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(Research Article)

Comparative Pharmacological Study of Fruits and Flowers Extract of *Withania somnifera*

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ABSTRACT

The in vitro antibacterial activity of Methanolic extracts of Withania somnifera (Solanaceae), was evaluated against seven Gram-negative bacteria, two Gram-positive bacteria and three fungi, using disk diffusion method followed by determination of minimum inhibitory concentrations by broth dilution method, against sensitive bacteria and fungi. Most of the extracts, at higher concentrations showed varying degrees of inhibitory activity against bacteria and fungi. The highest bio-activity was exhibited by the calyx extract against Pseudomonas aeruginosa. Staphylococcus aureus, Bacillus subtilis and Agerobacterium tumefaciens were the most sensitive pathogens after P. aeruginosa witch show maximum antimicrobial effects. Gentamycin and Ketoconazole, the standard antibiotics used were effective against the bacteria and fungi respectively.

Key Words: Antibacterial activity, Minimum Inhibitory Concentration, Zone of inhibition,*Withania somnifera* **,** Disc Diffusion Assay, Solanaceae.

INTRODUCTION

The use of higher plants and their preparation to treat infectious and non-infectious disease is an age old practices and are the only method available in the past. Though the use of natural sources like plant material for curing diverse forms of ailments leads to human civilization, the scientific analysis of different natural sources for their possible medicinal potency is comparatively recent origin[1]. Various plant extracts can serve both as potential antimicrobial crude drugs as well as a source of new anti-infective agents[2]. Antimicrobial resistance to anti microbial agents has lead to treatment failure and the shift of medical care from orthodox to herbal medicine. Most of the herbal medicines in use await validation of their claimed effects and possibly the development of novel antimicrobial drugs from them[3]. Natural plants derived compounds contribute a lot in fight against pathogens[4].

Withania somnifera (Family: Solanaceae) used in significant increase hemoglobin concentration, as well as increased hemolytic antibody responses towards human erythrocytes[5], increased phagocytic activity and prolonged survival time[6], Anti-inflammatory effect, analgesic effect, osteoarthritis[7], immuno-potentiating and myeloprotective effect[8], antifungal activity of Withania has been confirmed elsewhere, attributed to the withanolides.

Major causative agent of nosocomial infections is *S. aureus*[9], *E. aerogens* along with *E. coli. Klebsiella pneumonia* more frequently causes lung destruction and pockets of pus in the lung (known as empyema), respiratory infections, such as bronchitis, which is usually a hospital-acquired infection[10]. *P. merabilis* cause 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 systems[11,12].

Raoultella planticola has been determined to cause severe pancreatitis in one case[13]. *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[14,15]. *B. subtilis* can contaminate food; however, they seldom result in food poisoning. *E. aerogens* is a nosocomial and pathogenic bacterium that causes opportunistic infections including most types of infections. *Aspergillus* species are the most common mold causing severe infections[16,17].

The objective of present study is to evaluate the *in vitro* anti microbial properties of crude extracts of *Withania somnifera (W. Somnifera)* in methanol with gentamycin and ketoconazole against different species of bacteria and fungi.

MATERIAL AND METHODS

Experimental Design

Methanolic extracts of flower, unripen fruit, ripen fruit and calyx of *W. somnifera* were prepared with help of hot extraction method[18] in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay[19] against a few medically important bacteria and fungi. The fraction showing best activity was then used for determining of MIC by tube dilution method and minimum bactericidal/fungicidal concentration (MBC/MFC).

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 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 blender for 30 minutes[20]. Fine powder of each sample was stored in clean container to be used for Soxhlet extraction following the method[21] in methanol.

Preparation of Extracts

Plant samples were extracted with methanol solvent by using the Soxhlet apparatus for 18 hours at a temperature not exceeding the boiling point of methyl alcohol (65^{o}C). The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated at 40^{0}C by using an evaporator and stored in amber colour bottle for subsequent use in the further antimicrobial, anti-fungal and phytochemical analysis[22].

