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Comparison of antimicrobial activity of genetically transformed hairy roots of *Withania somnifera* with normal roots

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ABSTRACT

Withania somnifera, known as Ashwagandha, widely considered as Indian Ginseng, is a plant of repute in Indian system of traditional medicine. The present study was emphasized to investigate antimicrobial activity of hairy roots extarct of Witahnia somnifera with normal roots extract. Hairy root is a typical disease syndrome characterized by numerous, fast growing highly branched adventitious roots at the site of infection. Transgenic hairy roots were induced in W. somnifera by infecting leaf explants with two wild type strain of Agrobacterium rhizogenes ATCC 15834 and MTCC 4364 using MS media. The methanol extracts of hairy and normal roots of Withania somnifera were evaluated for the antibacterial activity and antifungal activity by agar plate disc-diffusion assay against bacterial stain S.aureus , B.subtilis, P.aeruginosa, E. Coli and fungal stain A. niger, A. fumigates, P. notatum, C. albicans . The drug extract concentration was taken 20mg/ml of methanol. The concentrations of standard drugs i.e. Erythromycin and Ketoconazole were 0.1 mg/ml, tested to find out the minimum inhibitory concentration (MIC). From these different extracts tested, the hairy root extracts were found to have potent antibacterial activity and antifungal activity. A synergistic increase in the antibacterial activity and antifungal activity of hairy roots was noticed when MIC was supplemented with these extracts. This study confirms the efficacy of hairy root extracts as natural antimicrobials.

Key words: *Withania somnifera*, Ashwagandha, Solanaceae, Antimicrobial activity, Transgenic hairy roots.

INTRODUCTION

Multi-drug resistance is world-wide problem attributed to extensive use of antibiotic, selection of pressure on bacterial stains and lack of new drugs, vaccines and diagnostic aids. These shortcoming leads to an urgent global call for new antimicrobial drugs, particularly from natural resources[1]. Majority of medicinal plant species are rich in bio molecule content which can cope with health hazards and recently antibacterial activities of many plant species have been reported. Ashwagandha [*Withania somnifera* L. Dunal (Solanaceae)] is an important medicinal plant, widely used as a home remedy for several diseases in India as well as other parts of the world. Historically, the plant has been used as an aphrodisiac, liver tonic, anti-inflammatory agent, astringent, and more recently to treat bronchitis, asthma, ulcers, emaciation, insomnia, and senile dementia [2].Clinical trials and animal research support the use of ashwagandha for anxiety, cognitive and neurological disorders, inflammation, and Parkinson's disease. Ashwaganda's chemopreventive properties make it a potentially useful adjunct for patients undergoing radiation and chemotherapy.It is described as an herbal tonic and health food in Vedas and considered as 'Indian Ginseng' in traditional Indian system of medicine. In fact, it is mentioned as an official drug in the Indian Pharmacopoeia as well (Indian Pharmacopoeia, 1985). Beside its use as general tonic several recent reports have demonstrated

immunomodulator and antitumor effect of ashwagandha as well as various parts of the plant have been reported to possess antiserotogenic, anticancer and anabolic properties and has beneficial effects in the treatment of arthritis, stress and geriatric problems. Recently, the plant was demonstrated to possess strong antifungal activity. However, to the best of our knowledge, no serious efforts have been made to test its antibacterial properties so far. The chloroform and ethanol (95%) extracts were reported to possess good anti fungal activity against *Aspergillus.niger* and weak anti bacterial activity against *E.coli, S.aureus* and *P.aeruginosa.* It can be found growing in Africa, the Mediterranean, and India. As a result of this wide growing range, there are considerable morphological and chemotypical variations in terms of local species. However, the primary alkaloids of both the wild and the cultivated species appear to be the same. The roots are the main portion of the plant used therapeutically [1-3].

Withania somnifera contain active ingredients like steroidal alkaloids and lactones known as withanolides. Withaferin A and withanolide D are the two main withanolides that contribute to most of biological activity of *Withania somnifera* [4]. In the present study, we established antimicrobial activity of ashwagandha roots against pathogenic bacteria and fungi. Although lot of work has been carried out on the medicinal application of *Withania somnifera*, there is still no information on comparison of antimicrobial activity of normal and hairy root extract. The study confirms that methanolic extract of hairy root possess strong antibacterial and antifungal properties.

