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EVALUATION OF ANTIBACTERIAL POTENTIAL OF SECONDARY METABOLITES PRODUCING BACTERIA ISOLATED FROM RHIZOSPHERE OF *CALENDULA OFFICINALIS*

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Abstract
The main objective of the present study was isolation and identification of bacteria and determination of their secondary metabolites producing ability that had inhibitory activity against pathogenic bacteria. The soil samples were collected from rhizosphere of *Calendula officinalis*. The samples were serially diluted and streaked on nutrient agar plates. The isolated species were morphologically and biochemically characterized. These isolates were evaluated for production of secondary metabolites. Antimicrobial activity of secondary metabolites was performed against pathogenic bacteria isolated from diabetic foot ulcer patients i.e. *Klebseilla pneumonia*, *Salmonella specie*, *Pseudomonas specie.* and *Shigella specie*. The bacteria isolated and identified from the rhizosphere were *Actinomycetes*, *Staphylococcus aureus*, and *Escherichia coli*. The secondary metabolites produced by these species were evaluated for their antimicrobial potential against *Salmonella spp*. *Pseudomonas* spp., *Klebseilla pneumonia* and *Shigella spp*. In crude form. *Actinomycetes* showed good results against *Salmonella* as compare to *S. aureus* and *E. coli*. These soil isolates had effective antimicrobial potential as crude form of secondary metabolites was evaluated in this study. Thus, it

is concluded from this study that these microbes and there products can be used in pure form for the development of novel potent antibiotics for agricultural or pharmaceutical purposes.

Keywords: Antibacterial, Secondary Metabolites, Bacteria, Rhizosphere, *Calendula officinalis*

INTRODUCTION

The soil microbes were of great impact as a factor hopeful the initial finding of drugs different categories of microorganisms such as protozoa molds and bacteria have to develop strategies to survive for limited nutrients in the soil. These microorganisms are acidophilus saprophytes free living nitrogen fixer autotroph, thermopiles, and pathogenic (Arifuzzaman *et al.*, 2010). The native people all around the world have been used natural product antimicrobials for centuries, the people began to fine out a single compounds that might be to kill disease causing bacteria. Plants and soil had been used to avoid injuries from becoming infected the Byzantine and Roman empires. All cultural groups have medicinal mythology that has some level of efficacy for any number of illnesses (Arifuzzaman *et al.,* 2010). The bacteria are the most abundant group usually more numerous than the four combined. Soil bacteria can be rod, (bacilli) cocci (spherical) spirilla (spirals), of these, bacillus are more numerous than the others. They are one of the major groups of soil bacterial population and are very widely distributed (Bong *et al*., 2000). The number and type of bacteria present in a particular soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matters contents, cultivation, aeration and moisture content. On the whole the soil is composed of five major components, these include; Mineral matter, Water, Organic matter, Air and living Organisms (Adebola *et al.,* 2010). The various component of the soil environment constantly changed and the quantity of these constituents are not the same in all soil but vary with locality. Living portion of the soil body includes small animals and microorganisms but it is generally considered that its microorganisms that plays the most important role in the release of nutrient and carbondioxide for plant growth (Abdulkadir *et al.,* 2012). Secondary metabolites is a prerequisite for the development of novel pharmaceuticals and this is an especially urgent task in the case of antibiotics due to the rapid spreading of bacterial resistance and the emergence of multi resistant pathogenic strains, which severe clinical problems in the treatment of infectious disease. The thematic series of on the biosynthesis and function of secondary metabolites deals with the discovery of new biologically active compounds from all kinds of source, including plants, bacteria and fungi and also with their biogenesis. Biosynthetic aspects are closely related to functional investigation, because the deep understanding of metabolic pathway of natural products. New secondary metabolites available from microorganisms may be used to optimize their availability by fermentation for further research and also for production in the pharmaceutical industry (Rajalakshmi and Mahesh., 2014). *Actinobacteria* represent a significant component of the microbial population in most soils including the mangrove region. This phylum of bacteria has been extremely useful to the pharmaceutical industry due to their seemingly unlimited capacity to produce secondary metabolites with diverse biological activities and chemical structures. Approximately 50% of *Actinobacteria* are from the genus *Streptomyces* and approximately 75% of commercially useful antibiotics are derived from this genus (Ding *et al.,* 2009). In recent years, the chances of discovering novel biologically active molecules from various known soil bacteria (including *Actinobacteria*) have reduced, implying that a saturation effect could be occurring. The isolation of known *Actinobacteria* such as Streptomyces from various environments was found to be producing similar compounds. Furthermore, the emergence of multidrug resistant pathogenic bacteria such as Methicillin Resistance *Staphylococcus aureus* (MRSA) and fungus has resulted in critical demand for new natural products and chemical compounds in pharmacology, which in turn has made the exploration of poorly exploited areas such as the mangrove environments essential to discover novel *Actinobacteria* and novel metabolites (Lee *et al.,* 2014). *Bacillus* spp. is Gram-positive bacteria found diversely in nature especially in soil. In extreme conditions such as high temperature, radiation and harsh chemical reagents, they can form endospores for survival. Besides spore forming, *Bacillus* spp. is also able to

