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Research Article

ESTIMATION OF BIO ACTIVITY OF ARIAL PARTS OF *WITHANIA SOMNIFERA* AGAINST THE BACTERIAL AND FUNGAL MICROBES

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ABSTRACT

We evaluated the bio-activity (antibacterial and antifungal) of Ethanol, acetone, Iso propyl alcohol, toluene and hexane extract of different arial parts (leaf and flower) of *Withania somnifera.* The dried and powdered parts were successively extracted using soxhlet assembly then antibacterial and antifungal activities were investigated with by both disc diffusion and serial dilution methods. The extracts of *W. somnifera* were evaluated or significantly inhibited six important bacteria (two Gram +ve and four Gram-ve bacteria) and two fungi *Staphylococcus aureus* (Gram +ve), *Bacillus Subtilis* (Gram +ve), *Escherichia coli* (Gram-ve), *Raoultella planticola* (Gram -ve), *Pseudomonas aeruginosa* (Gram-ve), *Enterobactor aerogens* (Gram-ve), *Candida albicans* and *Aspergillus flavus* to varying degrees. Leaf extracts of *W. somnifera* in different polar solvents showed highest activity in terms of inhibition zone, activity index, MIC, MBC/MFC and total activity. The inhibitory effect is very identical in magnitude and comparable with that of standard antibiotics. Gentamycin, the standard antibacterial drug used was effective in inhibiting these bacteria. The effect on *S. aureus* and *B. subtilis* were comparable to that of gentamycin. Ketoconazole, the standard antifungal used was effective against the fungi (*C. albicans* and *A. flavus*).

Keywords: *Withania somnifera*, *Raoultella planticola, Escherichia coli, Staphylococcus aureus* and *Candida albicans.*

INTRODUCTION

Withania somnifera used in significant increase hemoglobin concentration, red blood cell count, white blood cell count, platelet count and body weight as compared to controls, as well as increased hemolytic antibody responses towards human erythrocytes[1], Anti-inflammatory effect, analgesic effect, osteoarthritis[2], immuno-potentiating and myeloprotective effect[3], increased phagocytic activity and prolonged survival time[4], antifungal activity of Withania has been confirmed elsewhere, attributed to the withanolides.

Major causative agent of nosocomial infections is *S. aureus*[5], *E. aerogens* along with *E. coli. Raoultella planticola* has been determined to cause severe pancreatitis in one case[6]. *C. albicans* is notorious for causing candidiasis, it can affect the esophagus with the potential of becoming systemic, causing a much more serious condition, afungemia called candidemia[7,8]. *P. aeruginosa* is involved in respiratory tract, urinary tract[9], bloodstream, and central nervous system infections of nosocomial origin[10] and this pathogen is becoming resistant against gentamycin, ciprofloxacin[11], tetracycline, chloramphenicol, and norfloxacin[12]. *Bacillus Subtilis* can contaminate food; however, they seldom result in food poisoning. *E. aerogens is* a nosocomial and pathogenic bacteria that causes opportunistic infections incl-ding most types of infections.

MATERIAL AND METHODS

Experimental design

Crude extracts of different arial parts (leaf and flower) of *W. somnifera* (RUBL-20668) were prepared with a series of non polar to polar solvents by hot extraction method[13] in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay[14] against a few medically important bacteria, fungi and fungi. The fraction showing best activity was then used for determining of minimum inhibitory concentration (MIC) by tube dilution method[15] and minimum bactericidal/fungicidal concentration (MBC/MFC).

Collection of plant material

Different parts 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. Each shade dried plant part was powdered with the help of grinder. Fine powder of each sample was stored in clean container to be used for Soxhlet extraction following the method of Subramanian and Nagarjan[16], in different polar solvents selected.

Extraction procedure

Each plant part (10 gm) was sequentially extracted with different solvents (250 ml) according to their increasing polarity by using Soxhlet apparatus for 18 hours at a temperature not exceeding the boiling point of the respective solvent. The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated at 40^0C by using an evaporator. The residual extracts were stored in refrigerator at 4^0C in small and sterile glass bottles.

Drugs and chemicals used

Drugs: Gentamycin and Ketoconazole as standard antibiotics for bacteria and fungi respectively.

