"Phytochemical and in Vitro Antioxidant Assessment of Ashwagandha (W. somnifera) and Shilajit via DPPH and DMPD Assays"

Miss. Rutika Dhannajay Harshad, Miss. Amruta Sunil Gaikwad, Vighnesh Rahul Shimpi, Abhishek Ramesh koli, Shailesh Mahesh Ganbawale, Sushant Shailesh Savale

Biocyte Institute Of Research And Development, Sangli

DOI: https://dx.doi.org/10.51244/IJRSI.2025.12110173

Received: 06 December 2025; Accepted: 12 December 2025; Published: 23 December 2025

ABSTRACT

Ashwagandha (*Withania somnifera*) and Shilajit are widely recognized Rasayana (rejuvenating) substances in Ayurveda, known for their adaptogenic, antioxidant, and cytoprotective properties. Oxidative stress plays a major role in chronic diseases, and natural antioxidants from traditional medicinal systems have gained significant interest. The present study aimed to extract bioactive compounds from Ashwagandha roots and Shilajit resin using Soxhlet-assisted ethanolic extraction and evaluate their phytochemical composition and in vitro antioxidant activity. Qualitative screening revealed the presence of alkaloids, phenolics, flavonoids, terpenoids, and glycosides in both extracts. Quantitative estimations confirmed that both extracts contained considerable levels of total phenolics and flavonoids. Antioxidant activity was determined using DPPH and DMPD radical scavenging assays. Both extracts demonstrated dose-dependent free radical scavenging activity with significant inhibition percentages, indicating strong antioxidant potential. These findings support the traditional use of Ashwagandha and Shilajit as potent natural antioxidants and justify their application in stress management, anti-aging formulations, and oxidative-damage-related disorders.

Keyword: Ashwagandha (Withania somnifera), Shilajit, Rasayana ,Phytochemical screening, Total phenolic content (TPC) ,Total flavonoid content (TFC), Antioxidant activity

INTRODUCTION

Medicinal plants have played a crucial role in traditional healing systems and continue to serve as promising sources of therapeutic agents. Ayurveda, one of the oldest holistic systems of medicine, highlights several Rasayana herbs known for their rejuvenating, immunomodulatory, and antioxidant potential. Among these, **Ashwagandha (*Withania somnifera*)** and **Shilajit** hold significant therapeutic importance.[1]

Ashwagandha is widely recognized for its adaptogenic, neuroprotective, anti-inflammatory, and anti-stress properties. Modern studies demonstrate that Ashwagandha reduces oxidative stress biomarkers such as malondialdehyde (MDA) and enhances endogenous antioxidants including glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Similarly, Shilajit, a mineral-rich exudate formed from plant decomposition, is traditionally used for vitality, anti-aging, hypertension management, and tissue repair. It has been shown to reduce lipid peroxidation and significantly elevate total antioxidant capacity, SOD levels, and GSH content in various experimental models. [2]

Oxidative stress plays a major role in several chronic disorders such as neurodegeneration, aging, inflammation, and metabolic diseases. Therefore, identifying natural antioxidants from traditional medicinal sources has become a focused area of research. Ethanolic extraction is particularly effective for isolating phenolics, flavonoids, alkaloids, and other bioactive compounds responsible for antioxidant effects. [2]

In this context, the present study aims to evaluate the phytochemical profile and antioxidant activity of **ethanolic extracts of Ashwagandha and Shilajit** using Soxhlet extraction. Antioxidant potential was assessed

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

through **DPPH** and **DMPD** free radical scavenging assays, both widely accepted in vitro models for measuring electron-donating and hydrogen-donating ability of plant extracts. [3]

This study provides scientific evidence supporting the antioxidant potential of these Ayurvedic substances and contributes to their validation for therapeutic and nutraceutical applications.

MATERIALS AND METHODS

1. Materials

All chemicals used were of analytical grade. DPPH, DMPD, $FeCl_3$, Folin–Ciocalteu reagent, $AlCl_3$, sodium carbonate, methanol, ethanol, gallic acid, quercetin, and ascorbic acid were purchased from standard suppliers. Distilled water and glassware were used throughout the study.

2. Sample Collection and Preparation

2.1 Ashwagandha Roots

Fresh roots were shade-dried for 10–12 days and powdered (40–60 mesh).

2.2 Shilajit

Commercially available Shilajit resin/powder was cleaned and filtered to remove impurities.

3. Ethanolic Extraction Using Soxhlet Apparatus

3.1 Extraction Procedure

- 50 g of powdered sample (Ashwagandha or Shilajit) was placed in a Soxhlet thimble.
- Extracted using 70% ethanol (500 mL) for **6–8 hours** at 60–70°C.
- Extract was concentrated using a rotary evaporator at ≤40°C.
- Final extract was dried, weighed, and stored at 4°C.

3.2 Extract Yield Calculation

Yield (%) = Weight of dried extract /Weight of raw material ×100

4. Phytochemical Screening

4.1 Qualitative Phytochemical Tests

Standard phytochemical tests were performed for:

- Alkaloids (Dragendorff's test)
- Flavonoids (Shinoda test)
- Phenolics & Tannins (Ferric chloride test)
- Saponins (Froth test)
- Glycosides (Keller–Kiliani test)
- Terpenoids/Steroids (Salkowski test)

Observations were recorded as (+), (++), (+++), or absent (–).

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

5. Quantitative Phytochemical Analysis

5.1 Total Phenolic Content (TPC)

Determined using Folin–Ciocalteu method.

- Sample (20 µL) + diluted Folin reagent (100 µL).
- After 5 min, add 80 µL Na_2CO_3 (7.5%).
- Incubate for 30 min.
- Read at **765 nm**.
- Results expressed as **mg gallic acid equivalents (GAE)/g extract**.

5.2 Total Flavonoid Content (TFC)

Using $AlCl_3$ colorimetric assay.

- Sample (100 µL) + 2% $AlCl_3$ (100 µL).
- Incubate 10 min.
- Read at **415 nm**.
- Expressed as **mg quercetin equivalents (QE)/g extract**.

6. Antioxidant Activity

6.1 DPPH Radical Scavenging Assay

- DPPH (0.1 mM) prepared fresh in methanol.
- Sample + DPPH incubated 30 min in the dark.
- Absorbance measured at **517 nm**.

