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Antimicrobial study of in-vitro generated *Withania somnifera* leaf extract.

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Abstract: The objective of this work is to study the antimicrobial effect of in-vitro grown leaf extracts of *Withania somnifera* (L.) against some selected known microorganisms and the use of its constituents present there for the preparation of therapeutic compounds. This is evaluated by using zone of inhibition studies and minimum inhibitory concentration. The microorganisms used include *Shigella flexneri*, *Salmonella typhimurium*, *Vibrio cholera* and *Candida parapsilosis*. Inhibition zones of plant extracts were compared with standard antibiotics like Neomycin, and Kanamycin. Inhibition zones are revealed by methanol extract which is comparatively more than the ethanol extract. For methanol extract, *Salmonella typhimurium* shows the highest inhibition zone (25.13±0.52) whereas *Vibrio cholera* shows the least inhibition zone (21.33±0.53). The same extract shows maximum antimicrobial activity against *Salmonella typhimurium* followed by *Candida parapsilosis* and *Shigella flexneri*. These possibilities can build-up a novel idea in the preparation of pharmaceutical products.

Keywords: *Withania somnifera*, Antimicrobial activity, Leaf extract, Ayurvedic medicine, Pharmaceutical product.

1. Introduction

Withania somnifera was commonly known in India by different vernacular names like Indian Ginseng (English), Asgandha (Hindi), Ashwagandha (Bengali, Oriya, and Sanskrit) etc. There are about 23 species known to be widely distributed in the world, out of which only two species *W.coagulans* (L.)Dunal and *W.somnifera* (L.) Dunal are medicinally significant [1, 2]. The plants are widely used in Ayurvedic medicine in India [3]. The medicinal importance of this plant has a protracted history and is cited in Charaka Samhita. Various pharmacological researches have been made to examine the properties of ashwagandha in an attempt to confirm its practice as a multi-purpose therapeutic agent. The roots of this plant are the source of drugs and have got a wide range of application in the treatment of hiccup, female disorders, and cough, rheumatism, tuberculosis and exhibits excellent anti-tumour and antibacterial activities [4, 5]. It is used for its analgesic, antioxidant,

memory-improving, and the anti-inflammatory effect [6, 7].

1.1 Botanical Description of plant

It is a small-sized woody shrub that belongs to the family Solanaceae. The plant is erect and perennial about two feet in height. Roots are stout, fleshy, cylindrical, 1-2 cm in diameter and whitish brown. The branches of this plant are covered with miniature star-shaped hairs. Flowering nearly throughout the year. Leaves simple up to 5-10*3.6 cm, ovate, obovate, or oblong, entire, rounded or somewhat produced at the base, pubescent on the lower surface and glabrous on the upper surface. Flowers are bisexual, small, greenish or dull yellow, containing five sepals, petals and stamens each; the two-celled ovary has a single style and a bilobed stigma. The petals are united and tubular. The stamens are attached to the corolla tube having erect anthers which form a close column on a cone around the style.



Figure 1. *Withania somnifera* plant

1.2 *Withania somnifera* as a source of antimicrobial compounds

In the last few years, extensive study has been carried out on the screening of plant secondary metabolites for their therapeutic potential. At Central Drug Research Institute (CDRI), Lucknow, screening of a large number of plants for numerous therapeutic properties is being undertaken [9]. It is being expected that phytochemicals with suitable antibacterial efficiency will be used for the treatment of bacterial infection. Plant extracts and phytochemicals, both of which have antimicrobial properties, may play an important role in therapeutic treatments. Modern herbalists categorize Ashwagandha as an adaptogen, which increases the body's ability to withstand the stress of all kinds. It shows helpful effects on the endocrine, cardiac and central nervous systems. The predominant biochemical ingredients of Ashwagandha root are steroidal alkaloids and steroidal lactones in a category of parts referred to as Withanolides [10]. At present-day 12 alkaloids, 35 Withanolides and several sitoindosides from this plant have been isolated and studied. Withanolides serves as important hormone precursors. Withferin A has been receiving a good deal of attention due to its antibiotic and antitumor activities. It is isolated from the leaves of *Withania somnifera* (Fig. 1). *Withania somnifera* contains flavonoids and many active ingredients of the Withanolide class [11]. Chlorogenic acid is present in the leaves [12]. Lots of research has been undertaken on the Solanaceae family to evaluate their antimicrobial

