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Phytochemical profiling, antioxidant, antibacterial potential and proximate composition of *Withania somnifera* root extracts using different solvents

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Abstract

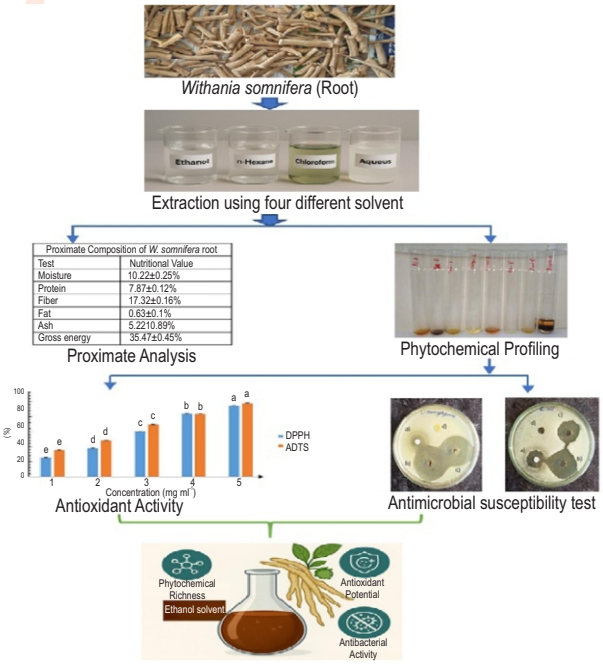
Aim: To evaluate the proximate composition, phytochemical profile, antioxidant potential, and antimicrobial activity of *Withania somnifera* root extracts using eco-friendly solvents.

Methodology: Roots of *W. somnifera* were collected, dried, and ground into fine powder. Extracts were prepared using different solvents (aqueous, n-hexane, chloroform and ethanol) and subjected to proximate analysis, qualitative phytochemical screening, antioxidant assays (DPPH and ABTS), and antimicrobial activity tests. Additionally, FTIR and HPLC analyses were performed to identify and quantify bioactive compounds.

Results: Proximate analysis showed: moisture content (10.22 ± 0.25%), protein 7.87%, fiber 17.32%, fat 0.63 %, ash 5.22 % and gross energy 3547 kcal kg^{-1} respectively. Phytochemical screening indicated that the ethanolic extract contained the highest concentration of bioactive compounds. Antioxidant assays showed strong dose-dependent radical scavenging activity, with the ABTS assay reported higher inhibition compared to DPPH. The ethanolic extract also exhibited significant antibacterial activity, with a maximum inhibition zone of 22 ± 0.6 mm against *Escherichia coli*. FTIR analysis confirmed the presence of functional groups characteristic of phenolic compounds, while HPLC analysis quantified withaferin A at 93.555 µg.

Interpretation: The ethanolic extract of *Withania somnifera* roots demonstrated superior phytochemical richness, potent antioxidant capacity and significant antibacterial activity. These findings highlight its potential application in the formulation of nutraceutical and pharmaceutical products.

Key words: Activity, Antibacterial, Bioactive compounds, Phytochemicals, *Withania somnifera*



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Introduction

Medicinal plants are a rich source of bioactive compounds that play crucial roles in healthcare, food preservation, and drug discovery. These compounds, including phenols, flavonoids, and alkaloids, exhibit diverse biological properties, such as antioxidant and antimicrobial activities, making them invaluable for utilization in the nutritional, pharmaceutical, and cosmetic sector (Granato *et al.,* 2017; Nikmaram *et al.,* 2018). Plant-derived antioxidants are extensively utilized to inhibit oxidation processes and extend the shelf life of food products, while antibacterial agents derived from natural sources offer promising alternatives to synthetic antibiotics, which are increasingly associated with the emergence of resistant bacterial strains. *Withania somnifera* (Ashwagandha), an important medicinal herb in Ayurveda and other traditional systems of medicine, has been used for over 3,000 years for its diverse therapeutic applications. It is often referred to as the "Queen of Ayurveda" or a "Rasayana herb" due to its adaptogenic, antioxidant, anti-inflammatory, and immuno-modulatory properties. The roots, seeds, and leaves of *W. somnifera* are widely employed in traditional remedies as aphrodisiacs, tonics, and stimulants. In addition to its indigenous presence in India, the plant is cultivated in Mediterranean regions, the Himalayas, Africa, and Australia (Mirjalili *et al.,* 2009; Verma *et al.,* 2011; Mir *et al.,* 2012; Langade *et al.,* 2019).

