ANTIBACTERIAL ACTIVITY OF *NERIUM INDICUM* AND *NERIUM OLEANDER* FLOWER EXTRACT AGAINST DIABETIC WOUND PATHOGENS

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Abstract : Diabetes mellitus, commonly referred to as diabetes was first identified as a disease associated with "sweet urine," characterized by elevated blood glucose levels (hyperglycemia) resulting from defects in insulin secretion, insulin action or both. Insulin is a hormone produced by the beta cells of the pancreas, which is required to utilize glucose from digested food as an energy source. Diabetic Wound Infections most frequent and serious complications of diabetes. Medicinal plants are source of great economic and thousands of species are known to have medicinal value and the use of different parts including root, stem, and flower and twigs exudates as the raw material for many herbal industries. Hundreds of plants species have been tested for antimicrobial properties and the medicinal value of these plants depends on bioactive phytochemical constituents that produce physiological action in the human body. The present study was carried to isolate and identify the pathogenic organisms from diabetic wound infections. From 30 diabetic wound samples, 65 isolates were isolated and identified most of them are *Staphylococcus aureus, Escherichia coli, Pseudomonas sps, Bacillus sps* and *Klebsiella sps*. The antibacterial activity of the *Nerium indicum, Nerium oleander* flowers, were tested using different solvents viz., ethanol, methanol, diethyl ether extract were prepared and tested for its antibacterial activity against the isolated bacteria viz., *Staphylococcus aureus, Pseudomonas sps, Escherichia coli, Bacillus sps,* and *Klebsiella*. The antibacterial activity of methanol extracts of *Nerium oleander* white flower at the concentration of 100µl was high (25mm) against *Staphylococcus aureus* whereas minimum activity was recorded (15mm) against *Klebsiella sps*. The ethanol extracts of *Nerium indicum* Red flower at 100µl concentration the activity was high (24mm) against *Staphylococcus aureus*. Minimum activity was (15mm) against *Pseudomonas sps*. The antibacterial activity of diethyl ether extracts of *Nerium oleander* Pink flower at 100µl concentration the inhibition zone was high (22mm) against *Staphylococcus aureus*. Whereas minimum activity (16mm) against *Pseudomonas sps*. The GC-MS analysis of the Methanolic plant extract of *Nerium indicum* indicated that the methanolic extracts of *Nerium indicum* (Red flower) contain highest concentration of Heptacosane which followed by Triacontane, Lucenin 2, Silicone oil, 2,2,3,3,4,4 Hexadeutero octadecanal, Mome Inositol, and 1,2-Benzenedicarboxylic acid, diethyl ester (CAS)..

IndexTerms **- Diabetis mellitus, Diabetic Wound Infection, *Nerium indicum, Nerium oleander*, Phytochemicals, Antibacterial activity.**

I. INTRODUCTION

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and post prandial blood sugar levels. The global prevalence of diabetes is estimated to increase from 4% in 1995 to 5.4% by the year 2025. It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025. Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. The incidence of diabetes is growing rapidly both in the India and worldwide. Diabetes is not a single disease. Rather, it is a heterogeneous group of syndrome characterized by an elevation of blood glucose caused by a relative or absolute deficiency of insulin. Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia, glycosuria, hyperlipidemia, negative nitrogen balance and sometimes ketonemia (Manisha Maish *et al*., 2011)

Diabetic foot infections (DFI) are commonly encountered problems in the practice of clinical medicine today. They are among the most frequent and serious complications of diabetes mellitus and responsible for most of the non-traumatic lower and limp amputations. In India, Diabetic foot infections are the most common diabetes related cause of hospitalization. (Vishnu Datta *et al*., 2014).

Foot infections in patients with diabetes cause substantial morbidity are frequent visits to healthcare professionals and may lead to amputations of a lower extremity. Diabetic foot infections require attention to local foot and systemic metabolic issues and co-ordinated management preferable by a multidisciplinary foot care team. The major predisposing factor to these infections is foot ulceration which is usually related to peripheral neuropathy (Benjamin *et al*., 2004).

The gram negative cocci especially *Staphylococcus aureus* is the predominant pathogen in diabetic foot infections. Patient who have chronic wounds or who recently have antibiotic therapy may also be infected with gram negative rods, and those with foot ischemia or gangrene may have obligate anaerobic pathogens. Wound infections must be diagnosed clinically on the basis of

local and occasionally systemic signs and symptoms of inflammation. The most common organisms involved in diabetic foot infections are *Pseudomonas aeroginosa, Staphylococcus aureus, Escherichia coli, Bacillus sps, Klebsiella species* (Younes *et al*., 2006)

The annual incidence of foot ulcers among patients with diabetes is approximately 2.5% with a prevalence of 4.10%. Diabetic foot ulcers have a major impact on the patient as well as healthcare system. These ulcers tend to heal slowly need intensive care and healing can be complicated by infection and gangrene, leading to long term treatment in hospital and or amputation. On first glance many diabetic foot ulcers seen relatively benign a skin defect of a few square centimeters, which as partly covered by callus or an eschew, in a patient who has few specific complaints. This lack of over symptoms may falsely reassure both the patient and the clinician, however the absence of symptoms, should actually be seen as a sign of severely diseased foot. (Vishnu Datta *et al*., 2014))

There is a general consensus among clinicians that diabetic patients are at increased risk of developing infection. This special vulnerability has been attributed to impaired leukocyte function associated vascular disease, poor glucose control and altered host response (Bhatia 2003) Once infection occurs, it is difficult to treat since the clinical cause of the infection is more fulminate and severe, and posses a greater threat to the glycemic status of the patient (Ishan *et al*., 2010).

