Research Article

In vitro Study of the Antibacterial Activities of *Withania somnifera* Leaf Extract against Human Pathogenic Bacteria

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ABSTRACT

The present work is designed to study the possibility of antibacterial effect of plant extracts against some known pathogens and the use of its constituents present there for preparation of pharmaceutical products. Studies on the antibacterial activity of leaf extracts of *Withania somnifera* (L.) was evaluated using zone of inhibition studies and minimum inhibitory concentration. The microorganisms used includes *Streptococcus mutans, Streptococcus pyrogens, Salmonella typhimurium* and *Vibrio cholerae*. This extract exhibits antibacterial activity against these experimental strains. Inhibition zones are revealed by methanol extract which are comparatively more than the ethanol extract. For methanol extract *Vibrio cholerae* shows the least inhibition zone (30.12 ±1.11 mm), where as *Streptococcus mutans* exhibits the highest inhibition zone (34.33±0.56 mm). Similarly the ethanol extract, *Streptococcus mutans* shows the highest inhibition zone (32.33±1.14 mm) and *Vibrio cholerae* shows the least inhibition zone (29.31±1.52 mm). Among the Gram-negative bacteria's *Salmonella typhimurium* was the most susceptible bacteria strain for both ethanol and methanol extract, where as *Streptococcus mutans* was also one of the most susceptible Gram-positive bacteria. This probability can open up a new vista in preparation of medicines. The use of plant extracts for specific pathogen can be authenticated by such experiments.

Keywords: *Withania somnifera*, Antibacterial activity, Human pathogenic bacteria, Leaf extract, Ayurvedic medicine.

INTRODUCTION

Withania somnifera (L.) Dunal is a medicinal plant widely used in Ayurvedic medicine in India [1]. In India, this plant is cultivated for medicinal purposes. It is regarded as one of the most useful herb having "vata" pacifying properties [2]. Roots of this plant are the source of drugs and have got a wide range of application in the treatment of hiccups, female disorders, cough, rheumatism and drowsiness. Besides roots, the other parts of this plant also useful for the treatment of inflammatory conditions, tuberculosis and exhibits excellent anti-tumor and anti-bacterial activities[3]. It is used for its analgesic effects, memory-improving effects, anti-inflammatory effects and antioxidant properties[4,5]. It is useful as an amoebicide, bactericide, diuretic, spasmolytic and contraceptive [6, 7]. The plant has been used for endocrine and cardiovascular activities and more recently to treat ulcers, emaciation, insomnia and senile dementia [8, 9].

Botanical Description of *Withania somnifera*

Withania somnifera (L.) Dunal, belongs to the family Solanaceae, is a small sized woody shrub popularly known in India by different vernacular names like Indian Ginseng (English), Asgandha (Hindi), Ashwagandha (Bengali, Oriya, Sanskrit), Asuragandhi (Tamil), Asvagandhi (Telugu), Asgand (Punjabi) (http://www.ayurvediccure.com/ashwagandha_herb.htm) etc. Though twenty-three *Withania* spp. are known to be widely distributed in world, only two species of *Withania* viz, *W.coagulans* (L.) Dunal and *W.somnifera* (L.) Dunal are medicinally significant[10,11]. *Withania somnifera* (L.) Dunal is a small and woody shrub, erect, greyish, perennial about two feet in height. Roots are stout, fleshy, cylindrical, 1-2 cm in diameter and whitish brown in colour. The stem and branches are covered with small star shaped hairs. Flowering nearly throughout the year. Leaves simple up to 5-10 x 3.6 cm, ovate, obviate, or oblong, entire, rounded or somewhat produced at base, pubescent on lower surface and glabrous on upper surface. Flowers are bisexual, inconspicuous, greenish or dull yellow in colour born on axillary umbellate cymes, comprising 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 and bear erect anthers which form a close column on cone around the style. Fruit 6 mm diameter, globose, smooth, red enclosed in an inflated covering. Seeds are kidney shaped and grey in colour [12].

