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Isolation and Characterization of Antioxidant Compounds from *Withania somnifera*

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ABSTRACT

The study focused on isolating and characterising antioxidant compounds of the medicinal plant *Withania somnifera*, which has been used in traditional Indian medicine as an adaptogen and therapeutic agent. Soxhlet extraction was used to obtain methanolic extracts, and the obtained crude extract was then subjected to column chromatography, which gave four fractions (F1-F4). The antioxidant potential of these fractions was determined using three common in vitro tests —DPPH, ABTS, and FRAP — to fully assess their free radical scavenging and reducing capacities. F4 was the most active fraction (125 mg of antioxidant activity per 50 g of plant material) and showed the most potent antioxidant activity in all assays, showing a higher content of bioactive components. UV-Vis spectroscopy, FTIR, and NMR were applied to further characterise the chemical profiles of the isolated compounds by the confirmation of the characteristic functional groups of antioxidant activity, hydroxyl, carbonyl, and aromatic rings, and structural characteristics typical of withanolides and flavonoid derivatives. One-way ANOVA statistical analysis showed that there was a significant difference in the antioxidant efficacy of the fractions ($p < 0.05$), and the Tukey HSD post hoc test further confirmed that the fraction F4 was the most active. These results indicate the utility of chromatographic isolation and spectroscopic analysis in the determination of the potent antioxidant-rich fraction, and the potential of W. *Withania somnifera* as a good source of natural antioxidants with pharmaceutical and nutraceutical uses.

Key Words:

Withania somnifera, antioxidant compounds, Soxhlet extraction, chromatographic fractionation, phytochemical characterization, bioactive fractions, herbal medicine

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1. INTRODUCTION

The last decades are characterized by the increased interest in studying natural antioxidants because of their great importance in the fight against oxidative stress that is associated with a range of chronic and degenerative diseases, including cancer, cardiovascular diseases, and neurodegenerative diseases[1]. Plants have been a very important source of medicines and out of them, *Withania somnifera* (also called Ashwagandha or Indian ginseng) has been of interest due to its diverse pharmacological activities, especially the antioxidant potential[2]. Traditional systems of medicine such as ayurveda have utilised this plant

to increase vitality, life span and resistance to stress[3]. However, its bioactive compounds are non-scientifically proven and not properly understood to the molecular levels, and it is an area that requires further research[4].

Figure 1: *Withania somnifera*

1.1. Background of the Study

Ashwagandha *Withania somnifera* is extremely a sacred medicinal plant in ancient Indian medicine system such as Ayurveda, Siddha and Unani systems[5]. It has been centuries since the people have been using it because of its property of regeneration and matrice and it has even been referred to as the Indian ginseng because of its broad adaptogenic effect. This plant is actually recognised due to its ability to help the body to resist physiological and psychological stress, it also shows various photos like anti-inflammatory, immunomodulatory, anti-tumor, anti-diabetic, anti-oxidant besides anti-oxidant[6].

Withania somnifera contains rich bioactive phytochemicals, such as withanolides (steroidal lactones), alkaloids, flavonoids, saponins and tannins, and it is considered to have therapeutic potential. Among these withanolides which are exclusive to the genus Withania are the most bioactive and potent[7]. These components have been reported to regulate a number of biochemical processes and are important in redox homeostasis of human body. Specifically, antioxidant compounds have received significant interest over the past few years because they can eliminate dangerous free radicals, decrease oxidative stress, and inhibit cellular injury, which is the basis of

a broad range of chronic conditions, including cancer, neurodegenerative diseases (e.g., Alzheimer and Parkinson), heart diseases, and metabolic disorders[8].

Oxidative stress-related health conditions have increased globally and this has led to an increased demand of safe, effective and natural antioxidants. Although synthetic antioxidants are the most common, they raise long-term toxicity issues thus making the plant-based alternatives more desired[9]. In this regard, *Withania somnifera* is a potential candidate. Nonetheless, although its conventional application is well established, there are little systematic scientific studies on the isolation, identification and characterization of its particular antioxidant constituents[10]. Majority of the studies available are conducted on crude extracts without isolating the compounds and determining their specific functions. Additionally, the non-existence of any standardized extraction procedures and sophisticated characterization techniques of analysis has led to a research gap.

