis

All the analysis were carried out in triplicate and the results were expressed as the mean ±SD. Correlation co-efficient (R) to determine the relationship between two or more variables among Radical Scavenging activity tests 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:

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

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

Table 1 was shown the antioxidant activities of the *H. nepalensis.* Various concentrations of leaf extracts were higher than those of stem extracts. OH scavenging activity of leaves of *H. nepalensis* was evaluated at 0.1 mg/ml was 26.7% and that of stems was 25.1% at same concentration (Table 1). The rates of antioxidant activities of the ethanol extracts were dependent on concentrations. OH scavenging activity of leaves of *H. nepalensis* was evaluated at 8.0 mg/ml was 78.8% and that of stems was 67.4% at same concentration. The all values of OH scavenging activity of leaves were lower than those of stems. However, the all groups for leaves and stems did not show a statistically significant difference ($p < 0.05$). DPPH scavenging activity of leaves of *H. nepalensis* was evaluated at 0.1 mg/ml was 82.5% and that of stems was 32.4% at same concentration. DPPH scavenging activity of leaves of *H. nepalensis* was evaluated at 8.0 mg/ml was 96.8% and that of stems was 57.2% at same concentration. The high antioxidant activity for DPPH found on leaf extracts. The all values of DPPH scavenging activity of leaves were lower than those of stems. The all groups for leaves and stems were shown a statistically significant difference ($p > 0.05$). Table 1 was shown the LOX activity of *H. nepalensis* extracts. The rates of LOX inhibition of the ethanol extracts were also dependent on concentrations. LOX inhibitory activity of leaves of *H. nepalensis* was evaluated at 0.1 mg/ml was 34.6% and that of stems was 17.2% at same concentration. LOX inhibitory activity of leaves of *H. nepalensis* was evaluated at 8.0 mg/ml was 67.6% and that of stems was 42.5% at same concentration.

Table 1: The hydroxyl radical and free radical scavenging effects and percent inhibition of lipoxygenase by *Hydrocotyle nepalensis* at different concentrations.

Concentration (mg/ml)	OH		DPPH		Lipoxygenase	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
0.1	29.69±1.61	25.08±3.14	82.53±2.15	32.42±2.71	34.57±3.78	17.21±3.82
0.5	44.46±6.71	36.64±5.77	90.73±1.23	37.49±3.80	38.82±4.55	22.64±4.52
1.0	55.18±3.84	46.96±4.25	92.37±1.68	41.47±3.94	45.90±3.49	25.93±3.84
2.0	67.94±5.09	54.77±4.19	93.46±0.61	48.22±4.28	54.58±4.09	28.96±3.55
4.0	71.95±1.95	62.08±3.71	94.75±0.68	53.19±3.35	60.07±4.41	36.64±4.09
8.0	78.80±3.07	67.36±1.60	96.80±0.27	57.19±3.57	67.63±4.84	42.45±4.41
t-test	0.921, $p < 0.05$		10.666, $p > 0.05$		3.311, $p > 0.05$	

Data represent the mean ± SD from three replicates.

When the nordihydroguaiaretic acid (NDGA) used as a negative control, extracts for leaves of *H. nepalensis* were 67.6% inhibitory effects on the activation of LOX and that of stem was 42.4% (Fig. 1).

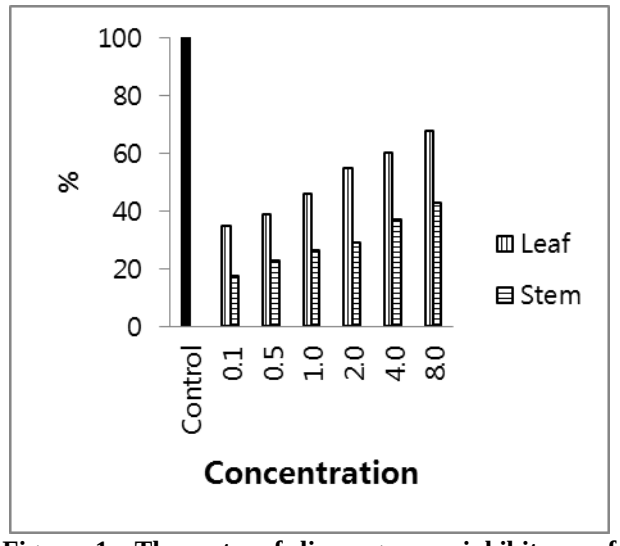

Figure 1: The rate of lipoxygenase inhibitory of nordihydroguaiaretic acid (negative control) and relative inhibitory rate of *Hydrocotyle nepalensis.*

