Volume 4, Issue 5, 2026

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

IN-VITRO ANTIOXIDANT ACTIVITY OF ASHWAGANDHA (*WITHANIA SOMNIFERA*) ROOT EXTRACT USING DPPH ASSAY

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DOI: https://doi.org/10.5281/zenodo.20229325

Keywords
anti-oxidant, nutraceutical, invitro study, extract ashwagandha

Article History
Received: 18 March 2026
Accepted: 27 April 2026
Published: 16 May 2026

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Abstract

Oxidative stress is a key mechanism in most of chronic diseases' pathogenesis. Medicinal plants are also an excellent source of natural antioxidants which can reduce the damage level caused by free radicals. In this present study, aims to assess in-vitro antioxidant properties of Withania somnifera methanolic root extract by employing DPPH assay. Withania somnifera has rich profile of nutritional and phytochemical, thus enhancing its pharmacological and therapeutic significance. It is rich in various biologically active components including withanolides, alkaloids, steroidal lactones, flavonoids, tannins, saponins, amino acids, as well glycosides. Ashwagandha also contains other secondary metabolites including naturally occurring sugars, fatty acids, which are important for metabolic and physiological functions, as well as minerals including iron, calcium, potassium, magnesium and zinc. The dried root powder was extracted with methanol and then used at concentrations ranges from 50 to 300 µg/mL. A dose dependent rise in radical scavenging activity was seen which was maximum at 300 µg/mL, 69%. The IC_{50} value was found to be about 140 µg/mL, which means it has high antioxidant potential. It has been concluded from this study that the local source Withania somnifera can be considered as natural antioxidants and further research with multi-solvent-extraction and multi-assay approach should be carried out.

1. INTRODUCTION

Oxidative stress is a biological phenomenon that is presented when the body is unable to manage the defense level between the development of reactive oxygen species (ROS) and its antioxidant activities. The R.O.S. are the molecules that are unstable and can cause damage to lipids, proteins, carbohydrates and nucleic acids important to cellular functions. Prolonged buildup of free radicals can result in cell dysfunction and damage to tissues, leading to development of various chronic & degenerative disorders like cancer, diabetes, cardiovascular diseases, neurodegenerative disorders & diseases, rheumatoid arthritis and aging (Jomova et al., 2023). Because of the dangers of oxidative stress, the search for natural antioxidant compounds that can neutralize these reactive species is the object of numerous studies and reduce oxidative damage.

In human body, oxidative stress is combated by both enzymatic and non-enzymatic antioxidant systems. However, with high oxidative loads, internal antioxidant systems can be compromised, and external supplements of

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ISSN: (e) 3007-1607 (p) 3007-1593

antioxidants are required (Hassanpour & Doroudi, 2023). Another category of antioxidants are the synthetic ones, which are widely used in the food and pharmaceutical departmens, but could cause chronic toxicity and/or carcinogenic effects after prolonged consumption. Accordingly, attention has turned to the use of naturally presented antioxidants from medicinal plants which are safer and biocompatible.

Antioxidants are substances that by preventing any damage to the cells, can arrest or decelerate the oxidative process. Many synthetic antioxidants have been used for a long time, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and are commonly used in the food and pharmaceutical industries; however, concern has been raised about the use of these antioxidants over the long term due to the potential for chronic toxicity and carcinogenicity (Stoia & Oancea, 2022). As a result, medicinal plants, which are characterized by their phytochemical richness and low side effects, have become the preferred choice of safer and more effective alternatives. The phenolic compounds, tannins, flavonoids, alkaloids & terpenoids are main antioxidants found in plants that have been proven to have significant antioxidant activity in many in-vivo as well in-vitro studies (Elshafie et al., 2023).

