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Effect of *Withania somnifera* leaf extracts as antibacterial agent against multidrug resistant bacteria

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Abstract

Objective: This study was designed to examine the phytochemical composition and to evaluate antibacterial potential of *Withania Somnifera* leaf against multidrug resistant bacteria & FT-IR Spectra of different bioactive compounds. **Materials and Methods:** Toluene, ethanol, and aqueous extracts of *W. Somnifera* (leaves) were subjected for *in vitro* antibacterial activity using agar well diffusion against different bacteria of clinical relevance including multi-drug resistant methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) *sps*. **Results:** The toluene extract resulted maximal active as antibacterial agent and found significantly higher active than aqueous extract ($P<0.05$). The difference between antibacterial activities of toluene and ethanol or ethanol and aqueous extract was statistically non significant ($P>0.05$). The maximum activity was observed against *Staphylococcus aureus.* The extracts (at least one) also showed antibacterial activity against *Bacillus cereus*, coagulase negative staphylococcus, *Streptococcus pneumoinae, Shigella dysenteriae*, Methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) sps. Minimum inhibitory concentrations of plant extract were found in the ranges of 0.156mg/ml to 0.625mg/ml for test strains. Phytochemical analyses revealed the presence of various metabolites like phlobtannins, tannins, steroids and alkaloids which may contribute for the antimicrobial action of leaves extract of *W. Somnifera.* The partial characterization of the crude extracts by IR spectral analysis revealed the possible presence of different bioactive compounds in the extracts. **Conclusion:** The leaves of *W. Somnifera* showed promising antibacterial activity against various clinically important bacteria and multidrug resistant MRSA and VRE

Keywords: Withania somnifera, Antibacterial activity, multi-drug resistance, IR spectral

Introduction

Infectious diseases account for one third of all deaths worldwide [1]. The emergence of multidrug-resistant bacteria have a great challenge to health authorities around the world especially in under developed countries; where adequate facility is not available to proper diagnose. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin resistant *enterococcus* sps (VRE) are the one of the most common nosocomial pathogens in throughout the world [2, 3]. MRSA and VRE strains are resistant to many other classes of antibiotics [3, 4], and markedly increases the morbidity and mortality in hospitalized patients [5]. Therefore to combat multi drug resistance it is necessary to discover new antimicrobial compounds.

Medicinal plants represent a rich source of antimicrobial agents. Plants are well known for medical importance and have been used as a good source of many effective drugs around the globe [6]. *Withania somnifera* (ashwagandha) belongs to family *Solanaceae* and is classically known for its rejuvenating benefits. The plant is known as Indian Ginseng for its wide range of therapeutic uses in Ayurvedic and other traditional systems of medicine. It is used for the treatment of tuberculosis, rheumatism, inflammatory conditions, and a potential antitumor agent [7, 8]. *W. Somnifera* is a important constituent of many herbal preparation used in a variety of musculoskeletal conditions (e.g., arthritis, rheumatism). It is also prescribed to improve vitality, energy and longevity and it also help

in prevention of diseases in athletes, and during pregnancy [9, 10]. More than 91 pharmaceutical products are known to be produced from this plant [11]. In present study we have focused to evaluate the antibacterial potency of the plant extract on additional pathogenic bacteria and multidrug resistant MRSA and VRE.

Materials and methods

Plant material

The leaves of the plant *W. somnifera* were collected from different area of Gwalior (geographical location: Lat. 26°.22' N and Long. 78°.18' E). The Plant was identified and the respective voucher specimens have been deposited in the Department of Biotechnology, MITS, Gwalior, India.

Preparation of leaves extract

The collected leaves of *W. somnifera* were washed with lukewarm water in bucket and allowed to dry. Dried leaves of *W. somnifera* crushed into powder form with the help of grinder. Powder were dissolved in different solvents (Toluene, ethanol and aqueous) and kept in shaker for 72 hrs at 24^0C -28^0C. Extract was filtrate out twice through cheese cloth and kept in incubator to evaporate the solvent. The dried extracts were collect in falcon tubes and kept in freeze for future investigation.

Tested Bacteria

The bacteria used in this study included Esherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 25923), Staphylococcus xylosus (MTCC 6149), Klebsiella pneumoniae (MTCC 2405), Enterococcus faecalis (ATCC 29212), Staphylococcus epidermidis (ATCC 12228), Streptococcus pyogenous (MTCC 442), Streptococcus pneumoniae (MTCC 655), Bacillus cereus (MTCC 430), Shigella dysenteriae (ATCC 9754), Methicillin resistant staphylococcus (MRS 906), Methicillin resistant staphylococcus (SJC902) Staphylococcus saprophyticus (MTCC 6155), Vancomycin resistant enterococci (VRE912), Methicillin resistant coagulase negative staphylococcus (MRCoNS), Methicillin resistant staphylococcus aureus (ATCC 700789).

