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RESEARCH ARTICLE

Antagonistic affects of Actinomycetes isolated from Tuti island farms (Central Sudan) against Fusarium oxysporum f.sp.vasinfectum a phytopathogenic fungus

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Abstract

Twenty five *Actinomycetes* species were isolated from thirty samples of soil collected from Tuti Island farms (Central Sudan) and Blue Nile bank using Starch Casein agar medium. Three genera were isolated, nineteen different species were belonging to *Streptomyces*, five species belong to *Actinomyces* and one species belong to *Arachnia* formerly *Actinomyces*. These species were screened and evaluated for their antifungal activity against *Fusarium oxysporum f.sp.vasinfectum*. The results showed that *Fusarium oxysporum f.sp.vasinfectum* has a resistance to the antagonizing effect, and it has an ability to destruct the secondary metabolites. The crude extracts of fermented actinomycetal broth were analyzed also by thin layer chromatography (TLC) technique and seventeen different antifungal of Actinomycetes were characterized.

1.Introduction

Search for new drugs against fungal infections is a major challenge to current research in mycotic diseases [1]. Among the different types of drugs, prevailing in the market antifungal drug is very small. The need for new, safe and more effective antifungal is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host. In recent years the microorganisms have become important in the study of novel microbial products exhibiting antimicrobial, antiviral, anti-tumor as well as anticoagulant and cardioactive properties. These active compounds may serve as model system in discovery of new drugs. Actinomycetes have occupied a prominent position in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites including antibiotics with diverse chemical structure and biological activities [2 and 3]. Thus, the proposed study is an effort to screen the Actinomycetes from Tuti Island (Central Sudan) having antifungal activity. Tuti Island is well known for its huge and unexplored diversity. This diversity can be explored for isolation and characterization of native Actinomycetes for antifungal metabolites. In order to screen a new antifungal compound, producer organism may be isolated and screened for, from different natural materials such as soils, which is based on the assumptions that samples from widely different locations are more likely to yield effective isolates and therefore hopefully effective metabolite.

The objectives of this study are to isolate the antifungal producer-Actinomycetes and characterize its metabolites according to very specialized methods and techniques.

Materials and Methods

Isolation of soil-borne Actinomycetes

Thirty-four random soil samples were collected from Tuti islands' farms and Blue Nile shore; and stored in sterile plastic bags. The soil samples were suspended in 25 ml of basal salt solution (5.0 g/l KH_2PO_4 and 5.0 g/l NaCl) [4]

and left at 28°C for 30 min. The soil suspension was diluted and heated at 50°C for 6 min. Subsequently, 0.1 ml of diluted soil suspension was spread onto starch-casein-agar plates (soluble starch 10.0 g/l; casein 0.3 g/l; KNO_3 2.0 g/l; NaCl 2.0 g/l; $MgSO_4.7H_2O$ 0.05 g/l; $CaCO_3$ 0.02 g/l; $FeSO_4.H_2O$ 0.01 g/l; KH_2PO_4 2.0 g/l; and agar 18.0 g/l), which were supplemented with 50 µg/ml of filter-sterilized cycloheximide to inhibit fungal growth, and incubated at 28°C for 12–14 days. Colonies of *Actinomycetes* on the agar plates were picked on the basis of their morphological characteristics and purified [5].

Identification of Actinomycetes

The identification of *Actinomycetes* was done on the basis of morphology of spore chain, pigment production, colour of aerial mycelium, colour of substrate mycelium, consistency, Gram's staining, growth on *Actinomycetes* media and growth on *Streptomyces* media. The potent *Actinomycetes* selected for further studies were characterized by morphological and biochemical methods. The microscopic characterization was done by Gram's staining. The mycelium structure, colour and arrangement of conidiospore and/or arthrospore on the mycelium were observed through the oil immersion (100X) objective. They were identified according to [6 and 7].

Extraction of antifungal from isolated Actinomycetes

Preparation of inoculate for fermentation process

Starch casein nitrate broth (10 ml) was prepared and then under aseptically conditions a loop full of purified growth added in the Starch casein nitrate broth. This broth was incubated at 28°C for 5 days. After 5 days the inoculums for fermentation process was ready for used [8].

Method for extraction

Upon the completion of fermentation, the medium was harvested and centrifuged to remove cells and debris. Filtrate was collected in a sterilized screw cap bottle. The filtrate was mixed with ethyl acetate in the ratio of 1:1 (v/v) and shaken vigorously for 1 h in a solvent extraction funnel. The solvent phase that contains antifungal compound was separated from the aqueous phase. Solvent phase was evaporated to dryness in water bath at 80 - 90°C and the residue is used to characterize the compounds [9].

