Cirramycin-B Antibiotic Production By *Streptomyces Cyaneus*-AZ-13Zc: Fermentation, Purification and Biological Activities

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Abstract: This work was carried out biosynthesis for specific of the bioactive substances that demonstrated inhibitory affects against microbial pathogenic, from *Streptomyces cyaneus,* AZ-13Zc. The active metabolite was extracted using ethyl acetate (1:1, v/v) at pH 7.0. The separation of the active ingredient and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antimicrobial agent have been investigated. This analysis indicates a suggested imperical formula of $C_{36}H_{59}NO_{12}$. The minimum inhibition concentrations "MICs" of the purified antimicrobial agent were also determined. The purified antimicrobial agent was suggestive of being belonging to 16-membered Macrolide group (Cirramycin-B antibiotic) produced by *Streptomyces cyaneus,* AZ-13Zc.
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1. Introduction

The genus *Streptomyces* are remarkable, and merit special consideration with regard to the morphological and metabolic differentiation phenomena they manifest during later stages of development (El-Naggar, 2007). *Streptomyces* species generally synthesize a sizeable number of diverse natural secondary metabolites, the best known of which are antibiotics currently used worldwide as veterinary and pharmaceutical industry (Saadoun and Gharaibeh, 2003). The macrolides are a group of antibiotics whose activity stems from the presence of a macrolide ring, a large macrocyclic lactone ring to which one or more deoxy sugars, usually cladinose and desosamine, may be attached. Macrolides belong to the polyketide class of natural products (Omura *etal.,* 2002). The chemical structure of these antibiotics was characterized by a ring consisting of not fewer than 12 carbon atoms and closed condensation of a number of acetate and propionate provided an alternative source of n-butyrate 2- methylmalonate and propionate building unites of the aglycones of the macrolide antibiotics monesin (Sathi *etal.,* 2001) The mechanism of action of the macrolides is inhibition of bacterial protein biosynthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl tRNA (Zhang *etal.,* 2007). This action is mainly bacteriostatic, but can also be bactericidal in high concentrations. Macrolides tend to accumulate within leukocytes, and are therefore actually transported into the site of infection (Selvin *etal.,* 2004). The clinically useful macrolide antibiotics could be conveniently classified into three groups based on the number of atoms in the lactone nucleus. Erythromycins A, B, C, D, E and F, oleandomycin, roxithromycin, dirithromycin, clarithromycin and flurithromycin are 14-membered macrolides whereas azithromycin is a 15-membered compound. 16-Membered macrolides include josamycin, rosaramicin, rokitamycin, kitasamycin, mirosamicin, spiramycin, cirramycin and tylosin (Min and Chang-Qin, 2005). The 16-membered macrolide antibiotics may be divided into three classes depending on the number and position of carbonyl groups in the molecule. The first class represented by leucomycins, spiramycins, possesses an aldehyde group attached to the six position of the ring structure through a methylene group. The second class includes the magnamycins, cirramycin, tylosin, and is similarly characterized by the aldehyde group attached to carbon 6 through a methylene group but also by a carbonyl group in position 9. The antibiotics of the third class, illustrated by chalcomycin and neutramycin, have only one carbonyl group, the keto function at position 9 (Nakagawa *et al.,* 1972). The cirramycin-B is active against Gram positive and Gram negative bacteria. The cirramycin-B has molecular weight 697.8 and empirical formula $C_{36}H_{59}NO_{12}$, and (U.V) absorption spectrum was recorded at 240 nm. The presence of an aldehyde group is apparent from the NMR spectrum

at 9.88 ppm, and positive reaction with molish's and fehling reactions (Min and Chang-Qin, 2005).

In the present study, the production of the bioactive substances that demonstrated inhibitory affects against microbial pathogenic, from *Streptomyces cyaneus,* AZ-13Zc were reported, along with some physico-chemical properties of secondary metabolites with high biological activities.

2. Material and Methods

2.1. Test organisms

A. Bacteria

Staphylococcus aureus, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Micrococcus luteus*, ATCC 9341.*Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145 and *Salmonella typhi* NCIMB 9331.

