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Activity of Antibiotic Producing Bacteria Isolated from Rhizosphere Soil Region of Different Medicinal Plants

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Abstract: The present study was carried out to explore the production of antibacterial agents from rhizosphere soil bacteria of medicinal plants and determine their activity against Gram-negative (*Pseudomonas aeruginosa*, and *Escherichia coli*) and Gram-positive (*Bacillus cereus*, and *Staphylococcus aureus*) bacteria. Soil samples collected from rhizosphere region of 11 medicinal plants were used to isolate and characterize antibiotic producing bacteria (APB). Those isolates (108) were primarily tested using Cross-streak method against test bacteria. This further led to antibiotic susceptibility test (AST) using cell free supernatant (CFS) extraction from cultures of APB obtained in the production media. Moreover, combinatorial effect of isolates' CFS with two organic acids (3% Acetic acid and 0.4 mg/ml Acetylsalicylic acid), two commercial antibiotics (0.016 mg/ml Augmentin and 0.128 mg/ml Doxycycline), and two pure antibiotics (10 mcg/disk Penicillin and 25 mcg/disk Carbenicillin) was in vitro evaluated using AST. Some of those combinations showed marked synergistic activity against test bacteria, specially CFS-Carbenicillin combinations, with variations in inhibitory zones. Additionally, the presence of acetic acid, lactic acid and citric acid in CFS of isolates APB was confirmed by HPLC analysis. Ultimately, in vitro antibacterial study for rhizosphere soil bacteria in this work suggests the possibility of using these bacterial metabolites in clinical infections caused by selected test bacteria, especially when they combine with antibiotics or organic acids.

Key words: antibiotic producing bacteria, cell free supernatant, antibiotic susceptibility test, multidrug resistance, high-performance liquid chromatography

1. Introduction

Soil is a rich source of microorganisms, producing a variety of secondary metabolites which are later identified for pharmaceutical compounds by researchers. Rhizosphere soil occupies a special place for microbial diversity, it is a thin layer of soil surrounding plant roots, and the root-occupied soil supports large active microorganism groups. Further, several environmental factors, including plant species, affect this diversity and composition of bacterial species in the rhizosphere. In particular, the rhizosphere soil of medicinal plants is more abundant with microorganisms, due to the influence of root exudates produced by the plant roots [1].

Antibiotics are agents that used therapeutically and sometimes prophylactically in the control of infectious diseases. The modern age of antibiotics began with Sir Alexander Fleming's discovery of penicillin in 1928. Since then, antibiotics have transformed modern medicine and saved millions of lives. The soil microorganisms are a major source of antibiotics. Over two-thirds of clinically approved antibiotics are natural compounds or semisynthetic derivatives. In addition, over 10000 different antibiotics have been isolated from cultures of Gram-positive and Gram-negative bacteria and of filamentous fungi. However, only about 100 of these have been commercially used to treat human, animal and plant diseases. The reason for this is that only compounds with selective toxicity can be used clinically [2].

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Overuse of antibiotics for maintaining human and animal health has been a major contributing factor to the rapid development and spread of antibiotic-resistant bacteria, which poses a challenge to global public health. Consequently, antibiotic resistance raises healthcare costs, the risk of failure in treatment and the rate of fatality. The results of novel antimicrobials and alternative therapeutic approaches are therefore urgently needed [3]. This study is with huge advantage to achieve that as it explores the production of antibacterial agents from rhizosphere soil bacteria of medicinal plants, and the combinatorial effect of their CFS with other antibacterial agents.

2. Material and Methods

2.1 Isolation of Bacteria

Soil samples were collected about 15 cm below the surface of the soil from rhizosphere region of medicinal plants that grow in Green house of Qatar University (25.3748543N, 51.4892503E). The plants were: A. *Aloe Vera*, B. *Ocimum*, C. *Thymus*, D. *Salvia*, E. *Ziziphus*, F. *Senna*, G. *Salvadora*, H. *Lawsonia*, I. *Moinga*, J. *Rosmarinus*, K. *Citrus*, L. Control (Blumenerde + Sand).

