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Enhanced Antiviral Activity of Isolated *Streptomyces Avermitilis* Extract in Conjugation with Gold Nanoparticles

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Abstract

Emerging and reemerging of viral outbreaks represent an ever-growing challenge to worldwide health. Nanoparticles have been attracting much interest due to their likely antiviral activity through interaction with viruses and are proposed to be one of the most promising solutions for detection, treatment, and prevention of viral diseases. Here, we report a gold nanoparticle conjugated extract from *Streptomyces avermitilis* shown to effectively inhibit hepatitis A [HAV], Adenovirus and Vesicular stomatitis virus [VSV] in cultured cells with half-maximal inhibitory concentrations of less than 4 nM and a selectivity index of greater than 550. A total of 20 strains of actinomycetes were isolated from agricultural soil for antiviral activity evaluation. Among which, [H1] exhibited a promising antiviral activity towards the three viruses. In an attempt to enhance the antiviral activity, Streptomyces extract was conjugated to 2.0 nm diameter gold nanoparticles. Although gold nanoparticles had low inhibitory effect on viral titer; the extract-gold nanoparticle conjugate displayed higher activity comparable to that of Streptomyces crude extract. This result support the hypothesis that multivalent molecules on the surface of gold nanoparticle can enhance potent therapeutics of active biological extracts due to interaction. We here introduce *Streptomyces avermitilis* H1 strain as a putative high antiviral producer with gold nanoparticle enhanced activity. More experimental efforts on animal models need to be performed to test for applicability of this developed conjugate in treatment of viral diseases.

Keywords: Antiviral activity; *Streptomyces avermitilis*; Viral assay; Gold nanoparticles, HAV; Adenovirus; VSV .

1. Introduction

Yet being the smallest known form of life, viruses are responsible for the most hazardous worldwide life-threatening outbreaks. Viruses cause human diseases including, Coronavirus, Chickenpox, Herpes, Influenza, Rabies, Smallpox, and AIDS. Particularly those with RNA as genetic material, can mutate rapidly and give rise to new types against which their hosts have little protection due to drug resistance [1].

Such as the case in Influenza virus as new vaccine is needed every year [2]. Major changes can cause pandemics as in coronavirus outbreak in 2019 and in 2009 when swine influenza spread to most countries [3,4]. Often these mutations take place when the virus has infected other animals such as bats, pigs and birds before spreading to humans. Usually, humans and animals eliminate the viral infection by the immune system, conferring lifetime immunity to the host for that virus [5]. However, some of the viruses overcome the immune system and cause more serious illnesses. Antiviral drugs can treat life-threatening infections but still have many limitations such as narrow spectrum being specific to special types of viruses as well as long term use might cause resistance to the drug [6].

So, the needs to explore new habitats for antiviral natural products are growing. Actinomycetes are the most economically and biotechnologically useful prokaryotes [7,8]. They produce antibiotics, antivirals, and other industrially important secondary metabolites [10-14]. Especially under the genus Streptomyces, are producers of approximately two third of all known bioactive compounds. In fact, 80% of the recognized antibiotics are sourced from this genus [15], from which highly commercial bioactive compounds have been isolated [16,17].

Adjustments of current antiviral compounds and development of new antiviral agents is a major area of research. Nanoparticle systems are intensively studied for antiviral therapy in the last decades [18-22]. They represent a growing challenge for the detection,

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prevention and treatment of viral infection [23]. There are two ways of best using nanoparticles in viral prevention. The first one is related to the nanoparticles modified with different organic molecules [18, 19]. The functionalized nanoparticles can affect the viruses due to chemical interactions between the molecules-functionalizes and molecules-receptors at the virus surface. The second direction is concerned with antiviral activity of 'pure' [non-functionalized] nanoparticles [20-32]. There are experimental results of antiviral action of Ag [20-24], Au [25,29], TiO_2 [26], $CuCl_2$ [27], CeO_2 [29-32], SiO_2 [28] nanoparticles against different viruses: influenza virus H3N2 and H1N1 [26,29], hepatitis B virus [20], herpes simplex virus [21,29], HIV-1 [22,24], dengue virus type-2 [27], Foot-and-Mouth disease virus [25] and vesicular stomatitis virus [32]. Microscopic studies demonstrated that nanoparticles are adsorbed on the virus surface [24,25], which leads to local transformations of the surface, such as agglutination of glycoproteins [24,25], thus preventing virus penetration into the cell. Nanoparticles also proved to control biological processes through transcription and gene regulation, as well as enzyme inhibition processes [33-35]. Their role in gene regulation involves ionic bonding between positively charged cationic ligands on the surfaces of nanoparticles and negatively charged anionic nucleic acids present in the DNA. Recently, a nanoparticle-DNA complex inhibited transcription by T7 RNA polymerase, signifying strong bonding in the complex [36].

