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Molecular Identification of *Streptomyces clavuligerus* and Structure Elucidation of Its Antimicrobial Efficiency

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Abstract

The present study was carried out for screening antimicrobial agents produced by soil Actinomycetes that demonstrated inhibitory effects against pathogenic microorganisms. Eleven Streptomyces sp. isolates were tested as antimicrobial producers, the most potent producer was isolate encoded C10S19 and was identified by molecular techniques as Streptomyces clavuligerus .The ethyl acetate extract was assayed by paper disk method and purified using thin layer chromatography (TLC). The structure elucidation of antimicrobial agent was performed using ultraviolet (UV), Infra-red (IR) and Mass spectra. Results revealed that Streptomyces clavuligerus produced clavulanic acid salt and holomycin that showed antagonistic activity against Gram positive Staphylococcus aureus ATCC 29213, Gram negative Escherichia coli ATCC 25922 and unicellular fungi Candida albicans ATCC 10231.

Key words: Streptomyces sp., antimicrobial activity, spectroscopic analysis.

I. Introduction

Actinomycetes are aerobic, spore forming gram-positive bacteria, belonging to the order Actinomycetales characterized with substrate and aerial mycelium growth (Lechevalier and Lechevalier, 1981). It has a high (G+C) ratio of the DNA (>55mol %). Actinomycetes possess sufficient distinctive features to present them into 'Kingdom bacteria' (Das *et al.*, 2012). They exhibit a range of life cycles which are unique among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem (Veiga *et al.*, 1983). Actinomycetes have proved their ability to produce a variety of bioactive secondary metabolites including antibiotics, antitumor agents, immunosuppressive agents and enzymes. These metabolites are known to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helminthic, anti-malarial and anti-inflammatory activities (Ravikumar *et al.*, 2011). Screening for antibiotic and non-antibiotic molecules among actinomycetes is therefor becoming increasingly important.

Streptomyces clavuligerus (S. clavuligerus) produces the β-lactam antibiotic, cephamycin C and the β-lactamase inhibitor, clavulanic acid (Hung *et al*., 2006). Clavulanic acid is a clinically significant inhibitor of β-lactamases, while the other clavam metabolites produced by *S. clavuligerus* demonstrate weak antibacterial and antifungal activities (Tahlan *et al*., 2004 a). Several other *Streptomyces* sp. have also been determined to be producers of clavulanic acid (Bignell *et al*., 2005; Jensen and Paradkar 1999). The combined use of clavulanic acid and broad-spectrum β-lactam antibiotics such as amoxicillin are an important therapeutic tactic to combat the rapid increase in β-lactam resistance (Tahlan *et al*., 2004 b). The cluster of genes for clavulanic acid biosynthesis is located downstream from the *pcbC* gene of the cephamycin C cluster in *S. clavuligerus* (Li *et al*., 2000).

The present study was carried out for screening antimicrobial agents produced by soil Actinomycetes that demonstrated inhibitory effects against standard microorganisms. The most potent producer isolate had to be identified by molecular techniques and the structure elucidation of the produced antimicrobial agent had to be performed .

II. Materials and Methods

1. Microorganisms isolates and media used:

The soil samples were collected from different Egyptian locations. The local isolates of Actinomycete strains were tested for their antimicrobial activity.
Growth media: ISP-2 medium: glucose, 4.0 g/L; malt extract, 10.0 g/L; yeast extract, 4.0 g/L; the pH was adjusted to 7 (Pridham *et al*.; 1957).
Production media: Starch casein medium: soluble starch, 10.0 g/L; casein, 1.0 g/L; and K_2HPO_4, 0.5 g/L; the pH was adjusted to 7 (Shirling and Gottlieb, 1966).

2. Test microorganisms:

For the investigation of the antimicrobial spectrum of the Actinomycete isolates, the following test microorganisms were included in the study: Gram negative bacteria: *Escherichia coli* ATCC 25922, Gram positive bacteria: *Staphylococcus aureus* ATCC 29213 and unicellular fungi *Candida albicans* ATCC 10231.