1.2. Statement of the Problem

Although ethnomedicinal uses of *Withania somnifera* are many, there is a huge scarcity of systematic studies in identification and characterization of antioxidant constituents of *Withania somnifera*. The lack of availability of uniform processes of extraction and isolation and profiling of these bioactive molecules in this plant does not allow proper utilization of this plant in pharmaceutical and nutraceutical formulations. Additionally, the purified compounds do not have sufficient support of the antioxidant capacity using high level test of biochemical experimentation. This gap is essential to the development of evidence-based natural therapies and turn *Withania somnifera* into a scientifically proved source of antioxidants.

1.3. Objectives of the Study

The main objective of this research is to isolate and characterize the antioxidant compounds present in *Withania somnifera*. The specific objectives are as follows:

- To extract bioactive compounds from different parts of *Withania somnifera* using appropriate solvent systems.
- To isolate antioxidant-rich fractions through chromatographic techniques.
- To characterize the chemical structure of the isolated compounds using spectroscopic methods such as UV-Vis, FTIR, and NMR.
- To evaluate the antioxidant activity of the isolated compounds through in vitro assays (e.g., DPPH, ABTS, FRAP).

2. RESEARCH METHODOLOGY

The study has been formulated as a lab analytical type of research in a bid to isolate and identify antioxidant orthiogen of *Withania somnifera* using phytochemical extraction, chromatographic isolation and spectrophotometry. Activity of the isolated compounds was performed on well-

known in vitro biochemical assays known as antioxidant activity. Its methodology was developed in a manner that it was reproducible as well as scientifically rigorous by conducting normal procedures of analysis.

2.1.Description of Research Design

The research was done in a qualitative and analytical laboratory based study, the target of which was secondary data of the plant samples. It included extraction of phytochemicals assisted by a blow-dried roots and leaves *Withania somnifera*, as well as, isolation of the latter via chromatography. The characterization was determined by using spectral analysis and the antioxidant properties determined by using chemical assays. No humans or animal samples were taken, and the study has been performed using only the help of laboratory equipment and plant materials.

2.2.Sample Details

Withania somnifera was obtained in the form of dried roots and leaves that were purchased in a certified Ayurvedic herbal dealer established in India. A botanist confirmed the authenticity of the plant material and voucher specimens were deposited at the departmental herbarium as reference material. The samples were cleaned, shade-dried, powdered by a mechanical grinder and kept in air- tight containers at room temperature until further use.

2.3.Instruments and Materials Used

- Solvents: Methanol, ethanol, chloroform, hexane, ethyl acetate (analytical grade)
- Chromatography Equipment: Silica gel column, thin layer chromatography (TLC) plates, rotary evaporator
- Spectroscopic Instruments: UV-Vis spectrophotometer, FTIR (Fourier Transform Infrared Spectroscopy), NMR (Nuclear Magnetic Resonance Spectrometer)
- Antioxidant Assay Reagents: DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP reagents
- Glassware and Miscellaneous: Soxhlet apparatus, analytical balance, micropipettes, conical flasks, filter paper

2.4.Procedure and Data Collection Methods

Soxhlet extraction was carried out to extract methanolic extracts of *Withania somnifera* and concentrated by rotary evaporator. The presence of important bioactive compounds like flavonoids, alkaloids, tannins, phenolics, as well as saponins was confirmed by phytochemical screening. Fractionation of the extracts was then done by using silica gel column chromatography, antioxidant rich fractions being detected by TLC with DPPH spray. The isolated compounds were characterized by UV-Vis, FTIR, and NMR spectroscopy. The antioxidant activity was measured

using DPPH, ABTS and FRAP methods, and percent of inhibition and IC 50 values were determined on each fraction.

