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Extraction and determination of antioxidant activity of *Withania somnifera* Dunal

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ABSTRACT

Antioxidant plays an important role in inhibiting and scavenging free radicals, thus, providing protection to human against infection and degenerative diseases. Now the modern research is directed towards "Natural antioxidants" from the herbal plants due to safe therapeutic. In the present paper we have investigated Antioxidant activity of extracts from Withania somnifera Dunal. for its free radical scavenging activity by adopting various in vitro methods. The extracts were investigated for the antioxidant activity using 2, 2 - diphenyl, 1- picryl hydrazyl (DPPH) radical scavenging activity, reducing capacity, competition with DMSO, Hydroxyl group reducing activity, estimation of total phenol and estimation of Ascorbic acid. The polar flavonoid extracted was found to have highest % of DPPH (83.07%) scavenging activity. The measurement of total phenolics by folin - ciocalteau reagent indicated that 20 mg of powdered Withania somnifera contain 0.115 g of phenols equivalent of catechol.

Keywords: Antioxidant, free radicals, folin – ciocalteau, *Withania somnifera* Dunal.

Abbreviations:

1} DPPH 2, 2, diphenyl 1- picryl hydrazyl
2} DMSO Dimethyl sulphoxide
3} O.D Optical density
4} WS Withania somnifera
5} DNPH 2, 4, dinitrophenyl hydrazine
6} EDTA Ethylene diamine tetra acetic acid
7} R.T Room temperature

INTRODUCTION

Many herbal plants contains antioxidant compounds which protects cells against degenerative effects of Reactive Oxygen Species (ROS) which is a free radical such as singlet oxygen, superoxide, peroxyl, radicals, hydroxy radicals [1;2]. The concept of oxidative stress is that, when a balance between ROS production and antioxidant defenses is lost, 'oxidative stress' result which through a series of events deregulate the cellular function and leads to various diseases such as aging, arthritis, asthma, carcinogenesis, diabetes, rheumatism and various neuro degenerative disease [3].

Antioxidants are substances that neutralize free radicals and their actions. There are natural antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, thioredoxin thiols, and disulphides bonding which form the buffering system in every cell. Alphatocopherols is a chain breaking antioxidant which prevents the propagation of free radicals reaction in all cell-membrane in human body. Ascorbic acid is also a part of normal protecting mechanism. Other non-enzymatic antioxidant includes carotenoid, flavanoid and related polyphenols, alpha lipoic acid, glutathione [4].

In India indigenous remedies have been used in treatment of various diseases such as diabetes, cancer etc. since sixth century B.C. *Withania somnifera* Dunal (Solanaceae) is used as rejuvenator in Ayuredic System of Medicine. Modern investigations have demonstrated anti- stress (adoptogen) effect of *Withania somnifera*. Antistress drugs are useful in management of stress related disorder such as arthritis, hypertension, diabetes and general debility [5].

The objective of the present study were to determine the antioxidant activity, total phenolic content, reducing power activity, hydroxyl group reducing activity, estimation of Ascorbic Acid. In DPPH radical scavenging method the free radicals, 2, 2- diphenyl - 1- picrylhydroazyl (DPPH) was used to find antioxidant (scavenging) activity of various extracts. The total phenolic content of the extracts was estimated by folin - ciocalteau test and total protein content by folin - lowry method.

MATERIALS AND METHODS

2.1. Plant materials and extracts:

The powdered form of *Withania somnifera* was purchased from Yogesh Pharmacy A-6/6 MIDC, Nanded- 431 603 (India). The Powder was extracted with different solvents for obtaining various kinds of fractions and extracts by hot continuous extraction method using soxhlet apparatus. Following are the solvents used for extraction of 100 gm *Withania somnifera* powder.

Solvents used for extraction		**Active principle extracts**
Ethanol + diethyl ether + N-Butanol	-	Saponin
Water	-	Tannin
Ethanol	-	Phenol
Methanol	-	Polar flavanoid
Ethyl acetate	-	Terpenoid
Chloroform	-	Non polar flavanoid

The extracts were concentrated by evaporating the solvents on boiling water bath. The dried extract thus obtained was used for the assessment of antioxidant activity through various *in vitro* models. Preliminary qualitative analysis was carried out to ascertain the presence of flavanoid, tannin, protein, etc.

