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Inhibition of the emergence of multi drug resistant *Staphylococcus aureus* by *Withania somnifera* root extracts

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ABSTRACT

Objective: To search systematically for an alternative therapy with compounds particularly from plant origin. **Methods:** Efficacy test of different root extracts of *Withania somnifera* (*W. somnifera*) (L) Dunal against multi drug resistant (MDR) *Staphylococcus aureus* (*S. aureus*) variants was performed following the agar well diffusion method. Evaluation of susceptibility pattern of the isolates was carried out by employing disk diffusion method using standard antibiotic disks. **Results:** *In vitro* study with *W. somnifera* root extracts was found to be effective against all the MDR *S. aureus* strains isolated from local and patient sources. Different root extracts of WS showed different degree of effectiveness against the isolates. **Conclusions:** The major active principles responsible for the antibacterial efficacy were mainly present in methanol (MeOH) extract and ethanol (EtOH) extracts as well as in butanol (BuOH) extract fraction. Amongst all the extracts the BuOH fraction was found to be most active against all the isolates but aqueous extract was the least active one. Finally it may be concluded that the antimicrobials from *W. somnifera* may raise an alternative therapy for MDR staphylococcal infections in near future.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most frequently isolated bacterial pathogens in hospital-acquired infections[1] as well as a common cause of community-acquired infections including endocarditis, osteomyelitis, septic arthritis, pneumonia and abscesses[2]. It is also a significant pathogen in economically important animals[3]. Staphylococcal resistance to first line drugs like synthetic penicillins has resulted in major problems to treat methicillin resistant *S. aureus* (MRSA) variants, which are increasingly common[4]. Of greater concern is the recent emergence of MRSA variants with reduced susceptibility to vancomycin, the antibiotic of last resort[5]. The emergence of vancomycin-intermediate *S. aureus* (VISA) variants raises specter of -untreatable staphylococcal infections, suggesting a search for alternative therapies[6]. Evidences also support the contribution of plant antimicrobials to treat the microbial infections[7].

Withania somnifera (*W. somnifera*) (L) Dunal, family solanaceae, commonly known in Bengali as Ashwagandha[8] is found throughout the drier region of India. It is a small perennial shrub of about 30 cm to 1.5 m in height. Dried roots and leaves are used as crude drug. Traditionally it is used internally to tone the uterus after miscarriage and for treatment of post partum difficulties. It is applied externally as a poultice to boils, swellings and other painful body parts. Immunomodulatory activity of Ashwagandha has been shown by some workers[9]. It is known for its cardioprotective property and also has hypoglycemic, diuretic and hypocholesterolemic effect[9]. Research suggests both roots and leaves of Ashwagandha possess antibacterial property[10]. The present communication

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reports the antibacterial property of *W. somnifera* root extracts against MDR *S. aureus* variants including VISA isolates in and around Kolkata, India during 2008-2009.

2. Materials and methods

2.1. Plant Materials

Whole roots of the plant, *W. somnifera* were collected from Purulia district of West Bengal in the middle of November 2009 and authenticated by the department of taxonomy, Shyamadas Baidya Shastrapith, Kolkata, West Bengal, India[11]. A voucher specimen was deposited in the department of Clinical and Experimental Pharmacology, School of Tropical Medicine, Kolkata. The freshly collected plant materials (root samples) were washed with tap water and finally with distilled water. Then the washed root samples were shade dried, pulverized mechanically to fine powder and stored in an airtight sterile glass container at 4 ℃ for future use. All experiments were performed with the same plant material within ten months from the date of collection.

2.2. Preparation of extracts

2.2.1. Aqueous extract

For aqueous extraction, 20 g of air-dried, powdered plant material was added to 200 mL double distilled water (DDH_2O) taken in a 500 mL conical flask (graduated) and boiled for 2 h. The volume of the extract was maintained to 200 mL by adding DDH_2O time to time. It was then filtered through cotton wool and centrifuged at 10 000 *g* for 15 min. The volume of the collected supernatant was 178 mL (approx). The solution thus obtained was evaporated to about 40 mL. This concentrated solution was quantitatively transferred to a 50 mL volumetric sterile flask and volume was made up with sterile DDH_2O. This solution was stored at 4 ℃ to use as water extract *W. somnifera*.

