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Comparative Study on Microbial Diversity from Urine Samples of Diabetic and Non-Diabetic Patients

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Abstract

Diabetes mellitus (DM), or simply diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. Microbial diversity of diabetic and non-diabetic patients' urine sample was examined. In our study we have isolated four isolates each from every sample. We have successfully isolated the isolates and performed biochemical tests for the screening of the identified isolates. Antimicrobial assay was performed in which our isolates showed resistance towards Penicillin antibiotics and sensitive towards Azithromycin, Tetracyclin and Ampicillin. We have also checked the antimicrobial potential of our isolates towards plant extracts in which we have used two anti-diabetic medicinal plants i.e. *Tinospora cordifolia* and C*atharanthus roseus.* Diabetic female patients isolate showed 17mm of ZOI for both the plant extracts only. The current study has therefore concluded that *T. cordifolia* and *C. roseus*, two medicinal plants, may aid in the discovery of novel medications that may be used as treatments for bacterial and diabetes ailments that are resistant to current medications.

Keywords: Urine, Diabetes Mellitus, Medicinal plants, Anti-diabetic.

1. Introduction

Urine testing has been a part of medicine for many centuries, with Hippocrates having written about urine examination as early as 400bc (Liao and Churchill et al., 2001).Urine is an unstable fluid; it changes composition as soon as it is eliminated through micturition. Accurate collection,

storage, and handling are crucial to maintaining the sample's integrity (Rai AJ. et al., 2010).Urine samples collected from the first void or "morning urine" are considered the best representative for testing. The urine accumulated overnight in the bladder is more concentrated, thus provides an insight into the kidneys' concentrating capacities and allows for the detection of trace amounts of substances that may not be present in more diluted samples. However, other types of urine specimens may be ordered according to specific purposes (randomly, 2-hours postprandial, 24-hour collection. Furthermore, urine should be ideally examined within the first hour after the collection due to the instability of some urinary components (cells, casts, and crystals). If not possible, the sample should be refrigerated at 4 degrees C for up to 24 hours, which will slow down the decomposition process. Any specimen older than 24 hours cannot be used for urinalysis (Wu X et al., 2010).

Diabetes mellitus (DM), or simply diabetes, is a group of metabolic diseases in which a person has high blood sugar, either because the body does not produce enough insulin, or because cells do not respond to the insulin that is produced. This high blood sugar produces the classical symptoms of polyuria (frequent urination), polydipsia (increased thirst) and polyphagia (increased hunger) (Chinmay D. Deshmukh et al., 2015).Diabetes is the seventh cause of death in the United States, and 30.3 million Americans, or 9.4% of the U.S. population, are living with diabetes.Diabetes is a metabolic disease that causes issues with several different organs and systems. DN, or diabetic nephropathy, is one of the most common and deadly consequences of diabetes. Renal biopsy remains the gold standard for early detection of diabetic neuropathy (DN), despite the identification of several biomarkers. Using renal blood oxygen level monitoring, blood oxygen level-dependent (BOLD), MRI is a noninvasive imaging diagnostic technique that can be used to evaluate the effectiveness of novel medications for diabetic nephropathy (DN) as well as the kidney oxygenation status and fibrosis process. According to recent research, noncoding RNAs, such as circular RNAs (circRNAs), long noncoding RNAs (lncRNAs), and microRNAs (miRNAs), all contributed to the development of DN and may one day be used as a therapeutic approach to manage the disease. Another typical diabetic consequence is dyslipidemia (Fei Hua et al., 2020). Type 1, type 2, and gestational diabetes—diabetes during pregnancy—are the three primary forms of the disease. Diabetes type 1 primarily affects children and teenagers. In 2017, there were 1,106,500 children with type 1 diabetes. Atypical thirst and dry mouth, frequent urination, exhaustion, incessant hunger, abrupt weight loss, bedwetting, and blurred vision are some of the signs and symptoms of type 1 diabetes (Leila Ismail et al., 2021).

