A Swamp Forest Streptomyces sp. KF15 with Broad Spectrum Antifungal Activity against Chilli Pathogens Exhibits Anticancer Activity on HeLa cell line

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Abstract

During the investigation, soil actinomycetes were isolated from Kathlekanu swamp forest and the crude ethyl acetate extract from the potent isolate KF15 was analyzed with GC-MS and HPTLC to identify bioactive metabolites. The crude extract was examined for *in-vitro* antifungal activity on pathogens of chilli; MTT cytotoxicity assay was performed against HeLa cell line to determine the anticancer potential. The isolate *Streptomyces* sp. KF15 exhibited antagonistic activity against fungal pathogens by inhibiting growth and altering growth pattern with increased antimicrobial activity in dose-dependent manner. GC-MS revealed many bioactive compounds and HPTLC depicted metabolite fingerprint. The IC_{50} of 99.85µg/ml indicated the high potential of KF15 extract to prevent proliferation of HeLa cells. Therefore, the findings of this study indicate that, the crude extract from *Streptomyces* sp. KF15 contains antifungal and anticancer metabolites; further study on purification could help in controlling many fungal diseases as well as cervical cancer in humans.

Introduction

The research on antibiotics from its first discovery by Alexander Fleming in 1928 to the present day has been an exciting and continuously developing research area. The revolution in the early fifties witnessed the discovery of almost all major groups of antibiotics. The major numbers of antibiotics discovered in this duration were extracted from *Streptomyces* species, representing about more than 75% of the total compounds discovered (Berdy, 2012). And also, this interval marked the beginning of cytotoxic, antiviral and enzyme-inhibitory metabolites discovery. But the constant evolution of bacteria to acquire antibiotic resistance has limited the use of most of the known antibiotics against the multidrug-resistant (MDR) pathogenic microorganisms (Raja and Prabakarana, 2011).

The increasing resistance of pathogenic microbes and the scarcity of potent antibiotics to battle against the infectious diseases has been the major concern worldwide (Kitouni et al. 2004). In spite of the prolonged inventory of accessible antibiotics in the market, antifungal agents are a miniature but remarkable group of drugs having a decisive role in the control of pathogenic fungi that cause life-threatening diseases in plants and animals, including humans (Thakur et al. 2007; Kekuda et al. 2015). Most of the currently using clinical antibiotics are either first-hand natural products or semisynthetic derivatives from microorganisms like actinomycetes or fungi. Major antibiotic products, comprising cephalosporins, polyketides, erythromycin and its derivatives, peptides, tetracyclines, vancomycin, fatty acids, and rifamicin were brought to light through actinomycete whole-cell antibacterial screening procedures (Baltz, 2007; Raja and Prabakarana, 2011).

Actinomycetes are the members of phylum *Actinomycetales* representing the major taxonomic unit within the bacterial kingdom. Actinomycetes are aerobic, gram-positive, and have DNA with more than 50% guanine and cytosine; they form immensely branched substrate and aerial mycelia (Chakraborty et al. 2021; Pallavi et al. 2021). The aerial mycelium forms chains of three to many spores at maturity and the colonies are discrete and lichenoid, leathery, powdery or butyrous in nature. Actinomycetes can produce a

vast variety of pigments accountable for the colour of the vegetative and aerial mycelia (Goodfellow et al. 2012).

Actinomycetes have diversified biological activities due to their capacity to produce variety of secondary metabolites. There are reports stating that various bio-active secondary metabolites from actinomycetes are highly useful in biomedical and pharmaceutical fields (Sharma et al. 2019; Nayaka et al. 2020). Apart from antibiotics, actinomycetes are recognized for the production of antitumour, antivirals, pesticide, insecticide, and antiparasitic agents that are proven to be effective against a wide range of pathogens and used extensively for clinical purposes (Hui et al. 2021). Therefore, the search for novel actinomycetes from unexplored regions or the habitats that has no/less human activity may lead us to the utilization of natural products from microbial origin with their diversified uses in industrial and clinical applications (Tan et al. 2019).

Capsicum annuum L. (Chilli) belongs to family *Solanaceae*, and is one of the world's predominant vegetable crops nurtured for its multiple uses as spice flavouring and food colouring agent. Apart from use as spice, chilli is used as antioxidants, capsaicin present in chilli helps to reduce obesity as well as diabetes. Furthermore, several vitamins and nutrients contained in chilli help to protect eyes, skin, and hearts from dysfunctions (Sonawane & Shinde, 2021). According to food and agriculture data from the last decade, India is the largest producer of chilli contributing about 38% of total production globally. Chilli is being heavily imported by many European and American countries and the demand is increasing year by year. However, a major hindrance to chilli production is diseases from pathogenic fungi and bacteria. The most common fungal pathogens infecting chilli during growing and post-harvesting season include *Botrytis cinerea*, *Fusarium* spp., *Colletotrichum* spp., *Sclerotium rolfsii*, *Alternaria* spp., *Phytophthora capsici* and *Aspergillus* spp. (Krasnov & Ziv, 2022).

Cancer is a significant public health concern worldwide and has second highest mortality rate by affecting all the age groups. It is a severe disease and has estimated deaths of more than 600,000 people last year in United States alone (Siegel et al. 2022). Cervical cancer is the fourth most common cancer in women worldwide and HeLa cell line is the oldest cancer cell line cultured; the highest percentage (58.2%) of cases reported from Asia and more than 400,000 women died of cervical cancer in 2020 (Singh et al. 2022). Naturally occurring compounds and bioactive products from microbes and plants are widely used for the therapy of numerous kinds of cancer as the adversity of radiotherapy and chemotherapy are increasing. Among microorganisms, *Streptomyces* species are known to harbor various bioactive metabolites that are rapidly used for cancer therapy (Kumar et al. 2021).

The current investigation is encouraged by the ability of *Streptomyces* sp. extract to control fungal diseases of plants and the potential of secondary metabolites from *Streptomyces* species as antitumor agents against cancer cell lines. Therefore, the present study was aimed towards the search for bioactive secondary metabolites from swamp soil *Streptomyces* sp. with antifungal and anticancer activity.

Material And Methods

Collection of soil samples

Twenty soil samples were gathered from Myristica swamp of Kathlekanu reserved forest (14.271382ºN, 74.743528ºE), Siddapura, Uttara Kannada. The soil samples were taken from depth of about 10 cm and collected through pre-monsoon, monsoon and post-monsoon seasons in numbered sterile zip-lock bags. The samples were brought to the laboratory and kept at 4℃ for further study.

Pre-treatment of samples

The soil samples were exposed to soil pre-treatment to eliminate the unwanted microbes and debris; for the easier isolation of actinomycetes. The soil samples were dried at room temperature for 7 to 8 h; and the larger soil particles were removed by sieving. The heat-treatment of soil at 70℃ for 20 min in a hot air oven was done to arrest the growth of fungi, gram negative bacteria and other undesirable microbes.

Pathogenic fungi and bacteria used

Pathogenic bacteria such as, *Staphylococcus aureus* (MTCC6908), *Escherichia coli* (MTCC40), *Shigella flexneri* (MTCC1457), *Enterococcus faecalis* (MTCC6845), and human pathogenic yeast such as, *Candida albicans* (MTCC227), *Candida glabrata* (MTCC3019) were purchased from NCIM, Pune, India. The fungal phytopathogens were procured from University of Agricultural Sciences, Dharwad and sub-cultured on potato dextrose agar (PDA) media for further use.

Isolation of soil actinomycetes

About 1 g each pre-treated soil samples were mixed with 10 ml of sterile distilled water to make uniform suspension and serially diluted up to 10^{-6}. About 100 µl suspension from each dilution was uniformly spread on the surface of different culture media like starch casein agar (SCA), starch agar (SA), actinomycetes isolation agar (AIA), international *Streptomyces* project (ISP)-2, and ISP-6 media. Every time, the media was added with 25 µg/ml of antifungal (amphotericin-B) and antibiotic (streptomycin) to prevent the growth of fungi and bacteria, respectively. All the plates were kept for incubation at 30℃ for 7 days until the colonies were emerged.

Primary screening for antagonistic activity

The primary screening of isolates was performed by following perpendicular streak method. The actinomycete isolates were grown along the diameter of the petri-plate with Muller-Hinton agar medium as straight lines and incubated at 30℃ for a week. Later, selected pathogenic bacteria were streaked perpendicularly to the actinomycete isolate; after incubation for 24 h at 37℃, the antagonistic activity of actinomycete isolates were recorded to select potential isolates with bioactivity.

Production and extraction of secondary metabolites

The actinomycete isolates displaying increased inhibition against pathogens in primary screening were selected for further study. The potential isolates were cultured in 500 ml of starch casein (SC) broth at 32℃ for 21 days at submerged fermentation condition. After incubation, Whatmann No. 1 filter paper

was used filter the culture broth to eliminate mycelial mass and other debris. Finally, the mixture of cell-free supernatant and ethyl acetate in equal volume (1:1) was prepared and kept in a separating funnel for 24 hr to get ethyl acetate extract.

