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COMPARATIVE PRIMARY PHYTO-PROFILE AND MICROCIDAL ACTIVITY OF *CENCHRUS CILIARIS* (ANJAN GRASS) AND *WITHANIA SOMNIFERA* (WINTER CHERRY)

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ABSTRACT
Crude extracts of different parts (root, stem, leaf and seed) of *Cenchrus ciliaris* (CAZRI-358) and (root, stem, leaf and flower) of *Withania somnifera* (RUBL-20668) and were successively extracted with polar to non polar solvents (water, chloroform and benzene) using soxhlet assembly. The extracts were then screened for their antimicrobial activity *in-vitro* against one gram positive bacteria (*Bacillus subtilis*), two gram negative bacteria (*Pseudomonas aeruginosa* and *Enterobactor aerogens*) and one fungus (*Aspergillus flavus*) by disc diffusion assay. Serial dilution method was used to determine minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC). Chloroform extract of leaves of both the plants showed highest activity, by *W. somnifera* (IZ-20.83±0.21 mm, AI- 1.389) and (IZ-20.67±0.24 mm, AI- 1.148) by *C. ciliaris* against *B. subtilis* and *P. aeruginosa* respectively.
Keywords: *Cenchrus ciliaris*, *Withania somnifera*, *Aspergillus flavus, Bacillus subtilis*, *Pseudomonas aeruginosa*, *Enterobactor aerogens* and Antibacterial.

INTRODUCTION

In recent times, there have been increases in antibiotic resistant strains of clinically important pathogens, which have led to the emergence of new bacterial strains that are multi-resistant[1,2,3]. Numerous studies have been carried out to extract and screen various natural products for antimicrobial activity[4,5,6] but more study is required due to non-availability and high cost of new generation of antibiotics with limited effective span have resulted in increase in morbidity and mortality[7], Therefore, there is an urgent need to search for new compounds from other sources with proven antimicrobial activity[8,9].

C_4 grasses are gaining attention in various field of research, as they are best suited to the present environmental conditions. C_4 grasses are more competitive under the conditions of high temperature, solar radiation and low moisture[10]. C_4 grasses are more efficient at gathering Carbon dioxide, utilizing nitrogen from the atmosphere and recycled N in the soil[11,12]. *C. ciliaris* L. (Poaceae) is highly nutritious grass and considered excellent for pasture in hot, dry areas and is valued for its production of palatable forage and intermittent grazing during droughty periods in the tropics. The grass, fed green, turned into silage, or made into hay is said to increase flow of milk in cattle and impart a sleek and glossy appearance. This grass has excellent soil binding capacity which helps to conserve soil in desert areas[13]. However, *C. ciliaris* is most suitable and highly nutritive grasses for desertic conditions, still no antimicrobial work yet have been done on this grass.

P. aeruginosa is involved in respiratory tract, urinary tract[14], bloodstream, and central nervous system infections of nosocomial origin[15] and this pathogen is becoming resistant against gentamycin, ciprofloxacin[16] tetracycline, chloramphenicol, and norfloxacin[17]. *Bacillus Subtilis* can contaminate food; however, they seldom result in food poisoning. *E. aeroge ns* is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. The present investigation evaluated the antibacterial and antifungal effects of crude extracts of *C. ciliaris* and *W. somnifera*. The study was carried out along with the standard drugs Gentamycin (for bacteria), Ketoconazole (for fungi).

MATERIAL AND METHODS

Experimental design: Crude extracts of different parts *of Cenchrus ciliaris* (CAZRI-358) and *Withania somnifera* (RUBL-20668) were prepared with a series of non polar to polar solvents (benzene, chloroform and water) by hot extraction method[18,19] in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay[20] against a few medically important bacteria and fungi. The fraction showing best activity was then used for determining minimum inhibitory concentration (MIC) by serial dilution method[18,21] and minimum bactericidal/fungicidal concentration (MBC/MFC).

Collection of plant material: Different parts of *C. ciliaris* (CAZRI-358) were collected in the month of August from the Central Arid Zone Research Institute, Jodhpur, Rajasthan and 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 transferred immediately to the laboratory cleaned with water and selected plant parts 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[22] 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 (Benzene < Chloroform < Water) 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. The extracts solutions were evaporated under reduced pressure at 40 °C.[23] The residual extracts were stored in refrigerator at 4^0C in small and sterile glass bottles. Percent extractive values were calculated by the following formula (Table 1).

$$\% \text{ yield} = \frac{\text{Weight of dried extract}}{\text{Weight of dried plant material}} \times 100$$

Drugs and chemicals used

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

Chemicals: Benzene, Chloroform, Water, Nutrient Agar (for bacteria) Sabouraud Dextrose Agar (for fungi).