B. Unicellular Fungi

Candida albicans, IMRU 3669**.**

2.2. Scaling up the optimal production of the antimicrobial agents by fermentation

Streptomyces cyaneus, AZ-13Zc was inoculated into 250 ml Erlenmeyer flasks containing 50 ml of liquid starch nitrate medium and incubated at 30ºC and 250 rpm on a rotary shaker. After 3 day this first- stage was transferred to 500 ml seed medium in a 2L conical flask and incubated under the same conditions for another 3 days of the production medium and the second stage was used as the inoculum for fermentation in 5L fermentor. The pH was adjusted at 7.0. The temperature was adjusted at 30ºC, the agitation at 250 rpm and aeration rate at 1vvm. Foam was suppressed by sterile sunflower oil (El-Tayeb *et al.,* 2004). Samples were taken every 4 hr.

2.3. Fermentation

Twenty-liter total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 r.p.m for 20 minutes. The clear filtrates were tested for their activities against the test organisms (Sathi *etal.,* 2001).

2.4. Extraction

The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator (Atta, 2010).

2.5. Precipitation

The precipitation process of the crude compound dissolved in the least amount of the solvent carried out using petroleum ether (b.p 60-80 °C) followed by centrifugation at 5000 r.p.m for 15 min. The precipitate was tested for its antibacterial activities (Zhang *etal.,* 2007).

2.6. Separation

Separation of the antimicrobial agent(s) into its individual components was conducted by thin layer chromatography using chloroform and methanol (24:1, v/v) as a solvent system (Atta *et al.,* 2009).

2.7. Purification

The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform and Methanol 8**:**2 (v/v), was used as an eluting solvent. The column was left for overnight until the silica gel (Prolabo) was completely settled. One-ml crude precipitate to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities (Atta *et al.,* 2009).

2.8. Physico-chemical properties of the antimicrobial agent

2.8.1. Elemental analysis

The elemental analysis C, H, O, N, and S was carried out at the micro analytical center, Cairo University, Egypt.

2.8.2. Spectroscopic analysis

The IR, UV, Mass spectrum, and NMR spectrum were determined at the micro analytical center of Cairo University, Egypt.

2.8.3. Reaction of the antimicrobial agent with certain chemical test

For this purpose, the following reactions were carried out: Molish's, Fehling, Sakaguchi, Ninhydrin, Ehrlish, Nitroprusside, Ferric chloride, and Mayer reactions (Atta *et al.,* 2009).

2.8.4. Biological activity

The minimum inhibitory concentration (MIC) could be determined by the cup assay method (Kavanagh, 1972).

2.8.5. Characterization of the antimicrobial agent

The antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc was identified according to the recommended international

references of (Umezawa, 1977; Berdy, 1974; Berdy, 1980a, b & c).

3. Results

3.1. Scaling up of the optimal production of antimicrobial agent using fermentation

The production of the antimicrobial agent from *Streptomyces cyaneus*, AZ- 13Zc was carried out in a 5L fermentor. The pH was adjusted at pH 7; temperature was adjusted at 30°C, and agitation at 250 rpm and aeration rate at 1vvm. Samples were taken every 4 hr. The activity of antimicrobial agent produced by *Streptomyces cyaneus*, AZ- 13Zc exhibited an increase in comparison to that produced in shake flasks. Results during fed-batch indicated that the dissolved oxygen concentration dropped gradually to about zero After 36 h. Also it was worthy to mention that the pH dropped to about 6.8 and was increased gradually again after 12 h. The activity of the antimicrobial agent production was began after 12 h. and increased until reached the maximum after 56 h incubation.

3.2. Fermentation and Separation of the antimicrobial agent

The fermentation process was carried out for three days at 30°C using liquid starch nitrate medium as production medium. Filtration was conducted followed by centrifugation at 5000 r.p.m. for 15 minutes. The clear filtrates containing the active metabolite (20 liters), was adjusted to pH 7.0 then the extraction process was carried out using Ethyl acetate at the level of 1:1 (v/v). The organic phase was collected, and evaporated under reduced pressure using rotary evaporator. The residual material was dissolved in the least amount of DMSO and filtered. The filtrates were test for their antimicrobial activities. The antimicrobial agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 4000 r.p.m for 15 minute where a brown powdered precipitate could be obtained. Separation of the antimicrobial agent(s) into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Among three bands developed, only one band at R_f 0.4 showed antimicrobial activity. The purification process through column chromatography packed with silica gel indicated that the most active fractions against the tested organisms ranged between 4 to 20 Fig. (1).