Characterization of potent isolate

Morphological characterization

The substrate and aerial mycelia colour, colony shape and pigmentation of isolate KF15 were recorded. The scanning electron microscope (SEM) analysis of isolate KF15 was also done to observe surface morphology of spore and arrangement of spore chain with the aid of a SEM instrument (JSM-IT500, JEOL, Japan).

Molecular characterization

The identification of isoalte KF15 by molecular approach was conducted by sequencing the 16S rRNA gene. The 16S ribosomal DNA was extracted by using HipurA *Streptomyces* DNA purification kit (MB527) according to the instructions manufacturer. To carry out the 16S rRNA gene sequencing, a thermal cycler (Applied Biosystems 2720, Thermal Cycler, USA) was employed along with forward (27F) and reverse (1492R) primers. The obtained PCR amplicons were validated by 1% agarose gel electrophoresis with a reference DNA ladder of 1 kb size. The 16S rRNA gene was subjected to nucleotide BLAST analysis and deposited at Genbank via NCBI website. Finally, the neighbour joining method was used to construct phylogenetic tree with the aid of MEGA7 software.

Biochemical characterization

The biochemical characterization was performed to study the nutritional and metabolic capabilities of the isolate KF15 and the tests were done by employing Vitek BCL card (VITEK test kit, Biomerieux, USA). The kit contains 64 microwells loaded with different substrates for the characterization of selected isolates.

Characterization of ethyl acetate extract

Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of ethyl acetate crude extract from isolate KF15 was examined to recognize the functional groups in the range of 400 cm^{-1} to 4000 cm^{-1} using a FTIR spectrophotometer (Nicolet 6700, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

High performance thin layer chromatography (HPTLC) fingerprinting

The ethyl acetate crude extract obtained from isolate KF15 was subjected to HPTLC metabolite fingerprinting analysis to determine the banding pattern of bioactive secondary metabolites. For HPTLC analysis, the dried ethyl acetate crude extract was used and re-dissolved in 0.5 ml of ethyl acetate solvent before analysis. Later, 4, 8, and 12 µl of extracts were spotted as separate samples on a pre-coated silica gel F_{254} aluminum plates. The plates were developed inside a twin-trough chamber with mobile phase containing the mixture of toluene: ethyl acetate: methanol: acetic acid, in the ratios of 5:3:1:0.5 by volume. Further, the plates were subjected for visualization under short UV (254 nm), long UV (366 nm); the derivatisation was done by using vanillin/sulphuric acid reagent and scanned under UV to visualize the band pattern.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of ethyl acetate extract from isolate KF15 was performed with the help of gas chromatograph and mass spectrometer (GCMS-QP2010 SE, Shimadzu, Kyoto, Japan) operating in electron ionization mode. About 2 ml ethyl acetate crude extract was introduced into an EC-5 column. Helium was used as inert carrier gas and a constant flow rate of 2 ml/min was set for the analysis with the increase in temperature at a rate of 20℃ per min up to 450 ℃.

Antimicrobial activity

The dried ethyl acetate extract obtained was dissolved in dimethyl sulfoxide (DMSO) to determine the antimicrobial potential of the bioactive secondary metabolites produced by the selected isolates. For antimicrobial activity, microbial pathogens were swabbed on Mueller-Hinton agar media to perform agar well diffusion technique; the working (stock) solution of 1 mg/ml of dried ethyl acetate crude extract was prepared and different volumes such as, 25, 50, 75 and 100 µl were poured in separate wells of 6 mm diameter. About 25 µg/ml of streptomycin and amphotericin B were taken as standards against bacteria and fungi, respectively. Finally, the clear zone formed around each well after 24 hrs of incubation at 37℃ was recorded in millimetre as zone of inhibition.

Antifungal activity

The primary screening for antagonistic activity against phytopathogenic fungi was determined by duel culture technique. The perpendicular streak technique was performed by streaking *Streptomyces* sp. KF15 in the centre of the plate along the diameter and on the sides, fungal pathogens such as *Fusarium* spp., *Colletotrichum* spp., *Aspergillus niger*, and *Sclerotium rolfsii* were inoculated. All the plates were kept for incubation at 28℃ for 4 days to examine antagonistic nature of KF15 by the formation of clear zone along the parallel diameter. The growth curve analysis and morphological changes of *Fusarium oxysporum* treated with KF15 crude extract was scrutinized by UV-Vis. spectrophotometer and SEM analysis. About 1 mg of extract was dissolved in 1 ml of 10% DMSO and combined with 10 ml of PDB; 1 ml of 10% DMSO was blended with 10 ml of PDB in a separate tube as a control. The tubes were inoculated with a loopful of *F. oxysporum* mycelia and the initial optical density (OD) was measured at 405 nm using spectrophotometer (UV-9600A, METASH Instruments, Shanghai, China). Then, the tubes were incubated at room temperature and OD was measured at 24 hr intervals up to 7 days to obtain

growth curve pattern. To detect morphological changes, separate eppendorff tubes with 1 ml of 10% DMSO and 900 µl of 10% DMSO with 100 µl crude extract from the stock was used. After 24 hr incubation, the mycelia content was observed under SEM to examine morphological changes.

Evaluation of anticancer activity by MTT assay

To determine the tumor suppressing capacity of KF15 extract on human cervical cancer (HeLa) cells, the HeLa cell line was acquired from National Centre for Cell Sciences, Pune, India and sub-cultured on DMEM-High Glucose media (#AL111, Himedia) containing 2 mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) and 10% fetal bovine serum (GIBCO, USA) medium. During the MTT assay, negative control indicated medium with cells but without the extract and medium with cells and 25 µg/ml of doxorubicin (#PHR1789, Sigma) indicated positive control. The different concentrations of KF15 extract from 25 to 125 µg/ml were poured into respective wells and incubated in a humidified 5% CO_2 atmosphere (Healforce, China) for 24 h at 37°C. After that, the used-up media was drawn out and the MTT reagent (# 4060 Himedia) was added and incubated for further 3 h. Then, about 100 µl of DMSO (#PHR1309, Sigma) was added to solubilize formazan crystals. Finally, the optical density was measured at 590 nm and the percentage growth inhibition was calculated and results were expressed as IC_{50} values using dose-response curve.

Results

Isolation and selection of actinomycetes

Total 31 actinomycete isolates were isolated from Kathlekanu swamp soil samples and were differentiated based on colony structure, mycelia colour and pigmentation. Then, the suspected isolates were sub-cultured repeatedly on petri dishes containing different culture media, but the best growth was observed in SCA media. The isolates were designated as KF1 to KF31 and maintained in SCA media slants for further study. For the selection of potent isolates, perpendicular streak technique was used and the antagonistic capability of the isolates was recorded (Table 1). The isolate KF15 was found out to be the most potent isolate among the 31 isolates, and exhibited considerable antagonistic potential against all of the pathogenic microbes used; therefore, isolate KF15 was employed for further analyses.

Table 1
Primary screening of isolates for antagonistic activity

Isolate	*S. aureus*	*E. faecalis*	*E. coli*	*S. flexneri*	*C. albicans*	*C. glabrata*
KF1	++	++			+	+
KF2	+	++	++			
KF3	+			+	+	+
KF4			+	+	+	+
KF5	++			+	+	+
KF6	++	++	+	+		
KF7			+	+	+	+
KF8	+	+			+	+
KF9	+	+	+	+	+	+
KF10	+	+	+	+		
KF11			+	+	+	+
KF12	++	++			+	+
KF13	+	+	+	+	+	+
KF14	+	++	+	+		
KF15*	**+++**	**+++**	**++**	**+++**	**+++**	**++**
KF16	+	+	+	+	+	+
KF17			+	+	+	+

KF18	**++**	**++**	**+**	**+**	□	□
KF19	+	+	+	+	+	+
KF20	+	+	+	+	+	+
KF21	+	+	□	□	□	□
KF22	+++	+++	+++	++	++	++
KF23	++	++	+	+	□	□
KF24	+	+	□	□	□	□
KF25	+	+	+	+	□	□
KF26	+	+	□	□	+	+
KF27	+	+	□	□	+	+
KF28	□	□	+	+	□	□
KF29	+	+	++	++	+	+
KF30	+	+	+	+	+	+
KF31	+	+	+	+	+	+
Note: "**-**" No activity; "**+**" Weak activity; "**++**" Moderate activity; "**+++**" High activity						

Extraction of secondary metabolites

The ethyl acetate extract was obtained by solvent separation method and the organic layer containing ethyl acetate fraction was collected in sterile screw capped bottle and gradually evaporated by pouring in large watch glasses. The dried ethyl acetate crude extract was scraped with the aid of sterile blade and stored in autoclaved eppendorff tubes for further characterization analyses.

Characterization of potent isolate

Morphological characterization

The aerial mycelium of isolate KF15 was grey in colour with the substrate mycelium showing slightly yellowish-white colour (Fig. 1A&B). There was no production of any coloured diffusible pigments in SCA media, but the isolate produced diffusible pigment of light brown colour in SC broth media. The colonies of isolate KF15 were flat, powdery with irregular margins. Further, the SEM analysis revealed that, the spores were produced from aerial hyphae and the spores were ovoid, smooth, arranged in spiral shaped spore chains (Fig. 1C).

