wjpmr, 2016, 2(2), 34-37

SJIF Impact Factor: 3.535

WORLD JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.wjpmr.com

Research Article

ISSN 2455-3301

WJPMR

PHYTOCHEMICAL STUDIES AND ANTIOXIDANT ACTIVITY OF *WITHANIA SOMNIFERA* PLANT ROOT PROTEINS

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Article Received on 20/12/2015 **Article Revised on 10/01/2016** **Article Accepted on 02/02/2016**

ABSTRACT

The phytochemical analysis and antioxidant activity of *Withania somnifera* root proteins was evaluated. The quantitative phytochemical study was done for the dialyzed ammonium suphate precipitated proteins. Free radical scavenging activity was evaluated using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and nitric oxide scavenging assay. The study reveals that, the *Withania somnifera* root proteins showed potent antioxidant activity in scavenging DPPH and Nitric oxide radicals in comparison with standard antioxidants like Ascorbic acid and BHA at a lower dose of 12 and 10µg respectively. The dialyzed protein precipitate contains more proteins and negligible amount of other phytochemicals.

KEYWORDS: Antioxidant, DPPH, Nitric Oxide, *Withania somnifera* proteins, Analysis.

INTRODUCTION

The knowledge about free radicals and reactive oxygen species (ROS) in biology is made a medical revolution in the field of health and disease management.[1] Though oxygen is an element indispensable for life, under certain situations has deleterious effects on the human body.[2, 3] Most of the harmful effects of oxygen are due to the formation of ROS, which have a tendency to donate oxygen to other substances.[4] Synthetic antioxidants are recently reported to be dangerous to human health.[5] Thus the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years.[4] The traditional diet, spices, and medicinal plants are rich sources of natural antioxidants, non-toxic and hence gaining importance.[6] *Withania somnifera*, commonly known as Ashwagandha, is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years. In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. Herein we considered the root the plant, isolated the proteins from the dried root and analyzed its antioxidant capacity in different model systems.

MATERIALS AND METHODS

BHA, Ascorbic acid, DPPH, were purchased from the Sigma Aldrich co. USA, Quercetin, Gallic acid, BSA were purchased from the Himedia Co. All the other chemicals and reagents were of Analar grade were purchased from the Merck Co., and S.d. fine chem., Mumbai, India.

Sample collection

The plant root of *Withania somnifera* was collected from authentic source, Karnataka, India. The collected roots were cleaned thoroughly with double distilled water and shade dried. The dried roots ground to powder (British Pharmacopea 100 mesh), stored in glass container for further studies.

Extraction

Aqueous extract was prepared by mixing 5g of powdered *Withania somnifera* root in 100ml of double distilled water, vortexed for 4 hours, centrifuged at 10000 rpm, supernatant collected. Later 65% ammonium sulphate was added to the supernatant and allowed to vortex for 24 hours. The precipitated protein was separated by centrifugation and subjected to dialysis against double distilled water using 2kDa cutoff molecular membrane for 72 hours with an interval of 6 hours and finally stored at -10°C.

Phytochemical analysis

The dialyzed protein extract was tested for the presence of bioactive compounds by adopting standard procedures.

Protein estimation

The protein estimation was carried according to Bradford's method[7] using BSA as standard and hexane extracts of different plant materials into a series of test tubes. Volume was made up to 100µl with distilled water and 900µl of Bradford's reagent was added to each tube. Absorbance was read at 535nm. Concentration of protein was calculated accordingly using standard graph.

Total phenols estimation

Total phenol content was determined according to the method of Folin Ciocalteu reaction[8] with minor modifications using Gallic acid as a standard (0-100µg). Various concentrations of hexane extracts ranging from 0-100µg were taken in series of test tubes & the volume was made up to 500µl with distilled water. 500µl of the Folin-ciocalteu reagent was added to each tube, the mixture was allowed to stand for 10 minutes followed by addition of l.0ml of 20% Sodium carbonate, incubated at 10 minutes at 37°C. Absorbance was read at 750nm and the concentration was calculated using the standard graph accordingly.

Carbohydrate estimation

Carbohydrate was done according to Dubois method.[9] 10 - 100 µg of the working standard solution was pipetted into a series of test tubes 200µl of the extracted sample was pipetted into two separate test tubes. The volume in each tube was made up to 1000µl with double distilled water. 1ml of 5% phenol was added to each tube followed by 5ml of 96% sulphuric acid, intensity of the colour was read at 520 nm. The amount of total sugar present in the given unknown sample solution was calculated using the standard calibration curve.