In an attempt to switch to viral prevention through natural product, we here isolated a strain of *Streptomyces avermitilis* with good tendency towards viral inhibition. Moreover, we illustrate the development of enhanced antiviral activity through conjugation of Streptomyces extract with gold nanoparticles.

2. Experimental

2.1 Sample collection

Different locations in Fayoum and Sharqiyah, Egypt, were selected for soil sampling. About 3 cm of soil surface was sampled using sterilized spatula and stored in dry sterilized polythene bags at 40° C until pretreatment [37]. Samples were suspended in 100 mL each of sterile saline and incubated at 28° C in orbital shaker for 30 minutes at 150 rpm. Samples were mixed with maximum speed vertexing and allowed to stand for few minutes. Serial dilutions up to 10^{-5} of samples were prepared using sterile saline [38]. To separate spores from vegetative cells, dilution 10^{-5} was subjected to 45° C water bath for 16 hours [39].

2.2 Sample preparation, actinomycetes purification and cultivation

The media employed for isolation and enumeration of actinomycetes were Actinomycete Isolation Agar Medium [40]: 5 g/L glycerol, 4 g/L sodium propionate, 2 g/L sodium casemate, 2 g/L KH_2PO_4, 0.1 g/L asparagine, 0.1 g/L $MgSO_4$. $7H_2O$,1 mg $FeSO_4.7H_2O$, 15 g/L agar, pH = 7.0; Kuster's Agar medium [40]: 10 g/L Glycerol, 0.3 g/L casein, 3 g/L KNO_3, 2 g/L K_2HPO_4, 2 g/L NaCl, 0.05 g/L $MgSO_4.7H_2O$, 0.02 g/L $CaCO_3$, 0.01 g/L $FeSO_4.7H_2O$, 16 g/L agar, pH = 7 ± 1; glycerol casein KNO_3 agar medium [1]: 10 g/L Glycerol, 0.3 g/L casein, 2 g/L KNO_3, 2 g/L KH_2PO_4, 2 g/L NaCl, 0.05 g/L $MgSO_4.7H_2O$, 0.02 g/L $CaCO_3$, 0.01 g/L $FeSO_4.7H_2O$, 18 g/L agar, pH = 7.8; and starch casein agar medium [41]: 10 g/L starch, 1 g/L casein powder, 15 g/L agar, 50% sea water, pH = 7.2± 0.2. All the media were supplemented with 0.050 mg/mL nystatin as the antifungal agent [42, 43]. Each soil sample dilution was plated on isolation media and incubated at 28° C for 7-10 days. Individual colonies were purified onto yeast extract-malt extract agar slants [39]. Pure cultures were obtained on yeast extract malt extract glucose medium [YMG] agar slants [44]: yeast extract, 4 g/L; malt extract, 10 g/L; glucose, 4 g/L; agar, 20 g/L; pH = 7.3. Finally, the Streptomyces isolation medium consisting of glucose: 5.0 g/L, L-glutamic: 4.0, KH_2PO_4: 1.0, $MgSO_4.7H_2O$: 0.7, NaCl: 1.0, $FeSO_4.7H_2O$: 3.0 mg, and agar: 25 g/L, was used for purification of Streptomyces colonies supplemented with nystatin at concentration of 0.050 mg/mL as the antifungal agent.

Selected strains were grown in liquid media in neutral [N] and extreme saline [S], conditions of cultivation for later testing for toxicity assay. Subcultures of single purified colonies were maintained on starch casein slants at 4℃ for long preservation until further use.

2.3 Preliminary screening for antibacterial activity

Isolated strains H1 to H20 were inoculated onto nutrient agar plates by streaking and incubated at 28 °C for 3 days. Five bacteria including *Salmonella typhi* PTCC 1609, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* PTCC 1533, *Bacillus subtilis* PTCC 1156, and *Klebsiella pneumonia* were used as test bacterial pathogens. A pure colony of test bacteria was transferred into fresh nutrient broth and incubated at 37 °C for 24 hours until the visible turbidity is reached. A sterile loopful of the bacterial suspension was then streaked perpendicular to the antagonist on the agar medium. The plates were incubated at 37 °C for 24 hours. The microbial inhibitions were observed by determining the diameter of the inhibition zones (Table 1). Isolate H1 was chosen as the most potent anti-microbially active against the tested bacteria for the next step analysis.