3.3. Physicochemical characteristics of the antimicrobial agent

The purified antimicrobial agent produces characteristic odour, their melting point is 228°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexane and benzene.

3.4. Elemental analysis

The elemental analytical data of the antimicrobial agent(s) revealed the following: **C**=61.59; **H**=8.5; **N**= 2.02., **O** = 27.89 and **S**=0.0**.** This analysis indicates a suggested imperical formula of $C_{36}H_{59}NO_{12}$

3.5. Spectroscopic characteristics

The infrared (IR) spectrum of the antimicrobial agent showed characteristic band corresponding to 13 peaks (Fig.2).The ultraviolet (UV) absorption spectrum of the antimicrobial agent recorded a maximum absorption peak at 240 nm (Fig. 3). The Mass spectrum revealed that the molecular weight is 697.4 (Fig. 4). The NMR-Spectrum could be also determined (Fig.5) It absorbs strongly in the ultraviolet region at 240 which is attributable to a conjugated carbonyl grouping. The infrared absorption bands are also indicative of the unsaturated carbonyl system in the agent. The presence of an aldehyde group is apparent from the NMR spectrum at 9.88 ppm.

3.6. Biochemical reaction of the antimicrobial agent

The reactions revealed the detection of certain groups in the investigated agent. The antimicrobial agent exhibited positive results with Molish's, Fehling and Mayer tests and negative results with nitroprusside, ninhydrin, ferric chloride, Sakaguchi, and Ehrlish reactions (Table 1).

Table 1, Summarizes the response of the antimicrobial agent to certain biochemical reactions.

Chemical test	Result	Remark
Molish's reaction	+	Present of sugar moiety
Fehling test	+	Present of free aldehyde or keto sugar
Ninhydrin test	-	Absence of free-NH_2 group
Sakaguchi reaction	-	Arginin is Absence
Nitroprusside reaction	-	Absence of Sulfur
Ferric chloride reaction	-	Absent of Di-ketons group
Ehrlish rection	-	Absence of indolic acid
Mayer reaction	+	Presence of nitro group

3.7. Biological activities of the antimicrobial agent

Data of the antimicrobial agent spectrum indicated that the agent is active against Gram-positive and Gram-negative bacterial and unicellular fungi (Table 2).

3.8. Identification of the antimicrobial agent

On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antimicrobial agent, it could be stated that the antimicrobial agent is suggestive of being belonging to Macrolide group (Cirramycin-B antibiotic) (Table 3).

Table 2, Antimicrobial spectrum of the agent by using paper disc diffusion method.

Test organisms		MIC (ug/ml) concentration
A- Bacteria		
a. Gram positive cocci		
Staphylococus. aureus	NCTC 7447	1.95
Micrococcus luteus	ATCC 9341	0.97
b. Gram positive bacilli		
Bacillus subtilis	NCTC 10400	0.97
c. Gram negative bacteria		
Escherichia coli	NCTC 10416	3.9
Salmonella typhi	NCIMB 9331	3.9
Klebsiella pneumonia	NCTC 9111	16.62
Pseudomonas aeruginosa	ATCC 10415	31.25
B- Fungi		
a- unicellular fungi		
Candida albicans	IMRU 3669	62.5

Table 3, A comparative study of the characteristic properties of the antimicrobial agent in relation to Reference antibiotic (Cirramycin-B).

Characteristic	Purified antibiotic	Cirramycin-B
1- Melting point	228°C	228-229°C
2- Molecular weight	697.4	697.4
3- Chemical analysis:		
C	61.59	61.57
H	8.50	8.40
N	2.02	2.01
O	27.89	28.02
S	0.0	0.0
4- Ultra violet	240	240
5- Formula	$C_{36}H_{59}NO_{12}$	$C_{36}H_{59}NO_{12}$

