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SCREENING, PARTIAL PURIFICATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUND FROM *STREPTOMYCES ROCHEI* SJKS1 ISOLATED FROM THE SEASHORE SOIL OF DHANUSHKODI

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ABSTRACT

Totality of 60 diverse Streptomyces colonies was isolated from soil samples acquired from 25 different geographical locations. Primary, secondary and tertiary screening methods were used to detect the isolates that possessed maximum antibacterial activity against both Gram positive and Gram-negative microorganisms. Solvent (ethyl acetate) extraction of metabolites of three best isolates was carried out followed by agar well diffusion assay. The potential isolate was morphologically, physiologically and biochemically characterized based on Bergey's manual of determinative bacteriology and International Streptomyces Project (ISP). Molecular characterization by 16S rRNA sequencing revealed that the isolate was Streptomyces rochei SJKS 1. Minimum inhibitory concentration (MIC) of the solvent extract from the isolate against Staphylococcus aureus ATCC 9144 and Escherichia coli ATCC 25922 was recorded to be 260 µg on comparison with the standards. Synergistic studies revealed a positive synergism between the solvent extract and the commercial antibiotics when used in combination against the test microorganisms. The probable mechanism of antibacterial action of the active compound was elucidated by membrane leakage studies and the ethyl acetate extract was further analyzed by thin layer chromatography (R_f value 0.12). UV-visible spectroscopic studies of the bioactive compound displayed a maximum UV absorption at 221 nm, a characteristic similar to that of polyene antibiotics. FTIR studies of the extract further revealed the functional groups of the antimicrobial compound.

KEY WORDS

Antimicrobial compound, FTIR, MIC, Streptomyces sp.

INTRODUCTION

Soil is an intensively exploited ecological niche and the microbial populations which inhabit the soil demonstrate enormous potential for antibiotic synthesis. The search for new antibiotics continues in order to combat the evolving pathogens, the ubiquitous antibiotic resistant microorganisms present in the environment and to advance pharmacological properties by developing safer, effective and broader spectrum compounds.

Among the diverse industrially significant microorganisms, actinomycetes are of chief importance and are primarily recognized as organisms of academic curiosity since they are capable of synthesizing many different bioactive secondary metabolites. *Streptomyces* is the best recognized genus of actinomycetes and soil is their most important habitat. *Streptomyces sp.* showcases a remarkable ability to elaborate different kinds of novel secondary metabolites [1,2]. The secondary metabolites obtained from *Streptomyces sp.* are industrially exploited as

antibacterial or antifungal agents, antitumor compounds, insecticides and herbicides. They synthesize a remarkable range of chemically distinct inhibitors of many different cellular processes which comprise of antibiotics, fungicides, modulators of immune response, and effectors of plant growth [3-5].

Currently, there is a need to expand the development of new microbial metabolites not only due to the increase in resistant microorganisms, but also due to the evolution of novel diseases and the toxicity of the currently used compounds. Also, the biosynthetic capacity of well-known microorganisms, such as *Streptomyces*, is not fully exploited and therefore, several strategies have to be employed to find new bioactive drugs from them.

Hence, the aim of the present work was to isolate *Streptomyces* from diverse soil samples, screen the ability of the isolates to produce antimicrobial compounds, select the most promising isolate, mass produce it followed by its morphological, cultural, and biochemical, physiological and molecular characterization. Partial purification of the antimicrobial compound obtained from *Streptomyces* by solvent extraction; detect active fractions by thin layer chromatography (TLC), analysis of the solvent extract by UV spectroscopic studies, determination of minimum inhibitory concentration (MIC) of the partially purified compound against the various test isolates, to predict the functional group of the antimicrobial compound by FT-IR and to study the synergistic effect of the antimicrobial compound and the commercially available antibiotics against the test microorganisms.

MATERIALS AND METHODS

Chemical and reagents

All the chemical and reagents, media used for the study were purchased from Hi-Media Laboratories, Ltd. Mumbai, India and Sigma-Aldrich Chemicals, Co., U.S.A.

Collection of soil samples and isolation of *Streptomyces*

Soil samples were acquired from a depth of 15 cm at various terrestrial locations in different parts of India. Starch casein agar medium was prepared and sterilized at 121°C in 15 lbs pressure for 15 minutes in an autoclave. Amphotericin B (50 µg/1000 ml) was then added to the sterilized medium to prevent fungal growth and poured into sterile Petri plates. One gram of the pretreated soil samples were suspended in 9 ml of sterile Ringer solution and were serially diluted up to 10^{-5} dilutions. Hundred microlitre of the diluted samples (10^{-3}, 10^{-4} and 10^{-5}) was spread over the agar plates in duplicates and the plates were incubated at 28±2°C for 7-10 days [6]. After incubation, the observed *Streptomyces* colonies were purified by streak plate method and maintained in SCA (Starch casein agar) slants at 4°C for further investigation.

