Antioxidant and antimicrobial activities of aqueous extract of Withania somnifera against methicillin-resistant Staphylococcus aureus

Vidhi Mehrotra¹, Shubhi Mehrotra¹, Vandna Kirar², Radhey Shyam², Kshipra Misra², Ashwani Kumar Srivastava¹, Shoma Paul Nandi¹*

¹Amity Institute of Biotechnology, Amity University, Noida 20103, U.P., India
²Defence Institute of Physiology and Allied Sciences (DIPAS), Timarpur, Lucknow Road, Delhi – 110054, India

ABSTRACT

Withania somnifera also known as Ashwagandha, has been an important herb in the ayurvedic and indigenous medical systems. The present study was designed to evaluate the antioxidant and antibacterial activity of aqueous root extract of W. somnifera against methicillin resistant Staphylococcus aureus (MRSA). Aqueous root extract of the plant was found to possess strong antibacterial activity against MRSA as revealed by the in -vitro agar well diffusion assay. The separation of the bioactive compounds from the plant extract was carried out using two dimensional thin layer chromatography (TLC) and contact bioautography. Two TLC spots were found to be bioactive against the pathogen with minimum inhibitory concentrations of 2.3 µg/µl and 5.2 µg/µl respectively. One spot was of alkaloids and the other one was a mixture of essential oil and phenolics. The antioxidant activity was estimated to be Trolox Equivalent Antioxidant Capacity of 9.83mg/gm of dry weight of extract and reducing power was 0.11mg/gm of dry weight of extract using ascorbic acid as standard. Our study suggests that the bioactive fractions separated from aqueous extract of W. somnifera is a potential source of antibacterial compounds with antioxidant property.

Keywords: Ashwagandha, contact bioautography, phenolics, flavonoids, essential oil
Drug resistance in human pathogenic bacteria has been reported from all over the world [1-4]. Methicillin-resistant Staphylococcus aureus (MRSA) is one such example which is responsible for several difficult-to-treat infections in human [5]. It may also be called as a multidrug-resistant Staphylococcus aureus or Oxacillin-resistant Staphylococcus aureus (ORSA). The present scenario of emergence of multiple drug resistance in human pathogens has necessitated a search for new antimicrobial substances from other sources including plants. The plant Withania somnifera (L.) Dunal, commonly known as “Ashwagandha” is well known for its therapeutic use in the ayurvedic system of traditional medicine [2,6,7]. It has been used as an antibacterial, antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent [8]. However there is no report of antibacterial activity of W.somnifera against MRSA.

The present study was undertaken to investigate the antioxidant and antibacterial properties of aqueous root extract of W. somnifera against MRSA. The plant extract was further studied by separation of bioactive compounds by TLC, its phytochemical characterization, stability and antioxidant activity.

MATERIAL AND METHODS

Preparation of the plant extract and antimicrobial activity assay:
Roots of W.somnifera were collected from Amity Herbal Garden, Manesar, India. They were washed with deionized water and kept for drying in the oven at a temperature of 45-50°C for 5-7 days till the weight became constant. The plant material was regularly examined to check for any fungal growth or rotting. The dried plant material (20g) was crushed to powder and soaked in 160ml of deionized water. Extraction was carried out using distillation unit at a temperature ranging from 80°C to 85°C for a time period of 8 to 10 hrs. The resultant extract was filtered using Whatman No. 1 filter paper and concentrated to 30ml using distillation unit under vacuum for 8 to 10 hrs. The concentrated extract so obtained was filtered by 0.22 µm membrane filter and stored separately in sterile glass vials at 4°C until use. Antimicrobial assay by agar cup diffusion was carried out as described by us previously [9] using vancomycin and water as positive and negative control respectively.

Thin-layer chromatography, contact bioautography, stability and identification of the chemical nature of the bioactive compound:
For TLC, 100µl of the extract was loaded on a precoated silica gel 60 F254 sheet (Merck, Darmstadt, Germany). Different solvent systems were used as mobile phase and finally chloroform: methanol: water (64:50:10) for first dimension (1-D) and Iso-propanol: 1N HCl (7:3) for second dimension (2-D) were optimized and used on the basis of the best separation obtained. After drying, the chromatogram was placed with silica layer onto the inoculated (MRSA) agar plate to enable diffusion. The plate was incubated at 37°C for 24hrs and the zone of inhibition was measured. Rf value of the bioactive spots were measured as the ratio of mobility of center of bioactive spot against the bacteria to the total distance traveled by the solvent front. The bioactive spots of W. somnifera extract were scrapped from the TLC plate and eluted in 25ml ethanol and kept for 24 hrs shaking. The solution was filtered using Whatmann filter paper No. 1, concentrated to 1ml volume and filtered through 0.22 µm membrane filter and...
bioassay was performed. To determine MIC, the eluted sample was serially diluted with the same solvent. It was assayed by the agar well diffusion method. The calculations were made accordingly after observing hairline zone of inhibition in the plates. Stablility of the antimicrobial fractions was checked at different temperature and pH. For temperature stability 100µl of crude extract was incubated separately at 4°C, 37°C, 60°C, 100°C for 1hr in a thermal cycler and then agar cup bioassay was performed. For stability at different pH TLC strips with bioactive spots were treated with 100µl of 100mM citrate buffer of pH 2.7 and 8, and followed by bioautography [9].

