“ANTIMICROBIAL EFFECT OF PLANT EXTRACTS ON ISOLATED MICROBIAL FLORA OF DIABETIC FOOT”

project submitted to Department of Microbiology

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Certificate

This is to certify that the minor research project entitled “ANTIMICROBIAL EFFECT OF PLANT EXTRACTS ON ISOLATED MICROBIAL FLORA OF DIABETIC FOOT” being submitted by S.Princy rose, I.Keziah, M.Rani priya, B.Deepthi, CH.Cheliciya of III BSC C.B.M, during the academic year 2019-2020 under my guidance to the coordinator RUSA.

Signature of the Head of the Department

Signature of RUSA coordinator

Signature of the principal

DECLARATION

I hereby declare that the project work entitled " **ANTIMICROBIAL EFFECT OF PLANT EXTRACTS ON ISOLATED MICROBIAL FLORA OF DIABETIC FOOT"** submitted to RUSA 2.0 scheme, under the guidance of **Smt.Sk. Khareemunisa,** Lecturer in Microbiology, Department of Microbiology(UG), Sri Y.N. college (Autonomous), Narsapur.

SIGNATURE OF PROJECT STUDENTS

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ABBREVIATIONS

mg – Milli grams

ml-Milli liters

Lts-Liters

M- Molar

% - Percentage

µg-Micro grams

µl – Micro liters

Gms - grams

˚C- Degree centigrade

Abstract

The present minor research project was undertaken to determine the bacteriological spectrum of diabetic foot and to assess their in vitro susceptibility to medicinal plant extracts. Diabetic foot contains poly microbes of both aerobic and anaerobic type. The bacterial inhabitants are highly resistant to antibiotics called multidrug resistant (MDR) bacteria. Natural antimicrobial agents derived from plant extracts with antimicrobial activity may be the option for treating the infection of the lesions to avoid further complications.

Diabetes mellitus is a chronic disorder affecting a large segment of population and also a major public health problem. India homes 33 million diabetics ranking highest in the world. 20% of all diabetic complication involve of feet. The burden of diabetic foot is set to size further in future since its contributory factors, such as peripheral neuropathy and vascular disease represent in >10% of the cases.

In view of the above, a prospective bacteriological study is necessary to assess the role of aerobic and anaerobic bacteria in diabetic foot ulcers. The antimicrobial spectrum of medicinal plant extracts would assist the clinicians in the therapy of this dreaded complication of diabetics.

Diabetic foot pus samples were collected from the patient after informed consent and processed in microbiological laboratory to identify possible bacterial species, their ability to interact with antibiotics were analysed in the first phase by Kirby Bauer method on Muller Hinton Agar, similarly the antimicrobial effect of medicinal plant extracts were also assessed and the results obtained are compared to identify suitable most effective therapy without much side effects .

By identifying the novel plant extracts with antimicrobial effect on pathogenic bacteria of diabetic foot we can able to devise suitable therapy to treat such complication and also suggest approaches such as nano particle based ointments with plant derived compounds as external application.

INTRODUCTION

ANTIMICROBIAL EFFECT OF PLANT EXTRACTS ON ISOLATED MIROBIAL FLORA OF DIABETIC FOOT

INTRODUCTION:

Diabetes mellitus represents a major public health threat worldwide. A serious complication of diabetes is the development of foot ulcers which, when they become infected, are the most common cause of diabetes-related hospital admissions and a leading cause of lower extremity amputation. In this review, we will update information on the diabetic foot microbiota together with the factors influencing its composition. We highlight the role of bacteria in the pathogenesis of diabetic foot ulcers. Based on current research evidence, we address the issue of differentiating infection from colonization. Finally, we emphasize the importance of the use of complementary culture and molecular-based methods for describing complex microbiota, with a view to overcoming their respective limits.