Withania somnifera (L.) Dunal belongs to the family Solanaceae, order Solanales, and its widespread applications and cultivation underscore its global significance. Phytochemically, *W. somnifera* is a treasure trove of bioactive compounds, including alkaloids (withanine, somniferine), steroidal lactones (withaferin A, withanolides), and phenolic compounds (John, 2014). These compounds contribute to its well-documented therapeutic properties, which include neuroprotection, anti-cancer activity, and antimicrobial effects (Che *et al.,* 2017). Despite these known benefits, there is limited systematic evaluation of how solvent polarity influences the extraction efficiency of these bioactive metabolites and their subsequent biological activities. To address these gaps, in this study proximate composition, phytochemical profile, antioxidant potential, and antimicrobial activity of *W. somnifera* root was analyzed by employing advanced characterization methods such as Fourier Transform Infrared (FTIR) spectroscopy and High-Performance Liquid Chromatography (HPLC). The study was conducted to identify and quantify key bioactive compounds, particularly withaferin A. Additionally, the antioxidant activity was evaluated through DPPH and ABTS assays, while antibacterial efficacy was assessed against *Escherichia coli* and *Listeria monocytogenes*.

Materials and Methods

Collection of test plant: *Withania somnifera* roots were procured from ICAR-CTRI, Vedasandur, Dindigul District, Tamil Nadu and later Identified and authenticated (Ref. No.: INSciR/Herbarium/0086) by a Botanist, at Institute of Natural Science Research, Devadanapatti, Theni District, Tamil Nadu, India. The roots were washed thoroughly with water, followed by rinsing with deionized water, air dried at ambient temperature, and pulverization into a fine powder using a mechanical grinder. The powder sample was preserved in tightly sealed jar at 4 °C for analyses.

Proximate composition: The proximate analysis of *W. somnifera* root powder was conducted to determine the moisture, fiber, protein, fat, ash, and gross energy content following the standard methods of AOAC (2005).

Extraction Methods: The powdered root sample (50 g) was extracted using a Soxhlet apparatus with four solvents: ethanol, aqueous, n-hexane, and chloroform at 80% concentration. The ratio of plant material to solvent was set at 1:10 (v/v). The extraction was carried out for 8–10 hrs at a controlled temperature of 60 °C. After completion, the extracts were cooled to room temperature, passed through Whatman No. 42 filter paper and condensed with a rotary evaporator (Rotavapor R-300, Buchi, Switzerland) at 65 °C for 5-6 hrs (Handa *et al.,* 2008). The obtained extracts were dehydrated and preserved at 4°C for further analyses. The extraction yield was calculated by the formula: Yield (%) = Weight of the concentrated extract (g) / Weight of the powder root (g) × 100.

Phytochemical analyses: Phytochemical analysis was conducted to identify the presence of major bioactive compounds in the extracts. The following qualitative phytochemical tests were performed as per the standard method of Harborn (1998): Tannins - Braymer's Test; Alkaloids - Hager's Test; Flavonoids - Shinoda Test; Steroids - Salkowski Test; Saponins - Foam Test; Phenols - Liebermann's Test; and Glycosides - Liebermann–Burchard Test. The presence (+) or absence (−) of each phytochemical constituent was recorded accordingly.

