

Evolution of Antimicrobial Activity of Leaf extracts of Winter Cheery(*Withania somnifera*)

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Abstract: The leaves of *Withania somnifera* were evaluated against some important bacteria of human and plants viz. *Proteus merabilis*, *Klebsiella pnemoniae*, *Agerobacterium tumefaciens* (plant pathogen) and one fungi *Aspergillus niger*. The dried and powdered leaves were successively extracted with a series of non polar to polar solvents using soxhlet assembly. The antimicrobial assay was done by both disc diffusion and serial dilution methods. Glacial acetic acid extract and toluene extract of *W. somnifera* show highest activity against *P. merabilis*, *K. pnemoniae* to varying degrees, by most of the extracts. Gentamycin, the standard antibacterial drug used was effective in inhibiting these bacteria. The effect on *P. merabilis* and *A. tumefaciens* were comparable to that of gentamycin. Ketoconazole the standard anti fungi used was effective against the fungi. The extract of *W. somnifera* also significantly ($P>0.005$) inhibited the bacterial and fungal growth. The inhibitory effect is very identical in magnitude and comparable with that of standard antibiotics used.

Keywords: *Withania somnifera*, *Proteus merabilis*, *Klebsiella pnemoniae*, *Agerobacterium tumefaciens* and *Aspergillus niger*.

INTRODUCTION

Various plant extracts have also been examined for their antibacterial activity with the objective of exploring environmentally safe alternatives of plant disease control.¹ Most of the herbal medicines in use await validation of their claimed effects and possibly the development of novel antimicrobial drugs from them.² Natural plants derived compounds contribute a lot in fight against pathogens.

Withania somnifera (solanaceae) are gaining attention in various field of research, as they are best

suited to the present environmental conditions. *W. somnifera* used for its anti-inflammatory effect³, Antioxidant⁴, memory-improving effects⁵, analgesic effect.⁶ 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.⁷

Aspergillus species are the most common mold causing severe infections.⁸ *K. pneumonia* more frequently causes lung destruction and pockets of

pus in the lung (known as abscesses). The mortality rate for untreated cases is around 90%. There may also be pus surrounding the lung (known as empyema), respiratory infections, such as bronchitis, which is usually a hospital-acquired infection.^{9,10} *P. merabilis* is a rod shaped bacterium causes obstruction and renal failure. It can also cause wound infections, septicemia and pneumonias, mostly in hospitalized patients. *A. tumefaciens* (Plant pathogen) uses horizontal gene transfer to cause tumors "crown gall disease" in plants. It can be responsible for opportunistic infections in humans with weakened immune system.^{11,12}

MATERIAL AND METHODS

Experimental design: Crude extracts of root of *W. somnifera* (RUBL-20668) were prepared with a series of non polar to polar solvents by hot extraction method¹³ in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay¹⁴ against a few medically important bacteria, plant pathogen and fungi. The fraction showing best activity was then used for determining of minimum inhibitory concentration (MIC) by tube dilution method^{15,16} and minimum bactericidal/fungicidal concentration (MBC/MFC).

Collection of plant material: Leaves of *W. somnifera* (RUBL-20668) were collected in the month of January from Jaipur district of Rajasthan. Plants samples were identified and deposited in the herbarium, department of botany, university of Rajasthan, Jaipur. The collected plant materials were separately shade dried for one week. Shade dried leaves were powdered with the help of grinder. Fine powder of leaves was stored in clean container to be used for Soxhlet extraction following the method of Subramanian and Nagarjan¹⁷, in different polar solvents selected.

Extraction procedure: Leaves (10 gm) were sequentially extracted with different solvents (250 ml) according to their increasing polarity (hexane < petroleum ether < toluene < benzene < iso propyl alcohol < chloroform < ethyl acetate < acetone < ethanol < glacial acetic acid < water) by using Soxhlet apparatus for 18 hours at a temperature not exceeding the boiling point of the respective solvent.¹⁸ The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated at 40°C by using an evaporator.¹⁹ The residual extracts were stored in refrigerator at 4°C in small and sterile glass bottles. Percent extractive

values were calculated by the following formula (table-2).

$$\text{Percent Extracts} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100$$

Drugs and chemicals used:

Drugs: Gentamycin (for bacteria) and Ketoconazole (for fungi)

Chemicals: hexane, petroleum ether, toluene, benzene, iso propyl alcohol, chloroform, ethyl acetate, acetone, ethanol, glacial acetic acid and water, Nutrient Agar (for bacteria), Sabouraud Dextrose Agar (for fungi).