Origin and Distribution

Ashwagandha is believed to have oriental origin. It is a green shrub distributed throughout the drier region of India, especially in wasteland ascending to an altitude of 2000 m in the Himalaya. *Withania somnifera*, which

translates in Assyrian to Ashwagandha, was suspected to be widely used back in Mesopotamia for its medicinal and narcotic properties. It is also found in the Mediterranean region in North America. In Africa it is found in the drier parts of the continent including South Africa and in several Indian Ocean islands. It is also found in Pakistan, Afghanistan, Srilanka, Congo, Egypt and Morocco. In India it is grown in Madhya Pradesh, Uttar Pradesh, plains of Punjab and North western parts likes Gujurat and Rajasthan [13, 14].

Withania somnifera as a source of antimicrobial compounds

There is a growing demand today for plant based on medicines, health products, pharmaceuticals, food supplements, cosmetics etc, in the International market. The antimicrobial properties of plants are due to secondary metabolites produced in them [15].Alkaloids, tannins, phenolics and flavonoids are some of the main classes of compounds that have considerable inhibitory effects on bacteria [16]. In the recent years, extensive research has been undertaken on screening of plant secondary metabolites for their therapeutic potential. At Central Drug Research Institute (CDRI), Lucknow, screening of a large number of plants for various therapeutic properties is being undertaken[17]. It is being hoped that phytochemical with adequate antibacterial efficiency will be used for the treatment of bacterial infection. The use of plant extracts with known antimicrobial properties can be of great significance in therapeutic treatments. *Withania somnifera* commonly referred as "Indian Ginseng"[18, 19].

According to Modern herbalists Ashwagandha as an adaptogen, increase the body's ability to withstand stress of all types. It shows positive effects on the endocrine, cardiac and central nervous systems. It helps body to produce its own thyroid hormones [20]. Steroidal alkaloids and steroidal lactones are the major constituents of Ashwagandha roots which are known as Withanolides. At present 12 alkaloids, 35 Withanolides and several sitoindosides from this plant have been isolated. The pharmacological activity of Ashwagandha has been attributed to two main Withanolides–Withaniferin A and Withanolide. Withanolides serve as important hormone precursors. Withaniferin A has been receiving a good deal of attention because of its antibiotic and antitumor activities. Withaniferin A has been isolated from the leaves of *Withania somnifera. Withania somnifera* contains flavonids and many active ingredients of the Withanolide class. Major alkaloids present in *W.somnifera* are somniferine, somnine, somniferinine, withanine, pseudo withanine, tropino, pseudotropine, choline etc.

Pharmacological industries have brought forth many new antibiotics since the last 30 years. Still, the resistance to these drugs in microorganisms is increasing. To reduce this problem, action must be taken against these resistant microorganism for which new drugs should be discovered which might be synthetic or natural. The synthetic drugs are mostly associated with side effects; hence more emphasis should be given to natural plant based drugs. Lot of research has been undertaken on Solanaceae family to evaluate their antimicrobial activity. The highest antibacterial potentials were observed for the sterols of fruits of *Euphorbia hirta* and total activity for root sterols of *Withania somnifera* was found to be highest against *Bacillus subtilis* and *Enterobacter aerogens.* Antimicrobial screening of ripen fruits of *Withania somnifera* was carried out by Kothari *et al*., in 2012 using glacial acetic acid and water extracts against *Proteus mirabilis*, *Klebsiella pnemoniae, Agrobacterium tumefaciens* and one fungi *Aspergillus niger.* Glacial acetic acid extract of *Withania somnifera* showed highest activity against *Agrobacterium tumefaciens* and water extract of *Withania somnifera* showed highest activity against *Klebsiella pneumonia.* According to Singh *et al*. alkaloid extract of different parts (root, stem, leaf and fruit) of *Withania somnifera* showed antimicrobial activity against *Enterobacter aerogens, Bacillus subtilis, Klebsiella pnemoniae, Agrobacterium tumefaciesns* and *Raoultella planticola* [21] .Results showed that stem alkaloid extract of *Withania somnifera* showed highest antimicrobial activity against *Enterobacter* aerogens and root extract of *Withania somnifera* showed significant activity against all test bacteria with MIC values of 0.039 mg/ml. The leaves of *Withania somnifera* were evaluated against some important bacteria of human and plant viz. *Proteus mirabilis*, *Klebsiella pnemoniae, Agrobacterium tumefaciens* and one fungi *Aspergillus niger*[22]. The different solvents used for extraction are water, acetic acid, benzene, toluene, chloroform, petroleum ether and hexane. Glacial acetic acid and toluene extract of *Withania somnifera* recorded highest activity against *Proteus mirabilis* and *Klebsiella pnemoniae* working on aqueous and methanolic root extract of W*ithania somnifera* against Gram positive and Gram negative bacteria and stated that, aqueous extract of *Withania somnifera* displayed maximum antimicrobial activity against the test microbes with zone of inhibition lying in the range of 33 – 50 mm [23]. The methanolic extract of *Withania somnifera* also displayed significant antimicrobial activity with the zone of inhibition between 15-38mm[24]. The methanolic extract of the plant were found to possess maximum inhibitory activity against *Salmonella typhimurium* [25] .

