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RESEARCH ARTICLE

ANTIVIRAL ACTIVITY OF SECONDARY METABOLITES PRODUCED BY STREPTOMYCES SPECIES ISOLATED FROM EGYPTIAN SOIL.

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Abstract

Virus infections pose significant global health challenges, due to the emergence of resistant viruses. Therefore, it is imperative that safe and conventional antiviral drugs be developed. In the present study, Actinomycetes isolated form Egyptian soil samples were used to investigate the possibility of acquiring antiviral activity from their metabolites by testing against three selected virus models. Results revealed that the sixth fraction purified from isolate (H47/60) metabolites induced significant reduction of virus infectivity titer recording 4.13 ± 0.181log $_{(10)}$ / ml, 2.79 ± 0.110 log $_{(10)}$/ ml and 3.38± 0.185 log $_{(10)}$/ ml for HAV, HSV-2 and VSV respectively. Further evaluation of the antiviral activity of the metabolite against three types of viruses was attempted by conjugation to silver nanoparticles.

Introduction:-

Actinomycetes are unicellular, gram-positive bacteria that belong to the order Actinomycetales with highguanine and cytosine (G+C) content in their DNA (Pahari et al., 2016). To our day, most of the discovered bioactive compounds were produced from actinomycetes (Janardhan, 2014). A wide range of actinomycetes secondary metabolites have various medical importance, for example: they yielded antibiotics, anticancer drugs, insecticides, herbicides and antiviral drugs (Arasu et al., 2016, Grasso et al., 2016). Actinomycetes are found in many habitats, such as freshwater, sea water, cold and warm blooded animals and composts; however, soil is their most important habitat (Van Elsas and Smit, 1995).

Previous studies reported the isolation of antiviral metabolites from actinomycetes,as a natural active compound that could offer high chemical variations with novel lead structures than standard combinatorial chemical compounds (Kitazato, et al., 2007). Yakugaku Zasshi (2004) found that Streptomyces microflavus strain produced at least 13 fattiviracin derivatives (FV-1 to FV-13) which possessed potent activity against enveloped DNA viruses such as the herpes family, HSV-1, and VZV and enveloped RNA viruses such as influenza A and B viruses, and three strains of HIV-1 (Divakar, 2013).

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Nanotechnology has been used to develop nanoparticle-based targeted drug carriers (Falanga et al., 2011; Hallaj-Nezhadia et al., 2010). The use of nanoparticles can be extended to develop antivirals that act by interfering with the viral infection, particularly during attachment and entry. The action of bactericidal effect of Ag+ ions in silver nanoparticles depends on suppressing the respiratory enzymes and electron transport components in addition to the interference with DNA functions (Li et al., 2006). In the present study, Three virus types were selected to cover both DNA and RNA viruses; Hepatitis A, Herpes simplex virus-2 and Vesicular Stomatitis representing positive-sense single-stranded RNA virus, double-stranded, DNA and negative -sense single-stranded RNA respectively.

The antiviral properties of silver nanoparticles were studied on the three virus models alone and in combination with the most potent antiviral metabolites extracted from Egyptian actinomycetes isolates, under optimized cultural conditions.

Materials and Methods:-

Collection of soil samples:-

Sixty soil samples were collected from different Egyptian localities (Table 1). The samples were withdrawn from a depth of approximately 20 cm using scoops (Mathur et al., 2012). The collected samples were then placed in polyethylene bags and labeled. The samples stored in the refrigerator (2-8°C) till further testing.

Isolation and purification of actinomycete isolates:-

One gram of each soil sample was serially diluted in distilled water followed by inoculation on starch casein agar medium plates to isolate Actinomycetes. The plates were incubated at 35°C for 72hrs (Srinivasan et al., 1991). White pin-point colonies, characteristic of actinomycetes were selected and purified into starch nitrate agar plates. The selected strains were further purified by multiple streaking methods, then the purified actinomycetes colonies were scraped from the agar plate, dipped into Trypticase Soya Broth medium. The flasks were incubated at 28±2°C in a shaker incubator at 200 rpm for 5-7 days followed by centrifugation at 14000 rpm at 4°C for 15 minutes (Bosserman et al., 2010). The crude extract was monitored for its safety on Vero cell line using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-dipheyltetrazolium bromidefor (MTT) assay (Stockert et al., 2012).

