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## Bio-efficacy of Unripen Fruits of Winter Cherry (*Withania somnifera*)

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### ABSTRACT

The unripen fruits of Winter cherry (*Withania somnifera*) were evaluated against medically important bacteria viz. *Proteus merabilis*, *Klebsiella pneumoniae*, *Aerobacterium tumefaciens* (plant pathogen) and one fungi *Aspergillus niger*. The dried and powdered unripen fruits were successively extracted with a series of non polar to polar solvents using soxhlet assembly. The antimicrobial assay was done by both disc diffusion assay (DDA) and broth dilution methods. Petroleum ether extract and toluene extract of *W. somnifera* show highest activity after glacial acetic acid extract against *A. tumefaciens* (plant pathogen) to varying degrees in the terms of high inhibition zone and activity index. Gentamycin, the standard antibacterial drug used was effective in inhibiting these bacteria. *A. tumefaciens* was the most susceptible organism in compare to the other organism. Gentamycin and Ketoconazole, the standard antibacterial and antifungal used was effective against the bacteria and 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:** Winter cherry, antibacterial, antifungal, Disc diffusion assay and broth dilution method.

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## INTRODUCTION

Now these days, drug resistance to human pathogenic bacteria has been commonly reported from all over the world. Consequently, new infections can occur in hospitals resulting in high mortality. Therefore, several medicinal plants have been tried against pathogenic microorganisms [1, 2]. Such plants should be investigated to better understand their properties, safety and efficiency [3]. According to World Health Organization [4] medicinal plants would be the best source to obtain a variety of drugs.

Winter cherry (*Withania somnifera*) is gaining attention in various field of research, as they are best suited to the present environmental conditions. *W. somnifera* used for its anti-inflammatory effect [5], analgesic effect [6], Antioxidant [7], memory-improving effects [8]. 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 [9].

*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 [10, 11]. *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 [12, 13].

## MATERIAL AND METHOD

### Experimental design

Crude extract of unripen fruits of *W. somnifera* (RUBL-20668) were prepared with a series of non polar to polar solvents by hot extraction method [14] in soxhlet assembly. Different extracts were then screened for antimicrobial activity by disc diffusion Assay [15, 16] 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 [17, 18] and minimum bactericidal/fungicidal concentration (MBC/MFC).

### Collection of plant material

Unripen fruits 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 unripen fruits were powdered with the

help of grinder. Fine powder of unripen fruits was stored in clean container to be used for standard Soxhlet extraction method [19] in different polar solvents selected.

### Extraction procedure

Unripen fruits (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 [20]. The obtained extracts were filtered by using Whatman No. 1 filter paper and then concentrated at 40°C by using an evaporator [21]. 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 (table2).

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

### 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, Muller-Hinton Agar Medium (MHA), 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, viz., *Proteus merabilis* (MTCC-3310), *Klebsiella pneumoniae* (MTCC-4030), *Aerobacterium tumefaciens* (MTCC-431) and *Aspergillus niger* (MTCC-282). Selected microorganisms were procured from IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on Muller-Hinton Agar Medium [22], 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  $1 \times 10^8$  CFU/ml for bacteria and  $1 \times 10^7$  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 [23]. 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) [24]. The

inhibition zones were measured and compared with the standard reference antibiotics [25, 26]. AI for each extract was calculated (Table 2).

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

### **Broth micro-dilution method**

Broth micro-dilution method [27] was followed for determination of Minimum inhibitory concentration (MIC) values 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  $1 \times 10^8$  CFU/ml and  $1 \times 10^7$  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 [28]. 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 [29]. 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 [30]. 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 [31]. 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 [32].