2.4 Characterization of actinomycetes isolate

2.4.1 Macroscopic analysis

Morphological and cultural characters of the selected actinomycete strain (H1) was studied by inoculating the selected strain for 5 days into International Streptomyces Project (ISP) Media 1 to 7 media [43]: ISP-1 (casein yeast extract agar), ISP-2 (yeast extract malt extract agar), ISP-3 (oatmeal agar), ISP-4 (inorganic salt starch agar), ISP-5 (glycerol asparagine agar), ISP-6 (Peptone yeast extract iron agar), ISP-7 (Tyrosine agar) and starch casein agar at 30 °C. (Figure 1 and Table 2).

Physiological characteristics of the actinomycete strain were studied with the use of Analytical Profile Index [API] for biochemical tests including citrate utilization, H_2S production, urea hydrolysis, indole production, acetone production, acid production from sugar (Table 2).

2.4.2 Microscopic analysis

The shape and size, mycelium structure and arrangement of spores of chosen strain were examined under light microscope through the oil immersion [100X]. Actinomycetes cells were also prepared for SEM [JEOL, JSM, 3060] according to the initial fixation and dehydration steps [45]. The cells were fixed at 24°C for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 [Sigma-Aldrich ChemieGmbh, Steinheim, Germany], dehydrated with a serial dilution of ethanol, and dried on a critical point dryer [HCP-2; Hitachi Co.]. The dried cell samples were examined using a SEM [JEOL, JSM, 3060]. [46] (Figure 2).

2.5 Molecular identification of Actinomycetes using 16S ribosomal RNA

The purified isolate was cultured in nutrient broth. The sample was centrifuged, washed twice with distilled water, the pellets were subjected to DNA extraction and PCR amplification. Genomic DNA was extracted according to the previously described method [47]. The 16S rRNA gene was amplified by PCR, using the universal prokaryotic primers 27F 5′-CAGCCGCGGTAATAC-3′, 1392R 5′-ACGGGCGGTGTGTAC-3′ to amplify a ~500-bp region. A single DNA fragment was extracted from the gel using a Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company, Germany. The partial 16S rRNA gene sequences were compared with sequences in GenBank with nucleotide-nucleotide BLAST [BLAST-N] to obtain the nearest phylogenetic neighbors [www.ncbi.nlm.nih.gov/BLAST/]. Sequences showing more than 97% similarity were considered to belong to the same operational taxonomic unit [OTU] [48]. Trees were constructed by neighbor joining within the program MEGA 3.1 and bootstrapped with 500 repetitions. These trees were used to obtain broader groupings supported by checks with the Ribosomal Database Project [RDP] database [49] (Figure 3, 4).

2.6 Extraction of active ingredients from actinomycetes

The actinomycete isolate was cultivated in 100 ml ISP2 broth medium for 7 days at 30°C and 250 rpm agitation. After centrifugation, ethyl acetate was added to the supernatant in the ratio of 1:1 [v/v] and agitated vigorously for 2 h at room temperature. The organic extract was evaporated to dryness using a Rotavapor [50]. The resulting dry extract was resuspended in 1 ml of methanol and subjected to biological assay [6 mm disc in diameter] against *S. aureus*. The control contained a disc of equal volume of methanol without the extract. The dry extract was dissolved in 1 ml of [NaCl 0.9%] instead of methanol.

2.7 Toxicity test of Actinomycetes

Hemolytic activity test was done for virus-free cell lines, to investigate the ability of six preparations of Actinomycetes biomass and Actinomycetes extracts on two different types of cultivation media, neutral broth and extreme saline to kill tested cells using two models, chicken fibroblast cells and 2% chicken erythrocytes. First, toxicity test of the six preparations were analyzed on chicken fibroblast cells [104 cells/well] following dehydrogenases activity detection [MTT-test], which is based on the ability of dehydrogenases of viable cells to reduce the non-colored form of 3–4,5-dimetilazol-2,5-diphenyl tetrazolium [MTT-reagent] to the blue form of crystal formazan. 0.5 µg/ml of MTT-reagent [Calbiochem, USA], dissolved in buffered saline was added. Then, 0.1 ml of MTT solution was placed in the plate wells previously filled with cell culture free of culture media. After 1-h wells were washed with buffered saline and then 0.1 ml of dimethyl sulphide [DMSO] was added. Optical density of the mixture in the wells was measured using spectrophotometer at 535 nm. Second, the preparation samples mixed with chicken erythrocytes in a 1:5 ratio, and then incubated for 2 h at 37°C. Equal volume of cold buffered saline at pH 7.2 was then added and centrifuged at 13,000 rpm for 5 min. Supernatants were separated and the optical density was measured at 412 nm using spectrophotometer.

