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Bhavna Dwivedi
Research Scholar, Center for Biotechnology and Microbiology Studies, APS University, Rewa, Madhya Pradesh, India

Sherendra Kumar Sahu
Faculty, Center for Biotechnology and Microbiology Studies, APS University, Rewa, Madhya Pradesh, India

Skand Kumar Mishra
Professor and Head, Department of Botany, MSG College, Rewa, Madhya Pradesh, India

Correspondence
Bhavna Dwivedi
Research Scholar, Center for Biotechnology and Microbiology Studies, APS University, Rewa, Madhya Pradesh, India

Antibacterial activity of *Withania somnifera* leaf extracts against Gram-positive bacteria

Bhavna Dwivedi, Sherendra Kumar Sahu and Skand Kumar Mishra

Abstract
Antibacterial property of different medicinal plants are well established and now- a- days they are using as an alternative medicine. In this experiment, the methanolic and aqueous leaf extract of *Withania somnifera*, commonly known as ashwagandha were evaluated for antibacterial activity against gram positive *Staphylococcus aureus*. High performance thin layer chromatography (HPTLC) based purified and characterized extracts were subjected to analysis disk diffusion inhibitory test and oxidative stress marker-reactive oxygen species (ROS) generation in bacteria. Results obtained indicated that extracts contained significant amount of phenolic, flavonoids and other phytoconstituents like alkaloid, tannin, terpenoids, steroids. However, the calculated minimum inhibitory concentration of extract was 100±7.5 µg. Further, a significant increased of ROS level was observed in treated bacteria concentration range from 20-800 µg/ml respectively (835±65, 1500.75±126.55, 2000±250.65, 4600±137.55, 4100±830.75, 4500±1200.59 with respect to control). Thus, the results suggested that extracts contained phenolic and flavonoid compounds which could be responsible for oxidative stress and bacterial death.

Keywords: *Withania somnifera*, antibacterial activity, flavonoids, methanolic extract

Introduction
The beneficiary effects of plants are well explained in Hindu mythology. Depending upon uses, plant kingdom is classified into medicinal and non-medicinal plants. Medicinal plants have been used to treat health problems and to prevent diseases from thousands of years. Secondary metabolites of plants are responsible for the biological properties of plant species [1]. India has rich diversity of plant species in a broad range of ecosystems. There are around 8000 of species which are considered as medicinal plant and give resources for villagers particularly tribal communities and Ayurveda for medicinal use [2].
Withania somnifera (WS) is well known important medicinal plant belongs under the Solanaceae family, commonly known as Indian ginseng, winter cherry and ashwagandha. In India, the plant has been reported to grow in Himachal, Jammu and Punjab and it is commercially cultivated in Madhya Pradesh, Rajasthan, Andhra Pradesh and Uttar Pradesh. Specific parts of plant like roots, stems, leaves etc. contain active constituents (phytochemicals) that used for the treatment of large number of human diseases. This medicinal plant exhibit anti-inflammatory, anti-oxidative, antimicrobial, anti-anxiety, immunomodulation, antihyperglycaemic, anticancer, cardiovascular protection, diuretic, adaptogenic, anti-stress and antiarthritis properties [3]. In pathogenic diseases there are a lot number of health issues caused by bacteria, fungus and other microbial agents. Root extracts of ashwagandha can be used as natural drug for the treatment of several infectious diseases caused by bacteria and fungi and leaf extract have potential as an antibacterial agent and effective in inhibiting the antibiotic resistant *S. aureus* strains.
This study distributed into these heads after collection of plant materials firstly extraction with different solvent then characterization and purification of fractions via high performance authenticate analytical techniques and further we evaluated the antibacterial activity (here, we used extreme pathogenic bacteria *Staphylococcus aureus*) of different plant extracts.

Materials and Methods
Collection of plant Material
We have used medicinal plant *Withania Somnifera* for this study. This plant is well described in different samhitas like Charak Samhita, Sushruta Samhita etc. for their medicinal values.We have collected the plant-leaf from Rewa, M.P. by taking help of expert botanist. The plant materials were cleaned with running tap water and sterilized by using commercially available volatile disinfectant agent.

