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A Comparative Study of *Tinospora cordifolia* Aqueous Extract's Antibacterial on Gram-Negative and Positive Pathogenic Bacteria

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ABSTRACT

The antimicrobial activity of *Tinospora cordifolia* aqueous extract was screened against gram-positive and negative multiple drug-resistant (MDR) bacterial strains. Gram-positive included *Bacillus cereus*, *Bacillus paranthracis* and gram-negative included *Aeromonas hydrophila, Aeromonas caviae* and *Aeromonas dhakensis*. These strains were isolated from wastewater treatment plants having domestic and hospital discharge and conferred resistance against most frequently used antibiotics. These multiple drug resistance bacterial strains showed sensitivity towards aqueous extract of *T. cordifolia*. The present study concluded that all selected bacterial strains showed a 17.9 mm lowest zone of inhibition and 33.9 mm highest in the case of gram-positive bacteria. Similarly, in the case of gram-negative bacteria, the lowest of 19 mm and the highest of 32.5 mm zone of inhibition.

Key words : Antimicrobial, multiple drug-resistant (MDR), aqueous extract, gram-positive, gram-negative

INTRODUCTION

Previously antibiotics provided the primary basis for the treatment of microbial infections including bacterial and fungal. The discovery of antibiotics gave people a belief in the medical fraternity since these were used as chemotherapeutic agents that help the eventual eradication of infectious diseases. Over use of antibiotics leads to the emergence and dissemination of a major factor that is the development of resistance against antibiotics in microorganisms (Mendelson *et al.*, 2016). This situation generated a worldwide therapeutic problem related to different microbial strains *Escherichia coli*, *Staphylococcus aureus*, *Haemophilus*, *Klebsiella pneumonia* and many others that produced beta-lactamase against beta-lactam antibiotics (Haberecht *et al.*, 2019). Antibiotic resistance in bacteria is a result of a single-step mutation that ultimately leads to multistep mutation like in the case of *Mycobacterium tuberculosis* and resistance against fluoroquinolones. Once any bacteria gain resistance, then these genes (resistant genes) spread through horizontal gene transfer (HGT) and spread widely among all. Conjugation, transduction and transformation are three main ways through which horizontal gene transfer occurs. These resistance genes are episomal in nature and Watanabe *et al.* proposed a term specific term for these "Resistant transfer factors" or R-factors. Plasmids an extrachromosomal organelle of bacteria carry these R-factors. Plasmids act as the main carrier in spreading resistance against antibiotics and act as the mobile genetic elements. Transposons and integrons are other such examples of mobile genetic elements. Recent years of research in genomics suggest resistance through horizontal gene transfer occurs more rapidly and spread wider than ever imagined (Nishiyama *et al.*, 2015; Hendriksen *et al.*, 2019; Pärnänen *et al.*, 2019). Environment acts as the main reservoir for the mobile genetic elements that include soil, aquatic environment, animal and hospital environment (Fouz *et al.,* 2020). Wastewater treatment plants (WWTPs) collect wastewater from domestic, industrial and hospital environments. Different research suggests hospital wastewater carries many folds of antibiotic-resistant genes (ARGs) in comparison to domestic and industrial waste.