2.1.1. Determination of DPPH radicals scavenging activity:

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH [6, 7].

Determination of DPPH radicals scavenging activity was estimated with the method used by Kato [5]. 1mM solution of DPPH in ethanol and also 1mg/1 ml extract solution in ethanol was prepared and 1.5ml of this solution was added to 1.5 ml of DPPH. The absorbance was measured at 517 m against the corresponding blank solution which is prepared by taking 3ml ethanol and control O.D. was prepared by taking 3ml of DPPH. The assay was performed in triplicates. Percentage inhibition of free radical DPPH was calculated based on control reading by following equation [8].

$$\text{DPPH scavenged (\%)} = \frac{(A_{con} - A_{test})}{A_{con}} \times 100$$

A_{con} - is the absorbance of the control reaction
A_{test} - is the absorbance in the presence of the sample of the extracts.

2.1.2. Reducing power assay [9]:

In this case 0.5 ml of different fraction (l mg/ml) was added to 3ml Pottassium ferrocyanide (1mM) solution. The mixture was shaken thoroughly and incubated for 10 minutes at Room temperature. Finally the mixture was tested spectrophotometrically at 700 nm using an appropriate blank of 3.5 ml of potassium ferrocyanide solution after every 10 minutes interval up to 30 minutes.

2.1.3. Determination of OH^- Scavenging Activity [10]:

The OH^- scavenging activity of different fraction was estimated by competiting with DMSO. The Assay was performed by adding 1ml of fraction mixed with 1ml of EDTA (0.1mM) and Fe^{3+} CCl_3COOH (167mM). The chemicals were prepared in 50 mM phosphate buffer of pH 7.4 and set as a blank by addition of all above except test sample and read the absorbance at 420 nm

$$\text{OH Scavenged (\%)} = \frac{(A_{con} - A_{test})}{A_{con}} \times 100$$

A_{con} - is the absorbance of the control reaction
A_{test} - is the absorbance in the presence of the sample of the extracts.

2.1.4. Total phenolics content [11]:
The total phenolic content of the extracts were determined by folin- ciocalteau reagent method. Dissolve the residue in known volume of the distilled water (5ml), then pipette out different aliquots (0.2-3ml) into test tubes, made up the volume of each tube to 3ml with distilled water. 0.5 ml of folin - ciocalteau reagent was added. After 3 minutes, 2 ml of 20% sodium carbonate was added. Mixed thoroughly, placed tubes in boiling water bath for exactly 1 minute. Cooled to room temperature and measured the absorbance at 650 nm against a blank. Prepared standard curves using different concentrations of catechol (1 mg/ml of distilled water). The total phenolic content was expressed in mg of catechol equivalents / mg of extract.

2.1.5. Estimation of ascorbic acid [12, 13]:
Ascorbic acid is also determined with spectrophotometrically. The dehydroascorbic acid alone reacts quantitatively and not the other reducing substances present in the sample extract. Thus this method gives an accurate analysis of ascorbic acid than dye method.

Ascorbic acid is first dehydrogenated by bromination by constant addition of bromine water drop wise to 10ml stock solution of ascorbic acid. Addition of bromine water was stopped when solution became orange to yellow. 10-100 µg standard dehydroascrobic acid was pipette out in a series of test tubes. Similarly, 0.1-2ml of aliquots of brominated water was pipette out (aliquots prepared by grinding 5g of sample material by mechanically in 20-50 ml of 4% oxalic acid solution, centrifuged, filtered and collected the sample. Bromine water was added drop wise till colour changes to oranges to yellow). Volume of each test tube made up to 3ml by adding D/W. 1ml of DNPH reagent was added, followed by 1-2 drops of thiourea. The blank was set with distilled water and incubated at 37^0C for 3 hrs. Whereas,7 ml of 80% H_2SO_4 was added for dissolving osazone crystals. The absorbance was measured at 540 nm, and a graph was plotted for standard ascorbic acid and calculated the ascorbic acid present in sample.