Chemicals: Ethanol, acetone, iso propyl alcohol, toluene, hexane, Nutrient Agar (for bacteria), Sabouraud Dextrose Agar (for fungi).

Micro-organisms: Test pathogenic microorganisms were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. (table 1)

Table 1: Name of the tested pathogens (bacteria, yeast and fungi)

S. No.	pathogens	Name of Pathogens	G+ve/G-ve	Specimen no.
1.	Bacteria	*Escherichia coli*	G-ve	MTCC-46
2.		*Staphylococcus aureus*	G+ve	MTCC-3160
3.		*Raoultella planticola*	G-ve	MTCC-530
4.		*Pseudomonas aeruginosa*	G-ve	MTCC-1934
5.		*Bacillus subtilis*	G+ve	MTCC-121
6.		*Enterobactor aerogens*	G-ve	MTCC-111
7.	Fungi	*Candida albicans*	-	MTCC-183
8.		*Aspergillus flavus*	-	MTCC-277

Screening for antimicrobial activity

Bacterial strains were grown and maintained on Nutrient Agar medium, while fungi were maintained on Sabouraud Dextrose Agar medium. Disc diffusion assay[17] 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 vaccuo so as to remove residual solvent, which might interfere with the determination. The IZ were measured and compared with the standard reference antibiotics. AI for each extract was calculated.

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

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens. 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 (serial dilution method)[17], 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. The turbidity of the test tube was interpreted as visible growth of microorganisms.

Determination of Minimum bactericidal/fungicidal concentration (MBC/MFC)

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[18]. MBC/MFC 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.[19,20]

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

RESULTS

Quantitative estimation

The preliminary phyto–profiling for the different parts of *W. somnifera* were carried out according to Bokhari[21] wherein the consistency was found to be sticky in all polar solvent extracts. The yield (mg/10g±S.D.) of the extracts was also analyzed wherein the highest yield was recorded for ethanolic extracts (1503.50±14.38) for flower and followed by leaf (1032.35±12.04) (Table 2).

Table 2: Preliminary phyto-profiling of different parts of *Withania somnifera*

Solvents	Parts	mg/10gm±S.D.	Color	Consistency
Hexane	Leaf	202.85±9.72	Dark green	Sticky
	Flower	261.20±8.24	Yellowish green	Sticky
Toluene	Leaf	336.95±9.33	Dark green	Sticky
	Flower	401.60±7.92	Yellowish green	Sticky
Iso propyl	Leaf	757.00±9.07	Dark green	Sticky
Alcohol	Flower	668.40±8.69	Yellowish green	Sticky
Acetone	Leaf	552.60±8.37	Dark green	Sticky
	Flower	376.90±6.72	Yellowish green	Sticky
Ethanol	Leaf	1032.35±12.04	Yellowish green	Sticky
	Flower	1503.50±14.38	Yellowish green	Sticky

Antimicrobial activity

Antimicrobial activity (assessed in terms of inhibition zone in mm* and activity index) of the different parts of *W. somnifera* extracts in different polar solvents, tested against selected microorganisms were recorded (Table 3). In the present study total nine extracts of selected plant were tested for their bioactivity, among which all extracts showed significant antimicrobial potential against test microbes. Highest antibacterial as well as antifungal activities were recorded for leaf extracts in different solvents. Highest antibacterial activity was recorded for toluene (IZ-20.83±0.29 and AI-1.042) against *S. aureus* followed by iso propyl alcohol and acetone extract (IZ-18.33±0.25; AI-1.018 and IZ- 16.83±0.24; AI-0.935) against *B. subtilis*. Highest antifungal activity was recorded for iso propyl alcohol extract (IZ-13.50±0.64 and AI-1.929) followed by ethanol extract (IZ- 12.67±0.22 and AI-1.810) against *C. albicans*. Most susceptible organism in the investigation was *B. subtilis, S. aureus* and *C. albicans* against which, most of the plant extracts showed inhibition zone.