produce secondary metabolite products which is an additional function to compete against other organisms. Bioactive compounds from *Bacillus subtilis,* bacteriocin-like substances, were reported to inhibit several clinical bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Salmonella typhi* and also a substance from *Bacillus licheniformis* could inhibit food spoilage bacteria. Moreover, the culture supernatant from *Bacillus* spp. isolated from soil named KW and SA was reported to contain N-acyl homoserine lactone that significantly decreased biofilm formation of *Burkholderia pseudomallei*. In addition, the *Bacillus* strain TKS1-1 in endospore form, was used to reduce the incidence of citrus bacterial canker. Some *Bacillus* spp. can produce several types of active compounds such as *B. amyloliquefaciens* FZB42 that has 8.5% of the genome dedicated for the synthesis of secondary metabolites (Chen *et al*. 2007). It can produce lipopeptides; surfactin, fengycin, bacillomycin D, polyketide (difficidin) and dipeptide bacilysin that can suppress growth of *Erwinia amylovora* (Chen *et al*. 2009). The aim of present study was to isolate and identify rhizospheric bacteria from soil samples of *Calendula officinalis* and determine the antibacterial activity of identified bacteria against clinical pathogens isolated from diabetic foot ulcer patients.

MATERIAL AND METHODS

Soli sample collection

The soil samples were collected from depth of 10 inches from the rhizosphere of *Calendula officinalis*, District Peshawar. The soil samples were collected in air tight polythene bags and were further processed at Microbiology Research Laboratory, Abasyn University, Peshawar, Pakistan.

Isolation of Bacteria

Serial dilution method was used for isolation of the different bacterial species. Soil sample (1 gm) was dissolved in distilled water (100 ml) to make stock solution. Then serial dilutions were made to produce up to 10^{-5} suspension. From each test tube, suspension (0.1 ml) was spread on sterilized nutrient agar media plates aseptically. The plates were incubated at 37 °C for 24 hours (Odeyemi *et al.,* 2020).

Purification and Identification of Bacteria

Bacterial purification involved subculturing and streak plate methods until pure colonies were obtained, considering size, pigmentation, form, opacity, margin, and elevation for colony morphology. Microscopy using Gram staining revealed bacterial characteristics. Biochemical tests included Triple Sugar Iron (TSI) for fermentation, Indole test for Kovac's reagent reaction, Citrate test for citrate utilization, Catalase test for bubble formation, and Urease test for media color change. Positive outcomes were indicated by pink TSI slant, cherry red indole, blue citrate medium, bubble formation, and light pink urea broth.

Preliminary Screening:

A preliminary screening was performed to determine the antibacterial potential of isolated bacteria. For this purpose, the pathogenic species were uniformly streaked in plates containing nutrient agar media, after this pustule of isolated bacteria were placed on it. The plates were incubated at 37°C and results were observed after 16 hours.

Secondary Metabolites Extraction

Nutrient Broth media was used to extract secondary metabolites. The flasks were incubated at 37 °C with constant shaking at 100 rpm for 3 days. Then ratio of ethyl acetate (EtOAc) added in the media was 1:1. The flasks were properly stirred so that the media components degrade finally. Filtration was made using Wattman filter paper after 2 to 3 hours. The filtrate was then placed in the separating funnel. It was mixed by turning upside down the bulb of funnel. The mixture was left to settle down for 10 minutes. The rotary evaporator was used to recover the EtOAc phase under vacuum pressure

at 45 °C. The same experiment will be repeated many times to extract sufficient quantity of crude metabolites for further study (Baazeem *et al*., 2021).