Micro-organisms: The organisms used in this study were three Gram-negative bacteria and one fungus.

Bacteria: *Proteus merabilis* (MTCC-3310), *Klebsiella pneumoniae* (MTCC-4030), *Agerobacterium tumefaciens* (MTCC-431)

Fungi: *Aspergillus niger* (MTCC-282).

Test pathogenic microorganisms were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. The reference strains of bacteria were maintained on nutrient agar slants, sub cultured regularly (after every 30 days) and stored at 4°C as well as at -80°C by preparing suspensions in 10% glycerol.

Screening for antimicrobial activity: Bacterial strains were grown and maintained on Nutrient Agar medium, while fungi were maintained on Sabouraud Dextrose Agar medium (SDA). Disc diffusion assay (DDA) was performed for screening. NA and SDA base plates were seeded with the bacterial and fungal inoculum, respectively (inoculum size 1×10^8 CFU/ml for bacteria and 1×10^7 cell/ml for fungi). Sterile filters paper discs (Whatman no. 1, 5mm in diameter) were impregnated with 100 µl of each of the extracts (100 mg/ml) to give a final concentration of 1 mg/disc and left to dry in vacuo so as to remove residual solvent, which might interfere with the determination. Petri plates were pre-seeded with 15 ml of growth agar medium and 1.0 ml of inoculum.²⁰ Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate with gentamycin (10mcg/disc) and ketoconazole (10mcg/disc) as standard for bacteria and fungi, respectively. The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were

incubated at 37°C for bacteria (24 h) and 27°C for fungi (48 h).²¹ The inhibition zones were measured and compared with the standard reference antibiotics.^{22,23} AI for each extract was calculated (Table 1).

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

Broth dilution method: Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. To measure the MIC values, various concentrations of the stock, 15, 7.5, 3.75, 1.875, 0.938, 0.469, 0.234, 0.117, 0.059, 0.029 mg/ml were assayed against the test pathogens. Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 15mg/ml final concentration and then two fold serially diluted; 1 ml of each extract was added to test tubes containing 1 ml of sterile NA media (for bacteria) and SDA (for fungi). The tubes were then inoculated with standard size of microbial suspension (for bacteria 1×10^8 CFU/ml and 1×10^7 cell/ml for fungi) and the tubes were incubated at 37°C for 24 h for bacteria and 28°C for 48 h for fungi in a BOD incubator and observed for change in turbidity after 24 h compared with the growth and in controls. A tube containing nutrient broth and inoculum but no extract was taken as control. The least extract concentration which inhibited the growth of the test organisms was taken as MIC. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control. Each extract was assayed in duplicate and each time two sets of tubes were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the test tubes. The MIC values were taken as the lowest concentration of the extracts in the test tubes that showed no turbidity after incubation.^{24,25} The turbidity of the test tube was interpreted as visible growth of microorganisms.

Determination of Minimum bactericidal/fungicidal concentration (MBC/MFC): Equal volume of the various concentration of each extract and nutrient broth mixed in micro-tubes to make up 0.5ml of solution. 0.5ml of McFarland standard of the organism suspension was added to each tube.²⁶ The tubes were incubated aerobically at 37°C for 24 h for bacteria and 28°C for 48 h for fungi. Two control tubes were maintained for each test batch.

These include tube-containing extract without inoculum and the tube containing the growth medium and inoculum. The MBC was determined by sub culturing the test dilution on Mueller Hinton Agar and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the Minimum bactericidal Concentration.²⁷ MBC was calculated for some of the extracts showed high antimicrobial activity against highly sensitive organisms.

