



Withania somnifera Chemical Constituents' In Vitro Antioxidant Potential and Their Response on Spermatozoa Parameters

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Abstract

Background: Phytomedicine is becoming more acceptable as an alternative medicinal approach in the modern era.

Objectives: The current study examined the antioxidant capacity and in vitro response of phytochemical constituents of *Withania somnifera* (Ashwagandha) on standard parameters of healthy volunteer semen.

Methods: The phytochemicals and their pharmacological response in a hydroethanolic (30:70 v/v) extract of *W. somnifera* roots were determined using standard protocols.

Results: The constituents included flavonoids, phenolic acids, alkaloids, and terpenoids were reported. High-performance liquid chromatography and Fourier-transform infrared spectroscopy determined a diverse array of biologically active chemical constituents in the extract. The extract of *W. somnifera* exhibits substantial antioxidant properties, including total antioxidant capacity, 2,2-diphenyl-1-picrylhydrazyl inhibition, H₂O₂ scavenging, and Fe³⁺ reducing potential ($P < .05$). The analysis of essential natural minerals explored adequate levels determined using atomic absorption spectrophotometer. Cytotoxic studies revealed significant thrombolytic, RBC membrane stabilization, and DNA damage protection activity ($P < .05$) while remaining non-mutagenic against *Salmonella typhi* TA98 and TA100. The best protective response of *W. somnifera* extract on human semen parameters ($n = 30$), such as total motility, progressive motility, and viability, demonstrated a significant ($P < .05$) improvement, particularly at the dose of 25 µg/mL and 50 µg/mL.

Conclusion: The study concludes that *W. somnifera* possesses favorable in vitro characteristics that could aid in the preservation of sperm during intrauterine insemination and in vitro fertilization.

Keywords

antioxidants, ashwagandha, Indian ginseng, intrauterine insemination, sperm preservation, in vitro fertilization, phytomedicine

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Introduction

It is widely accepted that more than 80% of the habitats of developing countries cannot afford allopathic medicines. However, those are dependent on traditional herbal drugs to treat various disorders, as reported by World Health Organization (WHO) (2003).¹ Potential herbal sources are also used to generate revenue worldwide, so the demand for medicinal plants has been increased day by day due to their therapeutic value for the ailment of many veterinary and medical complications.² The side effects of herbal drugs are very few when compared with allopathic medicines. Most Asian countries are blessed with a wide range of medicinal plants where the medicinal plants are still a long-standing being practiced to date. About 2000 species of medicinal plants in Pakistan have been identified and about 400 plants are practiced extensively as folk medicines having various therapeutic active ingredients.^{3,4}

Withania somnifera in the family of Solanaceae is usually called ashwagandha, Indian ginseng, poisonous gooseberry, Kanjaje in Hindi, or winter cherry. Several different species are morphologically similar in the variety *Withania*. The herb in Ayurvedic medicine is regularly used as a medicinal plant. It is accepted to treat various diseases affecting human health.⁵ The *W. somnifera* is commonly located in hot and dry climates (including Pakistan, India, and Iran).⁶ Leaves, roots, seeds, fruits, and flowers are used to treat impotence and enhance sexual desire with other conventional plants, either alone or in the polyherbal formulation.^{7,8} Several extracted phytochemicals with varying pharmacological and biological properties have been identified.⁹

This plant contains over 80 typical phytochemicals, including steroidal, alkaloids, saponins, glycosides, and volatile oil. Among these chemical ingredients, sitoindosides and withaferin A had the leading role in WS therapeutic effects.¹⁰⁻¹⁴ Ashwagandha has the potential to treat joint inflammation, nervous system disorders, diabetes, hyperlipidemia, attention deficit hyperactivity disorder, hiccup, obsessive-compulsive disorder, bronchitis, back pain, sleep deprivation, tumors, Parkinson's disease, tuberculosis, menstrual irregularities, and chronic liver disease.¹⁵ Ashwagandha roots possess antioxidant properties and treat infertility, the immune system and cardiovascular disease. Dried roots of the plant are generally utilized to treat nervous and sexual issues, and because of anti-inflammatory properties, ashwagandha improves sperm quality.¹⁶ The present study aimed to explore the in vitro antioxidant potential of phytochemical constituents of *W. somnifera* plant roots and its impact on spermatozoa parameters collected from healthy volunteers.

Material and Methods

Selection and Collection of Plant Material

The roots of *W. somnifera* were obtained from the Local market of Faisalabad-Pakistan and were identified and authenticated taxonomically from the Department of Botany, Government College University, Faisalabad-Pakistan (Ref#Bot-2019-7787). Roots were washed thoroughly using distilled

water, dried under shade and then grinded into a fine powder with the help of an electric grinder machine (Model CB 222, Cambridge, UK). Aqueous ethanol (30:70 v/v) extract was prepared following the protocol described by Sulaiman et al.,¹⁷ with some modifications. The extract was weighed and the final yield as a percentage yield of extract was calculated as

$$\text{Percent yield (\%)} = \frac{\text{Dried extract weight}}{\text{Dried plant material weight}} \times 100$$

Phytochemical Analysis

Analysis for identification of the major phytoconstituents was carried out as follow:

Qualitative Analysis

Various phytochemicals, including alkaloids, flavonoids, tannins, saponins, glycosides, steroids, and triterpenoids, were detected in the hydroethanolic extract by standard methods.¹⁸⁻²⁰

Quantitative Analysis

Total Phenolic Contents Estimation

The hydroethanolic extract was processed to estimate the total phenolic contents (TPC) using the Folin-Ciocalteu method described by Jain et al.¹⁸ Gallic acid was used to construct the standard curve, and a reading at 765 nm wavelength was taken to measure the intensity of the color complex. Total phenolic contents present in extracts was measured as mg equivalent to gallic acid (GAE) per mL of extract using the following formula

$$\text{Total phenolic contents} = C \times \frac{V}{M}$$

where C = concentration of gallic acid (mg/mL) taken from standard; V = volume of extract used in mL; M = weight of plant extract in grams.