The Ashwagandha (Withania somnifera) is part of the group solanaceae, one of the most important medicinal species, used in traditional Ayurvedic medicine. It is also called "Indian Ginseng" or also known as "Winter Cherry", it has rejuvenating and adaptogenic properties. Ashwagandha has been part of medicine for centuries in South Asia along the Middle East to treat stress, anxiety, fatigue, insomnia, inflammation, arthritis and neurological disorders (Such et al., 2016). Many parts of this plant, such as the berries, leaves, roots, and seeds, have medicinal significance, with the roots being regarded as the most valuable part because of their high content of bioactive compounds.

The use of Withania somnifera is not restricted to medicine, but also in nutrition and phytochemicals. The roots and leaves of Ashwagandha are rich in several biologically active compounds, such as withanolides (steroidal lactones), flavonoids, amino acids, iron, potassium, calcium, glucose, and essential fatty acids (EFAs) (Gaurav et al., 2023). Furthermore, the plant contains considerable amounts of phenolic compounds and natural antioxidants, which are believed to have therapeutic value. Ashwagandha is also believed to contain a number of energy-boosting and adaptogenic constituents which are believed to work for the benefits of body's overall functioning, physical performance and immunity. Additionally, the micronutrients and bioactive metabolites present in the plant make it a valuable herb in traditional and modern herbal medicine (Jamnekar et al., 2025).

The pharmacological properties of Withania somnifera are mainly attributed to the presence of biologically active constituents such as withanolides, sitoindosides, alkaloids, flavonoids, tannins, and steroidal lactones. The phytochemicals have a wide range of biological properties, such as antioxidant, anti-microbial, anti-inflammatory, immunomodulatory, anti-cancer, neuroprotective, and adaptogenic activities (Bashir et al., 2023). Of these properties, antioxidant activity is especially significant since oxidative stress is closely linked to cell aging and disease development. Ashwagandha extracts have been reported to efficiently scavenge free radicals and increase the antioxidant enzyme activities in biological systems and hence to protect from free radical-mediated damage (Gómez Afonso & Fernandez-Lazaro, 2023).

Many experimental researches have been conducted focusing on the antioxidant activity of Withania somnifera and various solvents for extraction and various antioxidant assays have been used. The various analytical methods is used widely in assessing antioxidant activity among which DPPH free radical scavenging assay is simple, rapid, sensitive, and reliable method. The DPPH assay involves the ability of antioxidant compounds to donate hydrogen atoms or electrons in order to neutralize free radicals, leading to a change in colour from deep violet to yellow. Methanolic and ethanolic extracts of

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ISSN: (e) 3007-1607 (p) 3007-1593

Ashwagandha have been shown to have strong free radical scavenging properties from the presence of phenolic and flavonoid compounds in previous studies (Waqas et al., 2025).

Despite the fact that many studies have been carried out on the antioxidant activity of Withania somnifera, scarcely any research has been conducted on the antioxidant activity of locally sourced Ashwagandha samples under standardized laboratory conditions. Additionally, the phytochemical makeup and the antioxidant activities of plant extracts can be affected by environmental factors, geographic differences, harvesting period, and extraction methods (Sun et al., 2025). There is a comparative dearth of information on locally available herbal samples as compared to commercially prepared formulations which have been the focus of most previous studies. Hence, the antioxidant activity of W. somnifera was evaluated to assess its medicinal value and support its traditional medicinal use.

2. METHODS & MATERIALS

2.1 Sample Collection and Authentication

The fresh root of W. somnifera was collected from the local market and were brought to the laboratory under the proper condition. The collected roots were washed well with distilled water to get rid of the dust and extra materials attached to them. The samples were then shaded dried at room temperature for about 10-14 days to reach constant weight. The dried roots were then ground in an electrical grinder to fine powder and kept inside a sealed container at RT for further experiments.

2.2 Preparation of Plant Material

The powdered plant material was sieved through a fine mesh to get a uniform particle size and to ensure the homogeneity of the sample. The prepared powder was protected from moisture, heat and direct sunlight to prevent degradation of phytochemical constituents. Approximately 20 g of the powdered root material was accurately weighed using an analytical balance for extraction and phytochemical investigations.

