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Production of Heliomycin from Actinomycete and Evaluation of its Antimicrobial Activities

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Abstract

Heliomycin (**1**) was isolated from the culture broth of actinomycete AB5. The structure of the isolated compound (**1**) was characterized by spectroscopic methods including NMR and mass spectrometry data.The influence of cultural parameters affecting the production of biomass including different culture media, pH values, temperatures and incubation periods were investigated. Maximum production of secondary metabolites (2.78 g/l) was found in the culture medium of Waksman at initial pH 7.5 for four days at 35ºC. The antimicrobial activity of heliomycin (**1**) was evaluated against Gram-positive bacteria, Gram-negative bacteria and fungi.

Key words: Actinomycete, secondary metabolites, heliomycin, NMR and antimicrobial.

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1. Introduction

Microbial natural products provides a huge numbersof approved drugs with diverse biological activities (Blunt et al., 2012; Citron et al., 2012; Raja et al., 2010).Among microorganisms, actinomycetes are the most promising group for production of antibiotics and other important bioactive substances (Cai et al., 2009).Actinomycetes are Gram positive, aerobic, mycelia bacteria with a high G+C nucleotide content (>55%) in their DNA. They are widely distributed in both terrestrial and aquatic habitats and form asexual spores. They are defined as prokaryotic organisms located between bacteria and fungi (Okami and Hotta, 1988).The filamentous actinomycetes species can produce about 70 - 80 % of all isolated bioactive compounds. As per literature, the most common representative genera

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of actinomycetes include *Streptomyces*, *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Frankia*,*Micrococcus*and *Micromonospora* (Goodfellow et al., 1988).

Heliomycin (**1**) was firstly isolated from the culture broth of *Streptomyces resistomycificus* (Brockmann and Schmidt-Kastner, 1951). It is an unusual aromatic polyketide that have anticancer (Adinarayana et al., 2006; Vijayabharathi et al., 2011),antibacterial and antiviral activity (Eckardt al 1972).Several studies revealed that heliomycin (**1**) inhibits RNA polymerase(Haupt et al., 2007), apoptosis (Shiono et al., 2002) and RNA and protein synthesis. Additionally, it acted as histone deacetylase inhibitor (Abdelfattah et al., 2018).Herein, we described isolation and structure elucidation of heliomycin (**1**) from actinomycete AB5. Optimization of culture conditions was studied to increase the yield and to reduce the cost of production.Additionally, the antimicrobial activity of heliomycin (**1**) against different pathogens was investigated.

2. Materials and Methods

2.1. Isolation and fermentation of actinomycete AB5

A sediment sample was collected from Ismailia Canal in the region of Mustard, Cairo, Egypt. Sample was collected during winter 2017 and kept at 4°C for further working up. One gram of wet sediment was dispersed in 9 ml of sterilized water and vortexed for 2 minutes. The sample was subjected to heat treatment at 60°C for 10 minutes to eliminate non-sporulating bacteria. To prevent the fungal and bacterial contaminants, cycloheximide (50 µg/ml) and nalidixic acid (75 µg/ml) were added to the medium. Following serial dilution (10^{-1}, 10^{-2} and 10^{-3}) of the suspension with sterilized water, a 100 µl of aliquot was spread on starch-casein agar (SCA)(Abdelfattah et al., 2016).The plates were incubated at 28°C for 15 days until the colonies appeared. Colonies that produce a yellow pigment were picked up, purified by repeated streaking on Waksman agar plates(Abdelfattah et al., 2017)and took a voucher number AB5. The strain was recognized as actinomycete by the formation of tough, leathery and yellow colonies that adhered to the agar surface. Well grown agar plates were used to inoculate 6x 250 cm^3 Erlenmeyer flasks each containing 100 ml of Waksman media. The flasks were incubated with 240 rpm for 4 days at 28°C.

2.2. Extraction and isolation of heliomycin (1)

The fermentation broth was harvested after 4 days and centrifuged at 4000 rpm for 10 min. The resulting mycelia cake was extracted three times with methanol. The water phase was extracted three times with ethyl acetate. As the TLC (DCM/10%MeOH) of both extracts from mycelia cake and water phase showed similar composition, they were combined and concentrated under reduced pressure. The brown crude extract was dissolved in methanol and left to stand overnight. A yellow precipitate separated out of the solution was observed. The precipitate was filtrated and washed three times with methanol to givea yellow solid.