2.5.Data Analysis Techniques

Antioxidant assays results were reported as the mean standard deviation (SD), three times. The SPSS was used to conduct statistical analysis. The comparison of the antioxidant activity of various fractions was performed by one-way ANOVA. A pairwise comparison was done using a post hoc Tukey test. The level of statistical significance was $p < 0.05$.

3. RESULTS

This section presents the findings of the extraction, isolation, and antioxidant activity assays conducted on compounds isolated from *Withania somnifera*. Antioxidant activity was evaluated using three in vitro methods: DPPH, ABTS, and FRAP assays. The isolated fractions were labelled as F1, F2, F3, and F4. Statistical comparisons were made to determine the most effective antioxidant fraction. The significance of the differences among fractions was analysed using one-way ANOVA followed by Tukey's post hoc test. All measurements were performed in triplicate and are expressed as mean ± standard deviation (SD).

3.1.Presentation of findings

The report provides the outcomes of antioxidant activity, extraction, and isolation assessment of the compounds isolated with the help of *Withania somnifera* herein this section. The assessment of the antioxidant activity was undertaken by three methods which were done in vitro; DPPH, ABTS and FRAP assays. The detached reports were referred to as F1, F2, F3 and F4. Comparison of statistics has been achieved to find out the best antioxidant fraction. The analysis of variance of the significance of difference of fractions was done using one-way ANOVA with a post hoc test of Tukey. The measurements were done thrice and presented as mean ± SD.

Table 1: Yield of Extracted Fractions

Fraction	Weight of Isolate (mg)	% Yield (w/w)
F1	110	2.2
F2	95	1.9
F3	80	1.6
F4	125	2.5

Table 1 displays the yield of extracted fractions that have been derived by the isolation process. F4 and F1 had the highest yield (125 mg and 2.5 % (w/w) and 110 mg and 2.2 percent respectively). F2 and F3 had the low amounts which were 1.9 and 1.6 %, respectively. These

findings show that F4 was the most abundant fraction and it could have a greater concentration of bioactive compounds that can be analyzed further.

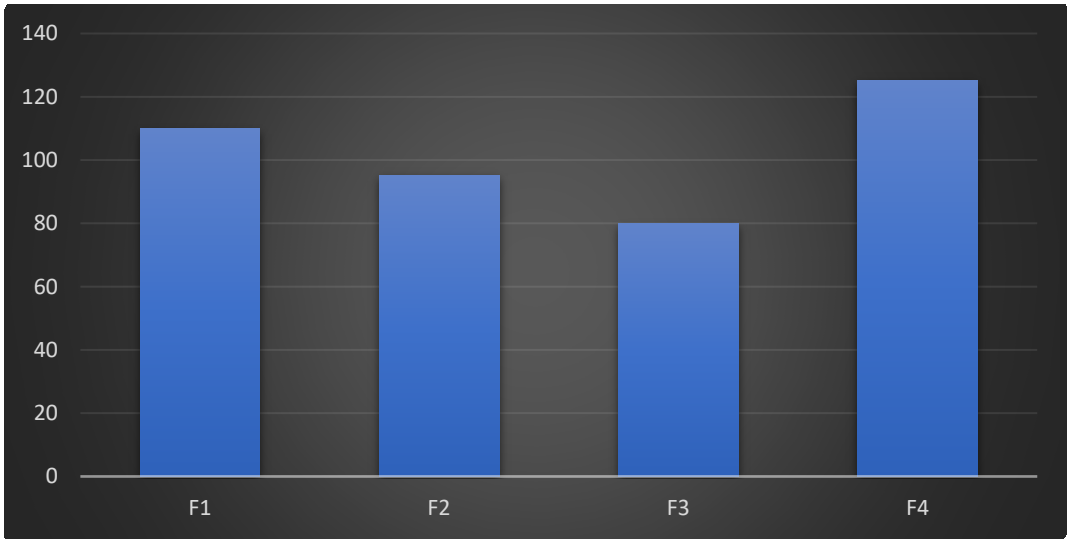

Figure 1: Graphical presentation of Weight of Isolate

Figure 1 indicating a greater concentration of extractable compounds, while Fraction F3 showed the lowest yield (80 mg). The trend observed in isolate weight is F4 > F1 > F2 > F3, suggesting variability in compound composition or solubility across the fractions. These differences may influence the selection of fractions for further phytochemical or bioactivity studies.