Drugs and Chemicals Used

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

Chemicals: Methyl alcohol, Muller-Hinton Agar Medium (MHA), Nutrient Agar (NA, for bacteria), Sabouraud Dextrose Agar (SDA, for fungi).

Micro-organisms

The organisms used in this study were seven Gram-negative bacteria, two Gram-positive bacteria and three fungi (Table-1). Selected microorganisms were procured from IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on Muller-Hinton Agar Medium[23], sub cultured regularly (after every 30 days) and stored at 4^{o}C as well as at –80^{o}C by preparing suspensions in 10% glycerol.

Preparation of Test Pathogens and Disc Diffusion Assay

Initial screening of different extracts for their antibacterial activity carried out using MHA and NA media did not reveal any significant difference, thus further studies were carried out using NA medium only[24]. Bacterial strains were grown and maintained on NA medium, while fungi were maintained on SDA medium. DDA was performed for screening by standard method[25]. Bacterial growth after a minimum of 18 hours and occasionally until 24 hours[26]. Activity index for each extract was calculated (Table-3).

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

Serial Dilution Method

MICs are considered as the “gold standard” for determining the susceptibility of the organisms to antimicrobials[27]. MIC of antibiotics was evaluated (thrice) using standard micro broth dilution method against *Escherichia coli* (gram negative) and *Staphylococcus aureus* (gram positive) organisms[28]. Bacterial and fungal suspensions were used as negative control, while broth containing standard drug was used as positive control.

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[29]. The tubes were incubated aerobically at 37^{o}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 MHA and further incubated for 24 h. The highest dilution that yielded no single bacterial colony was taken as the MBC[30]. MBC was calculated for some of the extracts showed high antimicrobial activity against highly sensitive organisms.

Total Activity (TA) Determination

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[31].

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

RESULTS AND DISCUSSION

1. Phyto-chemical Estimation

The phyto–chemical estimation for the *W. somnifera* were carried out according to Farnsworth[32] wherein the consistency was found to be sticky (in flower and calyx extract) and oily (in unripen and ripen fruit extracts). The yield of the extracts was also analyzed where in the highest yields were recorded for flower extract of *W. somnifera* (22.93%) (Table-2).

2. Antimicrobial Assay (ZOI, AI, MIC, MBC/MFC and TA)

Antimicrobial assay (assessed in terms of ZOI, AI, MIC, MBC/MFC and TA) of the Methanolic extracts of *W. somnifera* against selected microorganisms were recorded (Table-3 and 4). The antibacterial activity of the test samples was determined by measuring the diameter of zone of inhibition expressed in millimeter. The assay was repeated twice and mean of the three experiments were recorded. The diameter of the zone of inhibition was measured, commercial disc of antibiotics were used as positive control (standard) and experiment was done thrice[33].

(A) Calyx: Calyx extract show highest activity ZOI-17.67±0.24 mm, AI-2.209 followed by ZOI-16.33±0.25 mm, AI-0.907 against *P. aeruginosa* and *B. subtilis* respectively (Graph-1). Lowest MIC valve 0.938 mg/ml and highest total activity value 120.91 (table 4) were recorded against *P. aeruginosa.*

(B) Flower: Flower extract show highest activity ZOI-16.67±0.24 mm, AI-0.926 followed by ZOI-16.33±0.23 mm, AI-0.817 against *B. subtilis* and *A. tumefaciens* respectively (graph 1). Lowest MIC valve 0.938 mg/ml and highest total activity value 244.55 (table 4) were recorded against *P. aeruginosa, B. subtilis* and *A. tumefaciens.*

(C) Ripen fruit: Ripen fruit extract show highest activity ZOI-12.83±0.24 mm, AI-0.642 followed by ZOI-9.17±0.25 mm, AI-0.459 against *S. aureus* and *A. tumefaciens* respectively (graph 1). Lowest MIC valve 3.75 mg/ml and highest total activity value 36.93 (table 4) were recorded against *S. aureus, B. subtilis A. tumefaciens* and *C. albicans.*