2.2.2. Preparation of methanol (MeOH) extract

20 g of air-dried, powdered plant material was added to 200 mL of methanol (A.R) taken in a 500 mL conical flask. The flask was plugged with cotton plug and kept on a rotary shaker at 200-220 rpm for 24 h. It was then filtered through cotton wool and centrifuged at 10 000 *g* for 20 min. The supernatant was then collected and evaporated on a rotary evaporator under reduced pressure to yield a brownish mass. 300 mg of this brownish mass (methanol extract) was dissolved in 3 mL of pure DMSO (dimethyl sulphoxide). This solution (100 mg/mL) was labelled as *W. somnifera*(m).

2.2.3. Preparation of ethanol (EtOH) extract

To prepare ethanol extract, 96% ethanol was used and rest of the procedure was as described as for the MeOH extract. DMSO solution of the EtOH extract (100 mg/mL) was labelled as *W. somnifera*(e).

2.2.4. Preparation of normal butanol (n-BuOH) fraction

A part of methanol extract (1.5 g) was partitioned between normal butanol and DDH_2O. The normal butanol soluble fraction was separated and evaporated under reduced pressure. On complete removal of the solvent, the organic fraction (*n*-BuOH part) yielded a dark brown mass. 300 mg of this mass was dissolved in 3 mL of pure DMSO and the solution (100 mg/mL) was labelled as *W. somnifera*(b).

2.3. Microorganisms

A total of 100 local isolates (hospital environments and fingers of health care providers) and 50 clinical samples (pus/blood/urine) were placed under the study. Amongst the 100 local isolates and 50 patient isolates, multi drug resistance was observed in 15 and 5 *S. aureus* variants respectively. One vancomycin intermediate *S. aureus* (VISA) (MIC = 6 μg/mL)[12,13] was also found amongst the 15 locally isolated MDR variants.

2.4. Selection of MDR S. aureus variants

For selection of MDR varieties of *S. aureus* amongst 150 isolates, agar disc diffusion method (NCCLS; 2003, Approved standard M2-A8)[14] was employed. Standard antibiotic discs were used to evaluate the susceptibility pattern of the isolates. VISA confirmation was done following the agar dilution method (NCCLS; 2000, Approved standard M7-A5)[15].

2.5. Antibacterial assay

2.5.1. Agar well diffusion assay

For determination of antibacterial efficacy, different root extracts of *W. somnifera* were subjected to agar well diffusion following the method of Karou *et al*[16], 2006 with slight modifications. Selected *S. aureus* variants were first incubated at 35 ℃ on Mueller-Hinton agar (MHA) plates for 18 h. After incubation, microorganisms were suspended in sterile 0.85% saline (NaCl) water and the turbidity of the constituted organisms was adjusted to a turbidity of 0.5 McFarland standard (10^8 CFU/mL)[16]. Previously prepared MHA plates were then uniformly inoculated with the prepared bacterial suspensions. For the agar well diffusion, wells of 6 mm diameter were cut with the help of a cork-borer in the surface of the MHA plate(s) and 50 μL of the prepared aqueous solution as well as the prepared organic solutions (100 mg/mL) of the plant material [*W. somnifera*(m),

W. somnifera(e), *W. somnifera*(b)] were introduced into the individual wells separately[16]. The test compounds were then allowed to diffuse into the MHA at room temperature for half an hour before incubation. After overnight incubation of the plates at 35 ℃, the inhibition zone diameter (IZD) around the test wells were measured. Total zone diameter greater than 10 mm including the well was considered to indicate susceptibility of the microorganism to the test compounds. For all the bacterial strains pure solvents were used as controls. The experiments were done thrice and the mean values are presented.

3. Results

Agar well diffusion assay was performed with fifteen locally isolated varieties of MDR *S. aureus* (L_1–L_{15}) as well as with five patient isolates (P_1–P_5). Amongst the selected 20 MDR isolates (L_1–L_{15} & P_1–P_5), all strains except L_{14} were found to be sensitive to vancomycin but all were resistant to methicillin. L_{14} was detected as VISA. L_1–L_7 was also resistant to amoxycillin (10 μg/disc), amoxy-clav (20-10 μg/disc), cephazolin (30 μg/disc), cephalexin (30 μg/disc). Different degrees of sensitivity against gentamycin (30 μg/disc), ceftriaxone (30 μg/disc), cephoxitin (30 μg/disc), ciprofloxacin (5 μg/disc) and trmethoprim (10 μg/disc) were observed in L_1–L_7.