With reference to the literature obtained, our study is to find the *in vitro* antibacterial potentiality of *Withania somnifera* leaf extract against human pathogens which is yet not explored. The present work also aims to study the possibility of using the bioactive phytocompounds present in this plant for preparation of pharmaceutical medicines and to help those poor people who are in continuous use of natural plant products as their traditional medicines.

MATERIALS AND METHODS

Preparation of plant extract

The healthy plant leaves were collected and the percolation method was used to obtain the extracts of *Withania somnifera* leaves. The leaves were dried at room temp under shade condition for 15days. Then the leaves were powdered in grinder and sieved separately. Five grams of the material was soaked in 40 ml of acetone, ethanol and methanol in different conical flasks and left overnight in a rotary shaker with 500 rpm. After 24 hrs, the residues were filtered separately using eight layered clean cheesecloth. The extract were then transferred to the beakers and dried at room temperature for 24 hours. The dried extracts were taken out from the beakers to the eppendrof tubes separately and weighed to know the percentage yield of the extracts **(Table 1).** Then these were dissolved in appropriate volume of Dimethyl Sulfoxide (DMSO) to get a stock concentration of 50mg/ml. The samples were then stored at 4^0C in a refrigerator for future use. This method of obtaining plant extracts is known as percolation method.

Microbial strain and media used

For conducting this experiment, four bacterial strains were used i.e. two were Gram positive bacterial strains, namely *Streptococcus mutans* and *Streptococcus pyrogens* and two were Gram negative bacterial strains namely *Salmonella typhimurium* , *Vibrio cholera* **(Table 2)**. Nutrient agar media was considered as a suitable medium for bacterial growth. For preparation of 100ml of Nutrient broth, 0.5 gm of beef extract and 0.3gm peptone were used. For the preparation of nutrient agar medium, 1.5 gm of agar was added per 100 ml of nutrient broth.

Maintenance of pure culture

Pure culture of all bacterial strains were maintained on nutrient agar slants and stored under refrigeration. As per the requirement Nutrient broth cultures were prepared. Then these strains were taken separately under aseptic condition by inoculating a loop full of microbial strain for culture into the sterile nutrient agar slants. After 30 days the culture was transferred to fresh nutrient medium for sub-culturing.

Agar cup plate method

Agar cup plate method or ACPM was used for screening antibacterial activity of the extracts against the test pathogens [26]. Nutrient agar plates were prepared by pouring molten sterilized nutrient agar medium into petriplates followed by cooling at room temperature inside a laminar air flow chamber. The overnight grown test organisms were seeded over the plate using a sterile glass spreader. Wells of 0.8 cm diameter were punched over the agar plates using a sterile cork borer. Each well was filled with 100µl of plant extracts. The plates were incubated at 37°C for 24 hrs. Inhibition zones observed around each well after incubation period, confirms the antimicrobial activity of the respective extract. 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.

