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Characterization of an Antibiotic Produced by *Streptomyces violaceus* T_{118} and its Effect in Controlling Chocolate Spot Disease of Faba Bean Plant

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AN ANTIBIOTIC was extracted with n-butanol from the culture filtrate of *Streptomyces violaceus* T_{118} after 6 days of incubation, at 30°C, on starch nitrate medium at PH 7.0. The highest bio-chromatogram R_f value was recorded using ethyl acetate or (3%) NH_4Cl.

The physicochemical characteristics of the antibiotic showed that, it was yellow in colour, without characteristic odor, melting point 195~198°C, soluble in chloroform, butanol and dimethyl sulfoxide, while sparingly soluble in water and insoluble in acetone, cyclohexan, petroleum ether and ammonium chloride. Non reducing sugar, free NH_2 group or aromatic amines were present, while tyrosine, diketones or enolic group, free aldehyde nitro group and amino acids containing sulphur were absent. The elemental and spectroscopic analysis (I.R., U.V. NMR and Mass spectrum) showed that, the compound may be related to $C_{32}H_{38}N_2O_7$ with molecular weight of 562 gm.

It was found that the extracted antibiotic from *S. violaceus* T_{118} had a clear effect for controlling chocolate spot disease by concentration 85 and 90 µg/100ml, on both mycelium growth and disease severity on detached leaves and 100 µg/100ml on plants in pots (under green house conditions).

Keywords: *Streptomyces violaceus*, Antibiotic production, Chocolate spot disease, Faba bean disease.

Intensive use of chemical fungicides for production of ergonomically important plant against pests and disease caused by phytopathogenic bacteria and fungi has been lead to significant pollution in the environment and to many ecological disasters throughout the world. Therefore, the development of an alternative, more efficient and safe biological control methods of plant diseases is an important approach of agricultural biotechnology (Chet, 1987; Cook 1993 and Elad & Fokkema, 1994).

Antibiotics can be defined as products of microbial metabolism which is capable of inhibiting the growth and/or survival of pathogens and which is effective in low concentrations. Many fungi, bacteria and actinomycetes produce highly effective antimicrobial agents (Betina, 1983). Major emphasis is directed to streptomycetes. They are the most important antibiotic producers. Search for new antibiotic continues in order to control naturally resistant bacteria and fungi.

Streptomyces sp. are common soil microorganisms that are noted for their abilities to produce antibiotics and other secondary metabolites. About two thirds of the naturally occurring antibiotics are produced by *Streptomyces* sp. (Chatter and Hapwoord; 1989).

Recently, species of this genus have been investigated for their potential to control a wide range of plant pathogens (Daqun *et al.*, 1996).

The present work deals with the extraction, purification, determination of biological activities and some physicochemical characteristics of an antibiotic produced by *Streptomyces violaceus* T_{118} and effective in controlling chocolate spot disease of faba bean plant.

Material and Methods

Experimental organism

Streptomyces violaceus T_{118} (resistant to fungicide kocide 101) was isolated from soil cultivated with faba bean plants (Swelim *et al.*, 2002). It was found to be antagonistic to *Botrytis fabae* (the causal organism of chocolate spot disease).

Production of the antibiotic

250 ml conical flasks containing 50 ml of starch nitrate liquid medium were inoculated with spore suspension (5ml/flask) of *S. violaceus* T_{118} and incubated

at 28°C on a rotary shaker (200 rpm) for 6 days. Cells were separated from broth by centrifugation.

Extraction and purification

Six immiscible with water, solvents (butanol, hexane, benzene, petroleum ether, chloroform and diethyl ether) were tested for their extractability of the antibiotic from broth (Hussein, *et al.*, 1998).

Bio-chromatogramic technique, was used for the purification and determining the R_f values of the antibiotic mobility on paper strip chromatograms using different solvent systems.

Antimicrobial activity

The antimicrobial activities of the antibiotic were carried out by using dilution method as recommended by *Victorlorian* (1986). MIC and MDC were determined according to the method of Kavanagh, (1963) and Lorian and Atkinson, (1976).

Physico-chemical characteristics

The purified antibiotic was detected its colour and odor. The solubility and melting point (MP.) of the antibiotic were determined.