Diabetes mellitus represents an estimated prevalence in 2014 of 422 million patients [1].A serious complication of diabetes is the development of foot ulcers. Diabetic patients are believed to have a 12–25% lifetime risk of developing a foot ulcer [2]. Diabetic foot ulcers (DFUs), especially when they become infected, are the most common cause of diabetes-related hospital admissions [3]. Diabetic foot osteomyelitis develops in approximately 44–68% of patients admitted to hospital with a diabetic foot infection (DFI) and is the leading cause of amputation among such patients [4]. DFUs often develop due to a combination of extrinsic mechanical factors such as high plantar pressures or local trauma, plus intrinsic factors such as peripheral neuropathy, micro-vascular disease and impaired host immune response [5]. In patients with a healed DFU, significant independent risk factors for DFU recurrence during a 3-year follow-up period, despite intensive foot care, were: plantar ulcer location, presence of osteomyelitis, HbA1c > 7.5%, and C-reactive protein (CRP) > 5mg/l [6]. A DFI is defined by the presence of an inflammatory response and tissue injury that can run the clinical spectrum from simple, superficial

cellulitis to chronic osteomyelitis, as a consequence of interaction between the host and multiplying bacteria [7]. Factors significantly associated with the occurrence of DFI have rarely been studied. One prospective and multifocus study identified bone contact on probing, foot ulcer duration of longer than 30days,.

A history of recurrent foot ulcers, traumatic etiology of the ulcer and peripheral vascular disease as independent risk factors for DFIs from a multivariate analysis [8]. Another retrospective study of DFI reported that risk factors for DFIs were previous amputation, peripheral vascular disease and neuropathy but not the socioeconomic status nor the patient's knowledge of foot care [9]. Recent studies using molecular methods have confirmed that chronic wounds, including DFUs, have a polymicrobial nature that largely exceeds the identification capabilities of traditional culture methods [10–13]. However molecular methods are not routinely used in clinical settings and are also hampered by several biases [14]. Determining the specific role of the isolated bacteria is challenging but necessary to optimize the management of DFUs, especially when they are considered to be infected. In this review, we will update information on the diabetic foot microbiota together with the factors influencing its composition. We highlight the role of bacteria in the pathogenesis of DFUs. Based on current research evidence, we address the issue of differentiating infection from colonization. Finally, we emphasize the importance of using complementary culture and molecular-based methods to describe complex microbiota, with a view to overcoming their respective limits.

Culture-based methods:

Optimal management of DFIs requires identification of bacteria and antibiotic-susceptibility testing in order to adjust the antibiotic treatment. Traditional culture-dependent methods are usually performed in clinical practice. However, results are limited by the fact that these methods select for species that yield faster under restricted conditions. Indeed, in standard culture, *S. aureus* has been the most commonly isolated bacterium from DFIs in north-western countries [20].This does not take into account the failure to identify slow-growing, fastidious or anaerobic organisms, and does not necessarily reflect the most abundant or clinically important bacteria in DFIs [50]. Two large culture dependent studies on the ecology of DFIs sampling over 1266 patients returned similar results [39,51]. They identified mostly Gram-positive aerobic bacteria, primarily *Staphylococcus spp.* (24–35%) and especially *S. aureus* (47–55%). A higher incidence of Gram-

negative aerobes (*P. aeruginosa*, *Enterobacteriaceae*) and anaerobes was found in the most chronic wounds (Table S1) [39,62,63]. Anaerobes were reported as being of low abundance. However, in a recent study with optimized anaerobic conditions, a wide range of anaerobes were cultured from DFIs [52]. In older studies, anaerobes have been isolated from up to 95% of deep diabetic wounds; the predominant isolates being *Pepto streptococcus spp.*, Bacteroides spp., and Prevotella spp. [53,54]. This emphasizes the potential to enlarge the spectra of culture method bacterial identification with the use of the new approach, culturomics, which consists of large-scale culture conditions with colony identification by matrix-assisted laser desorption ionization time-of flight mass spectrometry or 16S rRNA PCR [14]. Promising strategies, such as a reduction in the sample transport time before inoculation and the use of antioxidant agents such as ascorbic acid and glutathione, will dramatically improve the culture of previously uncultured bacteria, including anaerobes [55]. Finally, as molecular tools are not available in most clinical settings, identifying the largest variety of bacteria including obligate anaerobes in pathological biofilm requires the use of accurate culture methods. These include optimal methods of wound sampling, specimen transport