For each sample, 1 gram was measured and diluted with 9 ml of sterilized distilled water, vortex, that is 10-1 dilution factor. Then, 1ml of the mixture was taken and diluted again with 9ml of sterilized distilled water, vortex, that is 10-2 dilution factor. Same process was repeated until 10-4 dilution factor. Hence, 0.5ml of 10-3 and 10-4 dilution was transferred to NA, MC Agar, and LB Agar. The sample was spread uniformly using a sterilized glass rod. Then, NA, MC Agar, and LB Agar plates were incubated at 37°C for 3 days [4].

2.2 Bacterial Test Species

Two Gram-negative bacteria (P. aeruginosa and E. coli) and two Gram-positive bacteria (B. cereus and S. aureus) were selected as test bacteria against isolated APB (Fig. 1). All of them were obtained from Department of Biological and Environmental Sciences except for P. aeruginosa; it was obtained as a result of culturing rhizosphere soil sample of plant B (*Ocimum*).

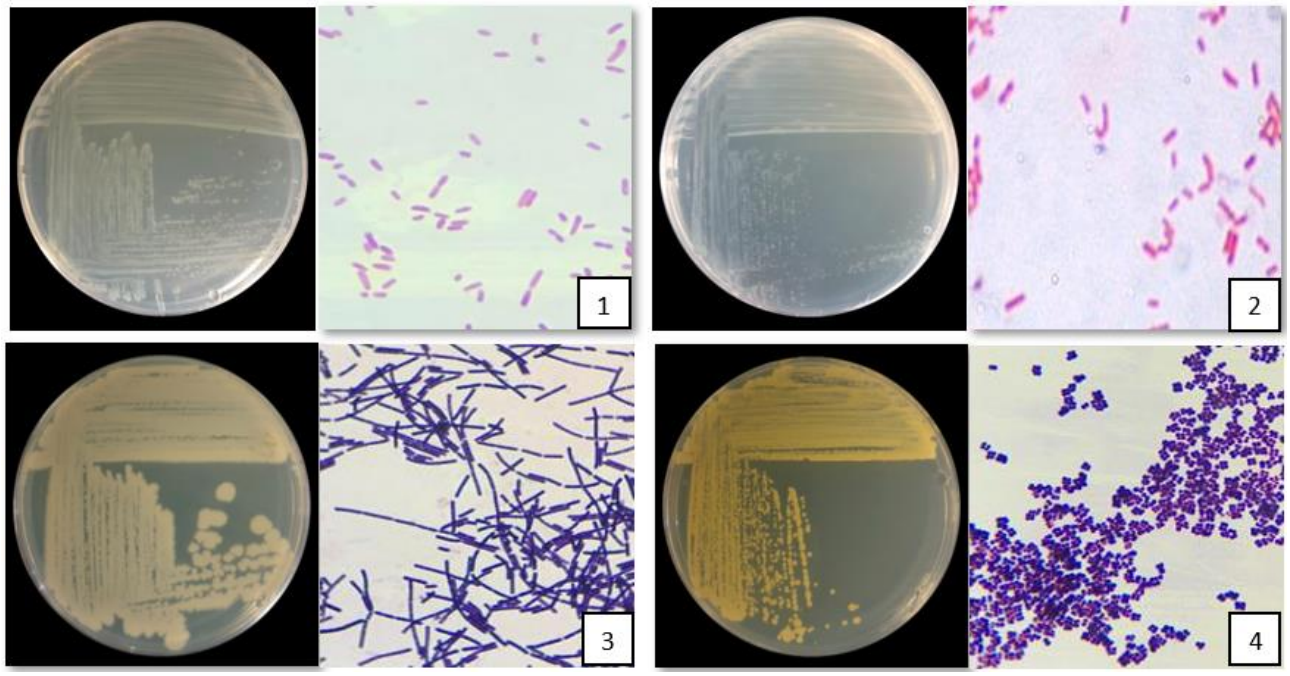

Fig. 1 Four selected test bacteria. 1: P. aeruginosa (Gram negative), 2: E. coli (Gram negative), 3: B. cereus (Gram positive), 4: S. aureus (Gram positive).