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Major bacterial phyla like Bacteriodates, Firmicutes, Chloroflexi, Actinobacteria, Proteobacteria, Acidobacteria were identified in treated wastewater that was further used in agricultural practices. According to WHO, industrialized countries have about 30% of the population that is affected by food-related disorders every year. Similarly, by 2050 multiple drug resistance will be the main reason for death all over the world. This situation became worse due to antibiotic abuse and misuse widely even if it's an educated or not. These untreated ARGs ultimately enter the human chain and cause severe infections and diseases. Aeromonads confer antibiotic resistance genes into their mobile genetic elements integrated into the plasmid region. Presence of several resistance genes including tetA, tetE, genes coded for tetracycline resistance located in the plasmid region. For streptomycin, there are sat1, aadA1 and aadA2 genes that provide resistance against streptomycin. For chloramphenicol resistance, there are specific genes like cat, catB2, catB3 and catB8. The gram-positive bacterium *Bacillus cereus* is a facultative anaerobe and produces toxin (Manyi-Loh *et al.*, 2018). Generally, this bacterium is found in different environments and showed its symptoms by contaminating food. Contaminated food causes gastrointestinal problems including diarrheal illness. The bacterium is also associated with respiratory tract infection viz., *Bacillus paranthracis*. Bacteria generally secrete a variety of toxins including phospholipase, hemolysins, tissue destructive exoenzymes and proteases. To tackle this major issue current research tried to provide an alternative to antibiotics. A natural plant herb, *Tinospora cordifolia* aqueous extract was used against different bacterial strains (Ilamkar *et al.*, 2020). The reason behind this was that natural products have been used all over the world for thousands of years in traditional medicine (Ruthiran *et al.*, 2016). There was prior knowledge for the treatment of herbal extractions for cardioprotective, anti-allergic, antiviral, antispasmodic, antibacterial and anti-cancerous. The plant provided a variety of bioactive compounds that show biocidal activity against pathogenic bacterial strains also. These herbal plants provided antimicrobial efficacy that is beyond belief in several disease treatments (Mulaudzi *et al.*, 2017). On earth, there as an estimate 10% of all flowering plants were used for the treatment of infections. Out of this 10%, only 1% have gained recognition in modern science. Herbal plants are rich in a diverse variety of secondary metabolites like alkaloids, tannins and flavonoids (Kite *et al.*, 2017). These are tested *in vitro* against their antimicrobial activity. The last few years of research provided the authenticity of herbal extract to cure gastrointestinal diseases, urinary tract infections, cutaneous infections and respiratory disease treatment (Bruno *et al.*, 2018). Medicinal plants are a great source of therapeutic drugs. The objective of the study was to investigate *T. cordifolia* aqueous extract on pathogenic bacterial strains including gram positives and gram-negatives.

MATERIALS AND METHODS

Fresh leaves of *T. cordifolia* were collected from the medicinal plant section of CCS Haryana Agricultural University Hisar, Haryana India. Leaves were washed and then dried in shade before grinding to form a coarse powder. Five g of this finely powdered leaves were put in 200 ml of triple distilled water (in the round bottom flask) for extraction by Soxhlet apparatus (Jain Scientific Glass Works, India, 18782) at 90±2°C temperature for 8 h. Two hundred ml extract was put in vacuum oven (Metrex Scientific instruments Ltd, New Delhi, India) for concentrated extract. It was then stored at -20 ^{0}C in the deep fridge (Remi Sales and Engg. Ltd).

To screen out the presence of phytoconstituents in aqueous extract Trease and Evans standard method with some modifications was followed. To identify the total number of components present in the aqueous extract preparative and analytical technique, thin-layer chromatography (TLC) method was used. Precoated silica gel normal phase TLC sheets (20 x 20 cm with 0.2 mm thickness) obtained commercially. A 100 ml mixture of ethyl acetate, methanol and chloroform was tried at different ratios (3 : 2 : 1, 2 : 1 and 1 : 1 v/v/v) for mobile phase eluents. The best chromatogram was obtained from ethyl acetate and methanol (2 : 1 v/v/v) and the spots were visualised under UV-transilluminator. The retardation factor (R_f) of the eluted spot was calculated as :

R_f = Distance travelled by sample/ distance travelled by solvent

Isolated herbal extract sample was run under Fourier-Transform Infrared Spectroscopy for identification of functional groups. Perkin Elmer Spectrum BX II version 10.6.2 was used for FT-IR analysis of samples.

Isolated microbial strains included both gram-positive and negative pathogenic bacteria. Gram-negative strains *Aeromonas caviae* (Accession no. CDBK01000019), *Aeromonas dhakensis* (Accession no.CDBH01000037) and *Aeromonas hydrophila* subsp. hydrophila (Accession no. CP000462). Gram-positive strains included *Bacillus cereus* (Accession no. AE016877) and *Bacillus paranthracis* (Accession no. MACE1000012). All the bacterial strains were isolated from the wastewater treatment plant including domestic and hospital discharge. Further their multiple drug resistance activity was also confirmed. Mueller-Hinton culture medium was used to study antibacterial activity by using the agar well diffusion method. Mueller-Hinton agar medium (Himedia Labs, Mumbai, India) was autoclaved and then transferred to Laminar Air Flow Cabinet (LAF). When culture media was slightly cooled down then poured into autoclaved disposable petri-plates (Tarsons). These plates were sealed with parafilm and placed into an incubator (Orbitex LT- orbital Incubator Shaker) at 37°C for 48 h to check for any bacterial or fungal contamination. For the preparation of fresh cultures, all the bacterial strains were separately grown in a nutrient broth medium (Himedia Labs, Mumbai, India) at 37°C in an incubator shaker at 150 rpm. Fresh microbial inoculum of 1.5×10^8 CFU/ml of each microbial strain was spread on separate petri plates with the help of L- shape sterilized disposable spreader (Tarsons). Pouring and spreading of microbial cultures was performed inside the Laminar airflow cabinet. Microbial cultures were dried for 20 min then with the help of a cork borer of 5 mm diameter wells were made in all petri plates. These wells were used to pour aqueous extract. Three different concentrations of aqueous extract were used to check antibacterial activity including 10, 50 and 100 µl. Tarsons micropipettes were used for measuring these concentrations and then added to wells. After adding the aqueous extract to each plate again plates were sealed with parafilm and transferred to an incubator at 37°C for 24 h. All of culturing experiment was performed in triplicates. The zone of inhibition was checked after 24 h to estimate antimicrobial activity.