3. Assay of antimicrobial activities of the isolated Actinomycete strains:

The antimicrobial activities of the isolated strains were assessed using the classical diffusion methods. All these methods were based on the visual observation of the inhibition zone around the microbial growth on an agar medium according to Cooper (1963 and 1972). The antimicrobial activity was studied by:-

3.1. Agar well diffusion methods:-

At the end of five days incubation of the producer isolated strains at 37^{o}C, the antimicrobial activities in the filtrates were assessed by using agar-well diffusion method. The antimicrobial activities of the isolates under study were determined by measuring the diameter of inhibition zone around the well (Oluwafemi and Debiri, 2008).

3.2. Paper-disc diffusion(Kirby-Bauer) method:-

After extraction of the antimicrobial secondary metabolite by solvent, the antimicrobial activity was monitored by the paper-disc diffusion method as reported by Cooper, (1972). The appearance of a clear inhibition zone around the disc in the inoculated plates is an indication of the antimicrobial activity.

4. Molecular identification:-

Extraction of genomic DNA:-

Chromosomal DNAs were isolated by a versatile quick- prep method of genomic DNA from G-positive bacteria (*Streptomyces* spp.*)* according to Pospiech and Neumann, (1995) and Song *et al*.; (1999) with some modification. The strains were inoculated in 25.0 ml of the Dox's broth medium at temperature 28°C under agitation speed 200 rpm over night. The cells were harvested by centrifugation until suitable amount of pellet. Then 1.0 ml of set buffer 75 mM NaCl, 25 mM EDTA, 20 mM tris–HCl, pH 7.5 was added and then tubes were centrifuged at 12.000 g/5min. The pellets was resuspended in 100 µl of Set buffer, and then 60µl lysozyme were added to a concentration 1.0 mg/l, mixed by vortex and incubated at 37 $^{\circ}$C for 30-60 min and centrifuged to remove supernatant. Then, 600.0 µl of 10 % SDS were added and incubated at 80 $^{\circ}$C after 5 min. 3.0 µl RNase were added and tubes were incubated at 37 $^{\circ}$C for 30 min. 200.0 µl protein precipitation solution (PPS) were added and kept in ice for 5 min then centrifuged at 12.000 g/5min. The supernatant was

transferred to a new eppindurf tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1/4 volume iso-propanol with gentle inversation, then centrifuged at 12.000 g/5 min. The pellets of DNA were transferred to a new epindurf tube, rinsed with 70% ethanol, centrifuged at 12.000 g/5 min, then dried under vacuum and dissolved in a suitable volume (100.0 µl) of TE buffer (100.0 mM NaCl, 1.0 mM EDTA , 100.0 mM tris-HCl, pH 8.00) or distilled water and stored at 2-8 °C.

Polymerase chain reaction (PCR) amplification of genomic DNA:-

The polymerase chain reaction was performed using primers designed to amplify about 1000 bp fragment of the DNA region of *Streptomyces*. The forward primer was StrepB, 5'-ACAAGCCCTGGAAACGGG T-3' and the reverse primer was SterF 5'-ACGTGTGCAGCCCAAGACA-3' (Rintala *et al*.; 2001and 2002 and Suutari *et al*.; 2001).

Amplification reactions were performed in 50 µl polymerase buffer containing 30 pecomoles of each primer, long of chromosomal DNA, 200µM dNTPs and 2.5 units of Taq polymerase. PCR was carried out for 30 cycles at 94 ºC for 1 min, followed by 50 ºC for 1 min and 72 ºC for 2 min. after completion, a fraction of the Polymerase chain reaction mixture was examined using agrose gel electrophoreses (Asuuble *et al*.; 1999) and the remnant was purified using QIA quick PCR amplification reagent (Qiagen).

DNA sequencing:-

The PCR reaction products were sequenced directly using a big Dye terminator reagent kit including Taq polymerase according to the protocol recommended by the manufacturer (Model 3130 automated DNA sequencer, Genetic Analyzer, Applied Biosystems, Hitachi, Japan).

Phylogenetic analysis*:-*

Blast program (www.ncbi.nlm.gov/blast) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREEVIEW program (Page, 1996).The chromatogram was viewed by FinchTv program.

5. Structure elucidation of antimicrobial agent produced by *Streptomyces clavuligerus*

5.1. Ultraviolet spectrum (UV) of the antimicrobial compound:

The UV spectrum analysis of the purified antimicrobial compound was recorded with T80+UV/VIS Spectrometer, PG Instrument Ltd.(UK) Range: 190-1000 nm.