Antioxidant activity of plant using DPPH and ABTS: The antioxidant potential of the ethanolic extract of *Withania somnifera* was evaluated using DPPHand ABTS radical scavenging assays, as described by Xiao *et al*. (2020). In the DPPH assay, 0.1 mM DPPH solution prepared in methanol was mixed with varying concentrations of the extract (1–5 mg ml^{-1}) and incubated in the dark for 30 minutes. The decrease in absorbance was measured at 517 nm using a UV–visible spectrophotometer. For the ABTS assay, the $ABTS^{\cdot}$ radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate and incubating the mixture in the dark for 12–16 hours. The resulting working solution was diluted to obtain an absorbance of 0.70 ± 0.02 at 734 nm, followed by mixing with different concentrations of the extract (1–5 mg ml^{-1}). The absorbance was then recorded at 734 nm, and the percentage of radical scavenging activity was calculated accordingly.

Antimicrobial susceptibility assays: The ethanolic and aqueous extracts were evaluated for antibacterial efficacy against *Escherichia coli* and *Listeria monocytogenes* by the agar well

diffusion technique (OIE, 2012). The bacterial cultures were maintained in nutrient broth at 37 °C and maintained to a turbidity corresponding to 0.5 McFarland standard (about 10^8 CFU m l^{-1}). Nutrient agar plates were inoculated with bacterial suspensions by evenly spreading 100 µl of the inoculum. Wells (4 mm diameter) were punched into the agar and filled with 100 µl plant extract solutions (50 mg m l^{-1}). Spectinomycin (100 µg) served as a positive control, while neat solvents were used as negative controls. The plates were maintained at 37 °C for 18 hrs, and the inhibition zones were recorded with a digital caliper.

Characterization of plant extract by FTIR: Fourier-transform infrared (FTIR) spectroscopy was conducted to analyse the functional groups present in the ethanolic root extract of *W. somnifera.* The evaluation was conducted utilising the Thermo Nicolet iS5 spectrometer (Thermo Fisher Scientific, USA) that was outfitted via an attenuated total reflectance (ATR) accessory. The spectra were obtained within the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1}.

Screening of bioactive compounds by HPLC: High-performance liquid chromatography (HPLC) was used to quantify withaferin A in the ethanolic extract. Dionex Ultimate 3000 liquid chromatograph (Thermo Fisher Scientific, Germany) with a reverse-phase Acclaim™ 120 C18 column (4.6 x 250 mm, 5 µm particle size), quaternary pump, and diode array detector was used for the analysis. The mobile phase comprised 0.1% formic acid (solvent A) and methanol (solvent B) with an average flow rate of 0.8 ml min^{-1}. The injection volume was 20 µl, and detection was performed at 270 nm. Retention times were compared with a standard solution of withaferin A for quantification (Ross *et al.,* 2009).

Statistical analysis: The experiments were carried out in triplicate and the data were expressed as mean±S.D. One-way ANOVA and Tukey's post hoc test was applied to identify significant differences ($p < 0.05$) among groups. SPSS software, version 20.0 (IBM Corp., USA), was used for statistical analysis.