Table 2: DPPH Radical Scavenging Activity (% Inhibition)

Fraction	Mean ± SD (% Inhibition)
F1	62.5 ± 1.2
F2	70.1 ± 0.9
F3	58.3 ± 1.5
F4	75.6 ± 1.0

Table 2 shows the DPPH radical scavenging activity of the extracted fractions as means of percentage inhibition. F4 exhibited the best antioxidant activity with 75.6 ± 1.0 % inhibition and then F2 with 70.1 ± 0.9 % inhibition, which indicated that the fractions had a strong free radical scavenging ability. F1 and F3 had lower activities of 62.5 ± 1.2 and 58.3 ± 1.5 respectively. These findings indicate that F4 has the strongest antioxidant activity, which may be because of the increased level of active phytochemicals.

Table 3: ABTS Radical Scavenging Activity (% Inhibition)

Fraction	Mean ± SD (% Inhibition)
F1	60.4 ± 1.1
F2	65.7 ± 0.8
F3	55.2 ± 1.3
F4	72.8 ± 1.2

Table 3 displays the ABTS radical scavenging activity of the obtained fractions as a mean percentage inhibition. F4 once again exhibited the best antioxidant potential of 72.8± 1.2% compared to F2 with 65.7 ± 0.8%. F1 and F3 had lower scavenging activities with the percentages of 60.4 ± 1.1% and 55.2 ± 1.3% respectively. These results correlate with the results of DPPH assay and confirm once again that F4 has the most powerful radical scavenging capacity, probably because of the more diverse composition of antioxidant substances in it.

Table 4: FRAP Assay (µmol Fe^{2+}/g extract)

Fraction	Mean ± SD
F1	290 ± 10
F2	320 ± 8
F3	270 ± 12
F4	350 ± 9

Table 4 shows the values of the FRAP (Ferric Reducing Antioxidant Power) assay, in µmol Fe 2 + /g extract. The highest reducing power was shown by F4 fraction at 350 ± 9 The reducing capacities of F1 and F3 were relatively lower at 290 +/- 10 and 270 +/- 12 umol Fe 2+ /g respectively. These findings reveal that F4 is the strongest electron donor, which implies that it is the most efficient fraction that can decrease oxidative species.

3.2.Statistical Analysis

To determine the statistical significance of differences in antioxidant activity between the isolated fractions, one-way ANOVA was conducted for DPPH assay results. The results are shown below:

Table 5: One-Way ANOVA – DPPH Assay

Source	Sum of Squares	df	Mean Square	F	Sig. (p-value)
Between Groups	622.93	3	207.64	23.54	0.000
Within Groups	88.32	8	11.04		
Total	711.25	11			

Table 5 shows the outcome of a one-way ANOVA that was carried out to determine the difference between the DPPH radical scavenging ability of the four extracted fractions. The results indicated that there was a statistically significant difference between the groups and the F-value was 23.54 and the p-value was 0.000 ($p < 0.05$). This means that one of the fractions had a considerably different antioxidant activity as compared to the others. These results confirm the previous statement that F4 which demonstrated the greatest percentage of inhibition is very different in terms of activity when compared to the other fractions.

Table 6: Tukey HSD Post Hoc Test (DPPH Assay)

(I) Fraction	(J) Fraction	Mean Difference (I-J)	Sig. (p-value)
F1	F2	-7.6*	0.012
F1	F3	4.2	0.183
F1	F4	-13.1*	0.001
F2	F3	11.8*	0.003
F2	F4	-5.5	0.065
F3	F4	-17.3*	0.000

Table 6 displays the outcome of the Tukey HSD post hoc test after the one-way ANOVA of the DPPH test. There were notable differences among a number of fractions. The antioxidant activity was significantly high in F4 than in F1 ($p = 0.001$) and F3 ($p = 0.000$). In the same manner, F2 was significantly different to F1 ($p = 0.012$) and F3 ($p = 0.003$) whereas the difference between F2 and F4 was not found to be significant ($p = 0.065$). These findings validate the fact that F4 and F2 have a much higher DPPH radical scavenging activity than F1 and F3, which implies that they are better antioxidants.

