ORIGINAL ARTICLE

Antibacterial and antifungal potential of some polar solvent extracts of Ashwagandha (Solanaceae) against the nosocomial pathogens

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The aim of the present study is to evaluate the antimicrobial (antibacterial and antifungal) effects of hexane, toluene, isopropyl alcohol, acetone and ethanolic extracts of different parts (root and stem) of *Withania somnifera* (RUBL-20668) in order to use it as a possible source for new antimicrobial substances against important human pathogens. The dried and powdered parts were successively extracted using Soxhlet assembly; then antibacterial and antifungal activities were investigated by both, disc diffusion and serial dilution methods. The extract of *W. somnifera* significantly inhibited some important bacteria (two Gram +ve and four Gram-ve bacteria): *Staphylococcus aureus* (Gram +ve), *Bacillus subtilis* (Gram +ve), *Escherichia coli* (Gram-ve), *Raoultella planticola* (Gram -ve), *Pseudomonas aeruginosa* (Gram-ve), *Enterobactor aerogens* (Gram-ve), one yeast *Candida albicans* and one fungi *Aspergillus flavus,* to varying degrees. Isopropyl alcohol, acetone and toluene extracts of *W. somnifera* showed highest activity against the pathogens. 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 *E. coli, R. planticola* and *S. aureus* were comparable to that of gentamycin. Ketoconazole, the standard antifungal used, was effective against the fungi. The inhibitory effect is very identical in magnitude and comparable with that of standard antibiotics used.

Key words: Antibacterial, antifungal, solanaceae

INTRODUCTION

Various plant extracts have also been examined for their antibacterial activity with the objective of exploring environmentally safe alternatives of plant disease control.[1,2] Antimicrobial resistance to antimicrobial agents has lead to treatment failure and the shift of medical care from orthodox to herbal medicine.

W. somnifera (Family Solanaceae) is one of the commonest plant species used for the treatment of candidiasis by root extracts. It is used for the treatment of arthritis, tuberculosis, cancer and sexually transmitted infections. It shows anti-inflammatory effect, analgesic effect, osteoarthritis,[3] immunopotentiating and myelo-protective effect,[4] increased phagocytic activity and prolonged survival time.[5] The antimicrobial properties of this plant species have been widely reported in literature.

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Major causative agent of nosocomial infections is *S. aureus*[6] along with *E. coli, E. aerogens* and *P. aeruginosa.*[7] *E. coli* is the culprit for human urinary tract infections.[8] *P. aeruginosa* is involved in respiratory tract, urinary tract,[9] bloodstream and central nervous system, and this pathogen is becoming resistant against gentamycin, ciprofloxacin[10] tetracycline, chloramphenicol, and norfloxacin.[11] *R. planticola* has been determined to cause severe pancreatitis in one case.[12] *C. albicans* is notorious for causing candidiasis; it can affect the esophagus with the potential of becoming systemic, causing a much more serious condition, a fungemia called candidemia.[13,14] *B. subtilis* can contaminate food; however, they seldom result in food poisoning.

MATERIALS AND METHODS

Experimental Design

Crude extracts of different parts *of W. somnifera* were prepared with a series of non-polar to polar solvents by hot extraction method[15] in Soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion assay[16] against a few medically important bacteria and fungi. The fraction showing best activity was then used for determining minimum inhibitory concentration (MIC) by tube dilution method[17] and

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minimum bactericidal/fungicidal concentration (MBC/MFC).

Collection of Plant Material

Different parts of *W. somnifera* 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 transferred immediately to the laboratory and cleaned with water, and selected plant parts were separately shade dried for 1 week. Each shade-dried plant part was powdered with the help of grinder. Fine powder of each sample was stored in a clean container, to be used for Soxhlet extraction following the method of Subramanian and Nagarjan[18] in different polar solvents selected.

Extraction Procedure

Each plant part (10 g) was sequentially extracted with different solvents (250 mL) according to their increasing polarity by using Soxhlet apparatus for 18 h 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°C by using an evaporator. The residual extracts were stored in refrigerator at 4°C in small and sterile glass bottles.

Drugs and Chemicals Used

Drugs

Gentamycin (for bacteria) and ketoconazole (for yeast and fungi).

Chemicals

Hexane, toluene, isopropyl alcohol, acetone and ethanol, nutrient agar (NA) (for bacteria), Sabouraud Dextrose agar (SDA) (for yeast and fungi).

Microorganisms

The organisms used in this study were four Gram negative bacteria and two Gram positive bacteria, namely [Table 1].

Test pathogenic microorganisms were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. The reference strains of bacteria were maintained on NA 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

Initial screening of different stem extracts for their antibacterial activity carried out using Mueller-Hinton and NA media did not reveal any significant difference. Thus, further studies were carried out using NA medium only. [19] Bacterial strains were grown and maintained on NA medium, while and fungi were maintained on SDA medium. Disc diffusion assay was performed for screening. NA and SDA base plates were seeded with the bacterial and fungal inoculum, respectively (inoculum size 1×10^8 colony forming units (CFU)/mL for bacteria and 1×10^7 cell/mL for fungi). Sterile filter paper discs (Whatman no. 1, 5 mm in diameter) were impregnated with 10 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 zones of inhibition (ZOI) were measured and compared with the standard reference antibiotics.[20] Activity index for each extract was calculated [Table 2].

