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

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 25mcg/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

OBJECTIVES

1. Isolate APB from rhizosphere soil of medicinal plants.
2. Examine the activity of isolates' CFS against four test bacteria.
3. Evaluate the combinatorial effect of isolates' CFS with two organic acids, two commercial antibiotics, and two pure antibiotics.
4. Analyze isolates' CFS for the presence of antibacterial compounds using HPLC.
5. Characterize isolated antibiotic producing bacteria.

LITERATURE REVIEW

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 (Dong *et al*., 2019).

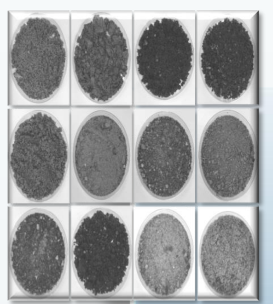
Antibiotics are agents that used therapeutically and sometimes prophylactically in the control of infectious diseases. The modern age of antibiotics began with Sir Alexander Fleming's discovery of penicillin in 1928. Since then, antibiotics have transformed modern medicine and saved millions of lives. 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 (Tamilarasi *et al.*, 2008).

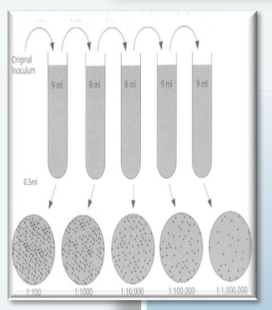
Overuse of antibiotics for maintaining human and animal health has been a major contributing factor to the rapid development and spread of antibiotic-resistant bacteria, which poses a challenge to global public health. Consequently, antibiotic resistance raises healthcare costs, the risk of failure in treatment and the rate of fatality. The results of novel antimicrobials and alternative therapeutic approaches are therefore urgently needed (Band and Weiss, 2014). 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.

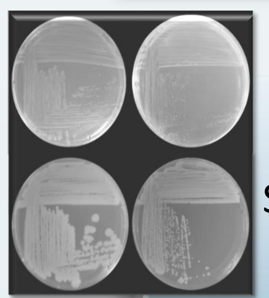
METHODOLOGY

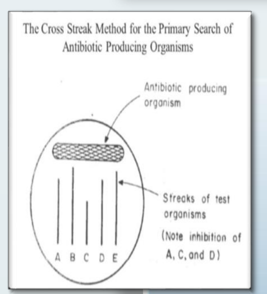
Table 1: Eleven medicinal plants names and their assigned code

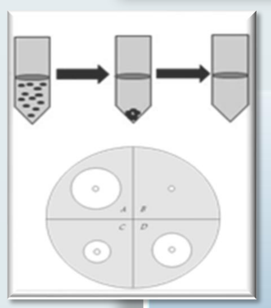
#	Plant code	Plant name
1	A	*Aloe Vera*
2	B	*Ocimum*
3	C	*Thymus*
4	D	*Salvia*
5	E	*Ziziphus*
6	F	*Senna*
7	G	*Salvadora*
8	H	*Lawsonia*
9	I	*Moringa*
10	J	*Rosmarinus*
11	K	*Citrus*
12	L (Control)	-

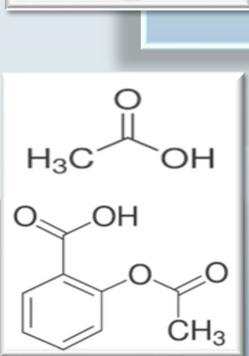

Soil sample collection

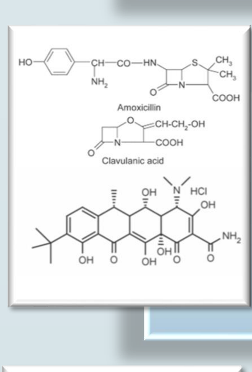

Culturing, and isolation of expected antibiotic producing bacteria (108)

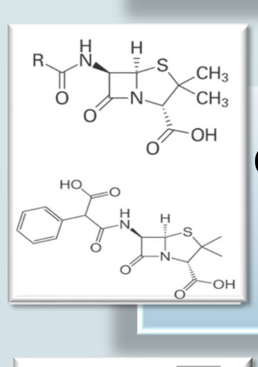

Selection of test bacterial species

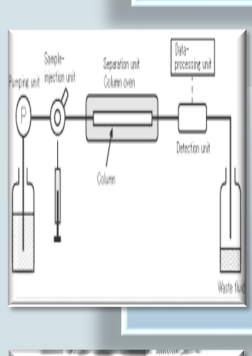

Primarily test - Cross-streak method

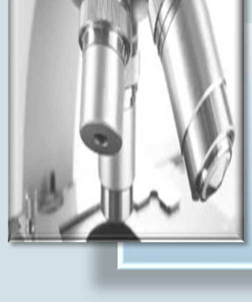

CFS disk diffusion method (12)