Results and Discussion

The proximate analysis of *W. somnifera* root revealed its nutritional composition, with 10.22% moisture content 7.87%. protein content, and 17.32% fiber content. The sample contained 0.63% fat, and 5.22% ash content. Additionally, the gross energy value was 3547 ± 0.45 kcal kg^{-1} (Table 1). These results are in close conformity with the reports of Hameed and Hussain (2015), who estimated 6.26% protein, 6.93% ash and 15.98% moisture contents. These values indicate that *W. somnifera* root is a promising source of dietary fiber and protein, with low fat and moderate mineral content, supporting its potential use as a functional food or dietary supplement (Wang *et al.,* 2021). Overall, these findings provide valuable insights into the nutritional characteristics of *Withania somnifera* root and serve as a foundation for further exploration of its applications in nutraceutical and therapeutic formulations. The phytochemical analysis of plant extracts showed the presence of saponins, tannins, phenols, glycosides, alkaloids, flavonoids and steroids in varying quantities in different solvents extracts. Ethanol was the most effective solvent, extracting the maximum number of bioactive compounds, followed by aqueous, chloroform and n-hexane extracts. Phenols were detected in all solvents, while terpenoids were exclusive to ethanolic extract and terpenoids present only in the ethanolic extract (Table 2). The above outcomes underline the significance of solvent polarity in the selective extraction of bioactive substances, as polar solvents like ethanol can dissolve a wider variety of plant secondary metabolites (Kokkaiah *et al.,* 2016). The findings of study closely align with the reports of Baskaran and Velu (2012), who recorded the presence major plant secondary metabolites— alkaloids, saponins, phenols, steroids, terpenoids and glycosides in the ethanolic extract of *Withania somnifera* root. Similar observations were reported by Sukumar *et al.* (2019), who highlighted the effectiveness of ethanol. The present study also emphasizes the remarkable bioactive potential of *W. somnifera* root extracts, particularly those obtained using ethanol as the solvent. The extraction yield of *W. somnifera* root showed variation across solvents, with ethanol extract producing the highest (18 ± 0.52%), followed by aqueous (15 ± 0.47%), chloroform (13 ± 0.21%), and n-hexane (10 ± 0.04%) extracts (Fig. 1). These results reflect the polarity differences among the solvents, supporting previous studies that highlight the effectiveness of polar solvents in extracting bioactive compounds from medicinal plants (Lapornik *et al.,* 2005; Do *et al.,* 2014; Chinnadurai *et al.,* 2019).

Table 1: Proximate composition of *W. somnifera* roots

Parameters	Nutritional value
Moisture	10.22 ± 0.25
Protein	7.87 ± 0.12
Fiber	17.32 ± 0.16
Fat	0.63 ± 0.1
Ash	5.22 ± 0.89
Gross energy	3547 ± 0.45 kcal kg^{-1}

All the values are expressed in % except for Gross energy. Values are mean of three replicates ±S.E.

Table 2: Phytochemical constituents identified in the extracts of *W. somnifera* using four different solvents

Compounds	N-hexane	Chloroform	Ethanol	Aqueous
Alkaloids	+	+	+	+
Saponin	-	-	+	+
Phenol	+	+	+	+
Tannin	-	+	+	-
Steroids	-	+	+	+
Terpenoids	-	-	+	-
Glycosides	-	+	+	-
Flavonoids	-	-	+	+

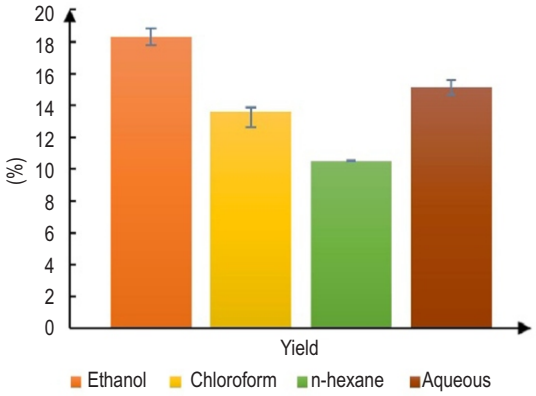


Fig. 1: Extraction yield of *W. somnifera* root obtained from different solvents. Values are mean of three replicates ±S.D.

