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Evaluation of Antimicrobial Efficacy of Tinospora Cordifolia Extract on Drug Resistant Pathogens

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KEYWORDS

Anti microbial strains, MIC- Minimum Inhibitory Concentration, zone of inhibition, MDR- Multi Drug Resistance, AST: Antimicrobial susceptibility test.

Abstract

The lightning-fast raise in drug-resistant pathogens represents an urgent threat to all over the world health, requiring the creation of innovative tactics to counter these formidable adversaries. In particular, the effectiveness of novel antimicrobial agents in the face of rising antibiotic resistance is examined in this study, which focuses on the assessment of antibacterial activity against drug-resistant pathogens.A thorough and interdisciplinary strategy that combined microbiological and molecular methods was used to achieve this. A range of in vitro susceptibility tests, such as determining minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) for different antibacterial compounds were performed on drug-resistant strains of bacteria that were isolated from clinical samples.

These investigations yielded significant findings about the genetic factors that influence antibiotic resistance as well as possible targets for completely novel antibacterial drugs. Using a panel of drug-resistant strains, the antibacterial activity of new compounds was assessed. These strains included multidrug-resistant strains resistant to Carbapenem and extended-spectrum β-lactamase (ESBL) producers. Using a variety of in vitro tests, the susceptibility of these pathogens to experimental antimicrobial agents was evaluated.

Promising antimicrobial plant components that are active against drug-resistant pathogens are revealed by the study's findings. These substances show promise in treating difficult infections that are resistant to drug. In order to conclude, assessing antibacterial activity against pathogens that are resistant to drugs is an essential task in the battle against antibiotic resistance. This study highlights the value of multidisciplinary strategies that combine drug development, genomics, and microbiology to find and assess viable remedies for this escalating public health emergency. The results of this study highlight the significance of ongoing research and innovation in this field and support ongoing efforts to create efficient treatments for drug-resistant infections.

INTRODUCTION:

The global health of humanity is seriously threatened by the rise of drug-resistant pathogens, such as microorganisms that are invulnerable to popular antibiotics (1). Researchers are exploring toward alternate strategies to fight bacterial infections as a consequence of this expanding issue. The assessment of plant extracts' antibacterial activity is one intriguing avenue for investigation. For centuries, plants have been a valuable source of natural compounds with potential therapeutic applications. Today, they remain an important tool in the search for novel antimicrobial agents. While some of those active ingredients aren't as effective as antibiotics on their own, they can work in conjunction with antibiotics to combat the bacteria's built-up resistance to them (2). It is true that the quantity and quality of bioactive secondary metabolites

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influence how well they are able to fend off various microbial strains (3).

A vital field of research with many important goals is evaluating the antibacterial activity of plant extracts against drug-resistant pathogens. Its primary goal is to recognize and utilize the antibacterial qualities of phytochemicals found in different plant species. Alkaloids, flavonoids, tannins, and essential oils are some of the phytochemicals that have shown promise in preventing the growth of bacteria resistant to antibiotics. The excessive consumption and misuse of antibiotics have been responsible for an upsurge in drug-resistant pathogens, resulting in the need for alternate treatment options essential. Plant extracts offer an appealing remedy because they might include new compounds that can successfully fight these resistant strains. These naturally occurring compounds are also good candidates for therapeutic development because they may not encourage additional antibiotic resistance.

The objective is to create bacterial infection therapies that are safe, efficient, and long-lasting in order to mitigate the threat that antibiotic resistance poses to global health. The Infectious Disease Society of America has already recognized the ESKAPE bacteria, also known as *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species, as being particularly dangerous due to their virulence and potential antimicrobial resistance (4).

It is imperative to take into account a number of variables in this situation such as the plant species selection, the extraction technique, the drug-resistant pathogen selection and the experimental design. These factors are essential to guarantee the validity and applicability of the study results. This overview gives a glimpse of the importance of evaluating *Tinospora* plant extracts for antibacterial activity against pathogens that are resistant to drugs. *Tinospora cordifolia* not only exhibits potential antibacterial activity against MDR bacteria with clinical origin but also against the bacteria that cause urinary tract infections. Due to the alkaloids, carbohydrates, and flavonoids found in this plant, it can successfully replace clinical isolates with invitro antimicrobial activity (5).