5.2. Infrared spectrum (IR) of the antimicrobial compound:

The IR spectrum of the purified antimicrobial compound was determined using a Fourier transform-infrared spectrophotometer (FTIR, Jasco 6100, Japan). The sample was ground with spectroscopic grade potassium bromide powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

5.3. Mass spectrum of the antimicrobial compound:

The mass spectrum analysis of the purified antimicrobial compound was recorded with Aglient (USA) mass spectrometer.

III. Results

1. Screening for the antimicrobial activity of Actinomycete isolates from different local soil sample:

As shown in table (1), results indicated that eleven Actinomycete strains were isolated from soil samples from different locations in Egypt. The isolates antimicrobial activity was tested against a wide range of test organisms (such as *Escherichia coli ATCC 25922, Staphylococcus aureus ATCC*

29213, and *Candida albicans ATCC 10231*). The most promising Actinomycete isolate that have the ability to produce antimicrobial metabolites against the above mentioned test organisms was encoded C10S19 which was isolated from the soil sample collected from a field of cabbage in Dakahlya .

Table 1: Screening for the antimicrobial activity of Actinomycete isolates from different local soil samples

Soil location	Isolate code	Antimicrobial activity of isolates against test organisms		
		E. coli	*S. aureus*	*C. albicans*
Saint Catherine rhizosphere of Caper	C1S16	+	+	-
El Azhar garden	C2S17	+	+	+
	C3S17	-	-	-
	C4S17	-	-	-
	C5S17	-	+	-
National research centre garden	C6S18	-	-	-
	C7S18	+	-	-
	C8S18	-	-	-
Field of cabbage at Dakahlya	C9S19	-	-	-
	C10S19	++	++	++
	C11S19	+	-	-

2. Molecular identification of the most potent *Streptomyces* sp.:

The resulted sequence was aligned with available, almost complete sequence of type strains of family *Streptomyces* and then with corresponding sequences of representative *Streptomyces* sp. In each case, the reference sequence was retrieved from the Gene Bank databases. The phylogenetic tree (diagram) revealed that the bacterial strain was closely related to *Streptomyces clavuligerus* with similarity matrix of 99%. (Fig. 1).

Finally, the phylogenetic analysis was performed after the alignment had been manually adjusted and verified with other related sequences then the phylogenetic tree was constructed by using neighbour-joining method in the MEGA program version 6. For data analysis, the phylogenetic trees of *Streptomyces clavuligerus* is presented in figure (1).

