

ORIGINAL ARTICLE

Inhibitory activity against pathogenic bacteria of streptomycetes isolated from natural soil resources

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Abstract

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Bacterial infections still remain a major public health problem worldwide, particularly caused by multidrug resistant bacteria. Most bacterial diseases can be treated with antimicrobial agents. However, these drugs have been used so widely and so long that the infectious strains have rapidly adapted to resist the drugs, making the drugs less effective. Each year, million people become infected with the antibiotic-resistant bacteria and die as a result of these infections. Hence, discovery of a new drug to combat these infections is urgently needed. Streptomycetes is major group of soil bacteria ubiquitous in natural resources. These bacteria have shown to be a remarkably rich source of natural products, accounting for the production of two-thirds of available antibiotics. Thailand has been a land rich in natural bioresources, including soil microorganisms. Therefore, the objective of this work is to search streptomycetes isolated from Thai natural resources for antibacterial activity. The authors have isolated fifty *Streptomyces* strains from soils collected from various geographic natural resources of Thailand. The 50 different isolates were determined for antimicrobial activity against various standard strains of pathogenic bacteria using agar plug methods. The infectious bacteria include *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*. The antimicrobial-producing *Streptomyces* isolates exhibited clear zone of inhibition around their agar plugs containing the colony.

Among the fifty *Streptomyces*, 10 isolates (20 %) (strains SMC2, SMC30, SMC47, SMC53, SMC56, SMC95, SMC146, SMC235 and SMC270) showed antimicrobial activities. Five strains (SMC30, SMC31, SMC56, SMC95, SMC270) showed growth inhibition against *E. faecalis*. Seven strains (SMC2, SMC30, SMC31, SMC53, SMC56, SMC235, SMC270) against *S. aureus*. Two strains (SMC31, SMC47) against *K. pneumoniae*. A strain of SMC47 against *A. baumannii* and *E. aerogenes*, and a strain of SMC146 against *P. aeruginosa*. The potent antibacterial agents of *Streptomyces* candidates were also subject to extraction and assay for the antimicrobial activity against the ESKAPE pathogens. The antimicrobial agents will be further isolated and purified. These will be useful as chemotherapeutics in the future.

Keywords: *Streptomyces*, antimicrobial, antibiotics, bacterial infection, agar plug method

การออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคโดยสเตรปโตมัยซิสที่แยกได้จากแหล่งทรัพยากรดินธรรมชาติ