The large number of synthetic drugs produced from pharmaceutical industries from time to time has lead to develop resistant to micro organisms that become major global issue in the treatment of infectious diseases. At present, there is an urgent and continuous need of exploration and development of cheaper as well as effective new plant based drugs with better bioactive potential with least side effects. Antimicrobials of plant origin have been proved to be effective in the treatment of infectious diseases. Simultaneously with lesser side effects, which are often associated with synthetic antibiotics (Danish Rizvi *et al*., 2011).

Natural products are important sources for biologically active drugs. There has been an increasing interest in the study of medicinal plants as natural products in different parts of the world. According to World Health Organization (WHO, 2003) more than 80% of the world's population relies on traditional medicine for their primary healthcare needs.

Use of herbal medicine in Asia, represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance.

Medicinal plants are a source of great economic value in the Indian sub continent. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country. India is rich in all 3 levels of biodiversity. In India thousands of species are known to have medicinal value and the use of different parts of several medicinal plants.

The different plant parts used include root, stem, flower and twigs exudates, while some of these raw drugs are collected in smaller quantities by the local communities and folk healers for local use, many other raw drugs are collected in larger quantities and treated in the market as the raw material for many herbal industries, although hundreds of plants species have been tested for antimicrobial properties, the vast majority of have not adequately evaluated. The medicinal value of these plants depends on bioactive phytochemical constituents that produce physiological action in the human body. (Avneesh kumar *et al*., 2009)

Nowadays drugs resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effects, on the host including hypersensitivity immune suppression and allergic reactions. The situation forced scientists to search for new antimicrobial substances.

Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for few and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs from medicinal plants for the treatment of infectious diseases. There are several reports on the antimicrobial activity of different herbal extracts in different regions of the world.

Nerium oleander L. *i*s an (Apocyanaceae family) beautiful free flowering shrub bearing different colors of flowers suited to sunny and dry localities. Apocyanaceae is commonly known as Gandeera is a large glabrous evergreen shrub with milky juice. *Nerium oleander* flowers (Rose and White coloured) are fragrant, calyx-lobes lanceolate, corolla 3.8 cm diam; fragrant, lobes rounded, Filaments hairy, appendages of anthers twice as long as the cells. Follicles 15-23 cm long, rigid at length separating. Seeds about 13 cm long-tipped with a coma of light brown hairs (Santhi *et al*., 2011)

Oleander is one of the most poisonous plants in the world and contains numerous toxic compounds. The most significant of these toxins are oleandrin and nerine, which are cardiac glycosides. They are present in all parts of plant, but are most concentrated in the sap, which can black out receptors in the skin causing numbness (Ravi kumar. A, and Deepukoushik yadav ., 2013).

The plant is used as a rat poison and as insecticide. The powdered leaves and bark are used as an insecticide. A green dye is obtained from the flowers. The plants is commonly used for informal hedging in the Mediteranean, used traditionally in treating dermatitis, abscesses, eczema, psoriasis, sores, ringworm, scabies, skin cancer, asthema, dye menorrheal, epilepsy, abortifacients, emetics and tuemollientmour.(Garima zibbu *et al*., 2010)

The leaves and flowers are cardiotonic, diaphoretic, anticancer, antibacterial, antifungal, and expectorant. *Nerium oleander* used in the treatment of cancer, the flowers, leaves, leaf juice or latex, bark and roots have been used against corn, warts, cancerous ulcers, carcinoma, ulcerating or hard tumours (Banerjee *et al*., 2011).

Nerium oleander L. shows terminal flower clusters that are available in different colors. Flowers are produced terminal heads and their colors. Each flower is about 5 cm in diameter with 5 petals, although some cultivators have double flowers. Oleandrine is anti-inflammatory, antitumoral, emollient and potentiality apoptopsis. The hydro alcoholic and aqueous extract of

the flowers is antinociceptive and cardiotonic. The patented ***oleander*** extract AntivirzelTM is also used in Africa as a treatment for HIV-AIDS. (Pankhurst.R *et al*., 2010)

Phytochemicals are divided into two groups which are primary and secondary constituents, according to their functions in plant metabolism. Primary constituents' compromise common sugars, amino acids, proteins and chlorophyll white secondary constituents consists of alkaloids, terpenoids and phenolic compounds. Earlier studies reported the various parts (Leaf, flowers, fixed opl, and stem) of *Nerium oleander* L. and *Nerium indicum* extracts biological activities, but very few literatures are available on antimicrobial activity of whole plant.

The aim of the present investigation was to study the antimicrobial activity and preliminary phytochemical screening of *Nerium oleander* L. and *Nerium indicum* flower extract against the bacteria isolated from diabetic wound sample.

II. MATERIALS AND METHODS

Collection of Samples

Clinical samples like wound swab and pus were collected from ulcers of diabetic patients from Government hospitals and reputed diabetic clinics in and around Rasipuram and Namakkal area. Sterile cotton swab were inserted into the deep wound and samples were collected in sterile screw capped tubes and immediately transported to laboratory. Thirty wound and pus samples were collected for this study.

Processing of samples:

50 ml of sterile nutrient broth was prepared, the samples containing cotton swab was inoculated into the broth and incubated at 37^{o}C for 24 hours. After incubation the samples were plated on the nutrient agar medium and the colonies developed were isolated and purified into pure cultures as per the standard procedure (Cappuccino, 2004).

The isolated bacteria were identified based on microscopic and biochemical characterization (Aneja, 2003).

Microscopic examination:

Gram staining and Biochemical Test

A smear of the isolate was made on to a clean glass slide, air dries and heat fixed. It was flooded with crystal violet solution and allowed to remain for 30 seconds. Then the slide was washed with running tap water for and then flooded with grams iodine solution and left for 30 seconds. It was then washed and added with 95% ethanol for decolourisation and washed gently with tap water add counter stain for 30 seconds with saffranin and washed. Then the slide was dried and observed under oil immersion microscope. (Aneja., 2003).

