ORIGINAL ARTICLE

Comparison of antioxidant activities of novel peptides isolated from *Azadirachta indica* and *Selaginella bryopteris*

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Abstract

Aim: The aim of this study was to compare the antioxidant activities of novel peptides isolated from *Azadirachta indica* and *Selaginella bryopteris*. **Material and Methods:** The novel peptides were isolated from the young leaves of *A. indica* and the fronds of *S. bryopteris* using inexpensive paper chromatography technique. The isolated peptides were studied for *in vitro* antioxidant activities. **Results and Discussion:** *In vitro* antioxidant studies showed that both the peptides are free radical scavengers and the radical scavenging activity increases with increase in concentration of the peptides. **Conclusion:** The peptide isolated from the fronds of *S. bryopteris* showed more free radical scavenging activity when compared with the peptide isolated from the young leaves of *A. indica.*

Key words: Antioxidants, Cu(II) ninhydrin, free radicals, peptides, scavenge

INTRODUCTION

Peptides are composed of amino acids joined by peptide bonds. Peptide bond formation involves the elimination of the elements of water between the carboxyl group of one amino acid and the amino group of another. The dipeptide so produced has terminal amino and carboxyl groups that can participate in further peptide bonds to extend the chain into a larger peptide called a protein. Peptides formed this way are called the alpha peptides.

The majority of peptides occurring in nature come under the category of alpha peptides. All amino acids and their carboxyl group derivatives such as esters and amides including small peptides produce a purple color with the classical ninhydrin reagent. This reagent was modified by us by adding cupric ion to distinguish qualitatively the carboxyl group derivatives of amino acids from the amino acids on paper after chromatography.[1] Amino acids produce a pink color and their carboxyl derivatives produce a yellow color with this reagent. We have used this method to identify the amino acid derivatives from the young leaf extracts of many plants.[2]

Selaginella bryopteris is a pteridophytic plant which is known for its remarkable resurrection capabilities. The dry plants have been used as a working remedy for several human health complications since centuries in India, particularly in the tribal areas. *Selaginella* is a lithophytic xerophyte that grows on the hills of tropical areas, particularly the Aravali mountain terrains from east to west in India. The plants grow luxuriantly during rains exhibiting a lush green velvety landscape. During summer, the plants undergo extreme desiccation. The fronds curl, become dry, and virtually dead. In this condition, they look like a closed fist hence often known in Unani as "*punjemariam*" or "*hathazori*." The dry plants when left in water unfold their fronds, turn green, and come back to active life. Several species of *Selaginella* have been used as potential herbal medicines.[3-6]

Azadirachta indica A. Juss is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants having a wide spectrum of biological activity. Neem is an evergreen tree, cultivated in various parts of the Indian subcontinent. Every part of the tree has been used as traditional medicine for household remedy

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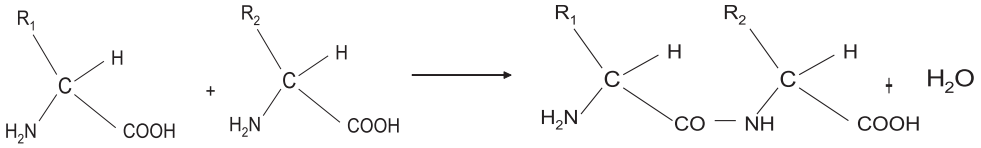

Amino acids

A Dipeptide

against various human ailments, from antiquity. Neem has been extensively used in ayurveda, unani, and homoeopathic medicine and has become a cynosure of modern medicine. The sanskrit name of the neem tree is "Arishtha" meaning "reliever of sickness" and hence is considered as "Sarbaroganibarini." The tree is still regarded as "village dispensary" in India.[7]

In our previous studies, we have reported the presence of peptides in *A. indica* and *S. bryopteris.* A tripeptide was isolated from *A. indica* with the sequence Ala-Phe-Cys and a tetrapeptide was isolated from *S. bryopteris* with the sequence Gly-Gly-Cys-His. Cysteine, a sulfur containing amino acid, is commonly present in both the peptides.[8,9] The present study has been carried out to find out and compare the antioxidant properties of the two peptides.