*Escherichia coli*is the prominent causative pathogen of UTI in both diabetic and non-diabetic populations, followed by coagulase-negative *Staphylococci* (CONs), *Enterococcus* species (spp.), *Candida albicans*, and non-albicans *Candida* spp (Rajesh Kumar et al., 2019).Patients with diabetes often have increased complications of UTI, including such rare complications as emphysematous cystitis and pyelonephritis, fungi (Ann Stapleton, MD 2002).*C. roseus i*s an important Ayurvedic medication in traditional medicine. It is potentially used in countries like India, South Africa, China and Malaysia for the healing of diabetes mellitus. Although, the molecular mechanisms behind this effect are yet to be exclusively explored. Due to the great antidiabetic and hyperlipidemic potential of *C. roseus*, and *T. cordifolia* commonly known as Guduchi, is a highly potent herb used in Ayurveda to combat diabetes and keep the function of various organs in harmony (Rohitsharma et al., 2015).

2. Materials and Methods

The whole study was done on "Comparative study on microbial diversity from urine samples of diabetic and non-diabetic patients" was conducted in the laboratory of Division of Microbiology at Career Point University, Hamirpur. Different material and methods acquired are discussed below:

2.1 Media :-isused for growth of microbes .
- CLED Agar (C.L.E.D)
- Muller Hinton Agar (MHA)

2.2 Chemical reagents, glass wares and plastic wares:
Analytical grade chemicals and reagents obtained from merck were used for the investigation the glass wares used were purchased from Perfit (India), and the plastics wares were purchased from Tarson (India).

2.3 Microbiological Methods
2.3.1 Maintenance: Bacterial culture was maintained on cled agar plate and store at 4°C.
2.3.2 Sterilization: The glassware and plastic were used were washed in detergent water, running tap water followed by rinsing in distilled water. The sterilization of glassware was carried out in hot air oven at 180°C All the media, distilled water and other material were sterilized by autoclaving and 121°C, 15 pounds per square inch pressure for 20 minutes. Laminar air flow chamber was used to maintain aseptic conditions during microbiological experiment like culturing, inoculation sampling etc.

2.3.3 Sampling and Site characterization
The urine samples were carried out from different sites of Hamirpur district of Himachal Pradesh from Awahdevi, Bhoranj, Dungrin and fill their medical consent forms. The samples were collected in a sterile container. After collection the samples were directly transported to the laboratory and store at 4°C. The sampling was done in the month of March 2024.

2.3.4 Macroscopic and Microscopic examination of urine (Wu X. et al.,2010).
Physical examination of urine: -describe the volume, color, clarity, odor, specific gravity.
Chemical examination of urine: - identify pH, Glucose, protein, ketone bodies, Nitrites.
Microscopic examination of urine: - detection of caste, crystal, and micro-organisms. (6)

2.4 Isolation of different bacterial isolates from urine samples of diabetic and non-diabetic patients.
Serial dilution method is used to isolation of bacterial isolates from collected urine sample of diabetic and non-diabetic patients (Avishai Ben-David et al., 2014). All the urine samples were diluted in the dilution range 10^{-1} to 10^{-9}.1 ml of urine sample was taken in 9 ml of sterile distilled water.Take 1ml of suspension from the tube and serially transfer to all the test tubes.Now take 1ml of sample suspension from the respective test tube and spread over the cled agar plate. Plates were incubated for 24 hours at 37°C.Colony forming units (CFU) were recorded on cled agar plates after 24hours.The average number per ml of urine sample was calculated as:

$$CFU = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Volume of sample plated}}$$

2.5 Screening of bacterial isolates from the urine samples of diabetic and non-diabetic patients.

Bacteria isolates were purified by using streak plate Method. Selected bacterial isolates were picked by using streak plate Method(Nabil Karah et al., 2020).Isolated bacterial isolates were screened for the microbial activity on the basis cled agar plate methods. Cled agar media was prepared.Sterilization was done at 121°C for 15 min.Then sterilized media was poured into petri plates.Isolated bacterial isolates were streaked on the cled agar platesThen incubate at 37°C for 24hours.After that examine the cled agar plates.