2.3 Primary Testing — Cross-Streak Method

A modified cross-streak method was used for antibacterial. Single streak of previously selected bacteria was made on surface of the NA and incubated at 37°C. Two days later, and after observing a good growth of the bacteria on the plates, the four selected test bacterial species were streaked at right angles to the original streak of selected bacteria and incubated at 37°C and the inhibition distance was measured after 2 days. A control plate was also maintained without streaking a line of selected bacteria, to assess the normal growth of bacteria.

2.4 Secondary Testing — CFS Disk Diffusion Method

For the four bacterial test species, a loopful of each bacterial culture was inoculated in 9 ml autoclaved distilled water and incubated at 37°C for 10 min. Then, four NA plates were swabbed; one for each aqueous culture, using sterile cotton swaps. Afterwards, a sterilized autoclaved paper disks were prepared from Whatman blotting paper with a hole puncher (5 mm) and soaked with CFS, each paper disk was placed on a labelled area on NA plate where a test bacterium had been swapped. The plate was incubated at 37°C and zone of inhibition was observed after 3 days.

2.5 Tertiary Testing — Solvent Extraction Disk Diffusion Method

Same procedure of disk diffusion method was applied, the only different is that paper disk was soaked with extracellular secondary metabolite extraction and intracellular secondary metabolite extraction.

2.5.1 Extracellular Secondary Metabolite Extraction

The prepared CFS was dissolved in 1-Butanol (1:1 Ratio) and was allowed to mix by inversion for about an hour. The mixture was spin at 10000 rpm for 10 min. The top layer was discarded and bottom layer containing metabolites was retained in the eppendorf tube. It was allowed to air dry and the mixture was dissolved in 50 µl of 100mM Tris HCl (pH 8).

2.5.2 Intracellular Secondary Metabolite Extraction

After obtaining of CFS, the resulted pellets were dissolved in 0.5 ml of the methanol and mixed gently by inversion for an hour. The pellet dissolved in methanol was centrifuged at 10000 rpm for 10 min. The top layer containing metabolites with methanol were transferred to new eppendorf tube. It was air dried and the mixture was dissolved in 50 µl of 100 mM Tris HCl (pH8).

2.6 Combined Antibacterial Activities Evaluation

Combinatorial effect of isolates CFS with two organic acids (Acetic acid and acetylsalicylic acid), with two commercial antibiotics (Augmentin and doxycycline), as well as with two pure antibiotics (penicillin and carbenicillin) was in vitro evaluated using agar well diffusion or disk diffusion method by measuring the zone of inhibition before and after combination.

2.7 High-Performance Liquid Chromatography

The obtained CFS of each isolate were analyzed for antimicrobial compounds, acetic acids, lactic acid, and citric acid using High-Performance Liquid Chromatography (HPLC). Standard stock solutions of acetic acid, lactic acid, and citric acid (5 mg/mL) were prepared and determined by HPLC, using WATER acquit UPLC with PDA detector at 210 nm. Mobile phase was isocratic elution by 100% of 1 mM H_2SO_4 + 8 mM Na_2SO_4 adjusted at pH 2.8. Flowrate was stable at 0.6 ml/min for 10 minutes. The used separation column was Agilent 3 mm 4.6 mm × 150 mm and the sample injection volume were 10 µL.

2.8 Characterization of Colony and Cell Morphology

The colony morphology of selected APB was noted with respect to shape, margin, elevation, size, texture, pigmentation and optical properties, they were Gram stained and observed under oil immersion in light microscope.

3. Results and Discussion

3.1 Rhizosphere Soil Values and Possibilities for the Antibiotics Production

Rhizosphere soil of medicinal plants is rich of microorganism as it is a dynamic site of biological activity in nature where many biochemical reactions occur for destruction of organic matters due to the influence of root exudates produced by the plant roots. Microorganisms gain advantage by releasing antibiotic agents to kill specific rivals and inhibit surrounding competing bacteria, leading to an increase through microbial biomass and rhizosphere development [5]. Studying microbiology of the rhizosphere in medicinal plants provides a better understanding of antibacterial agents' production which are later may identified for pharmaceutical compounds by researchers. In the present investigation, since rhizosphere soil is rich of microorganism that produce antibacterial agents; it was chosen as the source of ABP.