Antibacterial Activity

Nutrient agar medium was prepared and autoclaved at 121°C for 15 minutes at 15psi pressure. After autoclaving the nutrient agar medium was brought to Laminar Flow Hood and poured into sterilized petri plates and was allowed to solidify. After that the bacterial lawn was prepared onto the surface of nutrient agar medium with the help of sterile cotton swab by turning the plate at 60° between the streaks. Then specific numbers of wells were dug in the media with the help of sterile metallic borer. The wells were uniformly distributed in the petri plates at distance of 24mm from one another where Gentamycin was used as positive control while DMSO less than 1% was used as negative control. Test sample was prepared in concentration 3 mg/mL of DMSO and was added into respective wells. Then the petri plates were incubated for 16 hours at 37°C (Song *et al*., 2021).

Data analysis

Data was organized in Excel (latest version), and analysis was conducted using SPSS version 21.

RESULTS

The soil sample was collected from rhizosphere of *Calendula officinalis* and brought to Microbiology Research Laboratory, Abasyn University, Peshawar for isolation and detection of secondary metabolites producing bacteria. A total of 03 species were isolated after biochemical testing from rhizospheric region of *C. officinalis*. These species were *E. coli* (06) *Staphylococcus aureus* (04) and *Actinomycetes* (12) as shown in Figure 1.

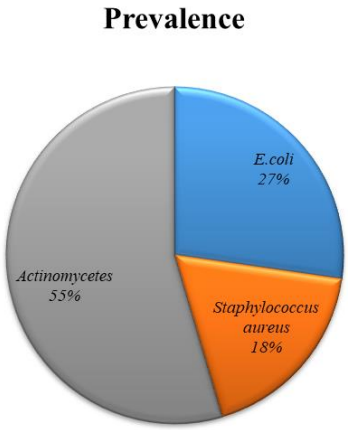

Figure 1. Prevalence of Bacteria Isolated from Rhizosphere

Identification of Bacteria

The growth of isolate on nutrient agar media after 24 hours of incubation was examined and the growth characteristics were observed. For microscopic observation, Gram staining technique was used and different structures of the isolate were observed as shown in Table 1 and 2.

Table 1. Gram reaction and Culture Characteristics of Isolated Bacterial Species

Isolates	Culture characteristics on Nutrient Agar	Microscopy			
		Color	Shape	Arrangement	Gram Staining
1. **1**	Round, convex, smooth, golden yellow, opaque colonies	Purple	Spherical/ cocci	Clusters, single	+
2. 2	Large, greyish white, smooth, opaque or translucent colonies	Pink	Rods	Pairs, Singles	-
3. 3	Circular, Greyish white, smooth, opaque-translucent colonies	Pink	Hyphae like Projections	singles	-

Table 2. Biochemical Test for Identification of Bacterial Species Isolated from Rhizosphere

Isolates	Cultural Characteristics, N.A Media	Shape	Coagulase	Citrate	Urease	Catalase	Oxidase	Indole	TSI	Gram Rx	Identified organisms
1	Abundant white thin Mucoid colonies	Cocci	+	+	+	+	-	-	K/K	+	*Staphylococcus aureus*
2.	Abundant opaque golden growth	Rod	-	-	-	+	-	+	A/A CO_2	-	*Escherichia coli*
3.	Form fungus	Rod	-	+		+	-	-	-	+	*Actinomycetes*

Key: + = Positive; - = Negative; A = Acid production; K = Alkaline reaction; H_2S = Sulfur reduction; A/A = Yellow/Yellow; A/ACO_2 = Yellow/Yellow with Carbon dioxide; K/A = Red/Yellow; K/K = Red/Red; K/A H_2S = Red/Yellow with Bubble precipitate; C.C = Cultural Characteristics; NA = Nutrient Agar, TSI= Triple Sugar Iron

Determination of Antibacterial Potential of Rhizospheric Bacteria

For anti-bacterial assay of extracted culture filtrate of rhizospheric bacterial species, the bacteria were isolated and identified from clinical samples of diabetic foot ulcer patients. A total of 30 samples were collected and among them 26 samples showed growth on the nutrient agar media, while 4 samples showed no growth. A total of four bacteria were obtained from the samples i.e., *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Shigella* and *Salmonella typhi* as shown in the Figure 2 and Tables 3 and 4.