Total Flavonoids Contents

The quantification of total flavonoids contents (TFC) was done following the protocol described by Pranuthi et al.,¹⁹ and optical density was measured using 510 nm wavelength. The TFC present in the hydroethanolic extract was calculated using a linear regression curve of Catechin (CE) as $\mu\text{g CE/g}$ of dried plants material.

High-Performance Liquid Chromatography for Phenolic Compounds

High-performance liquid chromatography (HPLC) for the determination of different phenolic compounds present in hydroethanolic plant extract was used following the method as described by Yue et al.,²¹ with minor modifications. Liquid chromatography

having C₁₈ column (250 × 4.6 mm internal diameter) of 5 μm film thickness and with an oven set at 30°C was used. Chromera HPLC system (Perkin Elmer, USA.) attached with Flexer Binary LC pump, UV/Vis LC Detector (Shelton CT, 06 484 USA) controlled by software V. 4.2. 6410 was used to analyze the data. Solvent A (70:30), composed of acetonitrile: methanol, respectively, and solvent B consisted of double distilled water with .5% glacial acetic acid, were used as mobile phase. Wavelength 275 nm was used to identify different phytochemical compounds using standards to compare the retention times and spiking.

Fourier-Transform Infrared Spectroscopy

Fourier-Transform Infrared Spectrophotometer (FTIR) was used to identify different chemical bonds or functional groups in the phytochemicals. Specific wavelength light absorbed by specific chemical bonds is the salient feature of the chemical bonds representing different functional groups. FTIR spectrometer (Model Bruker Platinum ATR with accessories A225/Q Platinum ATR Multiple Crystals CRY diamond and having interferogram size of 10 550 points) in the frequency range of 400–4000/cm available in the Central Hi-Tech Laboratory, Government College University, Faisalabad-Pakistan, was used as described in our previous study.²²

Trace Elements and Heavy Metals Estimation

An atomic absorption spectrophotometer determined the concentration of important trace elements and heavy metals in medicinal plants. The procedure recommended by the AOAC (2000)²³ consisted of two phases and nitric-perchloric acid was used to digest the sample. After digestion, Colagar et al.²⁴ protocol was used to determine trace and heavy metals (ppm) including Cu, Fe, Cd, Pb, Mg, Co, Zn, Ni, and Mn by an atomic absorption spectrometer (Aurora, Canada).

Determination of Antioxidant Potential of Selected Herb Using Different Assays

Total Antioxidant Capacity (Phosphomolybdenum Method)

Phosphomolybdenum assay was used to measure the TAC of selected plant hydroethanolic extract spectrophotometrically following the protocol described by Prieto et al.²⁵ The obtained results of TAC were expressed as ascorbic acid (used as standard) equivalents mg/g of the dry plant. Butylated hydroxytoluene (BHT) was used as reference controls.

2, 2-diphenyl-1-picrylhydrazyl Radical Scavenging Activity

Begum et al.²⁶ method was used to evaluate the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay to

determine the antioxidant capacity of plant extract against Vitamin C and BHT as standard. The following formula was used to calculate the percent DPPH inhibition after triplicate measurements of each selected medicinal plant.

$$\text{DPPH inhibition (\%)} = \frac{\text{Blank abs (A0)} - \text{Sample abs (A1)}}{\text{Blank abs (A0)}} \times 100$$

Hydrogen Peroxide (H₂O₂) Scavenging Potential

Ruch et al.²⁷ method to measure the H₂O₂ scavenging potential of *W. somnifera* hydroethanolic extract was used taking absorbance at 230 nm. Phosphate buffer saline (PBS) as blank and Vitamin C as standard were used to determine the percent hydrogen peroxide scavenging as

$$\% \text{ Scavenged [H}_2\text{O}_2] = \frac{[1 - \text{AS}]}{\text{AS}} \times 100$$

where AS= absorbance in the presence of the extract sample or standard.

Reducing Power Assay (FRAP Method)

To evaluate the antioxidant potential of the selected medicinal plant, the ferric reducing power assay (FRAP) method was used in which the reducing potential of substance Fe³⁺ (CN)₆ into Fe²⁺ (CN)₆ by direct electron donation is measured. Yadav et al.²⁸ protocol was used to determine the reducing potential of plant extract. Different concentrations of selected plant hydroethanolic extract as 25, 50, 100, 150, 200, 250, 300, 350, and 400 (μg/mL) were used to determine the dose-response effect. Wavelength 700 nm was used to take the absorbance of the mixture and the reducing potential of the plant extract is directly proportional to the absorbance of the reaction mixture.

In Vitro Evaluation of Toxicity of *W. somnifera* Extract

Hemolytic Assay to Determine Cytotoxic Potential of Plant Extract

To evaluate the safety of hydroethanolic plant extract, a hemolytic assay was used in accordance with Powell et al.²⁹ protocol with some modifications. Prior to the assay, RBCs were suspended in PBS at a concentration of 7.0 × 10⁸ RBCs/mL (using a hemocytometer). Absorbance against Triton X-100 (.1%) treated RBCs as positive control and PBS as a negative control was taken using Microwell Plate Reader BioTek, μ Quant (BioTek, Winooski, VT, USA) set at 576 nm wavelength and calculated as

$$\text{Percent hemolysis (\%)} = \frac{[Ae - Ap]}{Ad - Ap} \times 100$$

where Ae= the absorbance of plant extract; Ap= the absorbance of PBS; Ad=, the absorbance of DMSO (20%), used to make plant dilution.