2.8 Gold nanoparticle preparation and characterization

Gold nanoparticles were prepared using SCL Micelles as Nanoreactors. Aqueous SCL micellar solutions of PEO-GMADEA triblocks [0.1-5.0% w/v] were mixed with aqueous solutions of HAuCl4 at various HAuCl4/DEA stoichiometries. After stirring for 1 h,

an aqueous solution of excess sodium borohydride was added. The solutions immediately turned red, indicating the formation of colloidal gold.
Molecular weights and molecular weight distributions were measured by THF GPC using Viscotek instrument. The setup comprised a PLgel 3 ím MIXED-E 300 _7.5mm column, poly [methyl methacrylate] [PMMA] calibration standards, and a refractive index detector. Transmission electron microscopy [TEM] images were verified using a Hitachi 7100 microscope. Samples were prepared by dipping a Formvar-coated copper grid into an aqueous solution of SCL micelles, followed by air-drying at ambient temperature. All 1HNMR spectra were recorded on 1.0% w/v copolymer solutions in D2O using a Bruker Avance DPX 300 MHz spectrometer. Dynamic light scattering [DLS] studies were performed on a Brookhaven Instruments Corp. BI-200SM goniometer equipped with a BI-9000AT digital correlator using a solid-state laser [125 mW,ì]532 nm] at a fixed scattering angle of 90°. The intensity average hydrodynamic diameter, □Dh□, and polydispersity [í2/¡2] were calculated for each micellar solution before and after crosslinking by cumulants analysis of the experimental correlation function. [25,26]. The particle size distributions of the SCL micelles were also assessed using a Polymer Laboratories Particle Size Distribution Analyzer [PL-PSDA]. HDC is a relative technique, and the conversion from elution time to particle size involves calibration using a series of near-monodisperse polystyrene latexes [ex. Duke Scientific] as standards. Type I cartridge, with a range of 5-300 nm, was chosen, and the eluent flow rate was 2.0 mL min^{-1}. Shell crosslinked micellar solutions of 2.0-2.5% w/v at either pH 3 or 10 were filtered through a 0.45 in Whatman filter prior to analysis, and the sample injection volume was 20 íL.

2.9 Antiviral assay

2.9.1 Viral titration and viral infection

To ensure accuracy, each experiment was assayed in duplicate. Before 24-hour infection, 5×105 Vero cells were seeded in each well of a 12-well culture plate with standard growth medium [e.g., DMEM + 10% FBS + penicillin [100 U/ml] and streptomycin [100 µg/ml].]. Next day, 10-fold serial dilutions of viral sample [From 10-3 to 10-8] with serum-free growth medium [e.g., DMEM] was prepared. The infection solution with 0.1ml of diluted viral stock and 0.9 ml of complete growth medium was made containing FBS and antibiotics. The culture medium in Vero cell seeded 12-well culture plate was then replaced with previous infection solution. starting with most diluted so the same pipette tip can be used up the dilution series. Finally, the cells were incubated at 37℃ in 5% CO_2 for 48 hr.

2.9.2. Immunostaining of Infected Vero cells

AbFrontier's Adenovirus Titration Kit was used in the immunostaining method to quantify the virus stock Catalog # LF-RK0001. After 48 hr of infection, the culture medium was aspirated and cells were dried in hood for 5 min. Cells were then fixed by adding 1ml of ice-cold 100% methanol to each well and the plates were incubated at -20℃ for 10 min. Methanol was aspirated, and the wells were gently rinsed with 1ml of TBS-T. The final rinse from the wells were aspirated, and 0.5ml of anti-Ad5 antibody in TBS-T [2% BSA] was added and the mix was incubated for 1 hr at room temperature. Anti-Ad5 antibody was aspirated, and wells were gently rinsed 3 times with 1ml of TBS-T. Final rinse from the wells was aspirated, and 0.5ml of diluted anti-mouse antibody 1:1,000 in TBS-T [2% BSA] was added. Anti-mouse antibody was aspirated, and wells were gently rinsed 3 times with ml of TBS-T. After removing the final TBS-T rinse, 500 µl of staining Solution [10 ml of Development Buffer, 33 µl of Substrate A, 66 µl of Substrate B] was added to each well. The mix was then incubated at room temperature for 10 min then Staining Solution was aspirated, and ml of PBS was added to each well.

2.9.3. Calculation of Titer

Brown and black positive cells were counted using a microscope with a 20× objective [minimum of 3 fields; random selection], and the mean number of positive cells in each well was calculated. Infectious units [IFU/ml] from each well were calculated as follows:
[[infected cells / field] × [Fields/well]] / [[volume of virus] × [dilution factor]] dilution factor [counted dilution] = 10-6 dilution
viral titer = [[10 cells/field] × [466 fields/well]] / [[1.0 ml] × [10-6ml]] = 4.66 × 109 IFU/ml

2.9.4 Viral inhibition assay test

2.8.4.1 Antiviral assay for the actinomycete extract
The antiviral activity of the actinomycete extract was evaluated by the reduction of viral test [TCID50] calculated by the Reed & Muench statistical method [51]. The results were expressed in viral inhibition index [VII] calculated by the formula VII=B-A, where B is the virus titer in virus-infected controls [no sample], A is the virus titer in the test samples [52], and [PI] is a percentage of inhibition using antilogarithm values of virus titer as follow: PI=1-[antilogarithm test value/antilogarithm control value] x 100 [53].