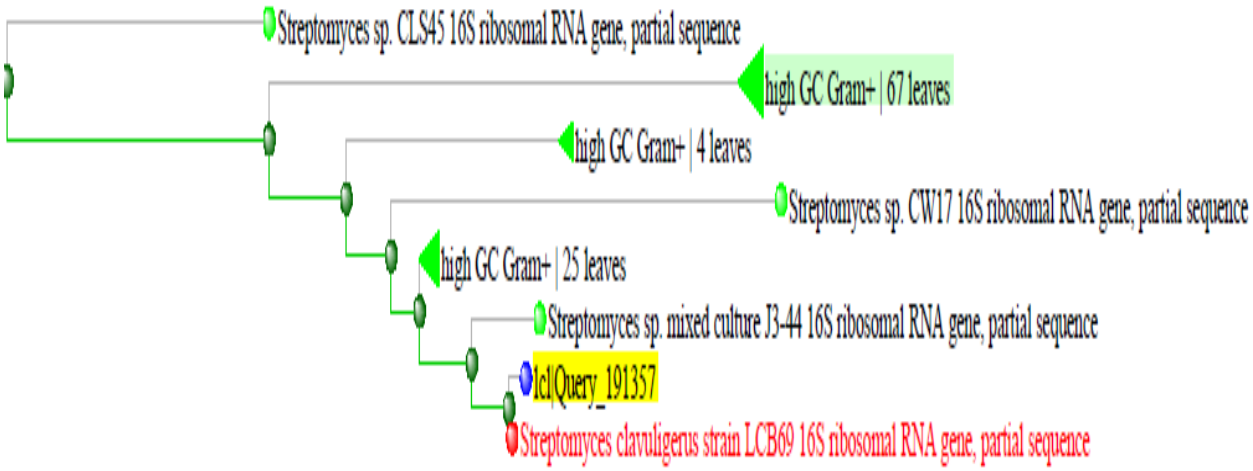


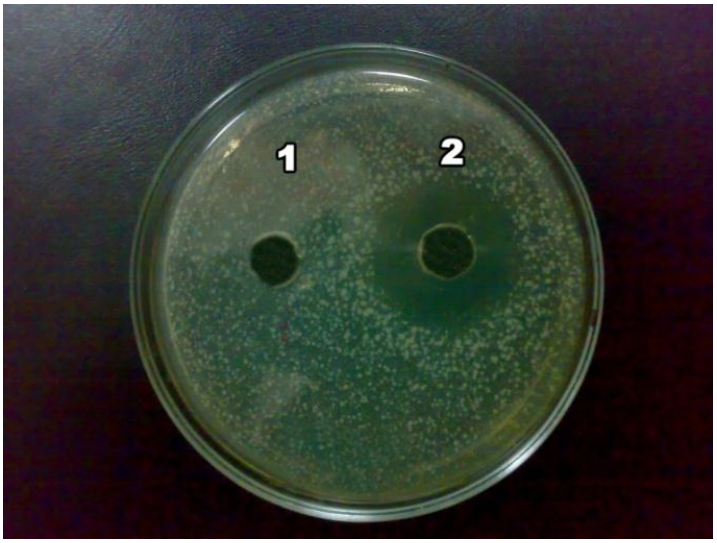
Fig. 1. The phylogenetic tree of local isolate *Streptomyces* spp.

3. Preparation of pure antimicrobial sample:

3.1. Production of antimicrobial compound from *Streptomyces clavuligerus*:

The fermentation process was carried out using liquid starch casein medium after incubation for 5 days at 30 °C .The culture was changing color from white to yellow at final pH 6.5, then filtered

through Whatman No. 1 filter paper and followed by centrifugation at 5000 rpm for 20 min .The clear filtrate was tested for activity against the test organisms and compared with a control (uninoculated culture medium).



1:control, 2:culture filterate

Fig. 2 Assay of culture filtrate against *Escherichia coli* ATCC 25922 as test organism and compared with control.

3.2. Extraction of antimicrobial compound from *Streptomyces clavuligerus* with organic solvents:

Various organic solvents were tested for the extraction of antimicrobial substance produced by the selected strain. It was observed that extraction with polar solvents such as methanol, di-ethyl ether and chloroform did not result in the removal of antimicrobial substance produced at the aqueous phase to the organic phase, while ethyl acetate extraction completely destroyed the antimicrobial substance activity. Antimicrobial substance was removed from the aqueous phase and recovered from the organic phase, then concentrated by rotary evaporator at 45°C , then assayed for its the antimicrobial activity using Paper-disc diffusion method and the inhibition zone (IZ) diameter was measured.

Table 2: Extraction of antimicrobial compound from *Streptomyces clavuligerus* with organic solvents

Organic solvent	Methanol,	Di-ethyl ether	Chloroform	Ethyl acetate
Diameter of inhibition zone(cm)	**0.0**	**0.0**	**1.3**	**2.7**

Paper-disc diameter = 0.6 cm

3.3. Purification of the antimicrobial compound produced by *Streptomyces clavuligerus*:

After extraction, the ethyl acetate extract tested with different TLC solvent systems such as ethyl acetate: hexane 1:1, ethyl acetate: methanol 1:1 and methanol: acetic acid 1:1. Results revealed that the best solvent system for separation was ethyl acetate: hexane 1:1. The purification of the active fraction from the culture broth of *Streptomyces sp* yielded 7 fractions from ethyl acetate extract as mentioned under material and method section and based on the complexity of each fraction. Fraction 5 was orange-yellow in color while fractions no 1, 2, 6 and 7 were faint in color. The activities of these fractions were assayed also against *Escherichia coli ATCC 25922* as a test organism.Fraction 5 exhibited the highest activity which is 2.0 cm diameter while in other fractions no inhibition was observed.