Biochemical characterization

The isolate *Streptomyces* sp. strain KF15 was characterized by using Vitek 2 BCL card to understand the nutritional parameters and enzymatic capabilities. The test depicted the positive results for 20 out of the total substrates used along with few negative results (Table 2).

Table 2
Biochemical characterization of potent isolate KF15

Well no.	Substrates	Mnemonic	Results
1	BETA-XYLOSIDASE	BXYL	+
3	L-Lysine-ARYLAMIDASE	LysA	+
4	L-Aspartate ARYLAMIDASE	AspA	+
5	Leucine ARYLAMIDASE	LeuA	+
7	Phenylalanine ARYLAMIDASE	PheA	+
8	L-Proline ARYLAMIDASE	ProA	+
9	BETA-GALACTOSIDASE	BGAL	+
10	L-Pyrrolidonyl-ARYLAMIDASE	PyrA	(-)
11	ALPHA-GALACTOSIDASE	AGAL	+
12	Alanine ARYLAMIDASE	AlaA	+
13	Tyrosine ARYLAMIDASE	TyrA	+
14	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	(-)
15	Ala-Phe-Pro ARYLAMIDASE	APPA	+
18	CYCLODEXTRIN	CDEX	-
19	D-GALACTOSE	dGAL	-
21	GLYCOGEN	GLYG	-
22	myo-INOSITOL	INO	-
24	METHYL-A-D-GLUCOPYRANOSIDE acidification	MdG	-
25	ELLMAN	ELLM	(-)
26	METHYL-D-XYLOSIDE	MdX	-
27	ALPHA-MANNOSIDASE	AMAN	+
29	MALTOTRIOSE	MTE	+
30	Glycine ARYLAMIDASE	GlyA	+
31	D-MANNITOL	dMAN	-
32	D-MANNOSE	dMNE	-
34	D-MELEZITOSE	dMLZ	-

Note: "**-**" No activity; "**+**" Weak activity; "**++**" Moderate activity; "**+++**" High activity

Well no.	Substrates	Mnemonic	Results
36	N-ACETYL-D-GLUCOSAMINE	NAG	-
37	PALATINOSE	PLE	-
39	L-RHAMNOSE	IRHA	+
41	BETA-GLUCOSIDASE	BGLU	(-)
43	BETA-MANNOSIDASE	BMAN	+
44	PHOSPHORYL CHOLINE	PHC	+
45	PYRUVATE	PVATE	+
46	ALPHA-GLUCOSIDASE	AGLU	+
47	D-TAGATOSE	dTAG	-
48	D-TREHALOSE	dTRE	-
50	INULIN	INU	-
53	D-GLUCOSE	dGLU	-
54	D-RIBOSE	dRIB	-
56	PUTRESCINE assimilation	PSCNa	-
58	GROWTH IN 6.5% NaCl	NaCl 6.5%	-
59	KANAMYCIN RESISTANCE	KAN	-
60	OLEANDOMYCIN RESISTANCE	OLD	-
61	ESCULIN hydrolyse	ESC	-
62	TETRAZOLIUM RED	TTZ	+
63	POLYMIXIN_B RESISTANCE	POLYB_R	-
Note: "**-**" No activity; "**+**" Weak activity; "**++**" Moderate activity; "**+++**" High activity			

Molecular characterization

The length of 16S rRNA gene sequence of isolate KF15 was 1494 base pairs and the accession number of OL913953 was obtained by depositing gene sequence to the NCBI database. Later, the 16S gene sequence of isolate KF15 was analysed with nucleotide BLAST, which disclosed 97.22% sequence similarity with *Streptomyces intermedius* strain WYM39 (Accession number: MH027581). Based on

similar gene sequences, a phylogenetic tree was established to understand the evolutionary relationship of *Streptomyces* sp. strain KF15 with the aid of MEGA 7.0 software (Fig. 2).

Characterization of ethyl acetate extract

Fourier transform infrared (FTIR) spectroscopy

The FTIR analysis hinted towards the presence of functional groups in KF15 ethyl acetate crude extract and the IR bands at 3390, 2926, 1636, 1383, 1101, 922, 827, 783 and 696 cm^{-1} represented various biochemical groups (Fig. 3). Further, the strong, broad peak at 3390 cm^{-1} was attributed to O-H stretching alcohols, and the medium intensity peak at 2926 cm^{-1} was correlated to C-H stretching alkanes. The broad, medium intensity peaks at 1636, 1383 and 1101 cm^{-1} were corresponded to C = C stretching di-substituted alkenes, O-H bending phenol and C-N stretching amine, respectively. However, C-Cl stretching halo compound, C-H bending 1,2,3-trisubstitued alkanes and C-Br stretching halo compound/ benzene derivatives were associated with the sharp, strong intensity peaks at 827, 783 and 696 cm^{-1}, respectively.

High performance thin layer chromatography (HPTLC) fingerprinting

The HPTLC fingerprinting of ethyl acetate crude extract of *Streptomyces* sp. strain KF15 displayed existence of bioactive compounds in the chromatograph as well as under UV light after derivatization (Fig. 4A-C). The analysis revealed prominent bands under short UV of 254 nm and long UV of 366 nm; the purple coloured bands were also visible after derivatization with vanillin sulphuric acid that confirmed the existence of bioactive metabolites. A total of 4 peaks were observed in the densitogram of *Streptomyces* sp. strain KF15 crude ethyl acetate crude extract along with % area and R_f values and the peak 4 exhibited highest area (31.24%) with R_f value of 0.88. The remaining peaks were 19.60, 22.99, and 26.16% in area and the R_f values were recorded at 0.52, 0.58, and 0.78, respectively.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS spectrum of *Streptomyces* sp. strain KF15 crude ethyl acetate crude extract displayed many peaks characteristic for possible bioactive compounds present, and the spectrum was compared with the compounds already deposited in the National institute of standards and technology (NIST) database to get nearest hit (Fig. 5). Totally, 20 compounds were identified by 20 major peaks depicted in the GC-MS spectrum; the compounds with different chemical nature were identified. The GCMS data revealed the presence of 6 straight chain alkanes, 6 branched alkanes, 1 cyclo-alkane, 1 bromine substituted alkane, 1 straight chain alkene, 1 organo-iodine compound, 1 fatty alcohol, 1 aliphatic alcohol, 1 phenolic compound, and 1 phthalate derivative in the spectrum (Table 3). Among the 20 compounds, 8 compounds with known biological activities were noted; the biological activities of remaining 12 compounds were not yet reported.

Table 3
Primary screening of isolates for antagonistic activity

RT 'min'	Area %	Compound Name	M.W.	Mol. Formula	Biological Activity
7.611	12.19	3,7-Dimethyldecane	170.33	$C_{12}H_{26}$	Not yet reported
8.740	4.00	Nonane, 4,5-dimethyl	156.31	$C_{11}H_{24}$	Not yet reported
11.101	5.52	1-Tridecene	182.35	$C_{13}H_{26}$	Antibacterial (Karanja et al., 2021)
13.438	9.93	2,6,10,15-Tetramethylheptadecane	296.6	$C_{21}H_{44}$	Not yet reported
13.810	2.80	Dodecane, 1-Iodo	296.23	$C_{12}H_{25}I$	Bioactive (Al-Rubaye et al., 2020)
14.655	11.23	Dodecane, 2,6,11-trimethyl-	212.41	$C_{15}H_{32}$	Not yet reported
14.920	5.02	2,3,5,8-Tetramethyldecane	198.39	$C_{14}H_{30}$	Not yet reported
15.083	2.27	Undecane, 4,8-dimethyl-	184.36	$C_{13}H_{28}$	Not yet reported
15.147	2.64	Decane, 2,3,5,8-tetramethyl-	198.39	$C_{14}H_{30}$	Not yet reported
18.927	5.28	Docosane	310.6	$C_{22}H_{46}$	Antibacterial and Antifungal (Lammers et al., 2021)
19.547	3.64	2,4-Ditert Butyl phenol	206.32	$C_{14}H_{22}O$	Antifungal (Kaari et al., 2022)
19.617	2.63	1-Butyl-2-ethyl cyclobutane	154.29	$C_{11}H_{22}$	Not yet reported
20.008	8.36	Tetradecane	198.39	$C_{14}H_{30}$	Antibacterial and Antifungal (Marimuthu et al., 2020)
23.849	3.20	Tridecanol, 2-ethyl-2-methyl	242.44	$C_{16}H_{34}O$	Not yet reported
24.792	5.43	Eicosane	282.5	$C_{20}H_{42}$	Antibacterial and Antifungal (Ahsan et al., 2017)
25.178	1.78	2-Bromotetradecane	277.28	$C_{14}H_{29}Br$	Not yet reported
29.100	3.10	Octadecane	254.5	$C_{18}H_{38}$	Antimicrobial (Nandhini et al., 2015)
29.417	6.15	Bis(tridecyl) phthalate	530.8	$C_{34}H_{58}O_4$	Bioactive
29.687	2.21	1-Decanol, 2-hexyl-	242.44	$C_{16}H_{34}O$	Not yet reported