Clot Lysis Assay for Thrombolytic Potential of Plant Extract

To evaluate the clot-dissolving potential of selected medicinal plant in vitro clot lysis assay was used following Rahman et al.³⁰ Fresh 1 mL venous blood from healthy volunteers (n = 10) (excluded those taking any type of anticoagulant) was taken into pre-weighed micro-centrifuge tubes as instructed by Institutional Research Scrutiny Committee (Ref. No. GCUF/DAS/19/1534). Streptokinase vial (1 500 000 IU) available in the market was used as positive control while PBS as a negative control for comparison and clot-dissolving potential was calculated as

$$\text{Percent hemolysis (\%)} = \frac{(\text{Clot initial weight} - \text{Clot final weight})}{\text{Clot initial weight}} \times 100$$

Mutagenicity/Genotoxicity Evaluation (AMES Test)

Because of its simplicity and relatively low cost, the Bacterial Reverse Mutation Test (Ames test) is recommended by several regulatory agencies (Organization for Economic Co-operation and Development, Food and Drug Administration, International Conference on Harmonization) to preclude genotoxic activity. It is used as a screening method in drug development. Bruce Ames developed the AMES test in the 1970s.³¹ Auxotrophic bacterial strains are commonly used to investigate the capability of a substance for genotoxicity through reverse mutations. *Salmonella typhimurium* two strains TA98 and TA100 were used using the fluctuation method to evaluate the genotoxicity of selected herb extract on incubating with the bacterial cell for up to 5 days in 96 well microplates. Revertant characteristic of genotoxic substance was expressed as change in color from blue (due to purple bromocresol) to yellow, representing the growth and metabolic activities of bacterial reverted strain. The results were represented as the number of revertants compared with controls. A probability test was performed to evaluate the results statistically.

DNA Damage Prevention Test

The DNA damage protection assay was used to determine the genoprotective capacity of the examined plant's aqueous ethanolic extract, following the protocol of Tian and Hua,³² with some modifications. Calf thymus DNA (Ct DNA) (.5 µg/ 3 µl) was treated with 5 µl of Fenton reagent [30% (v/v) hydrogen peroxide (4 µl), with 3 µ ferrous sulfates (2 mM)]

and with 5 µl of studied medicinal plant extract (100 µg/ml), respectively. Then, the DNA damage protection capacity of selected plant extract was evaluated by running the DNA on agarose gel electrophoresis and control DNA. Syngene GeneGenius Gel Light Imaging System was used for gel documentation.

In Vitro Spermatozoa Parameters

For in vitro spermatozoa parameters determination, healthy volunteers (time to pregnancy of 12 months) were selected (n = 30) for the collection of semen samples after taking written informed consent and semen samples were processed according to WHO protocol. Normal samples were selected for current analysis according to the WHO reference range.³³ Different doses of extract as 25 µg/mL, 50 µg/ML, and 100 µg/mL were used to determine the impact of selected medicinal Crude aqueous ethanolic extract. Physiological saline was used to dissolve the extracts in labeled Eppendorf's and kept overnight at room temperature. Kuma et al.³⁴ protocol with some modification was used to determine the in vitro effect of medicinal plant on semen parameters, including motility total (%), progressive motility (%) and viability (%). After collecting the semen and evaluating for standard parameters, equal volume (1: 1) of semen was mixed with plant extracts dissolved in .9% physiological saline. Then mix and incubate for 30 seconds at room temperature, then sperm motility total (%), as well as progressive motility (%), were examined under the light microscope (40X) at specific intervals like 0 min, 15 min, 30 min, 45 min, 60 min, and 120 min. The impact of studied medicinal plant on viability (%) of spermatozoa was also investigated using .1% Eosin Y stain in .9% physiological saline by incubating the semen, extract and stain (1:1:1) for up to 120 min following the protocol of Prakash et al. and Cheesbrough.^{35,36}

Statistical Analysis

The obtained results were reported as Mean ± SEM and were interpreted statistically using the one-way ANOVA test. Minitab 17 statistical software was used for the Probability test.³⁷

Table 1. Qualitative phytochemical constituents present in hydroethanolic extract of the studied medicinal plant.

Plants/phytochemicals	<i>Withania somnifera</i>
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	–
Glycosides	+
Steroids	+
Triterpenoids	+

“+” indicates the detection of phytoconstituent; “–” indicates non detection of phytoconstituent present.

Results

Alternative medicines for treating general health problems are becoming more popular and receiving more attention in drug development. *W. somnifera* roots gave extract yield as 22.19 ± 1.03 g/100 g dry weight of plant material on preparing the extract using 70% ethanol in water.

Qualitative and Quantitative Phytochemical Constituents Analysis

The results of different phytochemical constituents in the hydroethanolic extract given in Table 1 revealed the presence of flavonoids, tannins, alkaloids, saponins, steroids, glycosides and triterpenoids in the selected plant extract. The results of total flavonoid and phenolic contents determined during current research work are given in Table 2. Significant ($P < .05$) concentration of TFC and TPC was found in the hydroethanolic extracts of *W. somnifera* as (148.79 ± 6.39 μ g CE/g) and (372.81 ± 7.71 mg GAE/g), respectively.

High-Performance Liquid Chromatography for Phenolic Compounds. The results of HPLC-UV showed that various phenolic compounds including gallic acid (Rt = 2.786 min), catechin (Rt = 3.316 min), P-coumeric acid (Rt = 5.498 min), HB acid (Rt = 6.989 min), caffeic acid (Rt = 7.667), vanilic acid (Rt = 8.00 min), sinapic acid (Rt = 12.837) and ferulic acid (Rt = 13.297) are present in the roots extract of *W. somnifera* (Table 3 and Figure 1). All the phytochemical constituents identification was carried out by comparing the retention times (Rt) with the chromatographic peaks of reference standards. The chromatograph also represented some peaks with varying retention times, and these peaks were not identifiable. However, some unknown flavonoids might be present in the extracts, based on their chromatographic behaviors and UV spectra.

Fourier-Transform Infrared Spectroscopy. According to infrared spectroscopy in figure 2 and Table 4, the absorption peaks at range 4000 to 2500/cm was associated with the stretching vibration of single bond formed by hydrogen and other elements like O-H, N-H and C-H; peaks at 2500 to 2000/cm explored the absorption of light due to triple bond, for example, C \equiv C, C \equiv N; peaks at 2000–1500/cm represent the presence of a compound with double bonds including C=C, C=O while absorption peaks at 1500 to 400/cm consisted of many complicated bands and this part of the spectrum is unique to each compound also called fingerprint region. It is rarely used to identify particular functional groups like C-C, C-N, C-O, C-Cl, C-I, S-S, and N=O*.