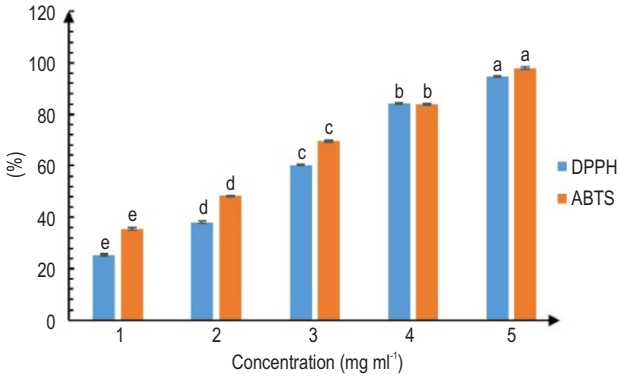
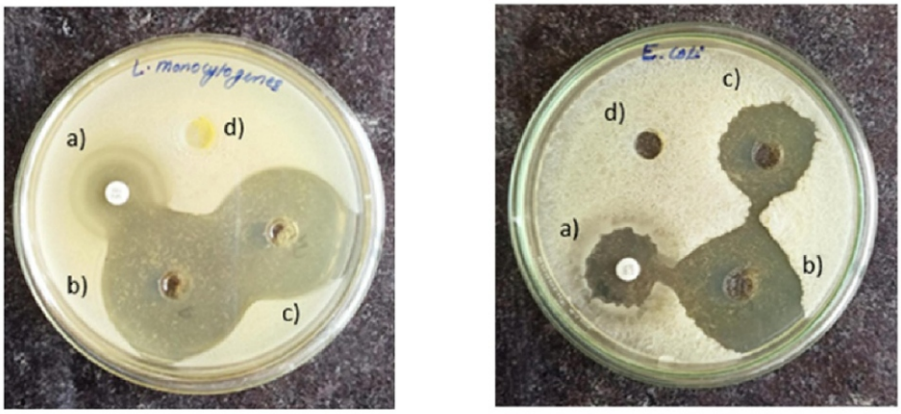


Fig. 2: Antioxidant activity (DPPH and ABTS assays) of ethanolic extract of *W. somnifera*. (Superscript represent significant difference among various concentrations).



(A) Zone of inhibition of *L. monocytogenes* (B) Zone of inhibition *E. coli*

Fig. 3: Zone of inhibition of *Listeria monocytogenes* (A) and *Escherichia coli* (B) treated with (a) Spectinomycin (positive control), (b) Ethanolic root extract, (c) Aqueous root extract and (d) Distilled water (negative control).

DPPH and ABTS assays confirmed the dose-dependent radical scavenging activity of ethanolic extract. At the highest concentration (5 mg m l^{-1}), the extract achieved 94.64% inhibition in the DPPH assay and 97.99% in the ABTS assay. Notably, ABTS activity remained consistently higher across all concentrations, highlighting the broader radical-quenching potential of the extract (Fig. 2). The strong antioxidant activity of the ethanolic extract is likely due to its high phenolic and flavonoid content (Vaishnav *et al*., 2025). Phenolic compounds function as electron donors, neutralizing free radicals, while flavonoids contribute by chelating metal ions and scavenging reactive oxygen species (Lal *et al.*, 2024). These findings corroborate with the reports of Nile *et al.* (2022) who recorded a higher level of antioxidant activity of ABTS and DPPH in the ethanolic extract of *W. somnifera*. Secondary metabolites like withanosides, withaferin, and phenolics present in the roots of *W. somnifera* determine the antioxidant activity. These findings align with the previous reports indicating that ABTS assay often demonstrate higher radical scavenging activity than DPPH assays, as ABTS can interact with both hydrophilic and lipophilic antioxidants (Munir *et al.*, 2022). These findings are consistent with Simur (2018) who reported higher antioxidant activities in ABTS assays, suggesting that ABTS provides a more comprehensive assessment of radical scavenging ability.