RT 'min'	Area %	Compound Name	M.W.	Mol. Formula	Biological Activity
36.677	2.61	1-Hexadecene	224.42	$C_{16}H_{32}$	Not yet reported

Antimicrobial activity

The ethyl acetate crude extract from KF15 was subjected to antimicrobial activity by using agar well diffusion method and the results revealed increased activity on test pathogens with increase in the volume (Fig. 6A-F). The results depicted that, the extract exhibited highest inhibition activity against *Enterococcus faecalis* with 14 ± 1.0, 15 ± 0.5, 15 ± 1.0, and 17 ± 1.0 mm inhibition zones along with *Candida glabrata* that showed 13 ± 0.5, 14 ± 1.0, 16 ± 0.5 and 18 ± 1.0 mm zones at 25, 50, 75, and 100 µl volumes of crude extract. The gram negative bacterial pathogens displayed moderate sensitivity to the extract compared to that of gram positive one by showing considerable inhibition zone at different volumes (Fig. 6G). The least sensitive pathogen was found out to be *Escherichia coli* with 10 ± 0.5, 12 ± 1.0, 13 ± 0.5 and 14 ± 1.0 mm inhibition zones at 25, 50, 75, and 100 µl volumes of extract, respectively.

Antifungal activity

The primary screening revealed noticeable inhibition of tested phytopathogenic fungi by KF15 proving its potential of antagonistic activity in duel culture method. The pathogenic fungi were unable to grow in the close vicinity of KF15 and clear zones on both the parallel sides of KF15 facing the fungi was clearly visible (Fig. 7A-C). The growth curve analysis revealed that, the fungi in control displayed normal pattern of growth curve with typical lag phase, log phase and stationary phase. Whereas, the pattern of growth curve in treated sample was shifted towards right with extended lag phase of growth. In control, the exponential growth (log phase) of fungus started just after 24 hr of inoculation; but, the fungus required more than 72 hr to start exponential growth in treated sample (Fig. 7D).

The SEM analysis of *Fusarium oxysporum* cells treated with KF15 extract showed damaged external morphology with rough surface compared to that of control cells having smooth outer cell surface. The hyphae and spores of control cells were smooth, tubular and turgid with intracellular matrix (Fig. 8A-C). SEM micrograph clearly displayed the damaged hyphae in the treated cells with fractures, aplanation and leakage of intracellular components along with other degenerative changes (Fig. 8D-F). The higher magnification images depicted the smooth surface, turgid nature of fungal spores with regular margins in control; whereas, the treated spores were shrunken, flaccid with rough surface and were having irregular margins. The treated spores were completely collapsed, evidenced by effusion of intracellular components ultimately leading to cell death.

Evaluation of anticancer activity by MTT assay

The KF15 ethyl acetate extract displayed escalated anticancer activity with increase in concentration to inhibit the growth of human cervical cancer (HeLa) cells, that is observed by the distinct changes in

shape, size, and other morphological details of the tumour cells (Fig. 9A-G). The MTT results presented the potential of KF15 to prevent the HeLa cells at different concentrations. The percentage inhibitions of 7.47 ± 1.55, 23.61 ± 1.83, 34.54 ± 1.83, 50.10 ± 1.98 and 64.32 ± 1.76 was observed at 25, 50, 75, 100 and 125 µg/ml, respectively (Fig. 9H). The KF15 extract displayed dose-dependent anticancer activity against HeLa cell line by effectively halting the cell proliferation. The IC_{50} value was determined to be 99.85 µg/ml against HeLa cells, and this result indicates the potential of KF15 extract to hinder the proliferation of cancer *in-vitro*. The deformed cells, blebbing of cell membrane and release of cell matrix were observed in the treated cells when compared with the healthy HeLa cells.

Discussion

The Kathlekanu swamp forest is one of the unique types of forests and that is microbiologically not explored by the researchers. Therefore, the actinomycetes from swamp soil may have increased antimicrobial capability by producing different kinds of novel compounds. The antagonistic potential could be attributed to various bioactive secondary metabolites produced by the isolate under optimum growth conditions. The earlier reports also suggested about the possibility of novel bioactive compound associated with the organism from rare habitats; the extreme or unexplored regions may harbour more number of potential actinomycetes with novel bioactive compounds (Chakraborty et al. 2021; Mohamed et al. 2021). The isolate KF15 was selected for mass culture using SC broth media and ethyl acetate solvent was used to extract the fermented culture broth. The earlier reports recommended the use of ethyl acetate for the extraction of secondary metabolites as it was recommended as the best solvent for the extraction of bioactive compounds produced by actinomycetes (Kumar et al. 2012; Vijayakumar et al. 2014).

Among the thirty one isolates, notable differences were observed in case of colony structure, aerial and substrate mycelia colour. The *Streptomyces* species were suspected based on the characteristic moist earthy odour, powdery and chalky appearance of colonies and formation of filamentous, heavily branched mycelia. These observations were in accordance with the earlier descriptions of genus *Streptomyces* reported in Bergey's manual of determinative bacteriology (Goodfellow and Williams, 1983; Goodfellow et al. 2012). As for the utilization of different substrates, it was noted that the isolate KF15 was capable of utilizing almost all the examined carbon sources; it was also eminent that the isolate exhibited the potential to produce various enzymes. Similar reports about utilization of carbon and nitrogen sources were recorded in the earlier reports on the biochemical characterization of many *Streptomyces* species (Taddei et al. 2006; Hussain et al. 2021). The gene sequence of the isolate *Streptomyces* sp. strain KF15 was blasted with the aid of nucleotide blast in NCBI database to collect matching strains and sequences. The isolate exhibited 97.22% sequence similarity with *Streptomyces intermedius* strain WYM39; the confirmation was attained by the construction of phylogenetic tree of related strains to understand evolutionary relationship. Similar report was recorded, where the molecular identification of the isolate *Streptomyces paradoxus* strain KUASN-7 was done via 16S rRNA gene sequencing along with the analysis of phylogenetic tree (Muthuraj et al. 2021).

The functional groups depicted by the FTIR analysis showed that, the bioactive compounds of KF15 may be classified into alkanes, alkenes, carboxylic acids, phenols and benzene derivatives. Similar reports about the FTIR analysis of ethyl acetate extracts from *Streptomyces* sp. CRB46 (Ambarvati et al. 2020), and *Actinomycetes* sp. (Alqahtani et al. 2022) suggested the presence of various functional groups such as alkanes, amines, phenols, carboxylic acids, and aromatic compounds. HPTLC fingerprinting analysis revealed the existence of overall chemical moiety in the extract. The major bands represented the possible bioactive compounds at different R_f values. Similar results were obtained in the earlier studies on HPTLC analysis suggesting HPTLC as a reliable chromatographic technique widely used for analysing many samples of dissimilar nature and composition; it is also used to identify and quantify bioactive compounds (Shekar et al. 2016; Anusree et al. 2019).

The GC-MS analysis revealed the peaks for 12 major compounds and among them, 6 compounds were reported to be biologically active in nature. The phenolic compound 2,4-di-tert-butylphenol was recommended to be having very good antifungal activity (Belghit et al. 2016), and the hydrocarbons namely, tetradecane, docosane, octadecane, and eicosane were reported to be having known antibacterial activity against pathogenic bacteria of both gram positive and gram negative nature (Ahsan et al. 2017; Marimuthu et al. 2020; Lammers et al. 2021; Nandhini et al. 2015). The organobromine compound 2-bromo dodecane was reported as a bioactive compound that was used for various biological activities (Al-Rubaye et al. 2020). Similar reports about bioactive compounds recognized by the GC-MS analysis of *Streptomyces* and other actinomycetes crude ethyl acetate crude extract demonstrated the presence of long chain alkanes, fatty acid methyl ester, fatty alcohol, pyrrolizidine and piperazinedione with well-known biological activities including broad spectrum antimicrobial activity and antioxidant potential (El-Naggar et al. 2017; Krishnamoorthy et al. 2020).

The antimicrobial activity of ethyl acetate crude extract from KF15 exhibited varied results against the tested bacterial pathogens and it was noticed that the higher inhibition zones were observed in case of gram positive bacteria when compared to gram negative bacteria. The difference in sensitivity towards the extract was attributed the dissimilarity in cell wall arrangement; the lipopolysaccharide present in the outer membrane acts as a protective barrier for gram negative bacteria. Whereas, the devoid of lipopolysaccharide protective layer in gram positive bacteria makes them susceptible to bioactive secondary metabolites (Reygaert, 2018; Pallavi et al. 2021). The earlier studies suggested that, the mechanism of action of antimicrobial compounds includes interaction of metabolites with the cytoplasmic membrane followed by the cell membrane damage and subsequent release of K^+, PO^{4-}, and nuclear contents from the matrix of the cell. Furthermore, the gram negative bacteria are suspected to utilize antimicrobial mechanisms like, drug target modification, limiting the drug uptake, drug efflux activation and drug inactivation to overcome the effects of antimicrobials (Biswas et al. 2022; Chakraborty et al. 2022).