MATERIALS AND METHODS

COLLECTION AND AUTHENTICATION OF DRUG MATERIAL

Plant material was collected from medicinal plant garden of Poona College of Pharmacy, Bharati Vidyapeeth University, Pune. It was authenticated by Agharkar Research Institute, Pune (Authentication No. 1946-2006).

INITIATION OF HAIRY ROOTS OF *WITHANIA SOMNIFERA*

The hairy root culture were initiated from *W. somnifera* leaf explants which was infected by *A. rhizogenes* (ATCC15834).The standard protocol was followed for establishment of genetically transformed hairy root cultures from *W. somnifera* leaf explants.[5,6]

COLLECTION OF THE DRUG EXTRACT

Ashwagandha root extracts were provided by the following Indian companies: Alchemy Chemicals, Ujjain; Amsar Pvt. Ltd., Indore; Ansar industries, Surat; Natural Remedies, Banglore; and Tulsi Amrit, Indore; Prashant Pharmaceuticals, Rajpipla; Green Pharmacy, Pune. All these extracts were procured from a standard supplier in India and were stored in suitable conditions.

MICROBIAL STRAINS USED

The bacterial strains used as test organism were Gram positive *Staphylococcus aureus* (NCIM No. 2079), *Bacillus subtilis* (NCIM No. 2063), Gram negative- *Escherichia coli* (NCIM No. 2345), *Pseudomonas aeruginosa* (NCIM No.2242) and fungal stain were *Aspergillus niger* (NCIM No.592), *Penicillin notaum* (NCIM No. 745) and *Aspergillus fumigates* (NCIM No. 623), *Candida albicans* (NCIM No. 3471) were collected from the Department of Pharmaceutical Biotechnology, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune.

Immediately they were sub-cultured by inoculating a loopful in Nutrient Broth (Hi Media, M002) and then incubated at 35-37°C for 18-24 hours. The bacterial stain were then streaked onto Nutrient agar plates for antibacterial activity and the fungal stain were streaked onto Sabourd's dextrose agar plates for antifungal activity, then plates were inverted and incubated at 35-37°C for 18-24 hours. They were then stored at 4°C till use.

SOLVENTS AND CHEMICALS

Alcohol, Beef extract, Yeast extract, Peptone, Sodium chloride, DMSO, Agar, Dextrose, Sabourd's dextrose agar media were used. All chemicals were purchased from Himedia laboratories, Mumbai.

BACTERIAL STOCK CULTURE:

A loopful of bacterial strain were transferred to nutrient medium and incubated overnight at 37°C.

FUNGAL STOCK CULTURE:

Fungi were cultured 3-5 days at 30°C in Sabouraud's dextrose agar medium.

EXTRACTION OF HAIRY ROOTS

Methanolic Extraction:

10 g of the hairy roots was weighed accurately and dissolved in 100 ml of methanol solution taken in a 250 ml round bottomed flask. This was then kept on a soxhlet apparatus and refluxed for 3 hours, after which it was allowed to

cool down to room temperature and filtered using a Whatman Filter paper. The filtrate was then collected and dried to dryness first on a water bath and then in an oven. After drying the residue was scraped out and was stored at 4°C till used for further analysis.

SAMPLE PREPARATION

The weighed amount of root extracts (20mg) were dissolved with 10 ml of methanol at room temperature

STANDARD PREPARATION

Stock solution of all standard drugs was prepared in distilled water at a concentration 1000 µg/ml. From that stock solution we take concentration 200 µg/ml.

STERILIZATION

The sterilization of media, culture tubes pipettes and other materials were done by autoclaving at 15 lb/sq. inch pressure at 121°C for 30 min.

