Production of Hygromycin-B antibiotic from *Streptomyces crystallinus*, AZ-A151: III. Fermentation, Extraction, Physicochemical Analysis and Biological Activities

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Abstract: This work was carried out for the biosynthesis of antimicrobial substance that demonstrated inhibitory effects against microbial pathogenic from *Streptomyces crystallinus,* AZ-A151. The active metabolite was extracted using ethyl acetate (1:1, v/v) at pH 8.0. The separation of the active ingredient of the antimicrobial agent and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis, spectroscopic characteristics and chemical reactions have been investigated. This analysis indicates a suggested imperical formula of $C_{15}H_{30}N_2O_{10}$. The minimum inhibition concentrations "MICs" of the purified antimicrobial agent were also determined. The purified antimicrobial agent was suggestive of being belonging to Hygromycin-B, antibiotic produced by *Streptomyces crystallinus,* AZ-A151.
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1. Introduction

Hygromycin B is an antibiotic produced by the bacterium *Streptomyces hygroscopicus*, a bacterium isolated in 1953 from a soil sample [McGuire, 1953]. The bacterial genus *Streptomyces* is a high G + C content, Gram-positive filamentous soil bacteria with a complex life cycle that includes morphological differentiation and spore formation [Del-Sol *et al.,* 2007]. Also, it has long been appreciated as a rich source for the production of various secondary metabolites including many pharmaceutically valuable compounds such as antibiotics, anti-cancer agents, immunosuppressant's and enzyme inhibitors [Myles, 2003 and Hindra and Elliot, 2010]. PCR-based methods have provided a rapid and accurate way to identify bacteria [Kohler *et al*., 1991; Beyazova and Lechevalier, 1993; Telenti *et al*., 1993; Mehling *et al*., 1995; Wilson *et al*., 1998 and Laurent *et al*., 1999]. In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful [Harvey *et al*., 2001 and Alves *et al*., 2002]. Moreover, PCR amplification, sequence of 16S rRNA gene, and phylogenetic analyses were performed too [Monciardini *et al.*, 2002].

It is well documented that the biosynthesis *Streptomyces* secondary metabolites is typically regulated via multiple regulatory pathways operating with several layers of complicated control systems [Lee *et al.,* 2005 and Chen *et al.,* 2010]. The comparison of rDNA sequences is a particularly powerful tool in streptomycete taxonomy. In addition, rDNA sequence comparisons have also been useful for answering questions concerning the horizontal transfer of genes within the genus *Streptomyces* [Huddleston *et al*., 1997].

In other report, the crude antibiotic was tested for number of components present by using precoated thin-layer chromatography (TLC) plates using ethanol: water: chloroform (40:40:20) solvent system. Also, purification of the antibiotic was carried out by column chromatography using silica gel [Augustine *et al*., 2005].

The actinomycins are characterized using a variety of analytical methods including ultraviolet visible spectroscopy system, infrared (IR), electrospray ionization mass spectrometry (ESI–MS) and tandem mass spectrometry (MS–MS) as well as various NMR techniques [Singh and Gurusiddaiah, 1984 and Kurosawa *et al*., 2006].

The absorption spectrum of active extracts in methanol were recorded in the UV region (210-400 nm) by using a UV-vis spectrophotometer (Cintra 40) and compared with those of known polyenic antifungal antibiotics [Thakur *et al*., 2007]. The selective production of hygromycin analogs by the Δhyg6 and Δhyg7 mutants has provided an opportunity to probe the importance of a methyl or methylene group on the aminocylitol ring for both in

vitro protein synthesis inhibitory activity and antibacterial activity [Palaniappan *et al.,* 2009].

In the present study, the production of the bioactive substances that demonstrated inhibitory affects against microbial pathogenic, from *Streptomyces crystallinus,* AZ-A151 were reported. The bioactive substance was extraction, purification, elemental and spectroscopic analysis and biological activities were determined.

2. Material and Methods

2.2. Test organisms

2.2.1. Gram Positive: *Staphylococcus aureus*, NCTC 7447.

2.2.2. Gram Negative: *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Salmonella typhi*.

2.2.3. Unicellular fungi: *Saccharomyces cerevisiae,* ATCC 9763.

2.2.4. Filamentous fungi: *Aspergillus flavus*, IMI 111023 and *Alternaria alternata*.

2.3. Fermentation

A loopful of the, *Streptomyces crystallinus,* AZ-A151 from the 6-day culture age was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of liquid starch nitrate medium (seven flasks). The flasks were incubated on a rotary shaker (200 rpm) at 35 ^{0}C for 10 days.

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 *et al.,* 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 antimicrobial activities [Atta *et al.,* 2009].

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 10**:**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 and Mass spectrum, were determined at the micro analytical center of Cairo University, Egypt.