MATERIALS AND METHODS

Sample Preparation for Free Radical Scavenging Activity and Antimicrobial Activity Assays

The Cu(II) ninhydrin positive compounds isolated from the fronds of *S. bryopteris* and young leaves of *A. indica* using paper chromatography[10] were evaporated to dryness at room temperature and dissolved in minimum quantity of distilled water and then used for free radical scavenging assays and antimicrobial activity studies.

Colorimetric Determination of Concentration of the Cu(II) Ninhydrin Positive Compounds

1 mL of the purified Cu(II) ninhydrin positive compound was added with 1 mL of Cu(II) ninhydrin reagent and the mixture was incubated at 40°C for 5 min. The yellow color produced was read at 420 nm. The amount of these compounds was determined using a standard graph constructed with L-glycyl glycine as the standard.

Free Radical Scavenging Activity of the Isolated Cu(Ii) Ninhydrin Positive Compounds

DPPH radical scavenging activity

Principle

Antioxidants react with DPPH and convert it to diphenyl-picrylhydrazine. The degree of discoloration from purple to yellow color was measured at 518 nm, which is a measure of the scavenging potential of antioxidant extracts.

1. Dot – Plot rapid screening assay[11]

   3 µL of extracts were spotted on thin-layer chromatography (TLC) plates and allowed to air dry. The TLC plates bearing the dry spots were placed upside down for 10 s in a solution of DPPH in ethanol. The spots exhibiting radical scavenging antioxidant activity showed up as yellow spots against a purple background.

2. Spectrophotometric assay[12]

   Aliquots of extracts and ascorbic acid 0.025, 0.05 and 0.1 mL were taken in separate tubes from 1mg/ml stock and volume was made up to 0.5 mL with ethanol. Samples were incubated with 50 µM DPPH in methanol for 20 min and O.D was measured at 572 nm and %inhibition was calculated in comparison with O.D of blank, that is, ethanol. The degree of discoloration indicates the scavenging potentials of the compounds.

Nitric Oxide Radical Scavenging Activity[13]

The reaction mixture (3 mL) containing 2 mL of sodium nitroprusside, 0.5 mL of PBS, and 0.5 ml of different concentrations of extract was incubated at 25°C for 5 h. Control extracts without the test compounds, but with equivalent amounts of buffer were added conducted in an identical manner. After 5 h, 0.5 mL of the incubation solution was removed from all the tubes and diluted with 0.5 mL of Griess reagent. The absorbance of the chromophore formed during diazotization coupling with NEDA was read at 546 nm.

Reduction Potential[14]

1 mL of extract with different concentrations was mixed with 2.5 mL of 1% potassium ferricyanide and 2.5 mL phosphate buffer pH 6.6. The mixture was incubated at 50°C for 20 min. After incubation, 2.5 mL of 10% TCA was added to it and centrifuged at 3000 rpm for 10 min. 2.5 mL of supernatant was taken; 2.5 mL water and 0.5 mL 0.1% ferric chloride were added to it. The absorbance of reaction indicated the reducing power of test samples.

Assay of Superoxide Radical Scavenging Activity[15]

0.5 mL of the extract with different concentrations was mixed with 0.2 mL of ethylenediaminetetraacetic, 0.1 mL of NBT, 0.05 mL of riboflavin, and 2.55 mL of phosphate buffer.

0.1 mL of DMSO was taken and the reagents were added in the similar manner and were treated as control. The absorbance was measured at 560 nm and the tubes were kept in sunlight for 30 min and the absorbance was again measured at 560 nm.