Preparation of extracts

Leaves were again cleaned and air dried under laminar airflow. Total 500 gm of leaves were grinded mechanically (by using laboratory grade grinder: speed 1000 to 5000 rpm for 10 minutes). Powder was further dried under vacuum dryer. The dried powder (100-200 gm) subjected to different solvents like water and methanol and kept for 12 hours at 55-60^{0}C under constant rotating condition in multimode extractor. Thereafter, the solution was filtered by using whatman no.-1 filter paper. Suspensions were centrifuged at 5000 rpm for 20 minutes. The clear supernatant collected and dried under rota evaporator to get different solvent extracted powder and stored at -20^{0}C until used for further analysis.

Qualitative analysis of phytochemicals

Leaf extracts were subjected for phytoconstituents evaluation by adopting standard methods. The following phytochemicals: alkaloids, tannins, phenols, terpenoids, steroids and flavonoids were analysed.

Alkaloid test

To evaluate the presence of nitrogenous-alkaloids compounds in extract, we adopted Meyer's reagent based precipitation method. Briefly, fractions were dissolved in 0.5M HCl in boiling water bath and filtered. Three drops of HCl containing extract solution was added into 1 ml of Meyer's solution and allowed to precipitate.

Steroid test

To identify presence of steroids in extract, 100 mg of each sample was dissolved into 2 ml of glacial acetic acid and then added 2 ml of concentrated H_2SO_4. The color changes from violet to greenish and/or blue denoting steroids.

Tannin test

To know the presence of tannins in extract, we measured 100 mg of extract and added into 10 ml of hot water, mixed well and followed by membrane filtration. After that, 2-3 drops of 0.1% ferric chloride solution was added into filtrate and observed to appear as brownish green color formation.

Terpenoid test (Salkowski test)

Extract (10 mg) was dissolved into 10 ml of Mili Q water. Three ml of extract was added into 2ml of chloroform and 3 ml of concentrated H_2SO_4 and allowed to form a brown layer. This colorful layer is the indication of terpenoids.

Test for phenolics

To know the presence of phenolics in different extract ferric chloride method were used. Briefly, a 100 µl of sample was added into 500 µl of 5% ferric chloride solution and mixed well. The solution was kept at room temperature for 30 minutes to develop colour (indicates presence of phenol).

Test for flavonoids

100 µl of sample was added into 500 µl of dilute ammonia solution. Mixture was gently shaken and concentrated H_2SO_4 (500 µl) was slowly added and incubated for at least 3-4 hours for development of yellow colour which indicated presence of flavonoids.

HPTLC based profiling of leaf extracts

We performed HPTLC based separation and purification for further experiments. Here, we used an advanced HPTLC machine (CAMAG, HPTLC) to perform the experiments. A 50 mg of lyophilized (different extract) powder was dissolved into 500 µl of n-hexane: ethyl acetate: water (7:2:1) ratio and carried out for further steps. We used 5 x 10 cm aluminium TLC plate which was pre coated with 0.2 mm silica gel 60F254 layer (E. Merck Ltd, Darmstadt, Germany) and kept in desiccators. Sample application was performed by spotting and drying method (spray technique) with a Hamilton micro syringe (Switzerland) which controlled by an automatic applicator (Linomat-V). The command was fixed according to experimental design in WIN CATS software (Version 1.3.0). For the light source, we used deuterium light for λ_{max} 254 and 366 nm and Tungsten light source for λ_{max} 620 nm and the slit dimensions were 6.00 X 0.45 mm. Here, we used solvent mixture; n-hexane, ethyl acetate and water (7:2:1) as a mobile phase to separate the plant analyses. After spot development, plate was kept into solvent saturated twin trough glass chamber (10 x 10 cm) and allowed to migrate. Then plate was scanned (UV light: λ_{254} nm) in camag TLC Scanner 3 equipped with digital camera SNR-Lens (DXA252: 223971607) and also with win CATS Software. Bands were excised from plate under UV light. Then, silica containing excised matters were dissolved into methanol and centrifuged at 10,000 × g for 30 minutes. Supernatant of different bands were collected and evaporated into rota evaporator for further experiments.

