Research Article

# *In vitro* Antimicrobial Activity and Qualitative Phytochemical Analysis of *Withania somnifera* (L.) Dunal Extracts

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**ABSTRACT**

The antibacterial activity of various solvent extracts of stem of *Withania somnifera* was evaluated against gram negative bacteria *Escherichia coli, Serratia marcescens*, *Pseudomonas aeruginosa* and gram positive bacterium *Bacillus cereus* by agar well diffusion method. While antifungal activity of same extracts was evaluated against *Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Penicillium chrysogenum* and *Trichoderma viridae*. All the extracts showed significant antimicrobial potential against different test bacteria. Most susceptible organism in the investigation was *S. marcescens* against which all of the plant extracts showed zone of inhibition. Maximum antibacterial activity was recorded for methanol extract of *W. somnifera* stem against *E. coli* (ZOI of 17.67±1.52 and AI of 0.974) followed by *B. cereus* (ZOI of 15 ± 1.0 and AI of 0.993). Ethyl acetate extract was least inhibitory towards all the bacterial strains. The range of MIC of extracts recorded was 3.125 to 50 mg/ml. In the present investigation lowest MIC value 3.125 mg/ml was recorded for methanol extract against *B. cereus* and *E. coli*. In case of antifungal study, most susceptible organism in the investigation was *T. viridae* against which all of the plant extracts showed inhibition zone. *A. flavus, A. oryzae* and *P. chrysogenum* were proven to be resistant against all the extracts used in the study. Lowest MIC value 50 mg/ml was recorded for methanol and ethyl acetate extracts against *T. viridae*. Qualitative phytochemical analysis indicated presence of different phytochemicals in different extracts.

**Keywords:** Antibacterial activity, Antifungal activity, Phytochemical analysis, *Withania somnifera*.

## INTRODUCTION

Natural products of higher plants may give a new source of antimicrobial agents with possibly novel mechanism of action.[1-4] Medicinal plants are a boon of nature to human mankind and have been used for centuries to cure a number of human diseases. In many parts of the world, medicinal plants are used against bacterial, viral and fungal infections. Evaluation of plants, bearing efficiency in healing various diseases is growing in recent years.[5] The primary benefits of using plant-derived medicines are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment.[6]

Since the discovery of antibiotics and their uses as chemotherapeutic agents, there was a belief in the medical fraternity that this would lead to the eradication of infectious diseases.[7] However, diseases and disease agents that were once thought to have been controlled by antibiotics are returning in new forms resistant to antibiotic therapies.[8] Incidents of epidemics due to such drug resistant microorganisms are now a common global problem posing enormous public health concerns of multi-drug resistant bacterial strains is increasingly limiting the effectiveness of current drugs and significantly causing treatment failure of infections.[9,10] Examples include methicillin-resistant Staphylococci, Pneumococci resistant to penicillin and macrolides, vancomycin-resistant enterococci as well as multi drug resistant Gram-negative organisms.[11]

*W. somnifera* (solanaceae) is gaining attention in various field of research, as they are best suited to the present environmental conditions. *W. somnifera* is used for its antioxidant[12], memory-improving effects[13], anti-inflammatory effect[14] and analgesic effect.[15] It shows relaxant and antispasmodic effects against several plasmogens on intestinal, uterine, blood vascular, bronchial and tracheal muscles. Withanolides possess remarkable antibacterial, anti-arthritic and immunosuppressive. The anti-tumor and radio sensitizing effects of *W. somnifera* have been studied.[16] The root of *W. somnifera*, known as Indian ginseng (Ashwagandha), has been described in Ayurvedic folk medicine to have potent aphrodisiac, sedative, and energy-enhancing tonic properties.[17-18] Moreover, it is beneficial in the treatment of arthritis, cough, geriatric problems, stress, rheumatisms and male sexual dysfunctions.[19-20]

Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial agents, a systematic investigation was undertaken to screen the potential antimicrobial activity of *Withania somnifera*.

## MATERIALS AND METHODS

### Collection of the plant samples

Fresh stem of *Withania somnifera* was collected from the botanical garden of G. J. Patel Institute of Ayurvedic Studies & Research, behind GIDC, New Vallabh Vidyanagar, Anand, Gujarat-388121, India. The taxonomic identities of these plants were confirmed by the taxonomist and the voucher specimen numbers of the

plants were preserved. Fresh plant material was washed under running tap water, air dried and then homogenized to fine powder and stored in airtight bottles at 4°C.