MATERIALS AND METHODS

Specimens (pus, wound exudates, or tissue biopsy) for microbiological studies were obtained from the ulcer region. Pus and exudates were collected from the margins and the base of the ulcer in 11 patients respectively using a sterile swab stick, which was then transported in a clean and sterile test tube. These specimens were immediately transported to the microbiology laboratory for further processing. Culture, isolation, antibiotic sensitivity and identification of the microorganisms were done according to the standard microbiological procedures. [20,21] Due to lack of resources, anaerobic culture was not done; therefore, results were analyzed for aerobic flora only.

Antimicrobial susceptibility testing of aerobic isolates was performed by the well diffusion method.

Composition of Muller Hinton Agar:

Beef extract- 2.00gm

Acid hydrolysate of casein- 17.50gm

Starch- 1.50gm

Agar- 17.00gm

Distilled water- 1000ml

pH 7.3+_0.1

Biochemical characterization

1) Oxidase test :

A small piece of filter paper was soaked in 1% Kovac‘s oxidase reagent and dried.

With the help of a loop a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate was taken and rubbed onto treated filter paper and observed for color changes.

Microorganisms are considered as oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

2) Catalase test:

The enzyme catalase converts hydrogen peroxide into water and oxygen, thus helping an organism to cope with toxic O* species. The catalase test is used to detect an organism‘s ability to produce catalase. With the help of a loop a small lump of bacterial colony from a fresh culture was taken and introduced into few ml of hydrogen peroxide in a test tube or into a drop of hydrogen peroxide on a glass slide and observed for the formation of bubbles due to release of oxygen. The organism which reacts is considered as catalase positive.

3) Indole test

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole.

Method:

a. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.

b. Incubate at 37°C for 24-28 hours in ambient air.

c. Add 0.5 ml of Kovac‘s reagent to the broth culture.

Expected results:

Positive: Pink colored rink after addition of appropriate reagent

Negative: No color change even after the addition of appropriate reagent.

3) MR-VP test

Methyl red test and Voges-Proskauer test both are done in methyl red–Voges-Proskauer (MR VP) broth, but the reagents that we add differs in terms of reaction.

Methyl Red (MR) Test:

 Positive methyl red test is indicated by the development of red color after the addition of methyl red reagent.

 A negative methyl red test is indicated by no color change after the addition of methyl red

Voges-Proskauer (VP) test:

1. Negative test is indicated by lack of color change after the addition of Barritt‘s A and Barritt‘s B reagents.
2. A positive Voges-Proskauer test is indicated by the development of red-brown color after the addition of Barritt‘s A and Barritt‘s B reagents.

5) Citrate Utilization Test:

Citrate utilization test is performed on Simmons citrate agar:

A. Negative citrate utilization test is indicated by the lack of growth and color change in the tube

B. A positive citrate result as indicated by growth and a blue color change.

Antimicrobial effect of Plant Extracts on Isolated Bacteria:

Procedure & Method for well diffusion:

- Agar well diffusion method is used to evaluate the microbial activity of plant extracts.
- Similarly to the procedure used in disk diffusion method, the agar plate surface is inoculated by spreading a 100 µl volume of the microbial inoculum over the entire agar surface.
- Then, a hole with a diameter of 6 to 8mm is punched aseptically with a sterile cork borer and a volume of 20-100 µl plant extract solution is introduced in to the well.
- The agar plates are incubated under suitable conditions depending upon the test microorganisms. (37˚ C)
- The Zone of Inhibition was measured using standard antibiotic zone measuring scale.