Trace Elements and Heavy Metals Estimation. In the current research work, mineral contents, both essential and toxic elements including Cu, Fe, Cd, Pb, Mg, Co, Zn, Ni, and Mn were also measured. The results revealed that the selected medicinal plant contained a significant ($P < .05$) concentration (ppm) of

essential minerals. The concentration of Cu in *W. somnifera* roots was 63.743 ± 4.33 , Iron (Fe) 768.051 ± 11.53 , Zinc (Zn) 39.284 ± 3.36 , Magnesium (Mg) level $59.207 \pm .88$, Cobalt (Co) 313.461 ± 4.10 , Nickle (Ni) 70.698 ± 3.04 , Cadmium (Cd) $4.507 \pm .35$, and Lead (Pb) 8.565 ± 1.03 are given in Table 5.

Antioxidant Activities of Selected Medicinal Plant Extract

The DPPH and H₂O₂ are given in Table 2, while figure 3A represents the phosphomolybdenum scavenge assay results. The potential of hydroethanolic extract to scavenge DPPH (%) and H₂O₂ (%) explored that this herb has significant ($P < .05$) potential to neutralize the free radicals. The phosphomolybdenum assay results revealed that increasing extract dose concentrations directly correlated with TAC calculated using the standard curve of Vitamin C and represented as ascorbic acid Eq mg/g in Figure 3A. Vitamin C was used as positive control that showed $90.15 \pm 5.93\%$ and $48.70 \pm 2.91\%$ percent DPPH radical and H₂O₂ scavenging activities, respectively (Table 2). The results of the ferric reducing antioxidant power (FRAP) assay was represented in Figure 3B, which revealed that the selected medicinal plant has significant ($P < .05$) potential to reduce the ferric ion to ferrous ion. Results also explored that the reducing capacity also increased with increasing the concentrations of extracts (Figure 3B).

In Vitro Evaluation of Toxicity of *W. somnifera* Extract

Cytotoxic Activity and Thrombolytic Activities. In vitro hemolytic analysis revealed that *W. somnifera* hydroethanolic extract has non-significant hemolysis ($P > .05$) as compared to a positive control (Triton X-100), which showed $90.37 \pm 6.955\%$ hemolysis. On the other hand, a negative control phosphate buffer saline causes $4.16 \pm .575\%$ RBCs hemolysis (Figure 3C). When comparing medicinal plant extracts to triton X-100, statistical analysis demonstrated that medicinal plant extract cause significantly ($P < .001$) less hemolysis of RBCs (Figure 3C). The results of clot lysis assay revealed that hydroethanolic extract of *W. somnifera* has significant ($P < .001$) clot lysis activity ($20.72 \pm 2.55\%$) on compared to a positive control ($73.6 \pm 7.70\%$) and negative control ($4.00 \pm .35\%$) on incubating for 90 min at 37°C. The results of thrombolytic activities as mean \pm SEM are shown in Figure 3D.

Mutagenicity/Genotoxicity and DNA Damage Prevention Tests. The results of the mutagenicity analysis of studied medicinal plants against TA98 and TA100 are given in Table 6 and Figure 4. The results of the Ames test explored that selected medicinal plants did not possess mutagenic activity or, in other words, *W. somnifera* is non-mutagenic. According to the Fold rule, to declare that extract is mutagenic, the number of positive wells should be double the number of positive wells in the background plate.³⁸ The results revealed that *W. somnifera* plates

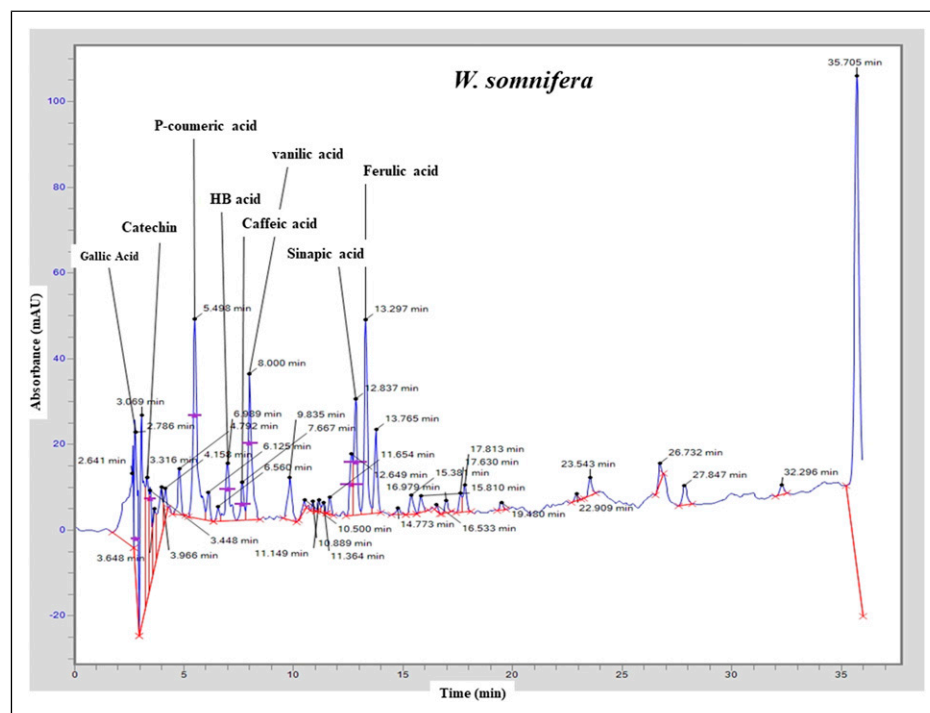
Table 2. Phytochemical constituents and antioxidant activities of *W. somnifera* hydroethanolic extract as mean \pm SEM of multiple determinations.

Plants\contents	<i>W. somnifera</i>	Vitamin C
TPC (mg GAE/g dry plants material)	372.81 \pm 7.71	–
TFC (μ g CE/g dry plants material)	148.79 \pm 6.39	–
H ₂ O ₂ scavenging activity (%)	21.65 \pm .71	48.70 \pm 2.91 ^A
DPPH inhibition (%)	49.04 \pm 1.03	90.15 \pm 5.93 ^A

“–” indicates not tested.

Table 3. Different phytochemical constituents identified using HPLC in the hydroethanolic extract of selected medicinal plant.