4. Structure elucidation of antimicrobial agent from *Streptomyces clavuligerus*:

4.1. Ultraviolet spectrum (UV):

The UV spectrum analysis of the purified antimicrobial compound was recorded with T80+UV/VIS Spectrometer, PG Instrument Ltd.(UK) Range: 190-1000 nm. The peaks at 201, 273 and 373 nm as presented in figure 3.

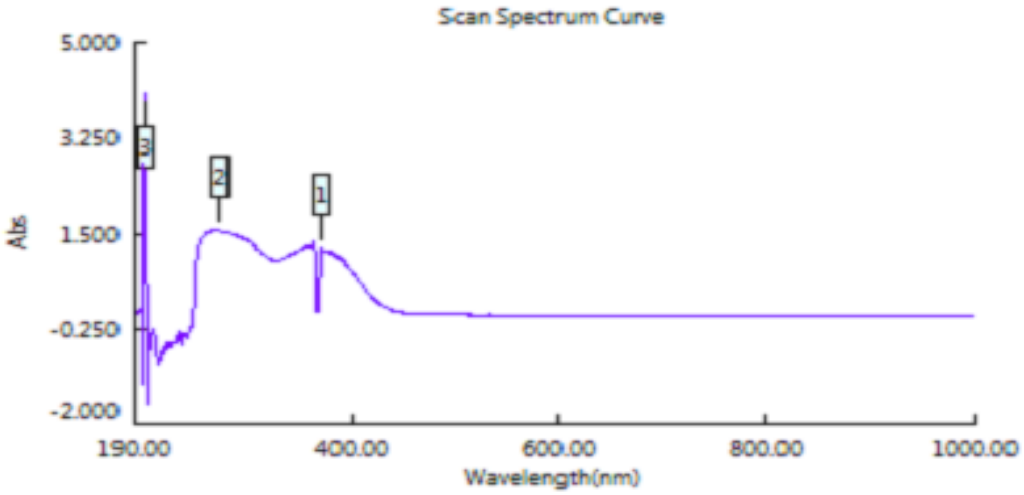


Fig. 3: Ultraviolet spectrum (UV) of the antimicrobial compound produced by *Streptomyces clavuligerus*

4.2. Infrared spectrum (IR):

The IR spectrum of the purified antimicrobial compound was determined using a Fourier transform-infrared spectrophotometer (FTIR, Jasco 6100, Japan). The sample was ground with spectroscopic grade potassium bromide powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

Comparing the IR spectrum data sheet of the sample and IR library of BIO-RAD showed that the tested sample contains alcohol group and carbonyl group of ester group. The IR chart as shown in fig. 4 showed absorbance band group R-CH_2OH at 1000-1075 indicated for C-O which is a primary alcohol, 1260-1350 indicated for OH and group R-CH_3 peaks from 1375-1380, 1435-1475, 2862-2882 and 2952-2972 indicated for C-H.