Soxhlet apparatus and its glasswares were purchased from Jain Scientific Glass Works, India. Vacuum oven used was of Metrex Scientific Instruments Ltd, New Delhi, India. All of the cultural media including nutrient broth and Meuller-Hinton agar medium were purchased from Himedia Labs, Mumbai, India. Autoclaved disposable petri dishes and spreaders were purchased from Tarsons. Incubator shaker of Orbitex LT- orbital Incubator Shaker used was of Scigenics Biotech Private Limited.

RESULTS AND DISCUSSION

Phytoconstituents screening showed the presence of alkaloids, steroids, amino acids and protein in aqueous extract. Contrarily, other components like carbohydrates, terpenoids, saponins, flavonoids and glycosides were absent in extract. Single spot was obtained on the chromatogram of thin layer chromatography. The retardation factor of the spot ranged between 0.23±0.11.

Band positions obtained from FT-IR analysis were 3427.59/cm strong O-H stretching, 2919.00/cm O-H/ N-H stretching, 2850.32/cm O-H/ N-H stretching, 2340.22/cm O=C=O stretching, 1737.32/cm C=O stretching, 1463.58/cm C-H bending, 1378.51/cm O-H bending, 1235.42/cm C-O stretching, 1171.35/ cm C-O stretching, strong ester, 1093.99/cm C-O stretching, and 729.90-719.90/cm C-Cl stretching (Table 1) with functional peaks (Fig. 1).

Table 1. FI-IR analysis of sample

Peak No.	Band position/ cm	Functional groups
1	3427.59	Strong O-H stretching
2	2919.00	O-H/N-H stretching
3	2850.32	O-H/N-H stretching
4	2340.22	O=C=O stretching
5	1737.32	C=O stretching
6	1463.58	C-H bending
7	1378.51	O-H bending
8	1235.42	C-O stretching
9	1171.35	C-O stretchings, strong ester
10	1093.99	C-O stretching
11	729.90	C-Cl stretching
12	719.90	C-Cl stretching

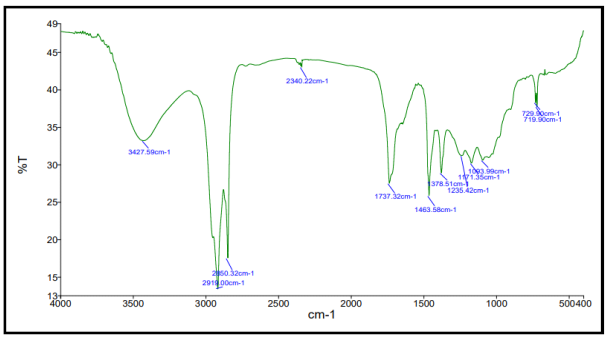

Fig. 1. Graphical peak of functional groups.

Gram-negative bacteria showed sensitivity towards the aqueous extract of *T. cordifolia*. All bacterium showed different results of extract sensitivity that ranged from lowest of 19 mm and highest of 32.5 mm zone of inhibition within 24 h of incubation (Fig. 2). *A. hydrophila* showed 21 mm of the zone of inhibition at a 10 µl concentration of aqueous extract and at a 50 µl concentration, it showed 29 mm of the zone of inhibitory activity. The highest antimicrobial activity was obtained at a 100 µl concentration of aqueous extract that was 30 mm in diameter of the zone of inhibition. *A. caviae* also known as Traveller diarrhoea showed a 19 mm diameter of zone of inhibition at a 10 µl concentration of aqueous extract which was the lowest among all. At 50 µl concentration of aqueous extract, it showed a 25.5 mm diameter zone of inhibition and at 100 µl showed a 27 mm diameter zone of inhibitory activity. *A. dhakensis* showed maximum sensitivity among all Aeromonads. At 10 µl concentration of aqueous extract, it showed a 27.2 mm diameter zone of inhibition, at 50 µl concentration 30.7 mm and at 100 µl concentration it showed a 32.5 mm diameter zone of inhibition which was the highest activity counted in 24 h.