**Preparation of plant extracts**

The plant extracts were prepared by proceeding with a sequential cold maceration method using hexane, ethyl acetate, methanol and distilled water as a solvent.[21]

50 gm of dried powder of plant material was soaked in 250 ml hexane for 24 hr at room temperature. This solution was filtered with the help of whatman No. 1 filter paper. The filtrate was collected in 15 cm petridishes and evaporated the solvent at room temperature. The solid dried extract was stored in 2 ml eppendorf tube and powder was used for antimicrobial assays. The filter cake was dried at room temperature and stored separately. The dried powder of filter cake was sequentially resuspended in 250 ml ethyl acetate, methanol and distilled water to prepare dried extract in each solvent. After extraction in each solvent remained filter cake was dried and further used with next solvent for extraction. All the dried extracts were stored at 4°C.

**Preparation of sample**

Samples for antimicrobial activity were prepared by dissolving 100 mg of each extract in 1 ml of dimethyl sulphoxide (DMSO).

**Microorganisms used**

Microorganisms were obtained from Department of Microbiology, ARIBAS, New V.V.Nagar, Gujarat, India.

Amongst four bacteria investigated three Gram negative bacteria were *Escherichia coli, Serratia marcescens* and *Pseudomonas aeruginosa* while a Gram positive bacterium was *Bacillus cereus.* All the bacterial strains used in the present study were maintained at 4°C on nutrient agar slants.

Tested fungal strains, including *Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Penicillium chrysogenum* and *Trichoderma viridae* were maintained at 4°C on potato dextrose agar (PDA) slants.

**Preparation of bacterial suspension**

Colonies of different strains of bacteria (*Escherichia coli, Serratia marcescens, Pseudomonas aeruginosa* and *Bacillus cereus*) were transferred to the different fresh nutrient broth in sterile conditions and were incubated at 37°C for 24 hr. These suspensions were preserved in 250 ml sterile flasks for use.

**Antibacterial activity by agar well diffusion method**

The antimicrobial activity the test extracts was carried out by agar well diffusion method.[22-23] For antibacterial assay, initially the stock cultures of bacteria were revived by inoculating in broth media and grown at 37°C for 18 hr. The agar plates of the Muller Hilton's Agar media were prepared. Each plate was inoculated with an aliquot (0.1 ml) of the bacterial suspension ($10^5$-$10^6$ colony forming unit "CFU"/ml) which was spread evenly on the plate. After 20 min, wells with 6 mm diameter were made with the help of sterile cork borer in the plates and filled with test samples of 100 mg/ml concentration. The positive and negative control wells were filled with Gentamycin (Standard drug at 10 µg/ml) and DMSO respectively. All the plates were incubated at 37°C for 24 hr. The antibacterial activity was assessed by measuring the diameter of the zone of inhibition (in mm).Triplicates was carried out for each extract against each of the test organism. Activity index for each extract was calculated using following formula.[24]

$$\text{Activity index (AI)} = \frac{\text{Inhibition Zone of the sample}}{\text{Inhibition Zone of the standard}}$$

**Antifungal activity**

Anti-fungal study was carried out through the same procedure as used in antibacterial study. Only difference was media used for antifungal study was potato dextrose agar (PDA) media.

For the antifungal activity, the stock cultures of fungi were revived by inoculating in broth media and grown at 27°C for 72 hr. The agar plates of the Potato Dextrose Agar media were prepared. Each plate was inoculated with an aliquot (0.1 ml) of the fungal suspension ($10^3$spores/ml) which was spread evenly on the plate. After 20 min, the wells with 6 mm diameter were made and filled with test samples of 100 mg/ml concentration. The positive and negative control plates with Fluconazole (10 mcg/disc)(standard drug) and DMSO were also prepared. All the plates were incubated at 27°C for 72 hr and then the diameter of zone of inhibition was noted. Triplicates were carried out for each extract against each of the test organism.[22] Activity index for each extract was calculated as per previous equation.[24]

**Determination of relative percentage inhibition (RPI)**

The Relative Percentage Inhibition of the test extract with respect to positive control was calculated by using the following formula.[25]

$$\text{RPI} = \frac{100\ (X-Y)}{(Z-Y)}$$

Where, X= Total area of inhibition of the test extract; Y= Total area of inhibition of the solvent and Z= Total area of inhibition of the standard drug.

The total area of the inhibition was calculated by using area = $\pi r^2$; where, r = radius of zone of inhibition.