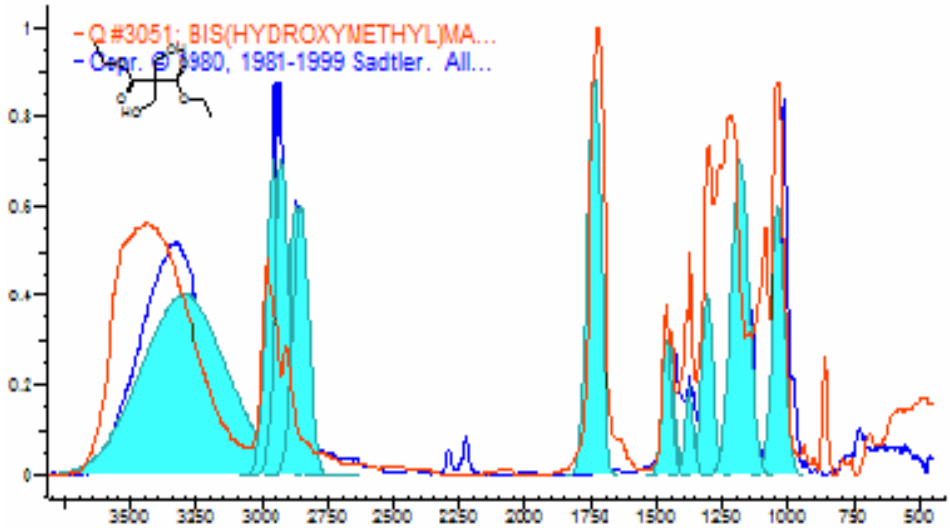


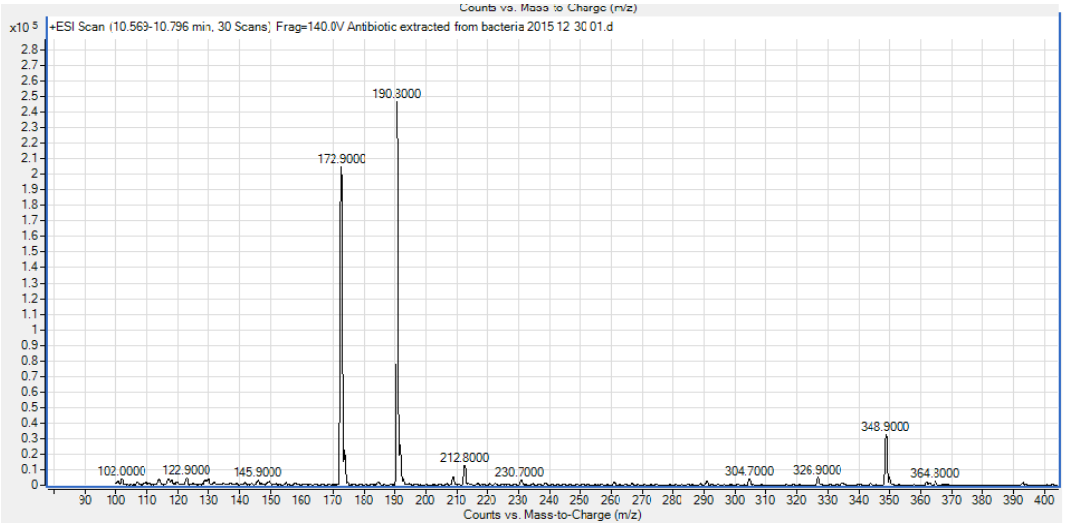
Fig. 4: Infrared spectrum (IR) of the antimicrobial compound produced by *Streptomyces clavuligerus*

4.3. Mass spectrum:

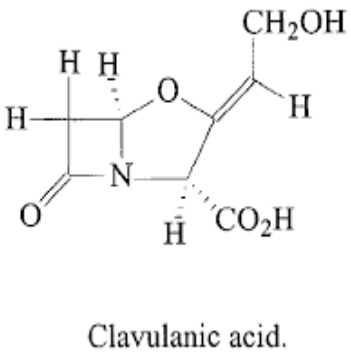
The mass spectrum analysis of the purified antimicrobial compound was recorded with Agilent Mass spectrometer (USA).

The mass spectra show clavulanic acid base peak at 218 m/z , clavulanic acid salt peak at 328 m/z, and finally clavulanic acid parent ion 190.8 m/z .Another peak at 212.8 (M^+) ,molecular weight of holomycin ,and peak at 172 m/z (M^+-CH_2CO) of holomycin.

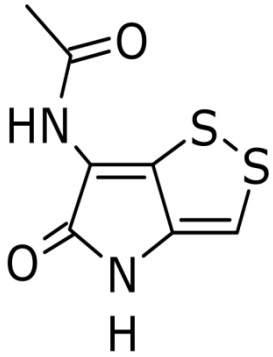
By performing preparative TLC extract ,sample had the same R_f ,when compared with the standard clavulanic acid.



The two secondary metabolites produced by *Streptomyces clavuligerus* are identified as shown in figure 6



Clavulanic acid.



Holomycin

Figure 6.The two identified secondary metabolites of *Sreptomyces clavuligerus.*

IV. Discussion

The main goal of the present research, was the isolation and characterization of antimicrobial agent producing microbes from diverse soil samples collected from different locations in Egypt. The results indicated that some of the studied isolates showed antagonistic effects against various tested organism ,especially the isolate encoded C10S19 isolated from the field of cabbage ,showed antimicrobial activity against wide range of tested organisms.

