ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

In-vitro antimicrobial and anticancer activities of methyl nonactate and methyl homononactate produced by Egyptian isolates *Streptomyces fulvissimus* FHM275 and *Streptomyces pratensis* FHM572.

Ferial M Rashad[1]*, Nadia H Abd El-Nasser[2], Insaf E Dawoud[1], Hayam M Fathy[1], Amal M Ali[2], and Fatima H Motawe[2].

[1]Department of Microbiology, Faculty of Agriculture, Cairo Univ., Giza 12613, Egypt.
[2]Department of Microbial Chemistry, National Research Centre, Doki, Giza 12311, Egypt.

ABSTRACT

The optimum conditions for maximal productivity of bioactive metabolite by *St. fulvissimus* FHM275 and *St. pratensis* FHM572 were as follow: 20% v/v loading volume at pH ranged from 7.0 to 7.5, 2% or 1% v/v of 5 or 7- day old spores suspension as inoculum size respectively, incubation at 30°C or 28°C respectively and agitation speed rate of 150 or 180 rpm respectively for 5 days. Unlike environmental factors, both *Streptomyces* strains attained the highest productivity of bioactive compounds from starch nitrate broth with starch, 20 ; potassium nitrate KNO_3, 2 and dipotassium hydrogen phosphate K_2HPO_4, 1g/l; which corresponding to C, N, P and K at concentrations of 7.8480, 0.2771, 0.1778 and 1.224 1g/l, respectively. Biosynthesis of bioactive molecules was inhibited completely in the absence of Mg^{2+}, although they were released in the absence of iron and NaCl, the productivity was enhanced on increasing their concentrations to the normal level of medium. With the surpass potency of *St. pratensis* FHM572, both broth filtrates showed broad spectrum of biological activities against human, agricultural pathogens and MDR clinical pathogens; the crude extracts (EA/CH3Cl, 1:1) gave notable antimicrobial activity against MDR with MIC values over the range of 0.34 and 3.11 mg/ml. Only, the purified pooled fractions PF2 and PFH2 which obtained from *St. fulvissimus* FHM275 and *St. pratensis* FHM572 showed antimicrobial and cytotoxic activities. PF2 is promising compound, it did not show any damage to the normal epithelial amnion cell line up to 100 µg/ml, but showed strong activity and highest selectivity against lung carcinoma A549 (IC_{90} up to 6.25 µg/ml), good activity against colon cancer HCT 116 (IC_{50} of 21. 4 µg/ml). PFH2 killed 90% of lung carcinoma A549, 50% of Caucasian breast adenocarcinoma MCF 7 and colon cancer HCT 116 at 6.25, 11.3 and 14.4 µg/ml, respectively. PF2 and PFH2 were identified as methyl nonactate ($C_{11}H_{20}O_4$) or methyl homononactate ($C_{12}H_{22}O_4$), respectively using spectroscopic techniques (MS, UV, IR, ^1H and ^{13}C NMR).

Keywords: *Streptomyces* spp., Antimicrobial activity, Cytotoxic activity, Methyl nonactate, Methyl homononactate, Structure elucidation.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

Natural products have traditionally played an important role in drug discovery and were the basis of most medicines dating back to ancient civilizations. Living organisms, including plants, microbes, and marines organisms, provide rich sources of chemically diverse bioactive compounds. More than 40% of the chemicals, identified as natural products, have not been chemically synthesized (Schwartsmann *et al*., 2002; Newman and Cragg 2012). In general, natural products including the microbial metabolites may be practically utilized in three different ways: applying the natural/fermentation product directly in the medicine, agriculture, or in any other fields; using as starting material for subsequent chemical or microbiological modification (derivatization); they can be used as lead compounds for chemical synthesis of new analogs or as templates in the rational drug design studies (Bérdy, 2005).

At present, new bioactive compounds were clearly needed to combat and overcome the development of resistance in pathogens, the evolution of new diseases, the existence of naturally resistant bacteria and the toxicity of some of the approved compounds. Actually, antibiotics/antitumor probably can never win the war; but fortunately, the search for new natural product drugs is not stopped (Demain and Sánchez, 2009; Lancini and Demain, 2013). Soil remains the richest versatile source for discovering new groups of microorganisms with industrial and clinical importance. Because of most antibiotics were produced by screening soil microorganisms, scientists have always believed that the soil is teeming with new and potent antibiotics because bacteria have developed novel ways to fight off other microbes (Ling *et al*., 2015). The top cultivable bioactive metabolites producers present in soil are the actinomycetes. These bacteria comprise about 10-33% of the total bacterial community present in soil. From the known antibiotics and similar bioactive microbial compounds, only about 150 compounds (<1%) are in direct use in the human, veterinary medicine and in agriculture. In the human therapy about one hundred compounds, most of them derived from actinomycetales species, are in direct practical use. Among actinomycetes, the genus *Streptomyces* is responsible for the synthesis of the majority of bioactive compounds with a range of biological activities that may eventually find current therapeutic applications as anti-infective, anticancer agents, immunosuppressive agents or other pharmaceutically useful compounds. Therefore, the potency of the *Streptomyces* species should not be underestimated. Their capacity to produce promising new compounds will certainly be unsurpassed and for a long time and they still have been producing the majority of the chemotherapeutically applied antibiotics (Osborne *et al*., 2000; Bérdy, 2005; Valli *et al*., 2012).

In a previous study, we have shown antimicrobial and cytotoxic activities of two *Streptomyces* species, *St. fulvissimus* FHM275 and *St. pratensis* FHM572, recovered from the rhizosphere soil sample of Egypt (Rashad *et al*., 2015). It showed broad spectrum of antimicrobial activity against a panel of Gram positive, Gram negative bacteria and fungi; the minimum inhibitory concentrations of their crude ethyl acetate/chloroform extracts confirmed their antimicrobial potencies (4.5 – 370 µg/ml). In addition, the crude extract of *St. fulvissimus* FHM275 showed antitumor activity only against colon cell line (HCT 116) and lung carcinoma cell line (A549) with IC_{50}: 15.5, 15.3 µg/ ml, respectively. However, *St. pratensis* FHM572 showed low activity against Caucasian breast adenocarcinoma (MCF7), moderate activity against hepatocellular carcinoma (HePG2), and strong activity against HCT 116 and A549 with IC_{50}: 70.6, 36.9, 16.1 and 7.5 µg/ ml. These results encouraged us to further evaluation of the biological activities against various multiple antibiotic resistant clinical isolates as well as anticancer activity of pure bioactive metabolites.

In the present study and in continuation of our previous work, the capability to synthesize the bioactive metabolites under different conditions of nutrition and cultivation by *St. fulvissimus* FHM275 and *St. pratensis* FHM572 was studied; their antimicrobial activity against multi-drug resistant clinical isolates was assessed; extraction, separation, purification of the bioactive compounds produced by each strain, elucidation of the cytotoxicity of pure compounds and the chemical structure of the pure active compound were conducted.

ISSN: 0975-8585

MATERIALS AND METHODS

Microorganisms

Bioactive compounds producing strains

St. fulvissimus FHM275 and *St. pratensis* FHM572 used in this study were isolated from cultivated soil in Egypt, identified, deposited in Gene Bank under the accession number of KM438035 and KM046933 and are available in Microbiological Resources Center (Cairo MIRCEN), Fac. Agric., Ain Shams University under nos. EMCC 1923 and 1924, in that order. The cultures were grown on starch nitrate and maintained at 4 °C in the same medium.

Test microorganisms

The following microorganisms were used as test organisms for antimicrobial bioassay during optimization studies: Gram positive bacteria (*Bacillus cereus*, *Bacillus subltis* ATCC 6633*, Staphylococcus aureus* ATCC 6538), Gram negative bacteria (*Escherichia coli* ATCC 19404, *Pseudomonas aeruginosa* ATCC 9027), yeast (*Candida albicans* ATCC 10231) and fungi (*Aspergillus flavus* NRRL A- 1957, *Aspergillus niger* ATCC 16404, , *Botrytis allii* NRRL A- 2502, *Macrophomina phaseolina* NRRL A- 62743, *Aspergillus terries*, *Rhizoctonia solani*). *B. cereus, A. terries* and *R. solani* were kindly provided by Culture collection of Cairo MIRCIN, Faculty Agric., Ain Shams University.

Multi-drug resistant clinical pathogens

Staph. aureus MRSA 43300 ATCC was obtained from Naval Medical Research Unit 3(NAMRU-3); and the other 9 clinical strains were kindly provided by Prof. Dr. Mervat Gaber Elanany, Dept. Chemical and Clinical Pathology, Faculty of Medicine, Cairo University; their antimicrobial resistant profile was determined by the clinical lab (Table 1).

Parameters controlling productivity of bioactive metabolites

The fermentation conditions in terms of environmental and nutritional requirements which affecting the productivity of bioactive metabolites by *St. fulvissimus* FHM275 and *St. pratensis* FHM572 were optimized by adopting search technique varying parameters one at a time. Starch-nitrate medium was used as a basal medium for the cultivation of both strains. It was composed of (g/l): starch, 20.0, KNO_3, 2.0, K_2HPO_4, 1.0, $MgSO_4.7H_2O$, 0.5, NaCl, 0.5, $CaCO_3$, 3.0, $FeSO_4.7H_2O$, 0.01, distilled water, 1.0 liter, pH 7.4. Unless otherwise stated, Erlenmeyer flasks were loaded with the basal medium in triplicate at 20 % v/v, inoculated with 2% v/v, incubated at 28 ^oC and 180 rpm for 5 days on a rotary shaker (New Brunswick Scientific, Edison, N. J., USA); each parameter optimized earlier was incorporated in subsequent experiments.