The swamp soil *Streptomyces* sp. KF15 exhibited considerable antifungal potential by effectively restricting the growth of fungal pathogens. The antagonistic nature of KF15 may be credited to the ability to produce and secrete secondary metabolites having antifungal activity that diffuses in the medium and

inhibits the further growth of the fungi (Li et al. 2021). The shift in growth curve pattern towards right-side indicates that, the fungus was unable to adjust to the conditions and moreover, the cells took more time for attaining maturity and to be ready to divide. Previous study involving the turbidimetric analysis of growth curve suggested that, the delayed lag phase was correlated with lower elongation rates of the formed hyphae and germination rates of conidia than those of control. This in turn, affected the lag phase by preventing the fungus in reaching critical turbidity which is measured by spectrophotometer to give higher OD (Meletiadis et al. 2003). The study also recommended that, turbidimetry was a brisk, non-destructive and low-cost method for examining the growth of filamentous fungi.

The effect of KF15 extract on the morphology of *F. oxysporum* was examined with the help of SEM imaging, and the results indicated clear evidence of morphological changes in treated cells. The earlier investigations on effect of *Streptomyces* extract on the morphology of fungal pathogens were also stated similar kinds of deformations and distortions in treated cells. The studies reported several levels of diastrophic and fractured mycelium and spores along with pores in treated cells. The results hinted at complete collapse and degeneration of spores due to decreased exopolysaccharide formation in outer membrane (Li et al. 2021). Another study reported the morphology of treated hypha showing exfoliated flakes, shrivelling, vacuolation and blistering; thickened cell wall and septum with partly ruptured cell membrane along with swollen cell nucleus was also observed (Zou et al. 2021).

The MTT assay results depicted the potentiality of KF15 extract to obstruct the growth of HeLa cells, and IC_{50} value of 99.85 µg/ml suggests that even at lesser concentration it was able to restrict cancer cell proliferation. The previous studies also reported increased anticancer activity of *Streptomyces* sp. extract against human breast cancer (T47D) cell line (Jaroszewicz et al. 2021), human lung cancer (A549) cell line (Kumar et al. 2021), and human cervical cancer (HeLa), hepatocellular (HepG2) and breast cancer (MCF7) cell lines (Taechowisan et al. 2021). Surprisingly, there was no or minimal significant adverse effect on the normal human lung cells and murine epithelial (L929) cells were observed in those studies indicating less adverse effect of microbial extract on normal or healthy cells. The reports also mentioned that, the bioactive compounds in the extract impart cytotoxicity mainly by interacting with DNA region having high G-C, topoisomerase-II activity inhibition, and breakage of single stranded DNA. The earlier studies also suggested that, the reactive oxygen species (ROS) instigates apoptosis in the tumour cells which finally leads to cell death (Ma et al. 2021).

Conclusion

Myristica swamp forest is one of the rare habitats that are under explored for novel bioactive microorganisms. The study investigated on isolation, characterization and application of actinomycetes from Kathlekanu swamp forest soil and the ethyl acetate crude extract of *Streptomyces* sp. KF15 revealed many bioactive metabolites in GC-MS analysis. The metabolite profiling showed the pattern in HPTLC and the duel culture method displayed the antagonistic potential of *Streptomyces* sp. KF15. The antimicrobial activity results hinted about the antimicrobial agents in the crude extract and the deformed fungal cells in SEM analysis confirmed the presence of antifungal metabolites in the crude extract.

Therefore, the output from this study concludes that, actinomycetes from an underexplored habitat harbors novel bioactive metabolites that help to combat against multidrug resistant pathogenic microbes. Further research on purification and structure elucidation could lead to the discovery of any novel antimicrobial compound.

Declarations

Author Contributions: Conceptualization, Sreenivasa Nayaka and Meghashyama Prabhakara Bhat; supervision, Sreenivasa Nayaka; methodology, Meghashyama Prabhakara Bhat; validation, Sreenivasa Nayaka and Raju Suresh Kumar; writing—review and editing, Meghashyama Prabhakara Bhat. All authors have read and agreed to the published version of the manuscript.

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Figures

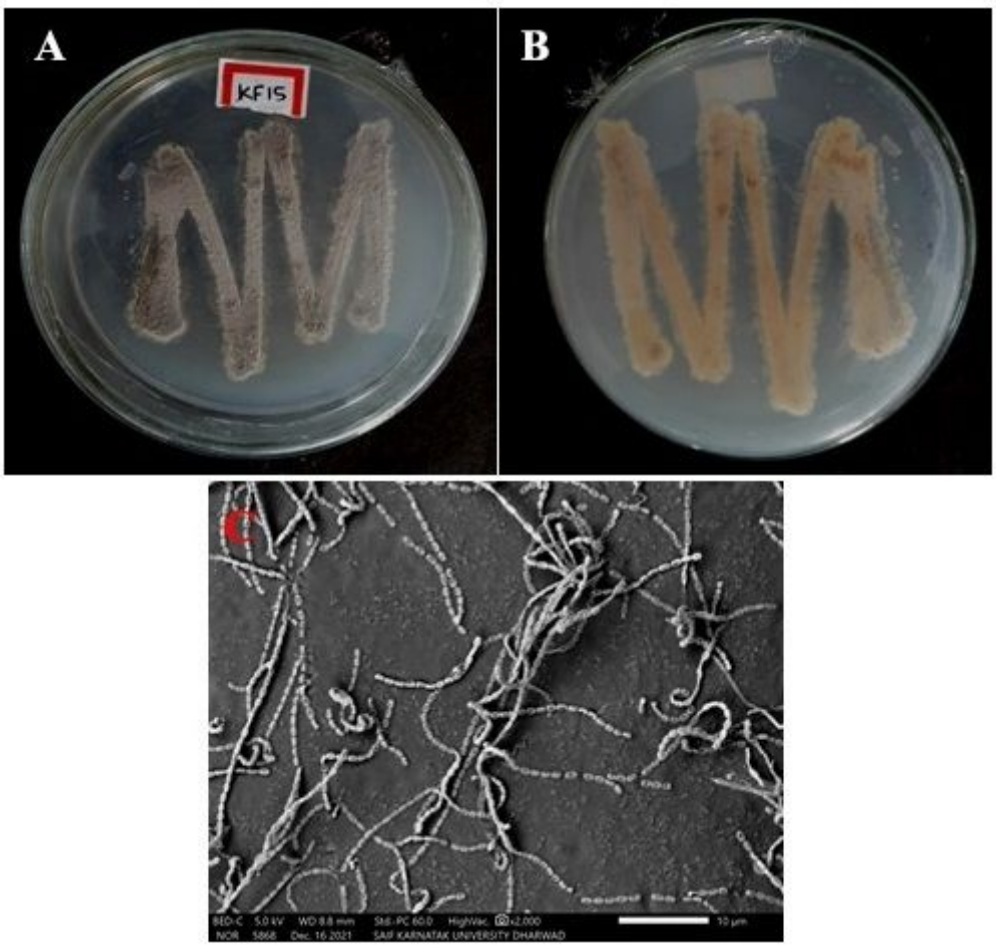

Figure 1

Morphological characterization of isolate KF15; **A)** Aerial mycelium, **B)** Substrate mycelium, and **C)** SEM micrograph