The spectroscopic analysis of the antibiotic deals with Elemental analysis (CNH ratio). Ultraviolet spectra (UV), Infrared spectra (IR), Mass spectrum and Nuclear Magnetic Resonance (NMR) were done at Cairo University Micro Analytical Center, Giza. Amino acids, sugar and protein contents were determined according to the methods of Block, *et al.*, (1958), Becker, *et al.*, (1964) and Lowery, *et al.*, (1951), respectively.

Some colour reactions were carried out on paper chromatograms of concentrated broth of the antibiotic to detect certain groups in the molecule (Plummer, 1978).

Effect of the antibiotic on detached leaves and potted plants

The effect of the antibiotic produced by *S. violaceus* T_{118} on detached leaves was studied according to the method of *Nawar and Kuti*, (1999). Detached leaves were inoculated with 10µ/1 spore suspension of *Botrytis fabae.* (250×10^3

spore/ml) or with different concentrations of the studied antibiotic. The severity of the disease was evaluated based on scale (0-9) (*Abou-Zeid*, 1985) after 24, 48,72 and 96 hr. (Abou-Zeid and Hassanein, 2000).

Also potted plants were inoculated by spore suspension of *B. fabae* (control) or with different concentrations of the antibiotic then covered by polyethylene bags and incubated at moist chamber. The results were recorded after 3, 7, 14 and 21 days (Abou-Zeid and Hassanein, 2000).

Results

Extraction and purification

Streptomyces violaceus T_{118} produces a yellow antibiotic in liquid shaken cultures of starch nitrate medium, it was extracted by counter current distribution using different water immiscible solvents at various pH values. N-butanol was the most efficient for the extraction of the substance at pH 4.0. The organic phase was collected, and evaporated under vacuum using a rotary evaporator. The extract was concentrated to obtain viscous deep brown syrup. The syrup was washed by chloroform several times to get rid of the yellow pigments which may interfere with the antibiotic activity of the product.

Bio-chromatogramic technique, was used for the detection of the antibiotic and to determine the R_f values using different solvent systems of which the rate of flow (R_f) ranged from 0.05 to 0.75, the highest value was recorded on using ethyl acetate (0.75) followed by 0.70 with 3% NH_4 Cl in water and n. Butanol acetic acid water 2 : 1 : 1 showed an R_f 0.60. (Fig. 1).

The yellow fluorescence band at (R_f 0.75) was collected in a clean glass beaker and elluted with pure chloroform. The solvent was filtered and concentrated. Subsequently further purification of the antibiotic was carried out using silica gel sheet developed by ethyl acetate.

Antimicrobial activity

The antimicrobial activity of the produced antibiotic (Table, 1) reveals that it is active against Gram positive bacteria (*i.e*) *Bacillus subtillus, Micrococcus luteus, Rhodococcus equi* with inhibition zone ranging from 13 mm to 19 mm, while it showed inhibition zone against gram negative bacteria (*i.e. Salmonella*

typhi, Escherichia coli and *Psudomonas aeruginosa* between 10 mm to 18 mm). On the other side it is suppressed the fungal growth by inhibition zone about 14 mm to 27.5 mm for some fungal species (*i.e. Candida albicans, Geotrichum candium, Fusarium solani, F. oxysporum, F. moniliforme, Botrytis cinerea* and *B. fabae*. It also noticed that, the product did not show any antimicrobial activities against *Saccharomyces cervisiae* on nutrient medium. Both MIC and MBC for the anti-microbial product were determined. MIC ranged from 10-100 µg/ml while MBC ranged from 10->100 µg/ml.