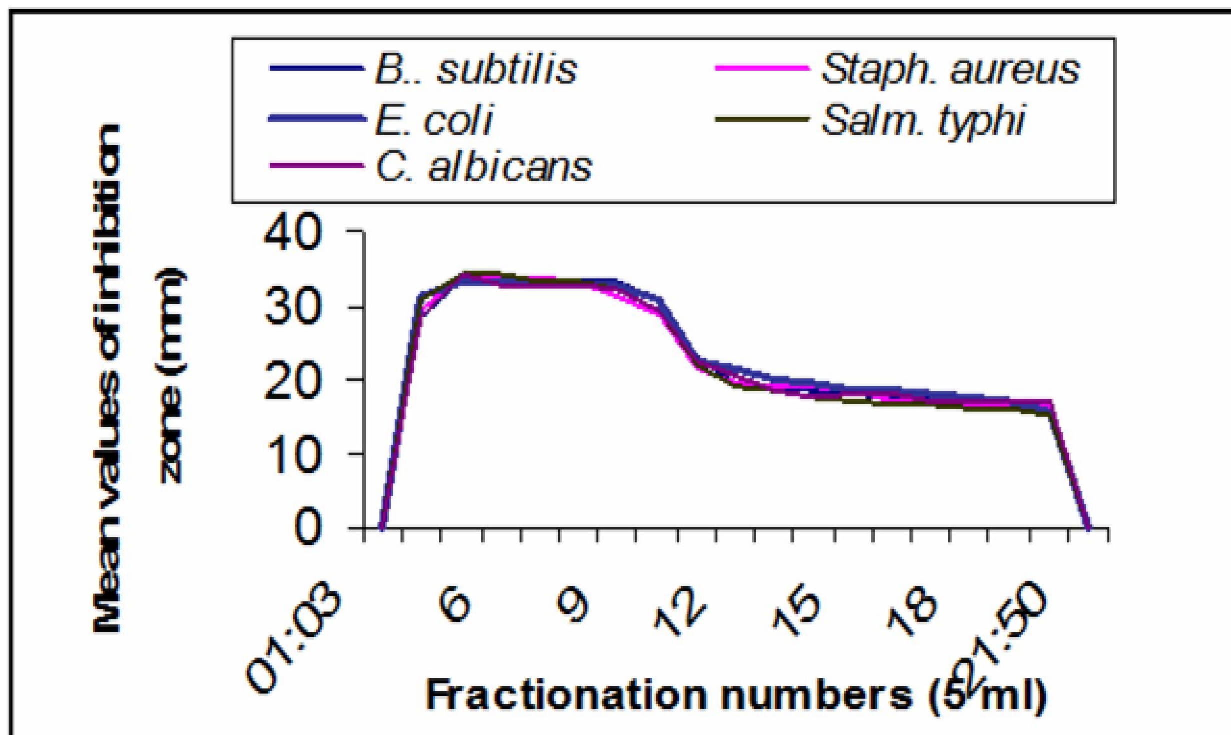

Figure 1. Antimicrobial activity of fractions obtained using silica gel column chromatography technique for antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc.