Hydroxyl Radical Scavenging Activity[16]

The reaction mixture contained 0.1 mL of deoxyribose, 0.1 mL of EDTA, 0.1 mL of H_2O_2, 0.1 mL of ascorbate, 0.1 mL of KH_2PO_4-KOH buffer, and various concentrations of different extracts in a final volume of 1.0 mL. The reaction mixture was incubated for an hour at 37°C. At the end of the incubation period, 1.0 mL of TBA was added and heated in a boiling water bath for 20 min. The pink color developed was measured at 535 nm in a spectrophotometer. The percent TBARS production for positive control (H_2O_2) was fixed at 100% and the relative percent TBARS was calculated for the extract treated groups.

RESULTS AND DISCUSSION

Free Radical Scavenging Assays

In recent times, there is an increasing interest in the role of free radical-mediated damage in the etiology of human diseases. In the status of normal metabolism, the levels of oxidants and antioxidants in humans are maintained in balance, which is important for sustaining optimal physiological conditions. Overproduction of oxidants in certain conditions can cause an imbalance, leading to oxidative damage to large biomolecules such as lipids, DNA, and proteins.[17] Oxidative damage to body cells and molecules has been widely postulated to be involved in the causation and progression of a range of chronic diseases, such as cardiovascular disease, neuronal disease, cataracts, and several forms of cancer. Human metabolism counts on an antioxidant defensive system involving enzymes and proteins to prevent these effects. However, the defenses can be overwhelmed in certain circumstances so that harmful effects occur. It is accepted that the intake of antioxidant substances reinforces defenses against free radicals. The use of synthetic antioxidants has been limited due to their toxicity.[18] Therefore, it is of great significant and necessity that research focuses on discovering potential natural, effective antioxidants to replace the synthetic ones. The peptides isolated from the fronds of *S. bryopteris* and from the young leaves of *A. indica* were tested for their free radical scavenging activity.

DPPH Radical Scavenging Activity

1. Dot – Plot rapid screening assay

   The positive DPPH test suggests that the samples are free radical scavengers [Plates 1 and 2].

2. Spectrophotometric assay

   The DPPH method is a valid, easy, accurate, sensitive, and economic method to evaluate scavenging activity of antioxidants of fruits and vegetables juices or extracts. When the antioxidants that can provide hydrogen exist, they donate hydrogen to the free radicals so that the radicals remove the odd electron to turn to unreactive ones.[19] DPPH assay is based on the concept that a hydrogen donor is an antioxidant. DPPH is one of the few stable and commercially available organic nitrogen radicals. The antioxidant effect is proportional to the disappearance of DPPH in test samples. A freshly prepared DPPH solution exhibits a deep purple color with absorption maximum at 570 nm. The purple color generally fades or disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals, that is, by providing hydrogen atoms or by electron donation, conceivably through free radical attack on the DPPH molecule and converted them to a colorless stable molecule 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine, resulting in a decrease in absorbance at 570 nm. Hence, the more potent the antioxidant more decrease in absorbance is seen.[20]

In *in vitro* antioxidant studies [Figure 1] of the two isolated peptide extracts, the extent of DPPH radical scavenging at different concentrations (2–10 µg/mL) of *S. bryopteris* and *A. indica* peptide extracts was measured, with ascorbic acid as the standard. The radical scavenging effect was found to increase with increasing concentrations. The control and the peptide extracts showed their maximum activity of 91.27%, 86.12%, and 80.01%, respectively, with IC50 values of 2 µg/mL, 3.5 µg/mL, and 4 µg/mL. There was a significant ($P < 0.05$) difference in radical scavenging, when the two peptide extracts were compared with control. The mean values of *S. bryopteris* were found to be closer to the