Primary Evaluation of Metabolites for existence of Antiviral Activity:-

Cytopathic inhibition assay was used for primary screening of crude metabolites (Mosmann, 1983) using three virus models (Hepatitis A virus, Herpes simplex-2 and vesicular stomatitis virus) supplied from R & D sector, Vacsera-Egypt. Virus infectivity titers were $10^{6.66}$, $10^{6.00}$ and $10^{7.66}$ $TCID_{50}$/ mL respectively determined according to Reed and Muench (Reed and Muench, 1938).

Vero cells (2×10^5/ml) provided by the Tissue Culture Department (VACSERA)pre-cultured for 24h in a humidified 37°C incubator with 5% CO2 in a 96-well microtiter plate. The safe concentration of extracted metabolite was used as 100µL/ml to treat pre-cultured 96-well Vero plate for 24h, untreated control plate was included. Treatment media was decanted and each of selected viruses under study was dispensed regarding virus dilution (10^{-1} - 10^{-8}) as 100 µL/ well. Infected cells were monitored daily using inverted microscope to record the wells that showed cytopathic effect (CPE), infectivity titers were determined according to Reed and Muench (Reed and Muench, 1938).

The isolate showing maximum antiviral effect was subjected for re-cultivation and optimization of culture conditions for antiviral metabolites extraction and identification.

Identification of selected isolate characteristics:-

The isolate that exhibited the most potent antiviral metabolite was identified on the basis of morphological, cultural and physiological and biochemical behaviors. Morphological and cultural characteristics were studied on different media following the instructions given by the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966). Different media were used including starch casein agar medium; ISP-2; ISP-3; ISP-4; ISP-5; ISP- 6 and ISP-7 (Shirling and Gottlieb, 1966).

Macroscopic and microscopic identification of the isolate:-

Macroscopic identificationincludes determination of aerial mass color, the color of substrate mycelium and presence of soluble colors other than melanoid pigments (Li et al., 2016), whereas microscopic identification of the isolate determines the micro-morphological characteristic of spore-bearing hyphae, spore morphology and surface (Shirling and Gottlieb, 1966).

Physiological characterization:-
Using carbon utilization test (Oskay et al., 2004); sodium chloride tolerance reaction (Sengupta et al., 2015); growth temperature and pH range; (Arul Jose et al., 2014).

Biochemical characterization:-
Using gram staining (Duraipandiyan et al., 2010); IMViC test (MacFaddin, 2000); H2S production test; motility test (Schau, 1986); Gelatin hydrolysis test; Nitrate reduction test (Murray B and Jorgensen, 2007); Catalase production test (Chun et al., 2000); Casein hydrolysis test (Wehr et al., 2004); Ornithine decarboxylase test (MacFaddin, 2000); Starch hydrolysis test (Garcia, 2010).

Phylogenetic identification:-
Molecular identification and Phylogenetic analysis done in Global institute research services solution (GIRSS) for isolate under study include: genomic DNA extraction (Miller et al., 1988), using Wizard Genomic DNA purification Kit (Promega), PCR amplification of the 16S ribosomal DNA using eubacterial universal primers F27 with the sequence 5'-AGAGTTTGATCMTGGCTCAG-3' and R1492 with the sequence 5'-TACGGYTACCTTGTTACGACTT-3' (Heuer et al., 1997), The PCR product was examined using 1.5% agarose gel in TBE buffer (pH 8.5), the PCR product was purified using PCR purification kit (Qiagen, Germany) and sequencing of the PCR products using an ABI PRISM 310 DNA sequencer and ABI PRISM Big Dye Terminator Cycle Sequencing in Vacsera sequencer unit (Frank et al., 2008). Sequences were then analyzed using Blast program to assess the DNA similarities (Altschul et al., 1990).

Optimization of culture Conditions of Selected Isolate:-
Optimization of culture conditions for enhancing metabolite antiviral Activity:-
The antiviral activity was enhanced by manipulating selected physical parameters, whereas pH levels of the basal media were adjusted from 5 to 10, followed by adjusting the optimum temperature for growth ranging from 20˚C to 50˚C and finally the effect of incubation period (Stockert et al., 2012).