Motility test (Hanging drop Method)

Bacterial motility can be observed, directly by placing a drop of culture on a cavity slide and viewing it under a microscopic field and noted the results. (Aneja., 2003).

Cultural characterisitics of isolates

The bacterial cultures were inoculated on to the surface of the different media to study the cultural characteristics of isolates. The culture media includes Nutrient agar, Macconkey agar, Mannitol Salt agar, *Pseudomonas* agar, Eosin Methylene Blue agar, Crystine Lactose Electrolyte Deficient agar, Muller Hinton agar and Biochemical Tests of Isolates such as Methyl Red test, Voges proskaur test, Citrate utilization test, Catalase test, Oxidase test, Carbohydrate fermentation test, Coagulase test, Urease test, and Triple sugar iron agar test

Antibiotic Sensitivity Test

Sterile Muller-Hinton agar plates were prepared and from the broth culture of the wound infection bacterial isolates, lawn culture was prepared by swapping the culture with sterile swab. Antibiotic disc were placed on the Muller Hinton plates and kept in the refrigerator for one hour to arrest the growth of the test organisms and to facilitate the diffusion of the antibiotics. Then the plates were kept in an incubator at 37^{0}C for 24 hours. After incubation the MHA plates were observed for the development of zone of inhibition. The zones of inhibition were measured in mm. (Bauer et al., 1966)

Antibacterial activity of plant extract:

Collection of plant sample

Fresh flowers from the selected plant *Nerium oleander* L. (Pink and white flowers) and *Nerium indicum* (Red flower) having medicinal value were collected from in and around Namakkal and Salem District. The plant materials were taxonomically identified. The plant flowers were freshly collected and the flowers were dries until all the water molecules evaporate and flowers gets dried well for gridding. After drying, the flowers were ground well using mechanical blender into fine powder and then transferred into airtight containers for further studies.

Extraction of plant

The dried flower materials were extracted with ethanol, methanol, and diethyl ether as solvent for 45 hours by soxhlet extractor. The extract was filtered while hot and concentrated in vacuum under reduced pressure using rotary flask evaporator. (Berlin et al., 2010)

Effect of medicinal plant extracts against pathogens (Agar Well Diffussion Method)

The Muller Hinton agar was inoculated with 100µl of the inoculums of test organism and poured into the petriplate. After the medium was solidified, a well was made in the plants with the help of a cork borer (0.85 cm). The plant extract was introduced into the well at various concentrations (50µl, 75µl, 100µl) and the plate was incubated at 37°C for 24 hours. Effect of plant flower extract on pathogens was determined by measuring the diameter of the zone of inhibition (Perez et al., 1990).

Phytochemical analysis of plant extrcts:

Qualitative chemical tests were carried out using extract from plant to identify the phytochemicals. A 10 mg/ml flower extract was used for the tests. The flower extract were analysed for the Alkaloids, Flavonoids, Steroids, Terpenoids, Carbohydrate, Phenols, Tannins, Glycosides, and Catachol.

Alkaloid:

A) MAYER'S TEST:

Alkaloids are basic nitrogenous compounds with definite physiological and pharmacological activity. Alkaloid solution produces white yellowish precipitate when a few drops of Mayer's reagents are added (Siddiqui and Ali, 1997). Most alkaloids are precipitated from neutral or slightly acidic solution by Mayer's reagent (Evans, 2002). The alcoholic extract was evaporated to dryness and the residue was heated on a boiling water bath with 2% hydrochloric acid. After cooling, the mixture was filtered and treated with few drops of Mayer's reagent. The samples were then observed for the presence of turbidity or yellow precipitation. (Bishnu Joshi et al., 2011).

B) WAGNER'S TEST:

Filtrates were treated with Wagner's reagent (Iodine in potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids (Prashant et al., 2012).

Flavonoids:

Lead Acetate Test:

Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids. (Santhi et al., 2011).

Glycosides:

A) BORNTRAGER'S TEST:

Extracts were treated with 3 drops of concentrated hydrochloric acid and heated on a boiling water bath for 10 minutes then cooled and add 1ml of chloroform and Ammonia. Formation of pink to red colour indicates the presence of glycosides. (Santhi et al., 2011)

B) AQUEOUS NAOH TEST:

A few drops of alcoholic neutral ferric chloride solution was added to the powdered flower sample previously dissolved in alcohol or distilled water. Formation of violet, Bluish green or bluish black colour indicated the presence of phenols.

Steroids:

SALKOWSKI TEST:

To a 0.5 gm of extract, 2 ml of chloroform was added and then concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colour formation at the interface was noted for the presence of steroids. (Prashant et al., 2011)

Terpenoids:

5 ml of each extract was added to 2 ml of chloroform and 3 ml of concentrated H_2SO_4 to form a monolayer of reddish brown coloration of the interface was showed to form positive result for the terpenoids. (Santhi et al., 2011)

Tannins:

5ml of extracts were added to few drops of 1% lead acetate solution. A yellow precipitates indicated the presence of tannins. (Santhi et al 2011).

Phenols:

FERRIC CHLORIDE SOLUTION:

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of Carbohydrate:

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

BENEDICT'S TEST:

Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars. (Prashant et al., 2011)

Catachol:

To 2 g of extract add Erlich's reagent and few drops of concentrated hydrochloric acid and the result was observed. (Berlin et al., 2010) and dried in desiccators. The concentrated extracts were obtained as residues. After which the residues were transferred into pre-weighed sample containers and were stored and later used for phytochemical screening an antimicrobial activity. (Santhi et al 2011)

III. RESULTS AND DISCUSSION

In the present study, 30 wound samples were collected from the diabetic patients from the hospitals at Rasipuram and Namakkal area. From these samples, organisms were isolated and identified by microscopic examination and biochemical tests and the results are presented below.