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 crystallinus,* AZ-A151 was identified according to the recommended international references of [Berdy, 1974; Berdy, 1980a b & c; Umezawa, 1977 and Umezawa and Hooper 1982].

3. Results

3.1. Fermentation and Isolation:

The *Streptomyces crystallinus*, AZ-A151 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g/l): Arabinose, 20; $NaNO_3$, 2.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.7; vitamin H, p.p.m and KCl, 0.5. The pH was adjusted at 8.0 before sterilization. After 10 days of incubation at 35 ^{O}C filtration was carried out through filter paper Whatman No. 1 and followed by centrifugation at 5000 rpm for 15 minutes. Only clear filtrates were tested for their antimicrobial activities.

The clear filtrates containing the active metabolite, was adjusted to pH 8.0 then 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 least amount of DMSO and filtered. The filtrates were test for their antibacterial activities.

The antimicrobial agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 4000 r.p.m for 15 minute. The fraction was test for antimicrobial activities.

Separation of antimicrobial agent 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 0.55 showed antibacterial activity. The purification process through column chromatography packed with silica gel, indicated that maximum activity was at fractions Nos. 14 to 23 (Fig. 1)

3.2. Physicochemical characteristics of the antimicrobial agent:

The purified antimicrobial agent produced by *Streptomyces crystallinus*, AZ-A151 produces characteristic odour, its melting points is 160 °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.

3.3. Elemental analysis:

The elemental analytical data of the antibacterial agent revealed the following data: **C**=46.43; **H**=7.46; **N**= 6.81; **O** = 39.43 and **S=0.0**. This analysis indicates a suggested calculated imperical formula of $C_{15}H_{30}N_2O_{10}$.

3.4. Spectroscopic characteristics:

The spectroscopic analysis of purified antimicrobial agent produced by *Streptomyces crystallinus,* AZ-A151, the infrared (IR) spectrum showed characteristic band corresponding to 21 peaks (Fig. 2). The ultraviolet (UV) spectrum recorded a maximum absorption peak at 225 nm (Fig. 3). The **Mass spectrum** showed that the molecular weight was 432.36 (Fig. 4).

3.5. Biological activities of the antimicrobial agent:

Data recorded in Table (1) indicated that the antimicrobial agent is fairly active against both Gram positive, Gram negative bacteria, unicellular and filamentous fungi. The minimum inhibitory concentration (MIC) of antibiotic produced by *Streptomyces crystallinus,* AZ-A151 was determined and results showed that MIC (µg/ml) against *Staphylococcus aureus*, NCTC 7447 (1.73), *Klebsiella pneumonia*, NCIMB 9111 (3.9), *Escherichia coli*, NCTC 10416 and *Salmonella typhi* (7.8)*,* for *Aspergillus flavus*, IMI 111023 and *Alternaria alternata* (31.25), and *Saccharomyces cerevisiae,* ATCC 9763 (62.5).

3.6. 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 Hygromycin-B antibiotic (Table 2).

Table 1. Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by *Streptomyces crystallinus,* AZ- A151 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).

Test organisms	MIC (ug/ml) concentration
	Antimicrobial agent produced by AZ-A151
***Staphylococcus aureus*, NCTC 7447**	**1.73**
Escherichia coli	**7.8**
***Klebsiella pneumonia*, NCIMB 9111**	**3.9**
Salmonella typhi	**7.8**
Saccharomyces cerevisiae	**62.5**
Aspergillus flavus	**31.25**
Alternaria alternata	**31.25**

Table 2. A comparative study of the characteristic properties of AZ- A151 antibiotic in relation to Reference Hygromycin-B antibiotic

Characteristic	Purified antimicrobial agent	Hygromycin-B
1- **Melting point**	160°C	160-180°C
2- **Molecular weight**	432.36	436
3- **Chemical analysis:**		
C	46.43	46.40
H	7.46	7.48
N	6.81	6.82
O	39. 3	39. 3
4- **Ultra violet**	225	No characteristic absorbance
5- **Formula**	$C_{15}H_{30}N_2O_{10}$	$C_{15}H_{30}N_2O_{10}$
6- **Active against**	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi.	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi.