2.1.6. Estimation of protein by Folin- lowry method [14]:
The protein can be estimated by using Folin -lowry method. To the one ml. of standard solution containing 10-100µg of protein sample (BSA) and approximately dilute unknown protein sample solution, add 4ml of reagent A that is sodium carbonate solution (2% W/V) in 0.1 N sodium hydroxide solution + reagent B that *is* $CuSO_4$ solution that is (1% W/V) + reagent C that is Sodium potassium tartarate (2% W/V) and mix it. After 10 minutes of incubation at Room temperature. Add 0.4 ml of folins reagent and vortex it. Run a blank with 1ml Distilled water along with standard protein and sample solution. After 30 min of incubation at R.T. read blue colour developed at 720 nm in UV- visible double beam spectrophotometer and record absorbance. Construct a calibration curve on graph paper, by plotting the protein concentration that is Bovine serum albumin (10-100µg) on X axis and absorbance on the Y axis.

2.1.7. Polyphenol oxidase enzyme extraction and inhibition [15]:
The enzyme was extracted by using salting out method (4^0C). The reaction mixture contained L-DOPA (4mM) citrate buffer (0.1M, PH. 4.8), enzyme extract and test fraction is added (1mg/ 1ml D/W) and incubated for 5min for detection of inhibition of PPO and absorbance was read at 470 nm.

2.1.7.1. *PPO enzyme extraction*:
Take 200g of chilled potato and peel it. Homogenate it in grinder using small volume of 0.1M citrate buffer PH 4.8. Filter through muslin cloth. Add equal volume of Ammonium sulphate. Incubate it at 0^0C for 30 minute for enzyme precipitation; centrifuge the filtrate at 5000 rpm for 10 min at 0-4^0C. Save pellet. Using 20ml of 0.1 M citrate buffer for dissolving precipitation, centrifuge at 5000 rpm for 10 min. collect supernatant and keep on ice bath.

2.1.7.2. *PPO enzyme inhibition assay*:
The control sample was 2ml of L-DOPA (4mM) + 1ml of extracted enzyme (PPO) + 1 ml of residual fraction (1mg/ml). All tubes were incubated for 5 min at R.T. 1 ml of sample + 3ml of buffer (0.1M, pH 4.8) were set as a blank. The absorbance was read at 470 nm in spectrophotometer within 2 and half hours at 4 ºC.

RESULTS

1) Determination of DPPH radicals scavenging activity:
DPPH is stable free radical at room temperature and accepts an electron / hydrogen radical to become a stable diamagnetic molecule [16]. The reduction capability of DPPH radical is determined by the decrease in its absorbance at 5l7 nm, induced by antioxidants. The decrease in absorbance of DPPH radical is caused by antioxidants, because of the reaction between antioxidant molecules and radicals, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity. [17]

A 1mg/ml solution of polar flavonoid, saponin , non polar flavonoid by chloroform , terpenoid, tannin exhibited 83.07% , 65.80%, 67.87%, 70.29% , 70.63% inhibition respectively. The results indicate that the extract reduces the radicals to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principle [18]. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution looses colour stoichiometrically depending on the number of electron taken up [19].

2)Reducing power activity:
Table 2 shows the reductive capability of the extracts to potassium ferrocyanide (standard) at different time period. For the measurement of the reductive ability, we investigate the Fe^{3+} - Fe^{2+} transformation in the presence of different extracts using methods of Lee [20]. The reducing capability of a compound / extract can be monitored by formation of blue colour at 700 nm. The highest reducing power activity was in tannin i.e. 0.070 for 10 min, where as non polar flavonoid showed 0.049 O.D. for 30 min.

3) Determination of OH^- scavenging activity:
To attack the substrate DMSO were generated by reaction of ferric - EDTA together with CCL_3- COOH and ascorbic acid. When the different extracts were incubated with the above reaction mixture, it could prevent the damage against free radicals .The results are shown in table 3. The highest inhibition was found in terpenoid (84.21%).

4) Total phenolics content:
Phenolic constituent are very important in plants because of their scavenging ability due to their hydroxyl groups [10]. The total phenolics content in *Withania somnifera* is 0.115g of phenols, by plotting O.D. on standard graph of catechol. It has been suggested that up to 1.0g polyphenolic compounds (from diet rich fruits or vegetables) ingested daily have remarkable inhibitory effect on mutagenesis and carcinogenesis in human [21].