RESULTS

In the 11 diabetic foot patients studied were men and women. The age ranged from 25 to 84 years . Among those having diabetic foot ulcers, a majority of patients (56.31%) were in the age group 51 to 70 years.

Among the bacterial isolates, gram-negative comprised of 76% and gram-positive accounted for 24%.Bacillus*. Spcs* , was the most common isolate, accounting for 21.67%; followed by *Staphylococcus. Spcs* and *Acenitobacter Spcs*.

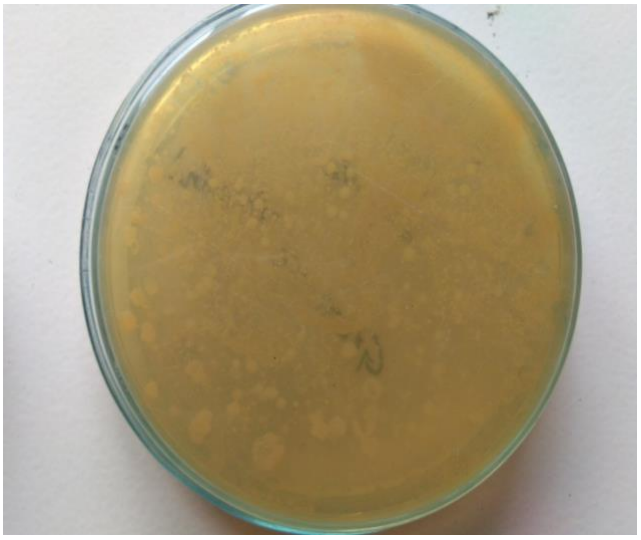
Staining Results:

1. *Acenitobacter: Gram –ve Bacilli*
2. *Staphylococcus: Gram +ve cocci*

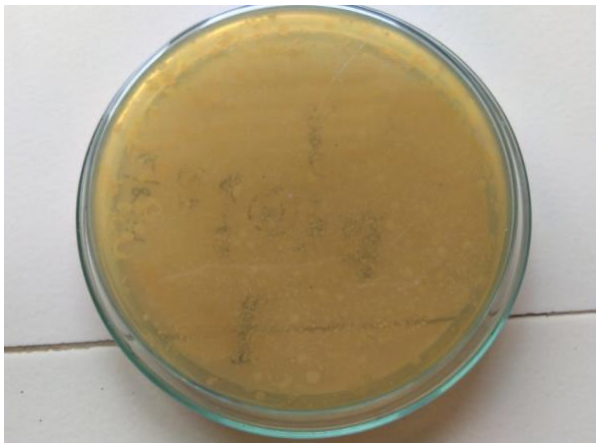
IMVC Test Results:

Bacterial Species	Oxidase	Catalase	Indole	Methyl Red	Voges Proskauer	Citrate
Acenitobacter	-ve	**+ve**	**+ve**	**-ve**	**-ve**	**+ve**
Staphylococcus	**-ve**	**+ve**	**-ve**	**-ve**	**+ve**	**+ve**