Antifungal analysis of isolated Actinomycete

The isolates were subjected to the bioassay techniques of modified Cross streak which was modified in our laboratory.

Modified Cross Streak method

All of the isolates were screened for their *in vitro* antagonism against *Fusarium oxysporum f.sp.vasinfectum*, according to the modified method [10]. Briefly, a loop full of isolated *Actinomycetes* strain was spotted as a circle on a SCA plate and incubated at 28°C for 3 days. A fungal mycelium from 3-days-old of each fungus was taken and transferred to an Actinomycetes-pre-grown SCA plate. The fungal mycelium was additionally placed on un-inoculated PDA plates separately as control treatment. The radial fungal growth in the direction of the antagonist in both the control and the dual culture plates was measured at 4 and 6 days after incubation of *Fusarium oxysporum vasinfectum*. The levels of inhibition were calculated by using the equation [11]:

Inhibition **(%)** = [(growth diameter in untreated control - growth diameter in treatment) X 100] / growth diameter in untreated control

Characterization of actinomycetal secondary metabolites using TLC

Fifty (50)µL di-methyl sulfoxide (DMSO) was added to each extracted sample mentioned previously, then the mobile phase (BAW) technique (butanol: acetic acid: water in ratio of 4:1:5) was Optimized, and test antibiotics was done by using 20 x 20 cm aluminium coated silica gel plate. Chromatogram was developed by loading 10 µl each fraction and running for half an hour. Spots on the plates were visualized under UV-Cabinet [12].

Results and discussion

Twenty-five Actinomycetes were isolated from the soil sample by using special medium (Starch-Casein agar) to enhance the growth of Actinomycetes and enable them to produce their pigment, and arranged into three main groups; the dominant group was Streptomyces which consists nineteen species (76%) followed by Actinomyces 5 species (20%), and Arachnia propionica (formerly; Actinomyces propionicus) 4%. The isolated species were identified by using Gram stain techniques to know the morphology, biochemical test, and salt tolerance. Isolated Actinomycetes were exhibit variable antifungal activities against test microorganism. The inhibitory percentage of Actinomycetes against F.oxysporum vasinfectum after 3 days incubation was 77.77% which shown by Actinomyces odontolyticus with inhibition zone 21mm and the lowest one was 11.11% by Actinomyces pyogenes, Streptomyces violaceus, and Streptomyces fulvissimus with inhibition zone of 11.11mm (Plate 1).

The results showed that F.oxysporum vasinfectum had high resistance to the antagonizing effect of Actinomycete; and it has an ability to destruct the secondary metabolites, and then used it as growth stimulators (Table 1b). The secondary metabolites of Actinomycetes were characterized by using Thin Layer Chromatography Technique and the retention factor (R_f) of the metabolites was recorded and used to know the correspondence antibiotic. Twenty isolated Actinomycetes shown produced antifungal drug grouped into seventeen groups, two isolated Actinomycetes produced protease inhibitors while another two produced acids (Actinomyces odontolyticus & Arachnia propionica) and one isolate was non-antibiotic producer (Actinomyces bovis) Table (2).

Conclusion

The results of a survey of a large number of Actinomycetes isolated from different substrates and cultured on special medium are concluded: 1. A large proportion of the Actinomycetes inhabiting natural substrates have the capacity to inhibit the growth of fungi and other microorganisms.2. The ability of these Actinomycetes to exert an inhibiting effect upon microorganisms is highly specific. This selectivity depends, not only on the strain of organism, but also on the medium in which it is grown and conditions of growth. 3. Antagonistic Actinomycetes produce a variety of antibiotics that vary in chemical nature, in antimicrobial action, and in their chemotherapeutic potentialities. 4. Some Actinomycetes produce more than one antibiotic substance. 5. Some antibiotics are produced by several different organisms. There is some evidence, however, that, although the same general type of substance may be formed by the various cultures, it may not possess exactly the same antibiotic spectrum; this suggests the possibility of variations in the chemical structure of the agent.