activity. In 2012, Kothari et al. used glacial acetic acid and water extracts to test ripening fruits of *Withania somnifera* for antimicrobial activity against bacterial strains *Proteus mirabilis*, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens*, and one fungal strain *Aspergillus niger*. Glacial acetic acid extract of *Withania somnifera* shows the highest activity against *Agrobacterium tumefaciens* and water extract of *Withania somnifera* shows the highest activity against *Klebsiella pneumoniae* [13]. Singh et al. (2012) found that alkaloid extracts from various parts of *Withania somnifera* (root, stem, leaf, and fruit) have antimicrobial activity against *Enterobacter aerogens*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens*, and *Raoultella planticola* [14]. The Result shows that stem alkaloid extract of *Withania somnifera* exhibit the highest antimicrobial activity against *Enterobacter aerogens* and root extract of *Withania somnifera* exhibits significant activity against all test bacteria. The leaf extracts of *Withania somnifera* were tested against some human and plant pathogens like *Proteus mirabilis*, *Klebsiella pneumoniae*, *Agrobacterium tumefaciens* and *Aspergillus niger*[15]. In 2011 Kumar et al., screened the leaf and root extract of *Withania somnifera* against some human pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis* and *Shigella sp.*) and Fungi (*Aspergillus niger* and *Trichophyton rubrum*). The leaf sample shows higher antimicrobial activity than the root sample [16]. Antimicrobial screening of *in-vitro* leaves of *Withania somnifera* was carried out by Sunderam et al., in 2011. The different solvents used for this extraction are

ethanol, ethyl acetate, dichloromethane and hexane. Antimicrobial activity was determined against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. Ethyl acetate extract of *Withania somnifera* has shown significant activity on *Staphylococcus aureus* and *Bacillus subtilis* [17]. While studying the antimicrobial activity of alkaloids of different parts of *Withania somnifera* against four bacterial and fungal strains namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Trichophyton mentagrophytes* were found to be sensitive against the extracts except *Aspergillus flavus* and *Aspergillus niger* [18].

According to the world health organization, medicinal plants would be the best source to obtain a variety of drugs [19]. It is observed that about 80% of peoples from developed countries use plant-based traditional medicine. Therefore, such medicinal plants must be examined to better understand their properties, safety and effectiveness [20]. Many plants have been used because of their antimicrobial properties. Hence, supplementary studies concerning the usage of plants as therapeutic agents should be highlighted; particularly those associated with the control of antibiotic-resistant microbes. The main objective of the present study is to find out the antimicrobial activity of in-vitro grown leaf extract of *Withania somnifera* and use it as a possible source of new antimicrobial substances.

2. Materials and Methods

The explants material was collected and brought to the laboratory. The materials were washed thoroughly with running tap water for 30 min. and long nodal segments were prepared. After that, the explant material was then surface sterilized with 0.1% of HgCl_2 for 5 mins, following 1% sodium hypochlorite for 1 min. in the laminar airflow hood. The explants were finally washed with sterilized distilled water for several times to lower the toxic effects of HgCl_2 . The solidified Murashige and Skoog basal medium was prepared, and its pH was adjusted then autoclaved at 121°C for 30 min. After sterilization procedure, on the cooling of the media, the explants were inoculated in solidified Murashige and Skoog basal medium containing auxins (1 gm/l IBA) and cytokinins (2 mg/l BAP). The culture was maintained in a culture room at $25 \pm 1^\circ\text{C}$ temperature under a 16h photoperiod of $35\text{-}50 \mu\text{m m}^{-2}\text{s}^{-1}$ photon flux density provided by cool relative

humidity. For the preparation of plant extracts in-vitro generated plant leaves were collected, and the percolation method was used to obtain the extracts of these plant leaves. The leaves were dried at room temperature under shade condition for 15 days after that the leaves were powdered in a grinder and sieved separately.

Five grams of the material was soaked in 40 ml of ethanol and methanol respectively in a different conical flask and left overnight in a rotary shaker. Next day different residues were filtered separately through eight layered clean cheesecloth. The extracts were dried at room temperature for 24 hours after that weighed to know the percentage yield of the extracts. Then these extracts were dissolved in an appropriate volume of dimethyl sulfoxide (DMSO) to get a stock concentration of 50mg/ml. The samples were then stored at 4°C in a refrigerator for future use.