ANTIMICROBIAL ACTIVITY BY AGAR DIFFUSION METHOD

The agar diffusion test was used to investigate antimicrobial effects of *Withania somnifera* hairy roots. In this method plates containing 10 ml of agar media and Sabouraud's agar medium were overlaid with 10 ml of inoculated stock solution of bacteria and fungi. Equidistant holes were made in the agar. 1ml volume of each sample (200µl µg/ml.) was pipetted into the agar wells. Standard compounds (200 µg/ml) were used as positive control and the negative control was methanol. After 24 hr. and 3-5 days incubation the diameter of the inhibition zones, (no growth) around the holes in the bacterial lawn were measured. A positive result was defined as an inhibition zone (halo size) of 9 mm or more around the holes, therefore indicating the presence of antibacterial substance in the extracts tested.[1,2,3]

RESULTS

DETERMINATION OF ANTIMICROBIAL ACTIVITY BY AGAR DIFFUSION METHOD:

The data obtained from agar well diffusion method of hairy roots extract and normal root extracts from different places of *Withania somnifera* (methanolic extract) were shown in following table.

Table No. 1. Determination of zone of inhibition in Bacteria:

Sr. No.	Sample	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*E. coli*
1.	TAI	1.2	0.9	1.4	1.1
2.	NRB	0.9	1.2	1.2	1.3
3.	AIS	1.0	1.0	1.0	0.9
4.	APLI	1.3	1.1	0.9	1.1
5.	PPR	0.8	1.3	0.7	1.3
6.	GPP	1.1	1.4	1.1	1.2
7.	HR	1.4	1.6	1.5	1.2
8.	ACU	0.8	1.1	1.2	1.5
9.	Standard	1.6	1.9	1.7	1.8
10.	Control	0.2	0.5	0.3	0.4

Table No. 2. Determination of zone of inhibition in Fungi:

Sr. No.	Sample	*A. niger*	*A. fumigates*	*P. notatum*	*C. albicans*
1.	TAI	1.4	1.2	1.3	0.9
2.	NRB	1.1	1.1	1.1	1.2
3.	AIS	1.3	0.9	1.2	1.1
4.	APLI	1.2	1.1	1.0	1.3
5.	PPR	1.1	1.2	0.8	1.4
6.	GPP	1.0	1.5	1.0	1.2
7.	HR	1.4	1.7	1.5	1.6
8.	ACU	0.9	1.0	1.2	1.1
9.	Standard	1.7	1.8	1.9	1.8
10.	Control	0.4	0.3	0.5	0.4

In antibacterial and antifungal activity extract samples were used in following sequence: 1. Tulsi Amrit, Indore (TAI); 2. Natural Remedies, Banglore (NRB); 3.Ansar industries, Surat (AIS); 4. Amsar Pvt. Ltd., Indore (APLI); 5. Prashant Pharmaceuticals, Rajpipla (PPR); 6. Green Pharmacy, Pune (GPP); 7. Hairy root extract; 8. Alchemy Chemicals, Ujjain (ACU).