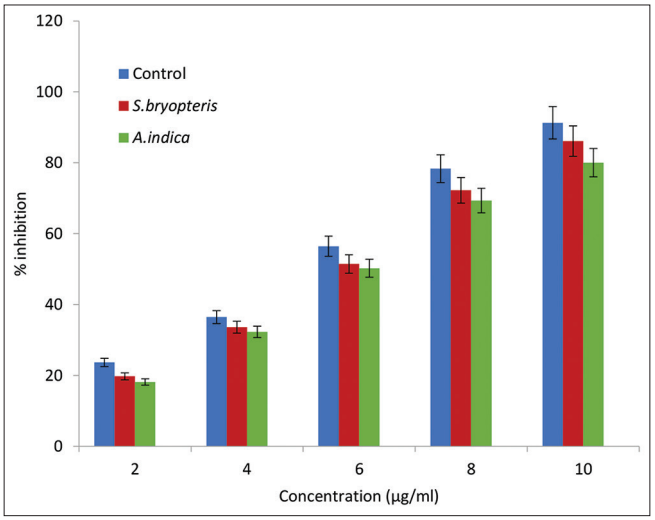

Figure 1: DPPH radical scavenging activity

control and indicated that it had more radical scavenging ability than *A. indica*.

Nitric Oxide Radical Scavenging Activity

Nitric oxide is an important chemical mediator generated by endothelial cells, macrophages, and neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders such as AIDS, cancer, Alzheimer's, and arthritis. Oxygen reacts with the excess NO to generate nitrite and peroxynitrite anions, which act as free radicals.[21] Figure 2 shows the nitric oxide radical scavenging activity of the peptides isolated from the fronds of *S. bryopteris* and from the young leaves of *A. indica.* The reduction of NO radical by the control and the two plant peptide extracts was found to be concentration dependent and the maximum scavenging effect was found to be 58.36%, 54.30%, and 50.54% for control, *S. bryopteris,* and *A. indica* peptide extracts, respectively, with the IC50 values of 6 µg/mL, 8 µg/mL, and 8.6 µg/mL. When the values of control and the two peptide extracts were compared, there was a significant ($P < 0.05$) difference at all the five different concentrations. When the values of *S. bryopteris* peptide were compared with the control at 4 µg/mL and 10 µg/mL, there was a significant ($P < 0.05$) difference, but at 8 µg/mL, there was no significant difference. This showed that peptide isolated from *S. bryopteris* had more potential than the peptide isolated from *A. indica* in nitric oxide radical scavenging activity.

Reduction Potential

The reducing property is associated with the presence of reductones and has been reported to have a direct, positive correlation with antioxidant activities of some plant compounds.[22] The presence of reductants such as antioxidant substances causes the reduction of Fe3+/ferricyanide complex to Fe2+/ferrous form. Therefore, the reducing power of the sample could be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[23] Samples with higher reducing power have better abilities to donate electrons. Free radicals form stable substances by accepting the donated electrons, resulting in the termination of radical chain reactions.[24] Absorbance of Fe3+ can be observed by measuring the O.D. values at 700 nm the reduction power of the extract increases with increase in concentration.[25] Figure 3 shows the reduction potential of the peptides isolated from the fronds of *S. bryopteris* and from the young leaves of *A. indica.* An increase in the absorbance of the control (ascorbic acid) and the two plant peptide extracts showed an increase in the reduction potential which is enhanced with increasing concentrations. Maximum absorbance was found to be 0.49, 0.40, and 0.37 for ascorbic acid, *S. bryopteris,* and *A. indica,* respectively. The absorbance values showed a significant ($P < 0.05$) difference when control was compared with *S. bryopteris* and *A. indica*. The mean values of *S. bryopteris* were found to be near to the control indicating that it has more reducing power than *A. indica.*

Superoxide Radical Scavenging Activity

Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as through non-enzymatic reaction such as autoxidation by catecholamines. Although superoxide radical is a relatively weak oxidant, it is known to be very harmful to cellular components because it is one of the precursors of more DNA, proteins, and other biological macromolecules, which, in turn, leads to cell or tissue injury associated with immune destruction, inflammation, aging, atherosclerosis, asthma, and carcinogenesis. Superoxide anions indirectly initiate lipid oxidation as a result of superoxide and hydrogen peroxide, serving as precursors of singlet oxygen and hydroxyl radicals.[26] Superoxide radical is involved in many pathological conditions. It mediates inflammatory tissue injuries in ischemia-reperfusion, arthritis, gout, and gastric ulceration. Superoxide radical has a low reactivity and a low capacity to penetrate the lipidic