Micro-organisms

(a) Bacteria: *Pseudomonas aeruginosa* (Gram-ve) (MTCC-1934),
Bacillus subtilis (Gram +ve) (MTCC-121),
Enterobactor aerogens (Gram-ve) (MTCC-111)

(b) Fungi: *Aspergillus flavus* (MTCC-277).

Screening for antimicrobial activity: Test pathogenic microorganisms were procured from Microbial Type Culture Collection, IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on Nutrient Agar medium, while fungi were maintained on Sabouraud Dextrose Agar medium. Disc diffusion assay[24,25] was performed for screening. Sterile filter paper discs (Whatman no. 1, 5mm in diameter) were impregnated with 100 µl of each of the extract (10 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. A bacterial suspension was prepared and inoculum size 1×10^8 CFU/ml was added for bacteria and 1×10^7 cell/ml for fungi to the sterilized medium before solidification. The media with bacteria was poured into sterilized Petri dishes under aseptic condition[26]. 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 hour) and at 27°C for fungi (48 hour)[27]. After incubation the average of IZ was recorded[28,29]. Inhibition zones were measured and average size was compared with IZ of standard reference antibiotics and Activity index for each extract was calculated by following formula and recorded (Table 1).

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

Determination of minimum inhibitory concentration (MIC): The Minimum inhibitory concentration (MIC) was evaluated as the lowest concentration with no visible growth of test pathogens[27]. To measure MIC, 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 serial dilution (1 ml of each extract was added to test tubes containing 1 ml of sterile Nutrient Agar media for bacteria and Sabouraud Dextrose Agar media 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 hour for bacteria and 27°C for 48 hour for fungi in a BOD incubator and were observed for change in turbidity and compared with the growth in controls[30,31]. A tube containing nutrient broth and inoculum but no extract was taken as control. 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.

Determination of Minimum bactericidal/fungicidal concentration (MBC/MFC): Equal volume of various concentration of each extract and nutrient agar were mixed in micro-tubes to make up 0.5ml of solution. Then 0.5ml of McFarland standard of the organism suspension was added to each tube[32]. The tubes were incubated aerobically at 37^0C for 24 hours for bacteria and 27^0C for 48 hours 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 Nutrient Agar followed by incubation. The highest dilution that yielded no single pathogen was taken as the Minimum bactericidal Concentration[33]. MBC was calculated for those extracts that had shown high antimicrobial activity against tested 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[30,34].

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

Statistical Analysis: Mean value and standard deviation were calculated for each test bacteria and fungi. Data were analyzed by one-way ANOVA and p values were considered significant at $p > 0.005$.[35]

RESULTS

Preliminary phyto–profiling: The preliminary phyto–profiling for the different parts of *C. ciliaris* and *W. somnifera* were carried out[23], wherein the consistency was found to be non-sticky in the high polar solvent extracts where as the low polar solvent extracts were found to be sticky in case of *W. somnifera* and vice-versa in case of *C. ciliaris*. The percentage yield w/w of the extracts was also analyzed, wherein the highest yield (%) was recorded in water extracts of leaves for both the plants (24.96 for *W. somnifera* and 55.60 for *C. ciliaris*). (Table 3)

Antimicrobial activity: Antimicrobial activity (denoted in terms of inhibition zone and activity index) of the plant extracts, tested against selected microorganisms were recorded (Table 1). In the present study total 24 extracts of different parts of selected plants were tested for their bioactivity. Twenty one extracts showed significant antimicrobial potential against test microbes. However three extracts showed no activity against any of the selected microorganisms at the tested concentration (one from *C. ciliaris* and two from *W. somnifera*). Most susceptible organisms in the investigation were *P. aeruginosa* and *B. subtilis* against which, most of the plant extracts showed inhibition zone. Maximum antibacterial activities were recorded for chloroform extracts of leaves in both the plants. In case of *C. ciliaris*, IZ of 20.67±0.24 mm and AI-1.148 against *P. aeruginosa* and in case of *W. somnifera*, IZ of 20.83±0.21 mm, AI 1.389, against *B. subtilis*. *C. ciliaris* and *W. somnifera* showed no bioactivity against *A. flavus*. It indicate that *A. flavus* is highly resistant pathogen.

MIC and MBC/MFC: MIC and MBC/MFC values (Table 2) were evaluated for those plant extracts, 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 for chloroform and water extracts of leaves against *P. aeruginosa* and *E. aerogens* by *C. ciliaris* as well as for chloroform extracts of leaf and stem against *B. subtilis* by *W. somnifera* indicating significant bactericidal potential of test extracts. MIC and MBC/MFC values were found equal for bactericidal nature of these plants.