Screening for antimicrobial activity

The test organisms used during the study were *Staphylococcus aureus* ATCC 9144, *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6051, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Klebsiella pneumoniae* ATCC 13883. Primary screening of the isolates by agar overlay method [7], secondary screening by cross streak method [8] and tertiary screening by agar well diffusion assay [9] were performed to spot the most potential isolate producing antimicrobial compound inhibiting the activity of the test microorganisms.

Antimicrobial metabolite production and extraction

The antimicrobial compound was recovered from the culture filtrate of the active isolates by solvent extraction method using ethyl acetate [10]. The inhibitory effects of the ethyl acetate extract on indicator organisms were studied by agar well diffusion assay. Wells measuring 4 mm in diameter were punctured on Mueller Hinton agar plates spread with 0.1 mL of overnight individual test bacterial cultures. The wells were loaded with 10 µl of the ethyl acetate extract in DMSO, 10 µl of the standard antibiotic Streptomycin (100 µg/ml), 10 µl of ethyl acetate and 10µl of distilled water. The plates were left undisturbed at room temperature for 2 hours and then incubated at 37˚C for 24 hours. The diameter of the inhibition zones around the wells was measured.

Determination of MIC

Broth tube dilution method [11] was employed for the determination of minimum inhibitory concentration of the antimicrobial compound obtained after solvent extraction against the test bacterial strains *S. aureus* and *E. coli*. The minimum inhibitory concentration or the lowest concentration of the substance that inhibited the growth or visual turbidity of the test organism was determined by measuring the OD at 600 nm using spectrophotometer.

Characterization of the potent isolate

The isolate was characterized based on morphological, biochemical, physiological criteria with the help of Bergey's Manual of Determinative Bacteriology [12] and International *Streptomyces* Project (ISP) [13]. The identity of the potential isolate was further confirmed by 16S rRNA sequencing. Disc diffusion method was carried out to determine the antibiogram pattern of the isolate [14].

Scanning Electron Microscope (SEM) analysis

A week-old *Streptomyces* culture which had been grown on starch casein agar media was used. It was centrifuged at 5000 rpm at 4°C and pellet was obtained. The pellet was fixed with 500 µl of 2.5% gluteraldehyde. This was allowed to stand overnight at room temperature and then the supernatant was discarded. Now the pellet was serially washed under gradient ethanol at 10%, 25%, 50%, 75% and 100%. About 50-100 µl of sterile distilled water was added to it. From this, 10 µl was coated on aluminum foil (1x1 cm) and air dried. The morphological features and the spores of the active *Streptomyces* isolate were observed over a range of magnifications under a scanning electron microscope [15].

Characterization of the extract

The partially purified compound from TLC was mixed with ethyl acetate and subjected to centrifugation. The pellet obtained after centrifugation was mixed with dimethyl sulfoxide (DMSO). This solution was used to perform agar well diffusion assay to confirm the antimicrobial activity of the purified compound as described earlier. The test organisms used were *S. aureus* and *E. coli*. UV-spectra of the active solvent extract were detected by dissolving it in methanol (1 mg/ml) followed by scanning in the range of 200-400 cm^{-1} using UV-visible spectrophotometer [16]. The active solvent extract was dried and subjected to fourier transform infra-red (FT-IR) analysis. The spectra were scanned in the range of 400 - 4000 cm^{-1}.

Leakage determination assay

Protein and nucleic acid leakage studies were carried out in order to witness the damage induced by the ethyl acetate extract in DMSO which results in the leakage of the bacterial cellular proteins and nucleic acids upon disruption of membrane integrity. The bacterial strains such as, *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, *E. faecalis* and *K. pneumoniae* were used during the study. The supernatant obtained was assayed to determine the protein concentration by Bradford method [17]. The nucleic acid leakage was determined by measurement of OD at 260 nm using UV-visible spectrophotometer following incubation and comparing the values with a standard graph.