RESULTS AND DISCUSSION

The present work was designed to study the possibility of antibacterial effect of plant extract against some known pathogen and the possibility of using the bioactive compounds present for preparation of pharmaceutical drugs. This probability can open up new vistas in preparation of medicines. Antibacterial activities of leaf extracts of *Withania somnifera* were evaluated in four human pathogenic bacteria species. Out of the four bacteria strains, two strain were Gram-positive viz. *Streptococcus mutans* (MTCC-497) and *Streptococcus pyrogens* (MTCC-1926) whereas two strains were Gram-negative viz. *Salmonella typhimurium* (MTCC-1252), *Vibrio cholera* (MTCC-3906) Ethanol and Methanol leaf extracts of *Withania somnifera* exhibited promising results against four bacterial strains (**Table-3**, **Figure 1, 2**). Inhibition zones ranged between 26.64 mm to 34.33mm. Inhibition zones exhibited by methanol extract were comparatively more in comparison to ethanol extract. For methanol extract *Vibrio cholerae* showed the least inhibition zone (30.12 ±1.11 mm), where as *Streptococcus mutans* exhibited the highest inhibition zone (34.33±0.56 mm). For the ethanol extract, *Streptococcus mutans* showed highest inhibition zone (32.33±1.14 mm) and *Vibrio cholerae* showed the least inhibition zone (29.31±1.52 mm). Among Gram-negative bacteria *Salmonella typhimurium* was the most susceptible bacterial strain for both ethanol and methanol extract, whereas *Streptococcus mutans* was the most susceptible Gram-positive bacteria. Inhibition zones of plant extracts were compared with standard antibiotics like Kanamycin, Neomycin, Gentamycin and Gatifloxacin (**Table 4**, **Figure-3, 4**).

Table 1: Percentage yield of methanolic and ethanolic leaf extracts of *Withania somnifera*.

Leaf Extract	Percolation method yields in (%)		
	Methanol	Ethanol	Acetone
	17.6	18.9	17.1

Table 2: Bacterial strains and diseases

Sl. No.	Bacterial Strains	Culture number	Type	Causing Disease
1	*Streptococcus mutans*	MTCC-497	Gram positive	Dental caries and Endocarditis
2	*Streptococcus pyogens*	MTCC-1926	Gram positive	Pharyngitis, Scarlet fever, Cellulitis, Impetigo and Erysipelas
3	*Salmonella typhimurium*	MTCC-1252	Gram negative	Typhoid fever, Intestinal inflammation, and Diarrhea
4	*Vibrio cholerae*	MTCC-3906	Gram negative	Severe watery Diarrhea

Table 3: Antibacterial effect of methanol, ethanol and acetone leaf extract of *Withania somnifera* against different Bacterial strains

Sl.No.	Test Bacterial Strains	Culture number	Diameter of zone of inhibition (in mm)		
			Methanol	Ethanol	Acetone
1	*Streptococcus mutans*	MTCC-497	34.33±0.56	32.33±1.14	28.78±1.12
2	*Streptococcus pyrogens*	MTCC-1926	32.33±1.14	30.66±0.56	29.21±0.56
3	*Salmonella typhimurium*	MTCC-1252	30.33±0.56	30.83±0.55	30.05±0.56
4	*Vibrio cholerae*	MTCC-3906	30.12 ±1.11	29.31±1.52	26.64±0.56

Table 4: Comparison of zone of Inhibition of methanolic plant extracts with Standard Antibiotics

Control set of Bacterial Strains	Diameter of zone of inhibition (in mm)				
	Methanol	Kanamycin	Neomycin	Gentamycin	Gatifloxacin
Streptococcus mutans	34.33±0.56	17.33±0.58	22.67±0.58	26±12	42.33±1.15
Streptococcus pyogens	32.33±1.14	17.67±0.58	21.67±0.58	28.33±0.58	42.33±0.58
Vibrio cholerae	30.33±0.56	16.33±1.53	21.33±0.58	26.67±0.58	43
Salmonella typhimurium	30.12 ±1.11	15.67±0.58	21.33±0.58	26.33±0.58	41.33±0.58