2.6 Morphological, Physiological and Biochemical characterization of bacterial isolates from urine samples of diabetic and non-diabetic patients.

The different isolates were identified on the basis of morphological, physiological and biochemical characteristics according to the Bergey`s manual of systematic bacteriology.

2.6.1 Morphological and physiological characterization

Morphological characteristics of isolates including Colony morphology, Colony elevation, Colony margin, Colony forms, Gram stain and cell shape were investigated.

2.6.2 Gram stain Four different reagents in the order of crystal violet, Gram`s iodine, Alcohol, and safranin were used for gram staining (Nishant Tripathi et al.,2023).Smear of the selected strain prepared on a glass slide.Air dry and heat fix, cover the smear with crystal violet for 1min.Slide was gently rinse with water and adding iodine for 30 seconds. Pour off the excess gram's iodine.Smear were decolorized with 95% ethyl alcohol and washed with water after 30 seconds.Adding a counter strain (safranin) for 30 seconds.After washing with water, the slide was dried and examined under microscope.

2.6.3 Biochemical characterization

For biochemical characterization, the isolated bacterial isolates were subjected to various biochemical test such as: -Indole, MR-VP, Citrate, Catalase and Oxidase.

2.6.4 Indole test: - determine the ability of the organism to convert tryptophan into indole (Charles Darkoh et al., 2015).Take a sterilized test tube containing 4 ml of tryptophan broth.Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.Incubate the tube at 37°C for 24-28 hours.Add 0.5 ml of Kovac's reagent to the broth culture.Observe for the presence or absence of ring.

2.6.5 MR-VP test: - The methyl red (MR) test is used to determine if an organism can produce stable acid end products from glucose fermentation. The Voges – Proskauer is used to determine if an organism produces acetylmethylcarbinol from glucose fermentation (ArchanaAnokhe et al.,2021).Take one tube of MR-VP broth.Using an inoculating loop, swish some of your assigned organism in the brothIncubate the tube for at least 48 hours.After the incubation period, vortex the tube and pour half of the broth into another clean test tube.Complete the tests on the two individual tubes as follows:

MR: to one tube add a drop or two of the pH indicator methyl red and swirl. If the tube turns red, the test is positive for mixed acid fermentation

VP: TO the other tube add three drops of Barritt's 1/A reagent and a drop of Barritt's 2/B reagent. Shake vigorously and watch the tube over the next hour or two. If the tube turns pink or red, the test is positive.

2.6.6 Citrate test: - detects the ability of an organism to use citrate as the sole source of carbon and energy (Dustin J. Van Hofwegen et al.,2014).Streak the slant back and forth with a light inoculum picked from the center of a well-isolated colony.Incubate aerobically at 35 to 37°C for 1-2 days.Observe a color change from green to blue along the slant.

2.6.7 Catalase test: - catalase is an enzyme that split hydrogen peroxide into oxygen and water (RayaneRafei et al., 2020).Heavy streaking of all the potentials on surface of nutrients agar slant. Incubate the tubes at 37°C for 24 hours.1ml of hydrogen peroxide was added over the growth of agar slant.Rapid appearance and sustained production of gas bubbles.

2.6.8 Oxidase test: - is used to determine whether an organism possesses the cytochrome c oxidase enzyme (WaelElamin et al., 2020).Use a loop and pick a well-isolated colony from a fresh (18- to 24- hour culture) bacterial plate and place onto a glass slide.Place 1 or 2 drops of 1% Kovács oxidase reagent on the organism smear.Observe for color changes. Microorganisms were oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms were delayed oxidase positive when the color changes to purple within 60 to 90 seconds.Microorganisms were oxidase negative if the color does not change or it takes longer than 2 minutes.