Synergistic activity of the antibiotics and the active extract [18]

To identify the synergistic activity, a combination of commercially purchased antibiotic and ethyl acetate extract loaded discs were placed on MHA plates spread with lawn culture of the indicator organisms, *S. aureus* and *E. coli*. The plates were then incubated at 30˚C for 48 hours following which the zones of inhibition were measured to determine the antimicrobial effect of the particular combination against the indicator microorganism.

During this study, one set of control comprised of plates loaded with the antibiotic discs and the other set of control included only the extract loaded discs. These were used to effectively compare and study the results of the synergism.

RESULTS AND DISCUSSION

Isolation of *Streptomyces*

In this study, soil samples collected were subjected for the isolation of *Streptomyces* sp. A total of 60 *Streptomyces* isolates were recovered from various soil samples using starch casein agar (SCA) as a growth medium. Sixty *Streptomyces* isolates (SJKS1 through SJKS60) with different morphotype were isolated from the soil samples (Table 1). They were then purified by streak plate method and were included for the course of screening.

Table 1: Isolation of *Streptomyces* from soil samples

Sample type	Source	Number of isolates	Number of positive isolates
Garden soil	College campus (DGVC, Chennai)	11	8
	Vapi (Gujarat)	5	3
	Mumbai	10	5
	Haryana	8	6
Marine soil	Marina beach (Chennai)	7	4
	N4 beach (Chennai)	7	4
	Dhanushkodi beach	3	2
	Juhu beach (Mumbai)	2	1
	Tithal beach (Gujarat)	4	3
Market soil	Koyambedu Market	3	2
	TOTAL	60	38

Screening for antimicrobial activity

Amongst the sixty isolates, 38 isolates (Table 2) showed inhibitory action against at least one test microorganism during primary screening by agar overlay method. This can be compared to report by Mohseni et al. [19] which stated that about 51% of the actinomycetes incorporated in his study had the ability to inhibit at least one test bacteria. Therefore, this study again provides evidence of the same.

Eleven isolates which showed inhibitory action against the indicator organisms were selected during secondary screening by cross streak method. A decrease in the isolates showing antimicrobial activity against more than two microorganisms was noticed. The result obtained in the present study was in agreement with the result of Bizuye et al. [20] who reported that in secondary screening 50% of the active strains showed weak antibiosis against certain test organisms.

Table 2: Inhibition patterns of the extracted compound

Sample	*E.coli* ATCC 25922	*S.aureus* ATCC 9144	*B.subtilis ATCC 6051*	*E.faecalis* ATCC 29212	*K.pneumoniae* ATCC 13883	*P.aeruginosa* ATCC 27853
	Inhibition Zone (mm)					
SJKS1	32	39	39	34	28	28
SJKS7	7	30	28	15	13	17
SJKS21	0	26	27	0	10	11
Standard antibiotic	14	33	31	22	25	18
Distilled water	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-

Extraction of antimicrobial metabolites using ethyl acetate

Starch casein broth was used to culture *Streptomyces* isolates in liquid medium. Shake flask fermentation is considered to be a better fermentation system when compared to static fermentation [21]. Ethyl acetate was used as a solvent to obtain the active extract of the selected isolates. Most of the studies confirm that the maximum antibacterial activity is found in ethyl acetate extracts when compared to xylene, methanol, ethanol, chloroform or acetone [22]. The antimicrobial potential of a compound obtained from ethyl acetate extract of *Streptomyces* strain RM42 has been reported by Remya and Vijayakumar [23]. Similar study has been performed by Mathur et al. [24] where the antimicrobial compound was extracted using ethyl acetate as a solvent. The compound extracted from SJKS1, SJKS7 and SJKS21 dissolved completely in ethyl acetate and DMSO. Maximum yield (1650 mg) of the compound after solvent extraction was given by the culture filtrate from

SJKS1 followed by S7 (150 mg) and S21 (90 mg). The extracted compounds from SJKS1, SJKS7 and SJKS21 isolates were assessed for their antimicrobial ability by agar well diffusion method.

Agar well diffusion

Agar well diffusion assay was carried out to perform tertiary screening, where the antibacterial activity of the culture media filtrates was tested against *S. aureus* and *E. coli*. It is always stated that isolates that show inhibitory activity on agar may not show such activity in broth culture [25]. This can be given as an explanation for the decrease in the number of active isolates during the tertiary screening. Based on the size of the inhibition zones, three isolates (SJKS1, SJKS7 and SJKS21) having strong activity against almost all the tested Gram positive and Gram-negative pathogens were selected. Compound from SJKS1 isolate exhibited maximum inhibitory effect against *S. aureus* (39 mm) and *B. subtilis* (39 mm), followed by *E. faecalis* (34 mm), *E. coli* (32 mm), *P. aeruginosa* (28 mm) and *K. pneumoniae* (28 mm) when compared to SJKS7 and SJKS21. Absence of clearance zone or presence of growth of the indicator organisms around the ethyl acetate well was noticed. This proved that the antimicrobial effect was due to the production of secondary metabolites by *Streptomyces* isolates and not because of ethyl acetate.