After selecting optimum conditions for the isolate growth the cell-free filtrate was mixed with equal volume of ethyl acetate (1:1 V/V) for extraction of the bioactive compound. The lower aqueous phase was discarded and upper organic phase was concentrated in a in a rotary evaporator at 40°C for 24 hours to obtain the crude extract (Gebreyohannes et al., 2013). The crude extract was then purified using column chromatography. Finally, obtained fractions were screened for antiviral activity; the fraction possessing antiviral activity was further purified and analyzed in Micro Analytical Center Cairo University.

Storage of the isolate: As glycerol stocks and lyophilized form (Kieser et al., 2000).
Partial characterization of the antiviral Metabolite:-
Performing spectral studies on purified band possessing antiviral activity as UV, IR, mass spectrum and ^{1}HNMR; all measurements were performed in Micro Analytical Center Cairo University.

Synthesis of Silver Nanoparticles:-
The silver nanoparticles (AgNPs) were prepared by chemical reduction method according to Ratyakshi and Chauhan (2009).

Solution A was prepared by dissolving 0.17 g AgNO3 in 100 ml distilled water to prepare 1 mM of AgNO3 stock solution; whereas solution B prepared by dissolving 0.3 g sodium citrate in 100 ml distilled water to prepare 1mM of trisodium citrate. Ten milliliter of solution A was diluted to 50 ml in a beaker and heated up while stirring at 400 rpm, after boiling 1ml of solution B was added and left on the heater till the formation of clear golden yellow color of AgNPs (Ratyakshi and Chauhan 2009). All Nanoparticles characterization (Table 4) and preparation processes were conducted at Agriculture research center, in the Central Lab of Nanotechnology and advanced materials.

Cytotoxicity assay ofsilver Nanoparticles:-
The cytotoxic activity of two-fold serial dilution of AgNPs against Vero cell lines incubated for 48 h in 5% CO_2 humidified incubator. Cell viability was evaluated by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay (Stockert et al. 2012).

Evaluation antiviral activity of purified fraction combined to AgNPs:-
Pre-cultured 96-well Vero plate treated with 100 µL/ml of purified antiviral extract combined to AgNPs safest concentration for 24hr, untreated control plate was included. Treatment media was decanted and virus models were dispensed regarding virus dilution (10^{-1} - 10^{-8}) as 100 µL/well. Infected cells were monitored daily for 7 days and infectivity titer was determined according to Reed and Muench (1938) as previous.

Statistical Analysis:-
All experiments were performed in triplicate, three independent experiments were conducted and the data was presented as mean ± SD. The two tailed t-test was used to evaluate antiviral activity related to reduction in virus infectivity titer. The results were considered statistically non-significant when p-value was > 0.05.

Results:-

Identification of selected isolate (H47/60) Characteristics:-
Characteristics of the isolate on starch nitrate agar medium (Fig 1) showed that the isolate as gram positive, possessing an earthy odor of actinomycetes.

Morphological characteristics of the isolate on different media recommended by the International Streptomyces Project (ISP) were shown in (Table 2), whereas physiological and Biochemical characteristics of isolate were shown in (Table 3). Microscopic examination of isolate (Fig 2) showed spore chains morphology under scanning electron microscope.

Phylogenetic identification of the isolate:-
The PCR product examined using 1.5% agarose gel was of 1500bp. Alignment of H47/60 isolate was identified as *Streptomyces Avermitilis* using Blast program (Query: 81873) and the sequence (436 bp) was deposited in gene bank database under accession number KY778695. The phylogenic tree of the isolate was shown in (Fig 3) as identified using DNA lasergene software.

Optimization of culture conditions for enhancing metabolite antiviral Activity:-
Streptomyces isolate (H47/60) showed significant increase in antiviral metabolite production at PH falling between 7 and 8 , incubation temperature falling between 25 ^{o}Cand 30 ^{o}C and six to seven days incubation period.

Cytotoxicity and antiviral effect of AgNPs:-
Cytotoxicity of synthesized AgNPs against Vero cells showed 87% cell death at concentration 1000 µg/mL and the inhibitory concentration 50% (IC50) was recorded at 62.5 µg/mL. In the meantime, the safe concentration was 15.6 µg / ml and was used to evaluate the antiviral potential but was found to induce mild decrease in virus titer.