Isolation:

30 diabetic wound samples were collected from these samples 65 bacterial cultures were isolated and purified. Further the purified isolates were analyzed for microscopic and biochemical characterization and the results are depicted in Table 1.

Colony morphology:

Among the 65 isolates, 18 isolates (S_{11}, S_{22}, S_{41}, S_5, S_{81}, S_{94}, S_{111}, S_{132}, S_{143}, S_{153}, S_{16}, S_{184}, S_{19}, S_{224}, S_{242}, S_{262}, S_{281}, and S_{303}) were large, circular, convicts, smooth, shiny, opaque colonies on nutrient agar.

Another 15 isolates (S_{21}, S_{63}, S_{83}, S_{92}, S_{172}, S_{183}, S_{203}, S_{21}, S_{223}, S_{241}, S_{251}, S_{261}, S_{263}, S_{292}, and S_{302}) were showed large opaque irregular fluorescent greenish pigmented colonies.

9 isolates (S_{21}, S_3, S_{61}, S_{112}, S_{151}, S_{181}, S_{221}, S_{252}, S_{271}) were showed small, smooth colorless and opaque colonies.

13 isolates (S_{12}, S_{42}, S_{64}, S_{82}, S_{93}, S_{131}, S_{141}, S_{152}, S_{201}, S_{231}, S_{272}, S_{293}, and S_{302}) were showed large dome shaped mucoid colonies.

Another 10 isolates (S_{13}, S_{32}, S_7, S_{91}, S_{10}, S_{12}, S_{144}, S_{171}, S_{222}, and S_{291}) were showed round, regular, mucoid, creamy colonies.

Preliminary Identification Test:

Microscopic observation:

Gram staining:

Among these 65 isolates 32 isolates (S_{12}, S_{21}, S_{32}, S_{43}, S_{52}, S_{63}, S_{74}, S_{82}, S_{102}, S_{12}, S_{14}, S_{152}, S_{162}, S_{172}, S_{182}, S_{192}, S_{201}, S_{211}, S_{213}, S_{232}, S_{244}, S_{251}, S_{262}, S_{263}, S_{271}, S_{272}, S_{283}, S_{291}, S_{294},S_{301},S_{303}) were Gram Negative rod.

27 isolates (S_{11}, S_{22}, S_3, S_{41}, S_{52}, S_{62}, S_{82}, S_{91}, S_{101}, S_{111}, S_{132}, S_{143}, S_{153}, S_{161}, S_{172} , S_{184}, S_{201}, S_{224}, S_{242}, S_{251}, S_{252}, S_{262}, S_{271}, S_{282}, S_{292}, S_{303}) were Gram positive cocci, 10 isolates (S_{13}, S_{21}, S_{34}, S_{52}, S_{61}, S_{92}, S_{101}, S_{161}, S_{182}, S_{212}) were Gram Positive rod.

Motility test:

Among 65 isolates 33 isolates were showed motile and the other 37 isolates are non-motile.

Identification:

Based on biochemical test and microscopic examination 18 isolates (S_{11}, S_{22}, S_{41}, S_5, S_{81}, S_{94}, S_{111}, S_{132}, S_{143}, S_{153}, S_{16}, S_{184}, S_{19}, S_{224}, S_{242}, S_{262}, S_{281}, S_{303}) tentatively identified as *Staphylococcus aureus*.

9 isolates as *Escherichia coli* (S_{21}, S_3, S_{61}, S_{112}, S_{151}, S_{181}, S_{221}, S_{252}, S_{271}.

15 isolates as *Pseudomonas* sps (S_{21}, S_{63}, S_{83}, , S_{172}, S_{183}, S_{203}, S_{21}, S_{223}, S_{241}, S_{251}, S_{261}, S_{263}, S_{292}, and S_{302}).

13 isolates as *Klebsiella* sps (S_{12}, S_{42}, S_{64}, S_{82}, S_{93}, S_{131}, S_{141}, S_{152}, S_{201}, S_{231}, S_{272}, S_{293}, S_{302}).

10 isolates colonies were identified as *Bacillus* sps. (S_{13}, S_{32}, S_7, S_{91}, S_{10}, S_{12}, S_{144}, S_{171}, S_{222}, S_{291}).

Among the isolates 27.69% was *Staphylococcus aureus*, 13.84% was *Escherichia coli*, 23.07% was *Pseudomonas* sps, and 20% was *Klebsiella* sps and *Bacillus* was15.38%.

Antibiotic Sensitivity of the Isolated Organisms

Staphylococcus aureus shows sensitivity for clindamycin and resistant for vancomycin, Ampicillin and other antibiotics viz., Tetracycline, ciprofloxacin, Erythromycin and Penicillin are intermediate. With regard to *Escherichia coli* all antibiotics are resistant. *Pseudomonas* sps shows resistance for all antibiotics except ciprofloxacin which shows intermediate. Ciprofloxacin showed sensitive against *Klebsiella* sps whereas other antibiotics did not inhibit the organism. Ciprofloxacin showed sensitive for *Bacillus* sps and other antibiotic are resistant (Penicillin and Ampicillin).

Antibacterial Activity of Medicinal Plants

In order to test the antibacterial activity the *Nerium indicum*, *Nerium oleander* flowers, using different solvents viz., ethanol, methanol, diethyl ether extract were prepared and tested for its antibacterial activity against the isolated bacteria viz., *Staphylococcus aureus*,, *Pseudomonas* sps, *Escherichia coli*, *Bacillus* sps, and *Klebsiella*.