Kokare et al. [26] discussed that during the process of screening for novel secondary metabolites *Streptomyces* isolates are often seen to show more activity against Gram positive bacteria than Gram negative bacteria. This is in agreement with the results obtained in the present study where maximum inhibitory activity was seen against Gram positive *S. aureus* and *B. subtilis* though considerably good activity was also seen against the Gram-negative *E. faecalis* and *E. coli*. Based on the results, from solvent extraction and agar well diffusion, one most potential isolate (SJKS1) was chosen. The MIC of the extracted compound against *S. aureus* and *E. coli* was found to be 260 µg/ml.

Characterization of the Potent isolate

The potent isolate (SJKS1) was isolated from the soil sample obtained from the sea shore of Dhanushkodi, Tamil Nadu, Chennai (Latitude 9° 10' N, Longitude 79° 28' E). The various physiological and biochemical characteristics of the selected isolate SJKS1 are recorded in Table 3. The colony morphology of the *Streptomyces* isolate (SJKS1) on various media was studied. The results are tabulated. Abundance of growth was visualized in SCA, ISP 1, ISP 2 and MSS. Absence of diffusible pigment was noticed in all the supporting media (Table 4).

Table 3: Physiological & biochemical characterization

Name of the test	Result
Gram Staining	Gram positive, grape like clusters
Sporophore Morphology	Spiral
Spore Texture	Smooth
Motility	Negative
Catalase Test	Negative
Oxidase Test	Positive
Gelatin Hydrolysis	Positive
Nitrate Reduction Test	Negative
Pectin Hydrolysis	Negative
Chitin Hydrolysis	Positive
Carbon Utilization	Positive
Melanin production	Negative
Sodium Chloride (NaCl) Tolerance Test	
1%	Negative
3%	Positive
5%	Positive
7%	Negative
9%	Negative

Table 4: Cultural characteristics of the isolate

Medium*	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
SCA	Abundant	White	Grayish brown	Absent
ISP 1	Abundant	Greenish brown	Greenish brown	Absent
ISP 2	Abundant	White	Dirty white	Absent
ISP 9	Poor growth	White	White	Absent
MSS	Abundant	Grayish white	Grayish white	Absent
SMK	Poor growth	White	White	Absent

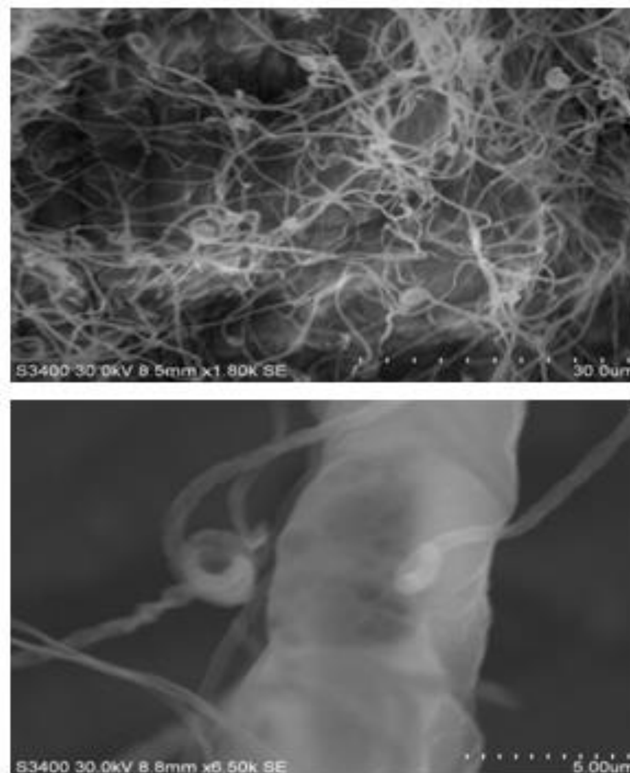
***(SCA - Starch Casein Agar; ISP 1 - Tryptone yeast Extract; ISP 2 -Yeast extract malt extract; ISP 9 - Carbon utilization medium; MSS - Modified soluble starch; SMK - Krasilnikov's synthetic agar)**