The antimicrobial activity of the extracts against these test pathogens was achieved by using the Agar cup plate method [21]. Nutrient agar plates were prepared and the microbial strains were seeded over the plate by using a sterile glass spreader. Wells of 0.8 cm diameter were made on the agar plate by using a sterile cork borer and filled with $100\mu\text{l}$ of plant extracts inside a laminar airflow chamber. Then these cultured plates were incubated at 37°C for 24 hrs. After the incubation period the Inhibition zones around each well were observed. The same procedure was followed for each extract and bacterial strain. The clear zone formed was measured and the average diameter of the inhibition zone was taken for evaluating the antimicrobial effect of the extracts.

Antimicrobial activities of leaf extracts were evaluated in 3 human pathogenic bacteria and one fungal species. The bacterial strain was Gram-negative viz. *Salmonella typhimurium* (MTCC-1252), *Vibrio cholera* (MTCC-3906), *Shigella flexneri* (MTCC-1457) and one of the fungal strains used was *Candida parapsilosis* (MTCC-2513). Inhibition zones of plant extracts were compared with standard antibiotics like Kanamycin, and Neomycin.

3. Results and Discussion

The percentage of yield of the extracts methanol and Ethanol solvent is 12 % and 8% respectively. Ethanol and Methanol extracts of in-vitro generated leaf of *Withania somnifera* exhibit promising results against all the bacterial and fungal strains (Table-1, figure-3 and 4). Inhibition zones ranged

between 19.8 to 25.13 mm. In contrast to ethanol extract, methanol extract shows a significant result. For ethanol extract, *Vibrio cholerae* shows the least inhibition zone (19.8 ±0.52) whereas *Shigella flexneri* exhibited the highest inhibition zone (22.6± 0.56). For methanol extract, *Vibrio cholerae* shows the least inhibition zones (21.33±0.53) whereas *Candida parapsilosis* shows the highest inhibition zone (22.5±0.56). Among the bacterial strain, *Salmonella typhimurium* is the most susceptible bacterial strain for both ethanol and methanol extract. Methanol extract has shown prominent inhibition zones against the fungus *Candida parapsilosis* (22.5±0.56)

Antimicrobial activities of in-vitro grown leaf extract of *Withania somnifera* (Fig. 2) were evaluated on three pathogenic gram-negative bacteria namely

vibrio cholerae, *Salmonella typhimurium* and *Shigella flexneri* and a fungal strain of yeast genus *Candida parapsilosis*. Ethanol and Methanol extracts show an inhibitory effect on microbial growth against all the bacterial and fungal strain. The test microbes were found sensitive to the leaf extract used in the experiment. Though the degree of sensitivity was variable.

The current work was intended to consider the possibility of the antimicrobial effect of plant extract against some known pathogens and the chance of utilizing the constituents present in there for the preparation of pharmaceutical products.

Table- 1. Antimicrobial effect of different solvent extracts of in-vitro generated *Withania somnifera* against microbial strain

Microbial Strain	Diameter of zone of inhibition (in mm)			
	Standard Antibiotics		Solvent extract	
	Kanamycin	Neomycin	Ethanol	Methanol
<i>Vibrio cholerae</i>	16.33±1.53	21.33±0.56	19.8 ±0.52	21.33±0.53
<i>Shigella flexneri</i>	17.33±0.58	21.67±0.58	21.6± 0.56	23.33±0.58
<i>Salmonella typhimurium</i>	15.67±0.56	21.33±0.58	21.4±0.58	25.13±0.52
<i>Candida parapsilosis</i>	---	---	20.2±0.55	22.5±0.56