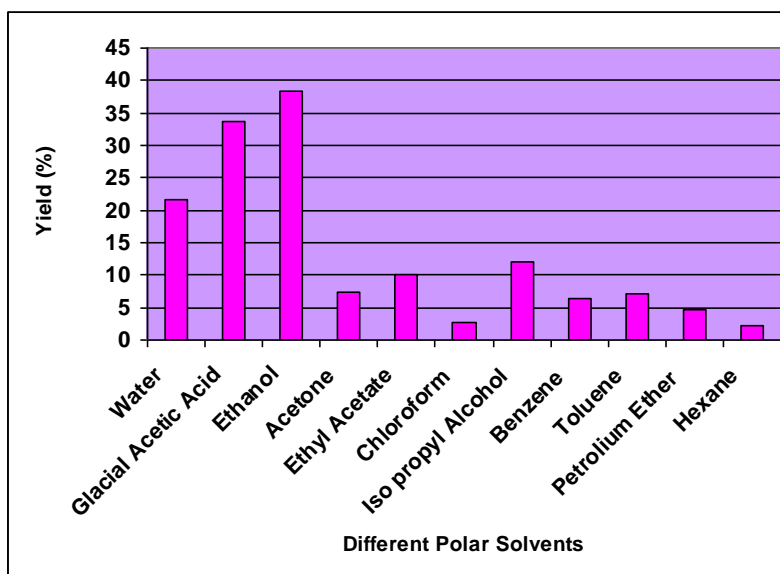
$$\text{Total Activity} = \frac{\text{Extract per gram dried plant part}}{\text{MIC of extract}}$$

### 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  $p > 0.005$  [23].

## RESULTS

**1. Qualitative and quantitative estimation:** The preliminary Phyto-profiling (Qualitative and quantitative estimation) for the unripen fruits of *W. somnifera* were carried out according to Farnsworth [33] wherein the consistency was found to be sticky in the high polar solvent extracts and oily in low polar solvent extracts. The yield (% w/w) of the extracts was also analyzed wherein the highest yield was recorded for ethanol extracts (38.34%) and followed by glacial acetic acid extract (33.73%) (Graph-1).



Graph 1: Total yield (% w/w) of Unripen fruit extracts of *Withania somnifera* in different polar solvents

**2. Antimicrobial activity:** Antimicrobial activity (assessed in terms of inhibition zone in mm, activity index and total activity) of the unripen fruits of *W. somnifera* extracts in different polar

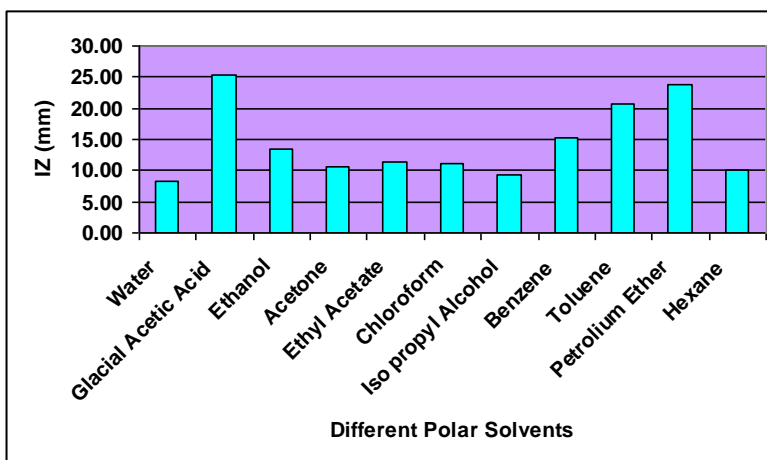
solvents, tested against selected microorganisms were recorded (Table 1). In the present study total eleven extracts of selected plant were tested for their bioactivity, among which all the extracts showed significant antimicrobial potential against test microbes. Most susceptible organisms in the investigation were *A. tumefaciens* against which, all of the plant extracts showed inhibition zone supported by Singh and Kumar [22].

**Table 1: Inhibition Zone (mm), Activity Index (AI) and Total Activity (TA) for Unripen fruit extracts of *Withania somnifera*.**

