ORIGINAL ARTICLE

# Antibacterial activity of flavonoids of *Withania somnifera* L.

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**Context:** Flavonoids from different parts (leaf, stem, root and fruits) of *Withania somnifera* have been screened for antibacterial activity against *Enterobacter aerogens, Bacillus subtilis,* Klebsiella pneumoniae, *Raoultella planticola* and *Agrobacterium tumefaciens*. **Materials and Methods:** Disc diffusion assay (DDA) was performed for antimicrobial screening. Minimum inhibitory concentration (MIC), minimum bactericidal (MBC) and total activity (TA) of extracts found sensitive against test pathogens have also been evaluated. **Results and Conclusions:** Most susceptible microorganisms in the present study were *R. planticola* and *A. tumefaciens*, which had shown susceptibility for almost all the extracts tested. Thereafter sensitive was shown by *K. pneumoniae*, *B. subtilis, E. aerogens.* However, highest inhibition zone was observed for *E. aerogens* (IZ 25.5 mm; AI 1.159 ± 0.023) followed by *R. planticola* (IZ 22 mm; AI 0.733 ± 0.133). Antimicrobial activity of flavonoids extracts of *W. somnifera* were carried out to validate the use of traditional medicinal herb and the results of this study tend to give credence to the common use of *W. sominfera* plant. The study provide platform for further studies in plant, so as to pinpoint specific alkaloids and/or flavonoids responsible for antimicrobial activity and might open new vistas in the therapeutic use of the plant in allopathy medicine too.

**Key words:** Antibacterial activity, flavonoids, minimum bactericidal, minimum inhibitory concentration, *W. somnifera*

## INTRODUCTION

The use of medicinal plants as a source for relief from illness can be traced back over five millennia to written documents of the early civilisation in China, India and the Near east, but it is doubtless an art as old as mankind. Neanderthals living 60,000 years ago in present day Iraq used plants such as holly hock. These plants are still widely used in ethno medicine around the world.[1,2] Medicinal plants still are rich source of antimicrobial compounds. There has been an increasing interest worldwide on therapeutic values of natural products. It is believed that the cure, to any debilitating human ailments may be found, among the world's flora in nature's pharmacy.[3] Phytochemicals have made significant contribution in maintaining human health. The significance of drugs derived from plants cannot be over emphasised with the recent trend of high percentage of resistance in microorganisms to the present day antibiotics.[4] Effort has been intensified by researcher towards a search for more plant source of antimicrobial agents. In the present study *Withania somnifera* has been selected for screening of flavonoids for antimicrobial activity.

*Withania somnifera* L. (Dunal) belongs to family Solanaceae and is classically known for its rejuvenate properties. The plant is used for the treatment of tuberculosis, rheumatism, inflammatory conditions and as a potential anti-tumour agent.[5] Many pharmacological studies have been conducted to investigate the properties of ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent.[6] For example anti-inflammatory properties have been investigated to validate its use in inflammatory arthritis[7] and animal stress studies have been performed to investigate its use as an anti-stress agent.[8-11] Several studies have also examined the anti-tumour and radio sensitising effect of *W. somnifera.*[12-14] The plant contains tropane alkaloids such as tropine, hygrine, anferine and a number of steroidal lactones known as with anolides. Although lots of work on different aspect has been done by different groups but screening of its flavonoids against the tested bacteria so far has not been done hence this aspect has been covered in the present study. The study provide platform for further studies in plant, so as to pinpoint specific flavonoids responsible for antimicrobial activity and might open new vistas in the therapeutic use of the plant in allopathy medicine too.

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## MATERIALS AND METHODS

### Plant Material

Different parts of *W. somnifera* (root, stem, leaf and fruits) were collected in the month of April from the western parts of India (Jaipur, Rajasthan). A voucher specimen has been submitted in Herbarium of Department of Botany, University of Rajasthan.