3.2 Primary Test

In primary test, it was observed that 12 bacterial isolates out of 108 isolates were antibiotic producers isolated from different 13 soil samples. This was determined by Cross-streak method, each bacterial isolate was streaked corresponding to the selected test bacteria: P. aeruginosa and E. coli (Gram-negative), B. cereus and S. aureus (Gram-positive), after two days, the detecting of any clear zone production in at least one out of four selected test bacterial colonies were chosen as a positive antibacterial activity of isolate (Fig. 2). The clear zones produced were due to the antibacterial agents' production that affect the growth of test species. In this test, only one isolate (I18) inhibited P. aeruginosa growth. Otherwise, most of APB were antagonistic against E. coli, B. cereus, and S. aureus in 50%, 25%, and 100% respectively.

Those results can be explained by the fact that strains of P. aeruginosa are known to utilize their high levels of intrinsic and acquired resistance mechanisms to

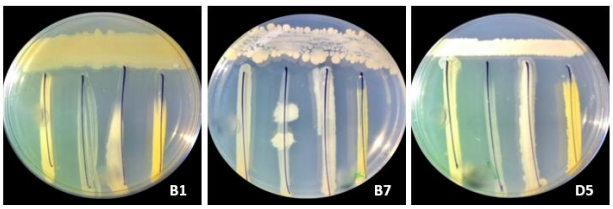

Fig. 2 Isolates that shows a positive antibacterial effect against four test bacteria.

counter most antibiotics. In addition, adaptive antibiotic resistance of P. aeruginosa is a recently characterized mechanisms which includes formation of thick biofilms, which contain a high level of the poly-saccharides alginate and thus altering its structure. These contributes to conformational changes in invading antimicrobial peptides when binding to them, which then oligomerizes, and this consequently hinders their ability to enter the biofilm. Moreover, formation of multidrug-tolerant persister cells, which is responsible for recalcitrance and relapse of infections [6]. On the other hand, Gram-positive bacteria are more susceptible to antibiotics because unlike Gram-negative bacteria, it carries only outer peptidoglycan layer which is not an effective barrier [7]. This may be the reason for all isolates to produce lager zones of inhibition against S. aureus and B. cereus which are Gram-positive bacteria.

3.3 Secondary Test

In secondary test, isolates' CFS was centrifuged and filtrated to test its antibacterial activity against test bacteria using disc diffusion method. In this test, CFS of the primary selected APB were antagonistic against Gram-positive bacteria (100%) compared to Gram-negative bacteria (33%) exhibiting inhibition zones ranging from 7.00 to 30.00 mm. Specifically, no CFS of APB had the ability to inhibit P. aeruginosa growth. While most of APB were antagonistic against E. coli, B. cereus, and S. aureus in 33%, 8%, and 100% respectively. Those results are strongly compatible with primary test with some antibacterial activity regression against P. aeruginosa, E. coli, and B. cereus, this result support study published by Kamat and Yelho (2012) which revealed that cross streak method

resulted in higher inhibition zones on indicator bacteria than those obtained by diffusion method due to difficulty in obtaining quantitative data, since the margins of the zone of inhibition are usually very fuzzy and indistinct [8].

3.4 Tertiary Test

In tertiary test, extracellular secondary metabolite and intracellular secondary metabolite were extracted from isolates CFS using 1-Butanol and methanol respectively. The same organic solvents were used by Pandey and Malviya [9]. 1-butanol is not highly soluble in aqueous solution, and it forms an immiscible layer, which helps to extract the extracellular compound more efficiently. Alcohols are the more commonly used solvents for efficient extraction of intracellular bioactive compounds from bacterial cultures [10]. After testing their antibacterial effect against each test species, it clear that neither P. aeruginosa nor B. cereus was inhibited by any of the two extraction. Also, E. coli was inhibited by extracellular secondary metabolites of some others (42%) but not intracellular secondary metabolites. S. aureus was inhibited by the two extraction, in which each targeted its growth differently, 67%, and 58% of extracellular secondary metabolites and intracellular secondary metabolites respectively inhibited the growth of S. aureus (Fig. 3). From this study, it is evident that secondary metabolites of isolates can overcome this resistance and inhibit S. aureus. It has been observed that mainly the extracellular secondary metabolite has given the best inhibition in comparison to the intracellular secondary metabolites. This result was not expectable since organic solvents are able to dissolve the secondary metabolites on the basis of their polarity and hence the secondary metabolites should show many folds increasing in the activity of the isolates [11].