Selected Medicinal plants	Phytochemical constituents	Retention time (min)	Area under curve	Height
<i>W. somnifera</i>	Gallic acid	2.786	364982.0	33353.0
	Catechin	3.316	297540.5	28849.9
	P-coumeric acid	5.498	612715.3	46027.5
	HB acid	6.989	183444.9	13449.1
	Caffeic acid	7.667	85820.3	8763.4
	Vanilic acid	8.000	397856.8	33322.8
	Sinapic acid	12.837	282621.8	26896.2
	Ferulic acid	13.297	450336.2	44925.1

**Figure 1.** Chromatogram representing different phytochemical constituents identified using HPLC in the hydroethanolic extract of *W. somnifera*.

showed minimum reversion of both *S typhi* TA98 and TA100 compared with positive controls (Table 6 and Figure 4). Moreover, results revealed comparatively to controls noticeable DNA damaging prevention potential. Figure 5 represented the

electrophoresis pattern of calf thymus (Ct) DNA after being treated with Fenton reagent and extract of selected herb to investigate DNA damage prevention activity against oxidative DNA damage.

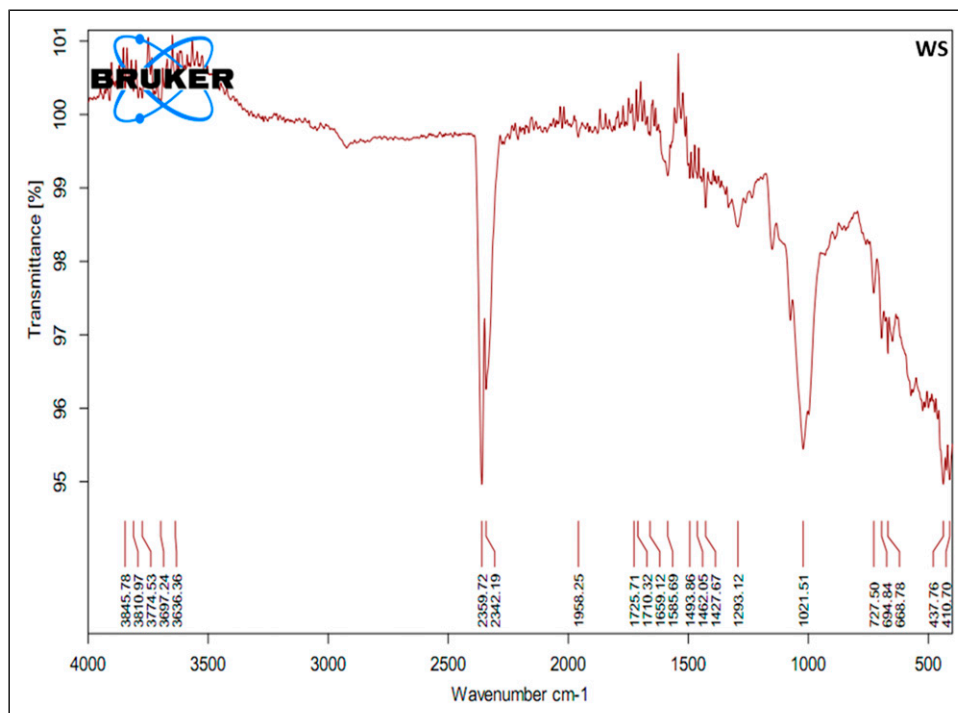


Figure 2. Fourier-transform infrared spectrophotometer (FTIR) graph representing different functional groups and possible phytochemical constituents identified using FTIR in the hydroethanolic extract of *W. somnifera*.

Table 4. Different functional groups and possible phytochemical constituents identified using FTIR in the hydroethanolic extract of *W. somnifera*.

Wavenumber (cm ⁻¹)/possible functional groups	<i>W. somnifera</i>
4000–2500/cm	3845
O-H, N-H, C-H	3810
Stretch vibration	3774
	3697
	3636
2500–2000/cm	2359
C=C, C=N	2342
Stretch vibration	
2000–1500/cm	1958
C=C, C=O, C=N	1725
Stretch vibration	1710
	1659
	1585
1500–400/cm	1493
C-C, C-N, C-O, C-Cl, C-I, S-S, N=O*	1462
Stretch, bend, or scissoring, rock vibration	1427
	1293
	1021
	727
	694
	668
	437
	410

In Vitro Spermatozoa Parameters

Results of different doses of plant extract on selective spermatozoa parameters revealed that there were significant ($P < .05$) differences among the control and plant extract (Figures 6B and C). Moreover, non-significant ($P > .05$) spermicidal activity was observed on incubating the semen with hydroethanolic extract of *W. somnifera* tested at different time intervals and normal saline as control (Figures 6A and D). Moreover, it was also reported that no significant ($P > .05$) reduction in motility of spermatozoa was observed in control-treated semen. It could be noted from figures 6B–D that incubation of the semen with the extracts of *W. somnifera* enhanced the motility and preserved the viability of spermatozoa for a longer time than control. Furthermore, comparing different doses of extract 50 µg/mL concentration has a more beneficial impact on motility and viability. At the same time, it was also found that a high dose of 100 µg/mL has toxic effects on selective spermatozoa (Figures 6B–D).

Discussion

It was well reported that the therapeutic applications like analgesic, antibacterial and antispasmodic were due to the alkaloids present or derived from medicinal plants³⁹ and the significant antioxidant potential is due to the presence of phenolic contents.⁴⁰ Most important groups like flavonoids as natural antioxidants are the critical natural phenolic agents. Findings revealed that reactive oxygen

species' detoxifying capacity is due to these natural flavonoids, which also can inhibit the low-density lipoproteins oxidations.⁴¹ The results of our study revealed the presence of various active constituents in *W. somnifera* extract. Gulati et al.⁴² reported the presence of alkaloids and tannins in the root extract of *W. somnifera*. Saxena et al.⁴³ also reported many other pharmacologically important constituents like alkaloids, steroids, starch, reducing sugars, amino acids, volatile oil, glycosides, withaniol, hentriacontane, and dulcitol in the roots.