%Inhibition = A control – sample A / control times ×100

6.2 DMPD Radical Cation Decolorization Assay

- DMPD + $FeCl_3$ prepared to form $DMPD^{\bullet+}$ radical ($A_{505} \approx 0.7–0.9$).
- Sample mixed with $DMPD^{\bullet+}$ and incubated 10 min.
- Read at **505 nm**.

%Inhibition = A control – sample A / control times ×100

RESULT

7.1 Ethanolic Extraction Using Soxhlet Apparatus

The dried powdered material of **Ashwagandha (Withania somnifera)** and **Shilajit** was subjected to continuous hot percolation using a Soxhlet apparatus with ethanol as the extraction solvent. After extraction, the solvent was evaporated, and the crude extract was weighed to calculate the percentage yield (w/w). The

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

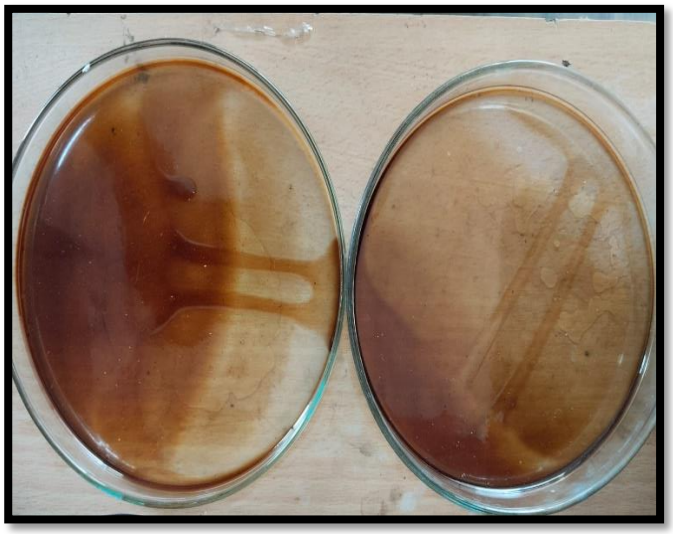
physical characteristics such as color and nature of the extract were also recorded.

Ashwagandha (*Withania somnifera*)

Shilajit

Ethanolic Extraction Using Soxhlet Apparatus Ashwagandha (*Withania somnifera*) & Shilajit

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025



Ethanolic Extraction Using Soxhlet Apparatus Ashwagandha (*Withania somnifera*)

Ethanolic Extraction Using Soxhlet Shilajit

Ethanolic Extraction Using Soxhlet Apparatus Ashwagandha (*Withania somnifera*)

Ethanolic Extraction Using Soxhlet Shilajit

% **yield w/w** of= ((y-x)/weight of sample) ×100

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

Table no. -1. Ethanolic Extraction Using Soxhlet Apparatus Ashwagandha (*Withania somnifera*) & Shilajit

Sr. no.	**Plant names**	**Colour**	**Nature**	**% yield w/w**
1	**Ashwagandha (*Withania somnifera*)**	Dark brown	Solid	**44.48**
2	**Shilajit**	Dark brown	Solid	**41.98**

The Soxhlet-based ethanolic extraction of Ashwagandha and Shilajit produced significant yields (44.48% and 41.98%, respectively). The color, nature, and high yield values confirm that ethanol is an effective solvent for recovering major phytochemicals from both materials.

7.2 Phytochemical Screening Of Ethanolic Extract Of Ashwagandha (*Withania somnifera*) & Shilajit

The ethanolic extracts of **Ashwagandha (*Withania somnifera*)** and **Shilajit** were subjected preliminary phytochemical screening to identify major classes of secondary metabolites. Standard qualitative tests were performed to detect alkaloids, flavonoids, phenolics & tannins, saponins, glycosides, and terpenoids/steroids.

Table no. -2. Phytochemical Screening Of Ethanolic Extraction of Ashwagandha (*Withania somnifera*) & Shilajit

Sr. No	**Phytochemical Screening**	**Ashwagandha (*Withania somnifera*)**	**Shilajit**
1	Alkaloids (Dragendorff's test)	+	+
2	Flavonoids (Shinoda test)	+	+
3	Phenolics & Tannins (Ferric chloride test)	-	-
4	Saponins (Froth test)	+	+
5	Glycosides (Keller–Kiliani test)	-	-
6	Terpenoids/Steroids (Salkowski test)	+	+

The phytochemical screening of ethanolic extracts of Ashwagandha and Shilajit indicates a rich presence of bioactive compounds such as **alkaloids, flavonoids, saponins, and terpenoids/steroids**. These constituents may play a major role in the antioxidant and therapeutic potential of both extracts. The absence of phenolics, tannins, and glycosides suggests that these compounds are either in negligible amounts or not efficiently extracted using ethanol in this study.

7.3 Quantitative Phytochemical Analysis OF Ethanolic Extract Of Ashwagandha (*Withania somnifera*) & Shilajit

7.3.1 Estimation of total phenolic contents

The total phenolic content (TPC) of the ethanolic extracts of **Ashwagandha (*Withania somnifera*)** and **Shilajit** was quantified using the **Folin–Ciocalteu method**, and results were expressed as **Gallic Acid Equivalents (GAE)**.