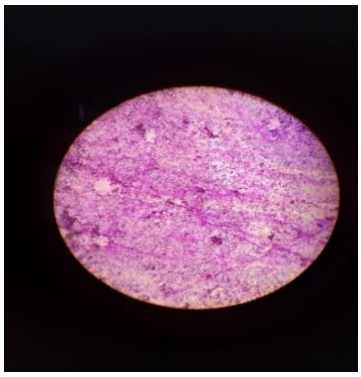
Isolated Bacteria



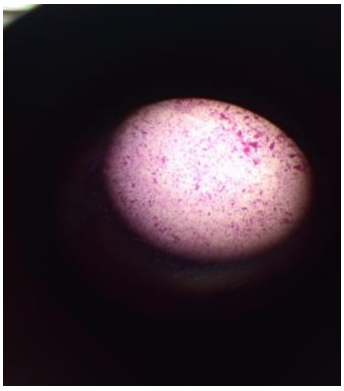
PUS SAMPLE



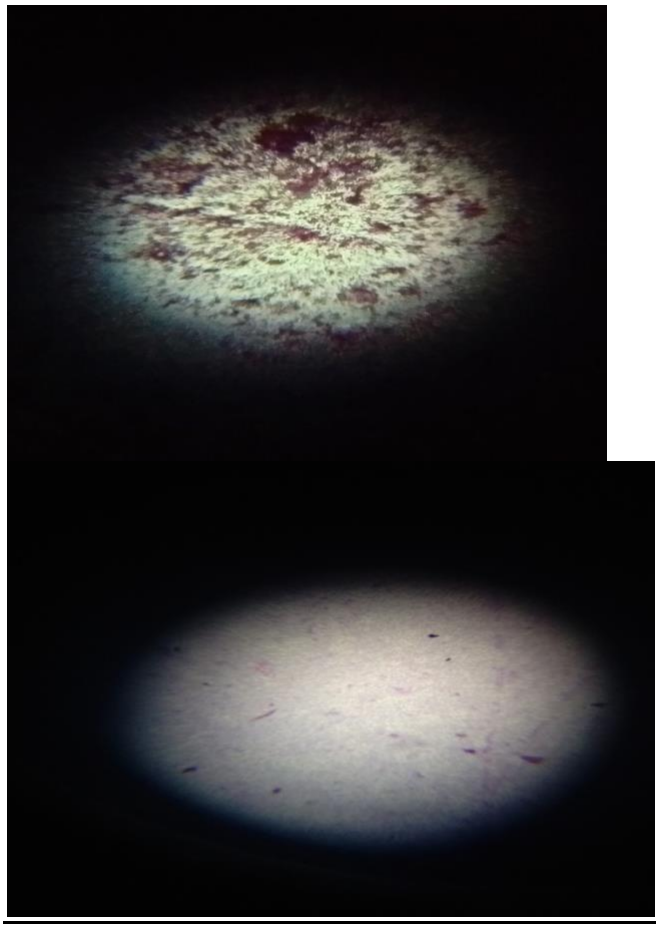
PUS SAMPLE: gram -ve



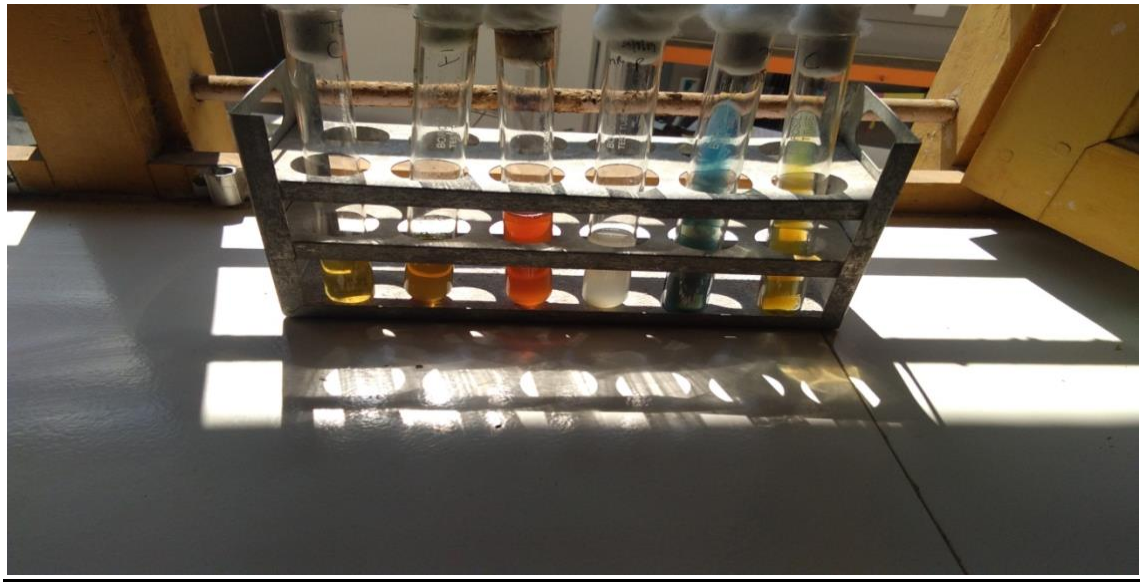
Gram +ve