Antibacterial activity

We used *Staphylococcus aureus* pathogenic bacteria, obtained from institute of microbial technology, Chandigarh, India. We followed the proper safety and others guideline (MTCC: 737) to maintain and perform the experiments in standard laboratory conditions. To test the anti-bacterial property of different fractions, we performed disc-diffusion antibiotic sensitivity test on *S. aureus* uniformed layer. One mg of different fractions were dissolved into 100% DMSO and marked as stock solution. Serial dilution (20, 40, 100, 400 and 800 µg/ml) of fractions were prepared from stock solution. Small round shape whatman filter paper-II was incubated with the diluted solution and followed by air drying. Fraction soaked-filter papers were placed on the layer of *S. aureus* (grown on nutrient agar plate-90 mm) and incubated at 37^{0}C for 12-24 hours. After 24 hours incubation, plates were observed and calculated the distance and diameter of inhibited zone. MIC was calculated on the basis on the minimum concentration able to inhibit bacterial growth. Along with this, we also measured the ROS generation (responsible for oxidative stress) in bacteria. Staining was done by DCF-DA (3µM/ml). After staining, fluorescence was measured by spectrofluorometer (excitation=435 nm and emission=535 nm). Results were expressed as relative fluorescence unit.

Results

Phytochemical analysis of water and methanolic extract suggested that they contained alkaloids, steroids, tannins and terpenoid along with phenol and flavonoids. Yellow precipitate in extracts confirmed the presence of alkaloids, similarly, formation of greenish color, brownish color and brown ring indicated the presence of steroid, tannin and terpenoid.

HPTLC investigation of both extracts confirmed the one or more flavonoid and phenolic compounds present in multiple samples respectively (Figure: 1). It was observed that methanolic extract (Which mentioned as Fraction-A and C) was the most endowed extract, consisting of ascorbic acid, gallic acid, hesperidin and rutin equivalent bands. In the same

images, it was observed that aqueous extract contained a significant amount of rutin, hesperidin and ascorbic acid equivalent bands (Fraction B: Figure: 1). However, both extract did not show any band with quercetin equivalent. The quantitative analysis also has shown that aqueous extracts contained bulky shoulder peaks in the region of Rf values from 0.05 to 0.14 which are the characteristic peaks of rutin, hesperidin and ascorbic acid respectively. Similar peaks observed in a methanolic extract with an extra Rf value 0.74 which collaborates with gallic acid.

To evaluate antibacterial property of fraction-A, B and C, we conducted disk diffusion method based minimum inhibitory concentration (MIC) calculation. Fraction-A only exhibited different degree of antibacterial activity with compare to control (Figure: 2). Table: 1 specifies the zones of inhibition in millimeter (mm) and corresponding MIC values in micrograms (µg). Further, we evaluated mechanisms of antibacterial activity of Fraction-A. Reactive oxygen species (ROS) is well known to kill pathogen. It can be observed that, Fraction-A showed an increasing trend for ROS generation in *S. aureus*, incrementing over a concentration range of 20 to 800 µg/ml of the fraction (Table: 2).