The results indicated that the antibacterial activity of methanol extracts of *Nerium oleander* white flower at the concentration of 100µl was high (25mm) against *Staphylococcus aureus*. Minimum activity was recorded (15mm) against *Klebsiella* sps. Whereas ethanol extracts of *Nerium indicum* Red flower at 100µl concentration the activity was high (24mm) against *Staphylococcus aureus*. Minimum activity was (15mm) against *Pseudomonas* sps.

The antibacterial activity of diethyl ether extracts of *Nerium oleander* Pink flower at 100µl concentration the inhibition zone was high (22mm) against *Staphylococcus aureus*. Whereas minimum activity (16mm) against *Pseudomonas* sps.

Phytochemical Analysis of Nerium indicum (Red Flower)

Phytochemical analysis of *Nerium indicum* Red flower extract was carried out and the results are presented in Table 7.

Alkaloids, Steroids, Phenols, Terpenoids were detected in Ethanol extracts of *Nerium indicum* flower. Whereas Glycosides, Flavonoids and Carbohydrates were absent.

Methanolic flower extract of *Nerium indicum* contain Alkaloids, Glycosides, Flavonoids, Steroids, Carbohydrates were not detected from the extract.

Diethyl ether flower extract of *Nerium indicum* contain Glycosides, Steroids, Tannins, Terpenoids, Carbohydrates, catachol were not detected from the extract.

Phytochemical analysis of Nerium oleander l. (Pink flower)

Phytochemical analysis was carried out the flower extract of *Nerium oleander* and the results are presented in Table 8. Ethanolic flower extracts contain Alkaloids, Flavonoids, Phenols, and Tannins.Whereas Glycosides, Steroids, Terpenoid, Catachol were absent.

Alkaloids, Steroids, Phenols, Tannins are detected in Methanol solvent of. *Nerium oleander*. Glycosides, Flavonoids, Terpenoids, Carbohydrates, Catachol were not detected from the extract.

The diethyl ether extract of *Nerium oleander* contain Alkaloids, Flavonoids, and Glycosides. Whereas Phenols, Carbohydrates, Steroids, Terpenoids, Tannins, Catachol were not detected.

Phytochemical analysis of Nerium oleander l. (White flower)

Phytochemical analysis *Nerium oleander* flower extract was carried out and the results are presented in Table 9. The ethanolic flower extracts contain *Nerium oleander* contain Alkaloids, Flavonoids, Glycosides. Phenols, Steroids, Terpenoids, Carbohydrates, Catachol were not present.

Glycosides, Flavonoids, Phenols, and Tannins are detected in Methanol solvents of *Nerium oleander* and Alkaloids, Steroids, Terpenoids, Carbohydrates and Catachol were absent.

The diethyl ether extract of *Nerium oleander* contain Alkaloids, Flavonoids, Glycosides, Phenols, and Tannins. Steroids, Carbohydrates, Terpenoids and Catachol were not present from the extracts.

GC-MS analysis of Nerium indicum (Red flower)

In the present study the plant extracts was subjected to GC-MS analysis for the identification of active metabolites. In Methanolic extracts of *Nerium indicum* and the results are presented in figure 1.

GC-MS analysis indicated that the methanolic extracts of *Nerium indicum* (Red flower) contain highest concentration of Heptacosane which followed by Triacontane, Lucenin 2, Silicone oil, 2,2,3,3,4,4 Hexadeutero octadecanal, Mome Inositol, and 1,2-Benzenedicarboxylic acid, diethyl ester (CAS) Table 10.

TABLE – 1

MORPHOLOGICAL CHARACTERISTICS OF ISOLATED BACTERIA ON DIFFERENT SELECTIVE MEDIA

Media	***Staphylococcus aureus***	***E.coli***	***Pseudomonas sps***	***Klebsiella sps***	***Bacillus sps***
Nutrient Agar	Large, Convicts, Circular, smooth, shiny opaque colonies	Small, smooth colourless opaque	Large opaque irregular green pigmented colony	Large dome shaped mucoid colonies	Round, regular mucoid creamy colonies
Macconkey agar	Lactose fermenting and non lactose fermenting colonies	Pink large colonies	Pale non lactose fermenting colony	Large mucoid lactose fermenting colony	Non lactose fermenting colonies
Eosine Methylene Blue Agar	-	Colony surrounded by green metallic sheen	-	-	-

Mannitol salt agar	Small yellow colonies	-	-	-	-
Cetrimide agar	-	-	Creamy white colonies	-	-
Blood agar	Large opaque β-haemolytic white golden yellow colonies	-	-	-	Haemolytic colonics

TABLE- 2

ORGANISMS ISOLATED FROM DIABETIC WOUND SAMPLES

Organisms	Number of Isolates	Percentage (%) of Isolates
Staphylococcus aureus	18	27.69
E.coli	9	13.84
Pseudomonas sps	15	23.07
Klebsiella sps	13	20.00
Bacillus sps	10	15.38

TABLE – 3

ANTIBIOTIC SENSITIVITY OF ORGANISMS ISOLATED FROM DIABETIC WOUND INFECTIONS

Name of the organism	Zone of inhibition mm in diameter						
	T	Va	Cf	Am	E	Cl	P
Staphylococcus aureus	16 (I)	13 (R)	17 (I)	-	20 (I)	24 (S)	25 (I)
E.coli	-	-	-	-	-	10 (R)	
Pseudomonas sps	12 (R)	-	18 (I)	-	11 (R)	10 (R)	11 (R)
Klebsiella sps	-	-	21 (S)	-	-	-	-
Bacillus sps	-	-	23 (S)	10 (R)	-	-	12 (R)