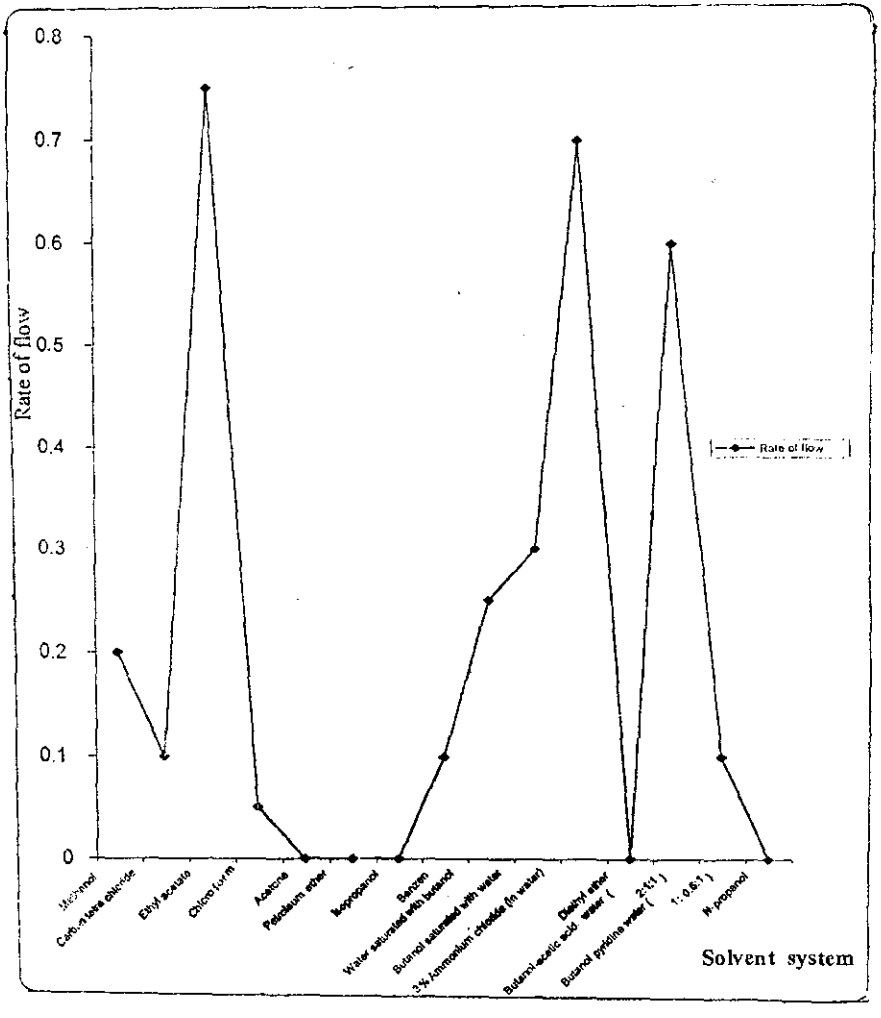


Fig. 1. The different rate of flow (R_f) values of the antimicrobial product obtained from S. violaceus T_{118} culture, when bio-chromatogram with various developing solvents.

TABLE 1. Antimicrobial potentialities of the antibiotic produced by *S. violaceus* T_{118}.

Test organism	MIC (μg/ml)	MBC (μg/ml)
Bacillus subtilis	10	10
Micrococus luteus	10	15
Rodococcus equi	10	10
Escherichia coli	30	35
Pseudomonas aeruginosa	25	25
Sallmonella typhia	35	35
Candida albicans	15	20
Geotrichum candium	20	25
Botrytis fabae	60	85
Fusarium oxysporum	> 100	> 100
F. moniliforme	65	> 100
F. solani	65	> 100

MIC = Minimum Inhibition Concentration.
MBC = Minimum Bacericedial Concentration.

Physicochemical characteristics

Physical properties

The antibiotic was found to be substance with no characteristic odor, soluble in chloroform, n. butnal and dimethyl sulfoxide but sparingly soluble in water and acetone, while insoluble in cyclohexan and petroleum either. Melting point was 195 ~ 198°C.

Spectroscopic analysis

Elemental analysis of the antibiotic showed that it contains carbon 77,2% hydrogen 6.6% nitrogen 2.6% and oxygen 13.6% which agrees with the formula $C_{32}H_{38}N_2O_7$ however molecular weight is 562 gm. Its mass spectra showed the presence of peak at λ 550 (10%). The most important peaks were m/z (abundance) 90.0 (26.97), 131.00 (4.49), 133.15 (14.61), 147.05 (64.66), 172.05 (2.25), 242.25 (2.25), 266.2 (100.00) and 390.45 (1.12) (Fig. 2).