The organism was identified based on 16S rRNA analysis. The NCBI BLAST search analysis of the 16S rRNA sequence of the strain with the sequences available in the data bank showed a maximum similarity of 100% with *Streptomyces rochei* strain SCSIOZ-SH09. The 16S rRNA sequence of the strain SJKS1 was submitted to the GenBank under the accession number MF767527. A phylogenetic tree based on 16S rRNA sequences was constructed by neighbor joining method to illustrate the phylogenetic relationship between the strain and other *Streptomyces* neighbors [27]. Evolutionary analyses were conducted in MEGA6 [28]. The *Streptomyces* isolate (SJKS1) was resistant to the antibiotic ampicillin and was most sensitive to tetracycline followed by gentamicin and penicillin.

Scanning electron microscope (SEM) analysis

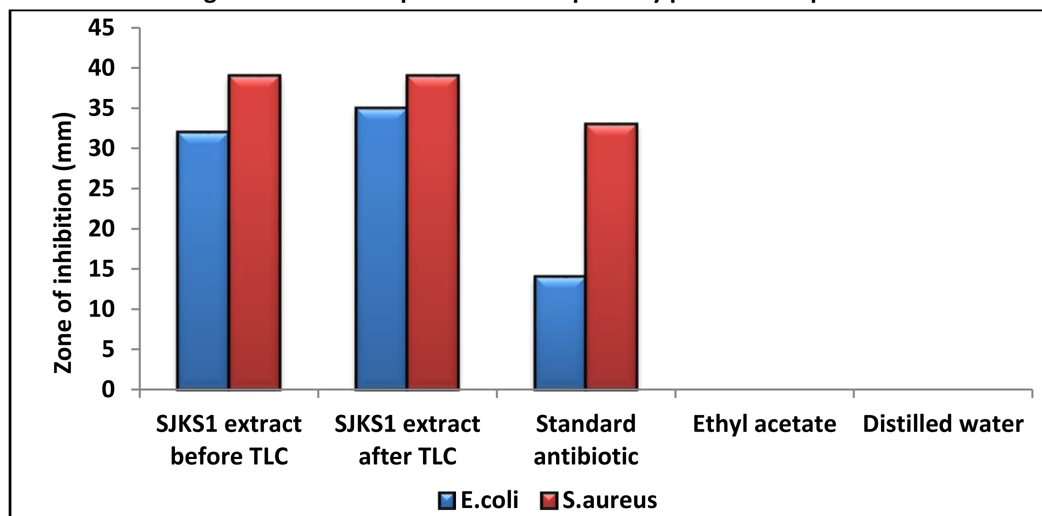
The SEM analysis revealed that the aerial hyphae of *Streptomyces* SJKS1 were differentiated into long spiral chains of cylindrical spores with a smooth surface [29,30]. The micro morphological characteristics such as spore surface ornamentation and spore chain morphology determined by scanning electron microscopic (Fig. 1) analysis further suggested that the isolate belonged to the genus *Streptomyces* [31].

Figure 1: Scanning electron microscopy of the *Streptomyces* isolate (SJKS1)



SEM analysis of *Streptomyces rochei* SJKS1 isolated from the sea shore of Dhanushkodi, Tamil Nadu, Chennai (Latitude 9° 10' N, Longitude 79° 28' E) showing the long spiral chains of cylindrical spores with a smooth surface.