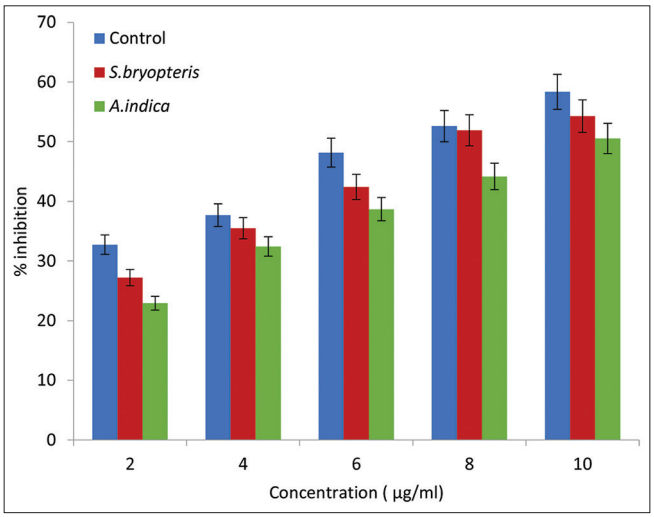

Figure 2: Nitric oxide radical scavenging activity

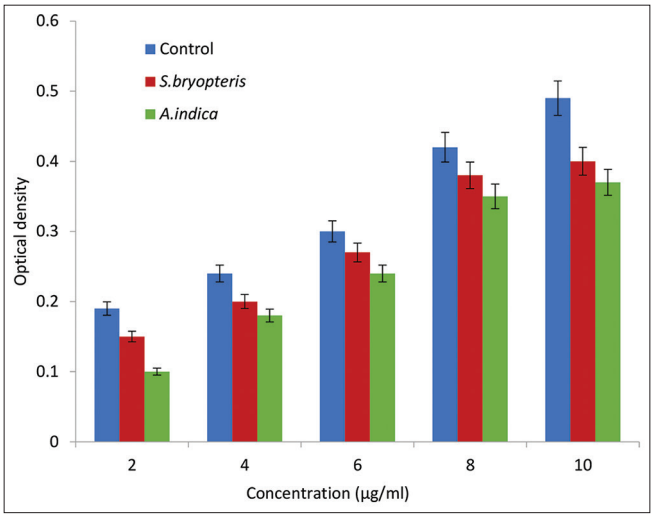

Figure 3: Reducing power assay

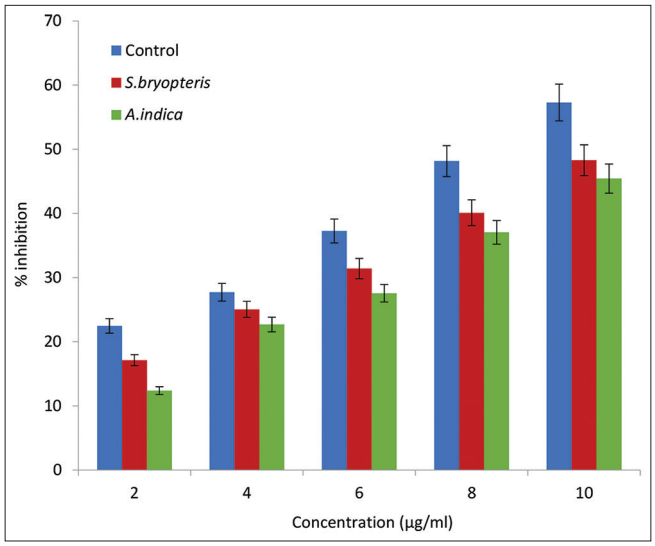

Figure 4: Super oxide radical scavenging activity

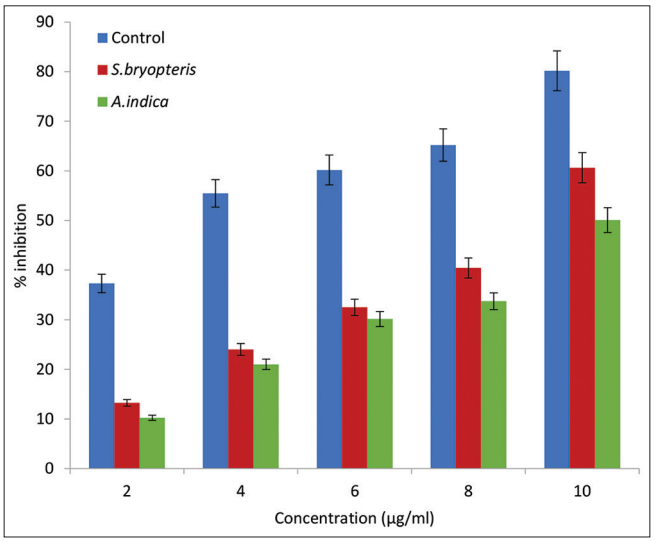

Figure 5: Hydroxy radical scavenging activity

membrane layer, but it can generate hydrogen peroxide and highly reactive hydroxyl radical, through Haber-Weiss reaction. Polyphenols present in medicinal plants scavenge superoxide radicals by the hydrogen donating capacity of their phenolic groups.[27] Figure 4 shows the super oxide radical scavenging activity of the peptides isolated from the fronds of *S. bryopteris* and from the young leaves of *A. indica.* The reduction of super oxide radical by the control and the two plant peptide extracts was found to be concentration dependent and the maximum scavenging effect was found to be 57.28%, 48.30%, and 45.44% for control, *S. bryopteris,* and *A. indica* peptide extracts, respectively, with the IC50 values of 8 µg/mL, 9 µg/mL, and 9.5 µg/mL. When the values of control and the two peptide extracts were compared, there was a significant ($P < 0.05$) difference at all the five different concentrations. The mean values of *S. bryopteris* were found to be near to the control indicating that it has more superoxide radical scavenging activity than *A. indica.*

Hydroxyl Radical Scavenging Activity

Hydroxyl radical is the most reactive species among oxygen radicals and can directly induce oxidative damage to biomolecules resulting in destruction of cell structure. Hence, scavenging activity of hydroxyl radical often serve as a main index for antioxidant activity evaluation of natural product. Hydroxyl radical is the major active oxygen centered radical formed