Environmental parameters

This included the effects of inoculum age, inoculum size, fermentation period, temperature, working volume, agitation and initial pH on the productivity of bioactive metabolites produced by each strain. To determine the effect of inoculum age, seed inocula were prepared by growing each strain on starch nitrate agar slant at 28 ^oC for 1- 10 days, then the slant cultures smashed and washed with 5 ml of sterile distilled water to prepare inocula. The effect of extending of incubation on the productivity of bioactive metabolites was examined daily up to 10 days following the previously mention technique. The optimum inoculum size was detected for each strain using 1, 2, 4, 8, 10, 12, 14 and 20 % v/v of spore suspension to inoculate triplicate flasks. The optimum temperature for maximum bioactive metabolites yield was measured by incubating the inoculated production medium at 25, 28, 30, 35 and 40^oC. To detect the optimum working volume or shaking speed for each strain, the flasks, in triplicate, were loaded at levels of 10, 20, 40 and 60 % (v/v), inoculated and incubated following the previously mention technique. The effect of shaking speed was tested by incubating the inoculated medium at 50, 150, 180, 200 and 250 rpm. To study the effect of initial pH value, fermentation medium was adjusted to 4.0, 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 using 1N HCl or NaOH. Then the medium was inoculated and incubated keeping all former experimental conditions at optimum.

Nutritional parameters

For optimizing nutritional requirements, an equimolar concentration (g/l) of different sources of carbon [cellulose, 17.40; cellobiose, 18.60; D- Fructose, 19.60; xylose, 19.60; maltose, 18.60; sucrose, 18,60; arabinose, 19.60; D- galactose, 19.60; lactose, 18.60; mannose, 19.60, mannitol, 19.80; D-glucose, 19.60; or glycerol, 20.0], nitrogen [$NaNO_3$, 1,70; NH_4NO_3, 0.89; NH_4Cl, 1.068; $(NH_4)_2HPO_4$, 1.32; $NH_4H_2PO_4$, 2.30; $(NH_4)_3PO_4$, 0.80; NH_4SO_4, 1.30; peptone, 1.87; urea, 0.6006; casein, 2.00 and yeast extract, 2.86] and phosphorus [KH_2PO_4, 0.78; Na_2HPO_4, 0.814; NaH_2PO_4, 0.6886; $NH_4H_2PO_4$, 0.6600; $(NH_4)_2HPO_4$, 0.7576 and $(NH_4)_3PO_4$, 0.8552] was added to the basal medium instead of starch, 20.0; KNO_3, 2.000 and K_2HPO_4, 1.0; in that order. Influence of various concentrations of the best source of C, N or P was studied. Besides the effect of different concentrations of $CaCo_3$, $MgSO_4.7H_2O$, NaCl and $FeSO_4.7H_2O$ on the productivity of bioactive compounds was also investigated; each parameter optimized earlier was incorporated in subsequent experiments.

In each experiment, the culture filtrate of each strain was assayed for the antimicrobial activity following the agar cup diffusion method in order to detect the optimum condition for the highest production of bioactive compound(s). After incubation, the contents in the flasks were aseptically filtered through sterile Whatman No. 1 filter papers; then the resulted filtrates were sterilized by filtration. A sterile cork borer was used to make holes (8.0 mm in diameter) in the plates seeded with the test organism, then only 0.1 ml of each filtrate was aseptically transferred into each hole. Then plates were kept at low temperature (4°C) for 2 h to allow maximum diffusion, and incubated at 30 °C for 1 and 3 days for bacteria and fungi, respectively. Antagonism was determined by measuring the size of inhibition zone around holes in millimeter; each result was an average of triplicate assays.

Antimicrobial activity against the multi-drug resistant nosocomial pathogens and MICs

Following all previous technique the *Streptomyces* strains were cultivated under all the optimized conditions, liquid cultures were aseptically filtered, sterilized and assayed for their antimicrobial activity against ten multi-drug resistant nosocomial pathogens. Minimum inhibitory concentrations (MICs) of the crude extracts were determined for establishment their antimicrobial activity. The MIC values were determined by broth tube dilution procedure using three-fold dilution method as previously described (Rashad *et al*., 2015).

Extraction and purification of the bioactive compounds

Each strain was grown under optimized fermentation conditions, the bioactive compound(s) from each culture filtrate was extracted by adding an equal volume of the ideal solvent system of ethyl acetate: chloroform (1:1) which exhibited the best bioactivity as previously obtained (Rashad *et al*., 2015). The crude extract of *St. fulvissimus* FHM275 was collected as dark yellow residue and *St. pratensis* FHM572 was collected as yellowish brown oily residue. The crude bioactive compound was dissolved in best solvent and purified using two different types of chromatography.

Thin Layer Chromatography (TLC)

TLC is a simple and quick chromatography; it is also usually used to determine the best solvent system for column chromatography in purification process. Crude bioactive compound(s) of each strain was dissolved in ethyl acetate: chloroform (1:1), spotted on TLC (Silica gel 60 F254, layer thickness 0.2 mm, E. Merck. Darmstadt, Germany) and developed in the gradient solvent system of petroleum ether: ethyl acetate ratios as a solvent system. The compounds were detected from their UV absorbance at 254 and 366 nm.

Silica gel Column Chromatography

Crude bioactive compound(s) of each strain was purified on a silica gel 60 for column chromatography, particle size 0.063- 0.200 mm (Merck, Germany) (5 by 20 cm), each crude bioactive compound(s) was dissolved in ethyl acetate: chloroform (1:1) and loaded on the top of the column. The mobile phase used was petroleum ether: ethyl acetate (60: 20, 60: 30, 50:50 v/v, respectively). The mobile phase run down and brought the mixture through the column by a gravity force. The components were distributed along the column and the colored components can be watched during the separation as colored bands on the

column or by using UV lamb. The drops from the bottom of the column were collected ; all separated fractions, each of 50 ml, were loaded on TLC, left to dry; then immersed in a cylindrical jar with cap containing petroleum ether: ethyl acetate (60:20) as a solvent system. The obtained fractions exhibited the same R_f value were collected and evaporated under vacuum and assessed for antimicrobial and antitumor activities.

***In vitro* cytotoxic activities bioassay**

Human normal and tumor cell lines and cell cultures

Anticancer activity of pure bioactive fraction was measured against human Caucasian breast adenocarcinoma (MCF 7), colon cell line (HCT 116), human hepatocellular carcinoma cell line (HePG 2) and lung carcinoma cell line (A549) as well as normal human epithelial amnion cells; all cell lines were obtained from ATCC. The bioassay procedure was conducted in the Bioassay-Cell Culture Laboratory, National Research Centre. Handling with cell lines was carried out under aseptic conditions in a laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA); HePG2, MCF7 and HCT116 cells were suspended in RPMI 1640 medium and A549 in DME medium. The media were supplemented with 1% antibiotic-antimycotic mixture (10,000 U/ml potassium penicillin, 10,000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37 ºC under 5% CO_2.

Cytotoxic activity

The viability of cells was determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay as described by Mosmann (1983). The assay is based upon the ability of mitochondria to catalyze the reduction of yellow MTT bromide to insoluble purple formazan, the concentration of which is measured spectrophotometrically. Cells were batch cultured for 10 days, then seeded at concentration of 10×10^3 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Medium was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of crude extracts and doxorubicin (adriamycin) as reference antitumor drug (positive control) to give final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml. After 48 h of incubation, medium was aspirated, 40 µl MTT salt (2.5 µg/ml) were added to each well and incubated for further 4 h at 37ºC under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200 µl of 10% sodium dodecyl sulfate (SDS) in deionized water were added to each well and incubated overnight at 37ºC. A positive control which composed of 100 µg/ml of adriamycin (doxorubicin) was used as a known cytotoxic natural agent that gives 100% lethality under the same conditions (Thabrew *et al*., 1997; Menshawi *et al*., 2010). The optical densities were measured using a microplate multi-well reader ELISA (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at a test wavelength 595 nm and a reference wavelength of 620 nm. Dimethyl sulfoxide (DMSO) is the vehicle used for dissolution of crude extracts and its final concentration on the cells was less than 0.2%. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. A probit analysis was carried out for IC_{50}, IC_{90} determinations using SPSS 11program. The percentage of change in viability was calculated according to the formula:

$$1- \frac{\text{Reading of extract}}{\text{Reading of negative control}} \times 100$$

Selectivity index (SI) was determined as the ratio of cytotoxicity (IC_{50}) on normal human epithelial amnion cells to each cancer cells (Mahavorasirikul *et al*., 2010).

Structure elucidation of purified bioactive compounds

The electron impact (Mass spectrum) spectrometric spectrum of the purified bioactive compounds were estimated using SSQ700-Finnigan mat. Scan speed: 0.5sec. EI= 70 ev. The ultraviolet (UV) and Fourier transform infrared (IR) spectra were estimated using SHIMADZU UV-2401PC UV- VIS PECORDING Spectrophotometer and FTIR- 6100, Jasco (Japan), Resolution 4 cm^{-1} respectively. Nuclear Magnetic Resonance

(NMR), the proton (^{1}H) and (^{13}C) NMR spectra were estimated using chloroform by Joel JNM- EX270, FF NMR system.