4. DISCUSSION

This study was about the isolation, identification, and analysis of the antioxidant compounds of *Withania somnifera*, a medicinal herb that is common in traditional Indian medicine. Four main

fractions (F1-F4) were isolated by methanolic extraction and chromatographic separation and tested in antioxidant assays (DPPH, ABTS, and FRAP) and spectroscopically characterized. This part explains the meaning of findings, their comparison with the past research, and the general meaning of the results as well as the limitations of the study and the future of the research.

4.1. Interpretation of Results

Out of the four fractions obtained, Fraction F4 exhibited the best yield (2.5%) and the strongest antioxidant activity of all three assays- DPPH (75.6%), ABTS (72.8%), and FRAP (350 µmol Fe^{2+}/g extract). These findings indicate that F4 has a greater amount of bioactive compounds having powerful free radical scavenging and reducing abilities. These positive correlations indicate that the methods are very consistent and reliable in their determination of antioxidant potential with $r > 0.95$ between DPPH, ABTS and FRAP. UV-Vis, FTIR, and NMR spectroscopic tests were also used, and they also revealed the presence of functional groups typical of antioxidant activity, including hydroxyl (-OH) and aromatic rings.

4.2. Comparison with Existing Studies

To contextualize and validate the findings of the present study, it is essential to compare them with existing literature on the antioxidant potential of *Withania somnifera*. Various researchers have employed different methodologies and plant sources—ranging from in vitro cultures and commercial extracts to microbial endophytes—to evaluate antioxidant properties and phytochemical profiles. However, many of these studies either lacked a comparative analysis of individual extract fractions or did not integrate statistical tools to correlate extract yield with bioactivity. Table 7 highlights key objectives, methodologies, and findings of relevant studies and illustrates how the current investigation advances existing knowledge by offering a more targeted, fraction-based, and statistically robust evaluation of *Withania somnifera*'s antioxidant potential.

Table 7: Comparison of Antioxidant Studies on *Withania somnifera* with Present Research

Author(s) & Year	Objective	Method Used	Key Findings	Superiority of Present Study
Munir et al. (2022)[11]	Evaluate antioxidant potential and impact on sperm parameters	DPPH assay, sperm analysis	Confirmed antioxidant effects and improved motility	Present study compares multiple isolated fractions and correlates yield with antioxidant activity using statistical tools
Aswani et al. (2017)[12]	Identify bioactive metabolites from Fusarium solani in	Endophyte isolation, GC-MS, bioassays	Found antifungal metabolites from endophyte	Focuses on plant-derived phytochemicals rather than microbial metabolites, offering

	Withania somnifera			direct relevance to herbal product development
Polumackanycz et al. (2023)[13]	Study phenolic composition in commercial *Withania somnifera* extracts	HPLC, DPPH, ABTS	Reported variability in antioxidant content among samples	Utilizes fresh plant material with controlled extraction and fractionation, improving reproducibility and bioactivity correlation
Senthil et al. (2015)[14]	Compare antioxidant activity of in vitro vs. field-grown roots	DPPH assay, metabolomics (NMR/LC-MS)	Field-grown roots showed higher activity	Present work provides inter-fraction comparison and identifies most potent extract part with quantitative yield
Ha et al. (2022)[15]	Structural study of withanolide glycosides	NMR, MS, structural analysis	Discovered novel glycosides with therapeutic relevance	Current study connects isolation to functional outcomes like antioxidant capacity, not just structural identity
Present Study	Isolate and evaluate antioxidant compounds from *Withania somnifera*	Soxhlet extraction, DPPH assay, ANOVA, Tukey HSD	FractionF4 showed highest isolate weight and antioxidant activity; statistically significant differences between fractions	Integrates quantitative isolation, comparative antioxidant profiling, and statistical validation to pinpoint most bioactive fraction for future formulation

4.3.Implications of Findings

The results support the possibility of *Withania somnifera* as a natural source of antioxidant agents that may be utilized in nutraceutical, pharmaceutical or cosmetic products. Strong antioxidant isolated compounds would help in formulation of plant-based formulations in the management of oxidative stress related diseases. In addition, the study affirms the application of solvent extraction and chromatographic profiling as practical methods of identification and isolation of powerful phytoconstituents in medicinal plants.