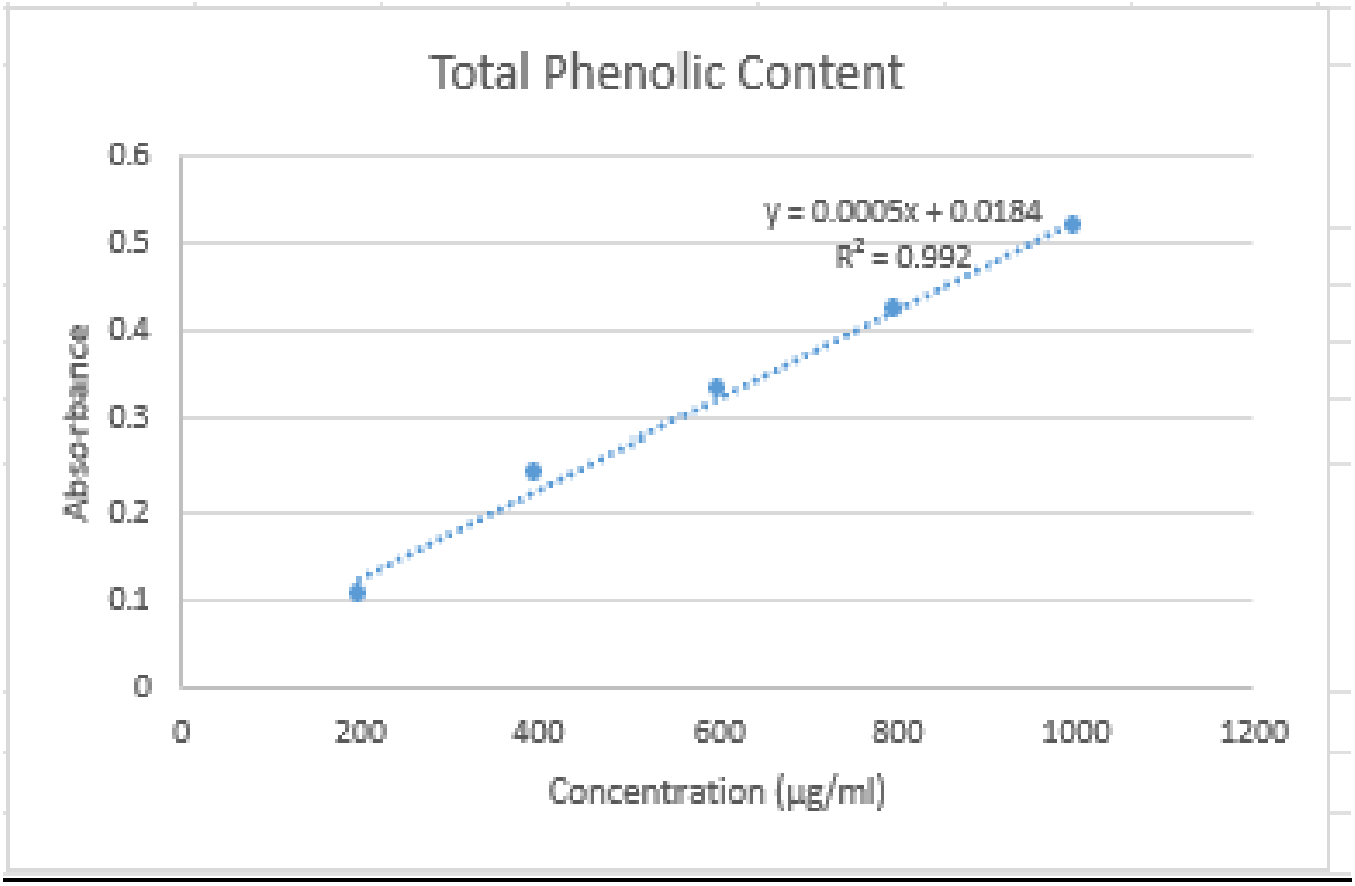
Table no. -3. Estimation of total phenolic contents Of Gallic Acid

Sr. no.	**Gallic acid Concentration (µg/ml)**	**Gallic acid Concentration (mg/ml)**	**OD**
1	200	0.20	0.102
2	400	0.40	0.241
3	600	0.60	0.332
4	800	0.80	0.421
5	1000	0.1	0.520

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

Table no. -4. Estimation of total phenolic contents Of Ethanolic Extraction of Ashwagandha (*Withania somnifera*) & Shilajit

Sr. NO	Samples at Concentration (1000µg/ml)	OD			Mean OD	Total phenolic contents (µg GA/g)	Total phenolic contents (mg GA/g) in ± SD
1	**Ethanolic Extract Of Ashwagandha (*Withania somnifera*)**	**0.17**	**0.18**	**0.14**	**0.16**	306.22 µg GA/g	**0.306 mg GA/g**
2	**Ethanolic Extract Of Shilajit**	**0.16**	**0.13**	**0.14**	**0.14**	267.91 µg GA/g	**0.268 mg GA/g**



Estimation of total phenolic contents Of Gallic Acid

7.3.2 Estimation of total flavonoid content

The total flavonoid content (TFC) of the ethanolic extracts was determined using the **Aluminium chloride ($AlCl_3$) colorimetric method**, with **quercetin** as the standard reference compound. Absorbance values of quercetin standards were recorded at 415 nm to construct the calibration curve. The flavonoid content of the samples was expressed as **µg quercetin equivalent (QE) per g of dry extract**.

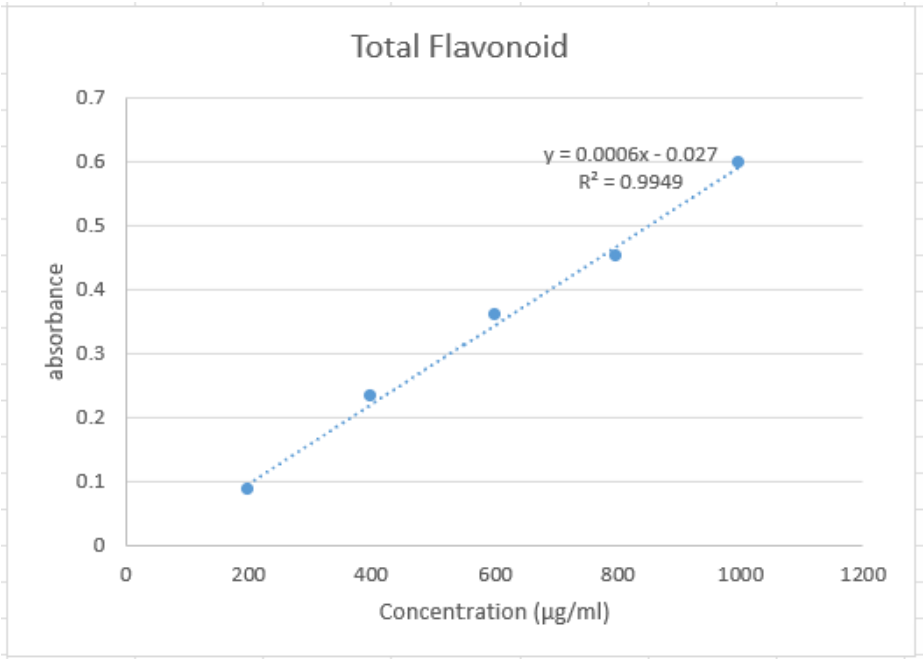
Table no. -5. Estimation of total flavonoid content of Quercetin

Sr. no.	Quercetin Concentration (mg/ml)	Quercetin Concentration (µg/ml)	O
1	200	0.20	0.102
2	400	0.40	0.241
3	600	0.60	0.332
4	800	0.80	0.421
5	1000	0.1	0.520

