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Isolation, characterization and evaluation of antimicrobial activity of Withanolide-A of *Withania somnifera*

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Abstract

The increasing prevalence of multidrug resistant strains of bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies. The WHO estimated that approximately 80% of world population relies mainly on traditional medicines, mostly plant drugs in their health care. About 30% of the currently used therapeutics is of natural origin. *Withania somnifera* Dunal is widely used in Ayurvedic medicine, the traditional medical system of India. The alkaloid (Withanolide –A) was obtained from 50% ethanol extract of *Withania somnifera* stem, subjected to column chromatography on silica gel eluted with a stepwise gradient of increasing order (Ethyl acetate: hexane). White crystals in ethyl acetate were obtained from ethyl acetate: hexane fraction. The aim of the present study was to investigate the in vitro antibacterial activity of the alkaloid, was screened against pathogenic bacteria of clinical origin including two species, *Pseudomonas* and *Staphylococcus aureus*. The growth inhibition was observed in four of the five tested pathogens at three different of Withanolide-A concentrations (100, 500, and 1000 ppm).The result indicates that the Withanolide–A exhibited significant antibacterial activity against the *Pseudomonas* and *Staphylococcus aureus*.

Key words: *Withania somnifera,* Withanolide-A, *Pseudomonas, Staphylococcus aureus*

1. Introduction

Some bacteria and fungi are extremely pathogenic causing serious human infections. The discovery of antibiotics to combat these pathogens marked a revolution in the twentieth century[1,2]. Unfortunately, because of the inappropriate usage of antibiotics in human and veterinary medicine, certain strains of bacteria and fungi have developed the ability to resist their antibiotics or change their infection process to penetrate the host cells[3,4]. Therefore, disease causing microbes that have become resistant to antibiotic therapy are becoming a public health problem. To substitute synthetic antibiotics, many of the modern and effective drugs have their origin in traditional folk medicine[5]. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; many of these isolations were based on the uses of the agents in traditional medicine. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care[6,7]. According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy[8]. The ethanobotanical data of plant prompted an investigation into its antimicrobial activity. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases[9,10]. The chemical diversity, structural complexity, lack of substantial toxic effects, and broad spectrum of antiviral and antibacterial activity of natural products, make them ideal candidates for new therapeutics[11,12]. *Withania somnifera* is an important medicinal plant in the Solanaceae family. The stem, roots are the main portions of the plant used therapeutically. Withanolides are main active constituents of *Withania somnifera*[13,14].

Thus, as part of a long-term evaluation for antimicrobial, we have conducted these studies on the potential antimicrobial effects of *Withania somnifera* alkaloids on the microbes. Therefore, the present study was planned to find out the antimicrobial potential of Withanolide-A of *Withania somnifera* for efficacy against human pathogenic bacterial strains like *Pseudomonas* and *Staphylococcus aureus*.

2. Materials and Methods

2.1 Collection of plant material

Withania somnifera a small, woody shrub 60-200 cm high, which is described under many common names such as Ginseng and Ashwagandha. It can be found growing in Africa, the Mediterranean, and India. *Withania somnifera* Dunal is widely used in Ayurvedic medicine, the traditional medical system of India. It is an ingredient in many formulations prescribed for a variety of musculoskeletal conditions and as a general tonic to increase energy, improve overall health and longevity, and prevent disease.

Withania somnifera stem was collected from campus of Zoology Department, and identified by a botanist from Department of Botany University of Rajasthan, Jaipur India (Herbarium No. RUBL19445).

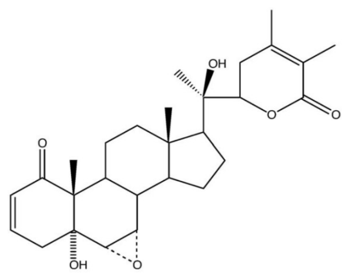
2.2 Extraction and isolation of Withanolide- A