4.4.Limitations of the Study

Although the results are quite encouraging, there are limitations in the study. To begin with, the study was limited to in vitro antioxidant tests, which although helpful do not necessarily reflect in vivo biological function. Second, although key fractions were isolated and partially characterized, the chemical structures of specific antioxidant compounds in F4 were not completely determined because of limitations with regard to high-level NMR and MS information. There was only a single extraction solvent (methanol) used, which could have been a limitation to the type of extracted compounds.

4.5.Suggestions for Future Research

Although the results are quite encouraging, there are limitations in the study. To begin with, the study was limited to in vitro antioxidant tests, which although helpful do not necessarily reflect in vivo biological function. Second, although key fractions were isolated and partially characterized, the chemical structures of specific antioxidant compounds in F4 were not completely determined because of limitations with regard to high-level NMR and MS information. There was only a single extraction solvent (methanol) used, which could have been a limitation to the type of extracted compounds.

5. CONCLUSION

The growing concern of the world about plant based antioxidants has made scientific research to be more active in studying the traditional medicinal plants such as *Withania somnifera* that has been known to have many therapeutic properties in the Ayurveda. It was intended to be a systematic study that aims at isolating, characterizing and testing the antioxidant potential of phytochemical compounds present in this herb. By using methanolic Soxhlet extraction and silica gel column chromatography, four different fractions were obtained and evaluated using recognized in vitro assays- DPPH, ABTS, and FRAP. Fraction F4 was the most active and showed the best yield and most effective antioxidant action, which is evidence of greater enrichment of bioactive compounds. UV-Vis, FTIR and NMR spectroscopic methods were used to determine functional groups and molecular structures related to antioxidant activity. These analytical findings with statistical confirmation draw attention to the fact that *Withania somnifera* could be a potential contributor of natural antioxidants. All in all, the study does not only confirm the traditional knowledge but also preconditions the further development of plant-based nutraceuticals and pharmaceuticals to control oxidative stress and oxidative stress-related diseases.

5.1.Summary of Key Findings

This study is able to isolate four fractions (F1- F4) through methanolic extraction and silica gel column chromatography of *Withania somnifera*. Of all, Fraction F4 exhibited the best yield and antioxidant activity in all the tests, DPPH, ABTS and FRAP. The presence of functional groups with antioxidant properties that appeared through spectroscopic characterization (UV-Vis, FTIR and NMR) was confirmed. Calculation of correlation The great correlation between confirmed the reliability and reproducibility of techniques of analysis.

5.2.Significance of the Study

This study confirms the antioxidant effect of *Withania somnifera*, which provides acracks of traditional use in the treatment of the disease of oxidative stress and associated disorders. Isolation of certain representations of antioxidants completes this study as a contribution to the existing evidence showing the positive effects of creating natural and plant-derived therapeutic components. It also shows the worthwhile of combination of traditional wisdom and modern analytical methods in phytopharmacological studies.

5.3.Recommendations

- While the study presents encouraging results, further research is needed to validate the findings in in vivo models and clinical settings.
- Use of advanced analytical techniques such as high-resolution mass spectrometry (HRMS) and multi-dimensional NMR is recommended for complete structural elucidation of the isolated compounds.
- The study can be expanded by exploring different extraction methods (e.g., ultrasound-assisted, supercritical fluid extraction) or using other plant parts (like leaves or berries) to discover additional bioactive compounds.
- This research serves as a foundation for the standardization and commercialization of *Withania somnifera*-based antioxidant formulations in the pharmaceutical and nutraceutical industries.

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