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

Table no. -6. Estimation of total flavonoid content of Ethanolic Extraction of Ashwagandha (*Withania somnifera*) & Shilajit

Sr. no.	Samples at Concentration (1000 µg/ml)	Absorbance (OD)	Mean (OD)	Flavonoid content (µg quercetin equivalent /g dry material)	Flavonoid content (mg quercetin equivalent /g dry material)
1	**Ethanolic Extract Of Ashwagandha (*Withania somnifera*)**	0.17 0.15 0.18	0.16	**260 µg QE/g dry**	**0.260 mg QE/g**
2	**Ethanolic Extract Of Shilajit**	0.16 0.14 0.15	0.15	225 µg QE/g	0.225 mg QE/g



Estimation of total flavonoid content

8. Antioxidant Activity Ethanolic Extraction of Ashwagandha (*Withania somnifera*) & Shilajit

The antioxidant potential of the ethanolic extracts of *Withania somnifera* (Ashwagandha) and Shilajit was evaluated using two complementary radical scavenging assays: DPPH and DMPD. Ascorbic acid was used as a standard antioxidant for comparison.

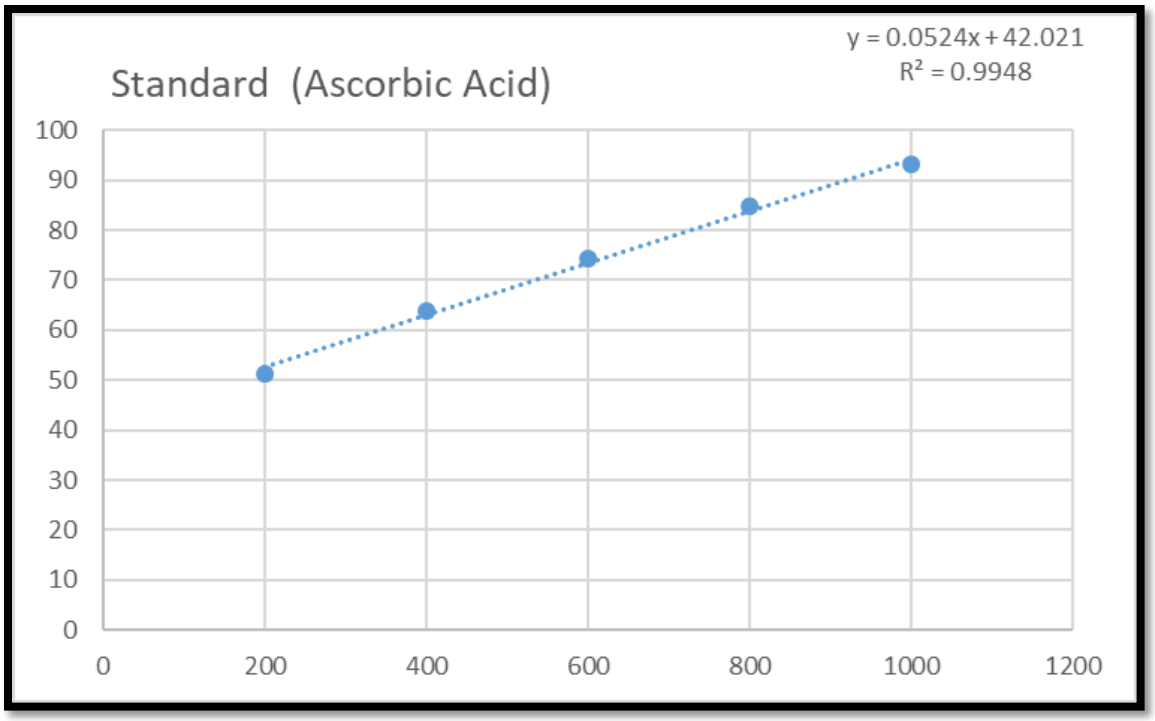
8.1 Antioxidant Activity by DPPH Method

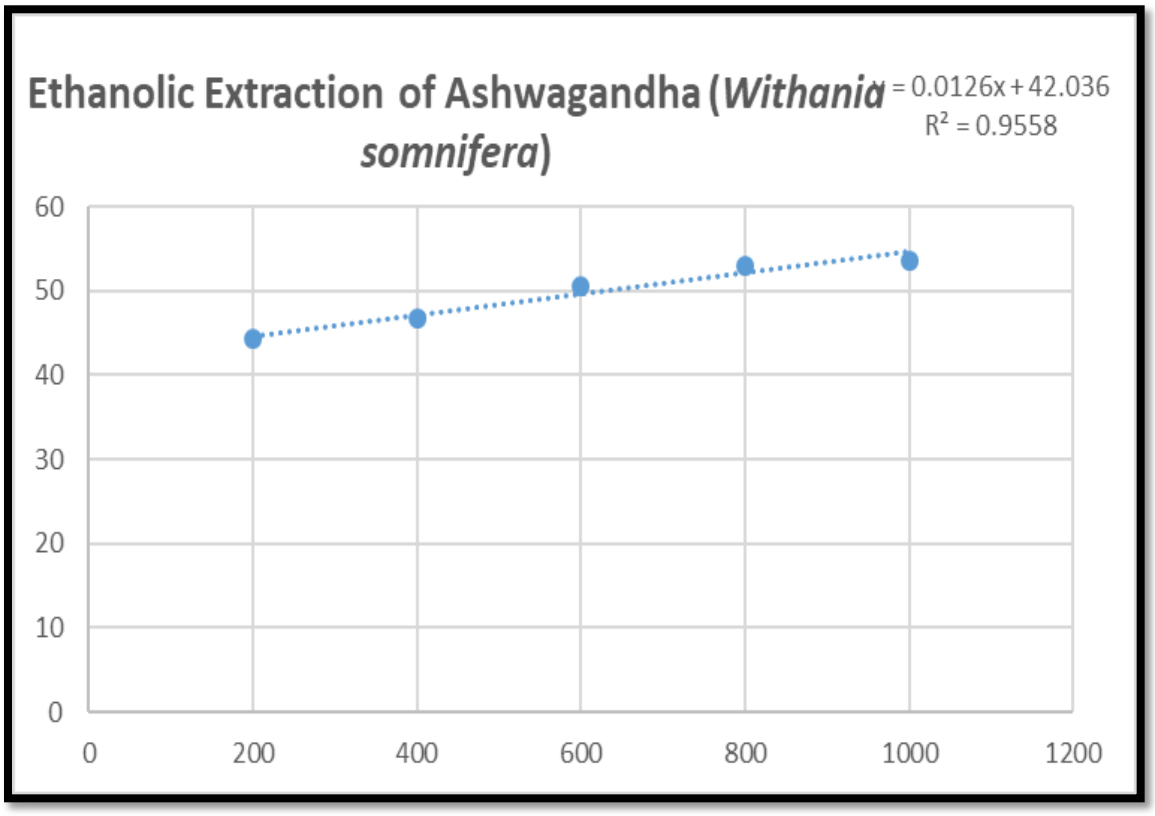
The antioxidant activity of ethanolic extracts of **Ashwagandha (Withania somnifera)** and **Shilajit**, along with a standard antioxidant (Ascorbic acid), was evaluated using the DPPH radical scavenging assay. The percentage inhibition was calculated based on the decrease in absorbance of DPPH reagent at 517 nm.