The ethanolic and aqueous roots extracts of *W. somnifera* showed significant antibacterial activity against *Escherichia coli* and *Listeria monocytogenes.* The ethanolic extract exhibited a larger zone of inhibition (22 ± 0.6 mm for *E. coli* and 18 ± 0.5 mm for *L. monocytogenes*) compared to the aqueous extract (14 ± 0.7 mm and 16 ± 0.4 mm). As a reference, spectinomycin, used as a positive control, produced inhibition zones of 12 ± 0.5 mm for *E. coli* and 20 ± 0.6 mm for *L. monocytogenes* (Fig. 3; Table 3). The potent antibacterial activity of ethanolic extract is attributed to high phenolic and flavonoid content. Phenolic compounds disrupt

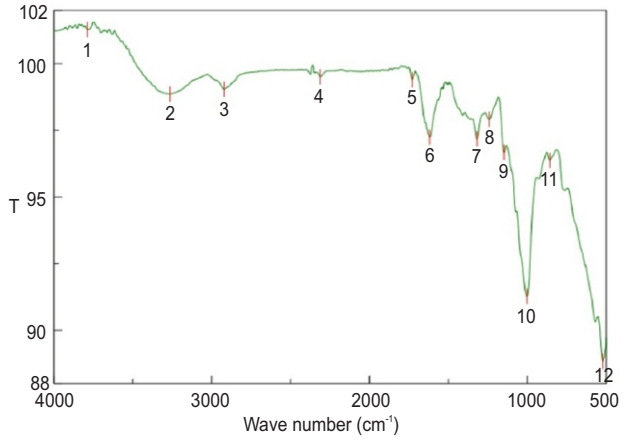


Fig. 4: FTIR spectrum of ethanolic extract of *W. somnifera* showing major functional groups.

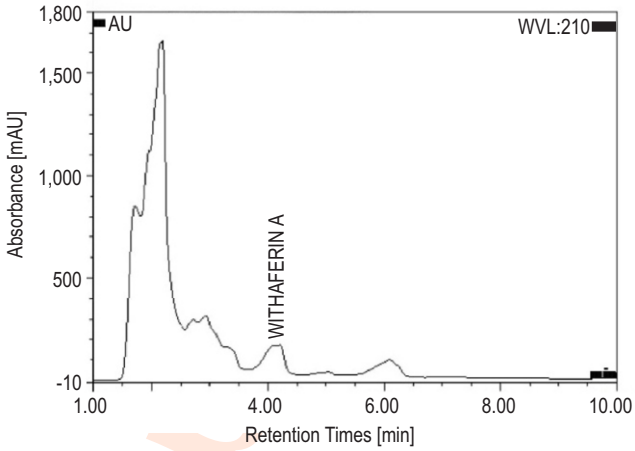


Fig. 5: HPLC chromatogram of ethanolic extract of *Withania somnifera*.

Table 3: Evaluation of antibacterial activity of W. somnifera extract

Extract	Zone of inhibition	
	E. coli	*L. monocytogenes*
Ethanolic root extract	22±0.6	18±0.5
Aqueous root extract	14±0.7	16±0.4
Spectinomycin	12±0.5	20±0.6

bacterial cell walls, increase membrane permeability, and inhibit essential bacterial enzymes, leading to cell death (Alam *et al.,* 2012). Similarly, flavonoids interfere with bacterial DNA replication and protein synthesis, further contributing to bacterial inhibition. These findings align with previous studies reporting strong antibacterial activity in methanolic stem extract of *W. somnifera* (Sinha, 2012; Dharajiya *et al.,* 2014). Furthermore, the ethanolic extracts often outperform aqueous extracts in antibacterial assays has been widely reported across different plant species, emphasizing the role of solvent polarity and phytochemical composition in determining antimicrobial efficacy (Ababutain, 2011).