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บทคัดย่อ

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การออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคโดยสเตรปโตมัยซิสที่แยกได้จากแหล่งทรัพยากรดินธรรมชาติ
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การติดเชื้อแบคทีเรียยังคงเป็นปัญหาสำคัญต่อสาธารณสุขทั่วโลก โดยเฉพาะอย่างยิ่งการติดเชื้อแบคทีเรียคือยาต้านจุลชีพหลายขนาน โรคที่เกิดจากการติดเชื้อแบคทีเรียส่วนใหญ่สามารถรักษาได้ด้วยยาต้านจุลชีพ อย่างไรก็ตามยาเหล่านี้เคยถูกนำมาใช้กันอย่างกว้างขวางและเป็นระยะเวลานาน ทำให้เชื้อมีการปรับตัวให้ทนต่อยาอย่างรวดเร็ว ส่งผลให้ยาที่ใช้รักษามีประสิทธิภาพน้อยลง ในแต่ละปีมีผู้ป่วยติดเชื้อแบคทีเรียคือยานับล้านคน และจากการติดเชื้อเหล่านี้นำไปสู่การเสียชีวิต ดังนั้นการค้นพบยาปฏิชีวนะใหม่สำหรับการรักษาจึงเป็นสิ่งจำเป็นอย่างเร่งด่วน สเตรปโตมัยซิสเป็นกลุ่มแบคทีเรียที่อาศัยอยู่ในดินพบได้ทั่วไปตามธรรมชาติ แบคทีเรียเหล่านี้ถือเป็นแหล่งที่อุดมสมบูรณ์ไปด้วยผลิตภัณฑ์ทางธรรมชาติที่ถูกนำมาใช้ในการผลิตยาปฏิชีวนะมากถึงสองในสามของที่มีอยู่ ประเทศไทยเป็นดินแดนที่อุดมไปด้วยทรัพยากรทางธรรมชาติรวมทั้งจุลินทรีย์ในดิน ดังนั้นวัตถุประสงค์ของการศึกษานี้ คือการค้นหาสารออกฤทธิ์ต้านจุลชีพจากสเตรปโตมัยซิส (*Streptomyces*) ที่แยกได้จากแหล่งทรัพยากรธรรมชาติของไทยได้ทำการแยกสเตรปโตมัยซิสจำนวน 50 สายพันธุ์จากดินที่เก็บรวบรวมได้จากแหล่งธรรมชาติที่แตกต่างกันทางภูมิศาสตร์ของประเทศไทย เชื้อจำนวน 50 สายพันธุ์ที่แตกต่างกันนี้ได้ถูกนำไปศึกษาการออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคสายพันธุ์มาตรฐาน ประกอบด้วย *Enterococcus faecalis* *Staphylococcus aureus* *Klebsiella pneumoniae* *Acinetobacter baumannii* *Pseudomonas aeruginosa* และ *Enterobacter aerogenes* โดยนำไปทดสอบด้วยวิธีการวางชิ้นวุ้น สเตรปโตมัยซิสสายพันธุ์ที่สร้างสารต้านจุลชีพได้จะแสดงการออกฤทธิ์ยับยั้งเป็นวงใสชัดเจนรอบชิ้นวุ้น

ผลจากการศึกษาพบว่า มีสเตรปโตมัยซิสเพียงจำนวน 10 สายพันธุ์ (คิดเป็นร้อยละ 20) ได้แก่สายพันธุ์ SMC2 SMC30 SMC31 SMC47 SMC53 SMC56 SMC95 SMC146 SMC235 และ SMC270 ที่แสดงการออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคได้ สเตรปโตมัยซิส 5 สายพันธุ์ได้แก่ SMC30 SMC31 SMC56 SMC95 และ SMC270 แสดงฤทธิ์ยับยั้งการเจริญของเชื้อ *E. faecalis* สเตรปโตมัยซิส 7 สายพันธุ์ได้แก่ SMC2 SMC30 SMC31 SMC53 SMC56 SMC235 และ SMC270 ยับยั้งการเจริญของเชื้อ *S. aureus* สเตรปโตมัยซิสจำนวนสองสายพันธุ์ได้แก่ SMC31 และ SMC47 ยับยั้งการเจริญของเชื้อ *K. pneumoniae* ส่วนสายพันธุ์ SMC47 ยับยั้งการเจริญของเชื้อ *A. baumannii* และ *E. aerogenes* และสายพันธุ์ SMC146 ยับยั้งการเจริญของเชื้อ *P. aeruginosa* สเตรปโตมัยซิสที่ผ่านการคัดเลือกมีประสิทธิภาพในการออกฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อแบคทีเรียก่อโรคได้ถูกนำไปทำการสกัดแยกสารเพื่อทดสอบกับเชื้อก่อโรคกลุ่ม ESKAPE ขั้นตอนต่อไปจะทำการแยกสารให้ได้บริสุทธิ์ ซึ่งสารที่ได้นี้จะมีประโยชน์เชิงเคมีบำบัดต่อไปในอนาคต

คำสำคัญ: สเตรปโตมัยซิส ยาต้านจุลชีพ ยาปฏิชีวนะ การติดเชื้อแบคทีเรีย วิธีการวางชิ้นวุ้น

