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Purification and characterization of the antimicrobial substance produced by *Streptomces misioensis* isolated from Egyptian soil

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Abstract: The antimicrobial substance was isolated from the fermentation broth of *Streptomyces misioensis*. It is active in vitro against *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, Sarcina lutea, Candida albicans* and *Saccharomyces cervisiae.* The production media for maximum yield of the secondary metabolites was g/l mannose 20, potassium nitrate 2.5, $(NH_4)_3PO_4$ 0.85 after 4 days at pH 8.The metabolites was extracted using ethyl acetate at pH 7 and purified by paper chromatography. The chemical structural analysis with UV, IR, MS and NMR spectral analysis confirmed that the compound produced by *Streptomyces misioensis* is related to isoprenoid compounds.

Introduction

Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances [1]. Streptomycetes is the largest genus of Actinobacteria and the tybe genus of the family Streptomcetaceae [2]. Streptomycetes are Gram positive and have high GC content [3]. Streptomycetes species are widely recognized as industrially important microorganisms for their ability to elaborate different kinds of novel secondary metabolites [4], notably antibiotic, antitumor agents and immunosuppressive agents [5]. Isoprenoids are the largest single family of natural compounds, with more than 23.000 known examples, and include industrially useful compounds such as flavors, antibiotics and plant hormones [6, 7, 8]. Actinomycetes have been known to produce several isoprenoid compounds, such as methyl isoborneol [9], pentalenene [10] and squalene- hopene [11]. Morever, several *Streptomyces* strains are known to produce poly ketide-isoprenoid hybrid compounds, such as furaquinocin [12], naphterpin [13], napyradiomycin [14] and marinone [15], all of which were reported to show biological activities. In present study, the production of the antimicrobial substances that demonstrated inhibitory effect against micro organisms from *Streptomyces misioinses* was reported. Also, extraction, purification and characterization of the antimicrobial substance were determined.

Material and Methods

Microbial strain

Streptomces misioensis was isolated from the soil at Qalubia Governorate in Egypt. The strain was maintained as slant culture using starch casein agar medium [16].

Test organisms

Gram positive bacteria as *Staphyloccus aureus, Bacillus subtils, Bacillus cereus* and *Sarcina lutea* and Gram negative bacteria as *Escherichia coli* and unicellular fungi such as *Candido albicans* and *Saccharomyces cerevisiae* were obtained from MIRCIN culture collection of the Faculty of Agriculture, Ain Shams University.

Fermentation

A loopful of the *Streptomces misioensis* from the 4 day culture agar was inoculated into 250 ml Erleumeyer flasks containing 50 ml of antibiotic production medium.The production medium composed of g/l mannose 20, potassium nitrate 2.5, $(NH_4)_3PO_4$ 0.85 at pH 8. The flasks were incubated on a rotary shaker (200 rpm) at 28^o C for 4 days. Twenty liter total volume was filtered through Whatman No. 1 filter paper, followed by centrifugation at 5000 rpm for 20 minutes. The clear filtrates were tested for their activities against the test organisms.

Extraction

The culture filtrates were adjusted at different pH values (2-10) and extracted with an equal volume of organic solvent 1:1(v\v). Different extraction solvents were tested for effectiveness, including ethyl acetate, butyl acetate, amyl acetate, chloroform, n-butanol, diethyl ether and benzene. The pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue. The residue was dissolved in the least amount of DMSO and filtrated.

Precipitation

The precipitation process of the crude compound was carried out using ethyl alcohol, acetone, saturated solution of ammonium sulphate and calcium chloride followed by centrifugation at 5000 rpm for 15 min.

Purification

The purification of the antimicrobial compounded was carried out using paper chromatography (Thick chromatography paper 46x57 cm Whatman 3MM), ethyl acetate was used as an eluting solvent. The active band was eluted by ethyl acetate and concentrated under vacuum.

Physicochemical properties of the antimicrobial substance

The elemental analysis C, H, O, N and S was carried out at the microanalytical National Research Center Dokki, Egypt.

Spectroscopic analysis

The IR, UV, NMR and mass spectra were determined at National Research Center Dokki, Egypt.

Characterization of the antimicrobial substance

The antimicrobial substances produced by *Streptomyces misioensis* was identified according to the recommended in national reference [17, 18, 19, 20, 21].

Results and Discussion

Fermentation, extraction and purification

The fermentation process was carried out for 4 days at 28^o C using the liquid production medium. After the incubation period, the filtration was conducted followed by centrifugation at 5000 rpm for 20 min .The supernatant was extracted with different extraction solvents. Maximum yield of the antimicrobial substance was observed with ethyl acetate (1:1, v/v). The organic layer 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 tested for their antimicrobial activities. The antimicrobial substance was precipitated by ethyl alcohol. The purification process was applied through paper chromatography using ethyl acetate as on eluting solvent.