FTIR spectral analysis of ethanolic extract of *W. somnifera,* as shown in Fig. 4, identified several key functional groups. The broad peak at 3262.97 cm^{-1} corresponded to O-H stretching, indicating the presence of alcohols. The peak at 2920.66 cm^{-1} represented CH_3 stretching, which is characteristic of ketones. The peak at 1241.93 cm^{-1} associated with C-H rocking, indicated vinyl group. The peak at 1001.84 cm^{-1} corresponded to C-O stretching, signifying the presence of acyclic secondary alcohols. Additionally, the peak at 857.20 cm^{-1} indicated O-O stretching, characteristic of peroxides, while the peak at 522.61 cm^{-1} represented the C-C skeleton of alkane. FTIR analysis further supports these findings by identifying functional groups such as O-H and C=O associated with antioxidant and antimicrobial properties. For example, O-H groups in phenols contribute to hydrogen bonding, enhancing interactions with microbial cell walls. Previous studies have identified similar peaks in *W. somnifera* extracts, confirming the presence of these functional groups (Aryal *et al.,* 2020; Trivedi *et al.,* 2021).

The retention time of approximately 4.0 minutes for Withaferin A was confirmed by running a standard Withaferin A sample under identical HPLC conditions. The concentration of withaferin A was quantified as 93.555 µg, corresponding to 187.111 µg g^{-1} plant sample. Additional peaks observed in the chromatogram suggested the presence of other secondary metabolites (Fig. 5). Quantification of withaferin A using HPLC underscore its role as a key bioactive constituent contributing to the observed biological activities. The sharp and distinct peak observed at 4.0 min further validates the purity and abundance of withaferin A in the extract. This early elution also reflects the compound's moderate polarity, enabling efficient separation under selected chromatographic conditions. Withaferin A has been extensively studied for its anti-inflammatory, anticancer, and antimicrobial properties, and its presence in significant quantities further highlights the pharmacological potential of *W. somnifera* (Behl *et al.,* 2020).

The ethanolic extract exhibited superior antioxidant activity, as demonstrated by DPPH (94.64%) and ABTS (97.99%) assays, and remarkable antibacterial efficacy against *Escherichia coli* and *Listeria monocytogenes.* These results underscore the ability of ethanol to break down plant cell walls, releasing a greater diversity of phytochemicals. The phytochemical composition reported in this study, including the presence of phenols, flavonoids, and withaferin A, is consistent with the previous findings on *W. somnifera.* The implications of these findings are far-reaching. The strong radical scavenging activity observed suggests that *W. somnifera* extracts could be utilized as natural antioxidants in food applications and

pharmaceutical industries to prevent oxidative degradation and manage oxidative stress-related diseases (Uddin *et al.,* 2018). The antibacterial efficacy of extracts show their efficacy as natural substitutes to synthetic, particularly in light of the growing global challenge of antibiotic resistance (Aryal *et al.,* 2020).

In addition to these practical applications, the presence of diverse bioactive compounds in *W. somnifera* underscores its value as a source of secondary metabolites for drug discovery and development (Mir *et al.,* 2012). Future research could explore the synergistic effects of these compounds, as combined action of multiple bioactive molecules often enhances therapeutic efficacy. This study demonstrate significant insights; however, it has limitations. The results derived from *in-vitro* experiments, although useful, do not entirely encompass the intricacies of biological systems. Future studies should prioritize *in-vivo* experiments to validate the pharmacological potential of *W. somnifera* extracts. The study highlights the rich phytochemical and bioactive potential of *W. somnifera* root extracts, especially the ethanolic extract, demonstrating strong antioxidant and antimicrobial activities. These findings validate its traditional uses and suggest applications in nutraceuticals, pharmaceuticals, and natural preservatives. Further research on bioavailability, *in vivo* efficacy, advanced extraction methods, and sustainable cultivation is essential to maximize its therapeutic and industrial potential.

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Authors' contribution: N. Kashyap: Conducted experimental and drafted the manuscript; **E. Suresh:** Manuscript writing, analysis and interpretation of data; **D. Agarwal:** Technical support for experiment; **A. Kathirvelpandian:** Revision and editing of manuscript; **P. Chidambaram:** Technical support; **J.R. Ramasre and A. Vaishnav:** Technical assistance.

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