It was well reported that oxidative stress generated due to the defective natural antioxidant defense system associated with

different diseased conditions.⁴⁴ So for the prevention of such conditions and to improve the natural antioxidant activities, synthetic chemicals or plants derived molecules like Vitamins A, C, E, carotenoids, and flavonoids with other supplementary foods are used.⁴⁵ Flavonoids and phenolic are important phytochemical constituents due to which medicinal plants have many therapeutic activities, including antioxidant potential. *W. somnifera* antioxidant potential is due to the presence of phytochemical compounds including ascorbic acid, phenolic compounds, alkaloids, anthocyanin, steroidal lactones, flavonoids, and tannin.⁴⁶

According to infrared spectroscopy in Figure 2, selected medicinal plant extract FTIR analysis revealed absorption peaks at 3500–4000/cm, which means hydrogen bonds in intra- and inter molecules. The absorption peaks at 3845, 3810, 3774, 3697, and 3636/cm were attributed to the asymmetrically stretching vibration of O-H, N-H, and C-H. The peak at 2359 and 2342/cm might be related to stretching vibration of C≡C and C≡N. The absorption peaks at 1958, 1725, 1710, and 1659/cm were attributed to the symmetrically stretching vibration of C=O. The absorption peaks at 1585/cm might be related to the stretching vibration of C=N and C=C. The peaks at 1400–1500/cm (1493, 1462, and 1427/cm) might be attributed to the strong to medium vibration of C=C and N=O functional groups. The absorption peak at 1293/cm was due to the stretching vibration of C-O. The peaks at 1021, 727, and 694/cm were attributed to the asymmetrically stretching vibration of C-O-C. The absorbance peak at 668/cm might be represented

Table 5. Mineral contents in the roots of *W. somnifera* as mean \pm SEM of multiple determinations.

Plants\ contents	<i>W. somnifera</i>
Copper (ppm)	63.743 \pm 4.33
Iron (ppm)	768.051 \pm 11.53
Zinc (ppm)	39.284 \pm 3.36
Magnesium (ppm)	59.207 \pm .88
Manganese (ppm)	80.384 \pm 1.85
Cobalt (ppm)	313.461 \pm 4.10
Nickle (ppm)	70.698 \pm 3.04
Cadmium (ppm)	4.507 \pm .35
Lead (ppm)	8.565 \pm 1.03

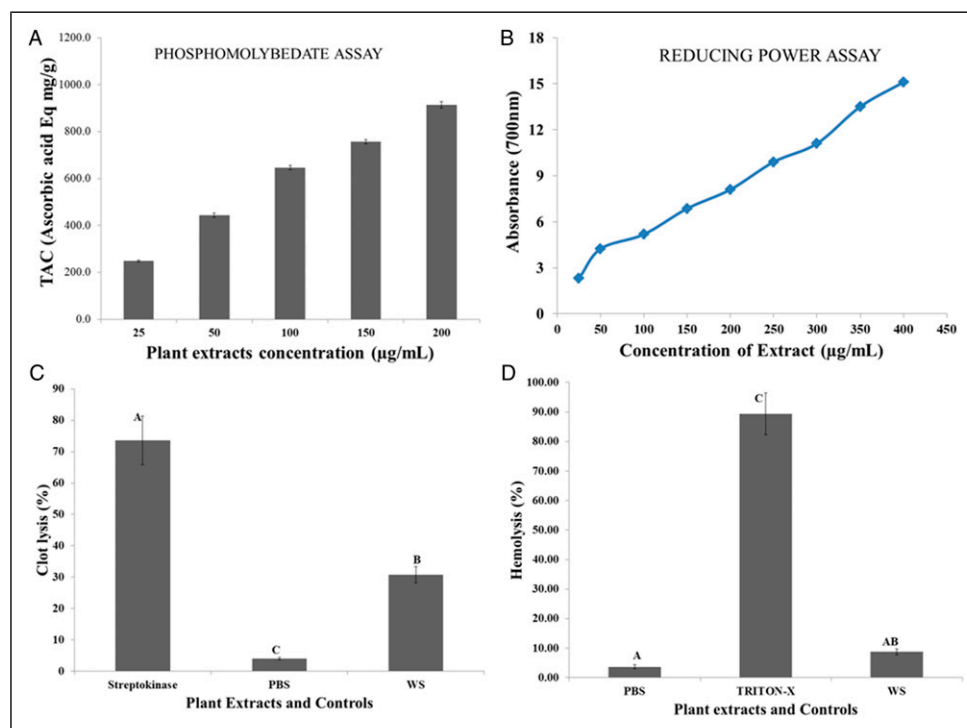


Figure 3. Graphical representation of (A) total antioxidant capacity (TAC) by phosphomolybdenum method. (B) Reducing potential investigated by FRAP (C). Thrombolytic activities (%) of selected medicinal plant. (D) Hemolytic activity (%) evaluated against washed RBCs. Results are mean \pm SEM of multiple determinations and controls. Different alphabets on the bars represent significance ($P < .05$) differences among tested plant extract and controls. WS= *Withania somnifera*.

aliphatic C-I stretching, while the peak at 437 and 410/cm might be related to S-S stretching vibration (Table 4 and Figures 2).²²

Bioelements are classified in different groups as Group I–Group V based on requirements and applications. Group, I included the elements that are the components to generate macromolecules like C, H, O, and N. Group II included the nutritionally vital minerals and principal elements. Group III elements are also known as Trace elements whose daily requirement is less than 100 mg. However, these are essential elements and serious diseases can develop in the deficiency of these elements. Group IV elements are called

additional trace elements whose pet role is unknown and possibly essential. Group V elements are toxic to the body and such elements enter the body through contaminated air, soil, water or food substances, including As, CN, and Hg.⁴⁷ The current research results explored an adequate amount of trace and essential elements in the roots of *W. somnifera*, which could be an important natural

Table 6. Mutagenic activity of selected medicinal plants against *S. typhi* TA98 and TA100.