### Extraction of Flavonoids

Collected plant parts were separately shade dried, finely powered using a blender and subjected to extraction following the method of Subramanian and Nagarjan.[15] Hundred grams of each finely powered sample was soxhlet extracted with 80% hot methanol (500 ml) on a water bath for 24 h and filtered. Each filtrate was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) using separating funnel. Petroleum ether fractions were discarded as being rich in fatty substances, whereas ethyl ether and ethyl acetate fractions were analysed for free and bound flavonoids, respectively. Ethyl acetate fraction of each of the samples was hydrolysed by refluxing with 7% $H_2So_4$ for 2 h (for removal of bounded sugars from the flavonoids) and filtered. The filtrate was extracted in ethyl acetate and washed with distilled water to neutrality. Ethyl ether (free flavonoid) and ethyl acetate fractions (bound flavonoids) thus obtained were dried *in vacuo* and weighed. The extracts were stored at 4°C and were re-suspended in their respective solvents to get 10 mg/ml concentration for antimicrobial assay.

### Selected Test Microorganisms

Pathogenic microorganisms selected for study include five bacteria, viz., *Enterobacter aerogens* (MTCC 2822), *Bacillus subtilis* (MTCC 121), *K*lebsiella pneumoniae (MTCC 4030), *Raoultella planticola* (MTCC 2271) and A*grobacterium tumefaciens* (MTCC 431). Selected microorganisms were procured from IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on 'Muller–Hinton Agar Medium' (Beef extract 2.0 g; Peptone 17.5 g; Starch 1.5 g; Agar 17.0 g; in 1000 ml of distilled water; Final pH 7.4 ± 0.2) at 37 ± 2°C.

### Antimicrobial Screening of Extracts

Disc diffusion assay (DDA) was performed for antimicrobial screening.[16,17] MH agar (for bacteria) and SD agar (for fungi) base plates were seeded with the standard inoculums size of bacterial ($1 \times 10^8$ CFU/ml). Sterile filter paper discs (6 mm in diameter) were impregnated with 100 µl of each of the extract (10 mg/ml concentration) to give a final concentration of 1 mg/disc, left to dry in vacuo to remove residual solvent, which might interfere with the determination. Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with streptomycin (1 mg/disc). The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were incubated at 37 ± 2°C for 24 h. Zone of inhibition (IZ) or depressed growth of microorganisms was measured and the 'Activity Index' (AI) for each extract was calculated as, Activity index = IZ produced by extract/IZ produced by standard; where IZ is inhibition zone.

### Minimum Inhibitory Concentration and Minimum Bactericidal/Fungicidal Concentration

Minimum inhibitory concentration (MIC) was determined for plant extract showing antimicrobial activity against test pathogens in DDA. Broth microdilution method was followed for determination of MIC values.[18] Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration and then added to broth media of 96-wells of microtiter plates using twofold serial dilution. Thereafter 100 µl inoculum of standard size was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. The microtiter plates were incubated at 37 ± 2°C for 24 h. Each extract was assayed in duplicate and each time two sets of microtiter plates were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of microtiter plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms. The minimum bacterial concentration/minimum fungicidal concentration (MBC/MFC) was determined by sub-culturing 50 µl from each well showing noapparent growth. Least concentration of extract showing no visible growth on sub-culturing was taken as MBC/MFC.

### Total Activity

Total activity (TA) is the volume at which test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g plant material by the MIC of the same extract or compound isolated and is expressed in ml/g.[19]

## RESULTS AND DISCUSSION

Results obtained in the study revealed that the tested plant extracts possess potential for antibacterial activity against *E. aerogens*, *B. subtilis*, *K. pneumoniae*, *R. planticola*, *A. tumefaciens* [Table 1 and Figure 1]. In the present investigation total eight extracts were tested, and all of them showed antibacterial activity. All selected five pathogens were inhibited by bound flavonoids of stem and root. Most of the extracts showed bioactivity against more than two

microorganisms tested. Bound flavonoids were found to be more potent than free flavonoids of the selected plant.