Statistical analysis

Data were statistically analyzed using analysis of variance (ANOVA) test (MSTAT-C Version 4, 1987).

RESULTS

Optimization of the fermentation conditions for production of bioactive compound(s)

Parameters controlling productivity of bioactive metabolites

Series of trials were directed to define the optimal fermentation conditions for achieving maximum productivity of bioactive compounds as evidenced by the size of inhibition zones against indicator microorganisms.

Environmental factors

The highest productivity was attained at the 5th and 7th days-seed inocula age of *St*. f*ulvissimus* FHM 275 and *St. pratensis* FHM572, as evidenced by the size inhibition zones, respectively (Fig. 1a). However, a decrease in the productivity was observed by increasing inocula age. The production of bioactive compounds started after 24 h and reached the maximum yields after the 5th day of incubation by both *Streptomyces* strains (Fig. 1b). After that, the productivity gradually decreased to become undetectable on the 9th and 10th day, respectively. Inoculation of the fermentation medium with 2 % (10^{6} spores / ml) of *St. fulvissimus* FHM 275 or 1% (10^{8} spores / ml) of *St. pratensis* FHM572 proved to be the optimum for obtaining the significant highest productivity. A concomitant decrease in productivity was observed at higher inoculum concentrations of both strains (Fig. 1c). Maximum productivity of bioactive metabolites was achieved at 30˚C with *St.* f*ulvissimus* FHM 275; but, *St*. *pratensis* FHM572 gave the highest production at 28˚C (Fig. 1d). Significant decreases in yields were observed by deviating the incubation temperature less or more than the optimum for each strain.

Aeration (flask loading and shaker speed) affected bioactive compounds productivity by both *Streptomyces* strains, it was altered by varying loaded volume / flask and the speed rate of shaker. The greatest significant productivity by both strains was attained at working volume of 20 % (v/v) and at the speeds of 150 and 180 rpm for *St. fulvissimus* FHM275 and *St*. *pratensis* FHM572, respectively. However, any drift of the working volume or shaking speed led to significant reduction of the yield of the bioactive compounds (Figs. 1e and1f). According to the inhibition zones (Fig. 1e), *St. fulvissimus* FHM 275 failed to synthesize bioactive compound(s) at pH 4.0, however its productivity was over the pH range of 5.0 – 9.0 with highest productivity at pH 7 to 7.5. Dissimilar behavior was observed with *St*. *pratensis* FHM572, where its antimicrobial activity was obtained over the pH range of 4.0 -9.0 with the uppermost productivity at pH 7.0. Deviance of initial pH to lower or higher the optimum values decreased the productivity.

Nutritional requirements

An equimolar of each of 13 carbon source was added instead of starch depending on the C\N ratio of the basal medium; based on the carbon source, the performance of each strain was variable in terms of the antimicrobial activity and antimicrobial spectrum against the target microorganisms (Fig. 2a). Nevertheless, starch was found to be the ideal carbon source that supported the highest productivity along with the highest activity by both strains. Starch at 20g/ l (C/N: 28.30) as a normal component in the basal medium was the idyllic concentration for obtaining the supreme productivity and potency of bioactive compounds either by *St. fulvissimus* FHM275 or *St. pratensis* FHM572 (Fig. 2b). The productivity was greatly reduced at starch levels lower or higher than 20 g/ l (C/N: 28.30).

All the tested organic and inorganic nitrogen sources supported the productivity of bioactive compounds by both strains (Fig. 2c), however, potassium nitrate (KNO_3) was pointed out as the best one. At 2.0 g/ l of KNO_3 (C/N: 28.30) as a normal component of basal medium, the highest productivity was achieved

ISSN: 0975-8585

by both strains (Fig. 2d). Also supreme productivity was obtained with K_2HPO_4 at a concentration of 1 g/l, the productivity was reduced beyond this level (Figs. 2e and 2f).

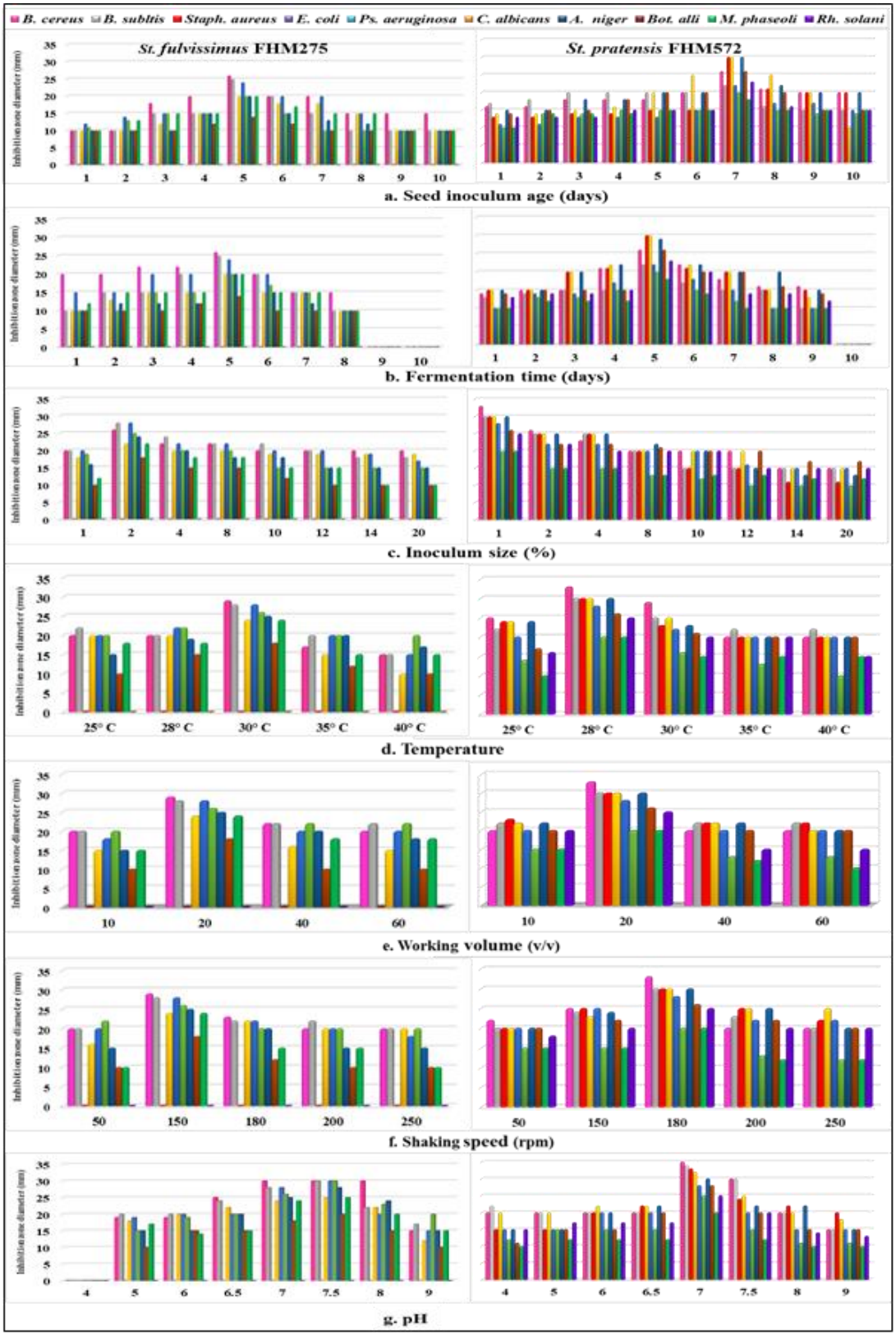


Fig. 1. Environmental parameters controlling productivity of bioactive metabolites.

Synthesis of bioactive metabolites by both strains were inhibited completely in the absence of $MgSO_4.7H_2O$, although, they were produced in the absence of NaCl and FeSO4. The highest productivity was

ISSN: 0975-8585

significantly achieved at a concentration of 0.5, 0.5 and 0.01 g/l, in that order. Optimal concentration required by both strains of $CaCO_3$ found to be 3 g/l, generally, the productivity was reduced by lowering the concentration under this level (Fig. 3a, b, c and d). It is worthy to mention that the growth of all the tested microorganisms was inhibited by the strain *St. pratensis* FHM572, however, *St. fulvissimus* FHM 275 showed no activity against *Staph. aureus* 25923 ATCC and *Rh. solani* MIRCIN as evinced by the antimicrobial activities.