Subsequent studies in this area are intended to deepen our comprehension of the possible medicinal advantages of compounds derived from plants which will ultimately aid in the creation of new strategies to fight bacterial infections that are resistant to drugs.

Materials and Methods:

Isolation and Identification of Antibiotic Resistant Bacteria:

Materials Required:

This experiment employed selective culture media from Hi-medium, India, including nutrient agar media (NA), nutrient broth media, MacConkey agar, EMB agar, Mueller–Hinton agar and Agar with Cetrimide,. To meet the supplier's specifications, all media were prepared. Antibiotic stock solution preparation:

In a 250 ml volumetric flask, 300 mg each of the following antibiotics were dissolved: Ampicillin(AMP), Amoxicillin clavulanate(AMC), Cefuroxime(CFM), Ceftriaxone(CTR), Imipenem(IMI), Gentamycin(GEN), Nalidixic Acid(NAL), Ciprofloxacin(CIP), Erythromycin(EN) and Tetracycline(TE). Within five minutes, the antibiotic had fully dissolved. In accordance with the manufacturer's instructions, the volumetric flask was sealed and kept at room temperature.

Sample collection:

Clinical isolates of *E.coli*, *Pseudomonas*, *Staphylococcus* and *Klebsiella* are collected from Government hospital in Khammam.

Confirmation of Resistant Bacterial Strains:

To verify the resistance of the various bacterial strains, the AST test was conducted using the disc diffusion method. In order to conduct the antibacterial susceptibility testing (AST), Mueller-Hinton agar (MHA) plates were prepared. The OD600 of a bacterial suspension in sterilized normal saline that was calibrated to the McFarland standard (0.5) was recorded. 10-15 L of bacterial suspension were poured and streaked onto the Mueller-Hinton agar plate to create a lawn of growth. Using forceps, antibiotic discs were placed on the bacterial lawn after streaking the plates. Plates were then incubated for 24 hours at 37 °C. After incubation, a metric ruler was used to gauge the zone of inhibition. The zone of inhibition was then compared to a previously provided standard zone of inhibition given by the Clinical Laboratory Standard Institute 2020 (6).

Confirmation by VITEK:

Resistant bacterial strains are also confirmed by VITEK instrumentation which gives the information on

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resistance against different antibiotics in terms of MIC and also gives information about identification tests, which also predicts the isolated organism. Marker genes which are responsible for resistance are also identified by using this method (7).

Materials required:

1. Source of microorganisms
2. Sterile Inoculating Loops
3. Supplemental Media: nutrient agar (NA), tryptic soy broth (TSB), trypticase soy agar with 5% Sheep Blood (BAP), and trypticase soy agar (TSA).
4. VITEK 2 Compact Identification cards (for example, GP, ANC, BCL, and GN): store at 2- 8°C in unopened original liner.
5. 75 mm × 12 mm clear polystyrene tubes (single use only)
6. DensiCHEK Plus Meter with McFarland Standards for calibration (0.0, 0.5, 3.0, and McF) .
7. Sterile saline solution (aqueous 0.45% to 0.50% NaCl, pH 4.5-7.0)
8. Bar-coded 10 well cassette card holders
9. Internal Carousel for card processing

Initiation of the V2C System:

The V2C Instrument is always "on"; the instrument will say "Ready" or "Not Ready" on the digital screen. Once the computer is initialized, the instrument will say "Ready." The V2C will not run if it is not on ready mode. Select VITEK 2 Compact to initiate the system from the upper left side of the screen. After the system is initiated, log onto the system using the appropriate user name and password. The system is now initialized and ready for data entry.