Free flavonoids of stem showed highest antibacterial activity (IZ 22 mm; AI 0.733 ± 0.133) against *R. planticola* with low MIC value of 0.039 mg/ml [Table 2 and Figure 2]. Bound flavonoids of stem showed same activity (IZ 10.75 mm) against *E. Aerogens* and *B. subtilis* [Figure 3]. Free flavonoids of leaf and root were found highly active against *A. tumefaciens* (IZ 18 mm; AI 0.643 ± 0.039 and IZ 15.25 mm; AI 0.544 ± 0.099, respectively) with low MIC values 0.039 mg/ml and 0.156 mg/ml, respectively. Most resistant microorganisms observed under present investigation were *E. aerogens* against which only seven extracts exhibited bioactivity. Bactericidal effect of free flavonoids of root was recorded against *K. pneumonia* and *Agrobacterium*. Free flavonoids of leaf and stem were found bactericidal against *B. Subtilis* and *R. planticola*, whereas bound flavonoid of stem was recorded bactericidal against *K. pneumoniae*. TA as a measure of potency was also determined. Most potent extract under study was free flavonoids of leaf, which showed high values of TA (269.23 ml/g) against *E. aerogens*, *K. pneumonia* and *A. tumefaciens* [Table 3].

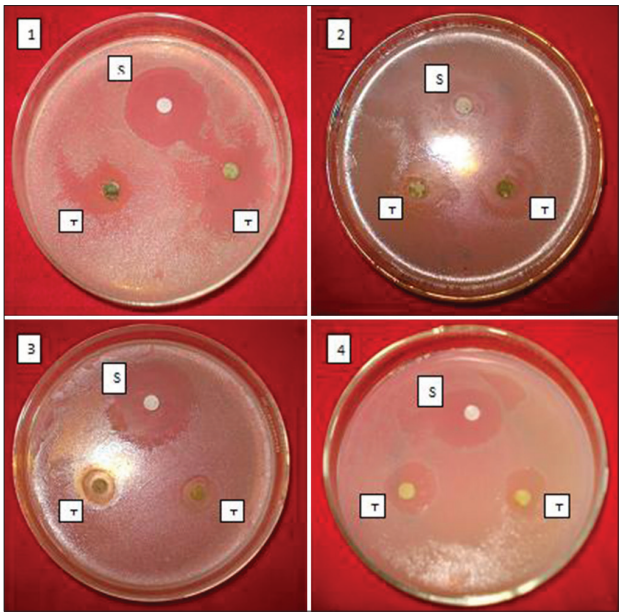


**Figure 1:** Inhibition zone of extracts of selected plants against microorganisms S: Standard Disc; T: Test extracts Disc. (1) *Withania somnifera*/Stem/E1/*Agrobacterium tumefaciens;* (2) *Withania somnifera*/Stem/E1/*Raoultella planticola;* (3) *Withania somnifera*/Root/E2/*Bacillus subtilis;* (4)*Withania somnifera*/Root/E1/*Klebsiella pneumoniae*

In the current investigation *W. somnifera* showed its antibacterial potential against test pathogens, which are being involved in a number of human diseases. W. somnifera has previously been studied for antibacterial and antifungal activities, but still the literature available is meagre. The methanolic extract was inhibiting the growth of bacteria P. aeruginosa, *E. Coli* and *S. aureus* than aqueous extracts.[20] Important compounds with aferin and with anolides were isolated from the methanolic extract of root of W. somnifera.[21] The methanolic extract of both leaves and root shows antibacterial activity, whereas only root extract in hexane shows antibacterial activity.[22] The previous finding also shown that the aqueous extract of W. somnifera inhibit the growth of Gram negative bacteria N. gonorrhea, which also supports the result because water is the most polar solvent and the with anolides are extracted in the water properly.The methanolic extract of the W. somnifera also inhibit the growth of *B. subtilis*, *E. coli*, P. Fluorescens and *S. aureus*.[23] Screening of the plant under investigation so far has not been worked out for flavonoids. Mostly the crude extracts have been screened, that too without MIC, MBC/MFC and TA determination.