Heliomycin (1): Yellow solid; (-)-ESI-MS: *m/z* (%) = 375 ([M-H]$^-$; 100); ^{1}HNMR ([D_6] DMSO, 600 MHz): δ_H = 14.55 (s, 1H, 7-OH), 14.36 (s, 1H, 3-OH), 14.07 (s, 1H, 5-OH), 11.40 (br. s, 1H, 10-OH), 7.23 (s, 1H, 11-H), 7.01 (s, 1H, 8-H), 6.34 (s, 1H, 4-H), 2.90 (s, 3H, 9-CH_3), 1.56 (s, 6H, 1-CH_3); ^{13}CNMR ([D_6] DMSO, 125 MHz): δ_C = 204.9 (C-2), 183.5 (C-6), 170.7 (C-3), 170.5 (C-5), 167.6 (C-7), 162.1 (C-10), 152.7 (C-11a), 152.1 (C-9), 142.2 (C-11c), 139.1 (C-9b), 128.5 (C-8), 128.4 (C-9a), 118.2 (C-11), 114.2 (C-11b), 107.1 (C-6a), 105.9 (C-5a), 102.1 (C-2a), 99.4 (C-4), 46.1 (C-1), 28.9 (2Me-1), 25.5 (Me-9).

2.3.Optimization of the cultural conditionsfor production of heliomycin (1)

2.3.1 Effect of media composition

Three different types of broth media were used in this study for evaluation of theoptimum conditions.

The compositions of these media were as follows: Starch casein medium (g/l): 10 g Soluble starch; 0.3 g Casein; 2 g K_2HPO_4; 2 g KNO_3; 0.05 g $MgSO_4.7H_2O$; 0.02 g $CaCO_3$ and 0.01 g $FeSO_4.7H_2O$. Waksman medium (g/l): 20 g Glucose; 5 g Beef extract; 5 g peptone; 3 g yeast; 5 g NaCl and 3 g $CaCO_3$. ISP2 media (g/l): 4 g Dextrose; 4 g Yeast and 10 g Malt extract. For all media used, the pH was adjusted to 7.5 before sterilization.One hundred ml of these liquid media was dispensed into each 250 mL Erlenmeyer flasks and autoclaved at 121°C for 20 min. The flasks were incubated at 28°C on a rotary shaker at 240 rpm for 96 h.

2.3.2 Effect of initial pH

To study the effect of pH on the production of secondary metabolites,the pH value of the culture media was adjusted to 2.5, 5.0, 7.5 and 10.0. The cultures were cultivated in Waksman and ISP2 media and incubated onrotary shaker(240 rpm)at 28°C for 96 h.

2.3.3 Effect of temperature

Four different temperatures was used for production of natural compounds (25, 28, 35 and 40°C). The strain was cultivated on Waksman medium at pH 7.5 under shaking at 240 rpm for 96 h.

2.3.4 Effect of incubation period

Production of secondary metabolites was measured at different incubation intervals (48, 72 and 96 h). The flasks were fermented in Waksman media at 28°C and pH.5 under shaking at 240 rpm.

2.4 Antimicrobial activity of heliomycin (1)

The susceptibility tests were performed according to NCCLS recommendations(Kiehlbauch et al., 2000). Two Gram-positive bacteria (*Staphylococcus aureus*and *Bacillus subtilis*), two Gram-negative bacteria (*Escherichia coli* ATCC 25955 and *Proteus vulgaris* ATCC 13315) and two fungi(*Aspergillus flavus* and *Candida albicans*) were used in the present study. The antimicrobial activities were performed by using disk diffusion method(Rabah et al., 2013). Gentamycin (4µg/ml) and ketoconazole (100µg/ml) were used as positive controls for bacteria and fungi, respectively. Heliomycin (**1**) was dissolved in dimethyl sulfoxide (DMSO) at concentration of 20mg/ml. Controls using DMSO were adequately done. The activity was determined by measuring the diameter of the inhibition zones after 24hours at 37ºC.