Actinomyces bovis show no effect against F.oxysporum vasinfectum while Streptomyces species1 was used Actinomycetal metabolites as growth stimulator (Table 1a & 1b). These are resembled to [5]. After 5 days of incubation, the inhibitory percentage was increased in some plates and decreased in others. Actinomyces bovis & Streptomyces species1 show no effect against F.oxysporum vasinfectum. The inhibitory percentage of Streptomyces violaceus, Streptomyces rochei, and Streptomyces hachijoensis was decreased into zero i.e. from 11.11, 18.52, and 17.14% respectively (Table 1a & 1b), and this might be according to the potent enzymatic mechanism of F.oxysporum vasinfectum to resist the antagonistic activities of mentioned Actinomycetes; these results are similar to the findings of [13]. The highest inhibitory percentage was 70% with inhibition zone 28mm (Plate 2) observed by Actinomyces odontolyticus and the lowest one was 7.50% with inhibition zone 3mm (Plate 3) observed by Streptomyces albus. These findings are similar to [14].

Plate 1: Show the antagonistic effects of Streptomyces violaceus against F.oxysporum vasinfectum

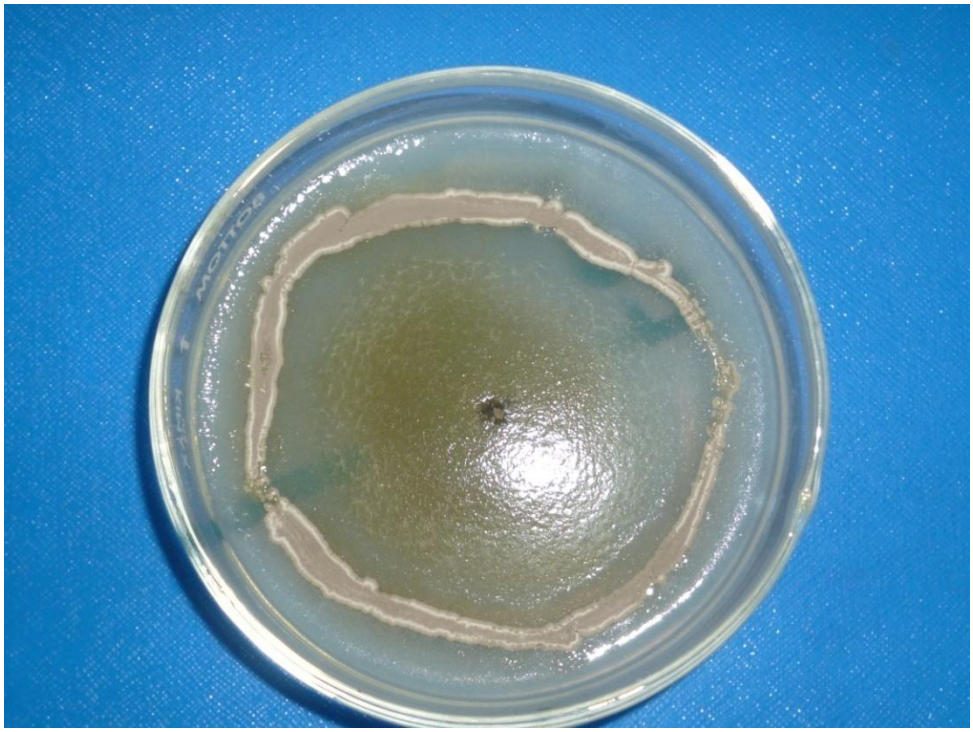

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Plate 2: Show the highest inhibitory percentage of Actinomyces odontolyticus against F.oxysporum vasinfectum

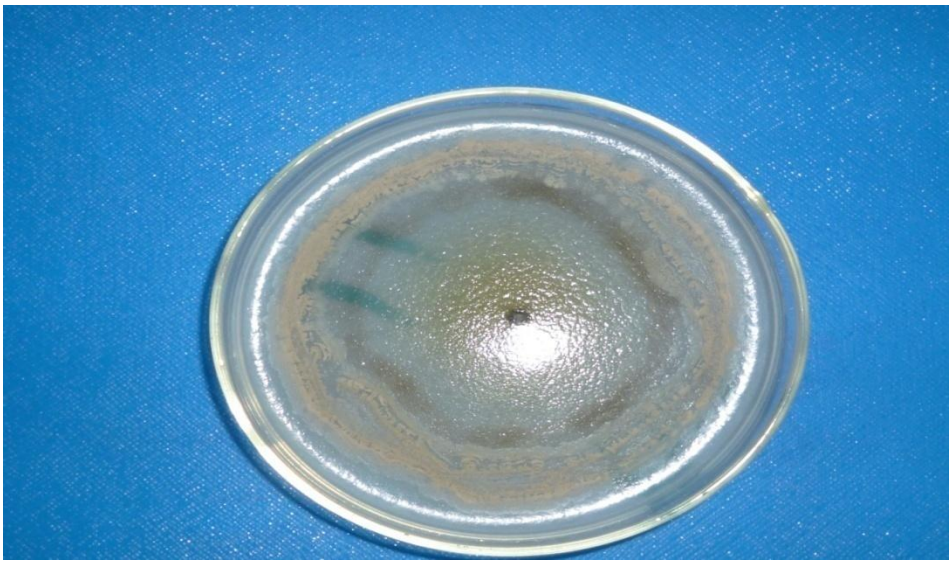

Plate 3: Show the lowest inhibitory percentage of Streptomyces albus against F.oxysporum vasinfectum.