S - Sensitive, R-Resistance, I-Intermediate,T -Tetracycline, Va-Vancomycin, E-Erythromycin, Cf - Ciprofloxacin, P-Penicilin, Cl- Clindamycin, Am-Ampicillin

TABLE – 4

ANTIBACTERIAL ACTIVITY OF *NERIUM INDICUM* FLOWER EXTRACT (Red flower)

Inhibition Zone (mm)									
Isolated orgs	**Ethanol Extract**			**Methanol Extract**			**Diethyl ether Extract**		
	50 µl Conc.	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**
S. aureus	18	19	24	17	19	20	18	20	22
Pseudomonas sps	15	16	19	16	18	22	12	15	16
E.coli	18	20	21	17	17	19	19	18	19
Bacillus	16	19	20	17	18	19	18	19	19
Kebsiella	15	18	19	16	17	17	16	16	16

TABLE – 5

ANTIBACTERIAL ACTIVITY OF *NERIUM OLEANDER* FLOWER EXTRACT (Pink flower)

Inhibition Zone (mm)									
Isolated orgs	**Ethanol Extract**			**Methanol Extract**			**Diethyl ether Extract**		
	50 µl Conc.	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**
S. aureus	16	18	20	18	19	20	19	20	22
E.Coli	18	20	20	18	18	20	19	19	20
Pseudomonas sps	14	17	18	15	18	19	16	16	21
Bacillus	17	19	19	17	18	19	18	19	19
Kebsiella	16	19	19	16	17	19	15	18	19

TABLE – 6

ANTIBACTERIAL ACTIVITY OF *NERIUM OLEANDER* FLOWER EXTRACT (White flower)

Inhibition Zone (mm)									
Isolated orgs	**Ethanol Extract**			**Methanol Extract**			**Diethyl ether Extract**		
	50 µl Conc.	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**	**50 µl Conc.**	**75 µl Conc.**	**100 µl Conc.**
S. aureus	16	20	22	18	20	25	19	20	23
E.Coli	18	20	20	20	20	22	18	19	20
Pseudomonas sps	16	18	22	18	20	22	16	19	22
Bacillus	18	18	19	19	20	20	19	19	19
Kebsiella	15	18	18	16	17	19	16	18	18

TABLE-7

PHYTOCHEMICAL CHARACTERS OF *NERIUM INDICUM* (Red flower) FLOWER EXTRACT

S. No	**Phytochemical Tests**	**Ethanol extract**	**Methanol extract**	**Diethyl ether extract**
1	**Test for Alkaloids**			
	Mayer Test	+	-	+
	Wagner Test	+	+	+
2	**Test for Glycosides**			
	Borntrager's Test	+	-	-
	Aqueous NaoH	-	-	+
3	**Test for flavonoids**			
	Lead Acetate test	-	-	+
4	**Test for steroids**			
	Salkowski's test	+	-	-
5	**Test for phenols**			
	$FeCl_3$ test	+	+	-

6	**Test for carbohydrates** Benedict's test	-	-	-
7	Tannins test	+	+	-
8	Terpenoids test	+	+	-
9	Catachol	+	+	-

TABLE-8

PHYTOCHEMICAL CHARACTERS OF *NERIUM OLEANDER* L. (Pink flower) FLOWER EXTRACT

S. No	Phytochemical Tests	Ethanol extract	Methanol extract	Diethyl ether extract
1	**Test for Alkaloids** Mayer Test Wagner Test	 - +	 - +	 + +
2	**Test for Glycosides** Borntrager's Test Aqueous NaoH	 - -	 - -	 - +
3	**Test for flavonoids** Lead Acetate test	 +	 -	 +
4	**Test for steroids** Salkowski's test	 -	 +	 -
5	**Test for phenols** $Fecl_3$ test	 +	 +	 -
6	**Test for carbohydrates** Benedict's test	 -	 -	 -
7	Tannins test	+	+	-
8	Terpenoids test	-	-	-
9	Catachol	-	-	-

TABLE- 9

PHYTOCHEMICAL CHARACTERS OF *NERIUM OLEANDER* L. (White flower) FLOWER EXTRACT

S. No	Phytochemical Tests	Ethanol extract	Methanol extract	Diethyl ether extract
1	**Test for Alkaloids**			
	Mayer Test	+	-	+
	Wagner Test	+	-	+
2	**Test for Glycosides**			
	Borntrager's Test	-	-	-
	Aqueous NaoH	+	+	+
3	**Test for flavonoids**			
	Lead Acetate test	+	+	+
4	**Test for steroids**			
	Salkowski's test	-	-	-
5	**Test for phenols**			
	$Fecl_3$ test	-	+	+
6	**Test for carbohydrates**			
	Benedict's test	-	-	-
7	Tannins test	-	+	+
8	Terpenoids test	-	-	-
9	Catachol	-	-	-

TABLE 10

Analysis of active ingredient of *Nerium indicum* methanol extract by GC-MS result