Sr. no	Sample code	Conc.	OD			Mean	Percent inhibition	Ic50
1	Control	-	1.756	1.764	1.785	1.768		
2	**Standard (Ascorbic Acid)**	200	0.858	0.855	0.878	0.864	51.16	152.27
		400	0.652	0.642	0.621	0.638	63.90	
		600	0.457	0.458	0.447	0.454	74.33	
		800	0.257	0.278	0.274	0.270	84.75	
		1000	0.125	0.122	0.118	0.122	93.12	

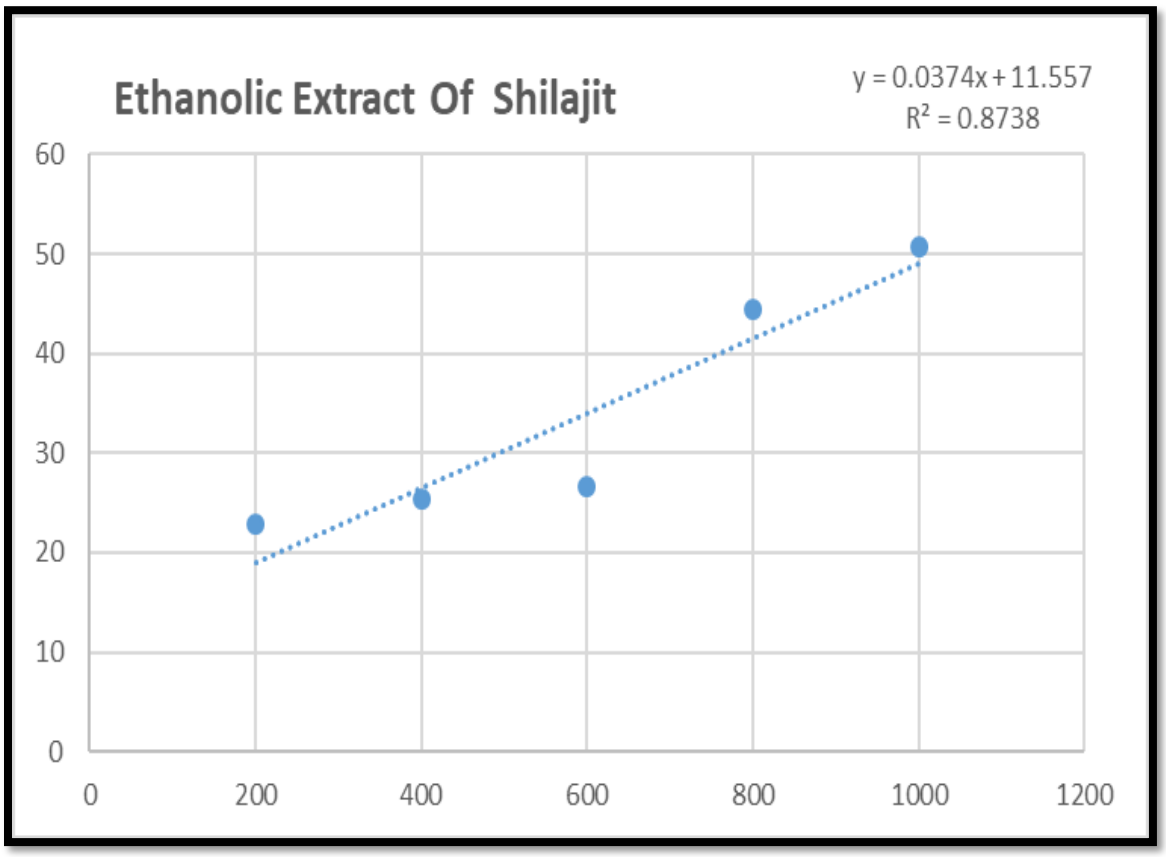
ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

3	**Ethanolic Extraction of Ashwagandha (*Withania somnifera*)**	200	0.989	0.997	0.974	0.987	44.20	632.06
		400	0.962	0.968	0.899	0.943	46.67	
		600	0.897	0.884	0.844	0.875	50.52	
		800	0.832	0.831	0.829	0.831	53.03	
		1000	0.822	0.819	0.818	0.820	53.65	
4	**Ethanolic Extract Of Shilajit**	200	1.356	1.369	1.369	1.365	22.83	1027.88
		400	1.258	1.245	1.459	1.321	25.32	
		600	1.156	1.158	1.574	1.296	26.71	
		800	0.986	0.985	0.974	0.982	44.49	
		1000	0.859	0.885	0.874	0.873	50.65	