**Determination of Minimum Inhibitory Concentration (MIC)**

Minimum Inhibitory Concentration (MIC) of different extracts with respect to different microorganisms was determined using broth dilution method.[24,26] Mueller Hinton broth (HiMedia, Mumbai) was used for the antibacterial study and potato dextrose broth for the

antifungal study (HiMedia, Mumbai). All the extracts dissolved in DMSO were first diluted to the highest concentration (100 mg/ml) to be tested and then serial two-fold dilution was made in concentration range from 1.56 to 100 mg/ml. For broth dilution, 0.1 ml of standardized suspension of a strain ($10^6$ CFU/ml) separately was added to each tube containing various extracts at concentrations of 0 (control), 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml in broth medium. The tubes were incubated at 37°C for 24 hr for bacterial strains and 27°C for 48 hr for fungal strains and observed for visible growth after vortexing the tubes gently. The lowest concentration of extract in a tube that failed to show any visible growth was considered as the MIC. A tube containing nutrient broth and inoculum but no extract was taken as a control. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control.

### Qualitative analysis of phytochemicals

The extracts were tested for the presence of alkaloids, tannins, Saponins, cardiac glycosides, steroids, phenols and flavonoids according to standard protocols for detecting the presence of different chemical constitutes in the plant extracts.[27]

### Statistical analysis

Mean value and standard deviation were calculated for each test bacteria and fungi. Data were analyzed by one-way ANOVA and p values were considered significant at $p > 0.005$.[24]

## RESULTS

Antimicrobial activity (denoted in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded. In the present study total 4 extracts of the stem of *Withania somnifera* having a concentration of 100 mg/ml were tested for their antimicrobial activity. All the extracts showed significant antimicrobial potential against different test microbes. Most susceptible organism in the investigation was *S. marcescens* against which, all of the plant extracts showed inhibition zone. Maximum antimicrobial activity was recorded for methanol extract of *W. somnifera* stem against *E. coli* (ZOI of 17.67±1.52 and AI of 0.974) followed by *B. cereus* (ZOI of 15±1.0 and AI of 0.993) (table 1). While the ethyl acetate extract showed antimicrobial activity only against *S. marcescens* and *E. coli.* Methanol extract was proven to have best antimicrobial activity as it showed significant inhibition of all the tested bacteria.

**Table 1:** Antibacterial activity (zone of inhibition - ZOI) of different extracts of *W. somnifera* stem

| Extract | Concentration (mg/ml) | Bacterial strains Zone of inhibition (mm) (mean ± SD) | | | |
|---|---|---|---|---|---|
| | | *Serratia marcescens* | *Bacillus cereus* | *Pseudomonas aeruginosa* | *Escherichia coli* |
| Hexane | 100 | 12.00±1.0 | 8.50±0.5 | - | - |
| Ethyl Acetate | 100 | 8.33±0.57 | - | - | 7.67±0.57 |
| Methanol | 100 | 10.33±1.15 | 15.00±1.0 | 9.50±0.5 | 17.67±1.52 |
| Aqueous | 100 | 8.67±0.57 | 8.33±0.57 | 10.17±0.76 | 11.33±0.57 |
| Gemtamycin | 10 µg/ml | 19.00±1.0 | 15.10±0.76 | 15.00±1.0 | 18.10±1.04 |
| DMSO | - | - | - | - | - |

Zone of inhibition (mm) (mean ± SD), (-) No zone of inhibition, Zone of inhibition including 6 mm well diameter, DMSO Dimethyl sulphoxide

**Table 2:** Antibacterial activity (activity index and relative percentage inhibition) of different extracts of *W. somnifera* stem

| Extract | Concentration (mg/ml) | *Serratia marcescens* | | *Bacillus cereus* | | *Pseudomonas aeruginosa* | | *Escherichia coli* | |
|---|---|---|---|---|---|---|---|---|---|
| | | AI | RPI | AI | RPI | AI | RPI | AI | RPI |
| Hexane | 100 | 0.631 | 39.87 | 0.562 | 31.68 | - | - | - | - |
| Ethyl Acetate | 100 | 0.438 | 19.22 | - | - | - | - | 0.423 | 17.95 |
| Methanol | 100 | 0.543 | 29.55 | 0.993 | 98.68 | 0.633 | 40.11 | 0.974 | 95.3 |
| Aqueous | 100 | 0.456 | 20.82 | 0.551 | 30.43 | 0.678 | 45.96 | 0.625 | 39.18 |

AI Activity index, RPI Relative percentage inhibition, (-) No inhibitory activity