Antibacterial activity

The agar diffusion method [12] modified by [13] was used to evaluate the antimicrobial activity. The solid media of Muller Hilton agar was prepared by dissolving 38 gm in 1 litter distilled water. About 25 ml of Muller Hilton agar (MHA) poured into Petri dish. The inoculums for bacteria were prepared from overnight grown culture and the turbidity was adjusted to 0.5 McFarland standards [14]. The 100 µl of bacterial inoculums were spread plated on MHA. The plates were dried and circular wells were prepared by micro tips. 50µl of plant extract added in agar well with the help of micropipette. The plates were covered by the parafilm and incubated at 37^0C for 24 h. the diameter of the inhibition zones were measured and the mean of the diameters of the inhibition zones were calculated.

Determination of Minimum Inhibitory Concentrations (MICs)

MICs were determined using broth dilution method for selected bacterial strains according to the Clinical and Laboratory Standard Institute (CLSI), USA guidelines [14]. The lowest concentration of plant extracts preventing bacterial growth was considered the MIC for that particular bacterial strain.

Phytochemical screening

Freshly prepared Ethanol, toluene and Aqueous extracts of leaves of the plant *W. Somnifera* were subjected to preliminary photochemical screening for detection of major constituents.

Infrared spectroscopy

The interpretation of infrared spectra involves the correlation of absorption bands in the spectrum of an unknown compound with the known absorption frequencies for types of bonds.

Results

The toluene (non-polar solvent) extract showed maximum antibacterial activity followed by ethanol (polar solvent) extract against the tested bacteria without statically significant difference ($P>0.05$) (Table 1). The antibacterial properties of toluene extract was significantly higher than aqueous extracts ($P<0.05$). S. aureus and E. faecalis found most susceptible to the polar and non-polar extracts of the leaves of W. somnifera. The extracts (at least one) also showed antibacterial activity against Bacillus cereus, coagulase negative staphylococcus, Streptococcus pneumoinae, Shigella dysenteriae, methicillin resistant Staphylococcus aureus (MRSA), methicillin resistant coagulase negative staphylococci (MR-CNS) and vancomycin resistant Enterococcus (VRE) sps. The antibiotic resistance profiles of ATCC 700789, MRS 906, SJC 902 (mecA positive) and VRE 912 (vanA positive) have been summarized (Table 2). The test strains were selected due to their resistance power against five different classes of antibiotics for each including β-lactams (methicillin), macrolides (erythromycin), lincosamides (clindamycin), sterptogramins (Pristinamycin), floroquinolones (ciprofloxacin), aminoglycosides (gentamicin) and glycopeptides (vancomycin). MIC Of plant extracts against were calculated against Vancomycin resistant enterococci, Methicillin resistant Staphylococcus, *Bacillus cereus* and found in the ranges from 0.156mg/ml to 0.625mg/ml (Table3).

Phytochemical analyses revealed presence of different chemical constituent alkaloid, phlobatannins, tannins and steroids in W.

Table 1. Screening of antimicrobial activity of *W. Somnifera* (leaves).

Bacteria	Zone of inhibition (mm)		
	Toluene	Ethanol	Aqueous
Staphylococcus aureus (ATCC 25923)	13	12	0
Streptococcus pneumoniae (MTCC 655)	0	0	7
Staphylococcus xylosus (MTCC 6149)	9	0	0
Streptococcus pyogenes (MTCC 442)	0	0	0
Staphylococcus saprophyticus (MTCC 6155)	12	0	0
Vancomycin resistant *enterococci* (VRE 912)	7	7	6
Methicillin resistant *staphylococcus* (MRS 906)	8	11	0
Methicillin resistant *staphylococcus* (SJC 902)	7	10	6
Methicillin resistant *staphylococcus aureus* (ATCC 700789)	12	0	0
Enterococcus faecalis (ATCC 29212)	7	7	6
Escherichia coli (ATCC 25922)	0	0	0
Bacillus cereus (MTCC 430)	9	6	6
Shigella dysenteriae (ATCC 9754)	10	0	9
Staphylococcus epidermidis (ATCC 12228)	11	0	5
Klebsilla pneumoniae (MTCC 2405)	0	0	0

Table 2. Antibiotic resistance pattern of Multidrug resistant bacteria.