S. No	Compound Name	Retention time (min)
1	TRIS TRIMETHYLSILYL ETHER DERIVATIVE OF 1,25-DIHYDROXYVITAMIN D2	4.69
2	QUERCETIN 7,3',4'-TRIMETHOXY	4.69
3	1,3-BIS(TRIMETHYLSILYLOXY)-2-TRIMETHYLSILYLAMI NO-3-[3',4'-BIS(TRIMETHYLSILYLOXY)PHENYL]-PROPAN ONE	6.97
4	1,2-Benzenediol (CAS)	8.05
5	1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline	9.44
6	N(1)-{4'-[3"-Oxo-4"-(p-fluorophenyl)-3",3"a,4",5"-tetrahydro-2"-methyl-(2H)-6"-indazolyl)]phenyl}-5-chloro-2-methoxybenzamide	11.88
7	6-(4-Chlorophenyl)-2,5,5-triphenyl-5,8-dihydro-6H-azeto[1,2-a][1,3]thiazolo[4,5-d]pyrimidine	12.46
8	**1,2-Benzenedicarboxylic acid, diethyl ester (CAS)**	14.42
9	Quinic acid	15.47
10	**MOME INOSITOL**	16.40
11	1-[[Bis(methylthio)methylene]acetyl]-2-(4-(4-methoxyphenyl)-1,3-butadienyl)cyclopropane	18.23
12	**2,2,3,3,4,4 HEXADEUTERO OCTADECANAL**	18.98
13	**SILICONE OIL**	19.31
14	c-4-Hydroperoxy-c-3-methoxycarbonyl-4-methyl-1,2,3,4-tetrahydrodibenzofuran-r-1,c-2-dicarboxamide	20.37
15	6-(4-Chlorophenyl)-2,5,5-triphenyl-5,8-dihydro-6H-azeto[1,2-a][1,3]thiazolo[4,5-d]pyrimidine	22.38
16	(R)-à-Methoxy-à-(trifluoromethyl)phenylacetic acid ester of (E,Rs)-2-[(1R,2S)-2-Hydroxycyclopentyloxy]-3-(p-tolylsulfinyl)propene	22.02
17	2(3H)-Furanone, 5-dodecyldihydro	24.44
18	1à-[(t-Butyldimethylsilyl)oxy]-(24R)[(methoxymethyl)oxy]vita min D3 tert-butyldimethylsilyl ether	25.99
19	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl	27.60

	ester	
20	**Lucenin 2**	28.00
21	**TRIACONTANE**	30.54
22	Stearic acid, 3-(octadecyloxy)propyl ester	31.72
23	Dodecanoic acid, 1,2,3-propanetriyl ester	32.17
24	**HEPTACOSANE**	33.11
25	Cyclodecasiloxane, eicosamethyl	33.41

Figure 1: Structure of *Nerium indicum* (Red Flower)

Figure 2: Structure of *Nerium oleander* (Pink Flower)

Figure 3: Structure of *Nerium oleander* (White Flower)

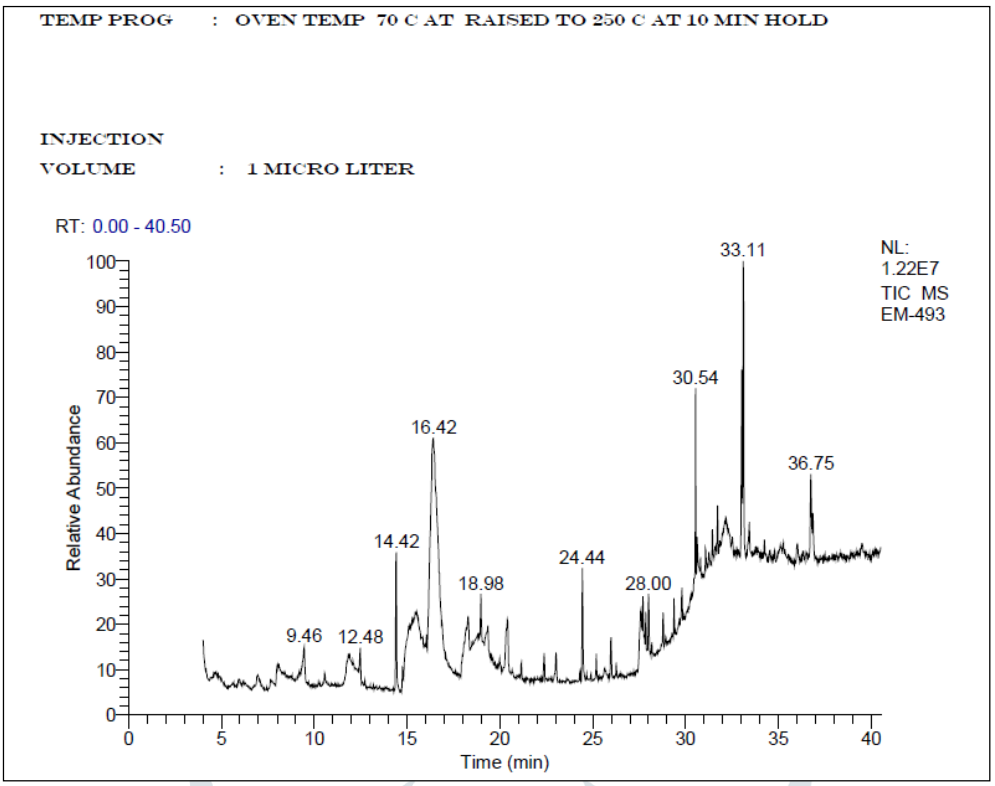

Figure 4- Analysis of active ingredient peak of methanol extract of *Nerium indicum by GC-MS*

IV. DISCUSSION

Fifteen percent of people with diabetes will develop a foot ulcer and 85% of major leg amputations being with a foot ulcer, carbuncles, boils and other skin infections may be hazardous, if not properly treated. Even a small cut may progress to a deep, open sore, called an ulcer. In most cases ulceration is a consequence of the awareness of trauma that can cause the breakdown of the skin.

In the present study 30 diabetic wound samples which are suspected for bacterial infection were analysed. From these samples, 65 isolates were isolated and identified.

Among these isolates, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sps, *Bacillus* sps, and *Klebsiella* sps were high in number. This is in accordance with Sivakumari and Santhi (2009). They isolated *Staphylococcus aureus*, *Pseudomonas* sps, *Escherichia coli*, *Bacillus* sps and *Klebsiella* sps from the diabetic wound sample.