CFS-organic acid (Acetic acid and Acetylsalicylic acid) combination agar well diffusion method


CFS-commercial antibiotics (Augmentin and Doxycycline) combination agar well diffusion method


CFS-pure antibiotics (Penicillin and Carbenicillin) combination disk diffusion method


HPLC analysis for antimicrobial compounds


Isolates Identification

RESULTS

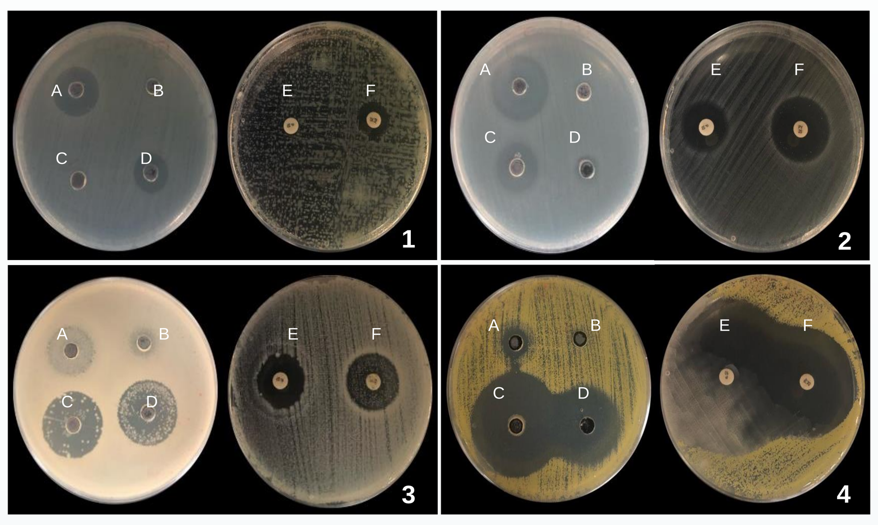


Figure 1: Activity of pure Acetic acid (A), Acetylsalicylic acid (B), Augmentin (C), Doxycycline (D), Penicillin (E), and Carbenicillin (F) antibacterial agents against *P. aeruginosa* (1), *E. coli* (2), *B. cereus* (3), *s. aureus* (4).

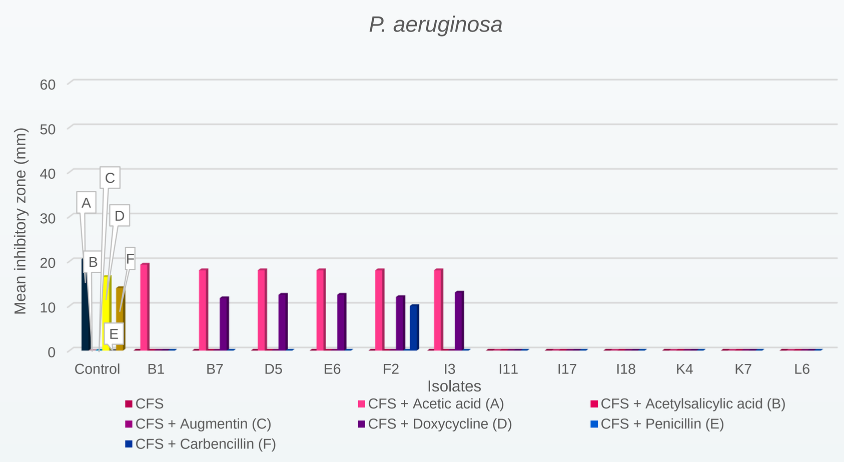


Figure 2: Comparative graph of CFS-organic acid/antibiotic combination antibacterial effect against *P. aeruginosa.*