2.9.4.2 Antiviral assay for the gold nanoparticle
The MTT staining assay as described by Mosmann [54] was used with minor modifications. After 24 h of

cell growth and monolayer formation in 96 well plates, the cells were infected with a previously determined dose of the three viruses of 50 µl per well. The viruses were adsorbed at room temperature for 2h at 5% CO_2. The viruses that did not adsorb were rinsed with Henks solution, then 200 µlof the 10-fold dilutions of the test nanoparticles were added. The control wells contained only fresh medium without nanoparticles. After 3-7 days of incubation at 37◦C and 5% CO_2, 20µl of MTT solution [5 mg/ml] were added to each well and incubated in an atmosphere of 5% CO_2 at 37 ◦ C for 4 h. The medium was then removed from the wells, and 150 µl of 96% ethanol was added to flush the dye from the cells. Samples were incubated at 37◦C with shaking for 15 min. Optical density of the samples was measured at a wavelength of 540 nm using Multiskan FC [Thermo Scientific, USA]. The optical density of each well was determined as compared to the optical density of the solvent as a control [54]. The percentage of inhibition of virus reproduction was determined by the following formula:

$$I = \frac{P_s - P_{av}}{P_{cc} - P_{av}} \times 100\%, \quad (1)$$

where Pcc is the average optical density of the cells control samples, Pav is the average optical density of the virus control samples, Ps is the average optical density of the test samples for a definite concentration of the substance.

The virucidal effect of nanoparticles was measured as follows: The viral suspension [virus with a titer of 7 × 107 PFU/ml, PFU is the plaque-forming units], was mixed with an equal volume of the mother liquor of the test nanoparticles in various dilutions and incubated at 37◦C for 1, 2, 3 and 4 h. Viral suspension plus equal volume of the medium without the nanoparticles was used as a control and incubated under the same conditions. The infectious titer of the virus was then evaluated through the cytopathic effect on the cells at the end point of the dilution. After 24 h, MDBK cells were infected with ten-fold serial dilutions of the viral material in a volume of 50 µl. Adsorption was carried out at 37◦C for 1.5 h, and 150 µl of a medium without serum was added. Control was made of non-infected cells. Plates in 5% CO_2 at 37◦C were maintained for 6 days until the pronounced cytopathic effect of the virus appeared. Virucidal effect was determined by reduction in the infectious titer of the virus by 50% or more, compared with the control. The results were expressed for three separate experiments. The statistical analysis was performed according to standard approaches with the calculation of the statistical error [standard deviation] using the Microsoft Excel 2007 software.

2.9.4.3 Antiviral assay for the conjugated extract with the gold nanoparticle

The MDBK cell line was purchased from the Cell Bank of the Chinese Academy of Sciences Cell Bank and cultured in RPMI medium containing 10% fetal bovine serum [FBS] and 1% penicillin-streptomycin. To observe the antiviral effect of nanoparticles, the three viruses were exposed to each nanoparticle suspension for 10 min and 60 min before infection of the MDCK cells. The antiviral effect of the nanoparticles was measured by WST-1 cytotoxicity assay through observing the optical density of treated cells at 450 nm [G.Biosciences. Cat. # 786-212, 786-857].

2.10 Statistical Analysis

All data are represented as means ± SEM of three replicates for each set. Comparisons were conducted using the Statistical Package for the Social Sciences software, version 17.0 [SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. Chicago: SPSS Inc]. Student's t-test and one-way analysis of variance [ANOVA] were used to evaluate the differences among groups. A P-value of < 0.01 or < 0.05 was used to designate a statistically substantial difference.

3. Results

3.1 Macroscopic examination

The 20 actinomycetes strains exhibited antibacterial activities against at least one of the tested bacterial pathogens using preliminary screening. The results are summarized in Table 1. Of all the 20 isolates, the best antagonistic actinomycete was selected for further studies. The potential isolate H1 exhibited highest activity against the five tested bacteria.

Morphological, cultural, and physiological characterizations of the selected isolate H1 were determined on the ISP media recommended by WAKSMAN6 and endorsed by the ISP7. Cultures were incubated for 14 days at 30°C. The color names used in this study were based on Color Standard [Nihon Shikisai Co., Ltd.]. Morphological and cultural characteristics of the isolate are shown in figure 1 and summarized in table 2.