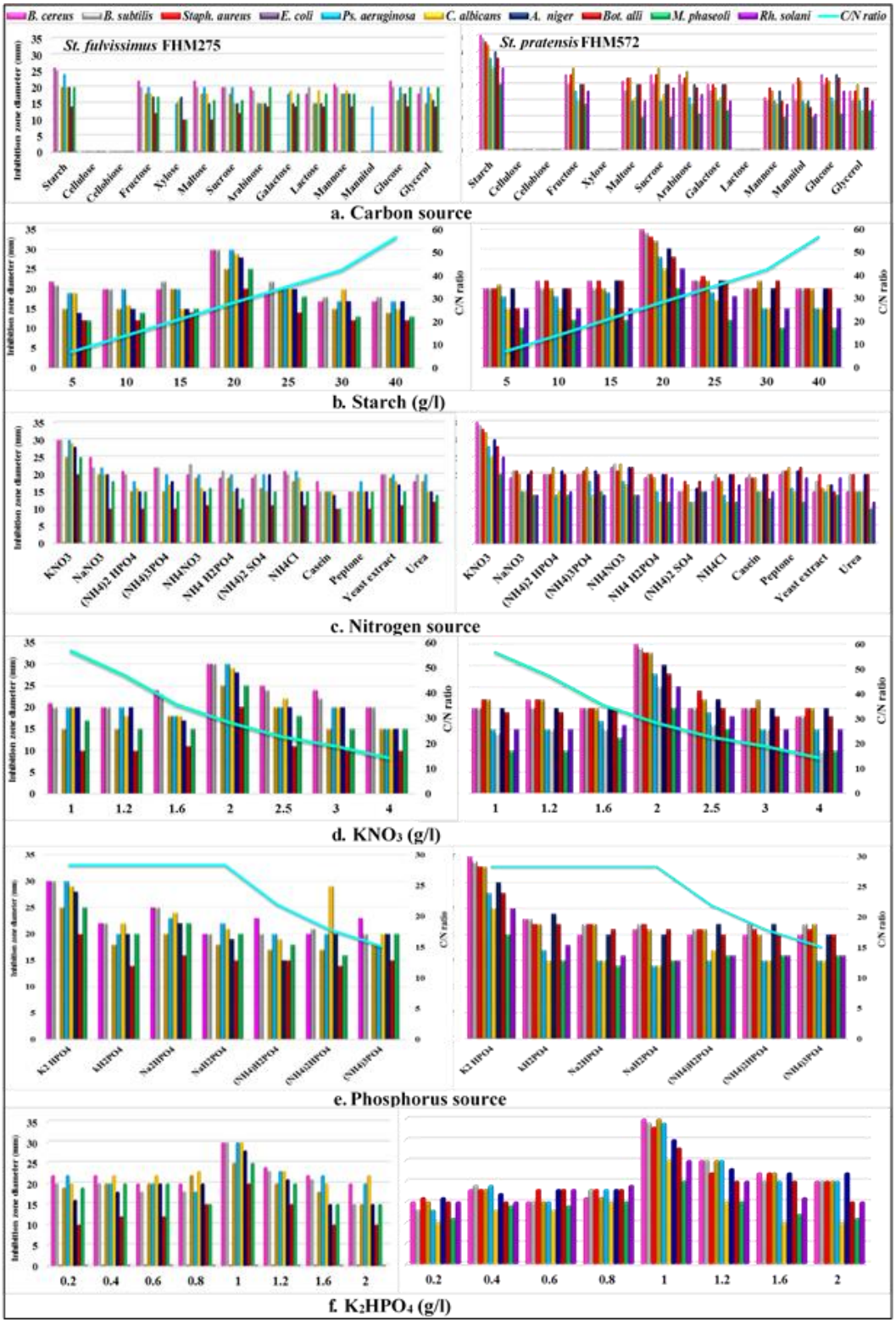


Fig.2. Effect of different carbon, nitrogen, phosphorus sources and concentrations on the productivity of bioactive compounds by *St. fulvissimus* FHM275 and *St. pratensis* FHM572.

ISSN: 0975-8585

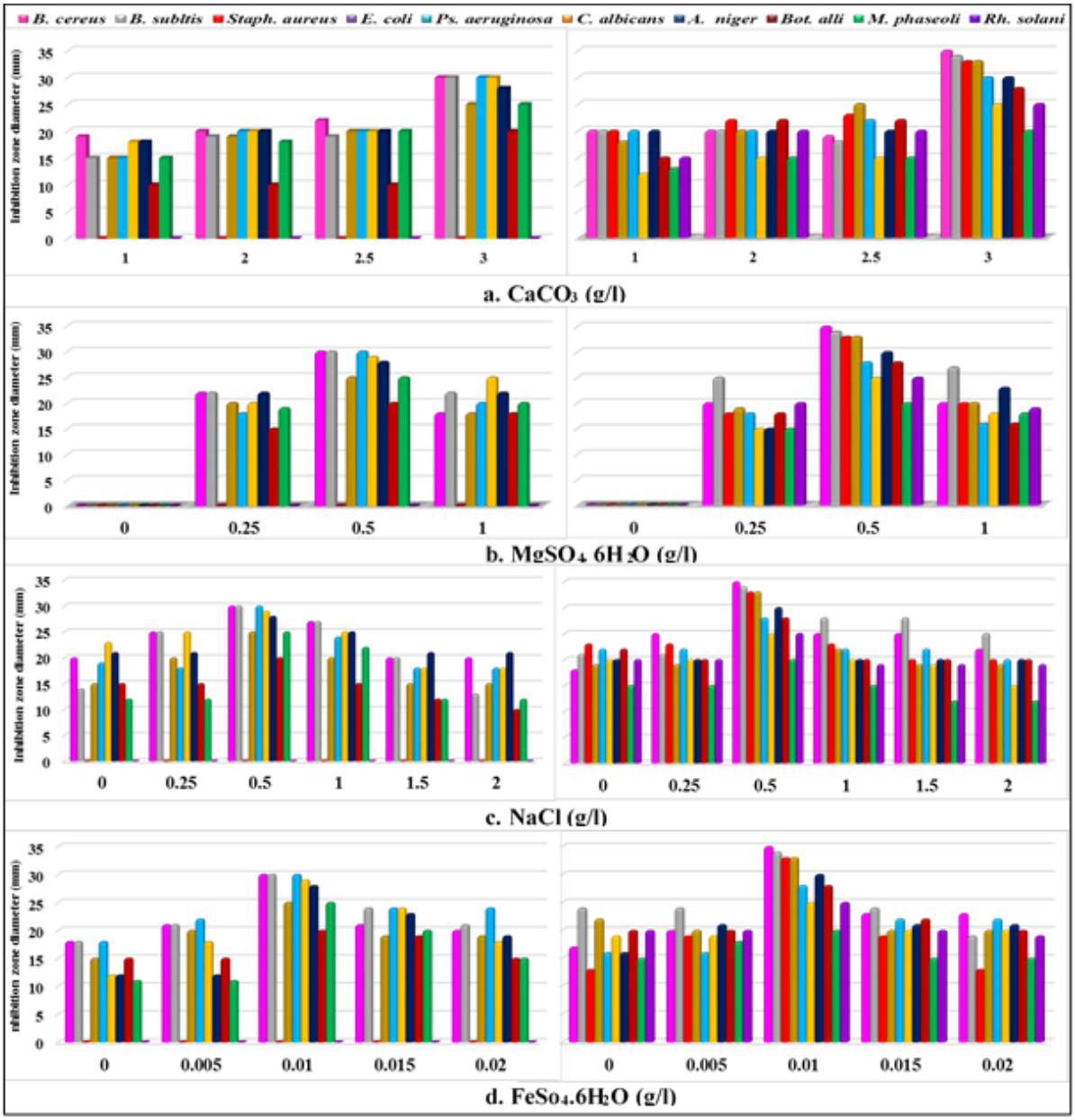


Fig. 3. Effect of different mineral concentrations on the productivity of bioactive compounds by *St. fulvissimus* FHM275 and *St. pratensis* FHM572.

Antimicrobial activity against the multi-drug resistant nosocomial pathogens and MICs

St. pratensis FHM572 exhibited strong inhibitory effects against the tested multi-drug resistant clinical isolates as evidenced by the inhibition zones that ranged from 18 to 26 mm; however, the *St. fulvissimus* FHM275 did not exhibit activity against *Enterococcus sp.* 128 and *Candida* 210 (Table 2). Neither *St. fulvissimus* FHM275 nor *St. pratensis* FHM572 showed any activity against MDR *Ps. aeruginosa* strains that showed resistance to almost all tested reference antibiotics (Table 1) or *Staph. aureus* 278. MICs of the crude extracts (EA/CH_3Cl, 1:1) ranged between 0.34 and 3.11 mg/ml; maximum activity of the crude extract of the strain *St. pratensis* FHM572 was obtained against *Enterococcus* 820, *Staph. aureus* 284 and *Candida* 210 which had the lowest MIC value of 0.34 mg/ml (Table 3). Always, *St. pratensis* FHM572 was generally more potent than *Streptomyces* FHM275 strain.