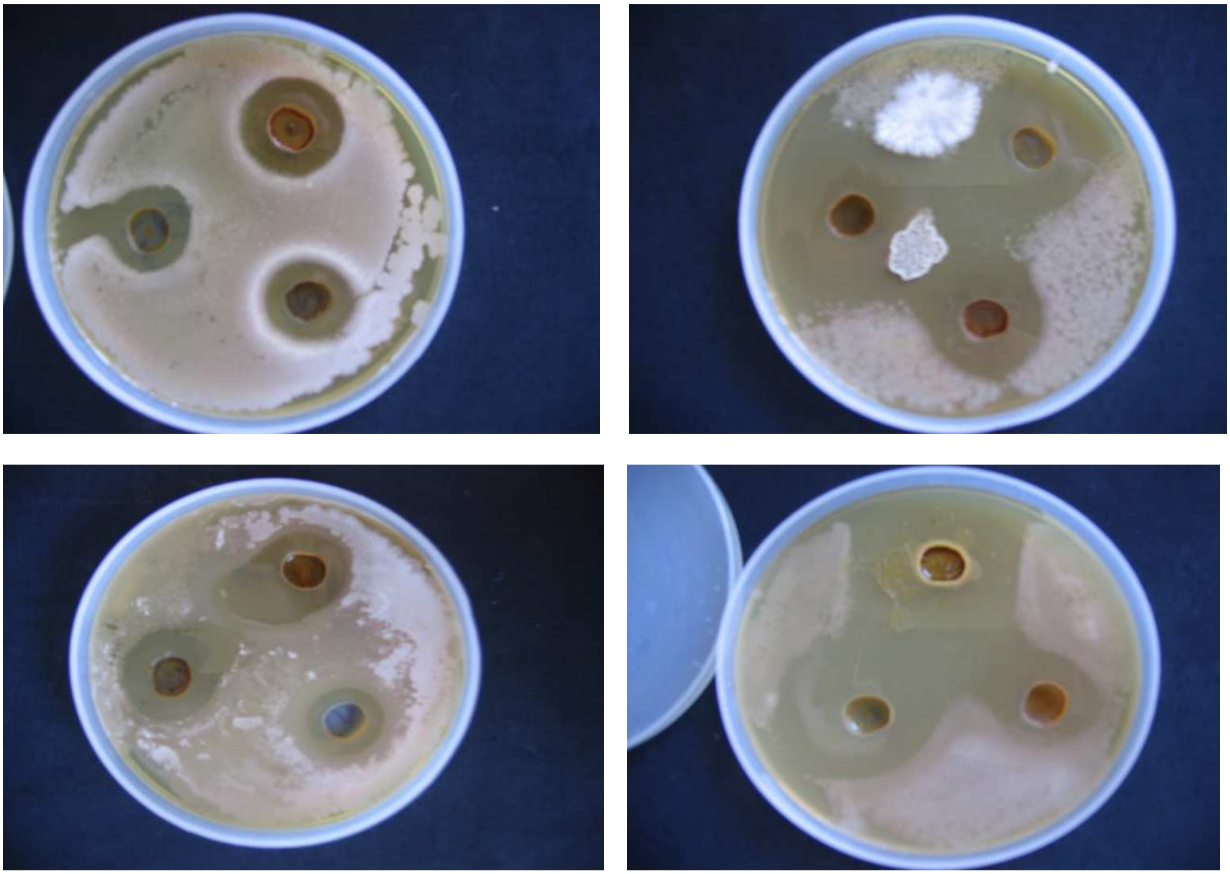

Figure1. Zone of Inhibition in Antimicrobial Activity

DISCUSSION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial and antifungal activity assay. Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings.

In the present study, the methanol normal root and hairy root extracts of *Withania somnifera* showed the activity against bacterial stain *S.aureus* , *B.subtilis, P.aeruginosa, E. Coli* and fungal stain *A. niger, A. fumigates, P. notatum, C. albicans* and extracts have been effectively proven for their utilization as source for antimicrobial compounds. The normal root extracts of *W. somnifera* exhibited inhibitory activity against all the strains while, only the hairy root extract of *W. somnifera* was more effective against bacterial stain *B. subtilis* and fungal stain *A. fumigates.*The antimicrobial activity of methanolic solvent extracts of roots of *W. somnifera* were evaluated by the disc diffusion method.

In present study, zone of inhibition was determined to compare antimicrobial activity of untransformed normal roots obtained from different manufacturers of *W.somnifera* with that of transformed hairy root extract. The zone of inhibition observed in hairy root extract was found to be having more antimicrobial potential than that of normal root extract. *W.somnifera* consist several withanolides which may be responsible for antimicrobial activity against bacteria and fungi.

The results of present investigation clearly indicate that the antibacterial and antifungal activity vary according to the manufacturers of the normal root extract with hairy root extract. Thus, the study ascertains the value of plants used in ayurveda, which could be of considerable interest to the development of new drugs.

CONCLUSION

The study thus concluded that hairy roots extract of *W. somnifera* was shown to have anti microbial activity and a good amount of the active constituent was present in the sample. Zone of inhibition is considerable factor to compare antimicrobial activity. Ayurveda since time immemorial has always known to show tremendous efficiency and are used in day to day life and have tremendous medicinal values. This study confirms the efficacy of hairy root extracts as natural antimicrobials.

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