3.2 Microscopic examination

Electron microscopy revealed that aerial mycelium was moderately short [40 to 60 pm in length], simply branched with spore-chains. Each spore-chain consisted of 20 or more spores and formed a coil with a few turns (Figure 2). Fragmentation of substrate mycelium revealed no sclerotium, sporangium nor flagellated spores. Spores were ellipsoidal to cylindrical, 0.5 to 0.6 by 0.8 to 1.2 pm in size, with smooth to slightly rough surfaces.

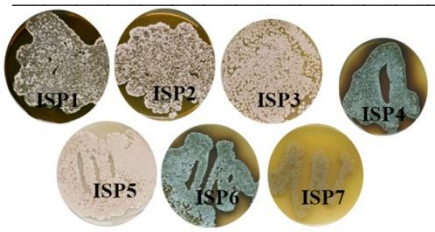

Figure 1: Morphological appearance of the isolate colonies on ISP1-7 media.

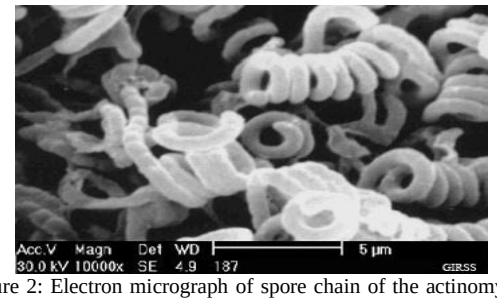

Figure 2: Electron micrograph of spore chain of the actinomycete isolate on yeast extract - malt extract agar, 10 days culture at 30°C. Bar represents 1 µm.

3.3 Molecular identification using 16S rRNA

509 base pair of DNA sequence were obtained using DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing. For DNA sequence similarities and phylogenetic analysis, sequence data was analyzed in the GenBank database by using the BLAST program available on the National Center for Biotechnology Information website [www.ncbi.nlm.nih.gov]. The unknown sequence was compared with the available sequences in the database to assess the DNA similarities [48]. The GenBank entry with the highest score from the BLAST search was downloaded. The result of PCR blasted with other bacterial sequences in NCBI showed similarity to the 16S small subunit rRNA. Edited sequences were used as queries in BLASTN searches [http://blast.ncbi.nlm.nih.gov/Blast.cgi] to determine the nearest identifiable match present in the complete GenBank nucleotide database. A bioinformatic tool, GeneDoc software, version 2.6.002, was used for more 16S rRNA gene sequence investigation. A total of 509 nucleotides of the partial sequence of *E. coli* species was 99% similar to the 16S ribosomal rRNA genes in other recorded strains of Streptomyces in National Centre for Biotechnology Information [NCBI]. The isolate was found to be similar to *Streptomyces avermitilis* strain 173267, Identities =99 %, Gaps = 0 %. (Figure 3 and 4).

For measuring antiviral activity of the crude extract of actinomycetes, virus titer was calculated before [V Titer] and after treatment [VTPT] with the crude extract in addition to virus/ crude extract log difference measured for the three viruses: HAV, Adenovirus and VSV (Table 3). When treated with Streptomyces crude extract [CE], results showed a decrease of 5.39, 6.0 and 4.28 post treatment compared to 5.8, 6.8 and 6.4 before treatment, indicating an activity percentage of 7.06, 11.7 and 33.12 with HAV, Adeno and VSV respectively. In the case of viral treatment with gold nanoparticles [GN], virus titer was decreased to 5.4,6.1 and 6.0 post treatment compared to 5.8, 6.8 and 6.4 before treatment, showing an activity percentage of 6.89, 10.29 and 6.25 with HAV, Adeno virus and VSV respectively. When treated with crude extract-nanoparticles conjugate [CNC], virus titer was decreased to 4.82, 5.0 and 3.8 compared to 5.8, 6.8 and 6.4 before treatment, Showing an activity percentage of 16.89, 26.47 and 40.62 with HAV, Adenovirus and VSV respectively.

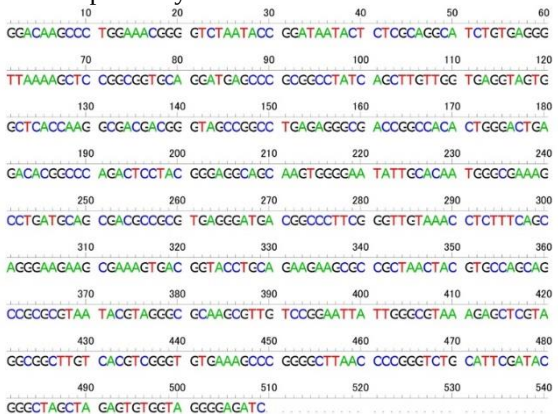

Figure 3: Nucleotide sequence of 509 bp of isolated 16S rRNA from Streptomyces isolate.