Total activity: Amount of extract isolated from each gram plant parts and total activity (TA) was calculated and recorded (Table 3). Total activity indicates the volume at which extract can be diluted with still having ability to kill microorganism. Most of the extracts showed high values of TA against *P. aeruginosa*, *B. subtilis* and *E. aerogens*, which proves the potential of extracts to inhibit growth of the test microorganisms, even at low concentration. Maximum TA values calculated were 133.10, 125.38, 59.31 ml against *P. aeruginosa*, *B. subtilis* and *E. aerogens* respectively.

Table 1: Showing Inhibition zone (mm)* and Activity index (AI) by different parts of *C. ciliaris* and *W. somnifera* against pathogens

Solvents	Polarity of Solvents	Plant Part	Test microorganisms							
			***Cenchrus ciliaris* (CAZRI-358)**							
			Pseudomonas aeruginosa		*Bacillus subtilis*		*Enterobactor aerogens*		*Aspergillus flavus*	
			IZ±S.D.	AI	IZ±S.D.	AI	IZ±S.D.	AI	IZ±S.D.	AI
Benzene	2.7	Root	8.33±0.24	0.694	-	-	-	-	-	-
		Stem	-	-	-	-	-	-	-	-
		Leaf	7.17±0.25	0.598	-	-	-	-	-	-
		Seed	7.33±0.29	0.611	-	-	-	-	-	-
Chloroform	4.1	Root	-	-	11.67±0.24	0.486	-	-	-	-
		Stem	-	-	8.5±0.64	0.354	-	-	-	-
		Leaf	20.67±0.24	1.148	-	-	-	-	-	-
		Seed	8.50±0.64	0.472	8.33±0.25	0.347	-	-	-	-
Water	9	Root	7.17±0.24	0.398	8.17±0.24	0.272	14.83±0.24	0.742	-	-
		Stem	8.33±0.26	0.463	12.33±0.28	0.411	15.67±0.22	0.784	-	-
		Leaf	7.67±0.26	0.426	12.33±0.26	0.411	16.33±0.27	0.817	-	-
		Seed	8.50±0.64	0.472	8.17±0.23	0.272	13.67±0.24	0.684	-	-
***Withania somnifera* (RUBL 20668)**										
Benzene	2.7	Root	7.17±0.27	0.896	12.17±0.25	0.676	-	-	-	-
		Stem	7.33±0.24	0.916	18.50±0.65	1.028	-	-	-	-
		Leaf	7.50±0.64	0.938	12.67±0.22	0.704	-	-	-	-
		Flower	-	-	-	-	-	-	-	-
Chloroform	4.1	Root	-	-	12.67±0.27	0.845	-	-	-	-
		Stem	-	-	15.50±0.65	1.033	-	-	-	-
		Leaf	-	-	20.83±0.21	1.389	-	-	-	-
		Flower	-	-	-	-	-	-	-	-
Water	9	Root	15.5±0.64	1.938	-	-	-	-	-	-
		Stem	11.67±0.24	1.459	-	-	-	-	-	-
		Leaf	14.17±0.28	1.771	-	-	-	-	-	-
		Flower	13.33±0.26	1.666	-	-	-	-	-	-

All values are mean±SD, n=3

Table 2: Showing minimum inhibitory concentration and (MBC/MFC) by different parts of *C. ciliaris* and *W. somnifera* in different polar solvents against pathogens

Solvents	Plant Part	Test microorganisms							
		***Cenchrus ciliaris* (CAZRI-358)**							
		P. aeruginosa		*B. subtilis*		*E. aerogens*		*A. flavus*	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Benzene	Root	1.875	3.750	-	-	-	-	-	-
	Stem	-	-	-	-	-	-	-	-
	Leaf	15	15	-	-	-	-	-	-
	Seed	15	15	-	-	-	-	-	-
Chloroform	Root	-	-	1.875	3.75	-	-	-	-
	Stem	-	-	3.75	7.5	-	-	-	-
	Leaf	0.938	0.938	-	-	-	-	-	-
	Seed	1.875	3.75	3.75	7.5	-	-	-	-
Water	Root	7.5	15	7.5	15	1.875	3.75	-	-
	Stem	3.75	7.5	3.75	7.5	0.938	1.875	-	-
	Leaf	7.5	15	3.75	7.5	0.938	0.938	-	-
	Seed	3.75	7.5	7.5	15	1.875	3.75	-	-
***Withania somnifera* (RUBL 20668)**									
Benzene	Root	7.5	15	1.875	3.75	-	-	-	-
	Stem	7.5	15	0.938	1.875	-	-	-	-
	Leaf	7.5	7.5	1.875	3.75	-	-	-	-
	Flower	-	-	-	-	-	-	-	-
Chloroform	Root	-	-	1.875	3.75	-	-	-	-
	Stem	-	-	0.938	0.938	-	-	-	-
	Leaf	-	-	0.938	0.938	-	-	-	-
	Flower	-	-	-	-	-	-	-	-
Water	Root	1.875	1.875	-	-	-	-	-	-
	Stem	3.75	7.5	-	-	-	-	-	-
	Leaf	1.875	1.875	-	-	-	-	-	-
	Flower	1.875	3.75	-	-	-	-	-	-