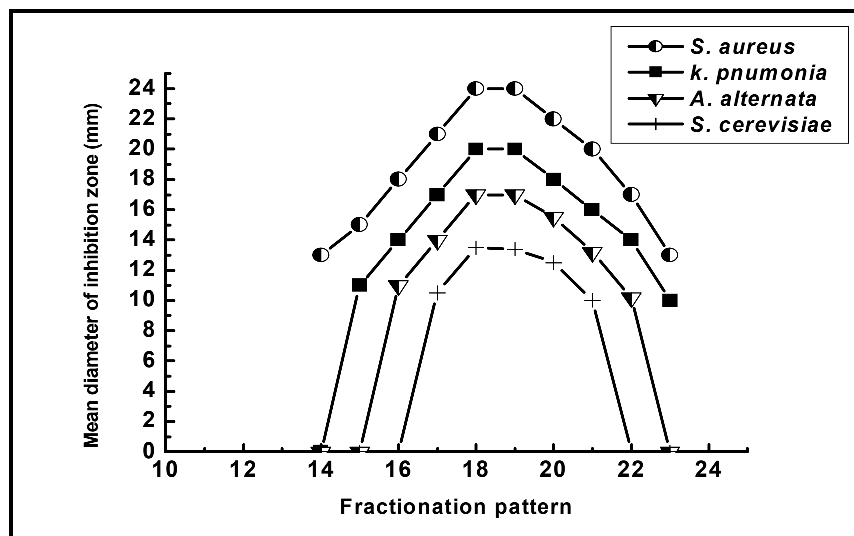

Figure 1. Fractionation pattern produced by *Streptomyces crystallinus*, AZ-A151.

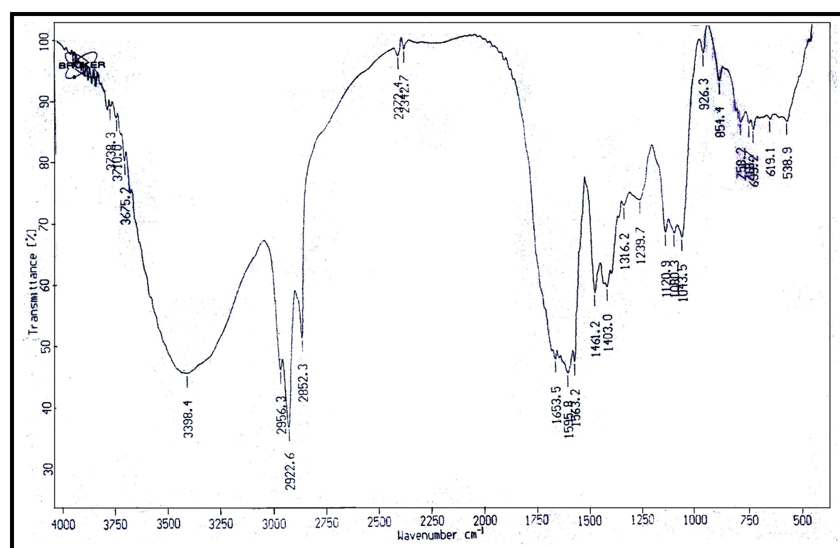

Figure 2: Infrared spectrum of the antimicrobial agent.