ISSN: 0975-8585

Table 1: Antibiotic sensitivity profile of nosocomial multi-resistant strains

Antibiotics / Potency/disk	*E.coli* 797	*E.coli* 799	*Ent* 128	*Ent* 820	*Sa* 274	*Sa* 284	*Pa* 71	*Pa* 76	*Can* 210
Inhibition of cell wall synthesis									
AMP10 µg		R	NT	R	R	R	R	R	
AUG (AMX 20µg + AMC 10 µg)		R	R	NT	R	R	R	R	
CPZ 75 µg	R	R					R	R	
SCF(SAM 30µg + CPZ 75µg)	S	S					R	R	
CRO 30 µg	R	R	R	R					
CXM 30 µg	R	R						R	
FOX 30 µg	S	S	R	R	R	R	R	R	
CAZ 30 µg	R	R	NT	R			R	R	
IMI 10 µg	S	S					R	R	
MEM 10 µg	S	S					R	R	
PTZ (PIP 100 µg + TZP 10 µg)	R	S					NT	R	
VA 30 µg			S	S	S	S			
CTX 30µg	NT	R	R	R			R	R	
TEC 30µg					NT	S			
CPM 30µg	R	R					R	NT	
Inhibition of cell membrane									
PB 300 U							S	S	
CO 10 µg							S	S	
Inhibition of DNA synthesis									
CIP 5 µg		R	I		R	R	R	R	
LEV 5 µg	R	R	S		R	R	R	NT	
NOR 10 µg	NT	R	NT	R			NT	R	
OFX 5µg					NT	R			
NA 30 µg		R					NT	R	
Inhibition of folic acid synthesis									
TS (TMP 125 µg + SMX 23.75 µg)	R	R	R	NT	NT	R	R	R	
Inhibition of protein synthesis by binding to 30S subunit									
AK 30 µg	NT	I			R	I	R	NT	
GM 10 µg	R	R	S	R	R	R	R	R	
DTX 30 µg					NT	S			
Inhibition of protein synthesis by binding to 50S subunit									
CD 2µg			R	NT	R	S			
E 15 µg			R	NT	R	S			
LZD 30 µg			NT	S	S	S			
Inhibition of macromolecules as DNA, RNA and protein									
NI 300 µg	NT	S	NT	R			NT	R	
Inhibition of cytochrome p									
Itraconazole 5 µg									S
Amphotericin-b 20 µg									R
Voriconazole 1 µg									R

Ent, Enterococcus sp.; Sa, Staph. aureus; Can, Cndida; Pa, Ps. aeruginosa. AMP: Ampicillin, Augmantin: (amoxicillin + clavulanic acid), Cpz : Cefoperazone, SCF: (sulbactam + Cefoperazone), CRO: Ceftriaxone, CXM: Cefuromixme, FOX: Cifoxitin, CAZ: Ciftazidime, IMI:Imipenem, MEM: Meropenem, PTZ: (piperacillin + tazobactam), VA: Vancomycin, CTX: Cefotaxime, TEC: Teicoplanin, CPM:Cefepime, AMP-b: Amphotericin b, PB: Polymyxin B, CO: Colistin, CIP: Ciprofloxacin, LEV: Levofloxacin, NOR: Norfloxacin, OFX: Ofloxacin, NA: Nalidixic acid, TS: (Trimethoprim + Sulfamethoxazole), AK: Amikacin, GM: Gentamicin, DTX: Doxycyline, CD: Clindamycin, ERYC: Erythromycin, LZD: Linzolid, NI: Nitrofurantoin, NT: not tested, ICZ: Itraconazole, VOR: Voriconazole. R: Resistant, S: Sensitive, I: intermediate, NT: not tested.

ISSN: 0975-8585

Table 2: Antimicrobial activity of the cultural filtrates of *St. fulissimus* FHM275 and *St. pratensis* FHM572 against multi-drug resistant nosocomial pathogens.

Nosocomial Pathogens	*St. fulvissimus* HFM275	*St. pratensis* FHM572
	Inhibition of zone diameter in mm	
E. coli 797	25	20
E. coli 799	20	26
Ps. aeruginosa 71	Not active	Not active
Ps. aeruginosa 76	Not active	Not active
Entero. sp. 128*	Not active	18
Entero. sp. 820*	18	20
Staph. aureus 278	Not active	Not active
Staph. aureus 284	18	18
Staph. aureus MRSA**	18	18
Candida 210	Not active	25

* *Enterococcus* sp.; ** ATCC 43300

Table 3: MIC values of bioactive crude extracts from cultural filtrates of isolates *St. fulissimus* FHM275 and *St. pratensis* FHM572.

Test organism	FHM275	FHM572	Resistance pattern to reference antibiotics µg/disk
	MIC mg/ml		
E. coli 797	3.11	3.11	CPZ, 75; CXM, 30 , CAZ, 30; PTZ (PIP 100 + TZP 10), CMP, 30; CRO 30' LEV, 5; TS (TMP, 125+SMX, 23.75); GM, 10.
E. coli 799	3.11	1.03	CPCZ 75; CRO, 30; CXM,30; CAZ, 30; CMP, 30; TS (TMP, 125+SMX ,23.75); GM, 10.
Enterococcus sp. 128	NA	3.11	CRO 30, FOX, 30; CTX 30.
Enterococcus sp. 820	0.34	0.34	AMP,10; CRO 30, FOX, 30; CTX, 30; GM, 10;
Staph. aureus 284	1.03	0.34	AMP, 10; AUG (AMX 20 + AMC 10), FOX, 30; CIP 5; LEV, 5; OFX, 5; TS (TMP, 125 + SMX, 23.75); GM, 10.
Staph. aureus MRSA	3.11	1.03	
Candida 210	NA	0.34	AMP-b, 20; VOR, 1

AMP: Ampicillin, AMP-b: Amphotericin b; Augmantin: (amoxicillin + clavulanic acid), CAZ: Ciftazidime, CIP: Ciprofloxacin; CPM:Cefepime; Cpz : Cefoperazone; CXM: Cefuromixme; CRO: Ceftriaxone; CTX: Cefotaxime; FOX: Cifoxitin; GM: Gentamicin; LEV: Levofloxacin; OFX: Ofloxacin; PTZ: (piperacillin + tazobactam); TS, (Trimethoprim + Sulfamethoxazole); VOR: Voriconazole.

Purification of bioactive compounds

Bioactive compounds produced by *Streptomyces* strains were separated and purified using silica gel column chromatography and TLC techniques, the compounds were sequentially eluted with petroleum ether: ethyl acetate in different ratios. From the crude extracts of *St. fulvissimus* FHM275, sixty fractions were separated and collected on the basis of their TLC profile as follow: twenty colorless fractions eluted with petroleum ether: ethyl acetate (60:20) and have similar R_f values were collected as PF1 and evaporated under vacuum; thirty yellow color fractions eluted with petroleum ether: ethyl acetate (60:30), each of which showed similar R_f value were collected as PF2 and evaporated under vacuum; ten pale brown color fractions showed similar R_f values were eluted with petroleum ether: ethyl acetate (50:50), collected as PF3 and evaporated under vacuum. Another, fifty fractions were separated from the crude extract of *St. pratensis* FHM 572 as follows: five colorless fractions with similar R_f values eluted with petroleum ether: ethyl acetate (60:20) were collected as FH1a and evaporated under vacuum; one more five similar colorless fractions eluted with petroleum ether: ethyl acetate (60:30) and found to have the similar Rf value of PFH1a were collected as PFH1b and evaporated under vacuum. Both the two groups PFH1a and PFH1b were pooled to comprise PFH1; additional forty oily yellow color fractions eluted with petroleum ether: ethyl acetate (50:50), each of which

showed similar R_f values, collected as PFH2 and evaporated under vacuum. All pooled fractions PF1, PF2, PF3, PFH1 and PFH2 were assessed for determination their antimicrobial and antitumor activities.

Biological activities of purified fractions

Antimicrobial activities

Each pooled fraction (PF) obtained from *St. fulvissimus* FHM275 or *St. pratensis* FHM572 was assessed to determine its antimicrobial activity using disk diffusion method. PF1 and PF3 of *St. fulvissimus* were not active while the yellow oily color compound PF2 was the major and the most potent one when checked against *Ps. aeruginosa* ATCC 9027*, Bacillus subtilis* ATCC 6633 *and Aspergillus niger* ATCC 16404; the inhibition zone diameters were 32, 33 and 30 mm, respectively. PFH1 that obtained from *St. pratensis* was not active while PFH2 was the major and the most potent one when checked against *E.coli* ATCC 19404, *B. cereus* and *A. niger*, the inhibition zone diameters were 33, 36 and 33 mm, respectively.

Cytotoxic activities of separated fractions

Following the MTT assay, all the fractions PF1, PF2, PF3, PFH1 and PFH2 were tested for their cytotoxicity. Fractions PF2 and PFH2 were active against tumor cell lines, however, PF1, PF3 and PFH1 were not. PF2 which obtained from *St. fulvissimus* FHM275 is promising compound and appeared to be the best. The compound did not show any damage to the normal cell line up to 100 µg/ml and showed strong activity and highest selectivity against lung carcinoma (A549), the complete inhibition that obtained up to 6.25 µg/ ml was in accordance with IC_{90}. This compound also showed good activity against colon cancer (HCT 116) with IC_{50} of 21. 4 µg/ml, although the value was higher than that obtained from crude extract (Table 4).

Table 4: Cytotoxic activities and selectivity index (SI) of the crude extract and purified compound from *St. fulvissimus* FHM275.

Bioactive Compounds	Human tumor cell line (s)												Normal human epithelial amnion cells	
	Caucasian breast adenocarcinoma MCF7			Colon HCT116			Hepatocellular carcinoma HePG2			Lung carcinoma A549				
	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}
	(µg/ ml)													
Crude*	NA	NA	-	15.5	28.4	3.62	NA	NA	-	15.30	26.20	3.67	56.1	97.0
Purified (PF2)	NA	NA	-	21.3	38.40		NA	NA	-		≤ 6.25	highest	NA up to 100	
DOXO**	26.10	48.90	1.98	37.60	65.10	1.38	21.6	37.9	2.39	28.30	55.6	1.83	51.7	87.8
Negative control	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA	-	NA	NA

IC_{50}: Inhibition concentration of the sample which causes the death of 50% of cells in 48 hrs; IC_{90}: Inhibition oncentration of the sample which causes the death of 90% of cells in 48 hrs; SI, Selectivity index = IC_{50} against normal cell line/ IC_{50} against each cancerous cell; *after Rashad *et al.* (2015); NA: not active;** Doxorubicin = adriamycin: Positive control.