The fresh dry stem *of Withania somnifera* (250 gm) was grind in a mixture, finally soocked in 50% ethanol for over night, boiling it for 24 hours and finally filtered with cotton gauze. The filtrate was concentrated under the reduce pressure at 50 ± 5 ºC to obtained ethanolic extract (15g) for isolation of alkaloids. The foure ethanol extract was combined and finally concentrated to one eighth of the original volume (125 g) under reduced pressure at 50±5°C. The concentrated extract was stirred with chloroform to remove chlorophyll. The chloroform extract was subjected to four times column chromatography on silica gel (1.2 mt., 250 mesh, 600×4 g, 0.580×4 L), eluted with a stepwise gradient of increasing order (5–10% each step and 2 L solvent eluted in each fraction). Ethyl acetate concentration in hexane was eluted. White crystals (200 mg) in ethyl acetate were obtained from ethyl acetate: hexane (1:3) fraction. The repeated crystallization in acetone from fractions was observed for thin-layered chromatography (Merck's silica gel 60F254 percolated glass plate) in system chloroform: ethyl acetate (4:1). The comparison of IR and NMR spectral data and melting point (280.50°C) of single 129 spotted compound showed the presence of Withanolide. They consists primarily of compounds known as Withanolide-A, which was believed to account for its extraordinary medicinal and antimicrobial activity. The Withanolide-A, have C28 steroidal nucleus with C9 side chain, with a six member lactones ring[15,16]. The percent of isolated alkaloids from shoot of *Withania somifera* is 0.08%. The details of isolated compound are given in table form (Table 1).

Table 1: Percent's of isolated of Withanolide-A from shoot of *Withania somnifera.*

S. No.	Amount of shoot of *Withania somnifera* (gm)	Amount of ethanol extract of *Withania somnifera* (gm)	Amount of isolated Withanolide A (mg)	% of isolated Compound
1	400gm	35gm	290 mg	0.07%
2	400gm	38gm	310mg	0.08%
3	500gm	45gm	370 mg	0.07%
4	500gm	50gm	410 mg	0.08%
5	500gm	50gm	424mg	0.08%
6	1000gm	90gm	890 mg	0.09%
7	2000gm	95gm	1810mg	0.09%
8	2000gm	190gm	1790mg	0.09%
9	2000gm	180gm	1750 mg	0.09%
Total	93000gm	773gm	8044mg	0.08% (Mean)

Fig. 1— Withanolide A or [5α, 20α(R)-dihydroxy-6α, 7α-epoxy-1- oxowitha-2, 24-dienolide]



2.3 Antibiotics

The commercial antibiotics Ampicillin was used to evaluate and control the pattern of antibiotic sensitivity of the different target strains. Ampicillin in stock solutions was freshly prepared at 10 mg/mL in sterile distilled water.

2.4 Bacterial Strains

Microorganisms were provided by the S.P. Biotech Jaipur. Two microorganisms were use in our investigated namely *Pseudomonas* (MTCC 4646) *Staphylococcus aureus* (MTCC1764). Both microorganisms were maintained at 4°C on nutrient agar slants.

2.5 Media Preparation

5g peptone was dissolved in 850 mL of double distilled water; 3g of beef extract was added in the solution, then after 15g of agar was dissolved in the solution, finally adjust pH to 7.0. Final volume made up to 1000 mL with distilled water and sterilized the medium in autoclave at 121°C for 20 minutes. Beef extract (3.0g) + Peptone (5.0g) + Agar (15g) + NaCl (5g).

2.6 Bacterial culture and susceptibility test

The antimicrobial activity of alkaloids was examined on Pseudomonas an Staphylococcus aureus using a modified Kirby-Bauer disc diffuse ion method. The appropriate molten agar media (Mueller Hinton or Luria Bertani) was inoculated the inoculums (1 x 108cfu/ mL) and poured into the Petri plate (Hi-media). For agar disc diffuse ion method, paper discs of 5mm diameter were prepared by keeping dipped for overnight in three different concentrations i.e. 100 ppm, 500 ppm, 1000 ppm of the test compound. Sterile paper discs of 5mm diameter (containing 500 ppm drug) along with one standard antibiotic containing disc were placed in each plate[17], after spreading of microbial strain. The plates were incubated overnight at 37°C. Microbial growth was determined by measuring the diameter of zone of inhibition with the help of inhibition zone scale (Hi-media).

2.7 Ethical aspects

The study was carried out under supervision of ethical committee of the University of Rajasthan department of Zoology Jaipur India.

3. Results

3.1. Antibacterial activity of Ampicillin

Ampicillin antibiotic were used as control for bacterial strains *Pseudomonas, Staphylococcus aureus.* Antibiotics showed no zone diameter of inhibition (ZDI) against both bacterial satin.