In L_8–L_{15} resistance were observed against amoxycillin (10 μg/disc), amoxy-clav (20-10 μg/disc), cephazolin (30 μg/disc), cephalexin (30 μg/disc), ceftriaxone (30 μg/disc), cephoxitin (30 μg/disc) and ciprofloxacin (5 μg/disc). L_8–L_{15} showed different degrees of sensitivity pattern against gentamycin (30 μg/disc). L_9–L_{15} was found to be resistant to trimethoprim (10 μg/disc) but L_8 was identified as sensitive to trimethoprim. P_1–P_5 showed resistance against amoxycillin (10 μg/disc), amoxy-clav (20-10 μg/disc), cephazolin (30 μg/disc), cephalexin (30 μg/disc), ceftriaxone (30 μg/disc), cephoxitin (30 μg/disc) except P_2. Only P_2 was sensitive to ceftriaxone and cephoxitin. All patient isolates were resistant to ciprofloxacin (5 μg/disc) except P_1 and except P_5 all were resistant to trimethoprim (10 μg/disc). P3 and P_5 were found to be resistant against gentamycin (30 μg/disc) but P_1, P_2 and P_4 were detected as sensitive. However the data reported in Table 1, shows antibacterial efficacy of aqueous solution [*W. somnifera*(a)] as well as the prepared solution of methanol extract [*W. somnifera*(m)] , ethanol extract [*W. somnifera*(e)] and the n-butanol fraction [*W. somnifera*(b)] of methanol extract of *W. somnifera* roots in terms of average IZD in mm. The aqueous extract showed least activity where as the *n*-BuOH fraction was found to be most effective. The MeOH and EtOH extracts showed moderate efficacy. Solvent controls did not show any significant zone to interpret.

Table 1

In vitro antibacterial activity of *W. somnifera* root extracts against MDR *S. aureus* variants expressed by IZD (mm).

Strains	*W. sormnifera*(a)	*W. sormnifera*(m)	*W. sormnifera*(e)	*W. sormnifera*(b)
L_1	12	16	15	21
L_2	11	15	14	20
L_3	12	16	15	20
L_4	11	15	14	19
L_5	11	15	14	19
L_6	12	16	15	20
L_7	12	16	15	20
L_8	11	15	14	19
L_9	10	15	14	19
L_{10}	10	15	14	19
L_{11}	11	16	15	20
L_{12}	11	15	14	19
L_{13}	10	15	14	19
L_{14}	10	15	14	19
L_{15}	11	15	14	19
P_1	11	16	15	20
P_2	12	16	15	21
P_3	10	14	13	19
P_4	11	15	14	19
P_5	11	15	14	19

L_1–L_{15}: Local isolates; P_1–P_5: Patient isolates; *W. sormnifera*(a): aqueous extract; *W. sormnifera*(m): DMSO solution of MeOH extract; *W. sormnifera*(e): DMSO solution of EtOH extract; *W. sormnifera*(b): DMSO solution of *n*-BuOH fraction of MeOH extract; IZD: Inhibition zone diameter.

4. Discussion

Since plants have co-evolved with pathogens, they justifiably have developed the chemical defense mechanisms against the parasitic microbes. Therefore, it is logical to anticipate the presence of a variety of compounds in plants with specific as well as general antimicrobial activity[19]. So far literature is concerned[18], we have several groups of phytochemicals like steroidal lactones, alkaloids, flavonoids, tannins etc that can be isolated from the plant, *W. somnifera*. The diversified bioactivity of the plant was attributed[21] due to the presence of these chemical constituents. The activity of the extracts from the plant material is largely dependent on the type of solvent used in the extraction procedure[22]. The traditional practitioners primarily make use of water as a solvent but in our study it was unequivocally established that the extracts with organic solvents were much more effective in comparison to aqueous and so it can be opined that the better solubility of the active components in organic solvents was the reason behind this[18]. These findings strongly suggest that the organic compounds present in MeOH and EtOH extracts as well as in BuOH fraction are the active principles[22]. The fact is that, the n-butanolic fraction of methanol extract, devoid of inorganic compounds (different salts) showed enhanced activity in comparison to the methanol extract. The major chemical constituents of the *Withania* sp, the withanolides, are a group of naturally

occurring C_{28}-steroidal lactone triterpenoids built on an intact or rearranged ergostane framework[22], in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring[23]. Presence of alkaloids and steroidal lactones (Withanolides) in the root extracts of *W. somnifera* might have played the key role to prove efficacy against MDR *S. aureus* variants. However further investigations are very much required to isolate the active principles in pure form and in our opinion, these bioactive principles may be the alternative therapeutic agents for MDR staphylococcal infections and thus may help preserve the efficacy of antibiotics.

Conflict of interest statement

We declare that we have no conflict of interest.

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