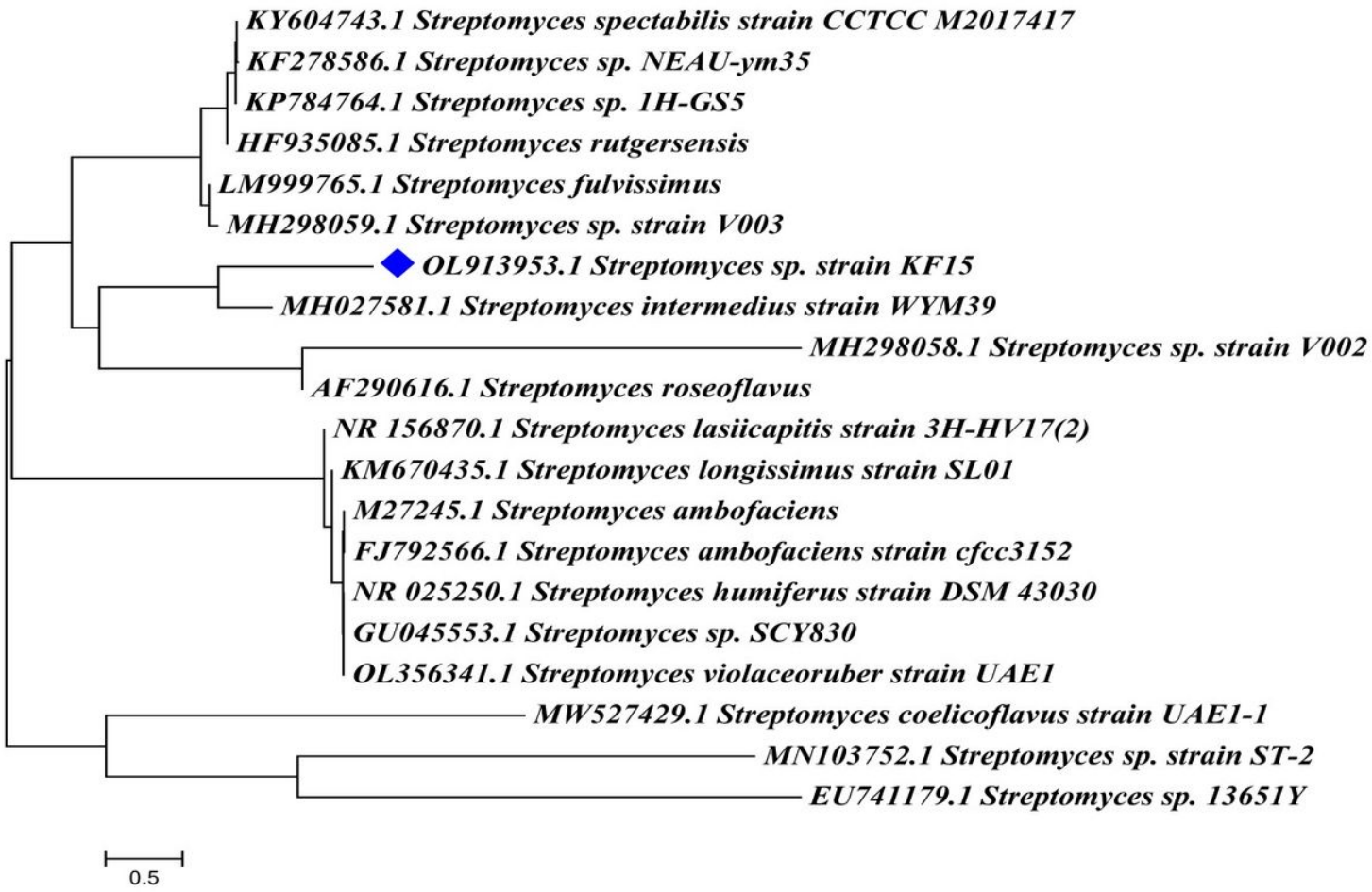

Figure 2

Phylogenetic relationship of KF15 with neighbouring *Streptomyces* species

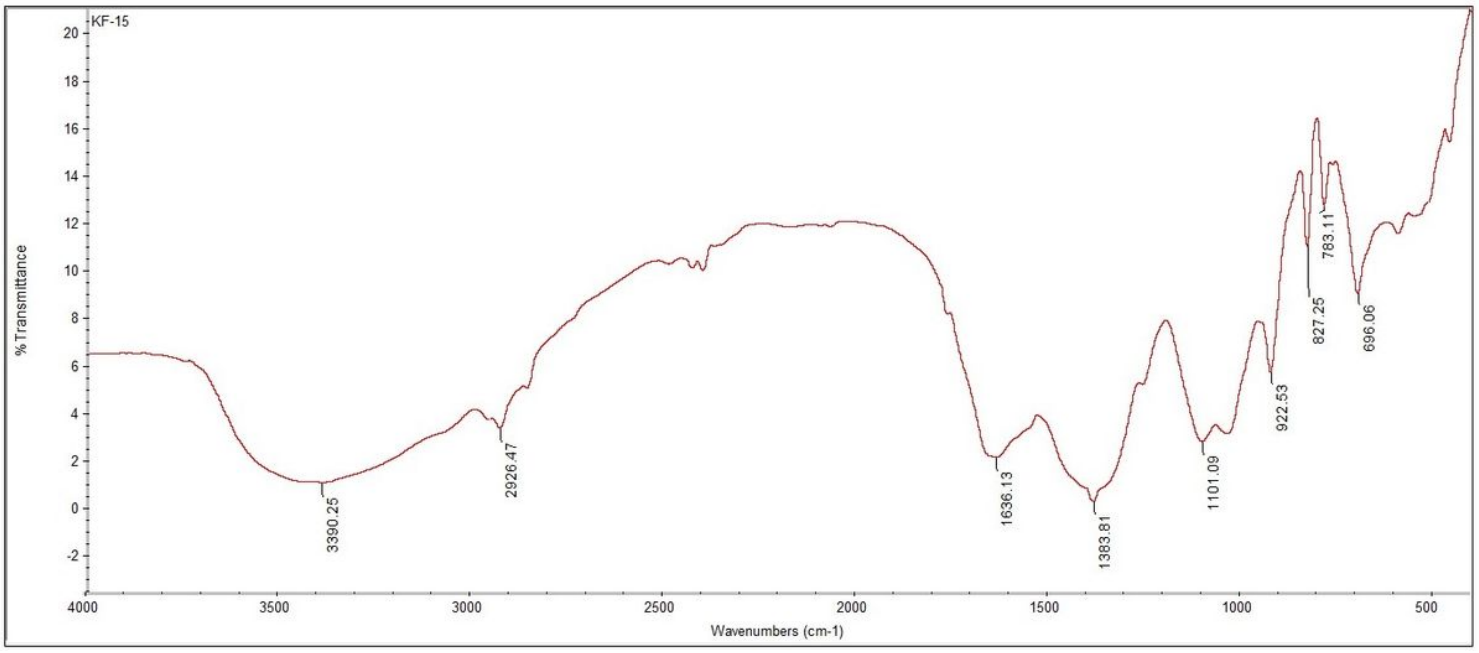

Figure 3

FTIR spectrum of KF15 ethyl acetate crude extract

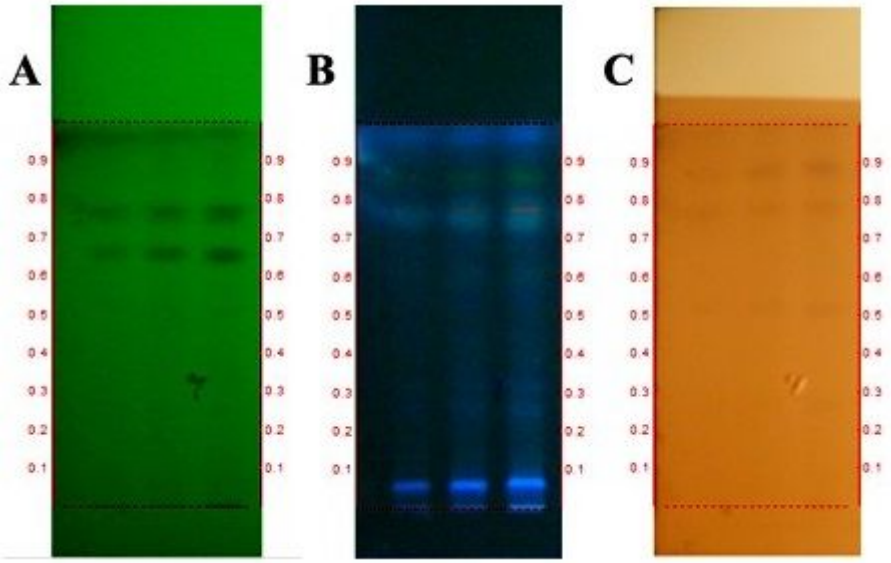

Figure 4

HPTLC fingerprinting of KF15 ethyl acetate crude extract; **A)** Under UV 254 nm, **B)** Under UV 355 nm, and **C)** After derivatisation