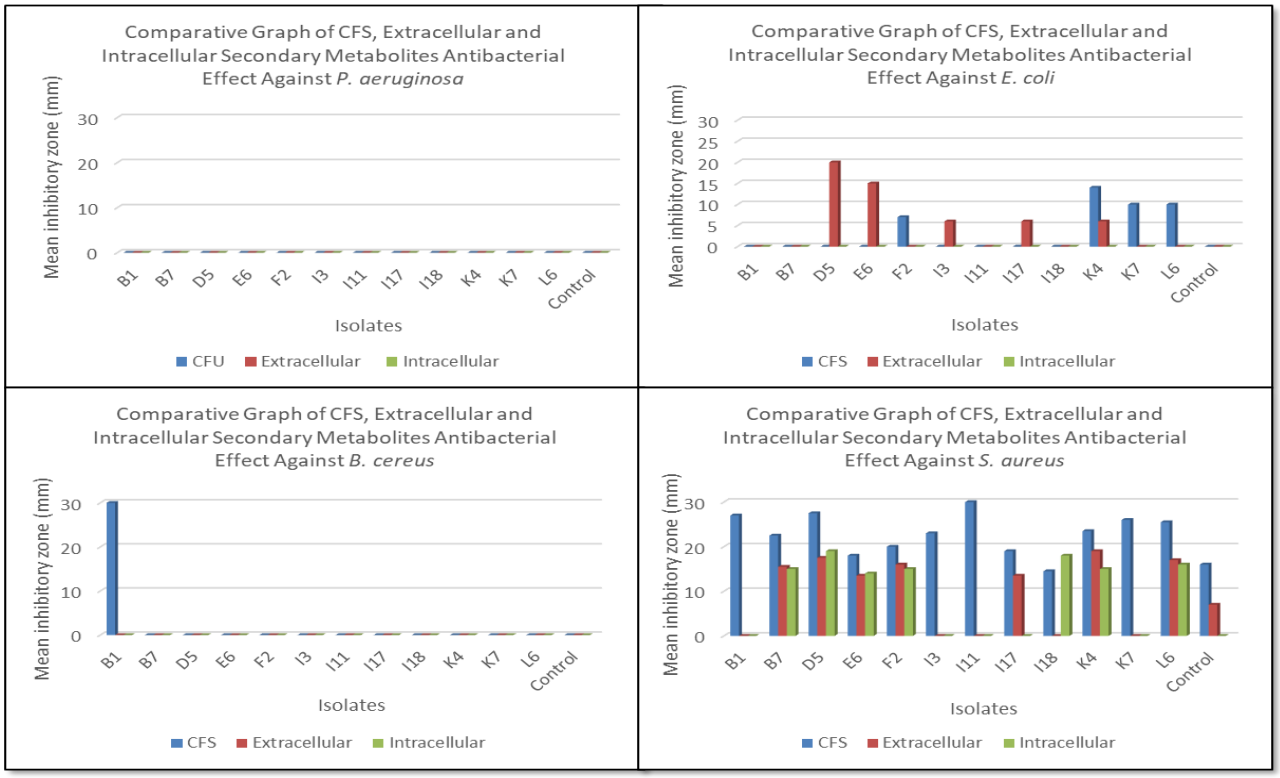

Fig. 3 Comparative graphs showing antibacterial effects of CFS, extracellular and intracellular secondary metabolites against selected test bacteria.