Figure 2: Inhibition patterns of the partially purified compound

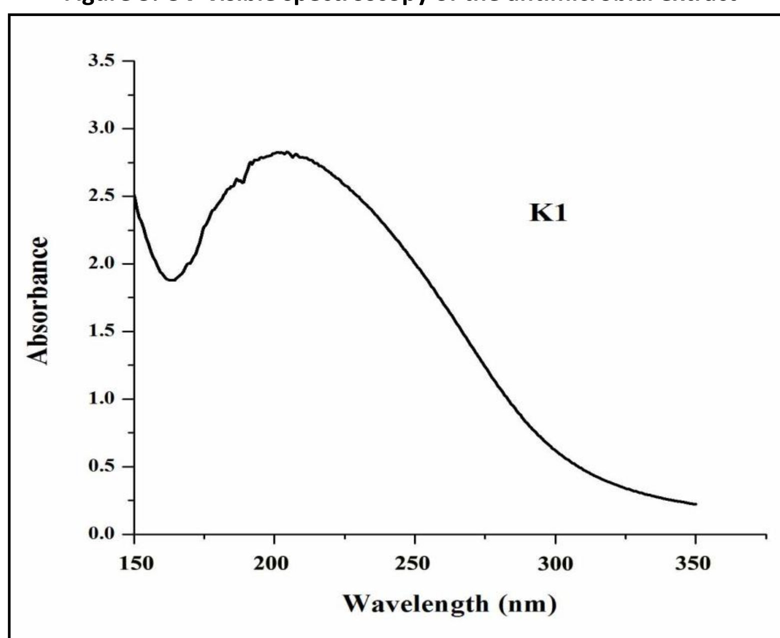


Characterization of the extract

Ethyl acetate extracted compound of the SJKS1 isolate was purified and separated by thin layer chromatography. Single separated band was observed and the Rf value was found to be 0.12 cm. The compound purified by TLC was subjected to agar well diffusion assay to detect and confirm its antimicrobial activity against the test organisms. It was analyzed from the results in Figure 2 that the activity of the SJKS1 active extract increased after TLC purification.

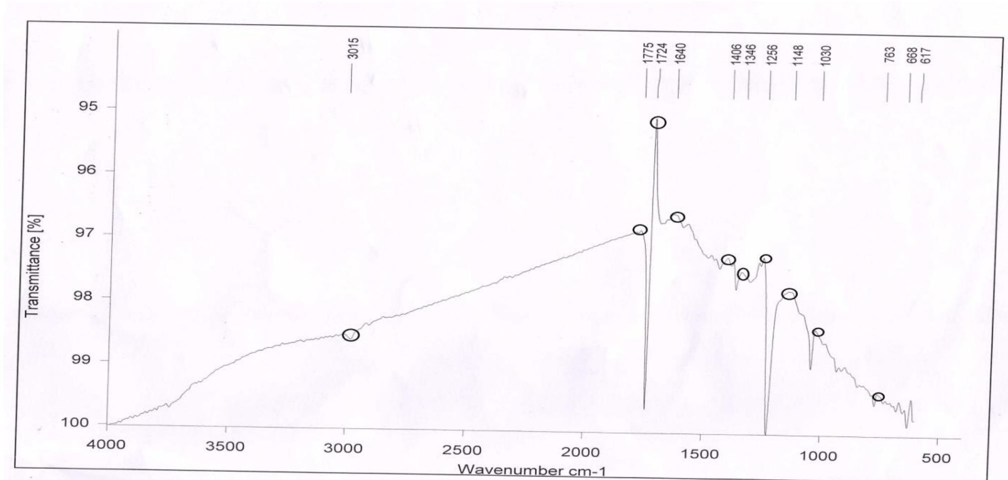
The partially purified antimicrobial compound after solvent extraction was dissolved in methanol and analyzed for UV absorption spectra. Maximum absorbance peak in the UV spectrum was at 221 nm (Fig. 3). The characteristics of the absorption peak indicate a polyene nature typically a trait of antibiotic producing species of *Streptomyces* bacteria. The spectral data are consistent with those obtained by Saadoun et al. [16] where it was interpreted that maximum absorbance peaks for solvent extracts from *Streptomyces* were obtained in the range of 215-270 nm. They were also highly polyene in nature.

Figure 3: UV-visible spectroscopy of the antimicrobial extract



The antimicrobial compound exhibited a maximum absorption peak at 221 nm.

Figure 4: FTIR spectrum of the antimicrobial compound



The FTIR spectrum of the active extract from the isolate was detected (Fig. 4). The peaks and the functional groups obtained during the FTIR analysis were classified. The ethyl acetate extract in its IR spectra displayed a broad absorption band at 3015 cm^{-1}. This peak is often weak, overlaps with C-H alkane absorption and is frequently diagnostic of unsaturation. Absorption at 1775 cm^{-1} and 1724 cm^{-1} confirms the presence of carbonyl group. The carbonyl stretching absorption is one of the strongest IR absorption. The peak at 1640 cm^{-1} indicates the presence of unsaturation and the possibility of an aromatic compound whereas the peak at 763 cm^{-1} shows an aromatic C-H bend. Olefinic alkene group is represented by peaks present at 1406 cm^{-1} and 1256 cm^{-1}. The peak at 1346 cm^{-1} signifies a saturated aliphatic compound and peak at 1030 cm^{-1} indicates a methyne group with C-C vibrations. Absorption at 1148 cm^{-1} is a characteristic of a C-O bond [32].