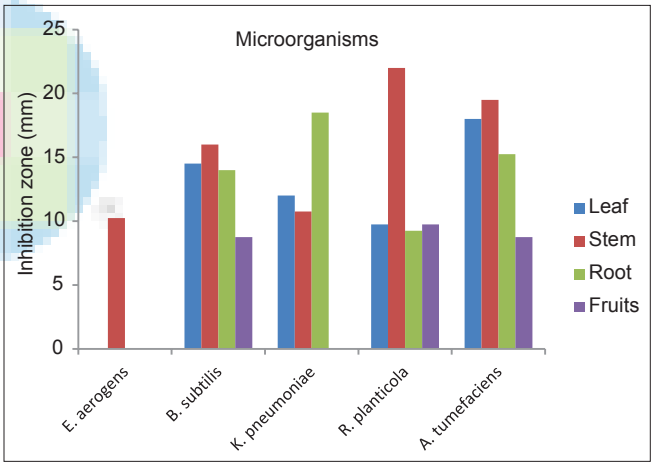


**Figure 2:** Antimicrobial activity of free flavonoids of *W. somnifera*

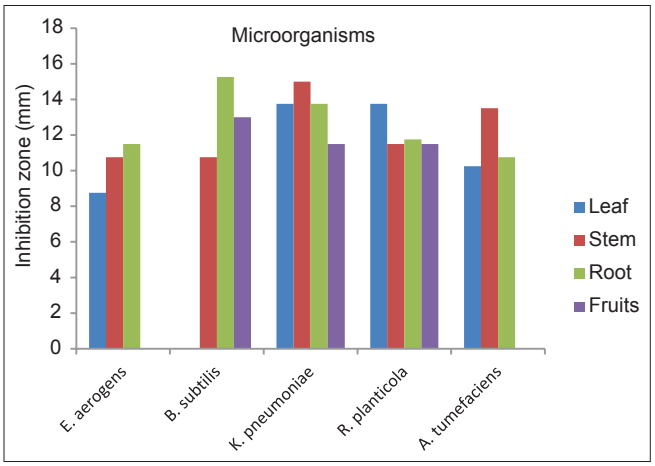


**Figure 3:** Antimicrobial activity of bound flavonoids of *W. somnifera*

The activity of plant extracts against both Gram positive and Gram negative bacteria may be an indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. From this study, it can be concluded that flavonoids (free and bound) of different parts of plant exhibited potential bactericidal properties. Present investigations together with previous studies provide support to the antibacterial properties of *W. somnifera*.

**Table 1: Determination of IZ and AI values of extracts of *W. somnifera* L**

| Microorganisms | | *E. aerogens* | | *B. subtilis* | | *K. pneumoniae* | | *R. planticola* | | *A. tumefaciens* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Plant parts** | **Extracts** | **IZ** | **AI** | **IZ** | **AI** | **IZ** | **AI** | **IZ** | **AI** | **IZ** | **AI** |
| Leaf | $E_1$ | - | - | 14.5 | 0.805±0.139 | 12 | 0.706±0.030 | 9.75 | 0.325±0.009 | 18 | 0.643±0.039 |
| | $E_2$ | 8.75 | 0.398±0.012 | - | - | 13.75 | 0.809±0.162 | 13.75 | 0.458±0.058 | 10.25 | 0.366±0.009 |
| Stem | $E_1$ | 10.25 | 0.466±0.012 | 16 | 0.889±0.112 | 10.75 | 0.632±0.015 | 22 | 0.733±0.133 | 19.5 | 0.696±0.054 |
| | $E_2$ | 10.75 | 0.489±0.012 | 10.75 | 0.597±0.097 | 15 | 0.882±0.088 | 11.5 | 0.383±0.033 | 13.5 | 0.482±0.054 |
| Root | $E_1$ | - | - | 14 | 0.778±0.167 | 18.5 | 1.088±0.147 | 9.25 | 0.308±0.008 | 15.25 | 0.544±0.099 |
| | $E_2$ | 11.5 | 0.523±0.023 | 15.25 | 0.847±0.014 | 13.75 | 0.808±0.044 | 11.75 | 0.391±0.025 | 10.75 | 0.384±0.063 |
| Fruits | $E_1$ | - | - | 8.75 | 0.486±0.014 | - | - | 9.75 | 0.325±0.009 | 8.75 | 0.312±0.027 |
| | $E_2$ | - | - | 13 | 0.722±0.028 | 11.5 | 0.676±0.029 | 11.5 | 0.383±0.017 | - | - |