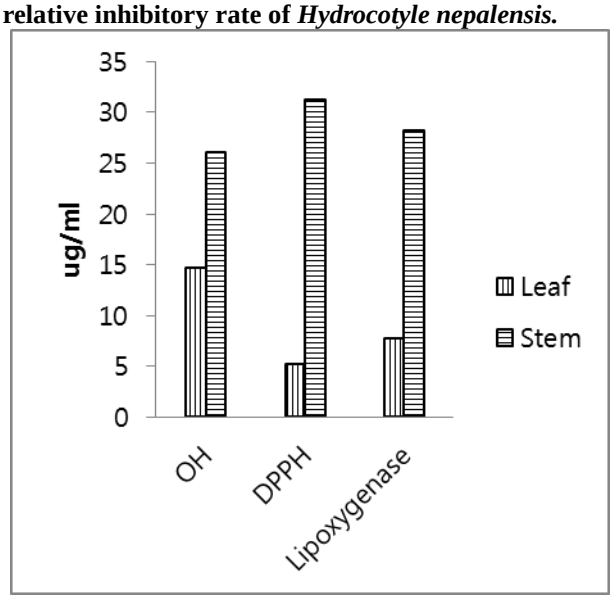

Figure 2: Inhibitory effects {IC_{50} (mg/ml)} on OH, DPPH, and lipoxygenase by *Hydrocotyle nepalensis* on 1.0 M.

The OH activity of leaves (IC_{50} = 14.61 μg/ml) was at the same levels as that of L-ascorbic acid and IC_{50} of stem was 26.02 μg/ml (Fig. 2). The DPPH activity of leaves (IC_{50} = 5.26 μg/ml) was at the same levels as that of L-ascorbic acid and IC_{50} of stem was 31.27 μg/ml. The LOX inhibition of leaves (IC_{50} = 7.76 μg/ml) was at the same levels as that of L-ascorbic acid and IC_{50} of stem was 28.23 μg/ml.

DISCUSSION

Normally free radical formation is controlled naturally by various beneficial compounds known as antioxidants. When there is deficiency of these antioxidants damage due to free radicals can become cumulative and debilitating. There are several nutrients in food that contain antioxidants. Vitamin C, vitamin E, and beta carotene are among the most commonly studied dietary antioxidants.

Herbal medicine is a major part of traditional medicine and has been used in medical practice since antiquity to cure human and other animal. Traditional medicine is the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, diagnosis, improvement or treatment of physical and mental illness.[9] About 60 to 85% of the populations of every country of the developing world rely on herbal or indigenous forms of medicine.[10] According to a survey by the National Center for Complementary and Alternative Medicine[11], herbal therapy or the usage of natural products other than vitamins and minerals was the most commonly used alternative medicine (18.9%) in United States when all use of prayer was excluded. 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.[12] The herbal plant is a common element of ayurvedic, homeopathic, and naturopathic medicine.[13]

Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals (such as Fe^{2+}) and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage *in vivo*.[14] In this study, hydroxyl radical scavenging activity of *H. nepalensis* was 78.8% (Table 1). In *Bauhinia vahlii*, the hydroxyl radical scavenging activity observed was in the range of 29.3-84.4% at the concentration of 80 µg/ml.[15] At 500 µg/ml of *Leucas linifolia*, hydroxyl radical scavenging showed 78% inhibition with an IC_{50} value of 150 µg/ml).[16]

DPPH scavenging activity of leaves of *H. nepalensis* was evaluated at 8.0 mg/ml was 96.8%. DPPH scavenging activity of leaves of *H. sibthorpioides* was evaluated at same concentation was 86.0%.[17] The IC_{50} of leaves for DPPH was 5.26 μg/ml and that of stem was 31.27 μg/ml (Fig. 2). The *Cucumis sativus* exhibited DPPH-free radical scavenging activity and IC_{50} was 14.73 μg/mL.[18] DPPH radicals of *Aegle tamilnadensis* were effectively scavenged by aqueous leaf extract (IC50 value of 82.05±1.02 µg/ml).[19]

The extract of *H. nepalensis* was 67.6% inhibitory effects on the activation of LOX (Table 1). The extract of *H. sibthorpioides* was 55.5% on the activation of LOX.[17] *Camellia sinesis*, *Rhodiola rosea*, and *Koelreuteria henryi* had notable significant inhibitory activities towards LOX.[1] These results show that these plants have some phytochemical constituents which may be active against the lipoxygenase enzyme.

We have shown that 4.0 mg/ml weight of ethanol *H. nepalensis* extract has inhibitory effect of lipoxygenase and antioxidants for DPPH. Anti-lipoxygenase activity of chosen antioxidant-rich plant materials can support their traditional use in folk medicine.[20] Higher level of antioxidant activity is observed in the ethanol extract of *H. nepalensis* leaves when compared with other cited species. Thus, these extracts can be considered as new sources of natural antioxidants.

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