Preparation of Inoculum:

The isolated inoculum is streaked onto agar plate. For cultures used on BCL and GN cards, the cultures are incubated for 18-24 h at 36±1°C. For cultures used on GP cards, cultures are incubated for 12-48 h at 36±1°C. All organisms to be identified are in pure cultures. Gram staining is performed using an isolated colony from a pure culture plate and documented the Gram stain reaction. The appropriate card is selected based on the Gram stain reaction and the organism's microscopic appearance. Allow the card to come to room temperature before opening the package liner. Aseptically transfered at least 3 ml of sterile saline into a clear polystyrene 12×75 mm test tube. Using sterile cotton swabs, a homogenous organism suspension is prepared by transferring several isolated colonies from the plates to the saline tube. The suspension is adjusted to the McFarland standard required by the ID reagent using a calibrated V2C DensiCHEK plus Meter.

The prepared suspensions are placed in the cassette. The density of inoculums is checked by using DensiCHEK plus meter .Density should be in the range of 0.5-0.63 for gram –ve and +ve cards.

The straw (in the V2C card) is inserted into the inoculated suspension tube in the cassette.

Then it is preceded for data entry. Then the cassette is placed in the filler box. Then results are obtained in terms of specificity of organism and biochemical information. It also gave the information on AST susceptibility.

Plant Extract Preparations:

Selected plants were identified and authenticated by the Taxonomist Prof.Musthafa, Head , Dept. of Botany, Kakatiya university.

Preparation of the *Tinospora Cordifolia* Leaf Extract:

Plant stems with leaves that were still fresh and in good health were gathered from various places. *T. cordifolia's* shade-dried components were ground into a powder, sieved, and then placed in an airtight container for future use. *Tinospora Cordifolia* powdered leaves were dissolved in 50 mL of distilled water, ethanol, or chloroform for about 48 hours while being frequently stirred in an airtight container. Then these are centrifuged at 5000rpm for about 10 min.The solvents were discarded and then put away for later use. An initial phytochemical screening was conducted on *Tinospora cordifolia* extract. Alkaloids, carbohydrates, glycosides, phytosterols, saponins, tannins, proteins, mucilage, flavonoids, terpenoids, and phytosterols were among the bioactive components screened in the plant extracts (8).

Stock Preparation

Dimethyl sulfoxide (DMSO), a universal solvent, was used to dissolve all of the obtained sequential extracts. Using DMSO, working solutions of various concentrations were created from these stock solutions. For additional research, these working solutions were kept in glass vials and kept at 4 °C.

Phytochemical Screening Tests:

Carbohydrates:

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Molisch's test: The extract was mixed with 2ml of Molisch's reagent, and the mixture was shaken properly. Then, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube, which was observed for the appearance of a violet ring at the interface.

Proteins:

Xanthoproteic test: One milliliter of concentrated nitric acid was added to 2–3ml of test solution in a test tube. A positive test is indicated by the formation of a white precipitate, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color. The solution is cooled before carefully adding ammonium hydroxide or sodium hydroxide in excess, whereby the yellow solution deepens into an orange color.

Amino Acids:

Ninhydrin test: Five drops of 0.2% ninhydrin solution in acetone were added to 1ml of amino-acid solution. The mixture was boiled over a water bath for 2 min and allowed to cool, before observing the formation of a blue color.

Flavonoids:

Alkaline reagent test: Two milliliters of 2.0% NaOH mixture was mixed with aqueous plant crude extract; a concentrated yellow color was produced, which became colorless upon adding two drops of diluted acid to the mixture. This result showed the presence of flavonoids.

Phenols:

Lead acetate test: To 0.2ml of extract, 2ml of aqueous sodium carbonate was added, followed by the addition of 0.2ml of Folin's reagent. A color change to blue or gray indicated the presence of phenols.

Alkaloids:

Test for alkaloids: To 2ml of extract, 2ml of concentrated HCl was added. Then, a few drops of Mayer's reagent were added. The presence of green color, white precipitate, or turbidity indicated the presence of alkaloids.