2.3 Preliminary Phytochemical Screening

The Withania somnifera roots methanolic extract was subjected to preliminary phytochemical analysis to determine its major constituents such as alkaloids, flavonoids, tannins, saponins, phenolics, glycosides and terpenoids using standard qualitative test (Desta, & Ferede, 2022).

2.4 Physico-Chemical Evaluation

Physico-chemical parameters of the powdered root material were determined according to standard pharmacognostic procedures to assess the quality and purity of the crude drug.

2.4.1 Determination of Moisture Content

An accurately weighed sample, about 2 g, was dried in the hot air oven at 105°C until the constant weight. The percentage moisture content was calculated using the difference in initial and final weights (Anandakumar et al., 2022).

2.4.2 Determination of Ash Value

About 2 g of powdered sample was placed in a silica crucible which was placed in furnace at 600°C until carbon-free ash was obtained. The residue (of sash) was cooled and weighed. The total ash content value was expressed as percentage w/w of the air-dried sample (Sukumar et al., 2020).

2.4.3 Determination of pH

Plant material was powdered and 1% aqueous solution was prepared using distilled water and the pH was measured with a calibrated digital pH meter (Sukumar et al., 2020).

2.5 Preparation of Methanolic Extract

Withania somnifera root material (20g) was extracted by soaking in methanol (200mL) and shaking every few hours at ambient temperature for 48 hours. The mixture was then filtered using Whatman filter paper No.1 and then the filtrate was concentrated under reduced pressure at controlled temperature in rotary evaporator. The crude extract was received and placed in sterile glassware and kept at 4°C for future use.

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2.6 DPPH Free Radical Scavenging Assay

The standard protocols of 2,2-diphenyl 1-picrylhydrazyl (DPPH) assay were applied with slight modification to assess the antioxidant activity of extract. A solution of 0.1 mM DPPH was made in methanol and protected from light exposure. Different concentrations of the extract at 50, 100, 150, 200, and 300 µg/mL levels were prepared. Equal volumes of DPPH solution and extract solution were mixed gently and incubated in the dark for 30 minutes at room temperature. A control solution containing DPPH and methanol without extract was also prepared. After 30 minutes of incubation, absorbance values were noted at 517 nm using a UV-visible spectrophotometer (Mauramo et al., 2021).

2.7 Calculation of Percentage Inhibition

According to Sharma et al. (2025), the percentage inhibition of DPPH free radicals by the extract was calculated using the following equation:

$$\%\, Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where:

- $A_{control}$= Absorbance of control solution
- A_{sample}= Absorbance of sample extract

2.8 Statistical Analysis

Each experiment was conducted with three repetitions and data was collected. The software Microsoft Excel & Statistix 8.1 were used for statistical calculations and graphical analyses (Steel et al.,1997). IC_{50} value was obtained by linear regression analysis for 50% inhibition of the concentration value.

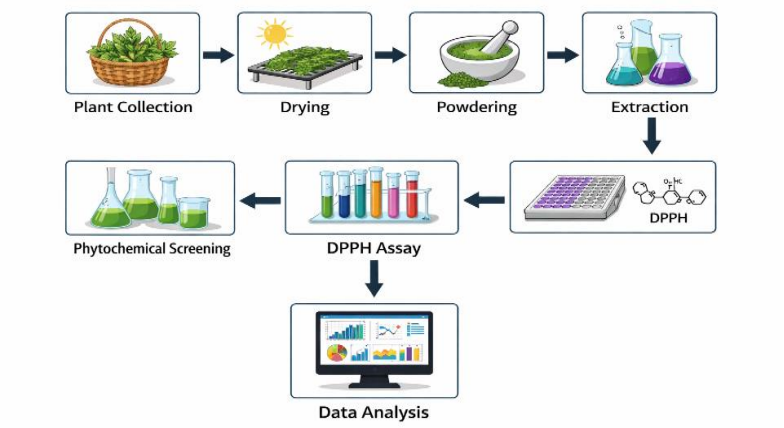

Figure 1: Flowchart of methodology to analyze the samples

3. RESULTS & DISCUSSION

3.1 Phytochemical Screening

Qualitative phytochemical analysis of methanolic extract of root of Withania somnifera showed presence of many Secondary metabolites with biological activity. The extract was found to contain alkaloids, flavonoids, tannins, saponins, phenolic compounds and glycosides. These phytochemicals are reported to exhibit antioxidant and therapeutic activities, some of which could be responsible for the free radical removal activity in the present study.