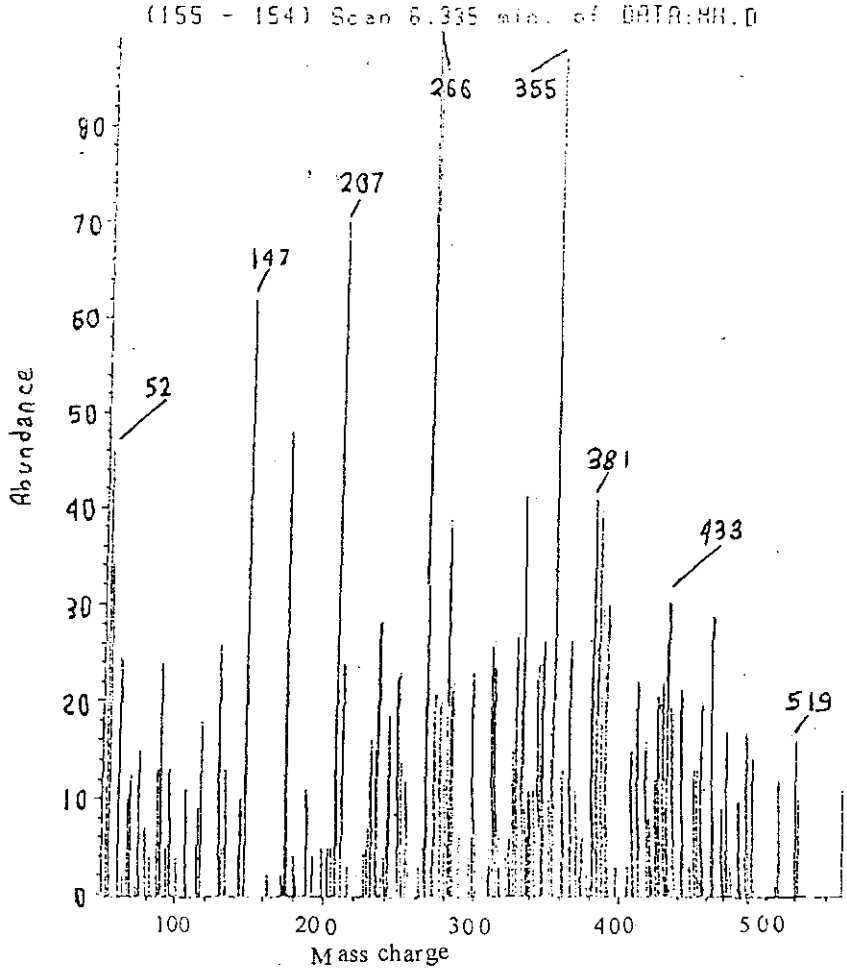


Fig. 2. Mass spectrum of the antibiotic produced by *S. violaceus* T_{118}.

The Ultraviolet spectrum showed a broad peak at λ_{max} 244 nm. (Fig. 3). The infrared spectrum I.R. showed absorption peaks indicating the presence of OH and/or NH groups (3417.6 cm^{-1}), aromatic C-H (3020 cm^{-1}), aliphatic C-H (2962 cm^{-1}) νC ≧O (1735.8 cm^{-1}) ν–CO-NH or C = N (1651.0 cm^{-1}) and νC = C (1519.8 cm^{-1}) (Fig. 4). Analysis of proton magnetic resonance (H^1 NMR) showed peaks for aromatic CH at σ 7.26(s), 7.51 (m), 7.73 (m), CH at σ 4.2 (m), CH_2 at σ 2.2 (s), CH_3 at σ 1.36 (q) and CH at σ 0.924 (l) (Fig. 5).

Chemical properties

The acid hydrolysate of the antibiotic showed that it contains non reducing sugar as a sugar moiety, proteins (0.4125 µg/ml). Amino acids were present.