Flavonoid estimation

Flavonoid estimation was done according to Cheon et al[10] by using Quercitin as a standard. Various concentrations (0-100µg) of hexane extracts were taken in test tubes. Made up the volume to 1.5 ml with 95% ethanol, then 100 µl of 10% of Aluminium chloride, 100µl of 0.1M of potassium acetate was added to each tube. The total volume was made up to 2.8ml of by using distilled water. O.D was measured at 415 nm and the concentration was calculated accordingly.

ANTIOXIDANT ACTIVITY

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The dose dependent DPPH radical scavenging activity was assessed according to the method of Shimada et al. with minor modifications (Shimada et al., 1992, Dinesha and Leela Srinivas, 2011). The *Withania somnifera* plant root crude extract and dialyzed proteins at a concentration at a range of 0-10µg each was mixed in 1 ml of freshly prepared 0.5 mM DPPH ethanolic solution and 2 ml of 0.1 M acetate buffer pH 5.5. The resulting solutions were then incubated at 37°C for 30 min and measured spectrophotometrically at 517 nm. BHA and Ascorbic acid (400 µM) was used as positive control under the same assay conditions. Negative control was without any inhibitor or *Withania somnifera* plant root crude extract and dialyzed proteins. Lower absorbance at 517 nm represents higher DPPH scavenging activity. The % DPPH radical scavenging activity of *Withania somnifera* plant root crude extract and dialyzed proteins was calculated from the decrease in absorbance at 517 nm in comparison with negative control.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined in a dose dependent way according to the method reported by Masuda et al with minor modification (Masuda et al., 1994). BHA (400µM) and ascorbic acid (400µM) standards in the range of 2 –10 µl were taken in respective tubes containing phosphate buffer saline, so that the volume in each tube was made up to 1ml. *Withania somnifera* plant root crude extract and dialyzed proteins were taken in the volume of 30µl (0 to 10µg) to the tubes containing phosphate buffer. For controls, volume was made up to 3ml with phosphate buffer saline and for the tests 1ml. Then 2ml of 10mM sodium nitroprusside was added to all the tubes except the controls. Nitric oxide radicals were generated from the samples spontaneously during the incubation period of 150 min. 0.5ml of the solution was taken from each tube to their respective tubes. To this 1ml of 0.33% sulphanilamide was added and was incubated at room temperature for 5 min., followed by the addition of 0.1% of NED to each tube. All the tubes incubated under diffused light for 30 min. The nitrite ions released were measured spectrophotometrically at 516nm.

Statistical Analysis

Results are expressed as Means ± S.D of five experiments. Student's t-test was used for statistical significance using SPSS (statistical presentation system software) for windows version 10.0.1 software, Inc. New York. A probability level of $P < 0.05$ was considered as statistical significance in comparing with relevant controls.

RESULTS AND DISCUSSION

The increase in ROS due to inflammation or environmental factors, are generally thought to increase mutations in DNA and thereby risk of cancer. The dual roles of ROS have been found in many types of autoimmune diseases.[14] The increased levels of ROS have been concerned in numerous chronic degenerative diseases such as cardiovascular diseases, cancers, type 2 diabetes, neurodegenerative diseases, obesity, and hypertension. During exercise also, it was found that, the increased level of ROS production via mitochondria. These adaptations include increased antioxidant defense, increased insulin sensitivity in muscle, and biogenesis of mitochondria.[15, 16] Here in as explained in the materials and methods, the proteins were isolated from aqueous extract of *Withania somnifera* plant root through ammonium sulphate precipitation. The precipitated proteins were subjected to dialysis against water for 72

hours with an interval of six hours. Further the crude aqueous extract and the dialyzed protein was subjected to phytochemical analysis to find the active ingredients which are responsible for their antioxidant activity. The antioxidant potential of the *Withania somnifera* plant root crude extract and the dialyzed proteins was studied using DPPH and Nitric oxide radical scavenging activity.