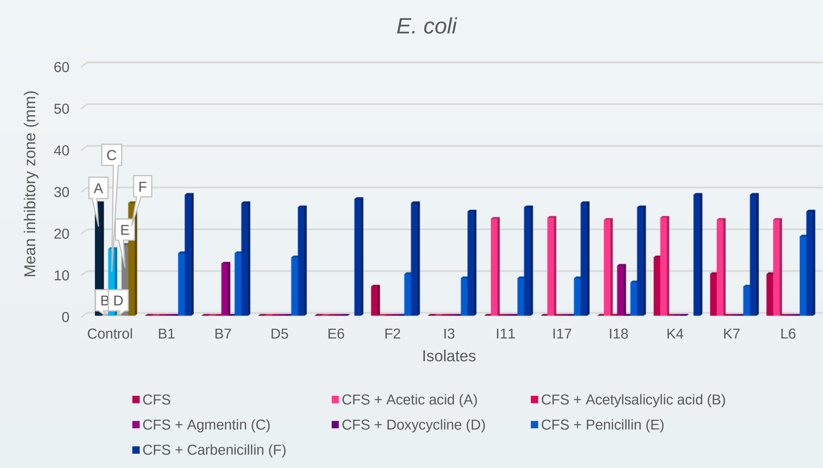


Figure 3: Comparative graph of CFS-organic acid/antibiotic combination antibacterial effect against *E. coli.*

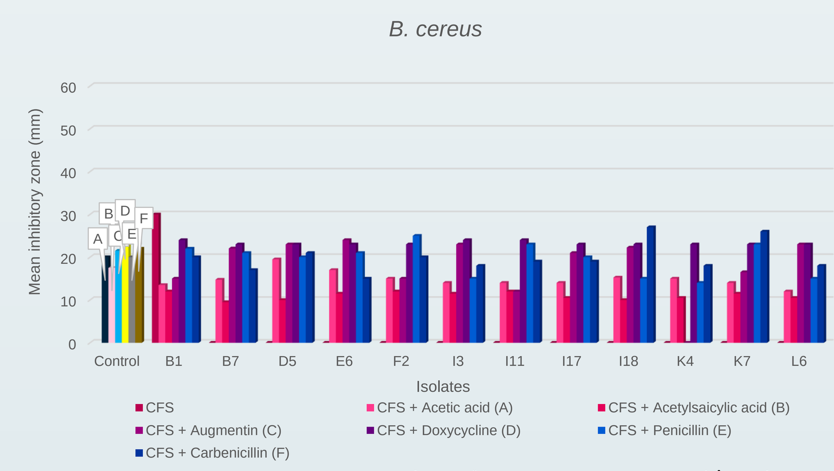


Figure 4: Comparative graph of CFS-organic acid/antibiotic combination antibacterial effect against *B. cereus.*

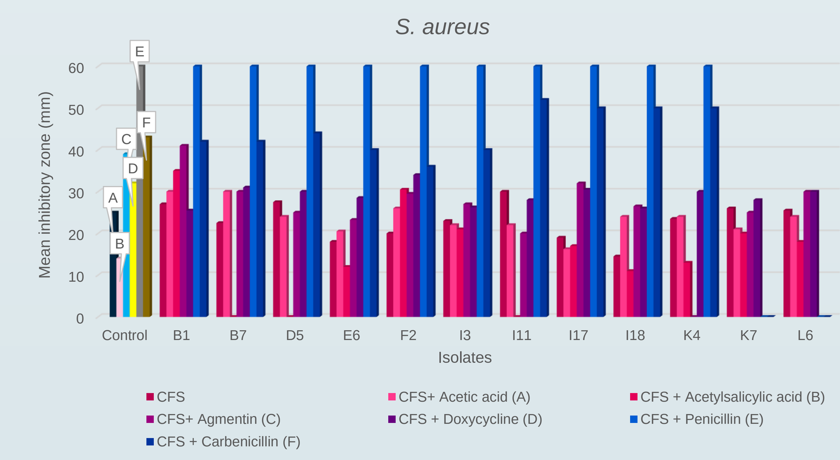


Figure 5: Comparative graph of CFS-organic acid/antibiotic combination antibacterial effect against *S. aureus.*