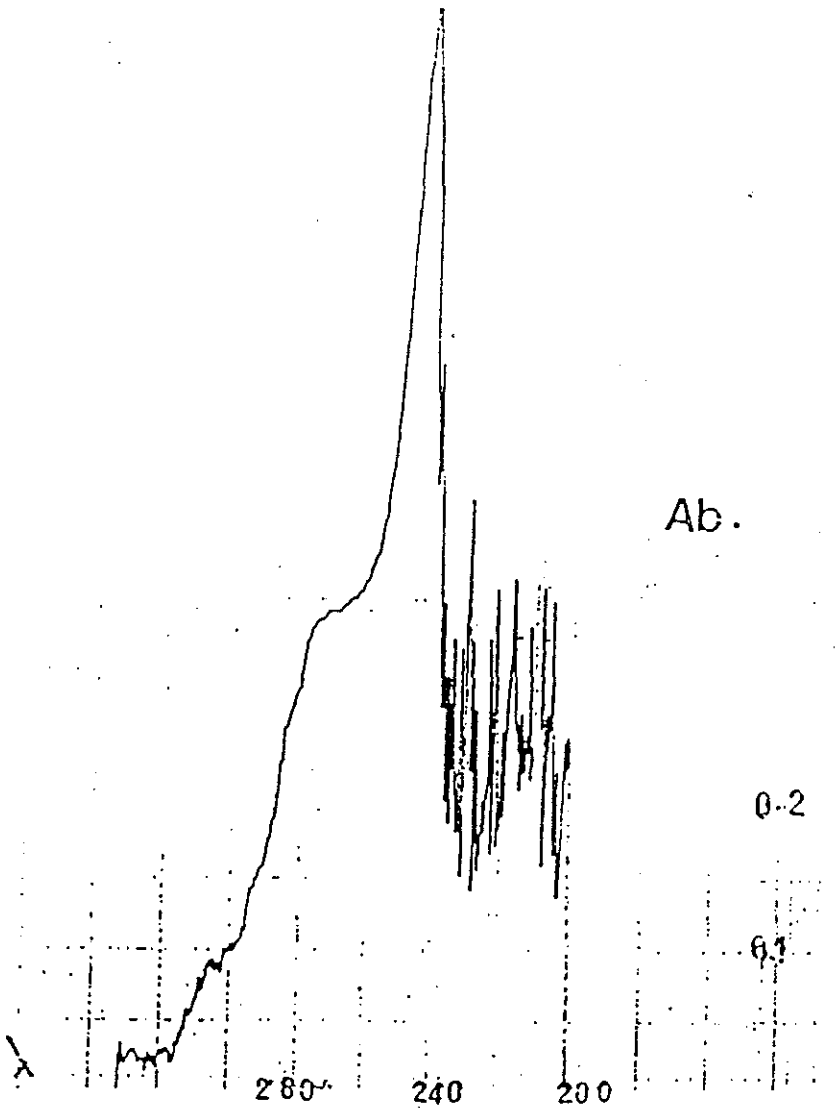


Fig. 3. Ultraviolet spectrum of the antibiotic produced by *S. violaceus* T_{118}.

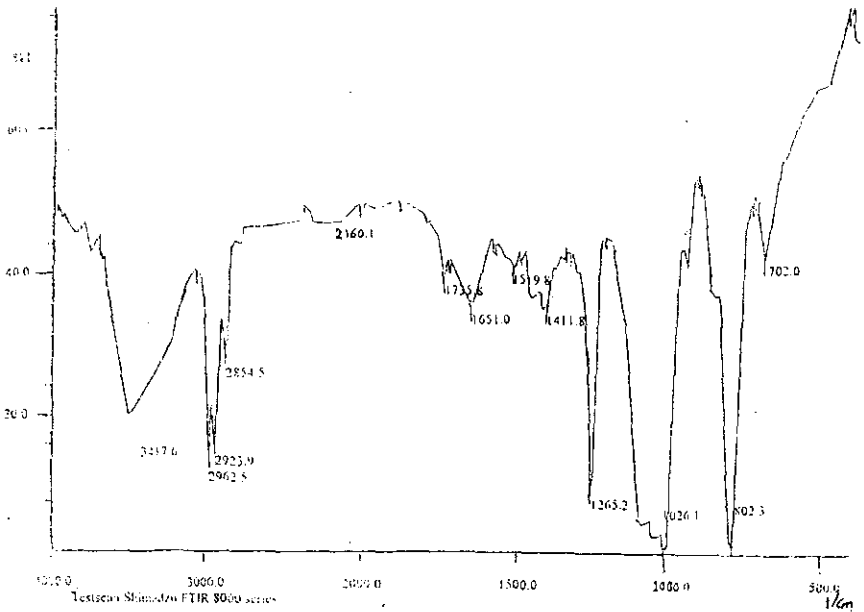


Fig. 4. *I.R.* spectrum of the antibiotic produced by *S. violaceus* T_{118}.