5)Estimation of ascorbic acid (Vitamin- C):
Ascorbic Acid / Vitamin C is also an important antioxidant which plays an important role in preventing free radicals. The table 2 shows that the *Withania somnifera* contains 44mg of Ascorbic acid per 100 gm of sample. The value was estimated by plotting the O.D.at 700 nm on standard Graph of ascorbic acid.

6)Estimation of protein by folin- Lowry method:
The protein content in the sample was estimated by Folin-Lowry method. It was 25 mg / 100g of sample.

7)Polyphenol oxidase enzyme extraction and inhibition:
The polyphenol oxidase is an enzyme which reduces the phenol concentration in our body. The patients of diabetes may fail to protect their phenolic content from such an enzyme. The extracts isolated from *Withania somnifera* shows an inhibitory activity against such a PPO. The highest inhibitory activity was shown by saponin having 74.66 % inhibition (Table 4).

1} DPPH radicals scavenging activity:
Observation Table

Sr. No.	Sample	O.D. at 517 nm
1.	Control	0.579

Observation Table:

Fractions(1mg/ml)	% of DPPH Scavenging activity
Polar flavanoid	83.07
Saponin	65.80
Non-polar flavanoid by chloroform	67.87
Terpenoid	70.29
Tannin	70.63

2} Reducing power activity:

Fractions (1mg/ml)	O.D. at 700 nm (10 min.)	O.D. at 700nm (20 min.)	O.D. at 700 nm (30 min.)
polar flavanoid	0.040	0.038	0.046
Saponin	0.020	0.024	0.034
Non-polar flavanoid by Chloroform	0.044	0.051	0.049
Terpenoid	0.048	0.019	0.021
Tannin	0.070	0.033	0.036

3} OH^- scavenging activity:
(Competition of test sample with DMSO)
Observation Table

Sr. No.	Sample	O.D. at 420 nm
1.	Control	0.095

Fractions(1mg/ml)	% of OH^- scavenging activity
polar flavanoid	62.10
Saponin	70.52
Non-polar Flavanoid by Chloroform	31.57
Terpenoid	84.21
Tannin	46.31

4} PPO inhibition assay:

Fractions(1mg/ml)	% of PPO inhibition
polar flavanoid	68
Saponin	74.66
Non-polar flavanoid by chloroform	26
Terpenoid	62
Tannin	28

DISCUSSION

Reactive Oxygen species (ROS)/ Oxidants formed in our body due to exogenous and endogenous factors are found to be responsible for many diseases [5]. Now the research is going on to reveal the potential of phytochemical antioxidants as health benefactors. This is due to their ability to neutralize the free radicals or ROS or oxidants responsible for the onset of cell damage. Synthetic antioxidants are found to be harmful to the health. Most of the natural antioxidants from plant source are safer to health and have better antioxidant activity.

There are many reports which support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases [22].

Plants are the potential source of antioxidants for example Tannin, saponin, Terpenoid, Polar flavanoid, phenol, ascorbic acid, Non- polar flavanoid and many more having the capability to scavenge the free radicals [24]. Their broad range of effects in biological system has drawn the attention of many experimental works. It has been proven that these mechanisms may be important in the pathogenesis of certain diseases and aging [25]. There are many reports that support the use of antioxidants supplementation in the reducing level of the oxidative stress and in slowing or preventing the development of complication associated with disease [21]. Many synthetic antioxidants have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plants constituents have proved to show free radical scavenging / antioxidant activity [22]. Flavanoid and other phenolic compounds of plant origin have been reported in scavengers and inhibitors of lipid peroxidation.

The hypoglycemic principles of *Gymnema sylvestre* isolated from saponin fraction of the plant are referred as the Gymnemosides and Gymnemic acid. Triterpene glycosides isolated from plants, inhibited glucose mechanism in muscle. [23]

CONCLUSION

In the present study, the antioxidant capacities of the plant extracts were analyzed using free radical scavenging activity (DPPH), Reducing power, Competition of test fraction with DMSO, etc.

The DPPH test is the oldest indirect method for the determining the antioxidant activity based on the ability of free radical 2, 2–diphenyl-1- picryl hydrazyl to react with hydrogen donors react with phenol. The fractions isolated

from *Withania somnifera* powder having ability to scavenge the free radical and useful to cure diabetes to some extent, all the fractions isolated from *Withania somnifera* Dunal. have antioxidant activity.

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