As shown in Table -1, the crude aqueous extract of *Withania somnifera* plant root crude is rich with Carbohydrates (24.43 ±0.63 mg/g), proteins (2.35 ± 0.11mg/g), phenols (2.71 ±0.03mg/g) and flavonoids (14.42 ±0.42 mg/g). The dialyzed protein precipitate was rich with proteins (2.12 ±0.02 mg/g) and contains negligible amount of phenols, flavonoids and carbohydrates. Further the crude extract and proteins of *Withania somnifera* plant root were subjected to study the DPPH radical scavenging activity in a dose dependent manner at a range of 0 to 14µg where along with proteins; BHA and Ascorbic acid were used as standard antioxidants. The *Withania somnifera* plant root proteins showed maximum scavenging activity of DPPH radicals at a dosage of 12 µg which is comparable with Ascorbic acid at 12 µg dosage. Further *Withania somnifera* plant root proteins were studied towards its capability in scavenging nitric oxide radicals in a dose dependent manner at a range of 0-10 µg. Here also BHA and Ascorbic acid are used as standard antioxidants. Here *Withania somnifera* plant root proteins showed more scavenging activity at a dosage of 10 µg and the results were comparable with Ascorbic acid which is little less. These *in vitro* study results confirm that, the root proteins of *Withania somnifera* plant are having good antioxidant activity and which are comparable with standard antioxidants like BHA and Ascorbic acid. Further purification of these proteins is required to find exact more active protein of *Withania somnifera* plant root.

Table -1: Proximate analysis of crude extract and dialyzed *Withania somnifera* plant root crude extract and dialyzed proteins.

Phytochemicals	Crude aqueous extract of *Withania somnifera* plant root (mg/g)	Dialyzed plant root proteins of *Withania somnifera*(mg/g)
Proteins	2.35 ± 0.11	2.12 ±0.02
Phenols	2.71 ±0.03	0.02 ±0.01
Flavonoids	14.42 ±0.42	0.02±0.01
Sugars	24.43 ±0.63	0.01 ±0.01

Protein, polyphenols, flavonoids, sugars, chlorophyll content in water extract of *Withania somnifera* plant root crude extract and dialyzed proteins were estimated as described in materials and methods and values are expressed in mg/g.
Results are shown as mean ± SD (n = 3).

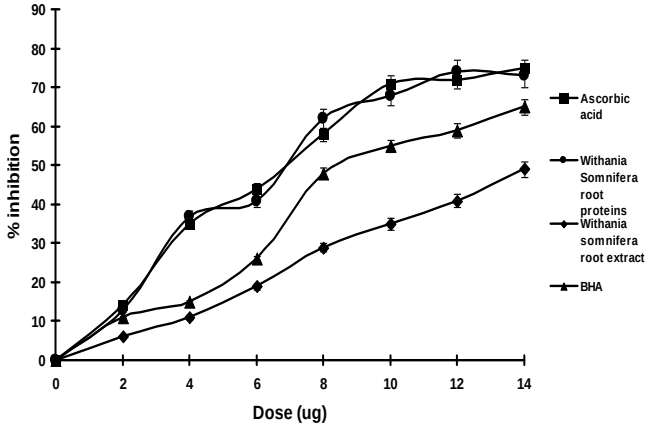

Fig. 1: Dose dependent DPPH radical scavenging activity of proteins from *Withania somnifera* plant root crude extract and proteins.

Dose-dependent DPPH radical scavenging activity of *Withania somnifera* plant root extract, proteins, BHA and Ascorbic acid. The control was without BHA, *Withania somnifera* crude extract, proteins and Ascorbic acid.. The DDPH radical scavenging activity was calculated as described in methods.
Results are shown as mean ± SD (n = 3).

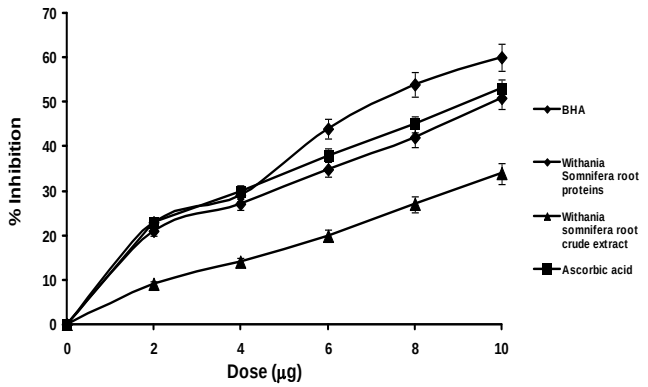

Fig. 2: Dose dependent Nitric oxide radical scavenging activity of proteins from *Withania somnifera* plant root crude extract and proteins.

Dose-dependent Nitric oxide radical scavenging activity of *Withania somnifera* plant root extract, proteins, BHA and Ascorbic acid. The control was without BHA, *Withania somnifera* crude extract, proteins and Ascorbic acid. The Nitric oxide radical scavenging activity was calculated as described in methods.
Results are shown as mean ± SD (n = 3).

ACKNOWLEDGEMENT

The authors gratefully acknowledge the Adichunchanagiri Mahasamstana Mutt and Shikshana Trust for providing facilities in AIMS- Central Research Laboratory, ABCRI for carrying out this work.

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