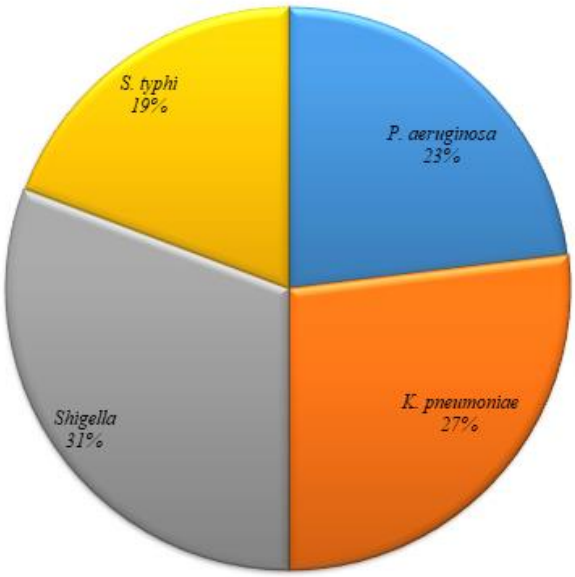

Figure 2. Prevalence of Bacteria Isolated from Diabetic Foot Ulcer Patients

Table 3. Gram reaction and Culture Characteristics of Isolated Bacterial Species

Isolates	Culture characteristics on Nutrient Agar	Microscopy			
		Color	Shape	Arrangement	Gram Staining
1.	Irregular, greenish blue, smooth, opaque-translucent colonies	Pink	Rods	Single or pairs	-
2.	Circular, Greyish white, mucoid, opaque-translucent colonies	Pink	Rods	Single, pairs or short chains	-
3.	Circular, Greyish white, smooth, opaque-translucent colonies	Pink	Rods	Pairs, singles	-
4.	Circular, Greyish white, smooth, translucent colonies	Pink	Rods	Pairs, singles	-

Isolated Bacterial Species

S. No	Bacteria	Biochemical Tests								
		Citrate	Indole	Urease	Oxidase	Catalase	Coagulase	Slant	Butt	Gas/ H_2S
1	*P. aeruginosa*	+	-	-	+	+	-	Alkaline/Alkaline	+	-
2	*K. pneumoniae*	+	-	+	-	+	Nil	Acid/Acid	+	-
3	*Shigella*	-	-	-	-	+	Nil	Alkaline/Acid	+	-
4	*S. typhi*	-	-	-	-	+	Nil	Alkaline/Acid	-	+

*Key: "-"= Negative, "+" =Positive, *Alkaline/Acid (Red slant/Yellow butt) = Dextrose fermentation, *Acid/Acid (Yellow slant/Yellow butt) =Dextrose, Lactose, Sucrose fermentation, *Alkaline/Alkaline (Red slant/Red butt) = Absence of carbohydrates fermentation, *Gas = Production of CO_2 and H_2

Antibacterial Activity of Secondary Metabolites Against Pathogenic Bacteria

The secondary metabolites of *Actinomycetes*, *S. aureus*, and *E. coli* showed good antimicrobial activity effect against *Pseudomonas aeroginosa*, *Klebseilla pneumonia*, *Shigella* and *Salmonella typhi* as shown in Figure 3.