3.5 Isolates CFS-Organic Acids/Antibiotics Combination Antibacterial Effect

Combinatorial activity was determined by combination of isolates CFS with two organic acids (3% Acetic acid and 0.4 mg/ml Acetylsalicylic acid), two commercial antibiotics (0.016 mg/ml Augmentin and 0.128 mg/ml Doxycycline), and two pure antibiotics (10 mcg/disk Penicillin and 25 mcg/disk Carbenicillin). The concentrations of agents were chosen based on their designation as minimum inhibitory concentration by previous articles. Since the composition and modes of action of CFS on microorganisms is different than organic acids and antibiotics, the combination of both agents could enhance antimicrobial effects. An antimicrobial combination has been utilized as an effective therapeutic strategy by using of various mechanisms of action [11]. In the present part of study, I put forward the hypothesis that whether treatment with combination of isolates CFS with organic acids and antibiotics have higher antibacterial activity against test bacteria or not. Keeping in view the application of rhizosphere soil bacteria to be used in conjunction with an organic acid or antibiotic. Organic acids and antibiotics with known antibacterial activity will produce beneficial or harmful effects when combined with conventional antibacterial, depending on the ratio in which the two components exist [12].

The first criteria was investigate the effect of those antibacterial agents without combination, the results indicated that P. aeruginosa exhibited complete resistance to Acetylsalicylic acid, Augmentin and Penicillin, this resistance could be caused by several mechanisms such as the formation of thick biofilms, which contain a high level of the polysaccharides alginate and thus altering its structure, these contributes to conformational changes in invading antimicrobial peptides when binding to them, which then oligomerizes, and this consequently hinders their ability to enter the biofilm [13]. While E. coli exhibited complete resistance to both Acetylsalicylic acid and

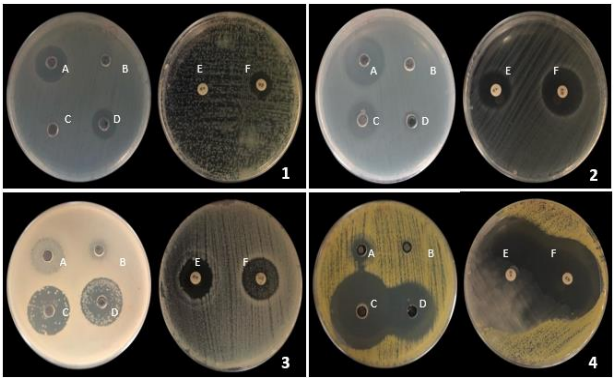

Fig. 4 Activity of pure Acetic acid (A), Acetylsalicylic acid (B), Augmentin (C), Doxycycline (D), Penicillin (E), and Carbenicillin (F) antibacterial agents against *P. aeruginosa* (1), *E. coli* (2), *B. cereus* (3), *s. aureus* (4).

Doxycycline. Otherwise, all bacterial test species where sensitive to the remained agents with different inhibitory zones when the susceptibility of them was examined.

Combination results showed that synergistic effect was verified from the combination of different CFS with the organic acids and antibiotics. In general, most of isolates CSF sharply lowered the inhibitory zones of antibiotics and organic acids against tested bacteria with some exceptions. For example, some CFS-Carbenicillin combination led to an increase of 2.00 mm inhibitory zone against P. aeruginosa, 1.00-4.00 mm inhibitory zone against E. coli, 4.00-5.00 mm inhibitory zone against B. cereus, 1.00-9.00 mm inhibitory zone against S. aureus when compared with the single use of CFS or Carbenicillin. Also, 66.7% of CFS-Penicillin led to an increase of 1.00-5.00 mm inhibitory zone against B. cereus. Furthermore, against S. aureus, 15% of CFS-Acetic acid led to an increase of 1.00-5.00 mm inhibitory zone, 50% of CFS-Acetylsalicylic led to an increase of 3.00-21.00 mm inhibitory zone, 8% of CFS-Augmentin combination (Only B1 CFS-Augmentin combination) led to an increase of 2.00 mm inhibitory zone, and 8% (Only F2 CFS-Doxycycline combination) led to an increase of 2.00 mm inhibitory zone.

3.6 HPLC Analysis

In Smulders study [14], separation, purification and identification of antimicrobial agents produced by APB were conducted by several techniques. And in this study, the presence of acetic acid, lactic acid and citric acid in CFS of isolates APB was confirmed by HPLC analysis. The organic acid acts by collapsing the electrochemical proton gradient, membrane lipids thus altering the cell membrane permeability which results in disruption of substrate transport. Alakomi also found that lactic acid, in addition to its antimicrobial property due to the lowering of the pH, also functions as a membrane-permeabilizing of the Gram-negative bacterial outer membrane and may act as a potentiator of the effects of other antimicrobial substances [15]. Thus, the used combinations might have acted cooperatively with each other, leads to a higher bactericidal effect of the combination in support of our findings.