Introduction

Infectious diseases have become a major public health problem worldwide, especially nosocomial infection¹. The causative agents are mostly a group of bacterial pathogens, called ESKAPE, which include *Enterococcus* species (e.g., *E. faecalis*), *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (e.g., *E. aerogenes*)²⁻⁴. The ESKAPE pathogens are top ten microorganisms frequently isolated from clinical specimens of patients (urine, pus, wound, etc.) admitted to hospitals as reported by WHO in 2014⁵. Most patients infected by the ESKAPE have history of prolonged illness with extended use of antibiotics, resulting in resistance to drug treatments and being at high risk of death⁶. Serious bacterial infections have still become a major global healthcare problem in the 21st century due to increasing antibiotic resistance, particularly multidrug resistance (MDR) and emerging new species of infectious agents. Recently, there are reports that revealed the danger of antimicrobial resistance to bacterial infected humans annually resulting in 23,000 deaths in the United States⁷⁻⁹ 25,000 deaths in Europe⁹⁻¹⁰ and 38,481 deaths in Thailand¹¹. The direct cost of antibiotics for treatments of antimicrobial resistance infections in Thailand (in 2010) was approximately 6,084 million Baht. Furthermore, the indirect costs of morbidity and mortality related to antimicrobial resistance infections were at least 40,000 million Baht.

The aforementioned health and economic impacts of antibiotic resistance promote discovering new antibiotics urgently needed for chemotherapy¹²⁻¹⁴. Until now natural products from a microbial origin

are a promising source of novel antibiotics¹⁵. Twenty five percents of discovered natural compounds were reported to be biologically active, 10% of which were derived from microbial sources¹⁶. There are approximately 22,500 bioactive compounds obtained from microorganisms, 45% of them (10,100) are produced by actinomycetes, 38% (8,600) by fungi, and 17% (3,800) by other bacteria. Among 10,100 bioactive compounds produced by actinomycetes, 75% are produced by streptomycetes (majority is genus *Streptomyces*) and the remaining 25% from the others (rare actinomycetes)¹⁷⁻¹⁹. Streptomycetes, Gram-positive filamentous bacteria with high G+C contents in their DNA (>55 mol %), are a distinct group of microorganisms producing diverse natural bioactive compounds. They are saprophytic and distributed widely in environment including soil, water and marine environments. They produce a number of important metabolites during the development of substrate to aerial mycelia²⁰⁻²². Therefore, streptomycetes (especially *Streptomyces*) are one of the most important sources of new bioactive secondary metabolites, such as antibiotics²³⁻²⁴. These bioactive substances have been verified and validated to be useful for human and veterinary medicines. These biological activities include antibacterial, antiviral, antimalarial, antiparasitic, antifungal, antiprotozoal, anticancer, antitumor, and immunosuppressant¹⁸.

Regarding to the problem of bacterial drug resistance, this work therefore attempts to isolate and elucidate the antibiotic activities of the local *Streptomyces* isolates against human pathogenic bacteria causing problems in public health. These *Streptomyces* have been isolated from Thai natural habitats and studied their antimicrobial activities against the

ESKAPE pathogens by agar plug method. Further, antimicrobials produced by the *Streptomyces* cultures have been preliminarily extracted with ethyl acetate and demonstrated their antimicrobial activities as well.

Methods

Bacterial strains and growth conditions

Fifty strains of *Streptomyces* species were previously isolated from soils collected from Thai natural resources. The bacteria were subject to identification under microscopic and macroscopic examinations, including colony morphology, mycelium and spore formations. The *Streptomyces* isolates were grown on International Streptomyces Project (ISP) medium No.3 at 28°C for 7 days and used in the agar plug assay. The standard bacterial pathogens as a test microorganism: *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae*, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 27853 and *Enterobacter aerogenes* ATCC 13048 were grown on tryptic soy agars at 37°C overnight. Single colony was inoculated to tryptic soy broth, then the culture was incubated at 37°C. After overnight cultivation, the culture broth was spread over entire surface of the Muller Hinton agar. The agar petri dish was air dried under sterile laminar flow before used in the agar plug assay.