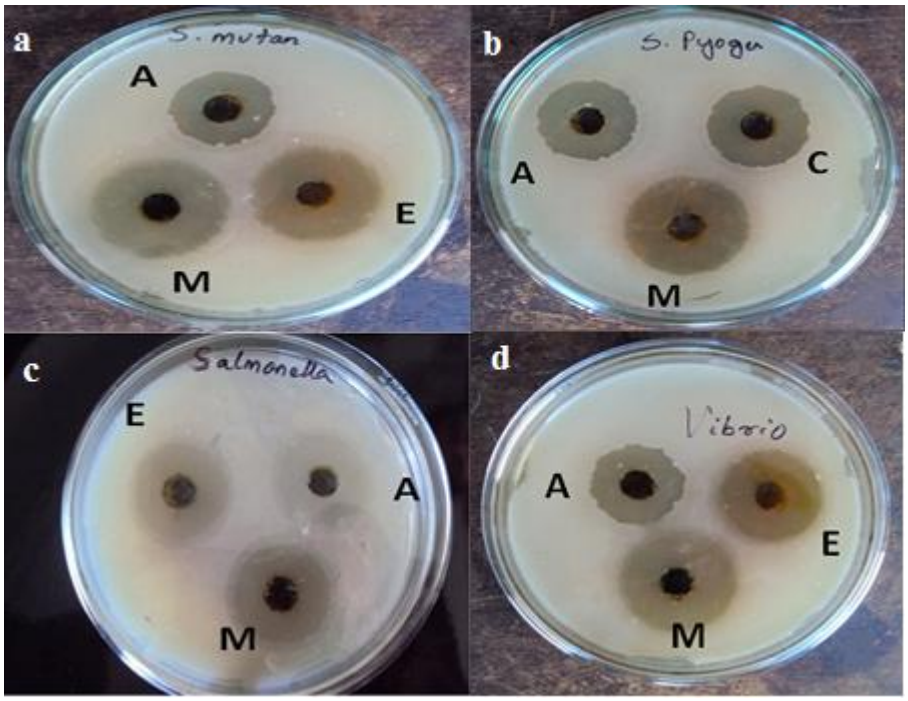

Figure 1: Inhibitory zones shown by *leaf* extracts of *Withania somnifera against*
a. *Streptococcus mutans*, b. *Streptococcus pyrogens*, c. *Salmonella typhimurium*, d. *Vibrio cholerae*

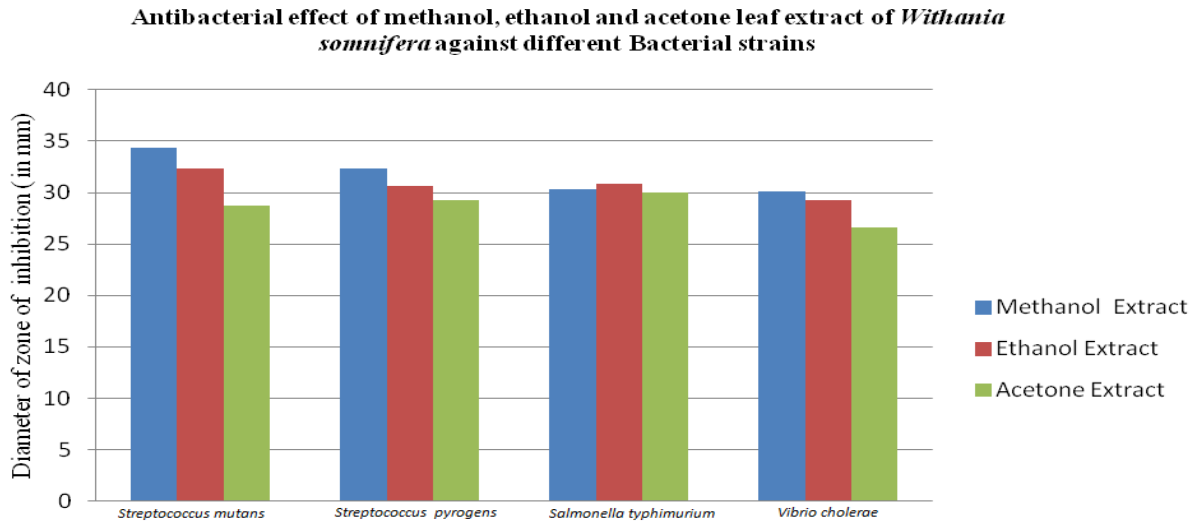

Figure 2: Antibacterial activities of different leaf extracts of *Withania somnifera.*