(D) Unripen fruit: Unripen fruit extract show highest activity ZOI-11.5±0.64 mm, AI-0.575 followed by ZOI-10.67±0.22 mm, AI-0.534 against *A. tumefaciens* and *S. aureus* respectively (graph 1). Lowest MIC valve 1.875 mg/ml and highest total activity value 77.11 (table 4) were recorded against *A. tumefaciens.*

In the present study total 12 pathogens were used for testing the bioactivity of *W. somnifera*, among which six pathogens showed significant antimicrobial potential. However, *E. coli*, *R. planticola, P. merabilis, E. aerogens, A. flavus* and *A. niger* were the most resistant pathogens. Most susceptible organisms in the investigation were *B. subtilis. P. aeruginosa, A. tumefaciens* and *C. albicans* against which, all the plant extracts showed zone of inhibition and lowest MIC valve and highest total activity value which supported the finding that plant extracts are usually more active against Gram positive bacteria than Gram negative[27,34-39]. The Gram-negative bacterial cell wall outer membrane appears to act as a barrier to many substances including synthetic and natural antibiotics[40]. 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[41]. Previous studies have noted alcohols to be reliable and consistent solvents for the extraction of antimicrobial substances from medicinal plants[42].

CONCLUSSION

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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Table 1: Name of the tested pathogens

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.		*Proteus merabilis*	G-ve	MTCC-530
8.		*Klebsiella pnemoniae*	G-ve	MTCC-3310
9.		*Agerobacterium tumefaciens*	G-ve	MTCC-431
10.	Fungi	*Candida albicans*	-	MTCC-183
11.		*Aspergillus flavus*	-	MTCC-277
12.		*Aspergillus niger*	-	MTCC-282

Table 2: Phyto-profile of Methanolic extracts of different parts of *W. Somnifera*

S.No.	Parts	Total Yield (%)	Color	Consistency
1.	Flower	22.93	Yellowish green	Sticky
2.	Unripen Fruit	14.46	Dark green	Oily
3.	Ripen Fruit	13.85	Green	Oily
4.	Calyx	11.34	Dark green	Sticky

Table 3: Zone of Inhibition (mm)* and Activity index of Methanolic extract of different parts of *Withania somnifera* against tested pathogens