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Table 3.1: Preliminary Phytochemical Constituents of *Withania somnifera* Root Extract

Phytochemical Test	Alkaloids (Mayer's Test)	Flavonoids	Tannins	Saponins	Phenolic compounds	Glycosides
Result	Present (+)	Present (+)	Present (+)	Present (+)	Present (+)	Present (+)

The preliminary phytochemical investigation of methanolic extract of root of Withania somnifera confirmed the availability of various bioactive compounds. The compound including flavonoids and phenols are two significant constituents found and have proven to possess antioxidant activity. These compounds can donate a H^+ ion or an electron to neutralize ROS and free radicals, thus reducing oxidative stress. Other bioactive compounds including alkaloids and tannins may also contribute the pharmacological activity of plant such as antimicrobial, anti-inflammatory property, protection on cellular activity. The extract contained saponins, which have been reported to have membrane-stabilizing and immunomodulatory properties, and glycosides, which are believed to play a vital role in the therapeutic effects of herb (Gaurav et al., 2023).

The results from the current study have been in line with the reported work by Sandhiya et al., (2022) on phytochemical composition of Withania somnifera. Phytochemical concentration may vary depending on the geographical origin, the climatic conditions, the time of the harvest and the extraction solvent used for analysis. These phytochemicals are detected and further confirm the medicinal value of Ashwagandha along with giving a scientific reason for the antioxidant activity observed in the other assays performed later.

3.2 Physico-Chemical Evaluation

The physico-chemical parameters of Withania somnifera roots (powdered) were underwent to evaluate the quality, stability and purity of crude drug material. Moisture content of sample was established as within acceptable limits thereby reducing chances of microbial contamination and decomposition during storage. The ash value gave an indication of the total inorganic matter in the plant, and the pH of the solution indicated the slightly acidic nature of the powdered sample.

Amongst the elements of herbal drug evaluation, the standardization of herbs is an important part of it, which helps in determining the quality, purity and authenticity of crude plant materials (Kherde et al., 2020). The moisture content of Withania somnifera root powder in the present study was 6.40 % which is comparatively low moisture content, indicating the proper drying condition and low possibility of microbial growth and/or enzymatic degradation during storage, which is similar to the findings by Jha et al. (2024).

Herbal materials containing high moisture content can be subjected to fungal contamination and degradation of bioactive compounds, so it is very important to keep moisture content low to ensure the stability and shelf life of plant materials (Santosh et al., 2021). The total ash value obtained in this study represents the total amount of inorganic residues remaining after incineration of the plant material. The ash content (4.80%) observed was within the range reported for medicinal herbs (i.e. compare, which indicates low contamination with extraneous matter like soil, sand or adulterants). Ash value is also recognized as one of the pharmacognostic parameters which can be used to determine the purity and quality of crude drugs (Beressa et al., 2021).

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Table 3.2: Physico-Chemical Parameters of *Withania somnifera* Root Powder

Parameter	Moisture Content	Total Ash Value	pH (1% aqueous solution)
Observed Value	6.40%	4.80%	5.9

The pH value of the aqueous extract was found to be slightly acidic, which may be attributed to the availability of phenolic compounds and other acidic phytoconstituents naturally present in the plant material. Similar physico-chemical characteristics of *Withania somnifera* have been reported in previous pharmacognostic investigations. These findings (as shown in Figure 2) confirm the acceptable quality and suitability of the collected plant material for further phytochemical and antioxidant analyses.