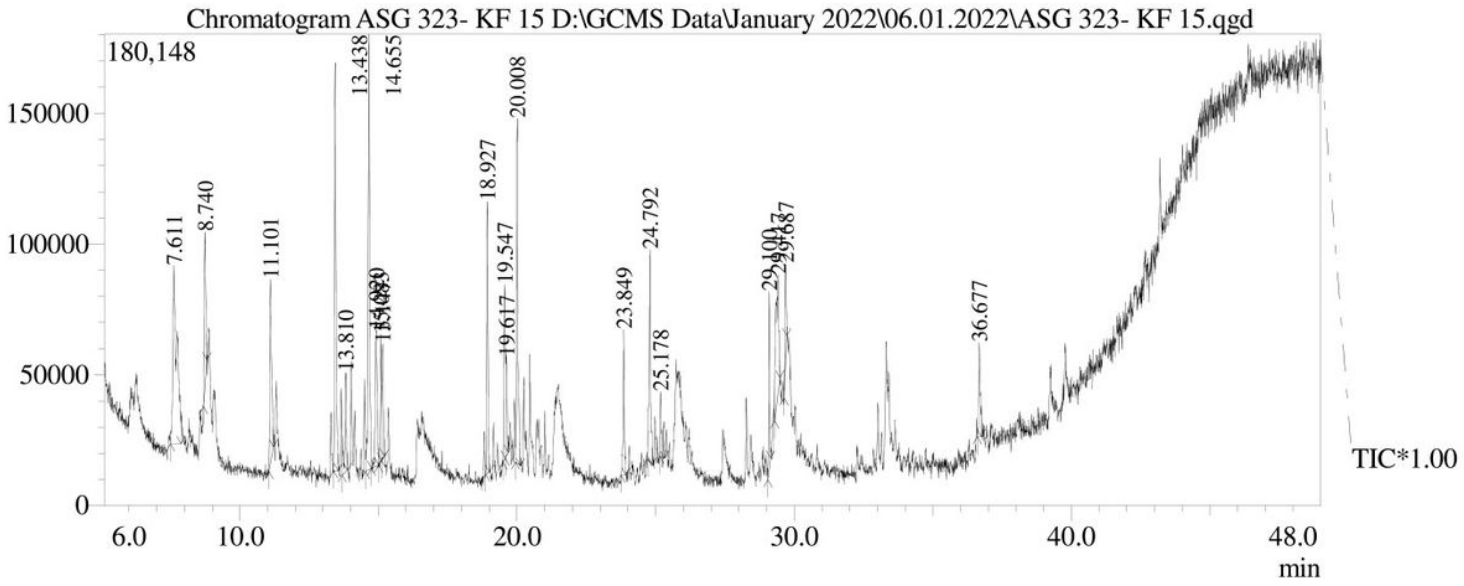

Figure 5

GCMS spectrum of KF15 ethyl acetate crude extract

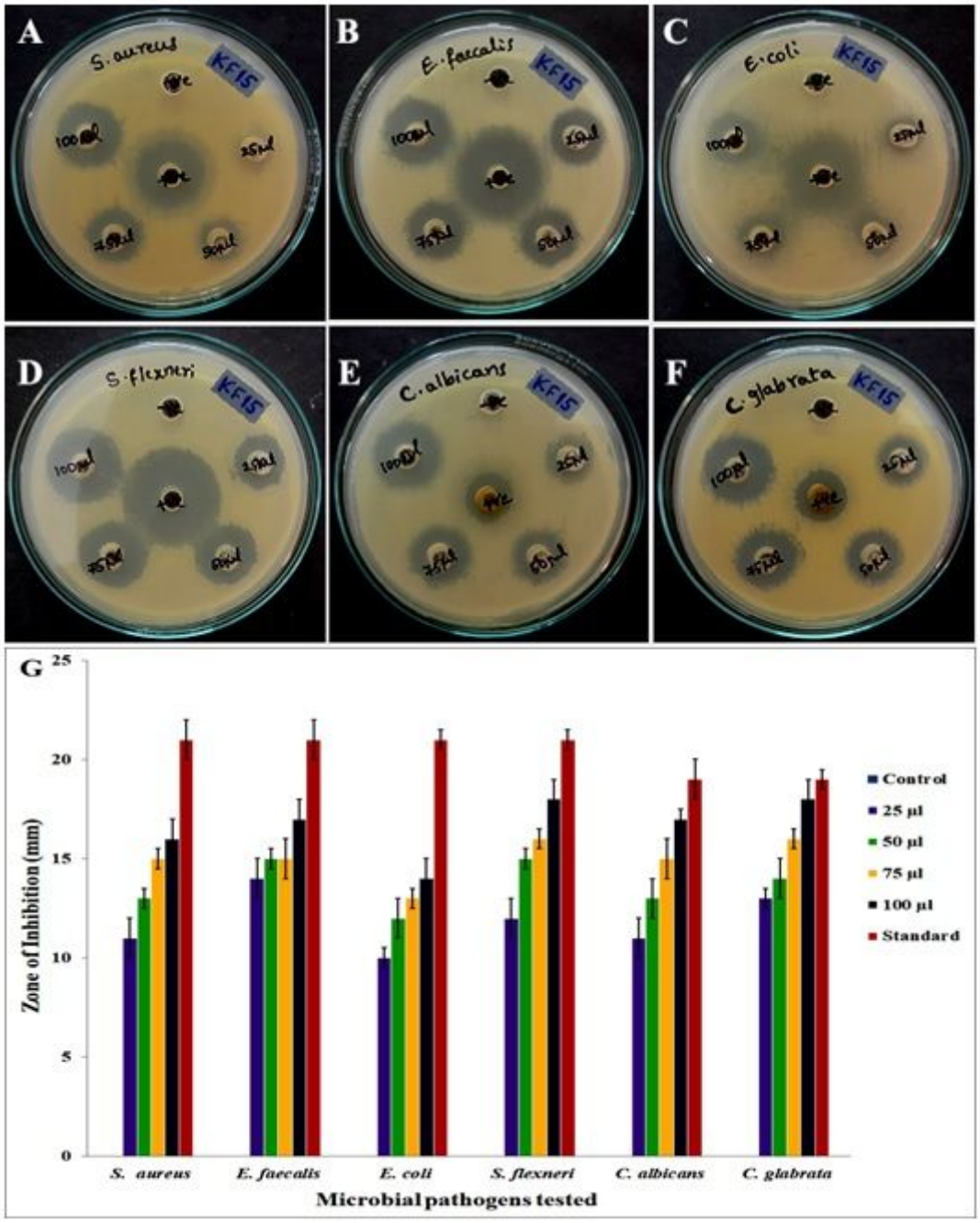

Figure 6

Antimicrobial activity of KF15 ethyl acetate crude extract; **A)** *S. aureus*, **B)** *E. faecalis*, **C)** *E. coli*, **D)** *S. flexneri*, **E)** *C. albicans*, **F)** *C. glabrata*, and **G)** Graph showing zone of inhibition against pathogens at different concentrations

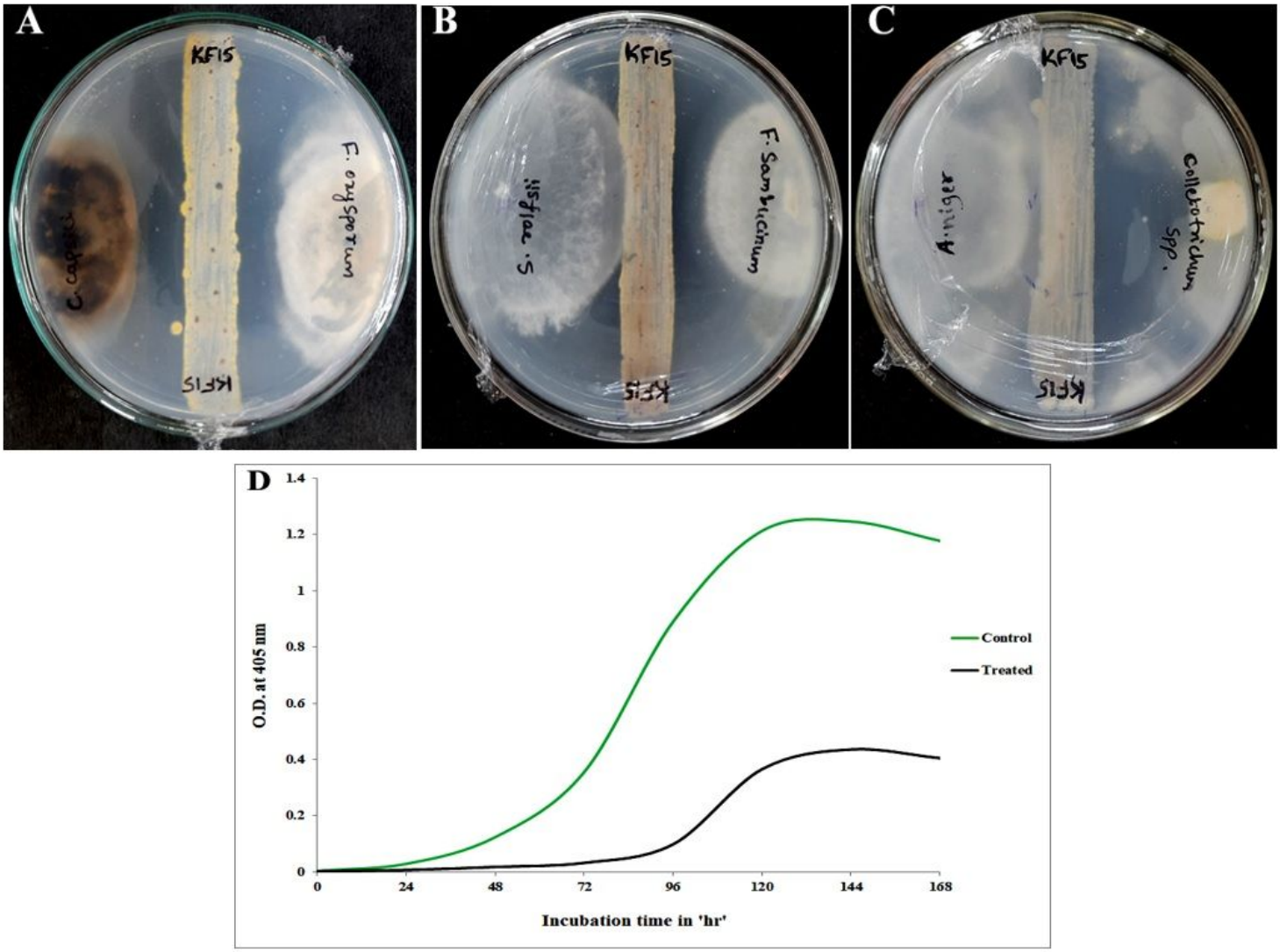

Figure 7

Antagonistic activity of KF15 by duel culture method; **A)** *Colletotrichum capsici* and *Fusarium oxysporum*, **B)** *Sclerotium rolfsii* and *Fusarium sambucinum*, **C)** *Aspergillus niger* and *Colletotrichum* sp., and **D)** Graph showing effect of KF15 extract on *Fusarium oxysporum* growth pattern