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

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

MTCC – Microbial type culture collection (IMTECH, Chandigarh, India)

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

Determination of Minimum Inhibitory Concentration by Serial Dilution Method

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): One was kept for incubation while another set was kept at 4°C for comparing the turbidity in the test tubes.[21] 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 MBC and MFC by Sub Culturing Method

The MBC/MFC 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 MBC.[22] MBC was calculated for some of the extracts that showed high antimicrobial activity against highly sensitive organisms.

Table 2: Zone of inhibition (mm)* and activity index of different extracts of *Withania somnifera* in different polar solvents against tested pathogens

Name of solvents (polarity)	Plant parts	ZOI (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*
Hexane (0.1)	Root	ZOI	-	-	9.33±0.26	8.17±0.24	8.50±0.64	-	8.33±0.27	-
		AI	-	-	0.467	0.681	0.567	-	1.190	-
Toluene (2.4)	Root	ZOI	-	-	-	9.67±0.24	8.50±0.65	-	7.33±0.24	-
		AI	-	-	-	0.806	0.472	-	1.047	-
	Stem	ZOI	7.33±0.25	12.33±0.20	7.17±0.25	8.33±0.25	8.17±0.28	-	8.17±0.22	7.67±0.28
		AI	0.528	0.617	0.398	0.694	0.454	-	1.167	0.675
Iso propyl alcohol (3.4)	Root	ZOI	8.67±0.27	14.33±0.23	7.33±0.25	-	9.17±0.26	-	7.50±0.64	-
		AI	0.721	0.717	0.367	-	0.509	-	1.071	-
	Stem	ZOI	-	9.67±0.24	8.17±0.27	-	11.67±0.23	7.67±0.22	8.17±0.24	-
		AI	-	0.484	0.409	-	0.648	0.652	1.167	-
Acetone (5.1)	Root	ZOI	-	-	-	10.67±0.27	10.50±0.64	8.5±0.27	-	-
		AI	-	-	-	1.067	0.583	0.711	-	-
	Stem	ZOI	-	8.33±0.25	-	7.17±0.24	10.67±0.25	-	8.50±0.65	7.33±0.22
		AI	-	0.417	-	0.717	0.593	-	1.214	0.651
Ethanol (5.2)	Root	ZOI	-	-	-	-	7.50±0.65	-	7.33±0.25	-
		AI	-	-	-	-	0.500	-	1.047	-
	Stem	ZOI	-	8.50±0.65	-	-	8.33±0.25	-	8.50±0.65	7.5±0.27
		AI	-	0.425	-	-	0.555	-	1.214	0.662

Abbreviations: All values are mean±SD, n=3 (P>0.005), ZOI: Zone of inhibition in mm±S.D. (including 6 mm of disc diameter), AI: Activity index

Total Activity Determination

Total activity (TA) 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.[23]

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

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.[21]

RESULTS

Qualitative and Quantitative Estimation

The preliminary phyto–profiling for the different parts of *W. somnifera* was carried out according to Bokhari[24] wherein the consistency was found to be sticky in the high polar solvent extracts whereas most of the extracts were found to be sticky. The yield (mg/10 g±S.D.) of the extracts was also analysed wherein the highest yield was recorded for stem extract (1073.4±11.49) followed by root extract (847.9±9.71) in ethanol [Table 3].

Table 3: Qualitative and quantitative estimation of different extracts of *Withania somnifera* in different polar solvents against tested pathogens

Solvents	Plant parts	mg/10 g± S.D.	Color	Consistency
Hexane	Root	157.25±7.87	Brown	Nonsticky
Toluene	Root	131.25±7.23	Brown	Sticky
	Stem	138.10±8.41	Dark green	Sticky
Isopropyl alcohol	Root	240.80±7.88	Brown	Sticky
	Stem	328.15±8.47	Dark brown	Sticky
Acetone	Root	141.75±5.82	Light brown	Nonsticky
	Stem	145.05±6.13	Dark green	Sticky
Ethanol	Root	847.90±9.71	Brown	Sticky
	Stem	1073.40±11.49	Green	Sticky

Antimicrobial Activity

Antimicrobial activity (assessed in terms of ZOI in mm* and activity index) of the different parts of *W. somnifera* extracts in different polar solvents, tested against selected microorganisms, was recorded [Table 2]. In the present study, a total of nine extracts of the selected plant were tested for their bioactivity, among which all these extracts showed insignificant antimicrobial potential against test microbes.

Antibacterial Activity

Highest antibacterial activities were recorded for root extract in isopropyl alcohol (ZOI - 14.33±0.23 and AI - 0.717) and followed by stem extracts in toluene extracts (ZOI - 12.33±0.20 and AI - 0.617) against *S. aureus* [Table 2].