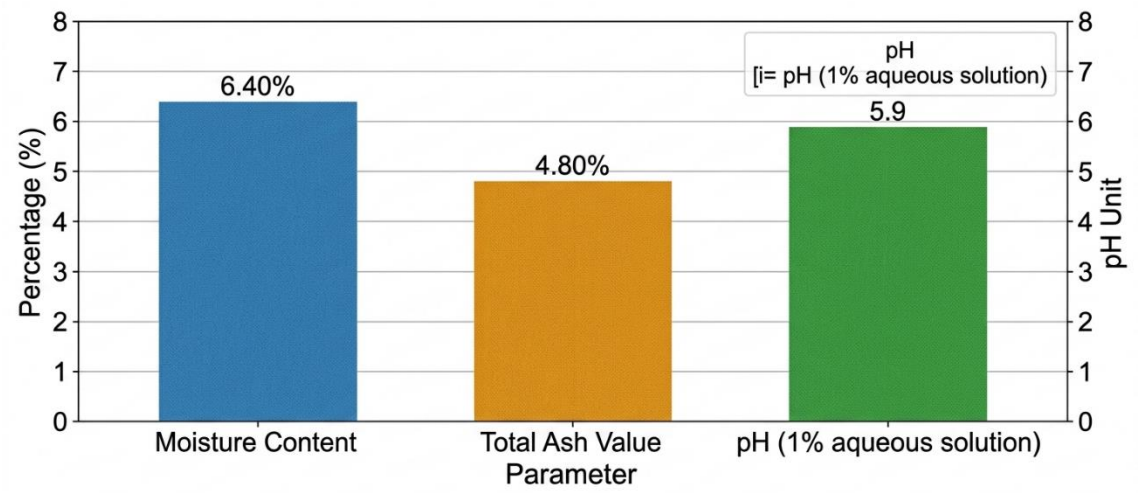

Figure 2: Analytical parameters (moisture content, total ash & pH) for test sample

3.3 DPPH Free Radical Scavenging Activity

Antioxidant activity of the extract of the root of Withania somnifera was studied using the free radical scavenging assay of DPPH. The results were noted at different antioxidant activity against all the concentrations investigated. The percentage inhibition was found to be dependent on the concentration of extracts with a higher value obtained at higher concentration level indicating concentration dependent on free radical scavenging effect. At 300µg/mL antioxidant activity was the maximum with 69% inhibition of DPPH radicals and the minimum was at 50µg/mL with 28% inhibition of DPPH radicals as shown in Table 3.3. The results have resemblance with the studies of Ganguly et al. (2018) who reported the increase of inhibition percentage with increase in concentration of extract solvent. The IC_{50} value of the extract was determined to be about 140µg/mL indicating moderate to strong antioxidant activity.

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Table 3.3: DPPH Scavenging Activity of *Withania somnifera* Root Extract

Concentration (µg/mL)	Absorbance (nm)	% Inhibition
50	0.620	28%
100	0.540	37%
150	0.460	48%
200	0.390	58%
300	0.310	69%

Based on the findings of the present study, W. somnifera showed good antioxidant property in the DPPH assay. The higher the extract concentration the higher the percentage inhibition, indicating the efficacy of the extract in the hydrogen atom/donation process or electron donation to scavenge free radicals as shown in Figure 3. The primary antioxidant activity observed is connected to the presence of phenolic compounds, tannins, flavonoids, and other phytochemicals which was observed due to the preliminary phytochemical screening.