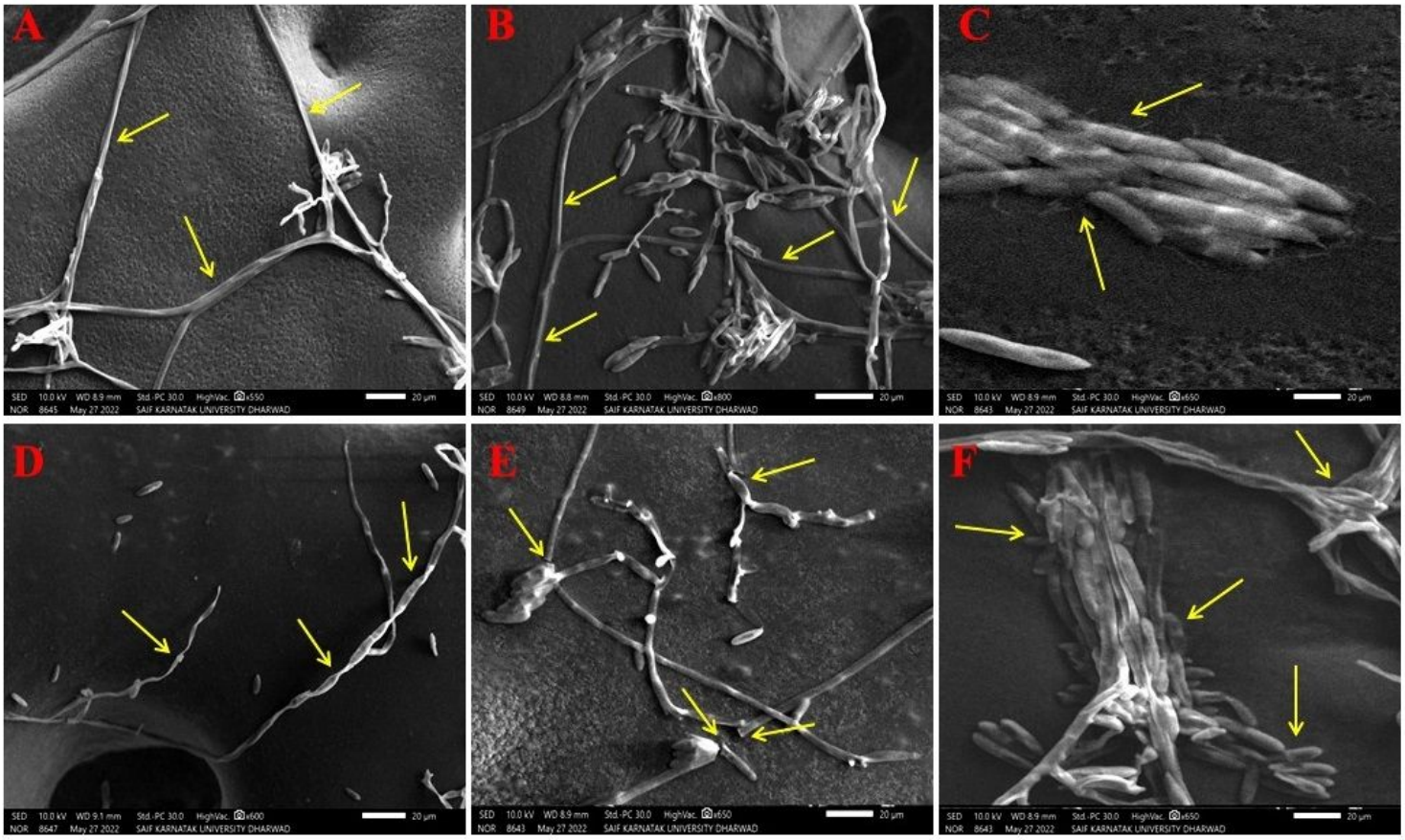

Figure 8

Effect of *Streptomyces* sp. KF15 crude extract on morphology of *F. oxysporum*; **A)** Control mycelium with tubular nature, **B)** Control mycelium with healthy spores, **C)** Healthy spores with turgid nature, **D)** Treated mycelium with aplanation, **E)** Treated mycelium showing collapse and breakage, and **F)** Treated spores showing aplanation

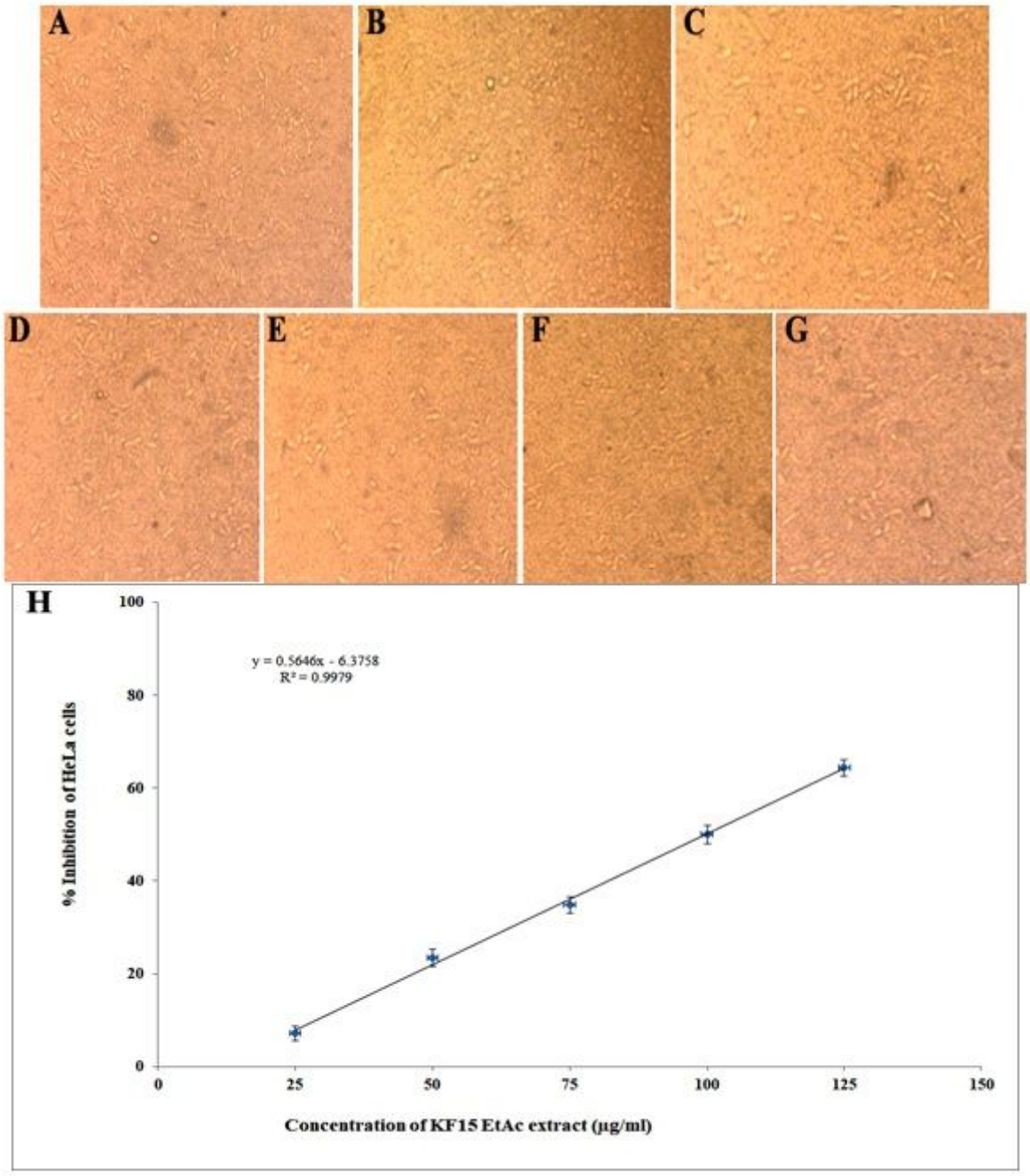

Figure 9

Anticancer activity of KF15 ethyl acetate crude extract; **A)** Control, **B)** Standard, **C)** 25 µg/ml, **D)** 50 µg/ml, **E)** 75 µg/ml, **F)** 100 µg/ml, **G)** 125 µg/ml, and **H)** Graph showing dose-dependent activity of KF15 extract