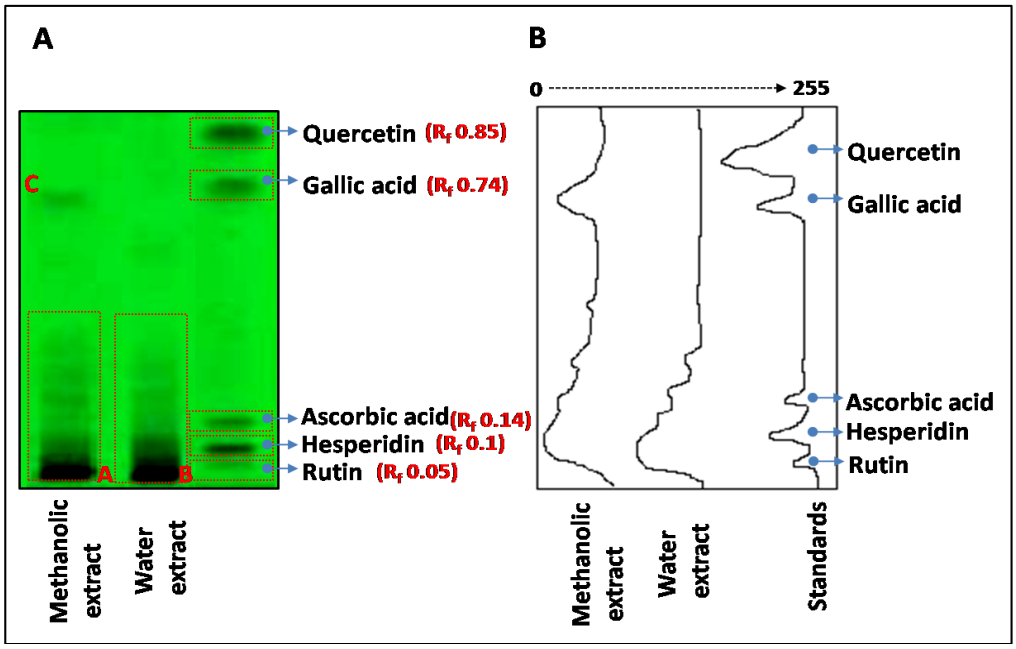

Fig 1: Analysis of methanolic and aqueous extract by HPTLC. A and C bands represented the fraction of methanolic extract having different Rf values (0.05, 0.1, 0.14 and 0.74). B band represented the fraction of aqueous extract having same Rf value, except 0.74.

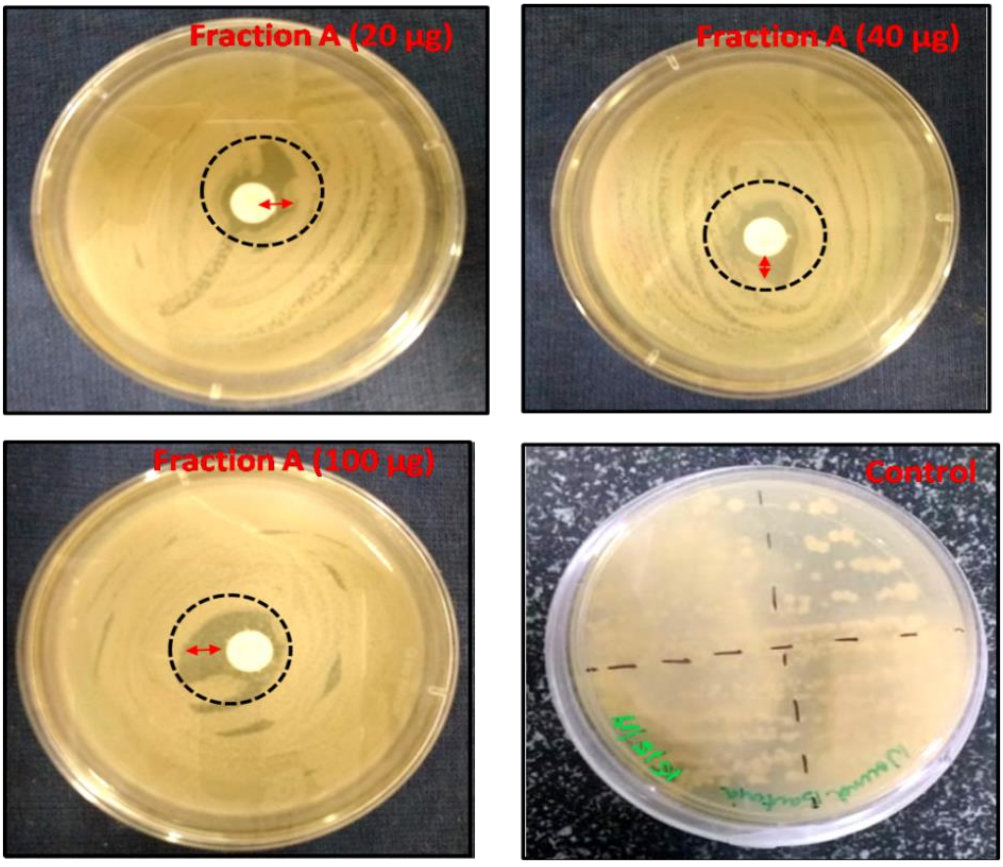

Fig 2: Different concentration of disks (white colour) shown zone of inhibition indicated as red arrow and compared with untreated control plate

Table 1: Diameters of inhibition zones (in mean (mm) ± SEM) after treatment with Fraction-A, B, C; MIC values (in mean (µg) ± SEM)

Fractions	Inhibition diameter and MIC values
A	3.5±0.2 mm**, 100±7.5 µg**
B	0.5±0.02 mm^{ns}, 0±0 µg^{ns}
C	0±0 mm^{ns}, 0±0 µg^{ns}
Control	0±0 mm, 0±0 µg

Table 2: Increased level of relative fluorescence intensity of Fraction A with compare to untreated control. Data are expressed as mean ± SEM, $^{**}p < 0.05$.