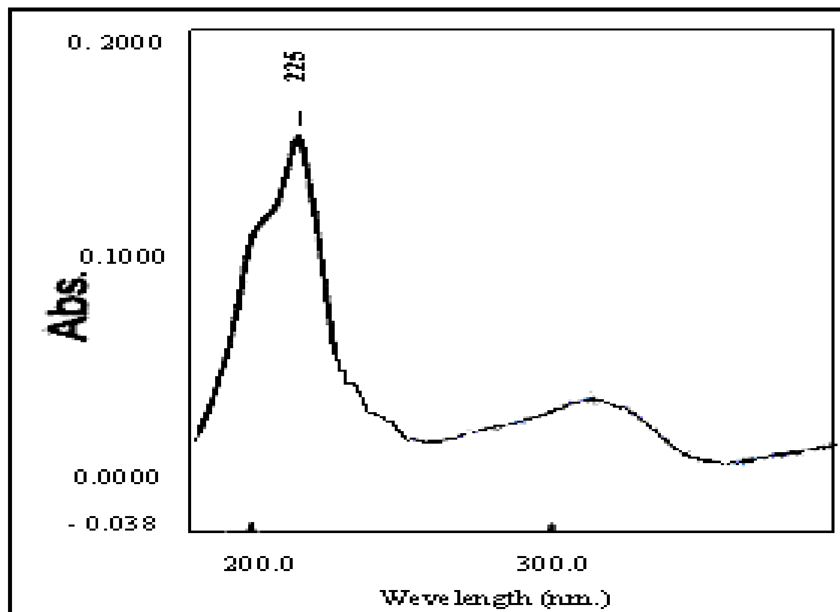

Figure 3. Ultraviolet absorbance of antimicrobial agent

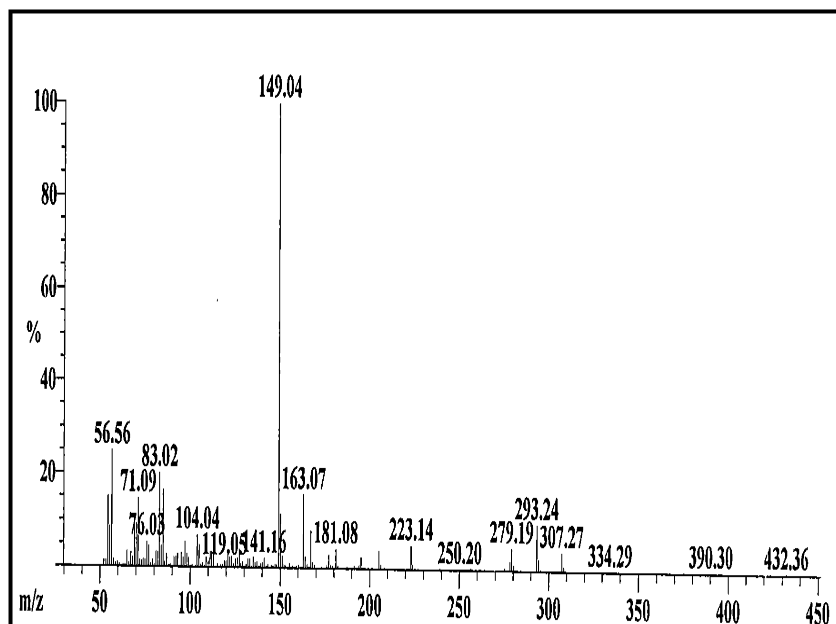

Figure 4: Mass spectrum of antimicrobial agent

4. Discussions

The aminoglycosides such as Hygromycin B are a large and diverse class of antibiotics that characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. The cyclitol is 2-deoxystreptaminestreptomycin, which has a streptidine moiety [Umezawa and Hooper, 1982]. Soil, in particular, is an intensively exploited ecological inhabitant, of which, produce many useful natural products, including clinically important antibiotics [Waksman, 1961 and Waksman, 1975]. Among soil inhabitants, actinomycetes, and more specifically streptomycetes, are of practical importance because they produce most of the useful natural antibiotics for medical use. Nevertheless, selective isolation of soil actinomycetes is important for understanding their ecological properties and for finding novel strains which can produce useful bioactive secondary metabolites. Therefore, various media and techniques have been developed for

selective isolation of actinomycetes [Hozzein *et al*., 2008].

The active metabolites were extracted by ethyl acetate at pH 8. Similar results were obtained by [Yoram, *et al.* 2006; Criswell *et al.* 2006; Sekiguchi, *et al.,* 2007 and Atta *et al.,* 2009].

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 fraction was obtained in the form of viscous syrup, and then tested for antimicrobial activities. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of chloroform and methanol (10:2, v/v), indicated that maximum activity was recorded at fraction Nos. 14 and 23. Many workers used a column chromatography packed with silica gel. Similar results were obtained by [Hitchens and Kell, 2003; Criswell *et al.* 2006 and Sekiguchi, *et al.,* 2007].

The physico-chemical characteristics of the purified antibiotic revealed that, melting point is 160^0C; and soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexan and benzene. Similar results were recorded by [Yanai, 2004 and Atta *et al.,* 2011]

A study of the elemental analysis of the antibacterial agent lead to an imperical formula of: $C_{15}H_{30}N_2O_{10}$. The spectroscopic analysis of purified antimicrobial agent produced by *Streptomyces crystallinus,* AZ-A151, the infrared (IR) spectrum showed characteristic band corresponding to 21 peaks. The ultraviolet (UV) spectrum recorded a maximum absorption peak at 225 nm. The Mass spectrum showed that the molecular weight was 432.36.

The biological activities (MIC) of the antimicrobial agent are fairly active against both Gram positive, Gram negative bacteria, unicellular and filamentous fungi. The minimum inhibitory concentration (MIC) of antibiotic produced by *Streptomyces crystallinus,* AZ-A151 was determined and results showed that MIC (µg/ml) against *Staphylococcus aureus*, NCTC 7447 (1.73), *Klebsiella pneumonia*, NCIMB 9111 (3.9), *Escherichia coli*, NCTC 10416 and *Salmonella typhi* (7.8), for *Aspergillus flavus*, IMI 111023 and *Alternaria alternata* (31.25), and *Saccharomyces cerevisiae,* ATCC 9763 (62.5). Similar studies were conducted by [Criswell *et al.*, 2006 and Sekiguchi, *et al.*, 2007].

Identification of antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being likely belonging to Hygromycin-B antibiotic [Berdy, 1974; Berdy, 1980a b & c; Umezawa, 1977 and Umezawa and Hooper 1982].

5. Conclusion

It could be concluded that: The Hygromycin-B antibiotic produced by *Streptomyces crystallinus,* AZ-A151 demonstrated obvious inhibitory affects against Gram positive and Gram negative bacteria and unicellular and filamentous fungi.

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