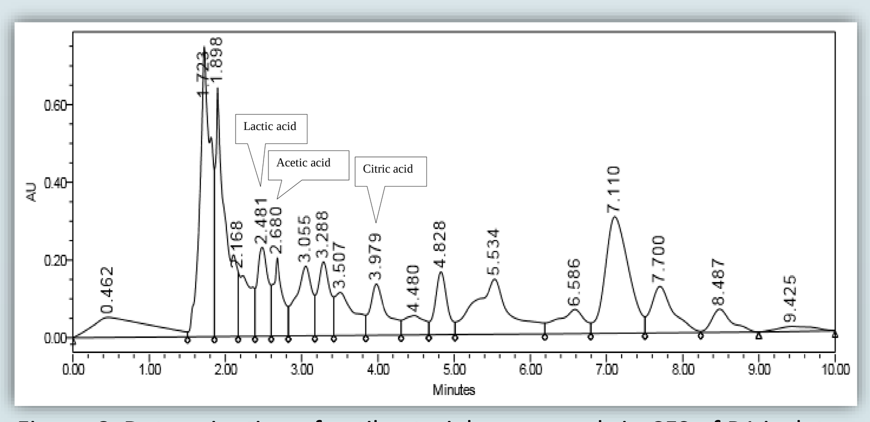


Figure 6: Determination of antibacterial compounds in CFS of B1 isolate using HPLC analysis.

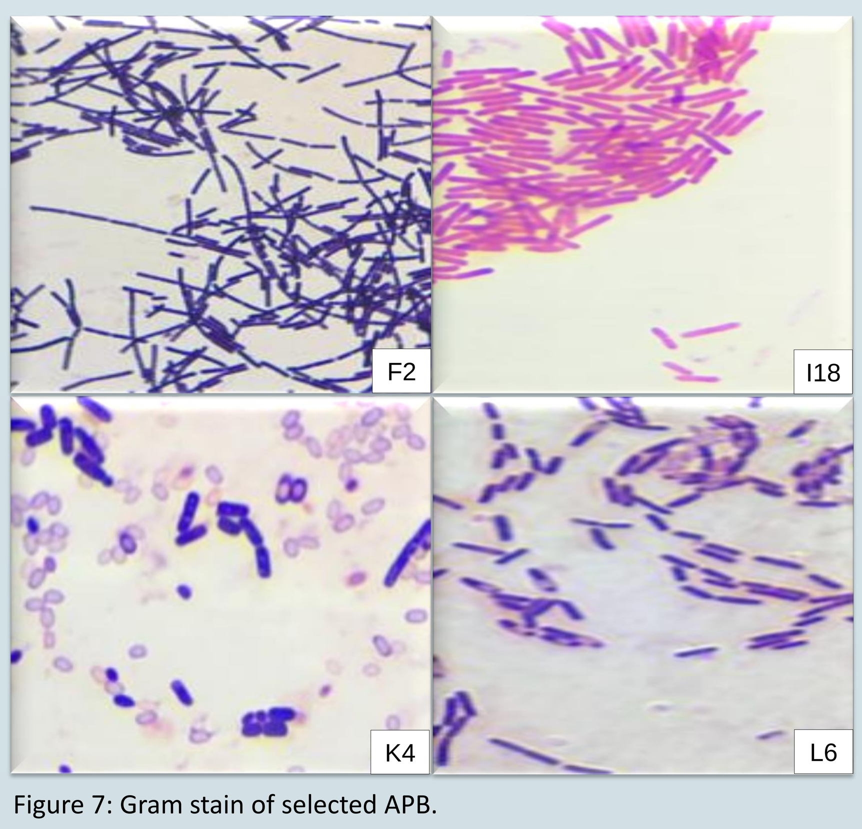


Figure 7: Gram stain of selected APB.

DISCUSSION

In the present study, *P. aeruginosa* was the most resistant test bacteria against all isolates' CFS, while *S. aureus* was the most sensitive one followed by *B. cereus*. 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 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 (Band and Weiss, 2014). Moreover, formation of multidrug-tolerant persister cells, which is responsible for recalcitrance and relapse of infections. 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 (Tamilarasi *et al.*, 2008). This may be the reason for all isolates to form lager zones of inhibition against *S. aureus* and *B. cereus* which are Gram-positive bacteria.

In previous studies, 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 as antibacterial agents 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 systems (Smulders *et al.*, 2007). Sgouras *et al.*, (2004) 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. 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.

CONCLUSION

A total of 12 *Bacillus* isolates associated with rhizosphere soil of medicinal plants have showed the ability to produce antibacterial compounds against 4 test bacterial species, especially *S. aureus* bacteria. In addition, combining Isolates' CFS with organic acid and antibiotics seems to enhance the inhibition of test bacteria. 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 and their contributing for the development of new safe and effective therapeutic and antibacterial agents, especially when they combine with antibiotics or organic acids. Further studies on molecular taxonomical characterization of the potential organisms, purification, and characterization of antibacterial compounds are warranted. Additionally, research work is necessary to isolate more APB from rhizosphere region of medicinal plants using different types of media, identify by RNA sequencing, leading toward comprehensive investigation for their mechanism of action.

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