Fig. (4) : I.R. Spectrum of the antibiotic produced by *S. violaceus* T_{118}.

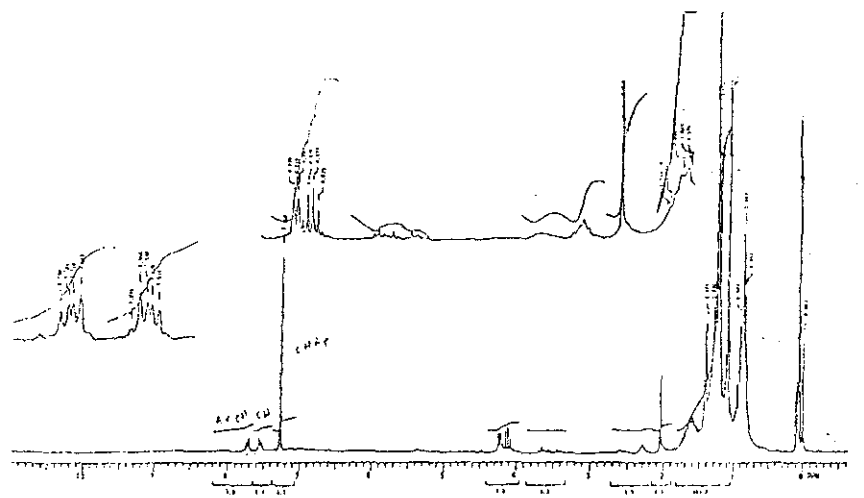


Fig. 5. Proton nucleus magnetic resonance of the antiobiotic produced by *S.violaceus* T_{118}.

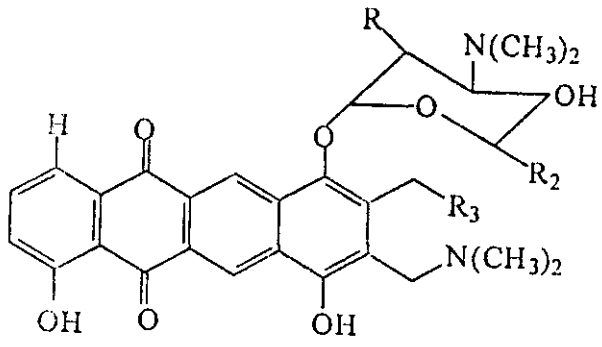
Behaviour of the antibiotic towards certain chemical tests and the proposed structure

From the data presented in Table 2, it was observed that, the antibiotic showed positive results with Molish's reaction Sakaguchi's reaction ninhydrin test, nitroprusside reaction and Tollen's reaction which indicate the presence of sugar moiety, arginine, free amine group (NH_2), and aromatic amine, respectively. However the remaining chemical tests gave negative results.

TABLE 2. Remarks on the behaviour of the antibiotic towards certain chemical tests.

Chemicals	Results	Remarks
Molish's Reaction	+ve	Presence of sugar moiety
Sakaguchi Reaction	+ve	Arginine is present
Ninhydrin test	+ve	Free-NH_2 group is present
Nitroprusside Reaction	+ve	Amines is found
Millon's Reaction	-ve	Tyrosine is absent
Ferric chlorid reaction	-ve	Diketones or enolic group aren't present
Fehling reaction	-ve	Free aldehyde and / or keto sugar are absent
Mayer's Reaction	-ve	Nitro group is absent
Tollen's reaction	+ve	Aromatic amine is present
Lead sulphide reaction	-ve	Amino acids containing sulphur are absent

From the listed data of the spectrum analysis the proposed chemical structure of such compound is illustrated in Fig. 6. It closely resembles to anthracyclines.



$(C_{32}\ H_{38}N_2O_7)$ M. wt 562 gm.