Phytosterols:

Salkowski's test: The chloroform extract was treated with concentrated H_2SO_4 and observed for the formation of a red color.

Saponins:

Foam test: A fraction of the extract was vigorously shaken with 20ml of water in a graduated cylinder for 15 min, which was observed for the presence of persistent foam.

Terpenoids:

Salkowski's test: About 5ml of extract was mixed with 2.0ml of chloroform and 3.0ml of concentrated H_2SO_4**.** A reddish-brown color at the interface indicated the presence of terpenoids.

Quinone:

HCl method: To 1.0ml of extract, a few drops of concentrated HCl was added. A yellowish-brown color indicated the presence of quinine.

Evaluating Antibacterial Effects of Plant Extracts:

Antibiotic Susceptibility Test for Plant Extracts:

For the AST test, the diffusion method was used to confirm the resistance of the various bacterial strains to plant extracts. Antibacterial susceptibility testing (AST) was carried out on Mueller-Hinton agar (MHA) plates. The McFarland standard (0.5) was prepared as a bacterial suspension in sterilized normal saline, and the OD600 value was recorded. 10-15µl of bacterial suspension were poured and streaked onto the Mueller-Hinton agar plate in order to create a lawn of growth on MHA plates. The plate was then left to dry for five minutes. After this, the agar media is cut into wells, and 10 to 15µl of the appropriate extracts are added to the wells. After that, the plates were incubated for 24 hours at 37 °C. A metric ruler was then used for the measurement of zone of inhibition of each antibiotic (9).

VITEK Analysis of Resistance Isolates of Clinical Area:

Clinical samples of 2 isolates of *E.coli*, 2 isolates of *Pseudomonas aeruginosa*, 2 isolates of *Klebsiella pneumonia* and 2 isolates of *Staphylococus aureus* are collected from Govt.hospital in Khammam. Their biochemical characterization and resistance profiles are collected from VITEK.

VITEK analysis confirmed *E.coli* isolates are resistant to Cefuroxime, Ceftriaxone, Cefepime,Ciprofloxacin and Trimetoprim. *Pseudomonas aeruginosa* isolates are resistant to Imipenem, Meropenem, Ciprofloxacin and Levofloxacin, *Styaphylococus aureus* strains are resistant to Benzyl pencillin, Oxacillin and Ciprofloxacin where as *Klebsiella pneumonia* isolates

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are resistant to Amoxicillin, Piperacillin, Cefuroxime, Ertapenem and Ciprofloxacin.

VITEK analysis confirmed the identity of bacterial isolates and recognized them as multidrug-resistant pathogens. This technology is crucial for accurately identifying the bacteria and understanding their resistance patterns.

Confirmation of Resistant Bacterial Strains:

Antibiotic susceptibility tests (AST) were used for the confirmation of resistant bacterial strains. Respective bacterial colonies were streaked on agar plates by using sterile swabs and after putting antibiotic discs on the bacterial-streaked plates, they were incubated at 37°C for 24 h. After incubation, the zone sizes were measured for each antibiotic against the respective bacterial strains.

AST of *E.coli:*

Antimicrobial	Disc con.	E1 Zone size(mm)	E2 Zone size(mm)	Resistant	Moderately sensitive	Sensitive	Interpretation
Amoxicillin	30µg	18	18	<=13	14-17	>=18	S
Cefuroxime	30µg	14	14	<=14	15-17	>=18	R
Ceftriaxone	30µg	12	12	<=13	15-20	>=21	R
Cefepime	30µg	12	12	<=14	15-17	>=18	R
Imipenem	10µg	16	16	<=13	14-15	>=16	S
Amikacin	30µg	17	17	<=14	15-16	>=17	S
Gentamicin	10µg	16	16	<=12	13-14	>=15	S
Ciprofloxacin	15µg	12	13	<=15	16-20	>=21	R
Trimethoprim	30µg	10	10	<=10	11-15	>=16	R

Antimicrobial susceptibility test of *E.coli*

This table shows the results for the E.*coli* antibiotic susceptibility test, in which the isolates are resistant to