Antifungal Activity

Highest antifungal activities were recorded for stem extracts in acetone and ethanol (ZOI - 8.5±0.65 and AI - 1.214) against

C. albicans and (ZOI - 7.675±0.28 and AI - 0.675) against *A. flavus* by stem extracts in toluene [Table 2].

MIC and MBC/MFC

MIC and MBC/MFC values [Table 4] were evaluated for those plant extracts, which were showing activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 1.875–15 mg/mL. In the present investigation, lowest MIC value 1.875 mg/mL was recorded for root extract in toluene against *P. aeruginosa* as well as stem extract in isopropyl alcohol and acetone against *B. subtilis*. Same lowest values were observed for root extract in hexane and stem extract in acetone against *C. albicans* [Table 4].

Bactericidal/Fungicidal Concentration

It is defined as the concentration of the antimicrobial that results in a 99.9% reduction in CFU/mL compared with the organism concentration in the original inoculum. MBC/MFC values were found higher than the MIC values of the extracts against microorganisms tested; indicate the bacteriostatic/fungistatic effects of the extracts whereas equal values indicate the bactericidal effect. In the present study, root/stem extract in isopropyl alcohol and root extract in acetone were found to bactericidal in nature against *S. aureus* and *P. aeruginosa* respectively [Table 4].

Total Activity

TA indicates the volume at which extract can be diluted with still having ability to kill microorganisms. Stem extracts in ethanol showed high values of TA 28.62 mL against *S. aureus* and *C. albicans*. Overall, the test pathogens were more sensitive to the isopropyl alcohol, acetone and

Table 4: Minimum inhibitory concentration and minimum bactericidal concentration/minimum fungicidal concentration of different extracts of *Withania somnifera* in different polar solvents against tested pathogens

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.*
Hexane	Root	MIC	-	-	3.75	3.75	3.75	-	1.875	-
		MBC/MFC	-	-	7.50	7.50	7.50	-	3.75	-
Toluene	Root	MIC	-	-	-	1.875	3.75	-	3.75	-
		MBC/MFC	-	-	-	3.75	7.50	-	7.50	-
	Stem	MIC	3.75	3.75	7.50	3.75	3.75	-	3.75	3.75
		MBC/MFC	7.50	7.50	15.00	7.50	7.50	-	7.50	7.50
Isopropyl alcohol	Root	MIC	3.75	3.75	7.50	-	3.75	-	3.75	-
		MBC/MFC	7.50	3.75	15.00	-	7.50	-	7.50	-
	Stem	MIC	-	3.75	3.75	-	1.875	7.50	3.75	-
		MBC/MFC	-	3.75	7.50	-	3.75	15.00	7.50	-
Acetone	Root	MIC	-	-	-	3.75	1.875	3.75	-	-
		MBC/MFC	-	-	-	3.75	3.75	7.50	-	-
	Stem	MIC	-	3.75	-	7.50	1.875	-	1.875	3.75
		MBC/MFC	-	7.50	-	15.00	3.75	-	3.75	7.50
Ethanol (5.2)	Root	MIC	-	-	-	-	7.50	-	3.75	-
		MBC/MFC	-	-	-	-	15.00	-	7.50	-
	Stem	MIC	-	3.75	-	-	7.50	-	3.75	3.75
		MBC/MFC	-	7.50	-	-	15.00	-	7.50	7.50

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*; *E.a.*: *Entrobactor aerogens*; *A.f.*: *Aspergillus flavus; C.a.*: *Candida albicans*; *B.s.*: *Bacillus subtilis*

Table 5: Total activity of different extracts of *Withania somnifera* in different polar solvents against tested pathogens

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	Root	-	-	4.19	4.19	4.19	-	8.39	-
Toluene	Root	-	-	-	7.00	3.50	-	3.50	-
	Stem	3.63	3.68	1.84	3.68	3.68	-	3.68	2.24
Isopropyl alcohol	Root	3.32	6.42	3.21	-	6.42	-	6.42	-
	Stem	-	8.75	8.75	-	17.50	10.83	8.75	-
Acetone	Root	-	-	-	3.78	7.56	3.48	-	-
	Stem	-	3.87	-	1.93	7.74	-	7.74	1.28
Ethanol	Root	-	-	-	-	11.31	-	22.61	-
	Stem	-	28.62	-	-	14.31	-	28.62	12.39

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

toluene extracts than to the hexane and ethanol extracts [Table 5].

DISCUSSION

Results of the present study showed that 9/9 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. Isopropyl alcohol and acetone extracts of *W. somnifera* express maximum antibacterial activities by suppressing the growth of all microbes under investigation.[25,26] In the present study, most of the extracts were found to be potent inhibitors of tested organisms except *R. planticola* and *E. coli.* Excellent antimicrobial activities were observed for isopropyl alcohol and acetone extracts, shown by low MIC and MBC/MFC values. MBC/MFC values were found higher than the MIC values of the extracts against microorganisms tested, indicating the bacteriostatic/fungistatic effects of the extracts. Gram positive bacterium, *B. subtilis,* and *C. albicans* fungus were the most susceptible organisms, which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative.[17,21,27,28]

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

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