Leakage determination assay

Leakage studies (Fig. 5 & 6) were performed to find out the mechanism behind the functioning of the antimicrobial compound. The membrane leakage was almost negligible at time zero. After 6 hours of treatment of the bacterial strains with the antimicrobial metabolites, protein and nucleic acid leakage increased.

Figure 5: Protein leakage determination of antimicrobial compound against different bacterial pathogens

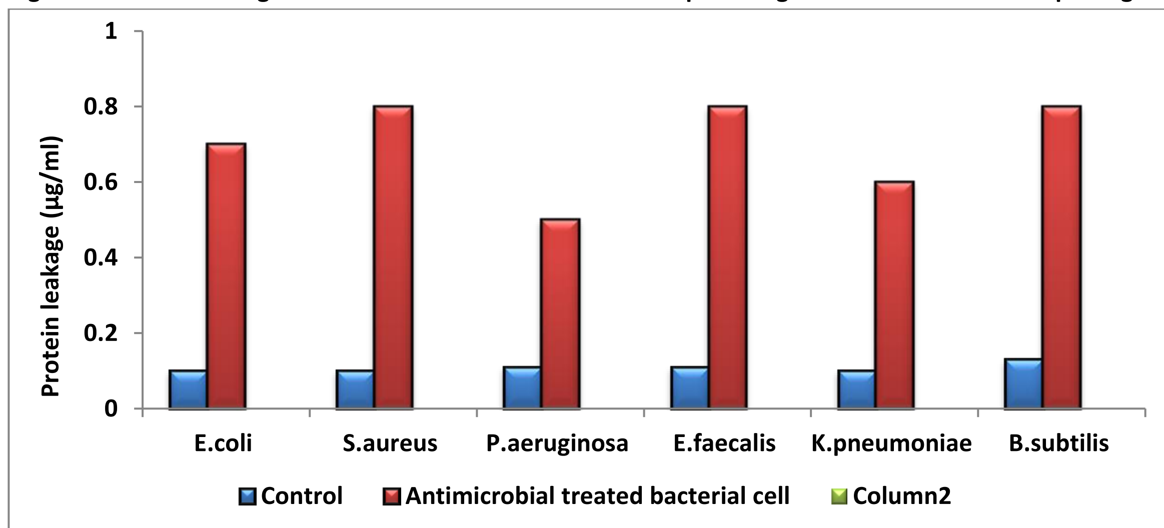
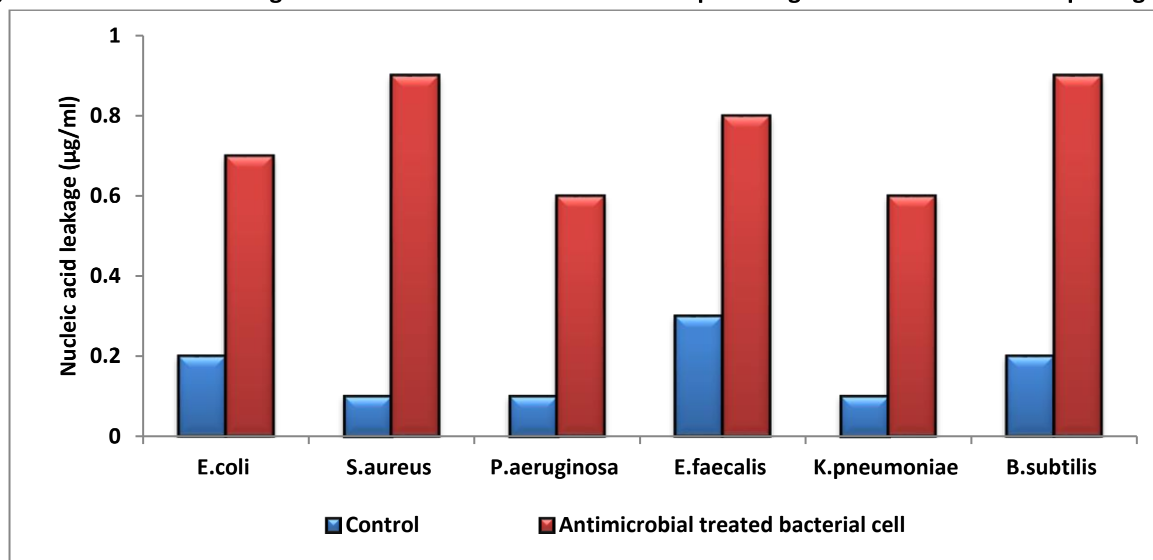


Figure 6: Nucleic acid leakage determination of antimicrobial compound against different bacterial pathogens



From the results, it was inferred that the antimicrobial metabolites may kill the bacterial strains by damaging their cell membrane. They affect the cell membrane integrity by causing leaking of the protein and nucleic acid from the intracellular cytosol. It was seen that the amount of protein leakage from the Gram-positive bacteria was higher than that of Gram-negative bacteria.