Ethnolic extracts and bacterial strains	Number of positive wells/total number of wells	Results
Mutagenic activity against TA98		
(a) Background	20/96	-
(b) Standard ($K_2Cr_2O_7$)	80/96	+
(c) <i>W. somnifera</i>	8/96	-
Mutagenic activity against TA100		
(a) Background	25/96	-
(b) Standard (NaN_3)	86/96	+
(c) <i>W. somnifera</i>	5/96	-

+, significant increase in the number of positive wells compared to the related control ($P < .05$); -, non-significant ($P > .05$) effect observed.

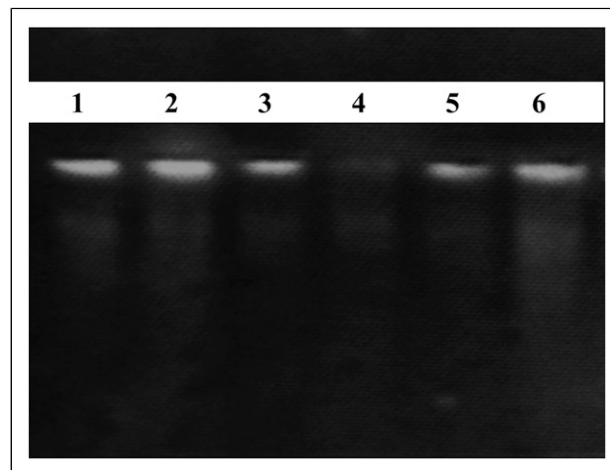


Figure 5. DNA damage prevention potential of *W. somnifera* roots extract using Ct DNA (calf thymus DNA). Lane 1 = untreated DNA, Lane 2= 2 mM $FeSO_4$, 30% H_2O_2 + DNA + 1 mM Quercetin, Lane 3 = 30% H_2O_2 + DNA, Lane 4 = 2 mM $FeSO_4$, 30% H_2O_2 + DNA, Lane 5 = 2 mM $FeSO_4$ + DNA, Lane 6 = 2 mM $FeSO_4$, 30% H_2O_2 + DNA + *W. somnifera* extract.

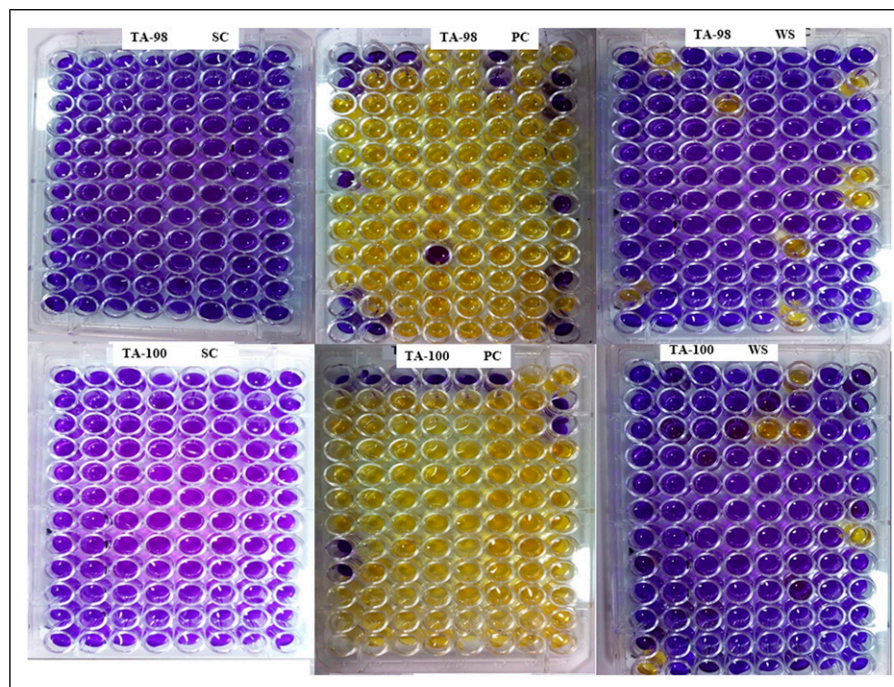


Figure 4. Mutagenicity evaluation of selected medicinal plant using *S. typhi* TA 98 and TA 100. SC= sterility control, PC= positive control, WS= *W. somnifera*; blue color indicates no bacterial convergent (no bacterial growth) and yellow color indicates bacterial convergent (bacterial growth).

supplement to manage diseases due to the deficiency of such vital ingredients.

Antioxidant Activities of Selected Medicinal Plant Extract

The phytochemical constituents acting as antioxidants can prevent the oxidation of substrates and help detoxify the free radicals like reactive oxygen species, which might be produced within the human body due to oxidation processes. When the human body cannot manage the oxidant agents due to a defective antioxidant system, oxidative stress is generated. Such conditions resulted in different metabolic and other healthcare disorders.⁴⁴ The findings are directly in the correlation of total phenolic and total flavonoid compounds in quantitative estimations. It was reported that the radical scavenging assay reactions primarily depend on hydroxylation and structure stability due to the donation of hydrogen by antioxidant compounds present in medicinal plants.⁴⁸ The results explored that the highest antioxidant potential of the extract might act as a natural antioxidant agent in the development of pharmaceutical preparations in foods and cosmetics as additives. Paul,⁴⁶ found that *W. somnifera* exhibited significant activities to scavenge the free radicals like DPPH,

ABTS⁺ radical, nitric oxide scavenging. Our research findings agree with Paul,⁴⁶ who determined that the ferric ions (Fe^{3+}) reducing the ability of WSI root extract was higher than the WSF root.^{46,49}

In Vitro Evaluation of Toxicity of *W. somnifera* Extract

Worldwide, herbal remedies have long been regarded harmless due to their natural origin, but new research demonstrates that this is not the case. So concern related to the safety of medicinal plants is becoming one of the emerging interests for researchers and the investigation of toxic aspects of herbs that might be associated with their usage, therefore, avoiding potential harmful effects.⁵⁰ The results of cytotoxic activity suggested that the selected medicinal plant has significant ($P < .05$) membrane-stabilizing (antihemolytic) potential. The safety of *W. somnifera* regarding toxicity, side effects and dosing is not yet well established, so the current study was the first time to explore the in vitro cytotoxic potential. As a result, it revealed that *W. somnifera* extract is non-toxic. It also described previously that *W. somnifera* has no herb to herb or drug interactions in practical doses.⁵¹⁻⁵³ Different thrombolytic drugs are used, but many of them were reported with a wide range of side effects like bleeding and embolism.⁵³ The