Antiviral activity of isolate extract and silver nanoparticles:-
Primary screening of Suez isolate H47/60 revealed reduction of test viruses infectivity titer. After optimization of isolate culture characteristics,the bioactive compounds from crude ethyl acetate extract were purified using Silica gel column chromatography according to (Afifi et al. 2012). Eight eluents were collected from the crude extract sample, antiviral activities of these eluents were performed, the sixth fraction (fraction no.6) showed noticeable antiviral activity. In mean while purified fraction obtained after optimization of culture conditions alone and in combination to silver nanoparticles exhibited noticeable reduction of virus infectivity titer (table 5).

Discussion:-

Vaccination policy is considered as a preemptive first line of defense against most viral infections by developing white blood cells that could combatthe introduced virus and acquire herd immunity (Heymann and Aylward, 2006).).Despite vaccines successes, there are some minor complications (i.e some allergic reaction, immunocompromised individuals are not recommended to use live-attenuated vaccines) (Omer et al., 2009).

Vaccines are considered ineffective on unstable viruses and with patients who has already been infected. These disadvantages were the idea behind modern antiviral drug design to reduce the possible likelihood of various side effects. The strategy of designing drugs that could prevent viral entry or inhibit virus uncoating is of great importance (Bishop, 1999). In our study we isolated actinomycetes from Egyptian soil sample; primary screening of Suez sample (47/60) revealed a noticeable reduction of test viruses infectivity titer. This could suggest that the

extracted metabolite had the ability to interfere with virus ability to infiltrate a target cell (Vero cell line). Several researchers were able to isolate antiviral metabolites from actinomycetes. Tsunakawa et al., 1992 isolated a new actinomycete strain, *Amycolaptosis Orientalis* No.Q427-8(ATCC53884) from a soil sample collected in Maharashtra state, India, which produced a complex of new antiviral antibiotics. The quatromicins structural studies suggested that they are a novel type of molecules unrelated to any known antibiotics. Each component of quatromicin exhibited antiviral activity against herpes simplex (Divakar, 2013).

Alignment ofthe 16S rRNA gene identified theisolate as *Streptomyces Avermitilis*. *Streptomyces Avermitilis* was recorded as industrial microorganism that produce diverse secondary metabolites like macrocyclic lactones, milbemycin, and avermectins (Komatsu et al., 2013).

Manipulating physical parameters of the culturing conditions was of great value in enhancing antiviral activity. Our result agreed with other researches including Bundale, S. et al., (2015) who developed an efficient fermentation process for the production of secondary metabolites from Streptomyces species by adjusting several cultivation parameters like pH, incubation period and temperature (Bundale et al., 2015).

AgNPs have been reported to possess anti-fungal, anti-inflammatory and anti-viral activity; Remya, RR. et al., (2015) proved that silver nanoparticles exerted antiviral activity against HIV-1 at non-cytotoxic concentrations and that the size and dose concentration of nanoparticles play an important role in inducing cytotoxicity (Remya et al., 2015). In his study on the cytotoxicity of AgNPs recorded 90.5% and 89.7% cell death against MCF-7 and Vero cell lines at 1000 mg/mL. The inhibitory concentration 50% (IC50) against MCF- 7 and Vero cell lines were observed at 7.19 mg/mL and 66.34 mg/mL respectively. These findings showed high similarities with our study, in mean time antiviral activity of silver nanoparticle at low concentration may be attributed to their activity at an early stage of viral replication (entry or fusion) by inhibiting the interaction between gp120 and the target cell membrane receptors (Lara et al., 2010).

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Table 1: Showing localities and numbersof isolates under study.

Cairo	11 samples
Assiut Governorate	5 samples
Kaliobeya	14 samples
Coastal Mediterranean area	10 samples
Kafr El Sheikh	4 samples
Suez	6 samples
Damietta	3 samples
Port Said	7 samples

Table 2:- Morphological characteristics of the isolate H47/60 on ISP Media.