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

Table 3: Preliminary phyto-profile and total activity of different parts of *C. ciliaris* and *W. somnifera* in different polar solvents against pathogens

Solvents	Parts	% Yield	Color	Consistency	Total activity of test pathogens			
					***Cenchrus ciliaris* (CAZRI-358)**			
					P. a.	*B. s.*	*E. a.*	*A. f.*
B	R	2.11	Yellow	Nonsticky	11.25	-	-	-
	S	1.97	Yellow	Nonsticky	-	-	-	-
	L	2.14	Yellow	Nonsticky	1.43	-	-	-
	Se	1.87	Yellow	Nonsticky	1.25	-	-	-
C	R	3.99	Brown	Sticky	-	21.28	-	-
	S	4.43	Yellow	Sticky	-	11.81	-	-
	L	4.56	Dark green	Sticky	48.64	-	-	-
	Se	5.36	Colorless	Nonsticky	28.59	14.29	-	-
W	R	4.26	Light yellow	Nonsticky	5.68	5.68	22.72	-
	S	2.78	Brown	Sticky	7.41	7.41	29.65	-
	L	5.56	Dark brown	Sticky	7.41	14.83	59.31	-
	Se	5.05	Coffee	Sticky	13.47	6.73	26.93	-
***Withania somnifera* (RUBL-20668)**								
B	R	1.56	Parrot green	Sticky	2.08	8.33	-	-
	S	0.68	Greenish brown	Sticky	0.90	7.22	-	-
	L	5.80	Parrot green	Sticky	7.74	30.94	-	-
	F	3.69	Green	Sticky	-	-	-	-
C	R	6.67	Dark brown	Nonsticky	-	35.57	-	-
	S	4.97	Dark green	Nonsticky	-	52.99	-	-
	L	11.76	Dark green	Sticky	-	125.38	-	-
	F	5.73	Dark green	Sticky	-	-	-	-
W	R	9.29	Pale green	Nonsticky	49.53	-	-	-
	S	14.73	Light green	Nonsticky	39.29	-	-	-
	L	24.96	Brown	Nonsticky	133.10	-	-	-
	F	21.95	Green	Nonsticky	117.07	-	-	-

B-benzene; C-chloroform; W-water; R-root; S-seed; L-leaf; Se-seed; F-Flower; *P. a. -Pseudomonas aeruginosa*; *B. s. -Bacillus subtilis*; *E. a. -Enterobactor aerogens*; *A. f. -Aspergillus flavus*

DISCUSSION

Resistance in microorganisms to many antibiotics has resulted in morbidity and mortality from treatment failure and increased health care costs. Though a number of antibiotics are available but increasing capability of microbes to develop multi drug resistance has encouraged research for new, safe and effective bioactive agents of herbal origin[36]. Results of the present study reveled that 21/24 plant extracts tested, inhibited the growth of selected bacteria and fungi, indicating broad spectrum bioactive nature of selected two plants (11/12 in *C. ciliaris* and 10/12 in *W. somnifera*). It indicates that *C. ciliaris* is more potential than *W. somnifera* as far as bioactivity increased. Water extracts in both the plants express maximum antimicrobial activities by suppressing the growth of all microbes under investigation. In the present study, most of the extracts of both the plants were found to be potent inhibitor of tested organisms. Excellent antimicrobial activities were observed for chloroform extracts of leaf in both the plants were indicated due to 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. Four extracts of *C. ciliaris* and five extracts of *W. somnifera* were found to be bactericidal in nature. Chloroform and water extracts of leaf in both the plants were recorded as bactericidal against *P. aeruginosa*, *B. subtilis* and *E. aerogens.* Gram positive bacteria *B. subtilis* was the most susceptible organism, which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative[18,24,30,37-39]. 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[40].

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 choice of selected plants by the pharmaceutical industries for preparing plant based antimicrobials drugs. *Cenchrus* grass easily grows in harsh climatic conditions or xeric conditions and requires less care; hence its use as raw material for preparing drugs would definitely be economical.

CONCLUSION

In the present study total 24 extracts of different parts of desert grasses were tested for their bioactivity, among which 21 extracts showed significant antimicrobial potential against test microbes. This paper thus provides a scientific basis for the use of these plant extracts in home-made remedies and their possible application against microorganisms such as *P. aeruginosa*, *B. subtilis* and *E. aerogens* that cause nosocomial infections. Further studies may lead to their use as safe alternatives to synthetic antimicrobial drugs. The demonstration of broad spectrum of *W. somnifera* and *C. ciliaris* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control. The effect of these plants on more pathogenic organisms, and toxicological investigations and further purification, however, need to be carried out.

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