Total activity (TA) determination: Total activity is the volume at which the test extract can be diluted with 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.¹⁸

$$\text{Total Activity} = \frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$$

Statistical Analysis: Mean value and standard deviation were calculated for each test bacteria, fungi and fungi. Data were analyzed by one-way ANOVA and p values were considered significant at $p > 0.005$.²⁸

RESULTS

1. Qualitative and quantitative estimation: The preliminary phyto-profiling (Qualitative and quantitative estimation) for the roots of *W. somnifera* were carried out according to Farnsworth²⁹ wherein the consistency was found to be sticky in all the extracts and water extract was non-sticky in nature. The yield (% w/w) of the extracts was also analyzed wherein the highest yield was recorded for glacial acetic acid extracts (26.71) and followed by water (24.96). (Table 2)

2. Antimicrobial activity: Antimicrobial activity (assessed in terms of inhibition zone in mm* and activity index) of the roots of *W. somnifera* extracts in different polar solvents, tested against selected microorganisms were recorded (Table 1). In the present study total eleven extracts of selected plant were tested for their bioactivity, among which all the extracts showed significant antimicrobial potential against test microbes. Most susceptible organisms in the investigation were *A. tumefaciens* and *P.*

merabilis against which, most of the plant extracts showed inhibition zone.

(a) Antibacterial activity: Highest antibacterial activity was recorded for glacial acetic acid extract (IZ-25.83±0.23 and AI-2.583) and (IZ-21.67±0.21 and AI-1.084) against *P. merabilis* and *K. pneumoniae* respectively and followed by toluene extract (IZ-20.33±0.22 and AI-1.017) against *A. tumefaciens*.

(b) Antifungal activity: Highest antifungal activity was recorded for glacial acetic acid extract (IZ-11.67±0.26 and AI-0.778) against *A. niger*.

MIC and MBC/MFC: MIC and MBC/MFC values (Table 3) were evaluated for those extracts, which were showing activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.489-15 mg/ml. In the present investigation lowest MIC value 0.489 mg/ml was recorded for glacial acetic acid extract against *P. merabilis* and *K. pneumoniae* and followed by 0.938 mg/ml for ethanol, ethyl acetate, chloroform and toluene extract against *A. tumefaciens* indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal show bactericidal and fungicidal activity (table 2).

Table 1: Inhibition Zone (mm)* and Activity index (AI) for leaf extracts of *W. somnifera*.

Polar Solvents	Bio-activity of Leaf extracts of <i>W. somnifera</i> against pathogens							
	<i>Proteus merabilis</i>		<i>Klebsiella pneumoniae</i>		<i>Agerobacterium tumefaciens</i>		<i>Aspergillus niger</i>	
	IZ	AI	IZ	AI	IZ	AI	IZ	AI
Water	11.17±0.26	1.117	-	-	7.33±0.24	0.367	-	-
Acetic acid	25.83±0.23	2.583	21.67±0.21	1.084	16.67±0.27	0.834	11.67±0.26	0.778
Ethanol	7.50±0.64	0.751	10.83±0.27	0.542	14.33±0.25	0.717	-	-
Acetone	8.33±0.22	0.833	9.33±0.23	0.467	7.17±0.25	0.359	-	-
Ethyl acetate	9.17±0.24	0.917	9.50±0.65	0.475	17.83±0.27	0.892	-	-
Chloroform	-	-	-	-	19.67±0.26	0.984	-	-
Iso propyl alcohol	-	-	-	-	16.50±0.64	0.825	-	-
Benzene	-	-	10.17±0.21	0.509	15.67±0.23	0.784	-	-
Toluene	-	-	13.33±0.26	0.667	20.33±0.22	1.017	-	-
Petroleum ether	-	-	-	-	9.17±0.24	0.459	-	-
Hexane	8.33±0.26	0.833	-	-	12.83±0.23	0.642	-	-

Abbreviations: All values are mean ± SD, n=3 (p>0.005), IZ= Inhibition Zone (mm±S.D.), AI=Activity index