Physicochemical characteristics of the antimicrobial substance

The purified antimicrobial substance produced by *Streptomyces misioensis* produces characteristic odor. The compound is freely soluble in ethyl acetate, acetone, amyl acetate, butyl alcohol and diethyl ether but insoluble in petroleum ether.

Elemental analysis

The elemental analytical data of *Streptomyces misioensis* showed the following: C=59.29, H=9.69, O=31.02, N = 0.0 and S= 0.0. This analysis indicates a suggested empirical formula of $C_6 H_{10} O_2$.

The antibiotic substance (B) produced from *Streptomyces misioensis* gives positive Libermann test indicating an isoprenoid aliphatic long chain compound. The IR showed absorption bands at cm $^{-1}$ 3474 (OH), 1722 for C=O, 1461, 1408, 1375, for cycloalkanes, 1275 for OH and C-O 2920, 2852, for CH, CH_2 and CH_3. The high UV absorption at nm 257, 277 suggested a highly oxygenated polyconjugated compound and this was inferred from its empirical formula $C_6 H_{10} O_2$.

The cited literature [22] revealed a patent for isolation of antibiotic compound from *Streptomyces misioensis* it was called BH890 but they did not suggest a structure. In another patent [23], it was claimed that this antifungal compound is related to polyene macrolide antibiotics. Some of them contain amino sugar in the glycosidic form, others are in the aglycone form. This is the case of our compound which showed absence of nitrogen in the elemental analysis. The spectral data of this compound is very closed to the polyen macrolide rimocidine isolated from *Streptomyces rimosus* [24] but in aglycone form in case of substance (B) produced from *Streptomyces misioensis* (Fig 1).

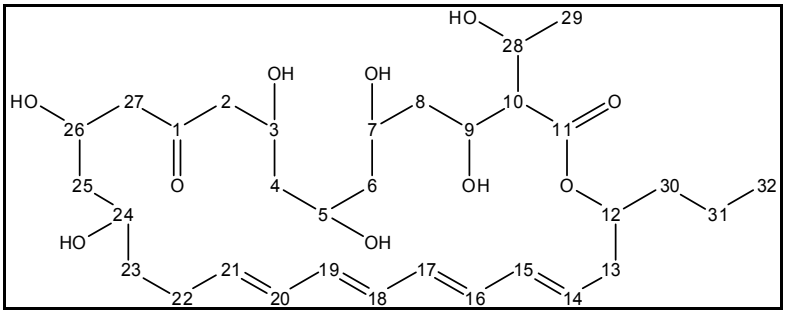

Fig. (1) Substance B rimocidinolide ($C_{32}H_{50}O_{10}$)

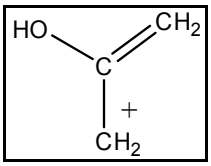

Fig (2) Base peak of substance B (C_3H_5O)

The HNMR and ^{13}C NMR of isolated antibiotic substance (B) produced from *Streptomyces misioensis* are listed in Table (1). The mass spectrum of this compound showed a base peak at m/z 57 due to fragment C_3H_5O in ionic form (Fig. 2). The mass does not show M^+ ion peak, but showed stable fragments at m/z 279 and 149 and show different losses of CH, CH_2 and CH_3 characteristic for isoprenoid cycloaliphatic compounds.

Table (1) ^{13}C and HNMR of antibiotic B

C_{13}/δ ppm				HNMR/ δ ppm			
1	174	17	131.1	1	----------	17	6.5
2	49	18	131.2	2	2.5	18	6.5
3	58	19	131	3	3.4	19	7.8
4	42	20	132	4	1.4	20	6.2
5	62	21	133	5	6.5	21	5.7
6	41	22	27	6	1.7	22	2
7	69	23	37.8	7	3.2	23	1.6

8	40	24	74	8	1.7	24	3.2
9	69.1	25	42	9	3.8	25	1.7
10	60	26	58	10	2.4	26	3.4
11	174	27	50	11	--------	27	2.7
12	71	28	61	12	3.9	28	4.2
13	34	29	22	13	2.3	29	1.2
14	130	30	36	14	5.7	30	1.5
15	131	31	16	15	6.2	31	1.3
16	131	32	14	16	6.5	32	0.96

Table (2) Biological activities of the antimicrobial substance by paper assay

Test organism	Diameters of inhibition zone (mm)
A- Bacteria	
Gram positive	
Staphylococcus aureus NCTC7447	19.0
Bacillus subtilis NCTC1040	21.0
Bacillus cereus NCTC8214	23.0
Sarcina lutea ATCC16424	15.0
Gram negative	
Escherichia coli ATTC9341	0
B- Unicellular fungi	
Candida albicans IMRU3669	18.0
Saccaromyces cerevisiae ATCC9763	23.0

Data of the antimicrobial substance spectrum indicated that the substance is active against Gram positive bacteria and unicellular fungi (Table 2).

Conclsion

To the best of our knowledge and believe this the first isolation of rimocidinolide (rimocidine aglycone) from *Streptomyces misioensis* isolated from Egyptian soil.

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