2.7 Antibacterial evaluation of isolated microbes from urine sample.

2.7.1 Antibiotics Susceptibility Test

Antibiotics susceptibility test was performed on Muller – Hinton agar using disk diffusion technique (Aswathy Suresh et al.,2017).Preparation of Nutrient broth. Dissolve 1.3g of nutrient broth powder in 100ml of distilled water.Mix and dissolved properly. Pour 9ml Nutrient broth in each test tube.Autoclave at 121°C for 15 min.Cool to room Temperature.Label the test tubes of nutrient broth with patient's name.Transfer colony of isolated microbes in a test tube of nutrient broth.Mix properly.Spread the inoculum on the MHA agar plate.Diffuse the antibiotics disc (Tetracyclin, Ampicillin, Penicillin, Azithromycin) on theMHA plate.Observe the zone of inhibhition.

2.7.2 Antibacterial evaluation of bacterial isolates using plant extracts.

Plants(*C. roseus* and *T. cordofolia*) have always been a very good source of drugs and many of the currently available drugs have been derived directly or indirectly from them (DK Patel et al., 2012).Label the test tubes of nutrient broth with patient's name.Transfer colony of isolated microbes in a test tube of nutrient broth.Mix properly.Incubate at 37°C for 24hrs.Observe turbidity.Again, take test tubes of fresh nutrient broth and label it.Transfer 1ml of overnight inoculum in a broth tubes.Spread the inoculum on the MHA agar plate.Make the 3 agar well on the MHA agar plate.Fill the agar well with plants extract of *C. roseus*, *T. cordofolia* andDMSO.Diffuse the antibiotics disc Tetracyclin, on the MHA plates.Observe the zone of inhibition.

3. Results and Discussion

3.1 Medical consent form of patients

Fill the medical consent form of both (Male & Female) diabetic and non-diabetic patients. In medical consent form of patients following things are mentioned: Name of patient, Age, Sex, Date of Birth, Type of Sample and Date of sample collection.

3.2 Sampling and Site characterization

The urine samples were carried out from different sites of Hamirpur district of Himachal Pradesh from Awahdevi, Bhoranj, Dungrin.

3.3 Collection of samples: The samples were collected in a sterile container. After collection the samples were directly transported to the laboratory and store at 4°C. The sampling was done in the month of March 2024 as shown in Table 1.

Table 1:- Macroscopic and Microscopic examination of Urine sample

Examination of urine	Diabetic Male	Non-Diabetic Male	Diabetic Female	Non-Diabetic Female
Volume	25ml	25ml	25ml	25ml
Colour	Yellow	Yellow	Dark Yellow	Yellow
Apperance	Clear	Clear	Cloudy	Clear
Glucose	50mg/dl	Nil	100mg/dl	Nil
Protein	Nil	Nil	Trace	Nil
pH	5	6	6	6
Microscopic Examination	Not identified	Pus cell identified	Full of pus cell	Not identified

3.4 Media preparation and isolation of microbes from collected samples

We prepare the CLED agar media for supports the growth of microbes. Then we use the pour plate method for solidification of agar on the plate. After that we perform the spreading techniques to count the number of isolated microbes present in the samples as shown in Table 2-5 respectively.

3.5 Isolation of bacterial isolates from collected samples: -

Table 2: - Morphological identification of bacterial isolates from diabetic male samples.

S. No	Dilution Factor	Colony pigmentation	Colony Elevation	Colony Margin	Colony Forms
1	10^{-2}	Creamy, yellowish	Flat, Raised	Entire, Undiluted	Circular, irregular
2	10^{-4}	Creamy, whitish	Flat, Raised	Entire	Circular
3	10^{-6}	Yellowish, Creamy	Flat, Raised	Entire, Undiluted	Circular, irregular
4	10^{-8}	Yellowish, whitish	Flat, Raised	Entire, Undiluted	Circular, irregular

Table 3: -Morphological identification of bacterial isolates from Non-Diabetic Male samples