3. Results

3.1. Isolation and structure determination of heliomycin (1)

The crude extract of the actinomycete AB5 showed an interesting yellow band on TLC. Working up of the strain resulted in the isolation of one yellow compound (**Figure 1**). The compound gave a yellow fluorescence band at 365 nm and a reddish-brown colouration after staining with anisaldehyde/sulphuric acid. The UV spectrum of **1** showed absorption maxima at 457, 320, 290, and 267 nm. Transitions from $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ are the factors responsible for the observed peaks in the electronic spectra of compound 1. The molecular weight of **1** was determined to be 376 Dalton by electrospray ionization mass spectrometry (ESI-MS) in the negative ion mode. The ^{1}H-NMR spectrum of compound **1** (**Figure 1**) revealed three broad signals at δ_H 14.28, 14.23 and 13.87each for one proton. This indicates the presence of three chelated hydroxyl groups in the molecule. A broad signal of one proton intensity appeared at δ_H 11.76 ppm, assigned for aromatic hydroxyl group. In the aromatic region, three proton singlets appeared at δ_H 7.15, 6.76 and 6.19 ppm. In aliphatic region, singlet of three protons at δ_H 2.79 is characteristic for aromatic methyl group. In addition to sharp singlet at δ_H 1.52 assigned for six protons indicated the presence of two magnetically equivalent methyl groups.

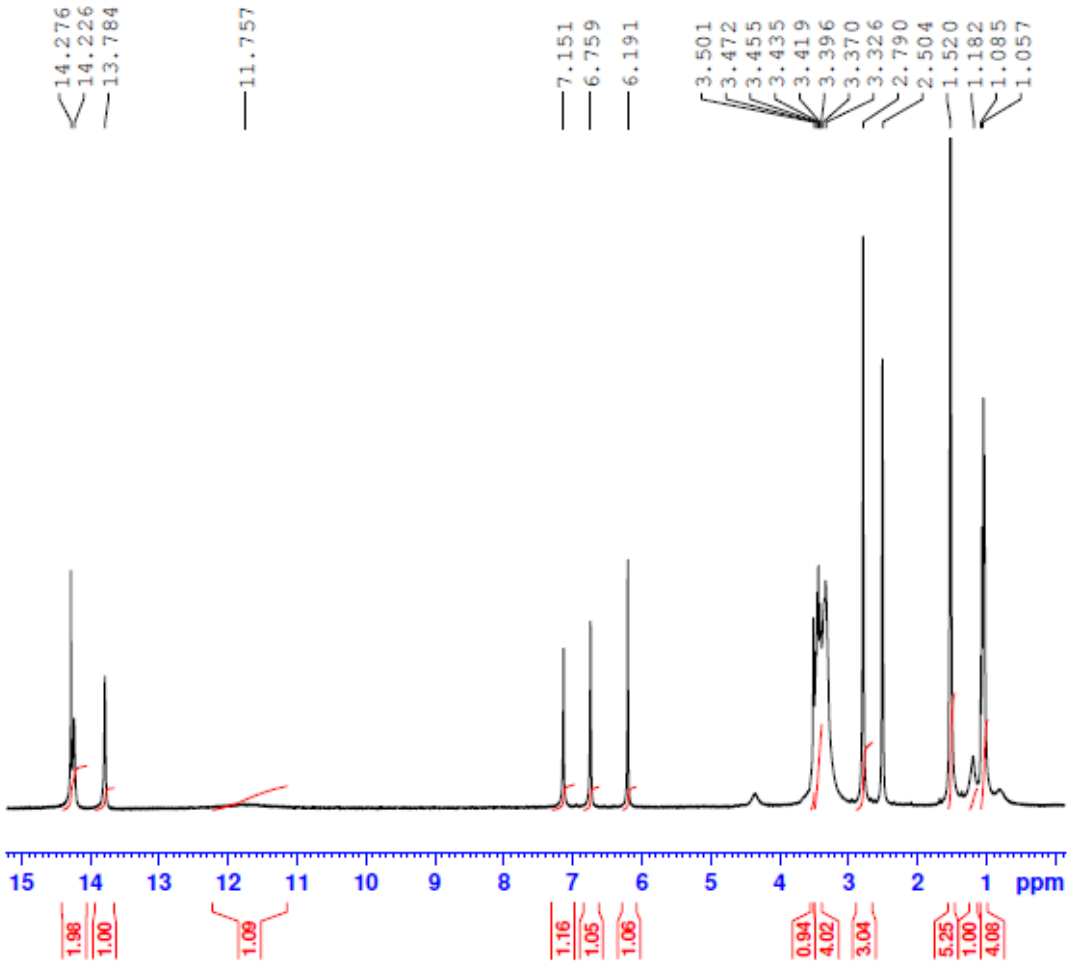

Figure 1: ^{1}H-NMR spectrum (400 MHz, DMSO-d_6) of heliomycin (**1**)

A search in SciFinder led to the assignment of **1** as heliomycin (**Figure 2**). The structure of compound **1** was also confirmed by comparison these spectral data with those in the literature (Kock et al., 2005).