Table 2: MIC and MBC/MFC of leaf extracts of *W. somnifera*

Polar Solvent	Total Yield (%)	Bio-activity of Leaf extracts of <i>W. somnifera</i> against pathogens							
		<i>P. merabilis</i>		<i>K. pneumoniae</i>		<i>A. tumefaciens</i>		<i>A. niger</i>	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
W	24.96	3.75	1.875	-	-	15	15	-	-
AA	26.71	0.469	0.469	0.469	0.469	0.938	0.938	3.75	3.75
E	10.32	7.5	7.5	3.75	7.5	0.938	0.938	-	-
A	5.53	1.875	1.875	3.75	7.5	15	15	-	-
EA	5.51	7.5	7.5	3.75	3.75	0.938	0.938	-	-
C	11.76	-	-	-	-	0.938	0.938	-	-
IP	7.57	-	-	-	-	1.875	3.75	-	-
B	5.80	-	-	3.75	3.75	1.875	1.875	-	-
T	3.37	-	-	3.75	3.75	0.938	0.938	-	-
PE	4.47	-	-	-	-	3.75	7.5	-	-
H	2.03	3.75	3.75	-	-	1.875	3.75	-	-

MIC - Minimum inhibitory concentration (mg/ml), MBC - Minimum bactericidal concentration (mg/ml)
MFC - Minimum fungicidal concentration (mg/ml), H-hexane, PE-petroleum ether, T-toluene, B-benzene, IP-iso propyl alcohol, C-chloroform, EA-ethyl acetate, A-acetone, E-ethanol, AA-glacial acetic acid, W-water.

Table 3: Phyto-chemical estimation and Total activity of stem extracts of *W. somnifera*

Polar Solvents	Color	Consistency	Total activity of Leaf extracts			
			<i>P. m.</i>	<i>K. p.</i>	<i>A. t.</i>	<i>A. n.</i>
W	Brown	Non-sticky	66.55	-	16.64	-
AA	Brick red	Sticky	569.61	570.83	284.95	343.37
E	Yellowish green	Sticky	13.76	27.53	110.12	-
A	Dark green	Sticky	29.47	14.74	3.68	-
EA	Dark green	Sticky	7.34	14.68	58.70	-
C	Dark green	Sticky	-	-	125.38	-
IP	Dark green	Sticky	-	-	40.37	-
B	Dark green	Sticky	-	15.47	30.94	-
T	Dark green	Sticky	-	8.99	35.92	-
PE	Dark green	Sticky	-	-	11.92	-
H	Dark green	Sticky	5.41	-	10.82	-

P. m. - *Proteus merabilis*, *K. p.* - *Klebsiella pneumoniae*, *A. t.* - *Agerobacterium tumefaciens*, *A. n.* - *Aspergillus niger*, H-hexane, PE-petroleum ether, T-toluene, B-benzene, IP-iso propyl alcohol, C-chloroform, EA-ethyl acetate, A-acetone, E-ethanol, AA-glacial acetic acid, W-water

Total activity: Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganism. Glacial acetic acid extracts showed high values of TA against all the microbes which prove the potential to inhibit the growth of the test microorganisms, even at low concentration. Maximum TA value was recorded 570.83 ml, 569.61 ml, 343.37 ml and 284.95 mg/ml against *K. pneumoniae*, *P. merabilis*, *A. niger* and *A. tumefaciens* respectively (table 3).

DISCUSSION

Results of the present study showed that 11/11 extracts tested inhibited the growth of selected bacteria and fungi, indicating broad spectrum bioactive nature of *W. somnifera*. It indicates that *W. somnifera* has broad spectrum bioactive nature. Glacial acetic acid and toluene extracts express maximum antimicrobial activities by suppressing the growth of all microbes under investigation. In the present study, most of the extracts were found to be potent inhibitor of tested. Excellent antibacterial and antifungal activities were observed by glacial acetic acid extracts were shown by low MIC and

MBC/MFC values. MBC/MFC values were found higher than the MIC values of the extracts against microorganisms tested; indicate the bacteriostatic/fungistatic effects of the extracts. *A. tumefaciens* bacteria were the most susceptible organisms.

Extracts under study not only inhibit the bacterial/fungal growth but the IZ developed, was more or less permanent when compared with the IZ developed by the standard drug used, as after sometime bacterial/fungal colonies could be easily seen in IZ developed by standard drugs. In the light of the fact that microorganism are becoming resistant against the drugs in use, present investigation is of great significance, as far as the future drugs are concerned and uses of selected plants by the pharmaceutical industries for preparing plant based antimicrobials drugs.

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