MIC and MBC/MFC

MIC and MBC/MFC values (Table 3) were evaluated for those plant extracts, which were showing activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.938-15 mg/ml. In the present investigation lowest MIC value 0.938 mg/ml was recorded by leaf extract in toluene, acetone and ethanol (against *B. subtilis* and *C. albicans*) indicating significant antimicrobial potential of test extracts. MIC and MBC/MFC values were found equal show bactericidal and fungicidal activity (table 4).

Total activity

Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganism. Leaf extracts showed high values of TA against *B. subtilis* and *C. albicans* which prove the potential to inhibit the growth of the test microorganisms, even at low concentration. Maximum TA value was recorded for ethanolic extracts of leaf 110.12 ml against *C. albicans* followed by acetone extracts of leaf 58.91 ml against *B. subtilis* (table 5).

Overall, the test pathogens were more sensitive to the glacial acetic acid extracts than to the ethyl acetate and Petrolium ether extract. This suggests that some of the active compounds in the crude extracts are polar and thus dissolved readily in glacial acetic acid while the ethyl acetate and Petrolium ether extract may have dissolved out non-polar compounds that possess less antimicrobial activity.

DISCUSSION

Results of the present study showed that 10/10 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. Leaf extracts of *W. somnifera* express maximum antibacterial and antifungal activities by suppressing the growth of all microbes under investigation.

Table 3: Inhibition Zone (mm)* and Activity index for different parts of *W. somnifera* against tested pathogens.

Solvents (Polarity Index)	Plant Parts	IZ(mm) and AI	Bio-activity of different extracts against pathogens							
			Escherichia coli	*Staphylococcus aureus*	*Raoultella planticola*	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Enterobactor aerogens*	*Candida albicans*	*Aspergillus flavus*
	Leaf	IZ	-	8.17±0.25	9.50±0.65	7.33±0.25	7.83±0.21	-	7.50±0.65	-
Hexane		AI	-	0.409	0.475	0.611	0.522	-	1.071	-
(0.1)	Flower	IZ	-	-	7.17±0.21	-	-	-	-	-
		AI	-	-	0.411	-	-	-	-	-
	Leaf	IZ	7.67±0.22	20.83±0.29	8.33±0.26	10.17±0.28	15.67±0.22	-	13.50±0.64	8.67±0.25
Toluene		AI	0.458	1.042	0.463	0.848	0.871	-	1.929	0.843
(2.4)	Flower	IZ	-	-	-	8.50±0.64	-	-	-	-
		AI	-	-	-	0.708	-	-	-	-
Iso	Leaf	IZ	8.33±0.27	12.33±0.25	8.50±0.64	-	18.33±0.25	-	12.67±0.21	7.33±0.23
propyl		AI	0.671	0.617	0.425	-	1.018	-	1.810	0.765
Alcohol	Flower	IZ	-	-	-	-	16.50±0.65	7.67±0.26	-	-
(3.4)		AI	-	-	-	-	0.917	0.483	-	-
	Leaf	IZ	-	11.17±0.24	-	8.33±0.25	16.83±0.24	-	10.67±0.22	-
Acetone		AI	-	0.559	-	0.833	0.935	-	1.524	-
(5.1)	Flower	IZ	-	-	-	-	12.33±0.26	-	-	-
		AI	-	-	-	-	0.685	-	-	-
	Leaf	IZ	7.5±0.24	14.83±0.24	-	7.17±0.25	14.67±0.22	7.33±0.21	12.67±0.22	8.33±0.24
Ethanol		AI	0.385	0.742	-	0.896	0.978	0.365	1.810	0.823
(5.2)	Flower	IZ	-	-	-	8.33±0.26	-	-	9.33±0.24	-
		AI	-	-	-	1.041	-	-	1.333	-