	Methicillin	Vancomycin	Erythromycin	Clindamycin	Prstinamycin	Gentamicin	Ciprofloxacin
PS1	R	S	R	R	R	R	R
PS2	R	S	R	S	R	R	R
PS3	R	S	R	R	R	R	S
PS4	R	R	R	R	R	R	S

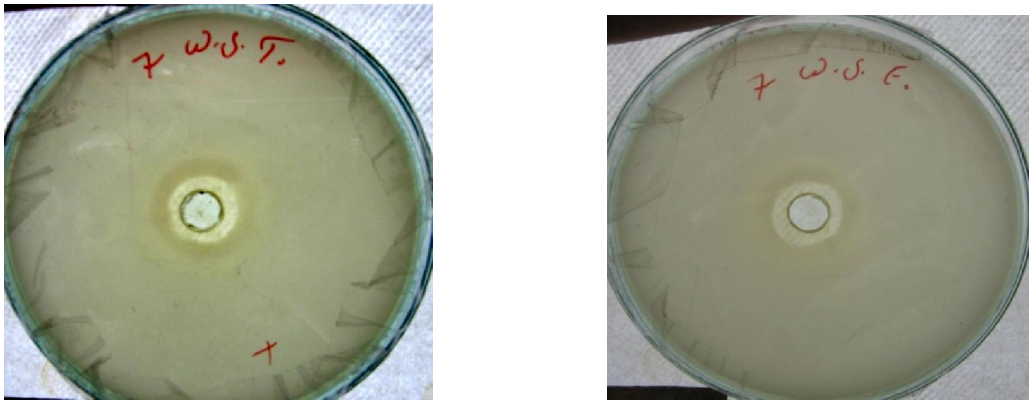
Where, **R:** resistant; **S:** sensitive and **PS1:** Methicillin resistant *staphylococcus aureus* ATCC 700789; **PS2:** Methicillin resistant *staphylococcus* MRS 906; **PS3:** Methicillin resistant *staphylococcus* SJC 902; **PS4:** Vancomycin resistant *enterococci* VRE 912.

Table3. Screening of MIC Of plant extracts against Vancomycin resistant *enterococci,* Methicillin resistant *Staphylococcus, Bacillus cereus.*

plant extracts→ bacteria ↓	*W. somnifera* (Toluene)	*W. somnifera* (Distilled water)	*W. somnifera* (Ethanol)
Vancomycin resistant *enterococci*	0.3125 mg/ ml	0.15625 mg/ ml	0.625 mg/ ml
Methicillin resistant *staphylococcus*	0.15625 mg/ ml	0.3125 mg/ ml	0.15625 mg/ ml
Bacillus cereus	0.3125 mg/ ml	0.15625 mg/ ml	0.15625 mg/ ml

Somnifera leaves extracts. The antibacterial activity of polar and non-polar extracts was stable during exposure of different Temperature ranges (27^0C-42^0C) against selected bacteria *B. cereus,* MRSA, VRE (Figure 1). The infrared (IR) spectra of the plant extracts were measured (as KBr discs) between 600 – 4000 cm-1 on FT-IR spectrophotometer. The important IR bands, such as (C-H), (C=C), (C-O) and (C-H) symmetric and asymmetric stretching, and stretching frequencies were studied to determine the presence of functional group in the distilled water and ethanol crude extracts. The antibacterial components of the extracts were also stable during exposure of different pH ranges for the earlier mentioned bacteria(figure 2, 3).

Figure 1. Screening of antimicrobial activity of *W. Somnifera* (leaves).



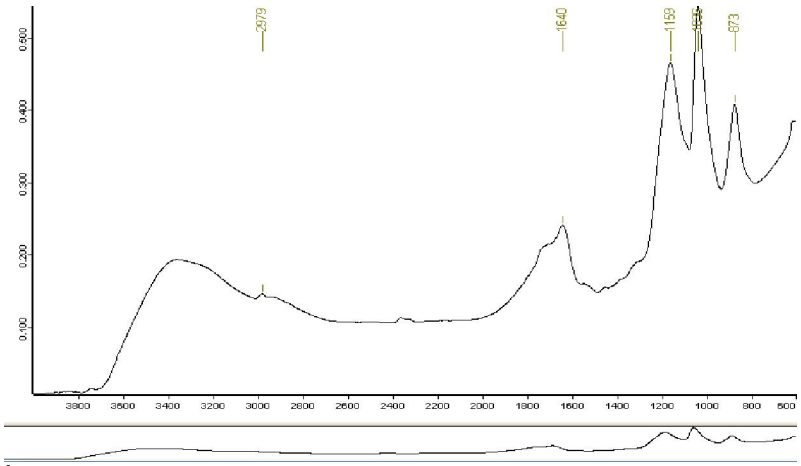
(a) (b)

Figure. Antibacterial activity of 10% *W. somnifera* extract against Vancomycin resistant *enterococci* in (a) Toluene (b) Ethanol.