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Cefuroxime, Ceftriaxone, Cefepime, Ciprofloxacin, Amoxicillin,Imipenem,Amikacin and Gentamicin. Trimethoprim and are sensitive to

AST of *P.aeruginosa*:

Antimicrobial	Disc con.	P1 Zone size (mm)	P2 Zone size (mm)	Resistant	Moderately sensitive	Susceptible	Interpretation
Cefepime	30µg	18	19	<=14	15-17	>=18	S
Imipenem	10µg	12	12	<=13	14-15	>=16	R
Amikacin	30µg	18	17	<=14	15-16	>=17	S
Ciprofloxacin	15µg	13	11	<=15	16-20	>=21	R

Antimicrobial susceptibility test of *P.aeruginosa*

This table shows the results for the *P.aeruginosa* antibiotic susceptibility test, in which the isolates are resistant to Imipenem and Ciprofloxacin and are sensitive to cefepime and Amikacin.

AST of *S.aureus*:

Antimicrobial	Disc con.	S1 Zone size (mm)	S2 Zone size (mm)	Resistant	Moderately sensitive	Susceptible	Interpretation
Benzyl pencillin	30µg	18	18	<=19	20-27	>=28	R
Oxacillin	30µg	13	13	<=10	11-12	>=13	S
Tetracycline	30µg	19	20	<=14	15-18	>=19	S
Erythromycin	10µg	13	12	<=13	14-22	>=23	R
Gentamicin	10µg	15	16	<=12	13-14	>=15	S
Ciprofloxacin	15µg	13	13	<=15	16-20	>=21	R

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Trimethoprim	30µg	09	09	<=10	11-15	>=16	R

Table 8: Antimicrobial susceptibility test of *S.aureus*

This table shows the results for the *S.aureus* antibiotic susceptibility test, in which the isolates are resistant to Benzyl pencillin, Erythromycin, Ciprofloxacin, Trimethoprim and are sensitive to Oxacillin, Tetracycline, Erythromycin.

AST of *K.pneumoniae:*

Antimicrobial	Disc con.	K9 Zone size (mm)	K10 Zone size (mm)	Resistant	Moderately sensitive	Susceptible	Interpretation
Amoxicillin	30µg	12	13	<=13	14-17	>=18	R
Cefuroxime	30µg	14	14	<=14	15-17	>=18	R
Ceftriaxone	30µg	12	12	<=13	15-20	>=21	R
Cefepime	30µg	12	11	<=14	15-17	>=18	R
Imipenem	10µg	11	11	<=13	14-15	>=16	R
Amikacin	30µg	17	17	<=14	15-16	>=17	S
Gentamicin	10µg	15	15	<=12	13-14	>=15	S
Ciprofloxacin	15µg	13	11	<=15	16-20	>=21	R
Trimethoprim	30µg	17	17	<=10	11-15	>=16	S

Antimicrobial susceptibility test of *K.pneumoniae*

This table shows the results for the *K.pneumoniae* antibiotic susceptibility test, in which the isolates are resistant to Amoxicillin,Cefuroxime ,Ceftriaxone,Cefepime,Imipenem and are sensitive to Amikacin,Gentamicin,Trimethoprim.

VITEK analysis confirmed the identity of bacterial isolates and recognized them as multidrug-resistant pathogens. This technology is crucial for accurately identifying the bacteria and understanding their resistance patterns.

Phytochemical Screening:

The results showed the presence of almost all important bioactive compounds which were being tested. These compounds were observed in variable concentrations in leaf extracts of *Tinospora cordifolia*.

For *Tinospora cordifolia* leaf extract:

The following Table shows the results of the phytochemical screening of *Tinospora cordifolia* leaves.

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The powder weights for the aqueous ethanol and chloroform solvents were 620mg, 560mg, and 520mg respectively. Most of the bioactive compounds were observed in the ethanolic and aqueous extracts of the respective parts of the plant.