3.2. Antibacterial activity of Withanolide-A of Withania somnifera stem

The antimicrobial activity of the alkaloids was quantitatively assessed by the presence of zone diameter of inhibition at three different concentrations of 100 ppm, 500ppm and 1000 ppm of Withanolide-A. Withanolide-A of *Withania somnifera* showed zone of inhibition against both bacterial strains used. The Withanolide-A, demonstrating the highest activity (15 mm ZDI) at 1000 ppm concentration followed by (13 mm ZDI) at the 500 ppm and (10 mm ZDI) at the 100 ppm for *Pseudomonas* respectively. While Withanolide-A also showed highest activity (12 mm ZDI) at 1000 ppm concentration, (10 mm zone diameter of inhibition) at the 500ppm and (≤10 mm ZDI) at the 100ppm for *S .aureus* respectively, (Figure 2 and 3).

Figure 2:Antibacterial activity of Withanolide-A on *Pseudomonas.*

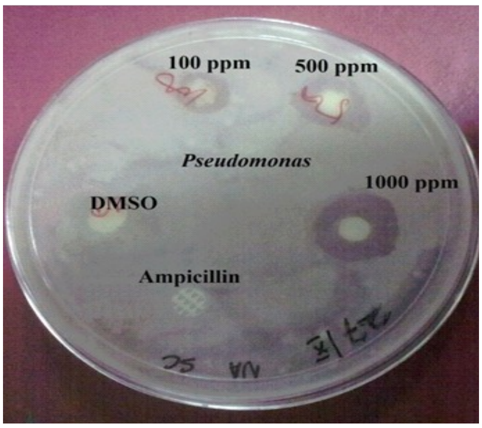
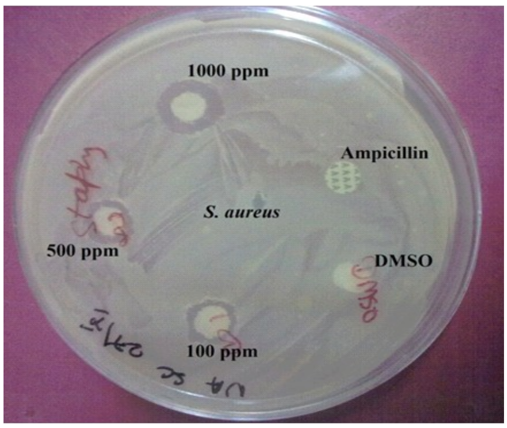


Figure 3: Antibacterial activity of Withanolide-A on *Staphylococcus aureus.*



4. Discussion

Phytochemical constituents such as steroids, alkaloids, flavonoids, tannins, phenol, and several other aromatic compounds are secondary metabolites of plants that serve a defense mechanism against prediction by many microorganisms, insects and other herbivores[18]. These secondary metabolites exert antimicrobial activity through different mechanisms[19]. Worked on steroidal extracted from some medicinal plants which exhibited antimicrobial activities on some bacterial isolates[20] also confirmed the antiviral property of steroids. Other secondary metabolite alkaloids which are one of the largest groups of phytochemicals in plants were observed in the Withanolide-A of *Withania somnifera*. One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms like bacteria[21]. The differences in the spectrum of activities of extracts show concentrations at which the extracts have the best antimicrobial activities[22]. Largest zones of inhibition were observed at 1000 ppm concentration against both bacterial stains. Data indicated that the pattern of inhibition depends largely upon the extraction solvent and plant part. Alkaloids provided more potent antibacterial activity as compared to aqueous ethanol extracts. The polarity of secondary metabolites and antibacterial compounds make them more readily extracted by organic solvents because Secondary metabolites are more soluble in organic solvents then water, and using organic solvents does not negatively affect their bioactivity against bacterial species suggesting that organic solvents are clearly better solvents of antimicrobial agents. Thus present study conformed potential value of the medicinally important plant by the presence of various compounds. The screening of plant extracts and plant products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic protypes. This plant may prove to be a rich source of compounds with possible antimicrobial activities, but more pharmacological investigations are necessary[23]. It is hoped that this study would direct to the establishment this compounds that could be used to invent new and more potent antimicrobial drugs from natural origin. Further work will emphasize the isolation and characterization of active principles responsible bio-efficacy and bioactivity.

5. Conclusion

The results confirm the validity of the use of *Withania somnifera* plant as medicine in ancient medicinal traditions and suggest that some of the plant extracts possess compounds with antimicrobial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens. It is quit safer to use as an herbal medicine as compare to chemically synthesized drug.

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