**Table 3:** Minimum Inhibitory Concentration (MIC) of different extracts of *W. somnifera* stem against different bacteria

| Test Organism | MIC in mg/ml | | | |
|---|---|---|---|---|
| | Hexane | Ethyl Acetate | Methanol | Aqueous |
| *Serratia marcescens* | 25 | 50 | 25 | 25 |
| *Bacillus cereus* | 50 | NA | 3.125 | 12.5 |
| *Pseudomonas aeruginosa* | NA | NA | 25 | 12.5 |
| *Escherichia coli* | NA | 50 | 3.125 | 6.5 |

MIC Minimum Inhibitory Concentration (mg/ml), NA Not assessed

**Table 4:** Antifungal activity (zone of inhibition - ZOI) of different extracts of *W. somnifera* stem

| Extract | Concentration (mg/ml) | Fungal strains Zone of inhibition (mm) (mean ± SD) | | | | |
|---|---|---|---|---|---|---|
| | | A.n. | A.f. | A.o. | P.c. | T.v. |
| Hexane | 100 | - | - | - | - | 8.67±0.57 |
| Ethyl Acetate | 100 | 7.67±0.57 | - | - | - | 9.83±0.29 |
| Methanol | 100 | - | - | - | - | 10.00±1.0 |
| Aqueous | 100 | - | - | - | - | 8.50±0.5 |
| Fluconazole | 10 mcg/disc | 16.16±1.04 | 21.33±1.15 | 16.00±1.00 | 18.66±1.52 | 22.33±0.57 |
| DMSO | - | - | - | - | - | - |

Zone of inhibition (mm) (mean±SD), (-) No zone of inhibition, Zone of inhibition including 6 mm well diameter, DMSO Dimethyl sulphoxide, A.n. *Aspergillus niger*, A.f. *Aspergillus flavus*, A.o. *Aspergillus oryzae*, P.c. *Penicillium chrysogenum*, T.v. *Trichoderma viridae*

**Table 5:** Antifungal activity (activity index and relative percentage inhibition) of different extracts of *W. somnifera* stem

| Extract | Concentration (mg/ml) | A.n. | | A.f. | | A.o. | | P.c. | | T.v. | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AI | RPI | AI | RPI | AI | RPI | AI | RPI | AI | RPI |
| Hexane | 100 | - | - | - | - | - | - | - | - | 0.388 | 15.07 |
| Ethyl Acetate | 100 | 0.474 | 22.52 | - | - | - | - | - | - | 0.44 | 19.37 |
| Methanol | 100 | - | - | - | - | - | - | - | - | 0.447 | 19.79 |
| Aqueous | 100 | - | - | - | - | - | - | - | - | 0.38 | 9.37 |

AI: Activity index, RPI: Relative percentage inhibition, (-): No inhibitory activity, A.n.: *Aspergillus niger*, A.f.: *Aspergillus flavus*, A.o.: *Aspergillus oryzae*, P.c.: *Penicillium chrysogenum*, T.v.: *Trichoderma viridae*

**Table 6:** MIC of different extracts of *W. somnifera* stem against different fungi

| Test Organism | MIC in mg/ml | | | |
|---|---|---|---|---|
| | Hexane | Ethyl Acetate | Methanol | Aqueous |
| *Aspergillus niger* | NA | 100 | NA | NA |
| *Aspergillus flavus* | NA | NA | NA | NA |
| *Aspergillus oryzae* | NA | NA | NA | NA |
| *Penicillium chrysogenum* | NA | NA | NA | NA |
| *Trichoderma viridae* | 100 | 50 | 50 | 100 |

MIC Minimum Inhibitory Concentration (mg/ml), NA Not assessed

**Table 7:** Qualitative analysis of phytochemicals present in different extracts of *w. somnifera* stem

| Name of phytochemical | Extract | | | |
|---|---|---|---|---|
| | Hexane | Ethyl Acetate | Methanol | Aqueous |
| Alkaloids | + | + | + | + |
| Saponins | + | + | + | + |
| Tannins | + | + | + | + |
| Sterols | - | + | + | + |
| Cardiac Glycoside | - | - | + | + |
| Flavonoids | + | + | + | + |
| Phenol | + | + | + | + |

(+) Present, (-) Absence

Antibacterial activity of different extracts of *W. somnifera* stem was compared with the antimicrobial activity of standard drug (Gentamycin) for evaluating relative percentage inhibition (table 2). The methanol extract of *W. somnifera* stem exhibited maximum relative percentage inhibition against *B. cereus* (98.68 %) followed by *E. coli* (95.30 %). Minimum relative percentage inhibition was observed in case of ethyl acetate extract against *E. coli* (17.95 %).