Type of Medium	Growth	Color of aerial mycelium	Color of substrate mycelium	Production of soluble pigment
ISP 2	Good	green grey	ochre brown	ochre brown
ISP 3	Good	green grey	ochre brown	none
ISP 4	Good	green grey	ochre brown	none
ISP 5	Good	green grey	ochre brown	ochre brown
ISP6	Good	None	ochre brown	none
ISP 7	Good	green grey	ochre brown	ochre brown

Table 3:-Physiological and biochemical properties of isolate

Physiological characteristics		**Biochemical characteristics**	
Carbon source utilization		Indole	positive
Glucose	+	Voges-Proskauer	positive
L-inositol	+	citrate utilization	positive
D-fructose	+	Gelatin	positive
Raffinose	+	Starch	positive
L- arabinose	+	casein hydrolysis	positive
D-mannitol	+	peptonization of milk	positive
Rhamnose	+	nitrate reduction	positive
Sucrose	−	H_2S production test	positive
Cellulose	−	Motility	negative
D-xylose	−	Methyl-red	negative
Sodium chloride tolerance test		ornithine decarboxylase	negative
2% w/v NaCl	+	urease test	negative
5% w/v NaCl	+		
7% w/v NaCl	−		
10% w/v NaCl	−		
Growth temperature range			
20°C	+		
25°C	++		
30°C	++		
35°C	+		
40°C	+		
50°C	−		
Growth PH range			
5	+		
7	++		
8	++		
9	+		
10	−		

- **For Carbon source utilization test:** (+): indicated that growth on tested carbon was significantly better than on basal while negative (−): indicates that the growth was similar to or less than growth on basal medium without carbon**.**
- The following grades were used to indicate the extent of growth for sodium chloride tolerance test, different temperature and PH ranges, whereas: −: no growth, +: moderate growth, and ++: good growth.

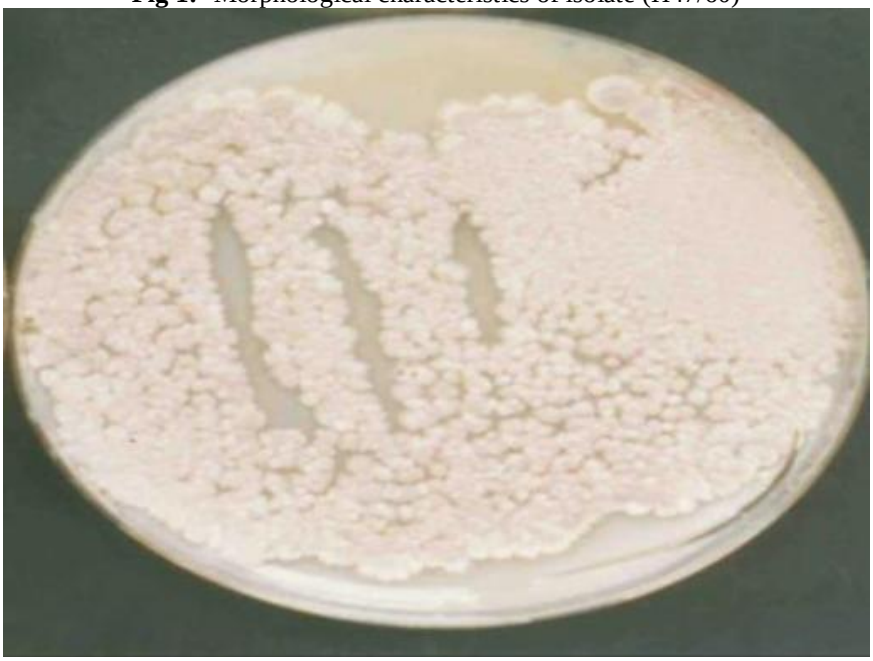
Table 4:- Silver nanoparticles characterization.

High-Resolution Transmission Electron Microscope (HRTEM)	Showed, spherical shaped particles with average size 5 ± 3 nm
Zeta sizer nano ZS	Showed the average particle size 5 nm and net charge -38.4 mV
Fourier Transform Infrared Spectroscopy (FTIR)	Showed a broad absorption band at 3425 cm^{-1} assigned for O−H stretching vibration. The presence of the sharp peak at 2922 cm^{-1} assigned to C–H stretching vibration. Also, sharp and strong absorption bands of N-H and C=O group appeared at 1636 cm^{-1} and 1429 cm^{-1} respectively, where 1058 cm^{-1} was assigned to C-N stretching
Spectrophotometer	Showed sharp characteristic absorption peak at 415 nm which corresponding to the plasmonic absorption band of spherical shaped silver nanoparticles

Table 5:- Screening of isolate metabolite antiviral activity.