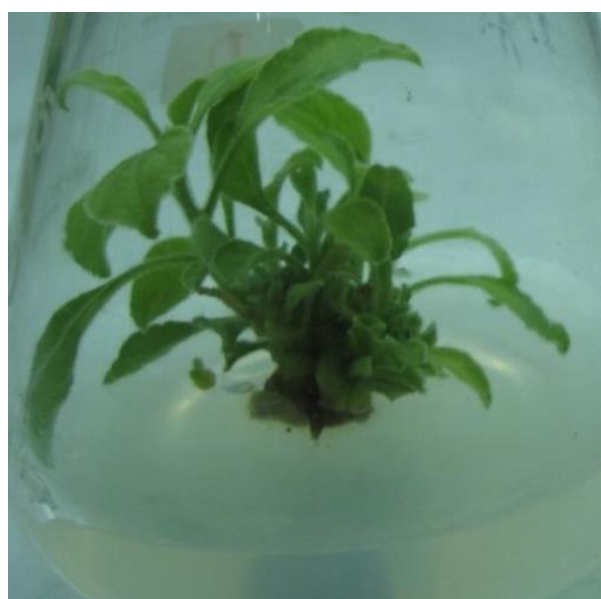


Figure-2. In-vitro generated *Withania somnifera*

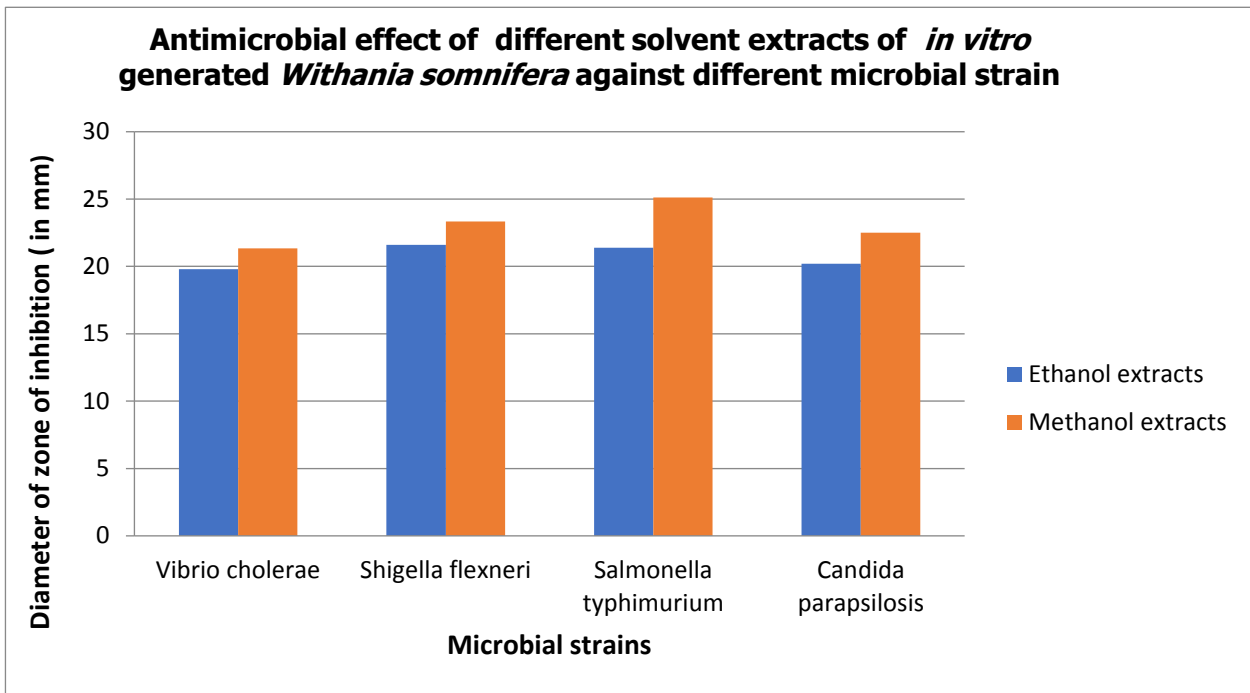


Figure 3. Antimicrobial effect of different solvent extracts against different microbial strain