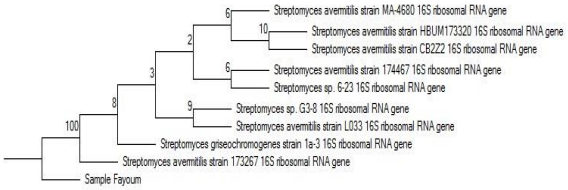

Figure 4: Phylogenetic tree for Streptomyces strain using the amplified 16S rRNA gene.

3.4 Evaluation of antiviral activity using cell culture and point assay

Interferon was used as a positive control to measure the antiviral activity in comparison with the three different previous treatments. Virus titer post treatment and virus/ interferon log difference was measured for the three viruses showing an activity percentage of 44.48, 54.11, and 46.56 with HAV, Adenovirus and VSV respectively. Activity percentage was calculated for all the treatments as shown in figures 5. To confirm that the antiviral activity of gold nanoparticles can be attributed to the inhibition of virus binding or fusion to the cells, a virus adsorption assay was performed [26]. One fusion inhibitor [Enfuvirtide] was included as control

specimen. Gold nanoparticles exhibited an IC50 of 0.43, 039 and 0.49 mg/mL with HAV, Adenovirus and VSV respectively, indicating inhibition the of binding to the cells. As expected, the fusion inhibitor inhibited virus adsorption. These results indicate that gold nanoparticles inhibit the initial stages of HAV, Adenovirus and VSV infection cycle with enhanced activity in conjugation with the *Streptomyces avermitilis* extract.

Table 1: Preliminary screening of actinomycetes twenty isolates for antimicrobial activity against five pathogenic bacteria.

	Test bacterial pathogens				
Isolate name	*Salmonella typhi* PTCC 1609,	*Staphylococcus aureus* ATCC 25923,	*Escherichia coli* PTCC 1533,	*Bacillus subtilis* PTCC 1156,	*Klebsiella pneumonia*
H1	+	+	+	+	+
H2	-	-	-	+	-
H3	+	-	+	-	-
H4	-	-	+	-	+
H5	+	-	-	-	-
H6	+	+	+	-	-
H7	-	-	-	-	+
H8	-	+	-	-	+
H9	+	+	-	-	+
H10	-	-	-	+	-
H11	+	-	-	-	-
H12	-	+	-	+	+
H13	-	-	+	-	-
H14	+	+	-	-	-
H15	+	+	-	-	-
H16	+	-	-	-	+
H17	-	+	-	-	+
H18	+	+	-	-	-
H19	+	-	-	+	+
H20	-	+	+	+	-

Table 2: Morphological and cultural characteristics of the isolate obtained with ISP 1-7 media.
A. The amount of vegetative growth and degree of formation of aerial mycelium are rated on a scale of excellent, good, moderate, poor, and none. B. The color codes and names used indicated are from the Color Harmony Manual, 4th ed. [6]

	Cultural characteristics		
Medium	A. Growth	B. Aerial mycelia	Diffusible pigment
Tryptone-yeast extract broth [TSP-1]	Good	Powdery Blackish-brown colored thick colonies	Black colored soluble pigments
[ISP-2]	Good	Thick powdery grey colonies	Slightly creamy- blackish colour,
Oatmeal agar [ISP-3]	Good	Powdery Creamish white-colored colonies	
Inorganic salt-starch agar [ISP-4]	Moderate	Powdery grey with waxy margin and convex surface.	Black colored soluble pigments
Glycerol asparagine agar base [ISP-5]	Good	Creamy thin colonies with striated surface	
Peptone yeast extract iron agar [ISP-6]	Moderate	Powdery grey thin colonies	Black colored soluble pigments
Tyrosine agar base [ISP-7]	Poor little mycelium growth.	Creamy lobe-shape, convex surface, and	

Table 3: Antiviral activity of four treatments on the three viruses in comparison to viral titer. Four treatments including interferon as a positive control, Streptomyces crude extract [CE], Gold nanoparticles [GN] and crude extract -nanoparticles conjugate [CNC] against three viruses HAV, Adenovirus and VSV are shown. For each treatment, viral titer post treatment [VTPT], as well as virus/ treatment log difference and activity percentage were measured.