Fig. 6.

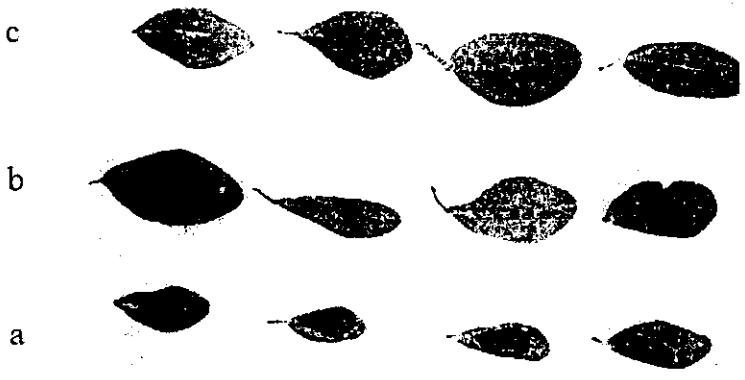


Fig. 7. Effect of antibiotic produced by *S. violaceus* T_{118} on chocolate spot disease (using detached leaves).
a) Control (nontreated) b) 85 µg/100ml c) 90 µg/100ml

Biological control of chocolate spot disease

** Detached leaves*

The data recorded in Table 3 and Fig. 8 showed that, there is an inhibition effect of the antibiotic produced by *S. violaceus* T_{118} when it was applied on detached leaves. The used anti-biotic reduced the bio-mass of the pathogen after 24 hr. of which symptom severity on treated leaves with *Botrytis fabae spore* suspension only (control) was high necrotic (disease severity were 2.4 - 8.2) during the experimental time, while the treated leaves with both *B. fabae* spore suspension and the antibiotic showed less necrotic effect (the disease severity was ranging between 1.0 to 1.6 and 0.8 to 1.4 by using 85 and 90 µg/ml, respectively) Generally using the antiboitic concentration 90µg/ml was better than 85 µg/ml.

TABLE 3. Effect of the antibiotic produced by *S. violaceus* T_{118} on chocolate spot disease using detached leaves on cultivar Giza. 3.

Treatment / Incubation time	Mean[a] of disease severity using scale (0-9) after [b]			
	24	48	72	96
85 µg/100ml	1.0	1.2	1.4	1.6
90µg/100ml	0.8	1.0	1.2	1.4
Control[c]	2.4	4.2	5.6	8.2

Treatment (T) 0.4 I x T 0.7 (L.S.D. 0.05) Incubation time (I) 0.4 Mean value of five replicates.
(b) Incubation time in hours.
(c)*B. fabae* spore suspension.

Potted plants (under green house conditions)

It's clear from the results given in Table 4` and Fig. 8 that, the all used concentrations of the antibiotic succeeded to reduce the disease severity on faba bean plant (c.v Giza 3) under green house conditions of which the sporulation potential (*B. fabae* spore suspension) covered the range between 3.2 to 9.0 on leaf surface (control), however the used concentration 100µg/100 ml was the best as it reduced the disease severity 0.8 to 2.0 compared to the control.

TABLE 4. Effect of the antibiotic produced by *S. violaceus* T_{118} on chocolate spot disease in green house conditions.

Treatment / Incubation time	Mean[a] of disease severity using scale (0-9) after [b]			
	3	7	14	21
85 µg/100ml	1.0	3.0	3.2	3.2
90µg/100ml	0.8	2.0	2.0	2.6
100µg/100ml	0.8	1.0	1.8	2.0
Control[c]	3.2	7.4	9.0	9.0

Treatment (T) 0.3 I x T 0.6 (L.S.D. 0.05) Incubation time (I) 0.3

(a) Mean value of five replicates. (b) Incubation time in days.

(c) *B. fabae* spore suspension.