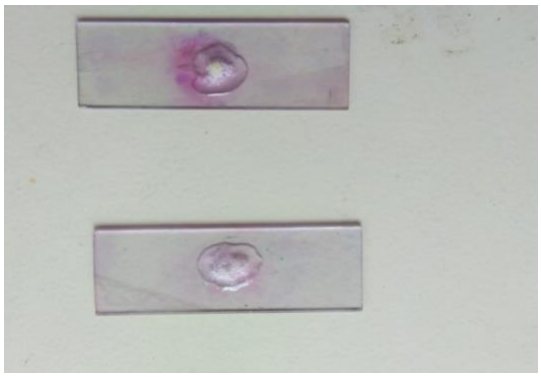
Gram-ve



Gram +ve Cocci **Gram -ve Bacilli**



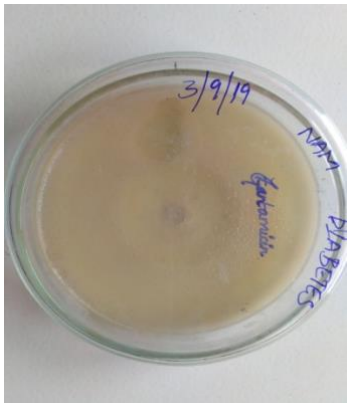
Imvic test: indole +ve, methyl red -ve, voges proskaur -ve, citrate utilization +ve



catalase test +ve

Medicinal plant extracts:

ANTIBIOTIC -CONTROL PLATES



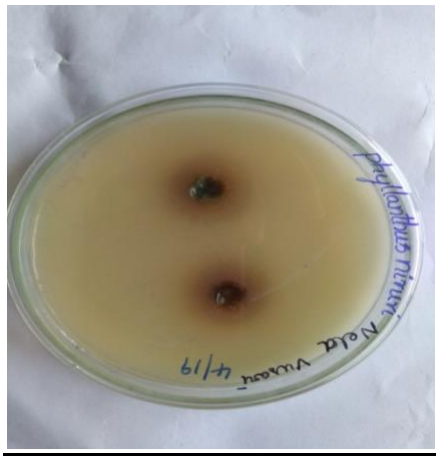
GENTAMICIN -35mm

AMIKACIN-40mm

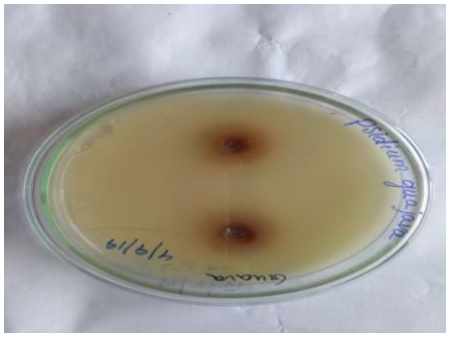
1.Clitoria ternatea – 16mm zone

2. Andrographis paniculata – 21mm zone

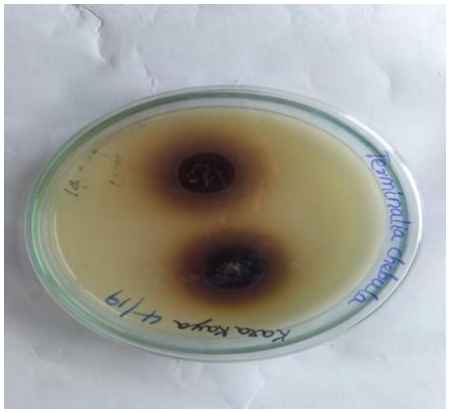
3.Phylanthus niruri -20mm zone



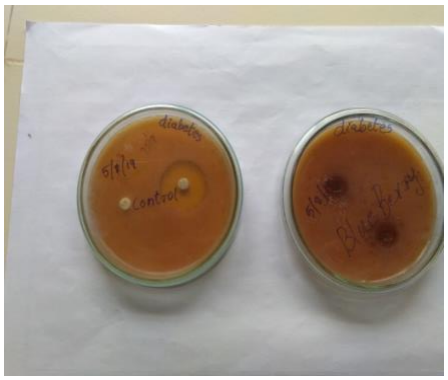
4. Psidium gujjava – 19mm zone



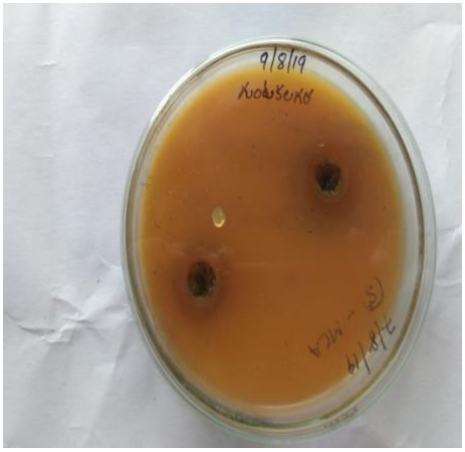
5. Terminalla chebulla – 30mm zone



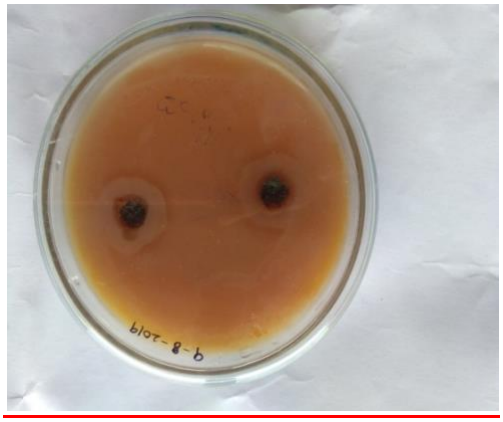
6. Blue berry- 16mm zone



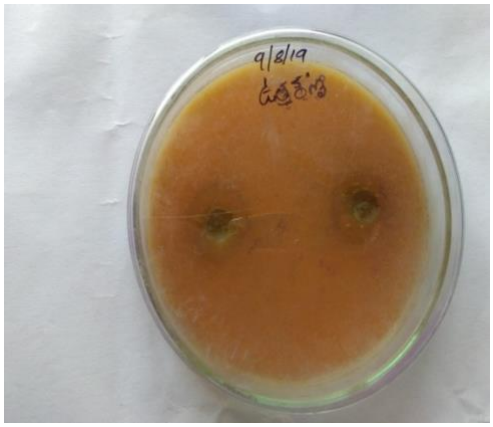
7. Calendulacea bhringaraja- 22mm zone



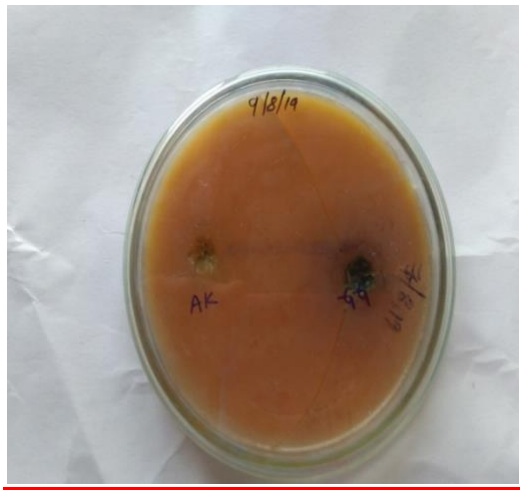
8. Azima tetracantha – 24mm zone



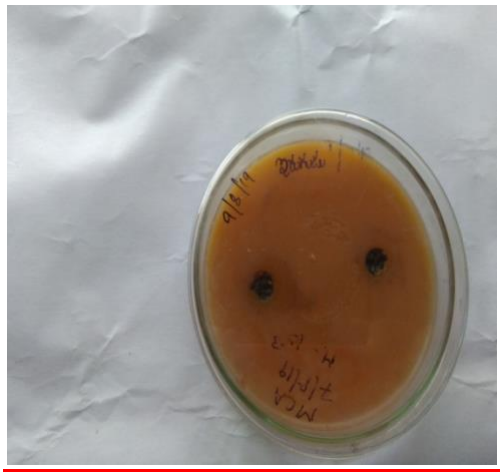
9. Achyranthus aspera -20mm zone



10. Aristalochia bracteata -17mm zone



11. Dalbergia latifolia - 20mm zone



12. Syzygium cumini -18mm zone

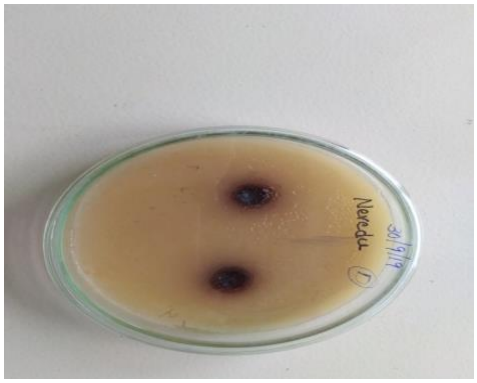


Table form:

Name of Plant Extract	Inhibition Zone for *Acenitobacter*	Inhibition Zone for *Stphylococcus*
Clitoria ternatea	16mm	11mm
Andrographis - paniculata	21mm	16mm
Phylanthus niruri	20mm	14mm
Psidium gujjava	19mm	14mm
Terminalla chebulla	30mm	22mm
Blue berry	16mm	14mm
Calendulacea bhringaraaja	22mm	19mm
cinnamom	18mm	17mm
Catharanthus roseus	20mm	20mm
Indeevara	17mm	20mm
Azima tetracantha	24mm	23mm
Aristalochia bracteata	17mm	16mm
Physalis minima	19mm	22mm
Dalbergia latifolia	20mm	17mm

Achyranthus aspera	19mm	20mm
Syzygium cumini	18mm	18mm
Clitoria ternatea	11mm	10mm

Discussion