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

Table 4: MIC and MBC/MFC of different parts of *W. Somnifera*

Name of Solvents	Plant Parts	MIC MBC/MFC	Bio-activity of different extracts against pathogens							
			E. c.	*S. a.*	*R. p.*	*P. a.*	*B. s.*	*E. a.*	*C. a.*	*A. f.*
	Leaf	MIC	-	3.75	3.75	7.5	7.5	-	3.75	-
Hexane		MBC/MFC	-	7.5	3.75	15	15	-	7.5	-
	Flower	MIC	-	-	15	-	-	-	-	-
		MBC/MFC	-	-	15	-	-	-	-	-
	Leaf	MIC	7.5	1.875	3.75	7.5	0.938	-	0.938	3.75
Toluene		MBC/MFC	15	1.875	7.5	7.5	1.875	-	0.938	7.5
	Flower	MIC	-	-	-	3.75	-	-	-	-
		MBC/MFC	-	-	-	7.5	-	-	-	-
Iso propyl	Leaf	MIC	7.5	1.875	3.75	-	1.875	-	1.875	7.5
Alcohol		MBC/MFC	15	3.75	7.5	-	1.875	-	1.875	7.5
	Flower	MIC	-	-	-	-	0.938	7.5	-	-
		MBC/MFC	-	-	-	-	0.938	15	-	-
	Leaf	MIC	-	3.75	-	7.5	0.938	-	1.875	-
Acetone		MBC/MFC	-	3.75	-	15	1.875	-	1.875	-
	Flower	MIC	-	-	-	-	1.875	-	-	-
		MBC/MFC	-	-	-	-	3.75	-	-	-
	Leaf	MIC	7.5	1.875	-	7.5	1.875	7.5	0.938	3.75
Ethanol		MBC/MFC	15	1.875	-	15	3.75	15	0.938	7.5
	Flower	MIC	-	-	-	3.75	-	-	1.875	-
		MBC/MFC	-	-	-	7.5	-	-	1.875	-

MIC - Minimum inhibitory concentration (mg/ml); MBC - Minimum bactericidal concentration (mg/ml); MFC - Minimum fungicidal concentration (mg/ml)

E. c. - Escherichia coli; *S. a. - Staphylococcus* aureus; *R. p. - Raoultella planticola*; *P. a. - Pseudomonas aeruginosa*; *B. s. - Bacillus subtilis*; *E. a. - Enterobactor aerogens; A. f. - Aspergillus flavus*; *C. a. - Candida albicans*

Table 5: Total activity of different parts of *W. somnifera*

Solvents	Plant parts	Total activity of different extracts against pathogens							
		E. c.	***S. a.***	***R. p.***	***P. a.***	***B. s.***	***E. a.***	***C. a.***	***A. f.***
Hexane	Leaf	-	5.41	5.41	2.71	2.71	-	5.41	-
	Flower	-	-	2.71	-	-	-	-	-
Toluene	Leaf	5.65	17.97	8.99	4.49	35.94	-	35.92	32.56
	Flower	-	-	-	10.71	-	-	-	-
Iso propyl	Leaf	6.43	40.37	20.19	-	40.37	-	40.37	29.91
Alcohol	Flower	-	-	-	-	71.3	5.86	-	-
Acetone	Leaf	-	14.74	-	7.37	58.91	-	29.47	-
	Flower	-	-	-	-	20.1	-	-	-
Ethanol	Leaf	5.67	55.06	-	13.77	55.06	6.34	110.12	27.43
	Flower	-	-	-	40.1	-	-	80.19	-

E. c. - Escherichia coli; *S. a. - Staphylococcus* aureus; *R. p. - Raoultella planticola*;

P. a. - Pseudomonas aeruginosa; *B. s. - Bacillus subtilis*; *E. a. - Enterobactor aerogens*

A. f. - Aspergillus flavus; *C. a. - Candida albicans*

In the present study, most of the extracts were found to be potent inhibitor of tested organisms except *R. planticola* and *E. coli*. Excellent antibacterial and antifungal activities were observed by toluene, iso propyl alcohol and acetone 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. Gram positive bacteria *S. aureus, B. subtilis* and *C. albicans* fungi were the most susceptible organisms, which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative[15,17,20,22,23,24].

In general, the Gram-negative bacteria have shown less sensitivity to plant extracts possibly as a result of their extra lipo-polysaccharide and protein cell wall that provides a permeability barrier to the antibacterial agent[25]. Susceptibility differences between Gram-positive and Gram-negative bacteria may be due to cell wall structural differences between these classes of bacteria. The Gram-negative bacterial cell wall outer membrane appears to act as a barrier to many substances including synthetic and natural antibiotics[26]. 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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