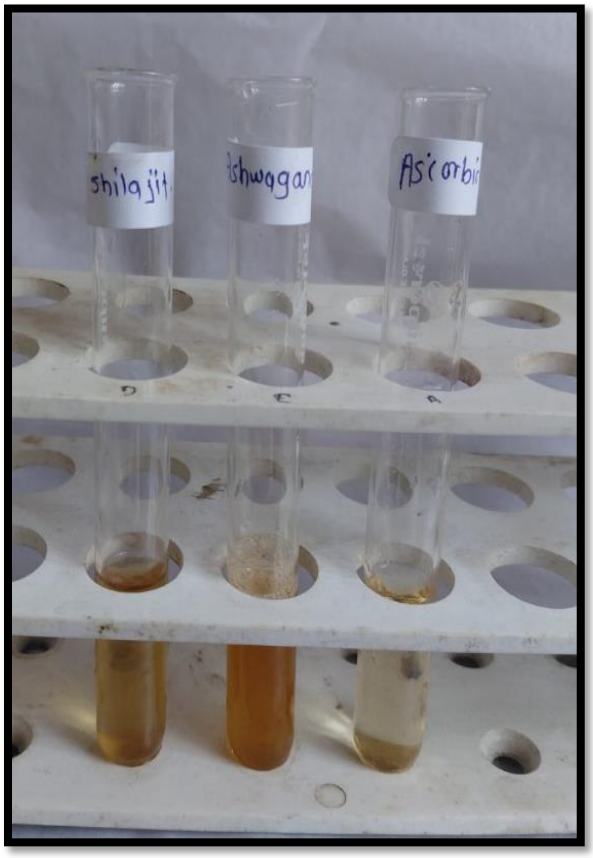


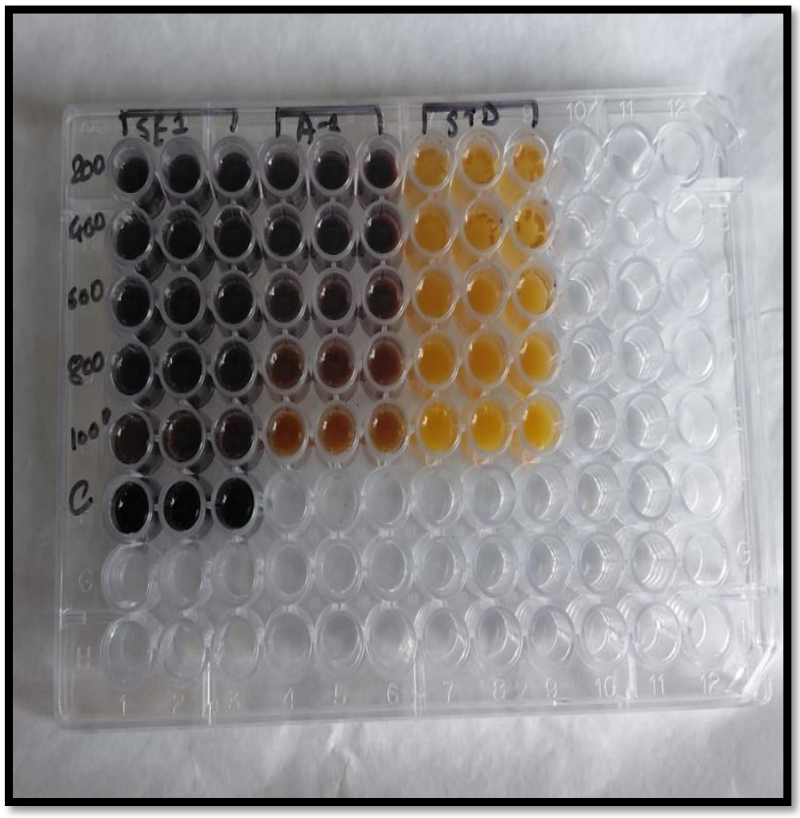


ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025



Sample	IC_{50} (µg/ml)	Antioxidant Strength
Ascorbic acid (Standard)	152.27	Very Strong
Ashwagandha (Ethanolic extract)	632.06	Moderate
Shilajit (Ethanolic extract)	1027.88	Weak



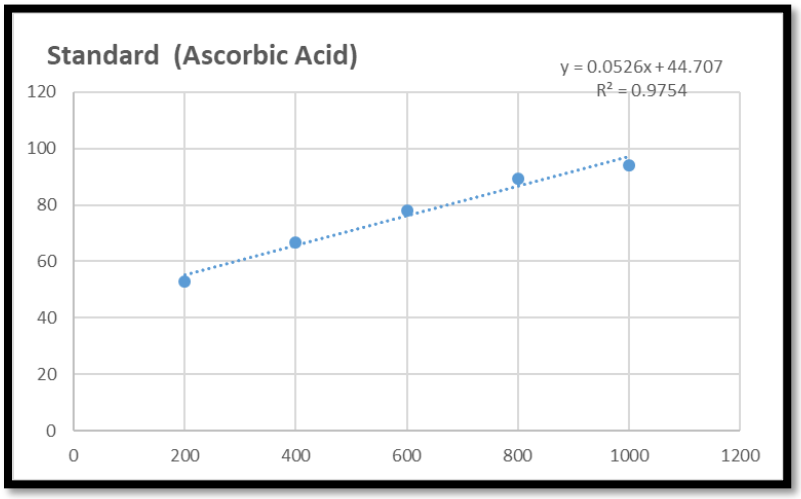


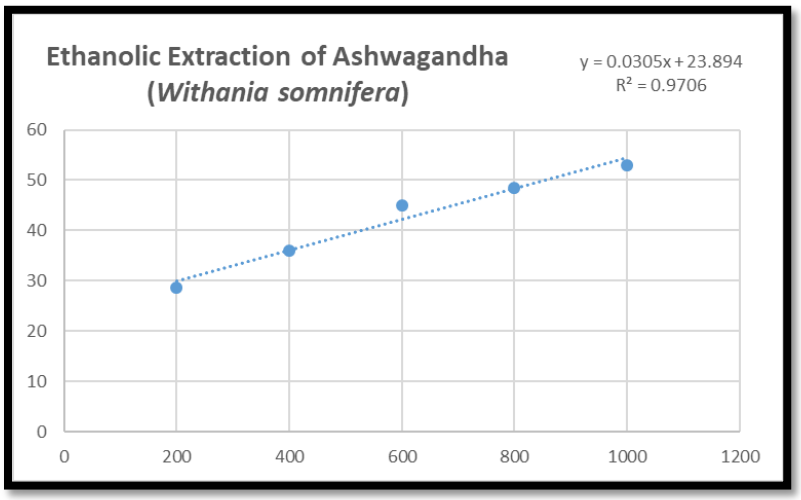
ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