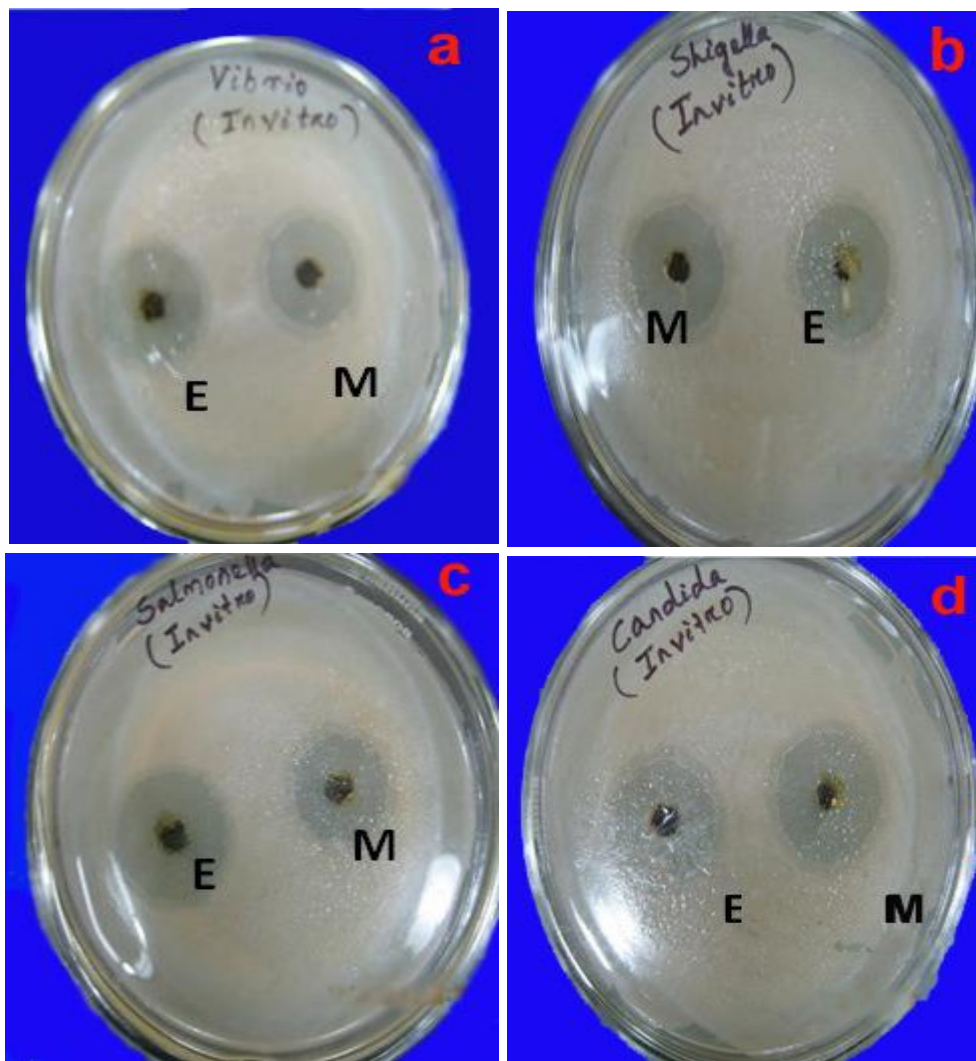


Figure 4. Inhibitory zones shown by leaf extracts of *Withania somnifera* against a. *Vibrio cholerae* b. *Shigella flexneri* c. *Salmonella typhimurium* d. *Candida parapsilosis*

4. Conclusion

In the present investigation the bioactivity evaluation of in-vitro generated *W. somnifera* leaves were carried out in forms of antimicrobial activity. *W. somnifera* is one of the most valuable medicinal plants used in human and veterinary disorders. Ethanol and Methanol extract of in-vitro collected leaves were prepared and antimicrobial activity in both bacterial and fungal were evaluated by Agar cup method. All the extract shows inhibitory effects against all the bacterial and fungal strain but, methanol extract reveals better result comparatively than the ethanol extract. Inhibition zones were comparable with that of standard antibiotics. The methanol extract shows maximum antimicrobial activity against *Salmonella typhimurium* followed by *Candida parapsilosis* and *Shigella flexneri*. Plant-based antimicrobial has enormous therapeutic potential as they serve the purpose with lesser side effects that are often associated with synthetic antimicrobial.

This work is further design for rapid multiplication of *W. somnifera* by developing a suitable protocol for *in-vitro* culture, so that sufficient plant materials will be available to derive plant extracts. Such work is also being helpful to the poor people who are in continuous use of natural plant products as their traditional medicines for long. The use of plant extracts for specific pathogen/diseases can be authenticated by such experiments. However, the biochemical composition of the extracts and the active constituents present in these extracts need to be examined for proposing their use in pharmaceuticals. This work could not be done within the limited scope of the laboratory and within a stipulated period. Those aspects need to assist in further study, to derive the full potential of the plants.

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Does the Article Screened for Similarity?

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Data Availability

No additional data are available.

Authors Contribution

Concept, methodology and manuscript preparation (S. K. Bahira and T. Moharana). Manuscript Review and Editing (R. R. Sahu and Tejashweta). All the authors have read and approved the manuscript

Conflict of interest

None of the authors have any conflicts of interest to declare.

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