IZ – Inhibition zone in mm (mean value; include 6 mm diameter of disc); AI – Activity index (IZ developed by extract/IZ developed by standard); ± – SEM; (-) – No activity. Extracts assayed in triplicate; $E_1$ is free flavonoids fraction; $E_2$ is bound flavonoids fraction, IZ of standard drug streptomycin against); *E. aerogens* (22 mm); *B. subtilis* (18 mm); *K. pneumoniae* (17 mm); *R. planticola* (30 mm); *A. tumefaciens* (28 mm)

**Table 2: Determination of MIC and MBC values of extracts of *W. somnifera* L.**

| Microorganisms | | *E. aerogens* | | *B. subtilis* | | *K. pneumoniae* | | *R. planticola* | | *A. tumefaciens* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Plant parts** | **Extract** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** | **MIC** | **MBC** |
| Leaf | $E_1$ | - | - | 0.078 | 0.078 | 0.156 | 0.312 | 0.312 | 0.625 | 0.039 | 0.078 |
| | $E_2$ | 0.625 | 1.25 | - | - | 0.078 | 0.156 | 0.039 | 0.078 | 0.156 | 0.312 |
| Stem | $E_1$ | 0.312 | 0.625 | 0.039 | 0.078 | 0.312 | 0.625 | 0.039 | 0.039 | 0.039 | 0.039 |
| | $E_2$ | 0.078 | 0.156 | 0.156 | 0.625 | 0.078 | 0.078 | 0.078 | 0.156 | 0.039 | 0.156 |
| Root | $E_1$ | - | - | 0.078 | 0.156 | 0.039 | 0.039 | 0.312 | 0.625 | 0.156 | 0.156 |
| | $E_2$ | 0.156 | 0.312 | 0.039 | 0.078 | 0.078 | 0.156 | 0.078 | 0.156 | 0.156 | 0.312 |
| Fruits | $E_1$ | - | - | 0.312 | 1.25 | - | - | 0.156 | 0.312 | 0.625 | 1.25 |
| | $E_2$ | - | - | 0.156 | 0.312 | 0.156 | 0.312 | 0.039 | 0.312 | - | - |

$E_1$ is free flavonoids fraction; $E_2$ is bound flavonoids fraction; MIC – Minimum inhibitory concentration (mg/ml); MBC – Minimum bactericidal (mg/ml)

**Table 3: Total activity of the extracts of W. *somnifera***

| Plant parts | Extracts | Quantity of extract mg/g dried plant part | Total activity (ml/g) | | | | |
|---|---|---|---|---|---|---|---|
| | | | *E. aerogens* | *B. subtilis* | *K. pneumoniae* | *R. planticola* | *A. tumefaciens* |
| Leaf | $E_1$ | 10.5 | - | 134.61 | 67.30 | 33.65 | 269.23 |
| | $E_2$ | 3.5 | 5.6 | - | 44.87 | 89.74 | 22.43 |
| Stem | $E_1$ | 5.5 | 17.62 | 141.02 | 17.62 | 141.02 | 141.02 |
| | $E_2$ | 3 | 38.46 | 19.23 | 38.46 | 38.46 | 76.92 |
| Root | $E_1$ | 7 | - | 89.74 | 179.48 | 179.48 | 44.87 |
| | $E_2$ | 6 | 38.46 | 153.84 | 76.92 | 76.92 | 38.46 |
| Fruits | $E_1$ | 15.5 | - | 49.67 | - | - | 24.8 |
| | $E_2$ | 6.5 | - | 41.66 | 41.66 | 41.66 | - |

E1 is free flavonoids fraction; E2 is bound flavonoids fraction

Therefore it can be used as antibacterial supplement in the developing countries towards the development of new therapeutic agents. Further pharmacological and clinical studies are required to understand the mechanism and the actual efficacy of these plant extracts in treating various infectious diseases. The demonstration of broad spectrum of antibacterial activity by *W. Somnifera* may help to discover new chemical classes of antibiotic substances that could serve as alternatives or second line treatment for infectious disease and their control.

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