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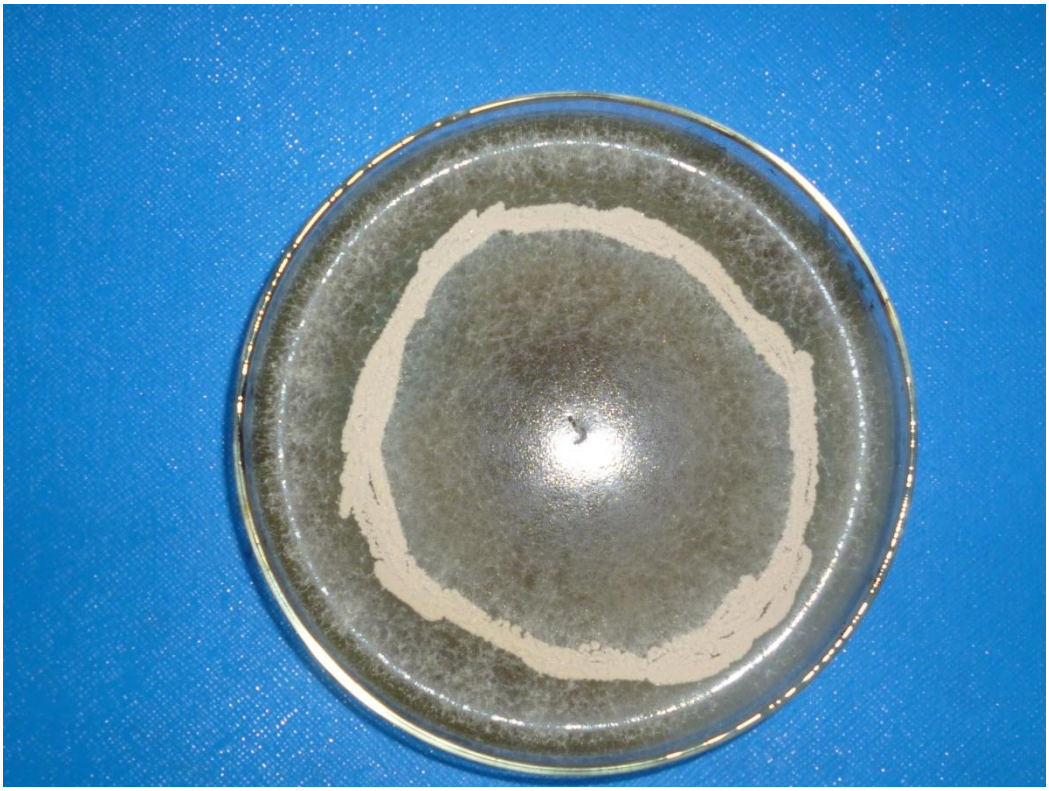

Table (1a): Inhibitory percentages of isolated Actinomycetes against the test moulds

Test organisms / Isolated Actinomycetes	Growth rate (mm) of Fusarium oxysporum vasinfectum after 3 days	I(%) against F.oxysporum vasinfectum after 3 days	Growth rate (mm) of F. oxysporum vasinfectum after 5 days	I(%) against F.oxysporum vasinfectum after 5 days
Actinomyces bovis	27/27	0.00	42/40	-5.00
Actinomyces odontolyticus	06/27	77.77	12/40	70.00
Actinomyces meyeri (1)	21/27	22.22	28/40	30.00
Actinomyces meyeri (2)	18/27	33.33	28/40	30.00
Actinomyces pyogenes	24/27	11.11	33/40	17.50
Arachnia propionica	20/27	25.93	31/40	22.50
Streptomyces hygroscopicus	56/70	20.00	59/87	32.18
Streptomyces species (1)	83/70	-18.57	87/87	0.00
Streptomyces rimosus	54/70	22.86	59/87	32.18
Streptomyces species (2)	59/70	15.71	58/87	33.33
Streptomyces filipinensis	45/70	35.71	47/87	45.98
Streptomyces griseus group (1)	47/70	32.86	46/87	47.13
Streptomyces griseus group (2)	60/70	14.29	54/87	37.93
Streptomyces canescus	49/70	30.00	52/87	40.23
Streptomyces griseus group (3)	62/70	11.43	72/87	17.24
Streptomyces albus	26/27	3.70	37/40	7.50
Streptomyces violaceus	24/27	11.11	40/40	0.00
Streptomyces rochei	22/27	18.52	40/40	0.00
Streptomyces fulvissimus	24/27	11.11	36/40	10.00
Streptomyces antibioticus	57/70	18.57	60/87	31.03
Streptomyces lavenduale	46/70	34.29	57/87	34.48
Streptomyces celluloflavus	48/70	31.43	63/87	27.59
Streptomyces noursei	52/70	25.71	55/87	36.78
Streptomyces griseus	47/70	32.86	54/87	37.93
Streptomyces hachijoensis	58/70	17.14	87/87	0.00