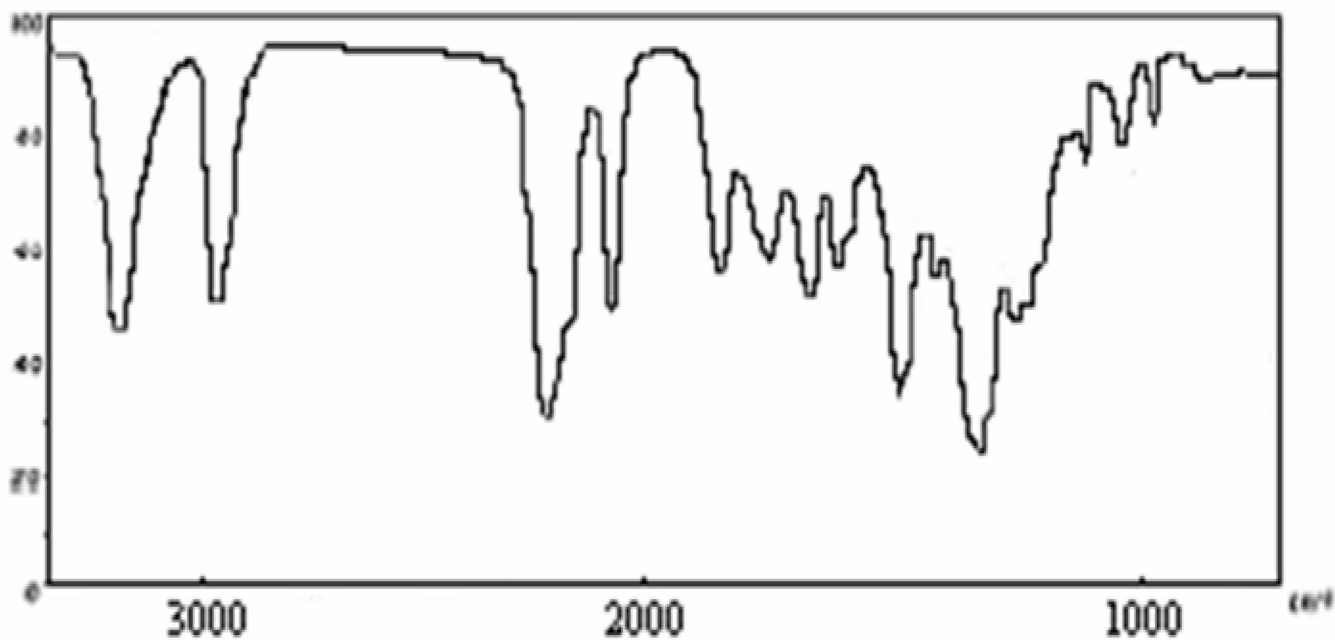

Figure 2. I.R spectrum of antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc.

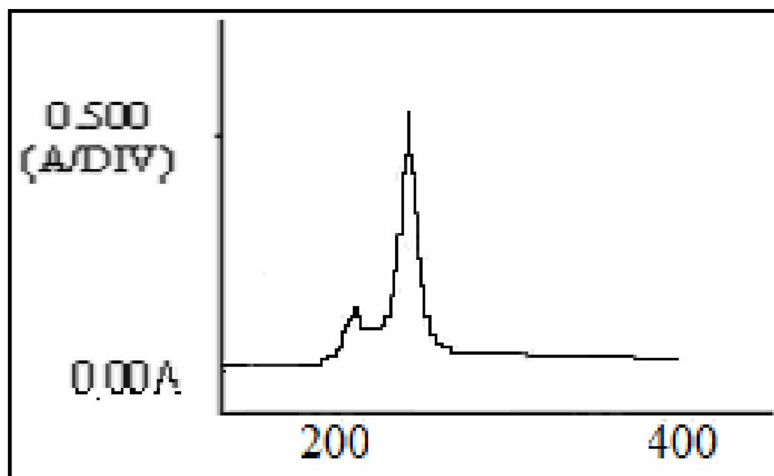

Figure 3. Ultraviolet absorbance of antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc.

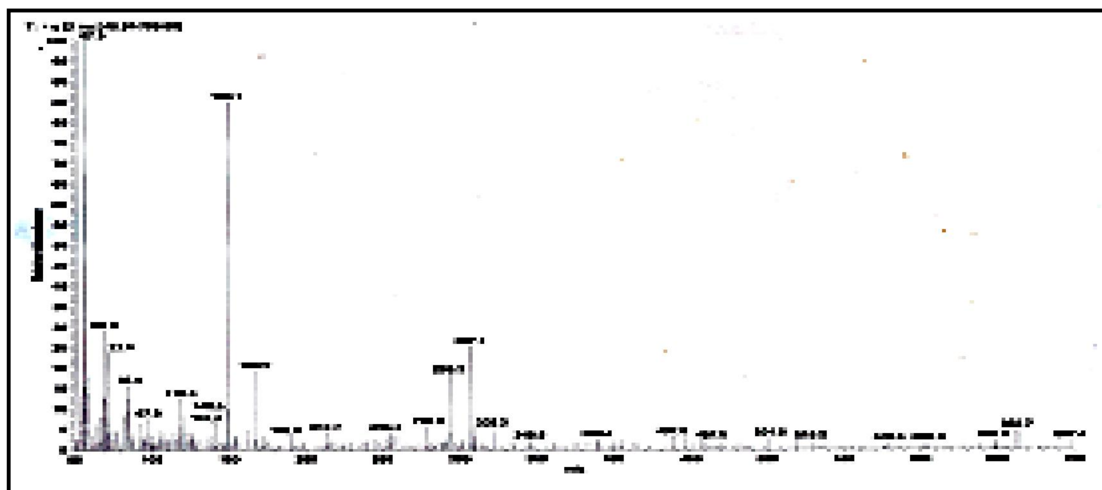

Figure 4. Mass-Spectrum of antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc.