S. No	Dilution Factor	Colony pigmentation	Colony Elevation	Colony Margin	Colony Forms
1	10^{-2}	Creamy, whitish	Flat	Entire	Circular
2	10^{-4}	Creamy, whitish	Flat	Filament	Circular
3	10^{-6}	Yellowish, Creamy	Flat	Undiluted, Filament	Circular, Filamentous
4	10^{-8}	Yellowish, whitish	Flat	Entire, Filament	Circular, Filamentous

Table 4: Morphological identification of bacterial isolates from Diabetic Female sample

S.No	Dilution Factor	Colony pigmentation	Colony Elevation	Colony Margin	Colony Forms
1	10^{-1}	Creamy, whitish	Flat	Entire	Circular
2	10^{-3}	Creamy, whitish	Flat	Entire	Circular
3	10^{-5}	Whitish	Flat	Entire	Circular
4	10^{-7}	Whitish	Flat	Entire	Circular

Table 5: - Morphological identification of bacterial isolates from Non-Diabetic Female samples

S. No	Dilution Factor	Colony pigmentation	Colony Elevation	Colony Margin	Colony Forms
1	10^{-1}	Creamy, whitish	Flat	Entire, Undiluted	Circular
2	10^{-3}	Creamy, whitish	Flat	Entire, Undiluted	Circular
3	10^{-5}	Creamy, whitish	Flat	Entire, Undiluted	Circular
4	10^{-7}	Creamy, whitish	Flat	Entire, Undiluted	Circular

3.6 Cultural identification of bacterial isolates from collected samples.

After isolation, we take 10^{-6} and 10^{-8} from Diabetic and Non-Diabetic Male and 10^{-5} and 10^{-7} from Diabetic and Non- Diabetic Female plates for the cultural identification because the colony count is more as compared to other dilution plate. Streak Plate Method is used for the cultural identification of bacterial isolates from collected samples. Streak Plate Method is used to isolate a pure colony from a single species of microorganism.

3.7 Gram stain of bacterial isolates from collected samples.

After cultural identification, we perform gram stain of collected samples. Gram stain is mostly used for the identification of bacteria.

Table 6:- Biochemical identification of bacterial isolates from collected samples

Type of samples	MR-VP Test	Indole Test	Citrate Test	Catalase Test	Oxidase Test
Diabetic Male	Positive	Negative	Negative	Positive	Negative
Non-Diabetic Male	Positive	Positive	Positive	Positive	Negative
Diabetic Female	Negative	Negative	Positive	Positive	Negative
Non-Diabetic Female	Positive	Positive	Positive	Positive	Negative

In our study from the biochemical characterization, we have reported the presence of *Staphylococcus aureus*, similarly Rawat et al., 2012 reported the similar results, where they found *E. coli*&*Staphylococcus aureus* in their urine samples from diabetic patients as shown in Table 6.

3.8 Antibacterial evaluation of isolated microbes from urine sample

We perform antimicrobial susceptibility test of isolated microbes from urine sample to check their sensitivity against the antibiotics. Antimicrobial susceptibility test is mostly done on MHA agar plate as shown in Fig. 1 and Table 7.

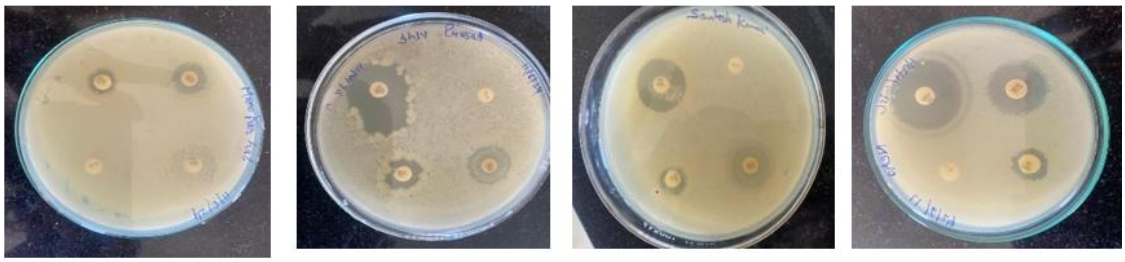

Fig.1: – Antibacterial evaluation of isolated microbesfrom urine sample.