Table 1 Concentration of antibacterial compounds in isolates' CFS using HPLC method.

#	AA (mg/ml)	CA (mg/ml)	LA (mg/ml)
B1	4.132514	-	1.802795
B7	8.213469	-	3.464614
D5	1.311849	-	-
E6	12.40003	-	1.02936
F2	6.598832	-	2.256804
I3	6.846362	-	2.989841
I11	5.979506	5.363399	0.030676
I17	11.06743	7.005317	1.48011
I18	9.371274	8.616311	2.002676
K4	1.818763	-	3.450215
K7	7.429332	-	1.498215
L6	7.486083	7.762448	1.366832

3.7 Colony and Cell Morphology

Table 2 Characteristics of colony and cell morphology of 12 antibiotic producing bacterial isolates.

		Colony Morphology						Cell Morphology		
Isolate	Media	Shape	Margin	Elevation	Size	Texture	Pigmentation	Gram stain	Shape arrangement	Spore forming
B1	NA	Circle	Entire	Raised	Small	Smooth	Pale green	-ve	Bacillus	No
B7	NA	Irregular	Curled	Flat	Moderate	Rough	Cream	-ve	Coccobacillus	Yes
D5	LB	Circle	Entire	Flat	Small	Smooth	Cream	+ve	Bacillus	yes
E6	NA	Circle	Curled	Raised	Moderate	Rough	Cream	+ve	Bacillus	No
F2	NA	Irregular	Curled	Raised	Moderate	Rough	Cream	+ve	Bacillus	No
I3	NA	Circle	Entire	Convex	Small	Smooth	Cream	-ve	Bacillus	Yes
I11	LB	Circle	Filamentous	Raised	Moderate	Smooth	Cream	+ve	Bacillus	Yes
I17	LB	Circle	Curled	Flat	Moderate	Rough	Cream	+ve	Bacillus	No
I18	NA	Circle	Entire	Convex	Small	Smooth	Off white	-ve	Palisadebacillus	No
K4	NA	Circle	Curled	Raised	Moderate	Smooth	Pale Pink	+ve	Bacillus	Yes
K7	NA	Irregular	Curled	Raised	Moderate	Rough	Cream	-ve	Coccobacillus	Yes
L6	LB	Circle	Entire	Flat	Small	Rough	Cream	+ve	Streptobacillus	Yes

(-ve): Grame negative. (+ve): Gram positive

4. Conclusion

In conclusion, a total of 12 Bacillus isolates associated with medicinal plant rhizosphere soil have demonstrated the ability to produce antibacterial compounds against four bacterial test species, especially S. aureus bacteria. The rhizosphere soil of medicinal plants is rich in microorganisms as it is a dynamic site of biological activity in nature where many biochemical reactions occur as microorganisms compete for organic matters. Because of its heterogeneous existence, rhizosphere soils are home to a wide and diverse population of bacteria; wide variations in biotic and abiotic soil conditions make its bacterial inhabitants adapt and establish survival

strategies and effective reproduction. Some of the most important strategies for this adaptation is the development of antimicrobials agents. Soil bacteria was therefore once the main source of antibiotic development and still retains its importance.

Nevertheless, the indiscriminate use of antibiotics and disinfectants in medicine and agriculture and their release into the environment has given birth to a crucial problem of MDR pathogenic microbes, and so we do need efficient metabolites that can be used as antibiotics to counter these resistant strains. Therefore, the combination of the action of CFS isolates with organic acid and antibiotics demonstrated the potential to reveal a new approach to their complementary antibacterial impact. This combination may lead to the production of new safe and effective antibacterial and therapeutic agents. Further research on molecular taxonomic characterization of the possible species, purification, and antibacterial compound characterization are warranted. Additionally, research work is required to extract more APB from the rhizosphere region of medicinal plants using different media types, identify by RNA sequencing, leading to thorough investigation for their action mechanism.

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