8.2 **Antioxidant Activity by DMPD Method**

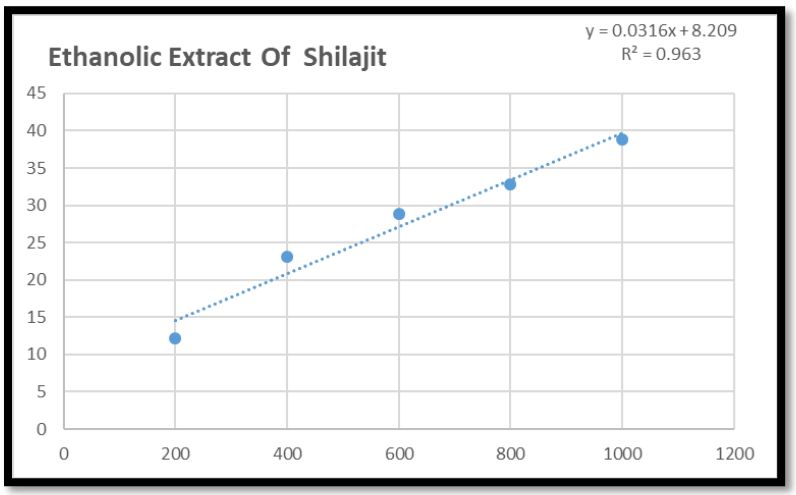
The antioxidant activity of the **ethanolic extracts of Ashwagandha (Withania somnifera)** and **Shilajit**, along with **Ascorbic Acid (standard)**, was assessed using the **DMPD radical cation scavenging assay**. Decrease in absorbance of DMPD•⁺ at 505 nm was used to determine % inhibition.

Sr. no	Sample code	Conc.	OD			Mean	Percent inhibition	Ic50
1	Control	-	1.588	1.589	1.684	1.620		
2	**Standard (Ascorbic Acid)**	200	0.758	0.755	0.778	0.763	52.86	100.62
		400	0.552	0.542	0.521	0.538	66.77	
		600	0.357	0.358	0.347	0.354	78.15	
		800	0.157	0.178	0.174	0.169	89.52	
		1000	0.089	0.098	0.099	0.0953	94.11	
3	**Ethanolic Extraction of Ashwagandha (*Withania somnifera*)**	200	1.123	1.124	1.222	1.156	28.63	855.93
		400	1.11	0.999	0.998	1.035	36.08	
		600	0.899	0.895	0.885	0.893	44.88	
		800	0.878	0.875	0.748	0.833	48.54	
		1000	0.789	0.789	0.71	0.762	52.93	
4	**Ethanolic Extract Of Shilajit**	200	1.425	1.412	1.435	1.424	12.11	1.322
		400	1.256	1.245	1.236	1.246	23.12	
		600	1.123	1.138	1.196	1.152	28.88	
		800	1.069	1.099	1.097	1.088	32.83	
		1000	0.989	0.987	0.997	0.991	38.83	

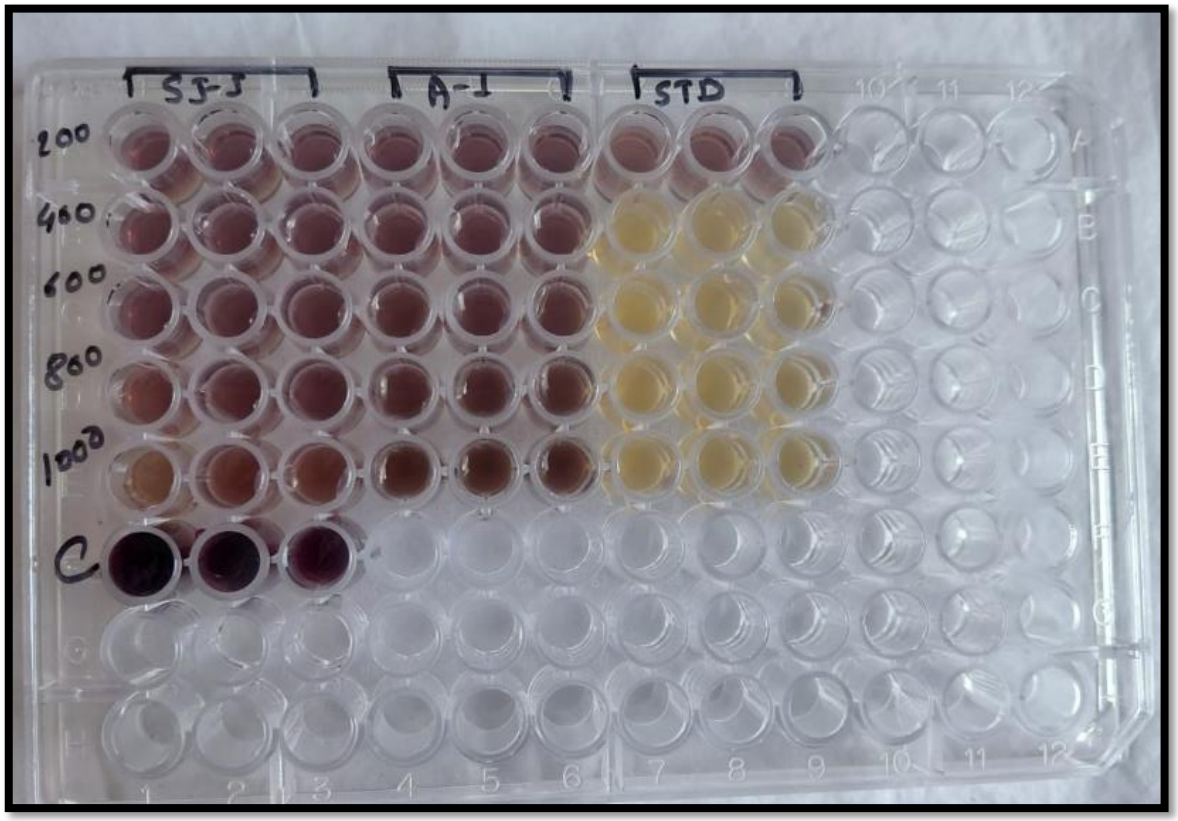




ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025



Sample	IC_{50} (µg/ml)	Antioxidant Strength
Ascorbic Acid (Standard)	**100.62**	**Very Strong**
Ashwagandha Extract	**855.93**	**Moderate**
Shilajit Extract	**1322** (approx.)	**Weak**



DISCUSSION

The Soxhlet-based ethanolic extraction of **Ashwagandha (Withania somnifera)** and **Shilajit** resulted in substantial yields of **44.48%** and **41.98%**, respectively, indicating that ethanol efficiently recovered the majority of extractable constituents. Both extracts were obtained as **dark brown solids**, which is consistent with the typical appearance of ethanol-extracted plant metabolites, including flavonoids, alkaloids, saponins, and terpenoids. The slightly higher yield observed in Ashwagandha may be attributed to its **higher content of ethanol-soluble alkaloids and flavonoids**, whereas Shilajit's complex resinous and mineral-rich composition could limit ethanol solubility. These findings underscore the suitability of **Soxhlet-assisted ethanol extraction** for efficient recovery of bioactive phytochemicals, which is essential for subsequent biological evaluations [1]

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XII Issue XI November 2025

Phytochemical Screening

Preliminary qualitative analysis revealed the presence of **alkaloids, flavonoids, saponins, and terpenoids/steroids** in both Ashwagandha and Shilajit extracts, whereas phenolics, tannins, and glycosides were not detected. The presence of alkaloids and flavonoids aligns with prior studies reporting their roles in **antioxidant, anti-inflammatory, and adaptogenic activities**[3]

Alkaloids can neutralize free radicals and reduce oxidative stress via electron donation.