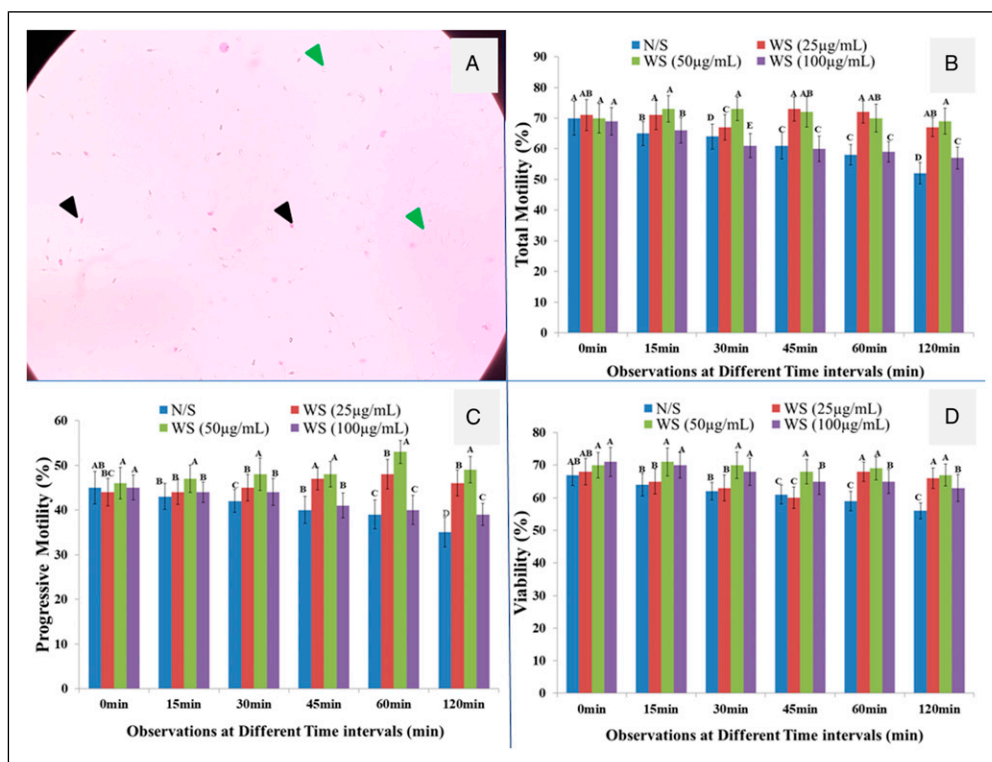


Figure 6. In vitro response of different concentrations of *W. somnifera* extract on Spermatozoa parameters. (A) Sperms stained with .5% eosin Y black color arrow heads represent the non-viable (pink color) sperms while green represent the viable sperms (un-stain). (B) Total motility. (C) Progressive motility. (D) Viability of spermatozoa treated with extract and normal saline as control at different time intervals. The values are mean \pm SEM of replicate determinations and bars share different alphabets are significantly ($P < .05$). N/S= normal saline (.89% sodium chloride); WS = *W. somnifera*.

clot-dissolving activities of the selected medicinal plant might be due to the presence of the secondary compounds. In phytochemical constituents analysis, a wide range of phytochemicals was identified.⁵⁴ Shahriar et al.⁵⁵ investigated the membrane-stabilizing and thrombolytic activities of the root extracts of *W. somnifera*. Sai Sandeep et al.⁵⁶ reported that *B. monnieri* Linn ethanolic extract leaf extracts have maximum (50.51%) thrombolytic activity.

According to the literature review, the selected medicinal plant was screened out for mutagenic activities for the first time. However, Khanam and Devi,⁵⁷ found that Ashwagandha root extract has a significant role in preventing DNA damage and ultimately replenishing the effect of oxidative stress imposed by a mutagen to prevent mutagenicity in mice.

In Vitro Spermatozoa Parameters

Due to the high antioxidant capacity and diversity of physiologically active constituents in selected herbs, their hydro-ethanolic extracts were evaluated in vitro for their effect on spermatozoa parameters. The beneficial properties of selected medicinal plants on selective spermatozoa parameters might be due to their potent antioxidant potential. Moreover, the presence of phytochemical constituents and an adequate amount of antioxidant minerals in *W. somnifera* has made its importance in treating infertility problems. According to the literature review, it was the first time investigating the in vitro improvement in the motility and viability of sperms using *W. somnifera* extract. However, many in vitro studies like *Acanthopanax senticosum* and *Astragalus membranaceus* extracts also showed improvement in the motility and viability of sperms collected from infertile males.⁵⁸ Our results also agree with the findings of Peng et al.⁵⁹ who used medicinal plants like *Rhizoma curculiginis* and *Radix Morindae officinalis*. The results revealed in vitro improvement in motility and the stabilization of sperm membranes. In vitro positive characteristics of natural herbs may help to restore the sperm functions prior to intrauterine insemination (IUI) and in vitro fertilization (IVF).

Conclusion

Medicinal plants with a wide range of therapeutic and nutraceutical properties are used mainly in developing countries to manage different disorders. The current study also revealed that *W. somnifera* has significant ($P < .05$) antioxidant activities due to a wide range of phytochemical constituents. Moreover, safety concerns in vitro study explored that hydroethanolic extract of the selected herb is non-toxic and non-mutagenic with membrane-stabilizing characteristics. By supplying varying amounts of the extract to spermatozoa, it has been observed that *W. somnifera* is a potential candidate for retaining spermatozoa parameters. However, the current findings revealed that *W. somnifera* has a beneficial in vitro impact. This natural herb might be helpful in storing/restoring the sperm functions for intrauterine insemination and in vitro fertilization. However, a

trial at a large scale and *In Vivo* study is required to declare the *W. somnifera* as an aphrodisiac medicinal plant.

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