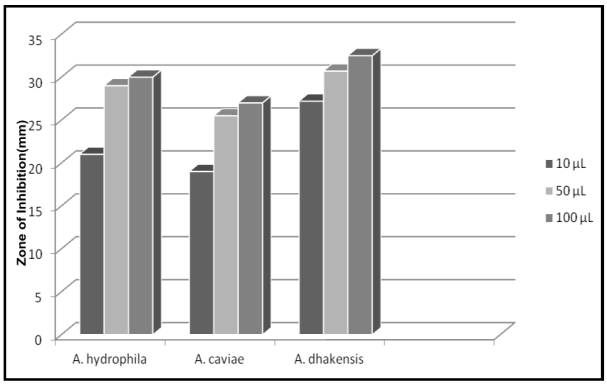

Fig. 2. Antibacterial activity of aqueous extract on gram-negative bacteria.

Gram-positive bacteria also showed sensitivity towards aqueous extract but some different results of diameter in the zone of inhibition were observed (Fig. 3). *Bacillus paranthracis* showed the highest sensitivity among gram-positive and negative bacteria. At 10 µl concentration of aqueous extract obtained the inhibition ring of 22.6 mm and 50 µl showed a 26.9 mm diameter of inhibition. But 100 µl showed quite high antibacterial activity of 33.9 mm diameter zone of inhibition. In the case of *Bacillus cereus*, the minimum inhibitory activity of *T. cordifolia* was observed. At a 10 µl concentration of *T. cordifolia* extract 17.9 mm diameter of zone of inhibition was observed and 50 µl showed 23.2 mm inhibitory activity. The highest 25.9 mm zone of inhibition was observed at 100 µl of aqueous extract. After 24 h incubation gradually the colour of Mueller-Hinton media started to change and after 36 h colour completely changed from pale yellow to light brown and during this time no bacterial growth was observed. The same observation was found in gram-negative bacteria.

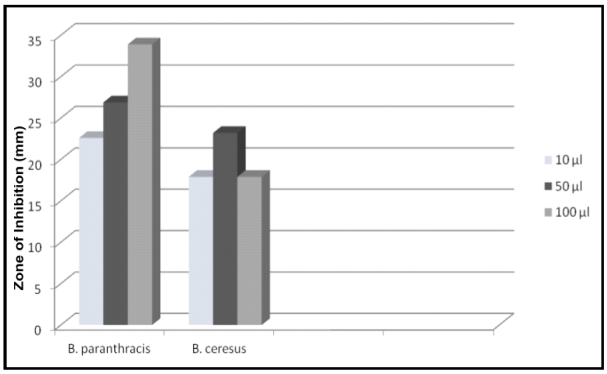

Fig. 3. Antibacterial activity of aqueous extract on gram-positive bacteria.

CONCLUSION

Microbial species isolated from wastewater confered resistance against frequently used antibiotics like chloramphenicol, tetracycline, streptomycin, ampicillin, rifampicin and piperacillin that's why these micro-organisms are considered multiple drug-resistant strains. FTIR results revealed the presence of ethers, carbonyl or alcohol, and aliphatic functional groups that provide therapeutic activity for the use of medicinal plants. Above study confirmed that aqueous extract can be used to treat bacterial infections caused due to gram-positive and negative bacteria. Plants and their parts like leaves, stems, flowers and roots have

strong bioactive compounds and these are easily available to common people. Herbal medicines could serve as a potential source due to the richness of secondary metabolites like tannins, terpenoids, alkaloids and flavonoids. Prior published data can be easily used at the processing time of bioactive compounds. The major need of today's medicinal therapy is to treat multiple drug-resistant bacteria. Current research used green synthesis based nano-particles to tackle multiple drug resistance. Plants that showed good results can be used. However, its main task is to determine the composition of active constituents that are providing antibacterial activity, their side effects and toxicity determination along with pharmaco-kinetics properties and evaluation of active required constituents.

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