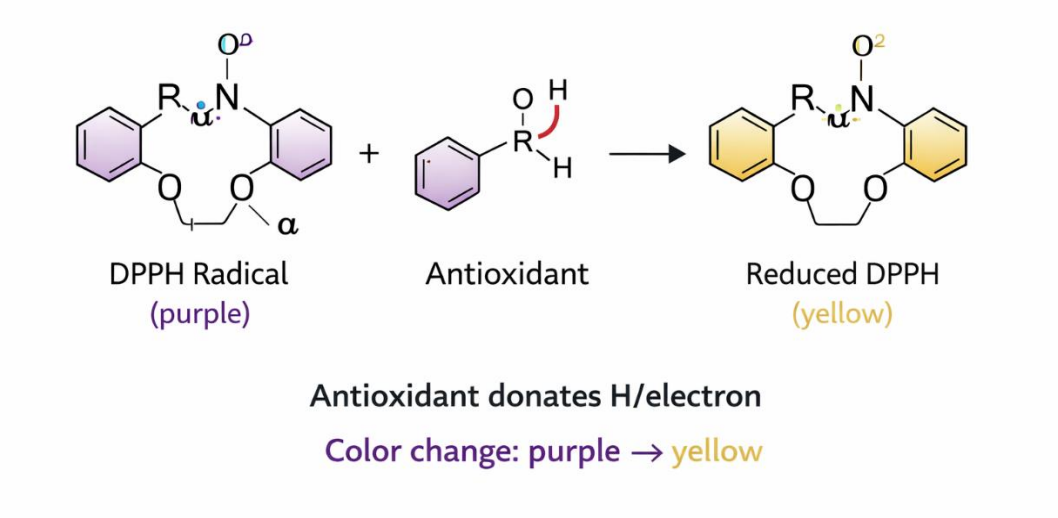

Figure 3: DPPH mechanism diagram

The DPPH assay has been well established as a quick and simple method for assessing the free radical scavenging ability of methanolic extracts of roots. The reduction of the purple-coloured DPPH compound to yellow coloured compound (diphenylpicrylhydrazine) form indicates antioxidant efficiency of the tested extract (Munir et al., 2022). The decrease in absorbance with rise in extract concentration was seen in the present study, thereby affirming the potential of Withania somnifera to scavenge free radicals from the literature (as shown in the above figure 4).

The IC_{50} value derived from this study also confirms the antioxidant activity of extract sample. The lower the IC_{50} value, the higher antioxidant activity of the sample is, because it takes less of a concentration to inhibit 50% of free radicals. The antioxidant activity observed has been reported in earlier studies and it can be inferred that this herb is rich in potent antioxidant content that can neutralize the damage caused by free radicals to the cellular components.

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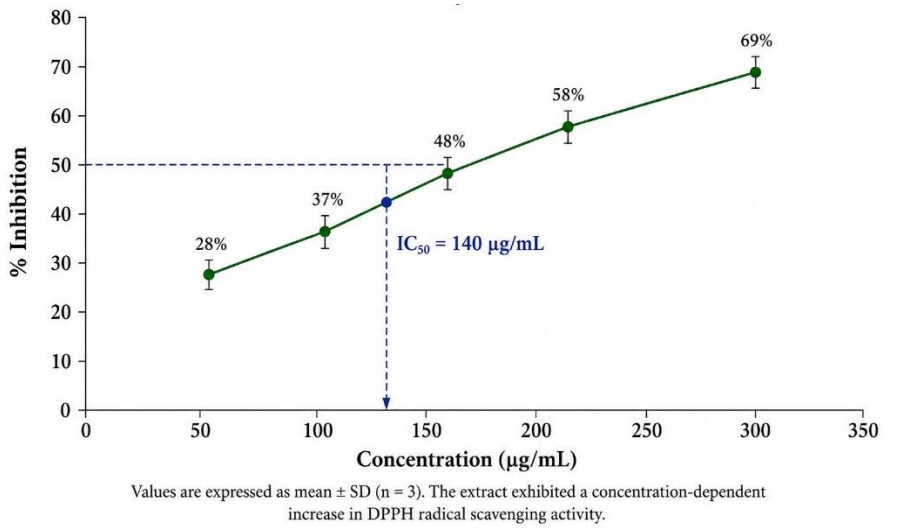
ISSN: (e) 3007-1607 (p) 3007-1593



Values are expressed as mean ± SD (n = 3). The extract exhibited a concentration-dependent increase in DPPH radical scavenging activity.