Screening the antimicrobial activity

Fifty strains of *Streptomyces* isolates were grown on ISP medium No.3 at 28°C for 7 days for study the antimicrobial activity according to the mycelial agar plug assay²⁵. Briefly, plugs of mycelium agar were cut with a sterile pipette tip and transferred

to the surface of the Muller Hinton agar previously spread with test microorganism. The medium plate was incubated at 37°C for 24 h. The antimicrobial activity was observed by the measurement of inhibition zone. Each isolated strain was screened for the antimicrobial activity in triplicate.

Disk diffusion assay

The *Streptomyces* strains that showed activity in the agar plug assay were selected to grow in a suitable medium for the production of antimicrobial metabolites. After seven days of cultivation, the cell-free culture broth was extracted twice with an equal volume of ethyl acetate. The ethyl acetate extract was dried, dissolved in a small volume of acetonitrile, and was utilized for the antimicrobial activity test using the standard disk diffusion assay²⁶. After incubation at 37°C for 24 h, the antimicrobial activity was visualized as mentioned previously. Three replicate analyses were performed on each sample to ensure the reliability and reproducibility of the results.

Results and Discussion

A total of different fifty *Streptomyces* strains isolated from diverse geographic natural environments of Thailand are shown in Figure 1. They were fast-to moderate- growing microorganisms and able to be cultured on the ISP medium No.3 at 28°C in order to be screened the antimicrobial activity against the human bacterial pathogens including *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes*. By the agar plug assay, ten *Streptomyces* isolates (20%) designated as strain numbers SMC2, SMC30, SMC31, SMC47,

Table 1 Antimicrobial activity of *Streptomyces* isolated from Thai natural environments against human pathogenic bacteria using agar plug method

*Gram-positive bacteria, **Gram-negative bacteria

Strain no.	Antimicrobial activity against test microorganisms					
	<i>E. faecalis</i> *	<i>S. aureus</i> *	<i>K. pneumoniae</i> **	<i>A. baumannii</i> **	<i>P. aeruginosa</i> **	<i>E. aerogenes</i> **
SMC2	-	+	-	-	-	-
SMC30	+++	++++	-	-	-	-
SMC31	+++	++++	+	-	-	-
SMC47	-	-	++	+	-	++
SMC53	-	+++	-	-	-	-
SMC56	+++	+++	-	-	-	-
SMC95	++	-	-	-	-	-
SMC146	-	-	-	-	++	-
SMC235	-	+	-	-	-	-
SMC270	++++	++++	-	-	-	-

SMC53, SMC56, SMC95, SMC146, SMC235 and SMC270 showed antimicrobial activities at different degrees of growth inhibition (Table 1). Five strains of *Streptomyces* including SMC30, SMC31, SMC56, SMC95, and SMC270 showed the growth inhibition of *E. faecalis* (Figure 2A). Seven *Streptomyces* strains SMC2, SMC30, SMC31, SMC53, SMC56, SMC235 and SMC270 showed antibacterial activity against *S. aureus* (Figure 2B). Two strains (SMC31 and SMC47) were observed to inhibit the growth of *K. pneumoniae* (Figure 2C). A *Streptomyces* strain SMC47 provided antimicrobial activities against both *A. baumannii* and *E. aerogenes* (Figure 2D, 2F). Finally, only one strain of *Streptomyces* (SMC146) exhibited the activity against *P. aeruginosa* (Figure 2E). These results pointed out the potential for these

Streptomyces as antimicrobials producers, mainly against Gram positive bacteria, however a few strains that have ability to produce antimicrobials against Gram negative bacteria were still discovered (Table 1). This finding is in agreement with the previous report²⁷.