In the present study 11 pus samples from patients suffering with diabetic foot were collected from clinical laboratory attached to a hospital. When cultured in vitro 02 isolates are predominant, *Acenitobacter & Staphylococcus.* .

The isolated bacteria are susceptible to antibiotics gentamicin and amikacin exhibiting an inhibition zone of 35mm and 40mm respectively.

The isolated bacteria when tested against plant extracts they have shown zone of inhibition ranging from 14mm to 30mm . A zone of inhibition greater than 13mm is considered as sensitive, so most of the plant extracts were capable of inhibiting the bacterial growth *in vitro.*

Out of 17 Plant Extracts tested 07 plant extracts have exhibiting greater inhibition of bacteria, like *Terminaelia chebula, Azima tetracantha, Catharanthus roseus, Andrographis paniculata, Bringha raja, Physalis minima, Ahyranthus aspera.*

Conclusion

Diabetic foot ulcer is a polymicrobial infection harbouring different bacteria. Multi drug resistance is one of the challenges faced by the therapists. To treat such infection alternate medicine which can be applied externally and have least side effects is highly appreciated, this project is such an attempt to counteract bacterial infections associated with diabetic foot, the plant extracts with nano particle base ointments will help to treat these infections more efficiently without any drug resistance. The efficacy of such drugs need to be tested and validated before application.

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