PFH2 that obtained from *St. pratensis* FHM 572 showed broad activities against different human cancer cell lines. Likewise PF2 compound, the PFH2 was strongly active and the highest selective against lung carcinoma cell line (IC_{90} up to 6.25 µg). This value was much lower than that needed to obtain an equal inhibition of normal human epithelial amnion cells (IC_{90}: 98.5µg/ml). PFH2 was also active against breast and colon cancer cell line, it killed 50% of cancer cells at values of 11.3 and 14. 4 µg/ml, respectively. Selectivity index was determined by comparing the cytotoxic activity (IC_{50}) against each breast and colon cancerous cell with that of the normal human cell, it

was greater than 3 which considered as highly selective (Table 5). The hepato carcinoma HepG2 appears to be the most resistant cell line to the tested purified compound and the purified PFH2 was lost its antagonistic effect comparing to crude extract from which it was separated. However, It worthy to mention that, except HepG2, the crude extracts were less effective than the purified compounds. Both were much more active when compared to the doxorubicin which used as reference control.

Table 5: Cytotoxic activities and selectivity index (SI) of the crude extract and purified compound from *St. pratensis* FHM572.

Bioactive Compounds	Human tumor cell line (s)												Normal human epithelial amnion cells	
	Caucasian breast adenocarcinoma MCF7			Colon HCT116			Hepatocellular carcinoma HePG2			Lung carcinoma A549				
	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}	SI	IC_{50}	IC_{90}
	(µg/ ml)													
Crude*	**70.60**	**114.40**	**0.84**	**16.10**	**29.30**	**3.70**	**36.9**	**71.7**	**1.61**	**7.5**	**14.8**	**7.93**	**59.5**	**105.4**
Purified (PFH2)	**11.30**	**25.90**	**4.50**	**14.40**	**27.60**	**3.50**	**97.4**	**150.7**	**0.53**		**≤ 6.25**	**highest**	**50.9**	**98.5**
DOXO**	**26.10**	**48.90**	**1.98**	**37.60**	**65.10**	**1.38**	**21.6**	**37.9**	**2.39**	**28.30**	**55.6**	**1.83**	**51.7**	**87.8**
Negative control	**NA**	**NA**	**-**	**NA**	**NA**	**-**	**NA**	**NA**	**-**	**NA**	**NA**	**-**	**NA**	**NA**

IC_{50}: Inhibition concentration of the sample which causes the death of 50% of cells in 48 hrs; IC_{90}: Inhibition oncentration of the sample which causes the death of 90% of cells in 48 hrs; SI, Selectivity index = IC_{50} against normal cell line/ IC_{50} against each cancerous cell; *after Rashad *et al.* (2015); NA: not active; Doxorubicin = adriamycin: Positive control.**

Structure elucidation of purified bioactive compounds

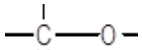
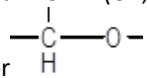
The purified bioactive compounds PF2 and PFH2 which produced by *St. fulvissimus* FHM275 and *St. pratensis* FHM572, respectively, were spotted on TLC using different solvent systems to evaluate the R_f values and subsequently to confirm their purity. Both PF2 and PFH2 were closely similar, whereas they gave nearly equal R_f values (0.8) using petroleum ether: ethyl acetate (60:20) as a solvent system, however they showed small differences with the other solvent systems (Table 6). Together PFH2 and PF2 gave positive Libermann test indicating an isoprenoid carbon-hydrogen skeleton. Their bio spectral data were, to some extent, comparable which indicate a specific difference in their close similar structure. PFH2 has a brilliant blue fluorescence under UV 365 ηm and has UV absorption at 240 and 280 ηm. Likewise PFH2, PF2 has a brilliant blue fluorescence under UV 365 ηm. The UV absorption spectrum of the compound PF2 showed bands at 245 and 280 within the same range as PFH2 (Figs. 4a and 4b). To high extent, the IR spectra of PF2 and PFH2 are similar (Figs. 5a and 5b). The IR spectrum of PFH2 showed the absorption bands cm^{-1} at 3428  (OH), 2922 and 2822 (CH, CH_2, CH_3), 1723 for C=O ester, 1633 for C=C, 1456 for CH_3, 1272 for ester formed 1040 for 

Table 6: R_f values of bioactive compounds in different solvent systems.

	PF2	PFH2
Petroleum ether: ethyl acetate (60:20)	0.80	0.80
Petroleum ether: methanol (96: 4)	0.43	0.45
Chloroform: methanol (40:10)	0.52	0.55

ISSN: 0975-8585

$$R_f = \frac{\text{Distance moved by the bioactive substance (s)}}{\text{Distance moved by the solvent system}}$$

The IR spectrum of the bioactive compound PF2 showed the adsorption peaks of a conjugated hydroxyl group (OH) at 3435 cm^{-1}, (CH, CH_2, CH_3) at 2923, 2854 cm^{-1}, ester group C=O at 3443 cm^{-1}, double bond C=C at 1634 cm^{-1}, methyl group CH_3 at 3435 cm^{-1} and 1110 for

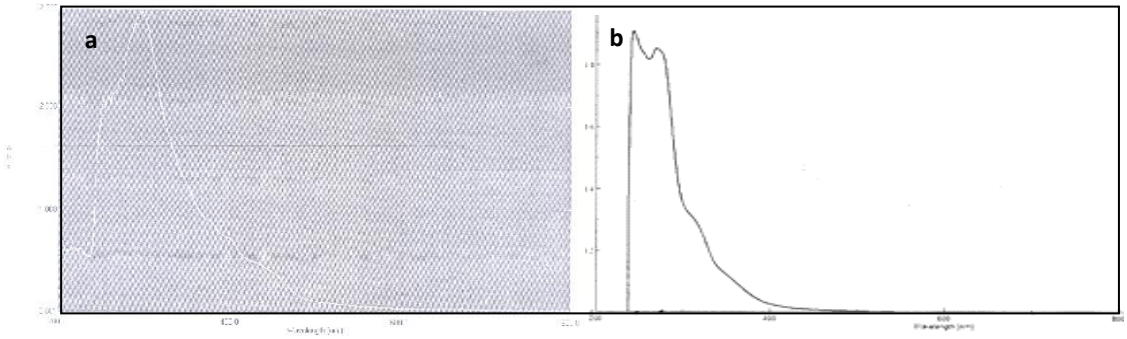


Fig 4: UV spectra of the bioactive compounds PF2 and PFH2 produced by:a) *St. fulvissimus* FHM275 and b) *St. pratensis* FHM572, respectively.

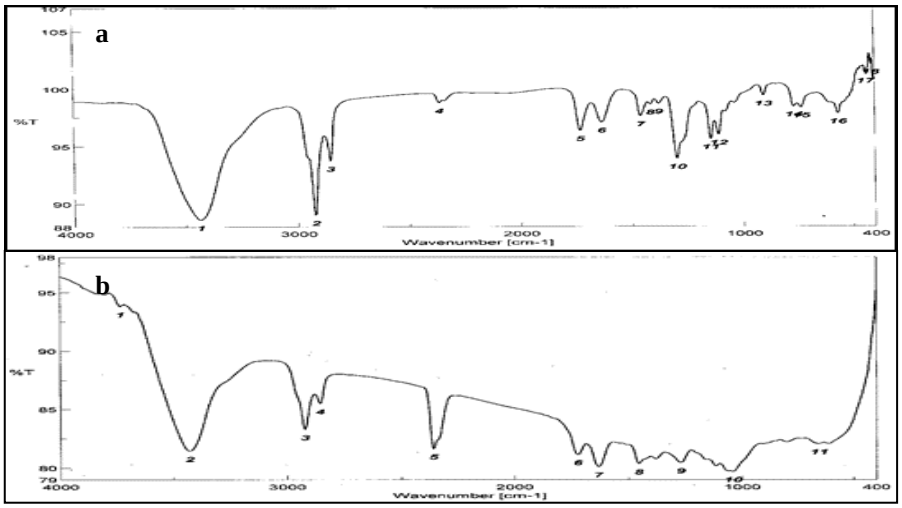
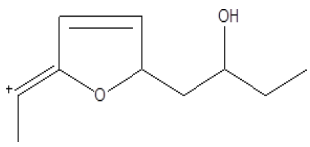
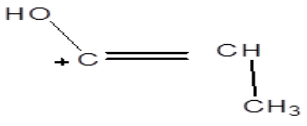


Fig 5: IR spectra of the bioactive compounds PF2 and PFH2 produced by: a) *St. fulvissimus*FHM275 and b) *St. pratensis* FHM572, respectively.