Table 7: – Antibacterial evaluation of isolated microbes from urine sample.

Type of patients	Zone of inhibition (mm) Azithromycin	Zone of inhibition (mm) Penicillin	Zone of inhibition (mm) Ampicillin	Zone of inhibition (mm) Tetracycline
Diabetic Male	17mm	Resistance	10mm	13mm
Non-Diabetic Male	23mm	Resistance	12mm	17mm
Diabetic Female	20mm	Resistance	10mm	15mm
Non-Diabetic Female	25mm	Resistance	10mm	17mm

3.9 Antibacterial evaluation of bacterial isolates using plant extracts.

We have use two anti-diabetic plants *C. roseus* & *T. cordifolia* for antibacterial evaluation of our isolates using plant extracts as shown in Fig 2 and Table 8.

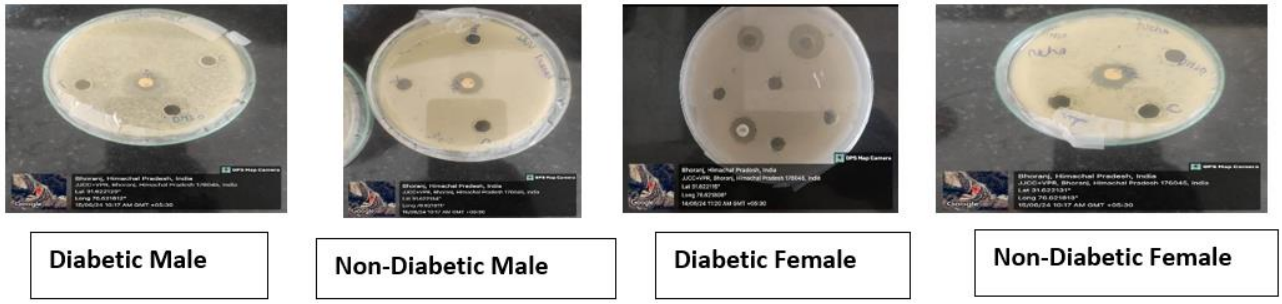

Fig 2:- Zone of inhibition of *T. cordifolia* and *C. roseus* plants extract against bacterial isolates.

Table 8:- Antibacterial evaluation of bacterial isolates using plant extracts.

Type of sample	Type of Plants	30ul zone of inhibition	50ul zone of inhibition	Tetracycline
Diabetic Female	*C. roseus*	13mm	17mm	18mm
Non-Diabetic Female	*T. cordifolia*	14mm	17mm	18mm

As per the report from Saleni et al., 2019 medicinal plants were used to treat various urine infection. So, in our study we have used two antidiabetic plants i.e. *C. roseus* &*T. cordifolia* were showed positive results only for diabetic female patients. In our study we have found 17mm of ZOI

using *C. roseus* plant extract similarly 17mm of ZOI was found using *T. Cordifolia* plant extract against the diabetic female isolates, so from our study we can confirm that, our isolates can be treated with chemically synthesized antibiotics as well as using herbal formulations.

Conclusion

We have successfully isolated one isolates each from diabetic male, female & non-diabetic male, female patients. All the isolates from urine samples showed resistance to Penicillin while Sensitive for Azithromycin, Ampicillin, Tetracyclin.Only Diabetic female isolate showed ZOI for anti-diabetic plant extracts. From our study we can conclude that our isolates can be treat with antibiotics as well as with herbal plant extracts.

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Data availability Data sharing not applicable to this article as no data-sets were generated or analyzed during the current study.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Competing interest The authors declare no competing interest.

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