Furthermore, the 10 isolated strains that showed antimicrobial activity in the agar plug assay were selected for production of the bioactive compounds in the fermentation broths. After extraction of the fermented broths with ethyl acetate, all extracts still showed the antimicrobial activity against the test microorganisms by the disk diffusion method (Figure 3). Further studies will attempt to isolate the substances responsible for their antimicrobial activities and elucidate their structures. The discovery of novel/

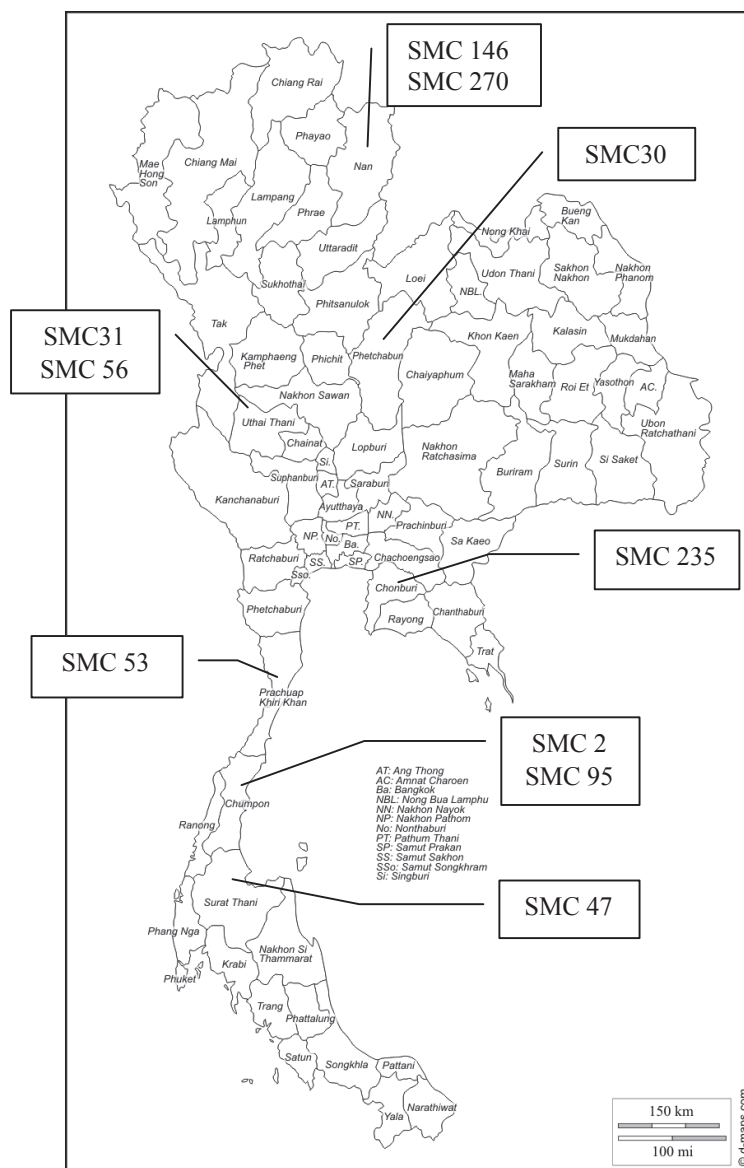


Figure 1 Geographic distribution of antimicrobial-producing *Streptomyces* strains SMC that were discovered in Thailand.