Table (1b): Antagonistic effect of isolated Actinomycetes against Fusarium oxysporum vasinfectum

Test organisms / Isolated Actinomycetes	Growth rate (mm) of Fusarium oxysporum after 3 days	Antifungal activity(mm) against F.oxysporum after 3 days	Growth rate (mm) of F. oxysporum after 5 days	Antifungal activity(mm) against F. oxysporum after 5 days
Actinomyces bovis	27/27	0	42/40	-2
Actinomyces odontolyticus	06/27	21	12/40	28
Actinomyces meyeri (1)	21/27	6	28/40	12
Actinomyces meyeri (2)	18/27	9	28/40	12
Actinomyces pyogenes	24/27	3	33/40	7
Arachnia propionica	20/27	7	31/40	9
Streptomyces hygroscopicus	56/70	14	59/87	28
Streptomyces species (1)	83/70	-13	87/87	0
Streptomyces rimosus	54/70	16	59/87	28
Streptomyces species (2)	59/70	11	58/87	29

Streptomyces filipinensis	45/70	25	47/87	40
Streptomyces griseus group (1)	47/70	23	46/87	41
Streptomyces griseus group (2)	60/70	10	54/87	33
Streptomyces canescus	49/70	21	52/87	35
Streptomyces griseus group (3)	62/70	8	72/87	15
Streptomyces albus	26/27	1	37/40	3
Streptomyces violaceus	24/27	3	40/40	0
Streptomyces rochei	22/27	5	40/40	0
Streptomyces fulvissimus	24/27	3	36/40	4
Streptomyces antibioticus	57/70	13	60/87	27
Streptomyces lavenduale	46/70	24	57/87	30
Streptomyces celluloflavus	48/70	22	63/87	24
Streptomyces noursei	52/70	18	55/87	32
Streptomyces griseus	47/70	23	54/87	33
Streptomyces hachijoensis	58/70	12	87/87	0

Table (2): Characterization of actinomycetal secondary metabolites using TLC

Isolated Actinomycetes	Rf	Antibiotic/ Chemical substance
Actinomyces bovis	0.00	No antibiotic produced
Actinomyces odontolyticus	0.21	Lactic acid
Actinomyces meyeri (1)	0.00	Protease inhibitor
Actinomyces meyeri (2)	0.00	Protease inhibitor
Actinomyces pyogenes	0.60	Tylosin
Arachnia propionica	0.49	Propionic acid
Streptomyces hygroscopicus	0.29	Nigericin
Streptomyces species (1)	0.98	Antimycin A
Streptomyces rimosus	0.40	Rimocidin
Streptomyces species (2)	0.93	Antimycin A
Streptomyces filipinensis	0.95	Filipin
Streptomyces griseus group (1)	0.83	Candicidin A
Streptomyces griseus group (2)	0.80	Candicidin A
Streptomyces canescus	0.33	Ascosin
Streptomyces griseus group (3)	0.70	Candicidin A
Streptomyces albus	0.89	Endomycin
Streptomyces violaceus	0.73	Mycetin
Streptomyces rochei	0.91	Borrelidin
Streptomyces fulvissimus	0.37	Chalcomycin B
Streptomyces antibioticus	0.96	Actinomycin
Streptomyces lavenduale	0.16	Streptothricin
Streptomyces celluloflavus	0.82	Thiolutin
Streptomyces noursei	0.25	Nystatin
Streptomyces griseus	0.44	Candicidin
Streptomyces hachijoensis	0.85	Trichomycin

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