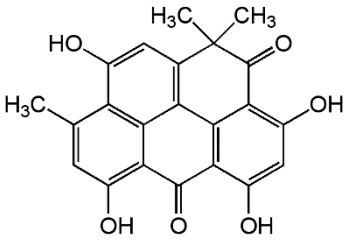

Figure 2: Chemical Structure of heliomycin (**1**)

3.2. Optimization of cultural conditions

To select the optimum media for the production of secondary metabolitesfrom actinomycete AB5, three different media were used. The influence of each media on the production of biomass was recorded (**Figure 3a**).Among the selected media, Waksman medium broth showed an increase in the amount of bioactive metabolites (1.46 g/l), followed by ISP2 broth (0.90 g/l) and starch-casine broth (0.29 g/l). The effect of initial pH of the culture media on production ofbiomass was investigated **(Figure 3b)**. The maximum yield (1.99 g/l) was obtained at initial pH of 7.5 followed by pH 10 (1.79 g/l) and pH 5 (1.73 g/l), respectively. At highly acidic condition (pH 2.5), the amount of crude extract was too little.The effect of different incubation temperature on the yield of the crude extract was reported in **(Figure 3c)**.The highest yield of biomass was observed at 35ºC (2.78 g/l). The production was gradually decreased at 28ºC (1.57 g/l) followed by 40ºC (1.03 g/l). Minor amount of biomass was observed at 25ºC. The effect of incubation time on the production of metabolites is shown in (**Figure 3d**). The maximum yield reached after 96 hours (2.11 g/l). Further decrease in the incubation time showed a gradual decrease in the production of biomass and the growth of actinomycetes. Therefore, optimum incubation time for the maximum production of secondary metabolite was at 96 hours. No growth was observed after 24 h of incubation.