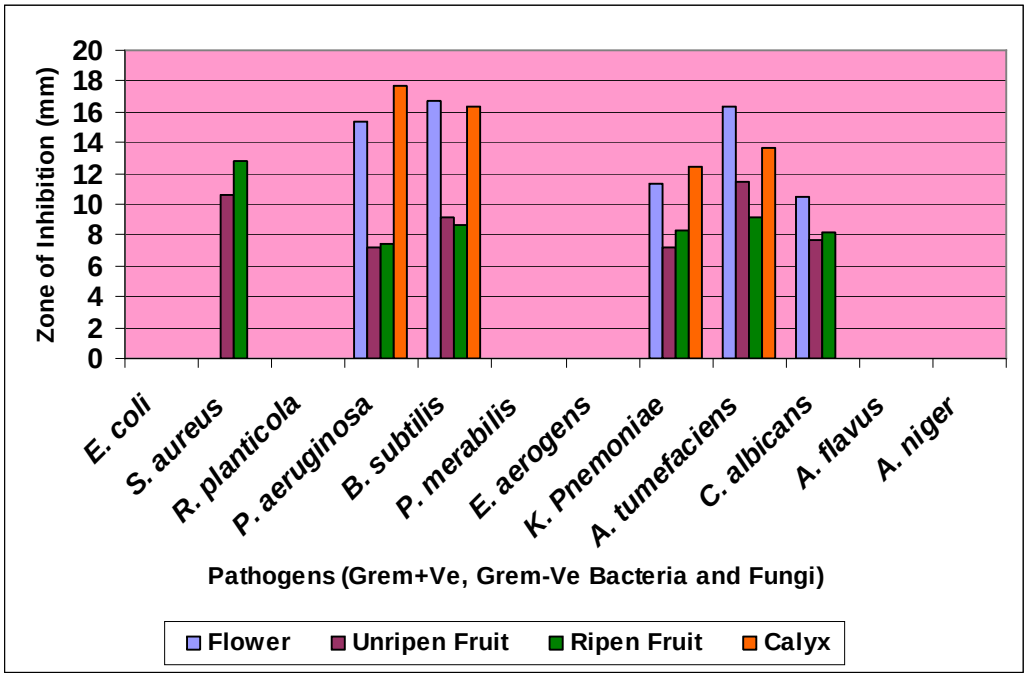
Test Pathogens	Bio-activity of different parts of *W. somnifera* against pathogens							
	Flower		Unripen Fruit		Ripen Fruit		Calyx	
	ZOI	AI	ZOI	AI	ZOI	AI	ZOI	AI
E. coli	-	-	-	-	-	-	-	-
S. aureus	-	-	10.67±0.22	0.534	12.83±0.24	0.642	-	-
R. planticola	-	-	-	-	-	-	-	-
P. aeruginosa	15.33±0.26	1.916	7.17±0.25	0.896	7.5±0.64	0.938	17.67±0.24	2.209
B. subtilis	16.67±0.24	0.926	9.17±0.23	0.509	8.67±0.22	0.482	16.33±0.25	0.907
P. merabilis	-	-	-	-	-		-	-
E. aerogens	-	-	-	-	-		-	-
K. Pnemoniae	11.33±0.29	0.567	7.17±0.24	0.359	8.33±0.23	0.417	12.50±0.65	0.625
A. tumefaciens	16.33±0.23	0.817	11.50±0.64	0.575	9.17±0.25	0.459	13.67±0.23	0.684
C. albicans	10.50±0.65	1.050	7.67±0.22	0.767	8.17±0.23	0.817	-	-
A. flavus	-	-	-	-	-	-	-	-
A. niger	-	-	-	-	-	-	-	-

*All values are mean ± SD, n=3, ZOI-Zone of Inhibition, AI-Activity Index

Table 4: Minimum inhibitory concentration (MIC), (MBC/MFC) and Total activity (TA) by Methanolic extract of different parts of *W. somnifera* against tested pathogens.

Test Pathogens	Bio-activity of different parts of *W. somnifera* against pathogens											
	Flower			Unripen Fruit			Ripen Fruit			Calyx		
	MIC	MMC	TA	MIC	MMC	TA	MIC	MMC	TA	MIC	MMC	TA
E. coli	-	-	-	-	-	-	-	-	-	-	-	-
S. aureus	-	-	-	3.75	7.5	38.55	3.75	3.75	36.93	-	-	-
R. planticola	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	0.938	1.875	244.55	7.5	15	19.28	7.5	15	18.47	0.938	0.938	120.91
B. subtilis	0.938	0.938	244.55	3.75	7.5	38.55	3.75	7.5	36.93	1.875	1.875	60.49
P. merabilis	-	-	-	-	-	-	-	-	-	-	-	-
E. aerogens	-	-	-	-	-	-	-	-	-	-	-	-
K. Pnemoniae	3.75	3.75	61.14	7.5	15	19.28	7.5	15	18.47	3.75	7.5	30.24
A. tumefaciens	0.938	0.938	244.55	1.875	1.875	77.11	3.75	3.75	36.93	1.875	3.75	60.49
C. albicans	1.875	1.875	122.28	3.75	7.5	38.55	3.75	3.75	36.93	-	-	-
A. flavus	-	-	-	-	-	-	-	-	-	-	-	-
A. niger	-	-	-	-	-	-	-	-	-	-	-	-

Note : MMC-Minimum microcidal concentration (MBC for bacteria and MFC for fungi)



Graph-1: Zone of inhibition (mm) of *W. somnifera*

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