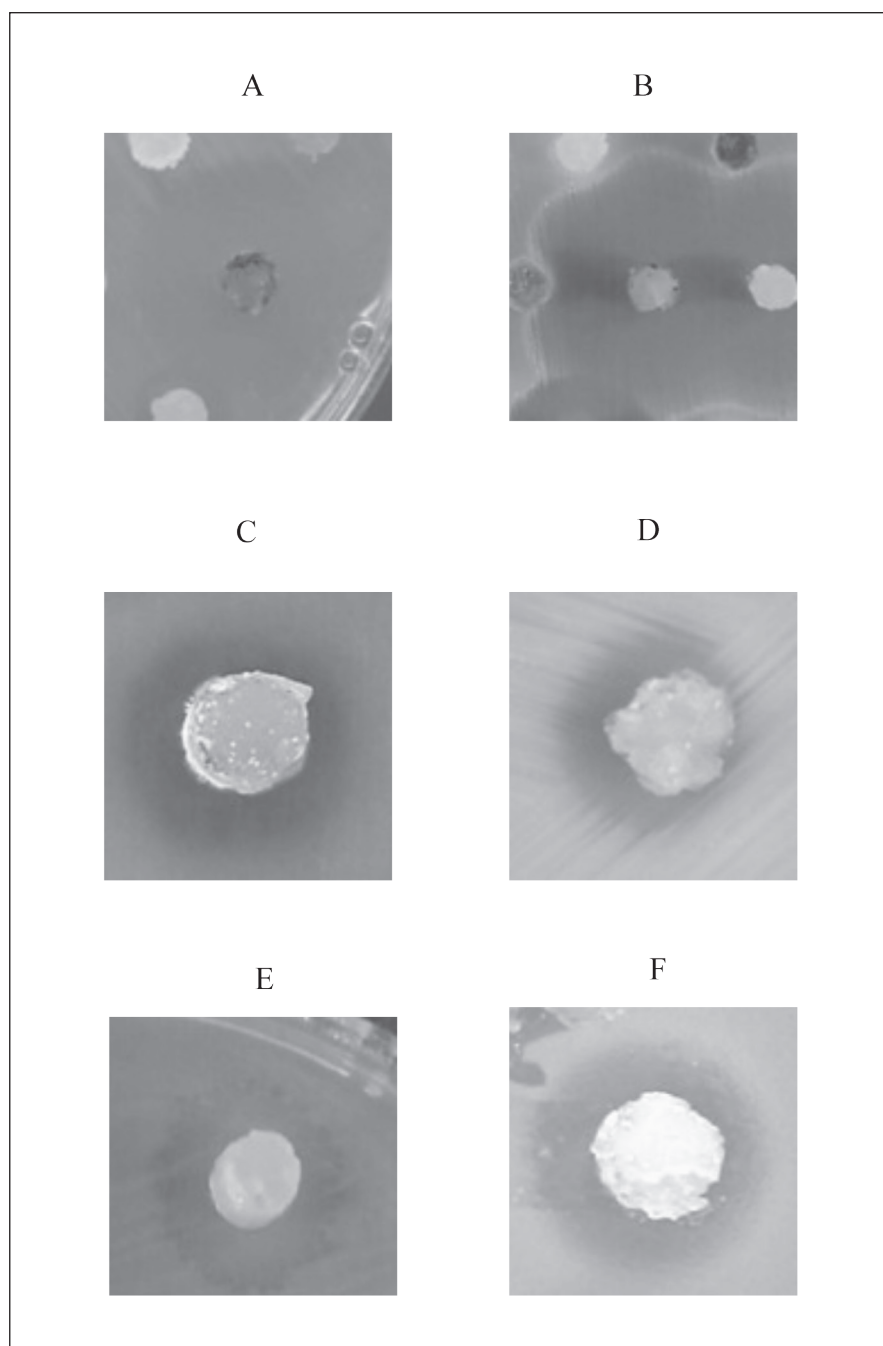


Figure 2 Screening of antibacterial producing *Streptomyces* using agar plug assay.
A: antibacterial activity against *E. faecalis*; B: antibacterial activity against *S. aureus*;
C: antibacterial activity against *K. pneumoniae*; D: antibacterial activity against *A. baumannii*;
E: *P. aeruginosa*; and F: antibacterial activity against *E. aerogenes*