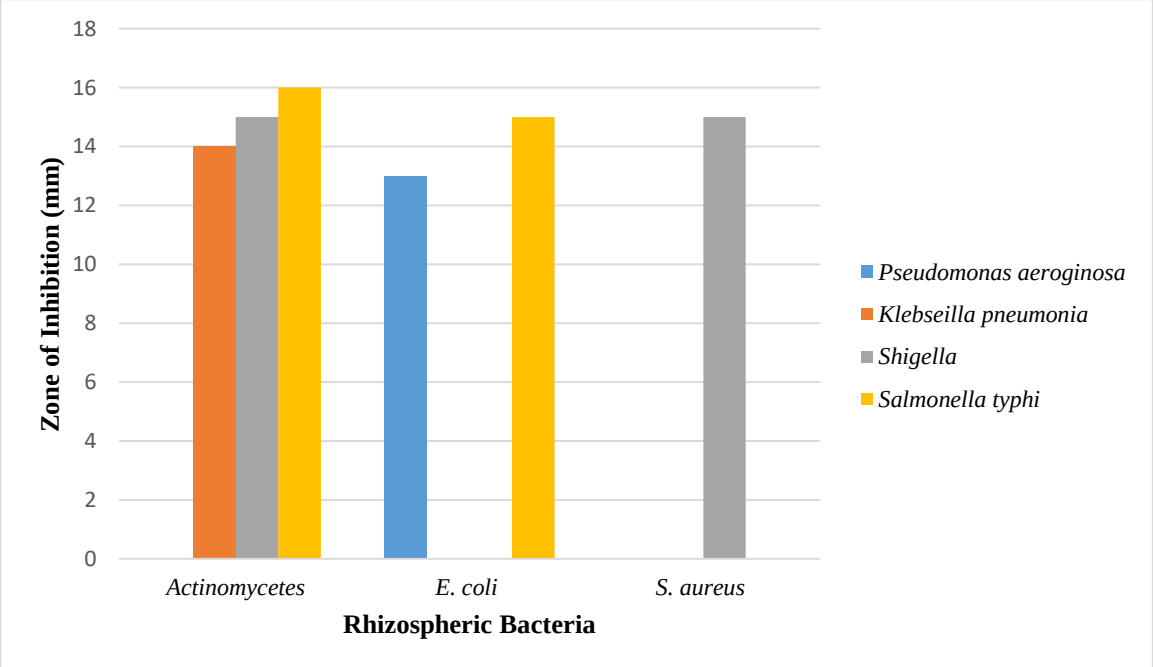

Figure 3. Secondary Metabolites Activity against Pathogenic Bacteria

DISCUSSION

Soil has been considered as a natural source of microorganisms with the ability to produce noble drugs. For this purpose, *Actinomycetes. E. coli*, and *S. aureus* has been isolated from soils for production of secondary metabolites. In this research work we isolated *Actinomycetes, E. coli* and *S. aureus* species from rhizospheric region of soil to test their ability to produce therapeutics with ability against pathogenic bacteria. The previous study reported that *Actinomycetes* produced a huge variety of antibiotics. They isolated different species from different parts of soil and they were searched for their inhibitory action on the strains of pathogenic microbes (Tiwari and Gupta, 2012) while in our study *Actinomycetes* also inhibitory action one pathogenic bacteria. According to Lee *et al.*, (2014) eighty-seven isolates were isolated from soil samples collected at 4 different sites. It was the first report to describe the isolation of *Streptomyces*, *Mycobacterium*, *Leifsonia*, *Microbacterium*,

Sinomonas, Nocardia, Terrabacter, Streptacidiphilus, Micromonospora, Gordonia, and *Nocardioides* while in our study 03 isolates were isolated from soil samples collected at different area of Peshawar. In our study, for the identification and characterizing of different bacteria from diabetic foot ulcer Gram staining also called Gram's method was used for the classification of bacterial species into two large groups i.e., Gran positive and Gram negative (Gupta and Keshari, 2017). Biochemical tests i.e., citrate, catalase, indole, TSI, urease, coagulase and oxidase test were performed in current study for the identification of bacteria. Whereas Shah *et al.,* (2022) and Zafar *et al*., (2023), also performed Gram staining, smear was used for the cytology detection of bacteria and showing absence and presence of bacteria in specimens, for the isolation specimens were plated onto chocolate agar, sheep blood 5%, phenyl ethyl alcohol (PEA) and MacConkey agar plate. The plate was under 10% CO_2 incubated at 37ºC and examined at 24 and 48 hours. In our study for determination of antibacterial activity, we prepared Muller Hinton Agar (MHA). The media was prepared and autoclave at 121℃ for 15 mins. The antibiotics (6mm) were placed on the surface of plates. The plates will be incubated at 37℃ for 24hrs. The same procedure was followed for the application of plant extract as instead of antibiotics wells will be made. The plant extract will be diluted with Dimethyl Sulfoxide (DMSO) for application in the wells. The extract was applied in different concentrations. Whereas, in the study of Sani and Aliyu. (2022), for the determination of the antibacterial activity agar well diffusion method was used. In previous study by Ceylan *et al.* (2008) five isolates were highly active against *S. aureus* strains including Methicillin resistant *Staphylococcus aureus* (MRSA). Twelve *Streptomycetes* isolates showed anticandidal activity against Candida albicans. On the other hand in the present research result three isolates (*Actinomycetes, S. aureus*, and *E. coli*) were highly active against *Salmonella spp*. *Pseudomonas*, *Klebseilla pneumonia* and *Shigella spp*.

CONCLUSION

In the present study *Actinomycetes, S. aureus*, and *E. coli* were isolated from rhizospheric region of *Calendula officinalis* and their antibacterial activity against *Salmonella spp*. *Pseudomonas*, *Klebseilla pneumonia* and *Shigella spp.*, was evaluated. The secondary metabolites of *Actinomycetes,* had significant activity against *Salmonella spp.* followed by *S. aureus*. Thus, it is concluded that rhizospheric region of *Calendula officinalis* has beneficial bacteria having antibacterial potential.

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