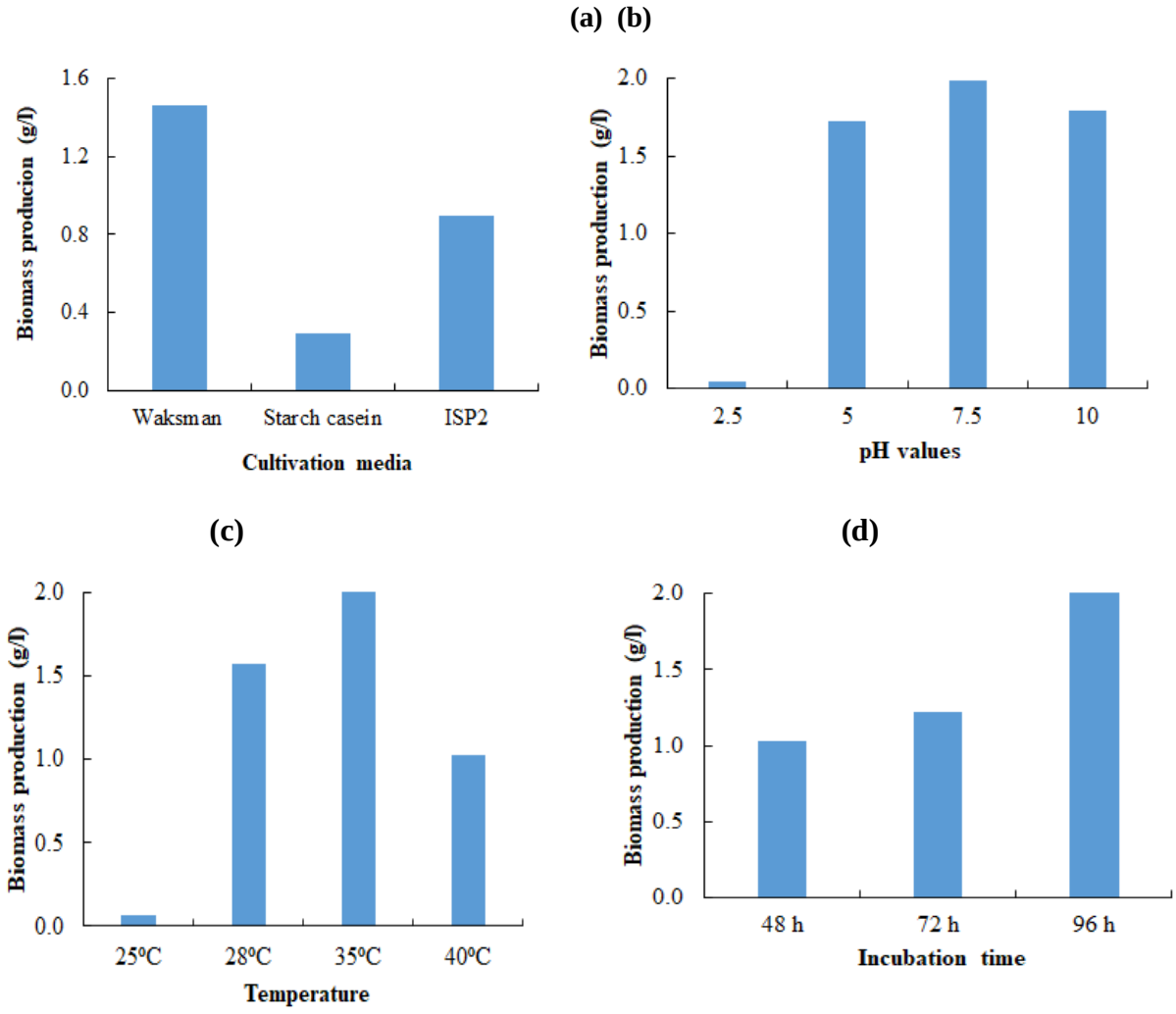

Figure 3: Effect of cultural conditions on the production of heliomycin (**1**); (a) composition of culture media, (b) pH values, (c) temperature and (d) incubation time.

3.3. Antimicrobial screening of heliomycin (1)

Heliomycin (**1**) was screened for its inhibitory activity against different human pathogenic bacteria and fungi (**Table 1**). It showed activities against all four different tested bacteria. On the other hand it was inactive against *Aspergillus flavus*and *Candida albicans*.

Table 1: Antimicrobial activity of heliomycin (**1**)

Test organism	Zone of inhibition (in mm)
Bacillus subtilis	12
Escherichia coli	10
Staphylococcus aureus	13
Proteus vulgaris	11
Aspergillus flavus	NZ
Candida albicans	NZ

NZ is no zone of inhibition
Average of triplicate determinations

4. Discussions

During our screening program for bioactive secondary metabolites from actinomycetes(Abdelfattah et al., 2018, 2016; Elmallah et al., 2017), the strain AB5 was isolated. The strain was found to produce an unusual polyketide **1** with benzo[c,d]pyrene ring. Heliomycin (**1**) was firstly isolated from the culture of *Streptomyces resistomycificus* by Brockmann and Schmidt-Kastner(Brockmann and Schmidt-Kastner, 1951). Its structure was established by classical methods and based on chemical and spectral data together with x-ray crystallographic analyses (H. Brockmann, E. Meyer, K. Schrempp, 1969; Höfle and Wolf, 1983). To choose the best conditions to produce heliomycin (**1**) from actinomycete AB5, several culturing media were used. Waksman medium was the best broth due to presence of enough amounts of sugar, yeast extract and other minerals. The pH 7.5 gave a maximum yield of compound **1**. It was reported that that most favorable range for growth and production of pigmented secondary metabolites from actinomycetes was established to be at pH 7.6 – 8 (Palanichamy et al., 2011). The pH is a significant factor that affects the physiology of microorganisms by influences nutrient solubility, enzyme activity, cell membrane morphology, by product formation and oxidative reduction reactions (Bajaj et al., 2009). Additionally, our results revealed the best temperature for growing of actinomycete AB5 as 35ºC for 4 days. Several literatures stated that optimum temperature for the growth of actinomycetes was in the range between 30ºC-40ºC (Vijayabharathi et al., 2012; Palanichamy et al., 2011). Ripa et al., 2009 reported that actinomycetes had the ability to produce bioactive compounds on the fourth day. The antibacterial activities of heliomycin (**1**) against Gram-positive and Gram-negative bacteria are in accordance with Adinarayana et al., 2006.Vijayabharathi et al., 2011 reported that heliomycin (**1**) exhibited a strong antimicrobial activity against *Enterococcus faecalis*and *Staphylococcus epidermis* and moderate activities against *Staphylococcus aureus*, *Salmonella*

typhii, *Klebsiella pneumoniae*, and *Bacillus subtilis*.

5. Conclusion

Aquatic actinomycete AB5 was isolated from a sediment sample collected from Ismailia Canal around the area of Mustard, Cairo, Egypt. The strain produced a yellow pigmented compound identified as heliomycin (**1**). The optimal production conditions were Waksman liquid medium, pH 7.5 for four days at 35ºC. The isolated compound (**1**) showed antimicrobial activity against gram positive and negative bacteria.

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