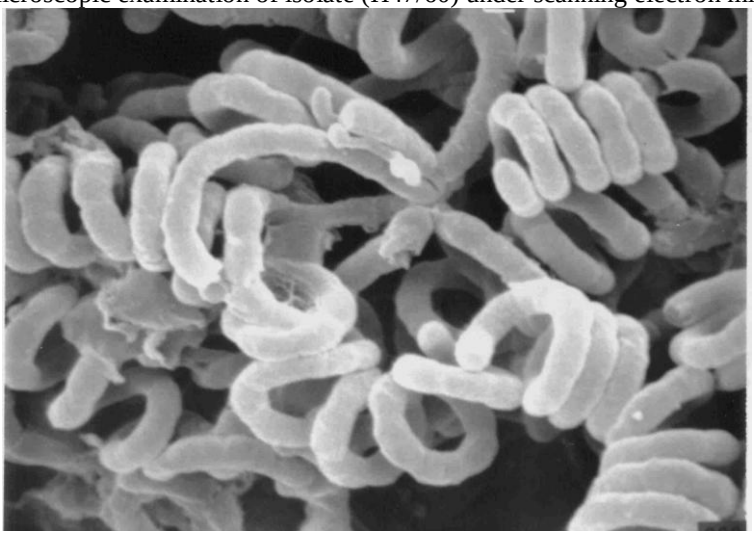
Antiviral activity	HAV	HSV-2	VSV
Silver nanoparticles	**0.53± 0.07 log $_{(10)}$ / ml**	**0.6 ± 0.042 log $_{(10)}$/ ml**	**0.69 ± 0.095 log $_{(10)}$/ ml**
Primary metabolite screening	**2.41±0.13 log $_{(10)}$ / ml**	**1.43±0.87 log $_{(10)}$/ ml**	**2.17± 0.022 log $_{(10)}$/ ml**
Purified fraction	**4.13± 0.181 log $_{(10)}$ / ml**	**2.79 ± 0.11 log $_{(10)}$/ ml**	**3.38 ± 0.18 log $_{(10)}$/ ml**
Purified fraction combined to silver nanocomposite	**4.58 ± 0.07 log $_{(10)}$ / ml**	**3.24 ± 0.07 log $_{(10)}$/ ml**	**3.99 ± 0.08 log $_{(10)}$/ml**

Fig 1:- Morphological characteristics of isolate (H47/60)



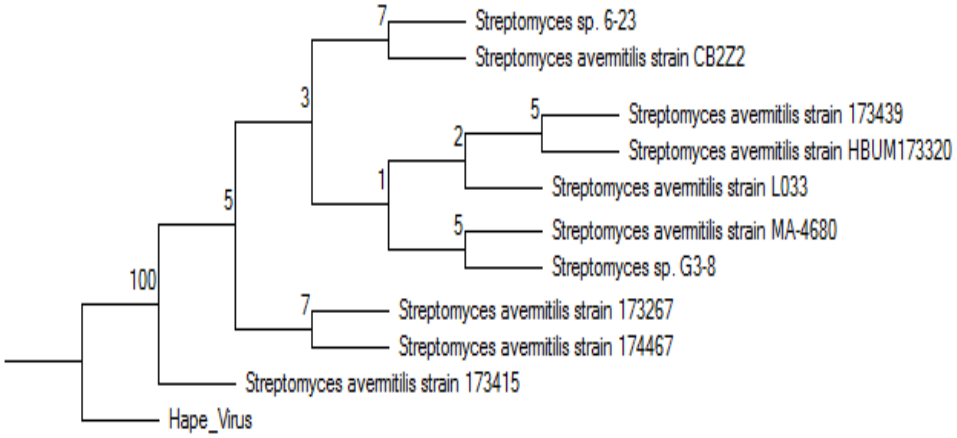
The colony on starch nitrate agar medium is whitish grey in color, mycelium was aerial and white in color; the substrate mycelium was light grey; colony elevation was raised with wrinkled surface and tenaciously adhering to the medium.

Fig 2:- Microscopic examination of isolate (H47/60) under scanning electron microscope.



Microscopic examination of spore chains appeared smooth with simple unbranched sporophore.

Fig 3:- The phylogenic tree of the isolate (H47/60)



The phylogenic tree obtained by using the Tree view program identified the isolate as *Streptomyces Avermitilis* strain.

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