MIC values were evaluated for those extracts, which were showing activity in diffusion assay (table 3). The range of MIC of extracts recorded was 3.125 to 50 mg/ml. In the present investigation, 3.125 mg/ml was recorded as MIC for methanol extract against *B. cereus* and *E. coli.*

Antifungal activity (denoted in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded. In the present study, total 4 extracts of stem of *W. somnifera* having concentration of 100 mg/ml were tested for their antifungal activity. Most susceptible organism in the investigation was *T. viridae* against which, all of the plant extracts showed inhibition zone. *A. flavus, A. oryzae* and *P. chrysogenum* were proven to be resistant against all the extracts used in the study. *A. niger* was inhibited by only ethyl acetate extract and resistant to other three extracts. Maximum antifungal activity was recorded for methanol extract of *W. somnifera* stem against *T. viridae* (ZOI of 10.00 ± 1.0 and AI of 0.447) followed by ethyl acetate extract against *T. viridae* (ZOI of 9.83±0.29 and AI of 0.440) (Table 4).

Antifungal activity of different extracts of *W. somnifera* stem was compared with the antifungal activity of standard drug (fluconazole) for evaluating relative percentage inhibition (table 5). The methanol extract of *W. somnifera* stem exhibited maximum relative percentage inhibition against *T. viridae* (19.79 %) followed by ethyl acetate extract against *T. viridae* (19.37%). Minimum relative percentage inhibition was observed in case of aqueous extract against *T. viridae* (9.37 %).

MIC values (Table 6) were evaluated for those extracts, which were showing activity in diffusion assay. The range of MIC of extracts recorded was 50 to 100 mg/ml. In the present investigation lowest MIC value 50 mg/ml was recorded for methanol and ethyl acetate extracts against *T. viridae.*

The methanol and aqueous extract showed the presence of alkaloids, tannins, saponins, cardiac glycosides, steroids, phenols and flavonoids. Glycosides and sterol were found to be absent in the hexane extract and cardiac glycoside was found to be absent in ethyl acetate extract (table 7).

## DISCUSSION

Plants are an important source of potentially useful structures for the development of new chemotherapeutic agents and the first step towards this goal is in vitro antibacterial activity assay.[28] Many reports are available on the antiviral, antibacterial and antifungal properties ofplants.[29,30] Some of these observations have helped in identifying the active principle responsible for such activities and in developing drugs for the therapeutic use in human welfare. Plants with possible anti microbial activity should be tested against an appropriate microbial model to confirm the activity and to ascertain the parameters associated with it. The effects of plant extracts on bacteria have been studied by a large number of researchers in different parts of the world.[31-33]

The quest for solutions to the global problem of antibiotic resistance in pathogenic bacteria has often focused on the isolation and characterization of new antimicrobial compounds from a variety of sources, including medicinal plants. This has seen several medicinal plants being screened for antimicrobial activities.[7]

Plants have traditionally provided a source of hope for novel drug compounds, as plant herbal mixtures have made large contributions to human health and well-being.[8]

The present study strongly demonstrated that the *Withania somnifera* plant has potent antibacterial activity. The results of the present study indicate that methanol extract of *W. somnifera* stem has maximum antibacterial activity followed by aqueous extract. Hexane and ethyl acetate extracts have less antibacterial activity as compared to methanol and aqueous extract. Methanol extract of leaf, root and stem of *W. somnifera* showed higher antibacterial activity.[34,35]

All the extracts used in the study were failed to inhibit *A. niger*, *A. flavus, A. oryzae* and *P. chrysogenum.* Only ethyl acetate extract slightly inhibited *A. niger, T. viridae* was most susceptible fungi which was inhibited by all the extracts used in the study. *Aspergillus* species are almost resistant to different extracts of *W. somnifera.*[24]

The presence of different phytochemicals was observed in different extracts of the stem, root and leaves of *W. somnifera.*[34,36] Presence of these phytochemicals in *W. somnifera* is indication of the presence of the bioactive compounds in the extracts used in the study. Phytochemicals present any plant parts give the antimicrobial activity to the plant.

## CONCLUSION

The results of our study allow us to conclude that the methanol and aqueous extracts of stem of *W. somnifera* exhibited significant antibacterial activity and support its ethno botanical importance in the treatment of some diseases as broad spectrum antibacterial agent. Antifungal activity of same extracts was only against *T. viridae.* It is scientifically justified that these extracts can be used to treat infectious diseases. However, crude extract need to be further purified through antibacterial activity guided fractionation to isolate and identify the compounds responsible for antimicrobial activity.

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