S.No.	Constituent of plant extract	Aqueous	Ethanol	Chloroform
1	Alkaloids	+	+	+
2	Flavonoids	+	-	+
3	Tannins	+	+	-
4	Saponins	+	+	+
5	Terpenoids	-	+	+
6	Phenol	+	+	-
7	Glycosides	-	-	-
8	Sugar	+	+	-
9	Aminoacids	+	-	-
10	Sterols	-	-	+

Phytochemical screening of *Tinospora cordifolia* leaf extract

AST of *Tinospora cardifolia* leaves on *E.coli:*

S.No.	Lab ID	Aqueous		Ethanol		Chloroform	
		Zone of inhibition	MIC	Zone of inhibition	MIC	Zone of inhibition	MIC
1	MDR11T(CI)	NIL	1/40	15	1/40	15	1/20
2	MDR12T(CI)	NIL	1/40	14	1/40	21	1/20

This shows that chloroform extract of *T.cardifolia* leaf is inhibiting the activity of drug resistance by *E.coli* at 1/20 dilution which indicates the concentration of 2.6µg.

AST of *Tinospora cardifolia* leaves on *P.aeruginosa*:

S.No.	Lab ID	Aqueous		Ethanol		Chloroform	
		Zone of	MIC	Zone of	MIC	Zone of	MIC

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		inhibition		inhibition		inhibition	
1	MDR24T(CI)	NIL	ND	13	1/40	13	1/40
2	MDR25T(CI)	NIL	ND	14	1/40	13	1/40

AST of *Tinospora cardifolia* leaf extract activity on *P.aeruginosa*

This shows that chloroform extract of *T.cardifolia* leaf is having the maximum inhibiting activity of drug resistance at 1/20 dilution.

AST of *Tinospora cardifolia* leaves on *K.pneumonia*:

S.No.	Lab ID	Aqueous		Ethanol		Chloroform	
		Zone of inhibition	MIC	Zone of inhibition	MIC	Zone of inhibition	MIC
1	MDR32T(CI)	NIL	ND	NIL	ND	NIL	ND
2	MDR33T(CI)	NIL	ND	NIL	ND	NIL	ND

AST of *Tinospora cardifolia* leaf extract activity on *K.pneumonia*

This shows that ethanol extract of *T.cardifolia* leaves having the maximum inhibiting activity of drug resistance on *K.pneumonia* in 1/40 dilution that indicates con. of 1.4µg.

AST of *Tinospora cardifolia* leaf extract on *S.aureus*

S.No.	Lab ID	Aqueous		Ethanol		Chloroform	
		Zone of inhibition	MIC	Zone of inhibition	MIC	Zone of inhibition	MIC
1	MDR39T(CI)	NIL	ND	18	1/40	22	1/40
2	MDR40T(CI)	NIL	ND	15	1/40	15	1/40

AST of *Tinospora cardifolia* leaf extract activity on *S.aureus*

This shows that ethanol extract of *T.cardifolia* leaves on *S.aureus* is having the maximum inhibiting activity at 1/40 dilution that indicates con.of 1.4µg.

Conclusion:

The antibacterial efficacy of *Tinospora cardifolia* plant extracts against these multidrug resistant pathogens was found to be promising. The findings in this study reveal the high resistance rates are obtained for clinical isolates of *Escherichia Coli*, *Pseudomonas* and *Klebsiella* and *Staphylococus*. Multiple drug resistance was common in gram negative isolates. These findings were also showing that resistant rates are high in clinical isolates..

VITEK findings also suggested that multidrug resistant bacteria are playing a vital role in spreading infections in communities. In order to prevent this traditional medicinal should be encountered to treat disease.The Multi Drug Resistant bacteria isolated from linical isolates in Khammam have demonstrated a good antibacterial activity when treated with chloroform and ethanolic *Tinospora* extracts. According to the study's findings, *Tinospora* leaf extract may be able to combat MDR bacteria, can be regarded as a natural source of bioactive compounds, and may be used as an alternative treatment for some infectious diseases triggered on by those human pathogenic bacteria.

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