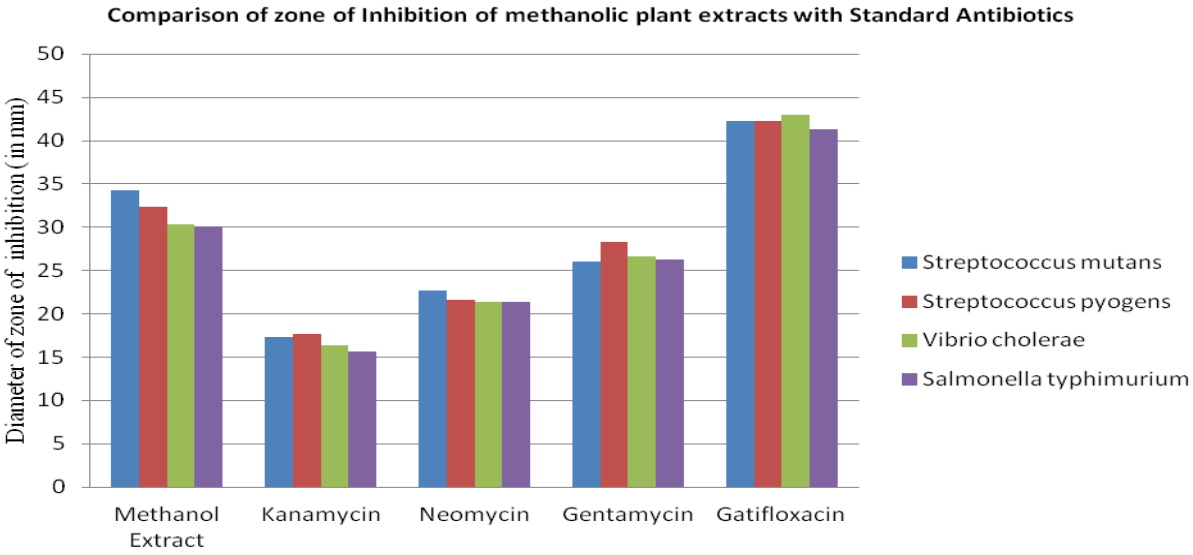

Figure 3: Comparison of zone of Inhibition of methanolic plant extracts with Standard Antibiotics

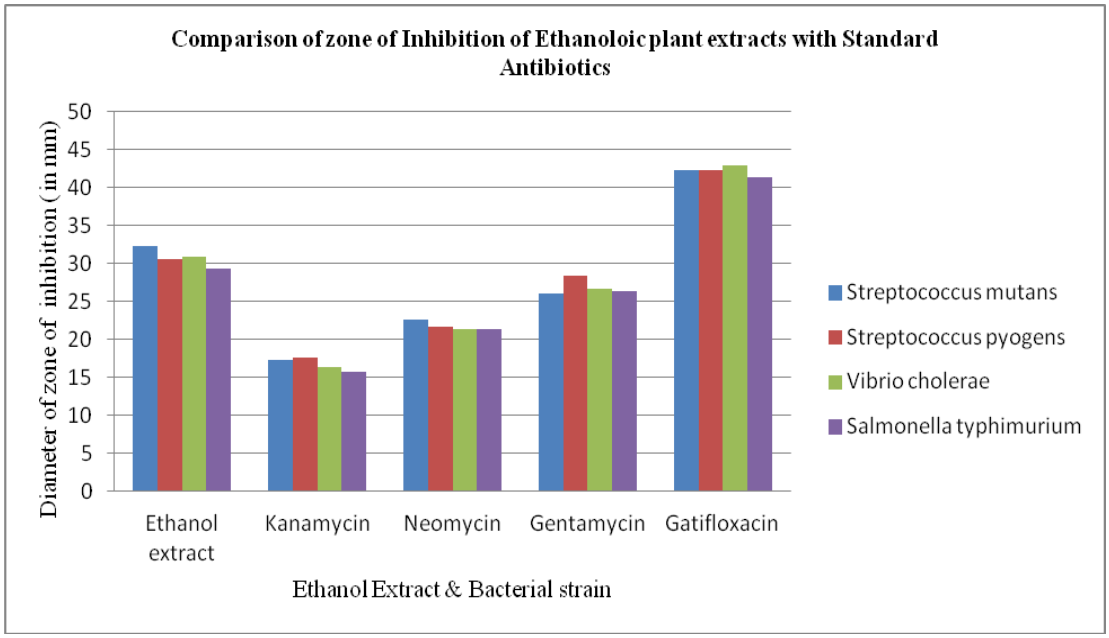

Figure 4: Comparison of zone of Inhibition of Ethanolic plant extracts with Standard Antibiotics