from the reaction of various hydroperoxides with transition metal ions causing lipid peroxidation and biological damage. If hydroxyl radical is generated near nucleic acids, it reacts with purine and pyrimidine bases and 2-deoxyribose, leading to mutations which play an important role in carcinogenesis, as well as in neurodegenerative and cardiovascular diseases.[28] Figure 5 shows the hydroxyl radical scavenging activities of the two plant peptide extracts. The scavenging of hydroxyl radical increased with increasing concentrations of two plant

Plate 1: Dot plot assay of the peptide isolated from *Selaginella bryopteris*

Plate 2: Dot plot assay of the peptide isolated from *Azadirachta indica*

extracts and maximum scavenging effect was found to be 80.18% for ascorbic acid, 60.62% for *S. bryopteris,* and 50.07% for *A. indica* at 10 µg/mL. The scavenging effect of *S. bryopteris* at 10 µg/mL was comparable to that of control at 6 µg/mL. The IC50 values of control and the two plant extracts were found to be 2 µg/mL, 6 µg/mL, and 10 µg/ml, respectively. There was a significant ($P<0.05$) difference in the hydroxyl radical scavenging activity of the two plant extracts as compared with the control (ascorbic acid). The mean values of *S. bryopteris* were found to be closer to that of the control indicating that it had more hydroxyl radical scavenging activity than *A. indica*.

CONCLUSION

In vitro antioxidant studies showed that both the peptides are free radical scavengers and the radical scavenging activity increases with increase in concentration of the peptides. The peptide isolated from the fronds of *S. bryopteris* showed more free radical scavenging activity when compared with the peptide isolated from the young leaves of *A. indica.*

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