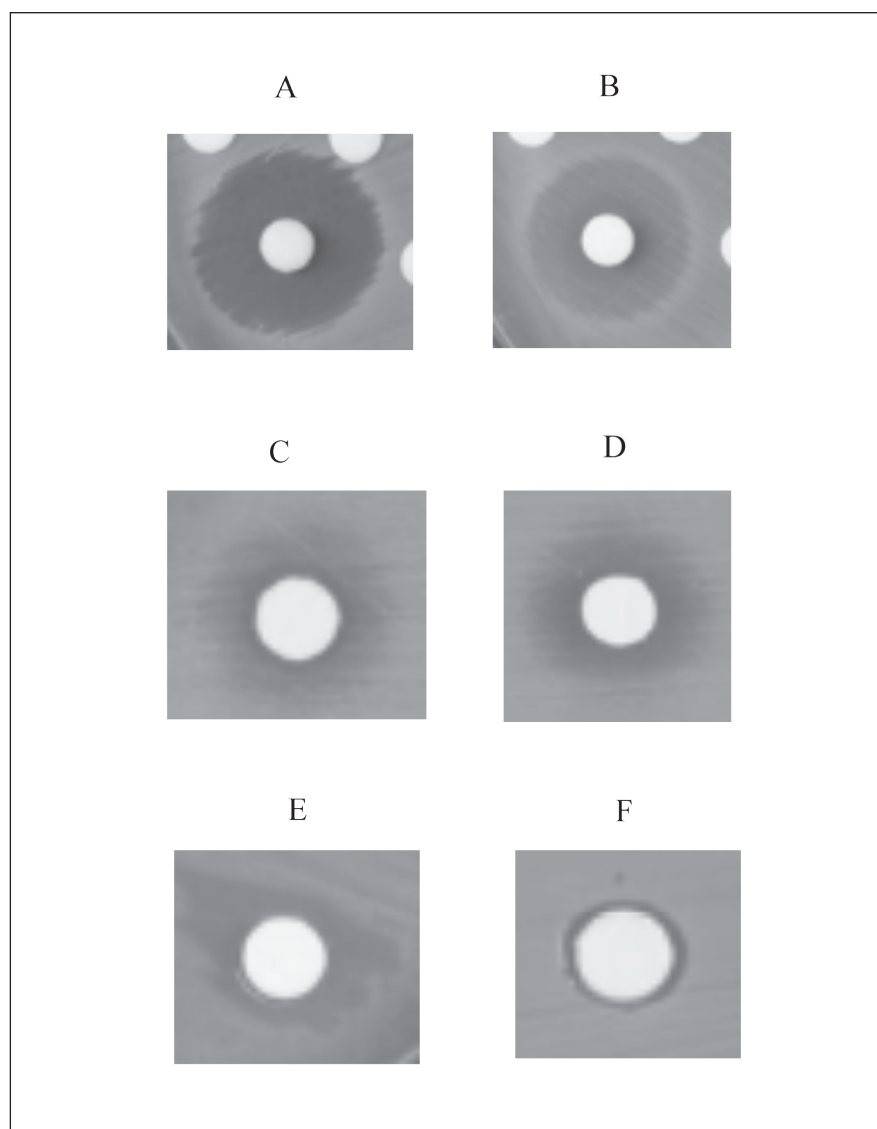


Figure 3 Disk diffusion assay for the antimicrobial activity of the ethyl acetate extracts against the ESKAPE pathogens.

A: antibacterial activity against *E. faecalis*; B: antibacterial activity against *S. aureus*;
C: antibacterial activity against *K. pneumoniae*; D: antibacterial activity against *A. baumannii*;
E: *P. aeruginosa*; and F: antibacterial activity against *E. aerogenes*

effective antimicrobials produced from local actinomycetes will have global impact on public health system, including reduction of commercial drug import and lowering the drug prices in the market. Another importance is the use of novel antimicrobial agent as a potential lead compound for further development of drug specific to particular human infectious diseases.

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