The mass measurements of the PF2 and PFH2 molecules (Figs. 6a and 6b) suggested the molecular formula as $C_{11}H_{20}O_4$, $C_{12}H_{22}O_4$ with a corresponding molecular weight of 216 and 230, respectively. The MS spectrum of PFH2 showed molecular ion at m/z 227 (M^+ -3) and a high stable fragment at m/z 167 in ionic form due to loss of 60 unit {$HCOOCH_3$ the ester radical} followed by m/z 149 due to loss of 18 unit (H_2O). The base peak at m/z 57 resulted from ionic fragment from the side chain $(C_3H_5O)^+$. The spectra showed different CH and CH_2 fragments specific for isoprenoid skeleton as follow:

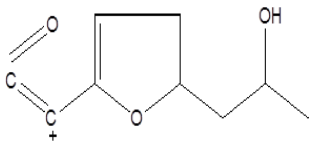




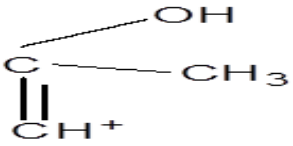
$C_{10}H_{15}O_2$ m/z 167 **m/z 57 base beak**

Mass spectrum of PFH2

ISSN: 0975-8585



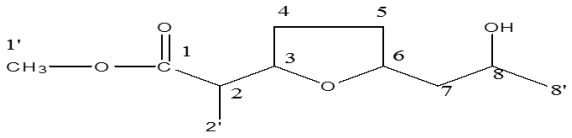
$C_9H_{11}O_3$ m/z 167



m/z 57 base beak

Mass spectrum of PF2

The spectrum of the PF2 molecule showed similar base peak as PFH2 at m/z 57 in ionic form but in different configuration and the same fragmentation pattern as PFH2 e.g. m/z 167 as follow:



Methyl nonactate (PF2)

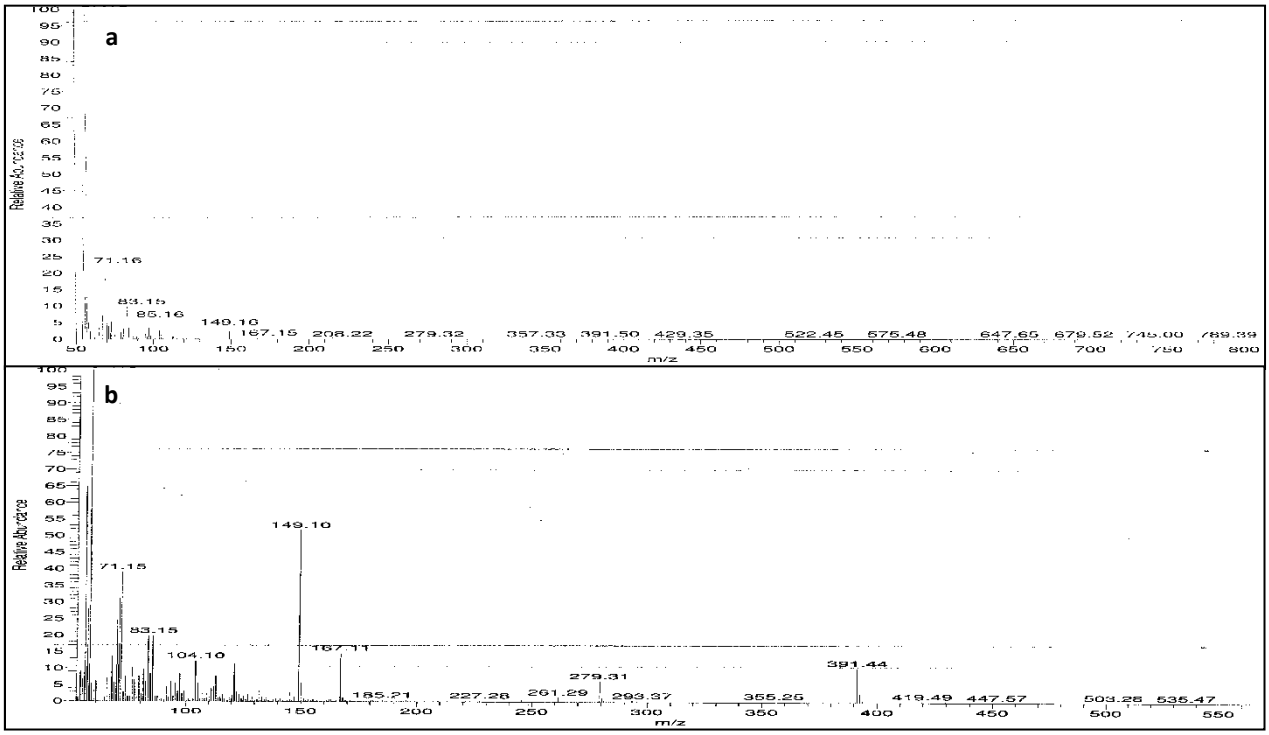
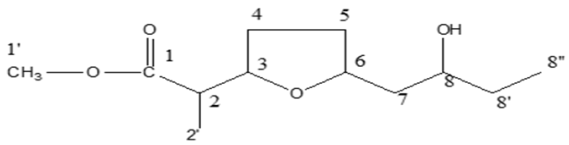


Fig 6: Mass spectra of the bioactive compounds PF2 and PFH2 produced by:a) *St. fulvissimus* FHM275 and b) *St. pratensis* FHM572, respectively.

The ^{1}HNMR and C^{13} NMR spectral analysis of both F2 and FH2 are presented in Table (7) and Figs. (7a, b and 8a, b). Built on the data base library, PF2 found to be methyl nonactate and PFH2 is methyl homononactate.



Methyl homononactate (PFH2)

ISSN: 0975-8585

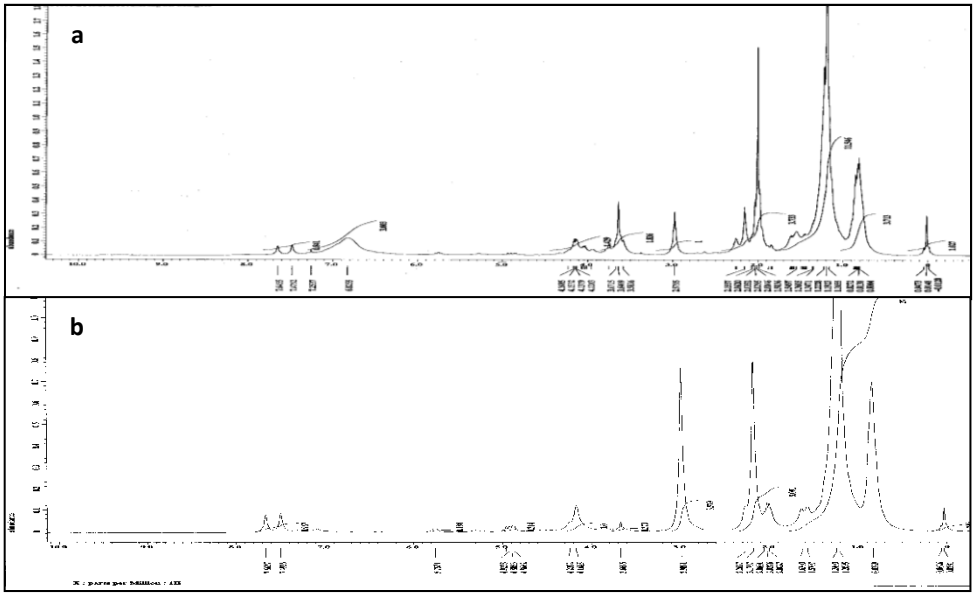


Fig 7: ^{1}H NMR of the bioactive compounds PF2 and PFH2 produced by: a) *St. fulvissimus*FHM275 and b) *St. pratensis* FHM572, respectively.

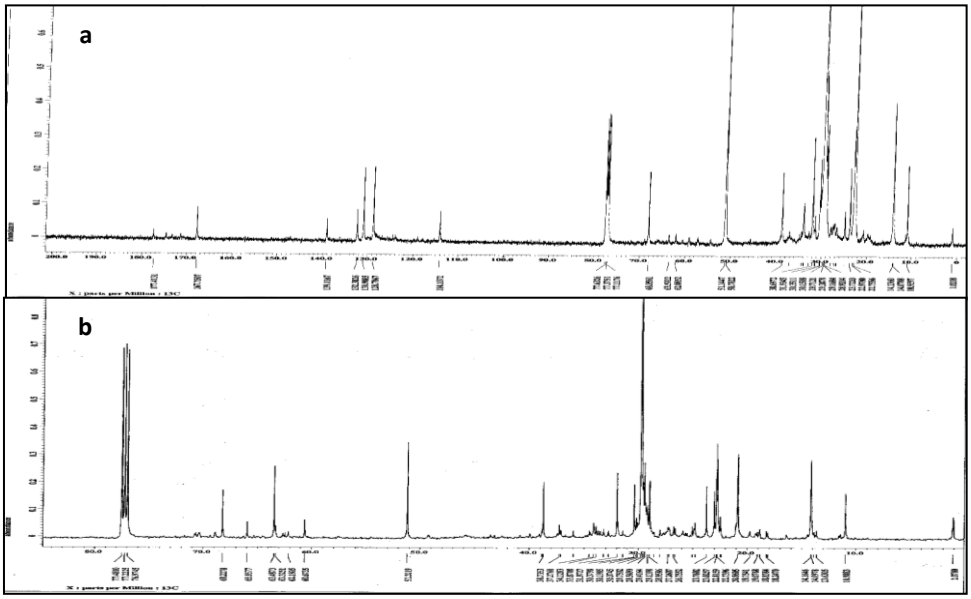


Fig 8: ^{13}C of the bioactive compounds PF2 and PFH2 produced by: a) *St. fulvissimus* FHM275 and b) St. pratensis FHM572, respectively.