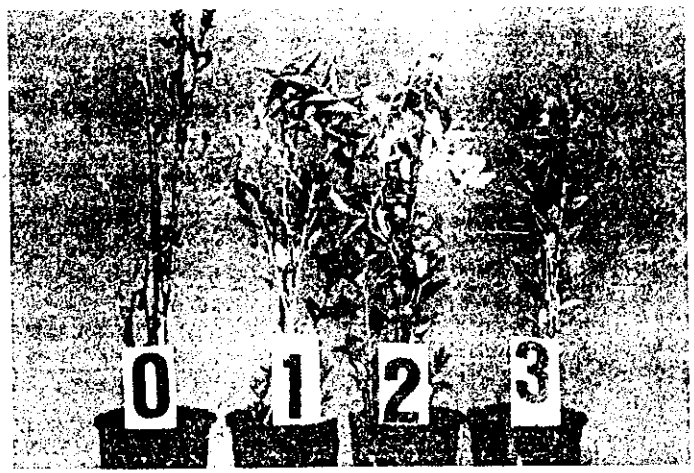


Fig. 8. Effect of antibiotic produced by *S. violaceus* T_{118} on chocolate spot disease under green house conditions

0) Control (nontreated) **1) 85 µg/100ml**

2) 90 µg/100ml **3) 100 µg/100ml**

Discussion

The antibiotic isolated from a culture broth of *Streptomyces violaceus* T_{118} was studied by using NMR, UV, Mass specrometry and analysis including elemental analyses. Several *Streptomyces spp* produce antibiotics which showed strong anti-fungal activity (El-Gammal 1985 and Ubukata, *et al.*, 1995).

The proposed structures of the antibiotic closely resemble those of anthracyclines 10-O Rhodo saminyl. The structural difference between the studied antibiotic and anthracycline is the absence of 2-OH and 2-CH_3 in α, β, γ,δ-unsaturated lactone moiety that was agree with Osamu & Yoshioka (1997). They isolated anthracycline A_{262} from culture broth of *Streptomyces violacues* A_{262}.

Antagonists used in biological control aimed at reduction of *Botrytis sp.* Both the disease severity and the bio-mass of pathogen were reduced in the presence of the used antibiotic, however disease severity on the control leaves or plants developed rapidly. This may be due to the reduction in bio-mass during the initial time of incubation or to the direct or indirect effect of the antibiotic on the enzymatic activity of the pathogen.

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خصائص المضاد الحيوى الناتج من أستربتوميسس فيوليشيس ١١٨ ط وتأثيره على مرض التبقع البنى فى نبات الفول البلدى

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أمكن استخلاص مضادا حيويا من العزلة أستربتوميسس فيوليشيس (١١٨ط) بعد ٦ أيام من التحضين عند درجة حرارة ٣٠°م وأس الهيدروجين ٧ وذلك على بيئة النشا والنترات وقد وجد أن كحول البيوتانول هو أنسب مذيب لاستخلاص هذا المضاد الحيوى كما أظهر مذيبى الإيثيل أسيتات وكلوريد الأمونيوم ٣٪ فى الماء أعلى قيمة R_f لهذا المضاد.

أظهرت الصفات الفيزيائية والكيميائية لهذا المضاد الحيوى أنه أصفر اللون ليس له رائحة مميزة ودرجة حرارة انصهاره ١٩٥-١٩٨°م، يذوب فى الكلوروفورم والبيوتانول - شحيح الذوبان فى الماء ولا يذوب فى الأسيتون، السيكلوهكسان الأيثر البترولى أو كلوريد الأمونيوم كما وجد أن الجلوكوز يوجد لى هيئة صورة غير مختزلة بالإضافة إلى وجود مجموعة الأمين الأروماتى ولم يستدل على وجود التيروسين، الكيتون الثنائى، ومجموعة الألدهيد الحرة أو الحمض الأمينى المحتوى على الكبريت.

بإجراء تحاليل العناصر والقياسات الطيفية والرنين النووى المغناطيس للبروتونات للمركب اقترحت الصيغة الكيميائية له وهى $C_{32}H_{38}N_2O_2$ والتى يمثلها الوزن الذرى ٥٦٠مم.

وجد أن لهذا المضاد الحيوى تأثيراً واضحاً على مرض التبقع البنى فى نبات الفول وذلك عند تركيز ٨٥. ٩٠/١٠٠ ملى وعلى كل من نمو الغزل الفطرى وشدة المرض فى الأوراق المنفصلة و١٠٠/١٠٠ ملى على النبات الكامل فى الصوبا.