Polar Solvent	Bio-activity of Unripen Fruit extracts of <i>Withania somnifera</i> against pathogens											
	<i>Proteus merabilis</i>			<i>Klebsiella pneumoniae</i>			<i>Agerobacterium tumefaciens</i>			<i>Aspergillus niger</i>		
	IZ	AI	TA	IZ	AI	TA	IZ	AI	TA	IZ	AI	TA
W	9.67±0.22	0.967	57.96	-	-	-	8.17±0.22	0.409	28.98	-	-	-
AA	8.33±0.24	0.833	89.94	-	-	-	25.33±0.24	1.267	720.69	13.50±0.65	0.9	374.76
E	-	-	-	-	-	-	13.50±0.65	0.675	204.48	-	-	-
A	-	-	-	-	-	-	10.50±0.65	0.525	39.97	-	-	-
EA	11.67±0.25	1.167	54.19	-	-	-	11.33±0.22	0.567	27.09	-	-	-
C	-	-	-	7.50±0.65	0.375	1.75	11.17±0.25	0.559	13.99	-	-	-
IP	-	-	-	-	-	-	9.33±0.26	0.467	31.99	-	-	-
B	-	-	-	8.33±0.26	0.417	8.52	15.33±0.25	0.767	34.08	-	-	-
T	11.50±0.65	1.15	38.67	7.50±0.64	0.375	4.83	20.67±0.23	1.034	77.30	-	-	-
PE	-	-	-	11.33±0.22	0.567	12.39	23.83±0.24	1.192	49.54	-	-	-
H	-	-	-	-	-	-	10.17±0.24	0.509	12.44	-	-	-

All values are mean±SD, n-3 (p>0.005), IZ-Inhibition Zone (mm±S.D.), AI-Activity index, TA-Total activity, 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

**(a) Antibacterial activity:** Highest antibacterial activity was recorded for petroleum ether extract (IZ-23.83±0.24; AI-1.192 and TA-49.54) and for toluene (IZ-20.67±0.23; AI-1.034 and TA-77.30) after the glacial acetic acid extract (IZ-25.33±0.24; AI-1.267 and TA-720.69) against *A. tumefaciens* followed by benzene extract (IZ-15.33±0.25; AI-0.767 and TA-34.08) against the same (Graph 2).



**Graph 2: Inhibition Zone (mm) of *Agerobacterium tumefaciens* in different polar solvents**

**(b) Antifungal activity:** Highest antifungal activity was recorded for glacial acetic acid extract (IZ-13.50±0.65; AI-0.9 and TA-374.76) against *A. niger*.

**Table 2: Primary Phyto-chemical Estimation, MIC and MBC/MFC of Unripen fruit extracts of *Withania somnifera*.**

Polar Solvents	Color of Unripen fruit extracts	Consistency	Total Yield (%)	Total activity of Unripen Fruit extracts of <i>W. somnifera</i>							
				<i>P. m.</i>		<i>K. p.</i>		<i>A. t.</i>		<i>A. n.</i>	
				MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
W	Light yellow	Non-sticky	21.73	3.75	3.75	-	-	7.5	15	-	-
AA	Brick red	Sticky	33.73	3.75	7.5	-	-	0.469	0.469	0.469	0.938
E	Greenish yellow	Sticky	38.34	-	-	-	-	1.875	1.875	-	-
A	Green	Oily	7.50	-	-	-	-	1.875	3.75	-	-
EA	Brownish Green	Oily	10.16	1.875	3.75	-	-	3.75	3.75	-	-
C	Dark green	Sticky	2.62	-	-	15	15	1.875	3.75	-	-
IP	Green	Oily	12.00	-	-	-	-	3.75	7.5	-	-
B	Black	Oily	6.39	-	-	7.5	15	1.875	1.875	-	-
T	Brown	Sticky	7.25	1.875	1.875	15	15	0.938	0.938	-	-
PE	Yellow	Oily	4.64	-	-	3.75	7.5	0.938	0.938	-	-
H	Green	Oily	2.33	-	-	-	-	1.875	3.75	-	-

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

**3. MIC and MBC/MFC:** MIC and MBC/MFC values were evaluated for those extracts, which were showing activity in diffusion assay. The range of MIC and MBC/MFC of extracts recorded was 0.469-15 mg/ml. In the present investigation lowest MIC value 0.469 mg/ml was recorded for glacial acetic acid extract against *A. tumefaciens* and *A. niger* followed by 0.938 mg/ml for toluene and petroleum ether 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).

## 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, petroleum ether, toluene and benzene extract 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 the above mention 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.

### CONCLUSION

Unripen fruit extracts of *W. somnifera* under the present study not only inhibit the bacterial as well as 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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