Abbreviations in figure:

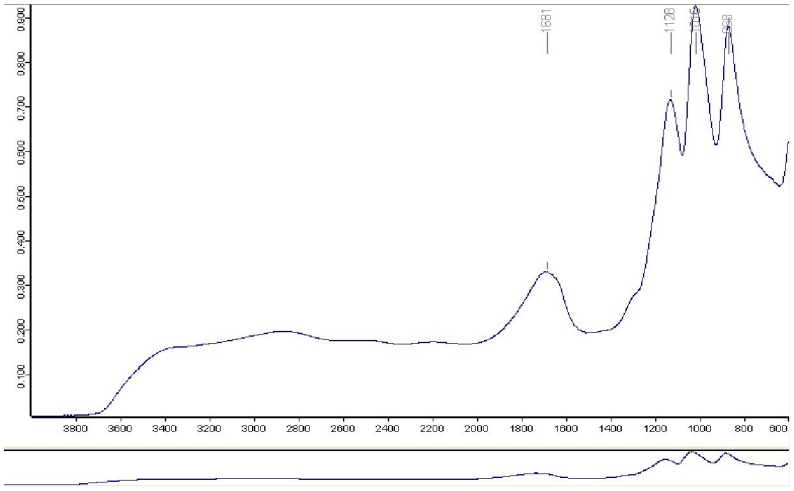
(a) W.S.T.-Withania somnifera in toluene.
(b) W.S.E.-Withania somnifera in ethanol.
(c) 7- vancomycin resistant *enterrococci*.

Figure 2. FT-IR Spectrum of *Withania somnifera* (Ethanol)



Sample 1 The band at 1640cm^{-1} represent C=C stretch of aromatic ring. the peak at 1640 cm-1 in sample 1 signify the possibility of an aromatic compound. The FT-IR spectrum of *W. somnifera* shows that there is presence of aromatic compound, because there is presence of strong absorption band in the region 900cm^{-1} – 650cm^{-1} (at 873cm^{-1}). A vibrational peak between at 2979 cm-1 are characteristic of a (C-H) symmetrical vibration of saturated hydrocarbon.The band at 1096cm^{-1} represent C-O band in plane of aromatic ring.

Figure 3. FT-IR Spectrum of *Withania somnifera* (D.W.)



Sample 2

The FT-IR spectrum of withania somnifera (D.W.) shows that there is presence of aromatic compound. because there is presence of strong absorption band in the region 900cm^{-1} – 650cm^{-1} (at 868cm^{-1}).The band at 1016cm^{-1} represent C-H band in plane of aromatic ring. The vibrational frequency (C-O) was observed in the spectra of all the extracts around 1128 cm-1. Deviation from this region to a higher wave number was observed which is indicative of a secondary amide. Vibrational peaks AT 1681 cm-1 in withania somnifera (D.W.) signify the possibility of an aromatic compound. Based on the physical state of the extracts and the characteristic features of the infrared vibrational peaks in the spectra, terpenoids, long chain fatty acids and secondary amine derivatives are possible compounds in the extracts.

Abbreviations in figure:

D.W.-Distilled water

Discussion

Improper and extensive use of antibiotics in medication, veterinary, agriculture is the source of multidrug resistance in bacteria worldwide [15, 16]. The infection of multidrug resistant bacteria represents more severity and longer hospitality [5, 17]. Thus antimicrobial resistance is responsible for increase the disease size, duration and case fatality rates. Researchers have difficult task to search safe and inexpensive drug with efficacy and make available for poor society. Therefore it is essential to switchover towards bioactive compound from plant origin to control the infectious diseases. In Ayurvedic and Yunani medicine, herbs are used to treat many infectious diseases [18]. In this study, antibacterial activity investigation focussed on W. Somnifera leaves give important clues in the identification and development of traditionally used herbal plants into modern drugs. The Ethanol and Toluene extracts of W. Somnifera leaves showed antibacterial activities against most bacterial strains used including the multidrug resistant MRSA and VRE. The aqueous extracts of W. Somnifera leaves also showed antibacterial activities against some bacterial strains. The results strongly suggest that the plant might have important compounds that can be used for the treatment of different bacterial ailments. In thermal stability of plant extracts, the compounds were stable under temperature 27^{0}C-42^0 C and pH 6-8. This work was only focused on antibacterial effects of extracts from leaves of W. Somnifera against different bacterial strains. Future investigation is required to isolate the active ingredients which may act as cost effective medicines.

IR spectra of crude extracts showed some similarities in their content due to the presence of some functional groups, but the different vibrational peaks of these functional groups in these extracts depicts that the extracts were different hence the diverse activity they exhibited against test organisms during the susceptibility screening (Figure 3).

Conclusion

The toluene, ethanol and aqueous extracts of the *W. Somnifera* leave have inhibitory effect against different bacteria and multidrug resistant MRSA and VRE. It can be further used for the treatment of various diseases caused by these microorganisms. The present finding highlights the importance of further investigation towards the goal of obtaining novel antimicrobial agent from the *W. somnifera*. However, further research is needed to identify bioactive compounds possessing antibacterial activity.

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