In order to study the chemical nature of the bioactive compounds after 1-D and 2-D TLC run, the solvents were allowed to dry and then, the sheets were sprayed with phosphomolybdic acid reagent (10g of phosphomolybdic acid powder in 100ml of absolute ethanol) to detect the presence of essential oils, Dragendorff Reagent (Solution A= 1.7g Bismuth nitrate in 100ml of H₂O: Acetic acid (4:1) mixed with Solution B=40% Potassium iodide, 5ml Solution A+ 5ml Solution B + 20ml Acetic acid + 70ml water) to detect the presence of alkaloids and 3% FeCl₃ in methanol was used to detect the presence of phenolics [10].

**Determination of phenolic content, antioxidant activity and reducing power of the plant extract**

The total phenolic and flavonoid contents of the aqueous extract was determined in terms of standard gallic acid and rutin respectively [11]. The total reducing power of the extract was calculated as per the method described by Zou et al [12] in terms of reducing ability of the extract to reduce Fe³⁺/ferricyanide complex to ferrous by using ascorbic acid as standard. Antioxidant activity of the extract was determined by ABTS [13], using trolox as standard. All the assay values were expressed as milligram of standard equivalents per gram of dry extract.

**RESULT AND DISCUSSION**

In the present study, the MRSA used was sensitive to vancomycin and resistant to ampicillin and we found that aqueous extract of W. somnifera contained antibiotic activity against MRSA. Antimicrobial activity of the extract was retained when treated at 4°C, 37°C, 60°C and 100°C for one hour (Fig. 1A) in a thermocycler indicating the presence of temperature stable bioactive component in the extract. Similarly, the extracts were stable over a range of pH 2.0, 7.0 and 8.0 (data not shown). The extracts were separated in TLC and the mobility of the bioactive components was monitored through contact bioautography as described by us previously [9]. When isopropanol: 1N HCL (7:3) was used as a running solvent bioautographed chromatogram showed the presence of at least two bioactive spots against MRSA (Fig 1B) in the extract with Rf values of 0.16 (spot 1) and 0.8 (spot 2) respectively, ethanol eluted TLC spots showed anti-MRSA activity in agar-cup assay (Fig 1C). The MIC values of the eluates were 2.3 µg/µl and 5.2 µg/µl for spot 1 and spot 2 respectively. Spot 1 showed the presence of alkaloids by developing orange color after spraying the TLC plate with dragendorff reagent (Fig. 1E). When the TLC plate was sprayed with phosphomolybdic acid, spot 1 did not develop any color, but spot 2 turned blue (Fig. 1E) indicating the presence of essential oils in that spot. Upon spraying with 3% ferric chloride, the spot 2 turned dark brown (Fig. 1E) indicating the presence of phenolics in spot 2. Thus spot 2 appears to be a mixture of both essential oil and phenolics.
Fig. 1 Anti-MRSA activity and phytochemical analysis of aqueous extract of *W. somnifera*. A, Agar-cup diffusion assay after treating the crude extracts at indicated temperature for one hour. B, Contact bioautography of 2D TLC strip. C, Anti-MRSA activity of TLC spot elutes. D, MIC of TLC spot elutes. E Spray detection of chemical nature of bioactive spots. Dragendorff reagent for detection of alkaloids (orange), phosphomolibdic acid reagent for detection of essential oils (blue) and ferric chloride reagent for detection of phenolics (brown).
It was also evident from the study that the crude aqueous extract has good amount of phenolics and flavonoids and posses reducing power. Total phenolic content as determined by Folin–Ciocalteu method in W. somnifera root extract was 0.6 mg gallic acid equivalent /g ($y=0.005x$, $r^2 = 0.9989$) of dry weight of extract and the total flavanoid was estimated to be 0.14 mg rutin equivalents per gram ($y= 0.0022 x$, $r^2 =0.9996$) of dry weight of W. somnifera extract with reference to standard. Total reducing power is measured to be 0.11 mg ascorbic acid equivalents per gram ($y= 0.0055 x$, $r^2 =0.9975$) of dry weight of W. somnifera extract with reference to standard curve obtained from ascorbic acid. Phenolics are associated with the antimicrobial efficiency in the plant as they cause the hyperacidification at the plasma membrane interface of the pathogen which potentially results in disruption of the $\text{H}^+$ - ATPase required for ATP synthesis [14]. Antioxidant activity determined by ABTS was measured in terms of Trolox Equivalent Antioxidant Capacity (TEAC) which was 9.83 mg/gm of dry weight of extract with reference to standard curve ($y= 0.0013 x$, $r^2 =0.9975$) (Fig.S1). According to Re et al [13] the result obtained in the ABTS assay is improved over the other antioxidant assays especially for aqueous extract. A good antimicrobial compound coupled with high antioxidant activity is a very interesting lead compound to fight with the present scenario of multidrug resistance in Staphylococcus aureus. It would have a dual role to inhibit the growth of MRSA and reduce the inflammation triggered by this pathogen by its high antioxidant activity. However further elucidation of the chemical structures of the biologically active compounds will be required along with studies in animal model to generate a potent drug.
Acknowledgement

Financial assistance was obtained from the Council for Scientific and Industrial Research, Government of India Scheme # 38(1116)/06/EMR-II. Authors are thankful to Mr. Z.H. Rizvi, retired senior horticulturist, Government of India, Archaeological Survey of India, Agra for identifying the plant and also to the Director, DIPAS for giving permission to carry out this collaborative work.

REFERENCES