Flavonoids are potent antioxidants capable of donating hydrogen atoms, chelating metal ions, and stabilizing reactive oxygen species (ROS). [3]

Saponins may enhance antioxidant activity indirectly by stabilizing cell membranes and improving the bioavailability of other compounds.

Terpenoids/steroids act as secondary metabolites that mitigate oxidative damage through radical scavenging and inhibition of lipid peroxidation.

The absence of detectable phenolics and tannins may reflect either low concentration in the extracts or **inefficient extraction by ethanol**, suggesting that sequential or alternative solvent extraction could improve recovery of these compounds for enhanced bioactivity studies. [3]

Quantitative Phytochemical Analysis

Total Phenolic Content (TPC)

The ethanolic extract of Ashwagandha contained **0.306 mg GAE/g**, slightly higher than Shilajit (**0.268 mg GAE/g**). Phenolic compounds are well known for their antioxidant effects, primarily through hydrogen donation and stabilization of ROS. The higher TPC in Ashwagandha is consistent with its **moderate radical scavenging activity** observed in both DPPH and DMPD assays. [4]

Total Flavonoid Content (TFC)

Flavonoid content followed a similar trend, with Ashwagandha (**0.260 mg QE/g**) exceeding Shilajit (**0.225 mg QE/g**). Flavonoids are particularly effective in neutralizing free radicals, chelating metal ions, and inhibiting lipid peroxidation. The TFC results correlate well with antioxidant assay outcomes, indicating that flavonoids are **major contributors to the observed activity**. [4]

Antioxidant Activity

DPPH Radical Scavenging Assay

Ashwagandha extract exhibited **moderate activity** (IC_{50} = 632.06 µg/ml), whereas Shilajit showed **weak activity** (IC_{50} = 1027.88 µg/ml). The trend observed corresponds well with TPC and TFC levels, highlighting **phenolic and flavonoid content as key determinants of DPPH radical scavenging**. The strong activity of ascorbic acid (IC_{50} = 152.27 µg/ml) validated the assay. [4]

DMPD Radical Cation Scavenging Assay

Similar results were observed: Ashwagandha (IC_{50} = 855.93 µg/ml) outperformed Shilajit (IC_{50} ≈ 1322 µg/ml), while ascorbic acid exhibited strong activity (IC_{50} = 100.62 µg/ml). The DMPD assay, which measures the ability to quench $DMPD^{\bullet+}$ cations, further confirmed that **Ashwagandha extract is more effective in radical neutralization** than Shilajit. [4]

Both assays demonstrated **dose-dependent activity**, consistently ranking antioxidant potential as **Ascorbic Acid > Ashwagandha > Shilajit**. The weak activity of Shilajit may be due to limited ethanol-soluble bioactive components or inherently lower radical scavenging potential in the ethanol-extracted fraction. [4]

Correlation Between Phytochemical Content and Antioxidant Activity

The results indicate a clear positive correlation between **TPC/TFC and radical scavenging activity**:

Ashwagandha: Higher TPC/TFC → Moderate antioxidant activity

Shilajit: Lower TPC/TFC → Weak antioxidant activity

This highlights phenolics and flavonoids as **primary contributors** to antioxidant potential. Additionally, alkaloids, saponins, and terpenoids may exert **synergistic effects**, further enhancing overall radical scavenging capacity [Ref]. The difference in absolute IC_{50} values between DPPH and DMPD assays reflects variations in radical types and solvent interactions, but both methods consistently rank the extracts' antioxidant potential.

Implications and Future Perspectives

The **moderate antioxidant activity** of Ashwagandha supports its **traditional use as an adaptogen and therapeutic agent** for oxidative stress-related disorders, including neurodegenerative diseases, inflammation, and metabolic conditions.

The weaker activity of Shilajit in ethanol extracts does not diminish its biological relevance; alternative extraction methods (e.g., aqueous or hydroalcoholic solvents) may enhance recovery of bioactive compounds.

These findings provide a **scientific rationale for the development of standardized Ashwagandha-based nutraceuticals** and encourage further mechanistic studies to explore its antioxidant and therapeutic potential.

Conclusion

The Soxhlet-assisted ethanolic extraction of Ashwagandha (Withania somnifera) and Shilajit successfully yielded substantial amounts of crude extracts (44.48% and 41.98%, respectively), demonstrating ethanol's efficiency in recovering major bioactive constituents. Both extracts contained key phytochemicals such as alkaloids, flavonoids, saponins, and terpenoids/steroids, which are known contributors to antioxidant and therapeutic activities. Quantitative analysis revealed that Ashwagandha exhibited slightly higher total phenolic (0.306 mg GAE/g) and flavonoid (0.260 mg QE/g) contents than Shilajit (0.268 mg GAE/g and 0.225 mg QE/g, respectively). This corresponded with its superior radical scavenging activity in both DPPH (IC_{50} = 632.06 µg/ml) and DMPD (IC_{50} = 855.93 µg/ml) assays, while Shilajit showed weaker activity (IC_{50} = 1027.88 µg/ml and ≈1322 µg/ml, respectively). These findings indicate a positive correlation between phenolic/flavonoid content and antioxidant potential. Overall, Ashwagandha demonstrates moderate antioxidant activity, validating its traditional use as an adaptogen and therapeutic agent against oxidative stress-related conditions. Shilajit, although showing weak activity in ethanol extracts, may yield greater bioactivity with alternative extraction methods. These results provide a scientific basis for the development of standardized Ashwagandha-based nutraceuticals and support further investigations into the mechanisms underlying their antioxidant and therapeutic effects.

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