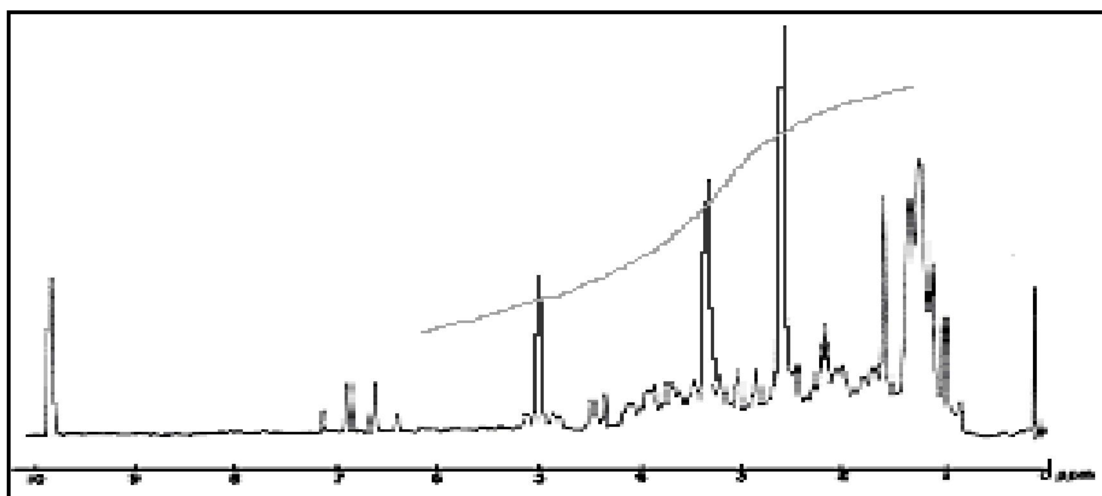

Figure 5. NMR-Spectrum of antimicrobial agent produced by *Streptomyces cyaneus*, AZ-13Zc.

4. Discussions

The active metabolites were extracted by ethyl acetate at pH 7. Similar results were obtained by (Criswell *et al.,* 2006 and Sekiguchi *et al.,* 2007). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process, where only one active fraction was obtained in the form of brown powder. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of chloroform and methanol (9:1, v/v), indicated that fractions activities was recorded from fraction Nos. 4 and 20. Many workers used a column chromatography packed with silica gel. Similar results were obtained by (Hitchens and Kell, 2003). The physico-chemical characteristics of the purified antibiotic revealed that, their melting point is 228 °C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexane and benzene. Similar results were recorded by (Yanai, 2004 and Yoram *et al.,* 2006). A study of the elemental analysis of the antibacterial agent lead to an imperical formula of: $C_{36}H_{59}NO_{12}$. The spectroscopic characteristics of the antimicrobial agent under study revealed the presence of a maximum absorption peak in UV. at 240 nm, infra-red absorption spectrum represented by 11 peaks. The Mass spectrum revealed that the molecular weight is 697.4 and NMR-spectrum was determined, which is attributable to a conjugated carbonyl grouping. The infrared absorption bands are also indicative of the unsaturated carbonyl system in the agent. The presence of an aldehyde group is apparent from the NMR spectrum at 9.88 ppm. The biochemical tests of the antimicrobial agent gave positive reaction with Molish's, Fehling and Mayer reactions. Similar results were recorded by (Pamboukian and Facciotti, 2004).

The MIC of antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against *Bacillus subtilis,* NCTC 10400 and *Micrococcus luteus* ATCC 9341, was 0.97 µg / ml, *Staphylococcus aureus,* was 1.95 µg / ml, *Escherichia coli* NCTC 10416, *Salmonella typhi* NCIMB 9331, was 3.9 µg/ ml, *Klebsiella pneumonia* NCTC 9111 was 16.623.9 µg/ ml, *Pseudomonas aeruginosa* ATCC 10415 was 31.25 µg/ ml and for *Candida albicans* IMRU 3669 was 62.5 µg/ ml. similar investigations and results were attained by (Khalifa, 2008).

Identification of the antimicrobial agent according to recommended international keys indicated that the antibiotic is suggestive of being likely belonging to Macrolide group (Cirramycin-B antibiotic) (Umezawa, 1977; Berdy, 1974; Berdy, 1980a, b & c).

5. Conclusion

It could be concluded that: The Cirramycin-B antibiotic produced by *Streptomyces cyaneus*, AZ-13Zc demonstrated obvious inhibitory affects against pathogenic microorganisms (Gram positive and Gram negative bacteria and unicellular fungi).

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