Similar results were observed in the case of nucleic acid leakage. This can be explained by the fact that the walls of Gram-negative bacteria are more complex than those of Gram-positive bacteria. The peptidoglycan layer around the Gram-negative bacterial wall is rich in lipids and it serves as a barrier to external agents that can destroy the cell [33].

Synergistic activity of the antibiotics and the active extract

The activity of two drugs in combination is greater to the sum of their independent activity when studied separately. This is called the synergistic effect. This effect of antibiotics combined with different secondary metabolites has been studied by Zhou and Peng [18].

Figure 7: Synergistic activity antimicrobial compound with different antibiotics

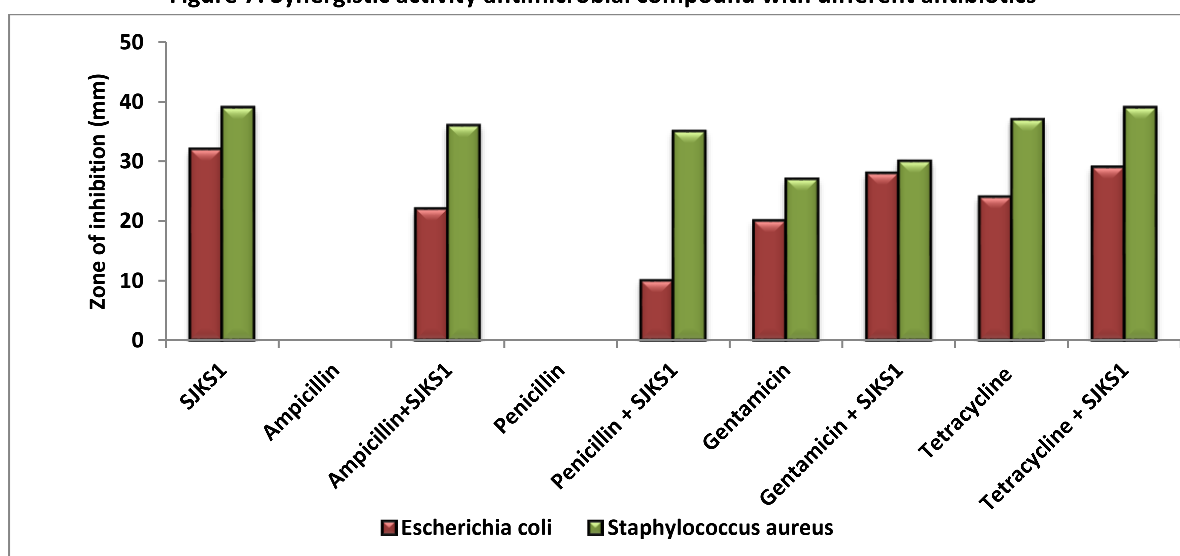


Figure 7 represents the results of inhibitory action of SJKS1, the individual antibiotics and the synergism of both against the test organisms *E.coli* and *S. aureus.* This test was performed to study if the synergism of any of

the antibiotic along with active extract from SJKS1 would result in an increased antimicrobial activity against the test organisms. It was detected that synergism gave better results in case of gentamicin combined with SJKS1 as well as tetracycline combined with SJKS1 (i.e) maximum inhibitory affect against test organisms was seen which was more than that of the individual inhibitory effect of the antibiotic or SJKS1 respectively.

CONCLUSION:

Based on the results of the present study, it is concluded that the isolate, *Streptomyces rochei* SJKS1 has shown the potential of producing an antimicrobial compound which has the ability to inhibit both Gram positive and Gram-negative microorganisms. In addition to this, synergistic activity of this antimicrobial compound along with the commercial antibiotics can yield better results and this factor can be exploited to yield a highly effective antimicrobial compound. If advanced purification and chemical characterization of the antimicrobial compound from this *Streptomyces* isolate is carried out, then this compound can offer a marked effect similar to those provided by the potent antibiotics commercially available in the market. Further, the activity of this antimicrobial compound against the drug-resistant microorganisms can be studied.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests.

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