Antibacterial activities of leaf extract of *Withania somnifera* were evaluated on four human pathogenic bacteria viz, *Streptococcus mutans, Streptococcus pyrogens, , Salmonella typhimurium and vibrio cholera.* Ethanol and Methanol extracts of plant leaves exhibit inhibitory effect on microbial growth against all the bacterial studies .However the extent of antimicrobial effect was quite specific for different groups of microbes. The test organisms were sensitive to the leaf extract used in the experiment. Previous works on this plant species have shown varied result against different bacterial and fungal strains. Previous report show that glacial acetic acid and toluene extract of *Withania somnifera* leaves recorded highest activity against *Proteus mirabilis* and *Klebsiella pnemoniae* among the used strains viz. *Proteus mirabilis*, *Klebsiella pnemoniae*, *Agrobacterium tumefaciens* and one fungi *Aspergillus niger* [27]. The leaf and root extract of *Withania somnifera* were experimented against some human pathogenic bacteria (*Escherichia coli*, *Bacillus subtilis* and *Shigella sp*.) and Fungi (*Aspergillus niger* and *Trichophyton rubrum*). Leaf sample showed higher antimicrobial activity than the root sample [28].Jain et al., (2011) working on aqueous and methanolic root extract of root extract of *Withania somnifera* against Gram positive and Gram negative bacteria and stated that, aqueous extract of *Withania somnifera* displayed maximum antimicrobial activity against the test microbes with zone of inhibition lying in the range of 33mm to 50mm.The methanolic extract of *withaniasomnifera* also displayed significant antimicrobial activity with the zone of inhibition lying in the range of 15mm to 38mm. [29]. In 2008 Kambizi et al, reported *in vitro* antimicrobial activity of water and methanol extracts from two plants, such as *Withania somnifera* and *Aloe ferox* on *Neisseria gonorrhea* and *Candida albicans*.The methanol extracts from both plants showed activity against *N. gonorrhoea*, while methanol extract of *Withania somnifera* was effective against *C. albicans*[30] .

Odisha is rich in biodiversity in general and phytodiversity in particular. The rich flora of the state is having many plant species whose utility has not been explored. Many of the plant species possess active components that have antimicrobial properties. Such plant should be identified. Their antimicrobial activity can be ascertained and the extract can be used to formulate new pharmaceutical and herbal medicine. While exploiting these plants for preparation of pharmaceutical drugs, attention must be given for rapid propagation, so that their number will not be reduced to a vulnerable one. The use of plant extracts for specific pathogen or diseases can be authenticated by such experiments. However the biochemical composition of the extracts and the active ingredients present in these extracts need to be analysed for suggesting their use in pharmaceuticals drugs. Such studies will not only open up new ways to search for alternative sources for medicines but also create awareness among common people who save many of the unknown phytodiversity from extinction.

CONCLUSIONS

Thus in the present investigation the bioactivity evaluation of collected *Withania somnifera* leaves was carried out in forms of antibacterial activity. The plant based antimicrobials have enormous therapeutic potential as they serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. *Withania somnifera* is one of the most valuable medicinal plant used in human and veterinary disorders. The leaf extracts of the plant were found to possess strong antibacterial activity against pathogenic bacterial species, as revealed by *in vitro* Agar cup method. All the extracts shows inhibitory effects against all the test bacterial strains. Inhibition zones were greater when it was compared with the standard antibiotics. The tested plant shows significant antibacterial activities against the bacteria *Salmonella typhimurium and Streptococcus mutans*. Both methanolic and ethanolic extract were found to possess maximum inhibitory activity against *Streptococcus pyrogens and Streptococcus mutans*. Therefore, the plant *Withania somnifera* acts as a reservoir of molecules to fight against the bacterial infections. These results are quite inspiring which indicate that, this herb should be studied more intensively to confirm these results and reveal other potential therapeutic effects. Hence, clinical trials by using plants like ashwagandha should be conducted.

Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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