Virus name	V titer	Crude extract CE			Gold nano particles GN			Crude extract conjugate CNC			Interferon		
		VTPT	Log Diff	Activity %	VTPT	Log Diff	Activity%	VTPT	Log Diff	Activity %	VTPT	Log Diff	Activity %
HAV	5.8	5.4	0.41	7.06	5.3	0.4	6.9	4.8	1.0	16.9	3.22	2.6	44.48
Adenovirus	6.8	6.0	0.8	11.7	6.1	0.7	10.3	5.0	1.8	26.5	3.12	3.7	54.11
VSV	6.4	4.3	2.2	23.12	6.6	0.4	6.3	3.8	2.6	40.6	3.42	3.0	46.56

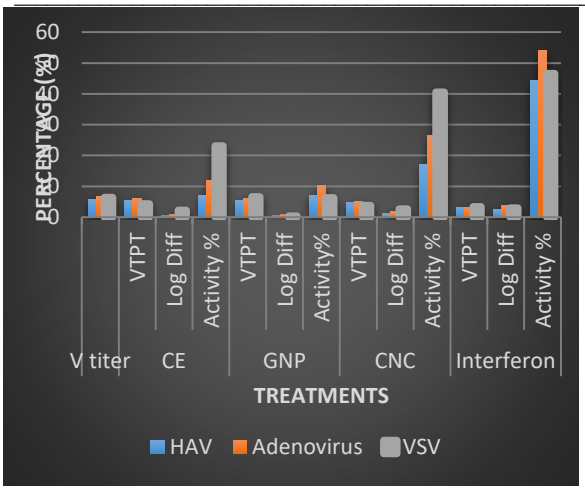

Figure 5: Evaluation of antiviral activity of four treatments on the three viruses in comparison to viral titer. Four treatments including interferon as a positive control, Streptomyces crude extract [CE], Gold nanoparticles [GN] and crude extract -nanoparticles conjugate [CNC] have been tested for antiviral activity on the three viruses HAV, Adenovirus and VSV. For each treatment, viral titer post treatment [VTPT], as well as virus/ treatment log difference and activity percentage were measured. The figure indicates that CE has an inhibitory effect on viral titer with the strongest action being towards VSV with 23.12% activity. CNC showed enhanced activity with more than twice as much activity as the CE.

4. Discussion

Though effective vaccines are available for most human viruses, viral mutants overcome the protection process in many cases. In addition, most if not all the currently used antiviral drugs have limitations and side effects [56]. Interferon has partial efficacy and a common incidence of opposing side-effects. Adefovir, entecavir, Nucleos[t]ide analogs, tenofovir, lamivudine are very effective as antiviral drugs, but long-term use often develops drug-resistance [57]. As a substitute, natural products have been continuously purified from plant and microbial origins to add to viral prevention and treatment due to their features of high diversity and specificity [57].

These gifted compounds have different mode of action by either obstructing viral antigens production or interruption of DNA replication. The exploration of new soils and habitats was recommended to screen for microorganisms able to produce bioactive compounds. In this context, the soil of Fayoum and Sharqiyah governorates, Egypt, have been used to screen for actinomycetes with promising antimicrobial activity. Here, we report isolation and molecular identification of a Streptomyces strain endowed with antiviral activity. The strain molecularly identified to be *Streptomyces avermitilis* has been tested against three viruses [HAV, Adenovirus and Vesicular stomatitis virus].

Gold nanoparticles have been proved to show promising activity in detection as well as inhibition of many viruses including HIV-1 [58]. Here, we investigated the antiviral action of *Streptomyces avermitilis* extract alone and in conjugation with gold nanoparticles against three viruses HAV, Adenovirus and VSV viruses in an attempt to enhance antiviral activity.

The main target for our study was based on the premised delivery of natural antiviral exudate produced by *Streptomyces avermitilis* to the site of viral replication in the host cell. We evaluated the use of old nanoparticles as a colloidal carrier system against three viruses in an antiviral assay in vitro. Our results revealed two main indications; Streptomyces extract had the highest antiviral activity percentages with VSV virus compared to Interferon which best affect Adenovirus. On the other hand, gold nanoparticles alone didn't exhibit advantage on the virulence of the three viruses. However, upon conjugation with the Streptomyces extract, noticeable increase has been detected in the antiviral activity percentage compared to the extract alone: with 58.22, 55.8 and 18.42 % increase against the three viruses HAV, Adenovirus and VSV viruses, respectively.

Nanoparticles, since they are not directly related to chemical interactions, give hope for the creation of drugs against which viruses cannot resist due to mutations. On the other hand, the effect of metallic nanoparticles on living organisms is not yet sufficiently studied. Therefore, when studying the antiviral properties of nanoparticles, one must bear in mind the problem of toxicity of nanoparticles. One of the ways to solve this problem is to use biologically synthesized nanoparticles.

Conclusion

We isolated and identified *Streptomyces avermitilis* as an antiviral producer with enhanced activity upon conjugation with gold nanoparticles against three viruses: HAV, Adenovirus and VSV viruses. Our study findings can be applied for characterization and emerging of new antiviral agents from the actinomycetes extracts.

Conflict of interest

The author declares there is no conflict of interest.

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