Figure 4: DPPH free radicals scavenging activity of Ashwagandha root extracct

The varying antioxidant concentrations of the different studies may be due to the solvents used, plant source, environmental factors, cut-down season and analytical method used. The good antioxidant activity observed in the present study can be attributed to extraction of compounds belonging to the classes of phenols and flavonoids, which are good antioxidants. Based on the results, the roots of Withania somnifera can be seen as a source of antioxidants and may be utilized as natural source for medicinal and nutraceutical applications in dealing oxidative stress related disorders.

3.4 Overall Interpretation of Study

The results from all the screening tests as well as physico-chemical analysis and antioxidant activity collectively confirm the biological efficacy of the extract of the root of Ashwaghandha with its rich secondary metabolites. While performing phytochemical elucidation, such compounds were detected and a significant correlation was observed between antioxidant performance of plant extract with DPPH activity, thus showing phenolics and flavonoids as two important ingredients in the plant extract antioxidant activity. Phenolic compounds have been found to donate hydrogen or electrons and thus to stabilize the ROS and to inhibit the oxidative chain reactions in biological systems.

The results of the physico-chemical parameters also showed that the plant material used in this work was suitable and of good quality and did not affect the biological effects observed in it. The moisture content is relatively low, and acceptable ash content indicates proper handling and authentication of the crude drug which is important for the reproducibility and reliability of results in pharmacognostic research.

The antioxidant activity in the present investigation lies in a similar range as reported by earlier studies, thus substantiating the consistency of use of Withania somnifera as a natural source of antioxidants. A slight difference in the IC_{50} value and the percentage of inhibition, however, could be attributed to the difference in the environmental conditions of growth, extraction method and solvent polarity, which all affect the yield of phytochemicals. The higher the polarity of the organic solvent, the more likely it is to extract phenolic and flavonoid compounds, and this is why methanol showed a relatively high antioxidant response in this study. It is concluded that from the results that a the plant extract of Ashwaghandha can be used as promising natural antioxidant agent.

4. CONCLUSION

The present study revealed that the extract of root (of Withania somnifera) possesses a

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ISSN: (e) 3007-1607 (p) 3007-1593

remarkable in-vitro antioxidant activity observed from the dose dependent scavenging activity in DPPH test. Phytochemical Screening revealed presence of important bioactive compounds that contributed to the antioxidant properties observed which included phenolic compounds, flavonoids, tannins and alkaloids. The physico-chemical evaluation showed the authenticity and quality of plant materials so that experimental results at the same time guarantee the reliability of experimental results. This calculated IC_{50} value suggested a moderate to strong antioxidant activity, which could help to reduce oxidative stress-induced damage. In general, the findings of this investigation gives research support to the traditional consumption of Withania somnifera as a natural source of antioxidants. In-vivo studies are recommended, however, for full coverage of the therapeutic potential, safety and mechanism of action of this study, advanced biochemical and clinical evaluations are recommended. Also, comparison studies with various extraction solvents and plant parts can give a better understanding for better optimization of its pharmacologic applications.

5. LIMITATIONS AND SCOPE

The present study focused only on in-vitro evaluation of Withania somnifera's root methanolic extract, hence it might not be a complete representation of its in-vivo biological effects. Only one solvent system and one antioxidant assay (DPPH) was used and no standard reference antioxidant was used for direct comparison which may limit comprehensive understanding of the results. Although these are limitations, studying can serve as a foundation for further studies. Future studies are needed to validate efficacy with in-vivo studies, multiple antioxidant assays, comparative solvent extractions and standard controls. Moreover, isolation of active compound(s) and clinical trials will further advance the understanding of its therapeutic application in preventing oxidative stress associated disorders.

6. ACKNOWLEDGMENT

All authors contributed equally to this research article.

7. CONFLICT OF INTEREST

None of the authors have any conflicts of interest to declare.

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