The soil microorganisms include various genera. Literature states that some common soil inhabitants are *Bacillus* sp. and *Streptomyces* sp. (Atlas and Bartha;1998) .The data indicated that *Streptomyces* sp. can be isolated from Egyptian soil. These species produce inhibitory substance of different nature. For example isolate encoded C10S19 was molecularly identified to be *Streptomyces clavuligerus* with similarity matrix of 99% and produces clavulanic acid and holomycin antimicrobials.

In the present study the main product produced by *Streptomyces clavuligerus* was clavulanic acid which is in agreement with several studies conducted by Atta and Yassen; 2014; Paradkar, 2013; Neto *et al*, 2005 and Fuente, *et al*., 2002 **.** The biosynthetic gene cluster of holomycin has been identified in *S. clavuligerus* and characterized biochemically and genetically according to Kenig and Reading, 1979 and Qin *et al*, 2013. The active metabolites were extracted by ethyl acetate at PH 7 with ratio 1:1 v/v. The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The crude extract was dissolved in ethyl acetate .The same method was used by Atta and Yassen,(2014) with slight difference which is the solvents used for extraction. Atta and Yassen collected the samples from Taif city, Kingdom of Saudi Arabia. They molecularly identified the *Streptomyces clavuligerus* with 98% similarity matrix with a different phylogenetic tree than that obtained in this study .Different geographical distribution of the isolated strains may explain the slight difference in the results obtained. Another difference is that Atta and Yassen extracted the antimicrobial agent using n-butanol while ethyl acetate was the organic solvent used in this research. Also they used a mixture of n-butanol:acetic acid:water in the ratio 3:1:1 whereas we used ethyl acetate :hexane mixture in the ratio 1:1 .This mixture was the most polar which caused significant separation of components into clear separate bands. An explanation of this is that our extract was partially purified using preparative TLC and was found to contain a mixture of two active metabolites ;clavulanic acid and Holomycin.The structure elucidation of these components was proved by using different spectrophotometric techniques. The absorbance bands obtained by UV and the functional groups illustrated in the IR chart show two estimated compounds of expected formula approximately C_{21} H_{25} N O_{10} S and C_{16} H_{23} N O_9.The mass spectrum showed clavulanic acid base peak at 218 m/z , clavulanic acid salt peak at 328 m/z, and finally clavulanic acid parent ion which appears at 190.8 m/z beside a peak of 212.8 (M^+) molecular weight of holomycin and peak at 172 m/z (M^+-CH_2CO) of holomycin .The clavulanic acid parent ion was supposed to be at 198 m/z. Thatis s why a further checking step was performed to prove that it is clavulanic acid by performing preparative TLC. The sample produced the same R_f ,when compared with the standard clavulanic acid. Clavulanic acid and Holomycin were mentioned together in literature before which is in concordance with our results. Mutants of *Streptomyces clavuligerus* with disruption in different genes for clavulanic acid biosynthesis produce large amount of holomycin which explains the production of dark yellow color ,most probably that of holomycin, on the fourth day of incubation. A possible cross regulation of two unrelated secondary metabolic pathways according to (Fuente *et al.*, 2002).

Currently the incidence of multidrug resistant organisms is increasing and compromising the treatment of growing number of infectious diseases. Many of these pathogenic organisms produce β lactamase enzyme capable of destructing β lactam ring in antibiotics like penicillins and cephalosporins.This explains why clavulanic acid became an important drug used altogether with amoxacillin for the required synergistic effect. Also Holomycin a member of dithiolopyrrolone natural products containing a unique heterobicyclic scaffold with an N-alkyl and an N-acyl substitution. Holomycin appeared to be active against rifamycin-resistant bacteria and also to inhibit the growth of the clinical pathogen methicillin-resistant *Staphylococcus aureus*. Holomycin also displays potent anticancer activities. Holomycin might be a lead molecule for the production of new hybrid compounds with higher activity and lower toxicity (Qin *et al.*,2013) .

Clavulanic acid and holomycin can be produced industrially from cultures of *Streptomyces clavuligerus*. The soil sample collected from the field of cabbage in Dakahleya is a promising soil sample for isolation of *Streptomyces clavuligerus* and therefore for the production of important therapeutic antimicrobial agents such as clavulanic acid and holomycin.

V. Acknowledgement.

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