Bacterial pathogens isolated from wound samples *Staphylococcus aureus*, showed sensitive for Clindamycin. Whereas *Bacillus* sps showed resistant to ampicillin and penicillin. Similar results were reported by Orji et al., (2009). The isolated diabetic wound pathogens were tested from susceptibility against antibiotics, few of the antibiotics were sensitive, but most of the antibiotics showed resistant against *Escherichia coli*, *Pseudomonas* sps, *Klebsiella* sps.

The present study was carried out to scientifically evaluate the use of *Nerium oleander* and *Nerium indicum* extract against isolated bacteria. Jawarkar, and Shrirao et al., (2010) observed the extracts of *Nerium indicum* have been reported to have various pharmacological effects like wound healing activity and antibacterial effects.

Antibacterial activity of various extracts of stem part of *Nerium oleander* was studied by measuring the zone of inhibition formed around the agar well. All the extracts showed good activity against *Pseudomonas* aeroginosa and *Bacillus* subtilis. Ethanol extract has activity against all the four microorganisms tested but have anti-bacterial activity Micrococcus leuteus only at high concentration. Thus the plant shows antimicrobial activity and can be a potent ingredient for herbal products (Garima and Gupta et al., 2011).

The flower extracts of *Nerium oleander* and *Nerium indicum* was tested against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sps, *Bacillus* and *Klebsiella* sps. Maximum antibacterial activity was measured from diethyl ether and methanol extract of *Nerium oleander* (Rose and white flowers) against *Staphylococcus aureus* and *Escherichia coli*, and Ethanol extract of *Nerium indicum* (Red flower) against *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella* sps.

With regard to *Nerium indicum* (Red) flower extract maximum zone of inhibition recorded against *Staphylococcus aureus* with ethanol as solvent. *Nerium oleander* (Pink and white) flower extract maximum zone of inhibition recorded against *Pseudomonas* sps and *Escherichia coli*. The similar results tested by Namian et al., (2013). They used *Nerium oleander* as anticancer and cell growth inhibitory activity.

Germi et al., (2010) reported that extracts of *Nerium oleander* leaf and flower had significant antioxidant, antibacterial and cytotoxic activity. It is suggested that further work be performed on the isolation and identification of the active compound from

Nerium oleander plant. The results indicated that *Nerium oleander* leaf and flower extracts could be considered as plant derived antibiotics, antioxidant and chemotherapeutic agents.

Rafi khan et al., (2011) reported the qualitative phytochemical investigation of aqueous extracts of flower of *Nerium oleander* showed the presence of Active chemical constituents such as Carbohydrate, Alkaloids, Flavonoids, Glycosides and Tannins. Absence of phytochemical such as Proteins, amino acids, Triterpenoids and Cholesterol.

In the present study In Methanolic extracts of *Nerium indicum* was subjected to GC-MS analysis for the identification of active metabolites. GC-MS analysis indicated that the methanolic extracts of *Nerium indicum* (Red flower) contain highest concentration of Heptacosane which followed by Triacontane, Lucenin 2, Silicone oil, 2,2,3,3,4,4 Hexadeutero octadecanal, Mome Inositol, and 1,2-Benzenedicarboxylic acid, diethyl ester (CAS).

V. Summary And Conclussion

Diabetic foot infections (DFI) are commonly encountered problems in the practice of clinical medicine. Diabetes is the underlying cause of lower extremity amputations in developed countries, and infection is the precipitating event for nearly 90% of these amputations. The present study was carried to isolate and identify the pathogenic organisms from diabetic wound infections.

From 30 diabetic wound samples, 65 isolates were isolated and identified most of them are *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas* sps, *Bacillus* sps and *Klebsiella* sps.

The flower extracts of *Nerium oleander* and *Nerium indicum* were tested against the isolated bacterial pathogens. *Nerium oleander* (white flower) showed maximum level of inhibition in methanol extract against *Staphylococcus aureus* and *Pseudomonas* sps. *Nerium oleander* (Pink flower) showed maximum level of inhibition in diethyl ether extract against *Staphylococcus aureus* and *Escherichia coli*. Flower extract of *Nerium indicum* in an Ethanol extract showed maximum zone of inhibition against *Staphylococcus aureus*, *Pseudomonas* sps and *Bacillus* sps, Whereas the other solvents recorded minimum level of inhibition against the selected isolates.

In Nerium flower extract Alkaloids, Flavonoids, Phenols, Glycosides, Steroids, Terpenoids, Tannins are present in the all solvent extract.

Among the five organisms tested, zone of inhibition was maximum in Nerium flower extract of ethanol solvent against *Staphylococcus aureus* compare to other solvents. Alkaloids, Flavonoids, Glycosides and Steroids are presented in the ethanol solvent of Nerium flower extracts.

In the present study the plant extracts was subjected to GC-MS analysis for the identification of active metabolites. In Methanolic extracts of *Nerium indicum* and the results are presented.

GC-MS analysis indicated that the methanolic extracts of *Nerium indicum* (Red flower) contain highest concentration of Heptacosane which followed by Triacontane, Lucenin 2, Silicone oil, 2,2,3,3,4,4 Hexadeutero octadecanal, Mome Inositol, and 1,2-Benzenedicarboxylic acid, diethyl ester (CAS).

From this study it concluded that diabetic bacterial wound infection can be treated with *Nerium oleander* and *Nerium indicum* flower extracts of ethanol, methanol and diethyl ether. Since its exhibited favourable antibacterial activity, phytochemical and pharmacological studies will be needed to isolates the bioactive compounds.

VI. References

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