Fraction A (Concentrations µg/ml)	Relative fluorescence intensity
Control (Untreated)	835±65
20	1500.75±126.55
40	2000±250.65**
100	4600±137.55**
400	4100±830.75**
800	4500±1200.59**

Discussion

Plants have medicinal properties because of the presence of phytochemical constituents. Phytochemicals also known as secondary metabolites naturally synthesize and stored in different plant parts such as leaves, vegetables, roots and stems. Apart from these, phytochemicals have defense mechanism, protect plant from various diseases and environmental hazards [4]. Very interestingly, while these compounds are used in mammalian system then they act as the antifungal, antimicrobial, antiatherosclerotic, antileukemic, anticlastogenic, stimulate immune system, modulate hormone metabolism and strongly influences cellular homeostasis [5]. In present investigation we were also able to identify several flavonoids, phenolic, alkaloid, tannin, steroid and terpenoid compounds presents in leaf extract on the basis of biochemical characterization. HPTLC results reveal the presence of flavonoid and phenolic like molecules. As, these components are very common phytoconstituents and present in most of the medicinal plants and we also observed the similar components in ashwagandha leaf. Thus we expected anti-bacterial property of leaf extract. Several reports suggested that methanolic root extracts of *W. somnifera* inhibit growth of *Escherichia coli* and *Enterococcus* [6]. Similarly, we also observed the anti-bacterial activity of leaf extract against *Staphylococcus aureus*. Fraction A only exhibit a different degree of antibacterial activity compare to others and 100 µg/ml concentration is most effective against *S. aureus* strain.

The mechanisms what we have been identified to kill bacteria that molecules presents in extracts induce level of reactive oxygen species and thus it elevated levels of oxidative stress. It is also reported that a constant elevation of ROS leads to damage macromolecules and created pore in bacterial membrane and thus death occur. In this study, we also observed the similar components presents in leaf extract, thus it could be expected that the suppression of glycolysis in *staphylococcus aureus* occurred after treatment and induced death and considered as another reason. However, *W. somnifera* root extract exhibited antibacterial effect by creating membrane pore against *Escherichea coli* and *Salmonella typhimureum* which tested *in vitro* and *in vivo* in Guinea pigs [7, 8]. As Fraction-A can exhibited the antibacterial activity with compare to other fractions. The probable reasons behind of these are the some kind of active flavonoids which are only presents in high amount in this fraction are responsible for positive anti-bacterial function.

Conclusion

Ashwagandha is already a well known medicinal plant. Results of this study concluded that the methanolic extracts of leaf of this plant showed a considerable antibacterial activity against *S. aureus.* Therefore, these fractions could be used as a potential drug candidate (phytomedicine) and need higher mammalian study for better resolution.

References

1. Dar RA, Shahnawaz M, Qazi PH. General overview of medicinal plants: A review. The Journal of Phytopharmacology. 2017; 6(6):349-351.
2. Singh R. Medicinal plants: A review. Journal of Plant Sciences. 2015; 3(1):50-55.
3. Singh N, Bhalla M, Jager De P, Gilca M. An overview on Ashwagandha: A Rasayana (Rejuvenator) of Ayurveda. Afr J Tradit Complement Altern Med. 2011; 8(5):208-213.
4. Wadood A, Ghufran M, Jamal SB, Naeem M, Khan A, Ghaffar R *et al*. Phytochemical Analysis of Medicinal Plants Occurring in Local Area of Mardan. Biochem Anal Biochem. 2013; 2(4):1-4.
5. Khoo HE, Azlan A, Kong KW, Ismail A. Phytochemicals and Medicinal Properties of Indigenous Tropical Fruits with Potential for Commercial Development. Evid Based Complement Alternat Med. 2016; 2016:1-20.
6. Mathur V, Vats S, Jain M, Bhojak J, Kamal R. Antimicrobial Activity of Bioactive Metabolites Isolated from Selected Medicinal Plants. Asian J. Exp. Sci. 2007; 21(2):1-6.
7. El-Boshy MES, Abdalla OM, Risha A, Moustafa F. Effect of *Withania somnifera* extracts on Some Selective Biochemical, Hematological, and Immunological Parameters in Guinea Pigs Experimental Infected with E. coli. ISRN Veterinary Science. 2013; 2013:1-6.
8. Jain P, Varshney R. Antimicrobial activity of aqueous and methanolic extracts of *Withania somnifera* (Ashwagandha). J Chem. Pharm. Res. 2011; 3(3):260-263.