Table 7: ^{1}H NMR and ^{13}C data for bioactive compounds PF2 and PFH2

No.	δ_H ppm		$\delta\ ^{13}C$ ppm	
	PF2*	PFH2**	PF2	PFH2
C_1	———	———	177.000	176.000
C_2	2.900	2.970	44.000	44.000
C_3	4.200	4.170	77.000	77.000
C_4	1.600	1.980	28.900	28.900
C_5	2.002	2.006	30.300	30.300
C_6	4.100	4.100	68.000	68.200
C_7	1.570	1.500	38.600	38.700
C_8	4.920	4.910	77.600	77.480
$C_1^{/}$-CH_3	3.660	3.640	45.000	51.200
$C_2^{/}$- CH_3	1.630	1.190	14.000	14.000

$C_8^{/}$	———	1.360	———	29.700
$C_8^{/}$- CH_3	1.209	———	29.7000	———
$C_8^{//}$- CH_3	———	0.8270	———	10.900

* produced by *St. fulvimissus* FHM275.
** produced by *St. pratensis* FHM572.

DISCUSSION

The capability of *Streptomyces* to synthesize the bioactive compounds is not a stable trait but can be greatly influenced under different conditions of nutrition and cultivation. Therefore, study on the production of biological active agents usually involves a search strategy to determine the most suitable culture condition. Biosynthetic capacity is regulated mostly by two control levels, the first is the nature and concentrations of nutrients; the second is the environmental factors (Waksman, 1961; Saudagar and Singhal, 2007).

In the present study, fermentation parameters were firstly manipulated to institute the ideal conditions for obtaining the maximal productivity of bioactive compounds from *St. fulvissimus* FHM 275 and *St. pratensis* FHM572. Our results are in conformity with the findings by many researchers who found that inoculum age and size play an essential role in the performance of the bioprocess results and strongly was strain dependent. While some of them obtained highest productivity with spore inocula, the others found vegetative inocula was the most suitable conditions for maximum productivity (Elibol *et al*., 1995; El-Enshasy *et al*., 2000; Mukhtar *et al*., 2012). A concomitant decrease in the yield which observed at lower or higher inoculum concentrations might be attributed to the effect of inoculation volume on the growth and then the metabolites accumulation (Hunt and Stieber, 1986; El-Enshasy *et al*., 2000; Song *et al*., 2012).

The exact time for biosynthesis commencement or reaching the maximum productivity for bioactive compounds was inconsistent and also strain dependent (Sujatha *et al*., 2005; Reddy *et al*., 2011; Bundale *et al*., 2015). The elaboration of bioactive metabolites found to be in parallel with microbial growth; it was early started and reached supreme on the 5th day by *St. fulvissimus* FHM275 and *St. pratensis* FHM572, then decreased by extending the fermentation time. A drastic reduction in productivity after 9 or 10 days, respectively, may be due to the degradation of the compound (Gross and Morell 1971). Both *strains* showed a relatively narrow range of incubation temperature for moderately good productivity (25 to 40 °C) with ideal temperature of 28 and 30 °C for maximum productivity, respectively. In view of the previous studies, the optimum temperature for the best productivity was found to be variable according to the tested producer strain, it was over the range of 24 to 37 °C. Deviation from the optimum temperature resulted in reduction of the bio-production yield. Such adverse effect is attributed to the retardation of the metabolic processes (Augustine *et al*., 2005; Sujatha *et al.*, 2005; Oskay *et al.*, 2011; Song *et al*,. 3012; Uddin *et al*., 2013).

The initial pH value of the fermentation medium is markedly affect the activity of several enzymes that catalyze metabolic reactions and regulate the biosynthesis of secondary metabolites in *Streptomyces* species (Srinivasan *et al.*, 1991; Elmahdi *et al*., 2003). In the light of previous studies, there was a broad range of optimum pH levels ranged from 5.0 to 10 (Crawford *et al.*, 1993; Bundale *et al*., 2015) however, the majority found the optimum pH for best bio production in *Streptomyces* cultures was near neutral (Augustine *et al*., 2005; Sujatha *et al.*, 2005; Oskay *et al*., 2011; Reddy *et al*., 2011) and slight basic (El-Naggar *et al*., 2003; Mukhtar *et al*., 2012). The present study confirmed that the effect of pH is strain dependent, the productivity of *St. fulvissimus* FHM275 was obtained over the pH range of 5-9 with the optimum of 7-7.5; while, *St. pratensis* synthesized the bioactive compounds over pH range of 4.0 -9.0 and optimum of 7.0.

An obvious correlation between aeration (working volume, agitation speed) and productivity of bioactive compounds was previously observed by many researchers. Evidence presented from the obtained results showed the aeration requirement was also strain dependent while the utmost productivity was attained at 20 % (v/v) loading volume at speed rates of 150 rpm or 180 rpm by *St. fulvissimus* FHM275 and *St. pratensis* FHM572, respectively. It has been found that oxygen limitation can lead to a slow-down of metabolism or in some cases a complete change in metabolism (El-Enshasy *et al*., 2000; Büchs, 2001; Augustine *et al*., 2005).

The current study showed that the composition of the production medium is directly influenced the performance of *Streptomyces* strains; changes in the nature or concentrations of carbon, nitrogen and

phosphorus sources as well as minerals significantly affected growth and then the biosynthesis of bioactive metabolites. Carbon and nitrogen sources are commonly used as growth and energy substrates to synthesize enzymes and bioactive secondary metabolites through fermentation. However, inorganic phosphorus is generally the main growth-limiting nutrient, it often regulates the synthesis of extracellular enzymes and secondary metabolites; it is virtually exhausted during growth before the onset of biosynthesis (Demain, 1989; Sánchez and Demain, 2002; Juan, 2004; Sánchez *et al*., 2010). In general, there is no agreement in the fermentation literature concerning the favorite nature and/or concentration of various sources in terms of its influence to get the highest productivity by actinomycetes. Such influence is highly specific for every strain and even for another secondary metabolite produced by the same strain (Shapiro, 1989; Lee and Demain, 1997; Sanchez and Demain, 2002; Jonsbu *et al.,* 2000; Jonsbu *et al.,* 2002; Saudagar and Singha, 2007).

Minerals are indispensable components for microbial growth and subsequently bioactive metabolites production. Some of them are clearly added, however, the others are present as impurities with some medium constituents. Calcium, magnesium, iron and sodium chloride are vital constituents for any fermentation medium; all of them are physiologically required and involved in multiplicity of cellular reactions. Calcium carbonate acts as buffering agent to control the pH of the fermentation media besides its own effect on antibiotic production (Abbas and Edwards, 1990). Mg^{2+} is required for protein synthesis, and its depletion may restrict enzyme synthesis and activity (Fraústo da Silva and Williams, 2001). Moreover, the stimulatory effect of Mg^{2+} ions may reflect its role as an ammonium ion-trapping agent, thereby eliminating nitrogen catabolite repression in fermentations (Omura *et al*., 1980; Byrne and Greenstein, 1986). Iron is also a crucial element for all living cells because it is incorporated as a cofactor in many metalloproteins involved in vital metabolic pathways. Sodium chloride has a profound effect on the production and release of bound antibiotic from the mycelium, conversely, some studies revealed that NaCl was without any effect on the antibiotics productivity (Perlman and Langlykke, 1949; Basak and Majumdar, 1975; Pelczer *et al*., 1993). Some metals were found to be essential for growth and/or productivity; however, the others were not required. This might explain the conflicting results that obtained by many authors (Basak and Majumdar, 1975; Abbas and Edwards, 1990).

The present findings indicated the dependence of the biosynthesis on the fermentation medium composition. Unlike environmental factors, nutrition requirements of both strains were consistent. Among all the different carbon, nitrogen and phosphorus sources assessed, the highest productivity by *St. fulvissimus* FHM 275 and *St. pratensis* FHM572 was achieved with starch,20 ; KNO3, 2 and K2HPO4, 1g/l; which corresponding to C, N, P and K at concentrations of 7.8480, 0.2771, 0.1778 and 1.224 1g/l, respectively. The present results also showed the necessity of the biosynthesis of active metabolites to the presence of Mg^{2+}; although the bioactive compounds were released in the absence of iron and NaCl, the productivity was enhanced on increasing their concentrations.

The antibiotic activities of the culture filtrates of *Streptomyces* strains during optimization studies exhibited a wide spectrum of activity against common pathogenic bacteria and fungi. *St. fulvissimus* FHM275 showed activity against *B. cereus, B. subtilis, E. coli, Ps. aeruginosa, C. albicans, A. niger, Bot. allii* and *M. phaseolina*. However, *Streptomyces pratensis* FHM572 exhibited a wider spectrum of antagonistic potential against the aforementioned tested microorganisms in addition to *Staph. aureus* ATCC 25923**,** *R. solani.* Due to the rapid emergence of multi-drug pathogens causing important community acquired infections that represents seriously worldwide medical crisis, there is an increasing demand for obtaining an effective antibiotic with broad spectrum. Therefore, it was important to assess the antimicrobial potentiality produced by these *Streptomyces* strains under optimized conditions against MDR clinical isolates. *St. fulvissimus* FHM275 was active against nosocomial multidrug strains of *E. coli* 797, *E. coli* 799, *Enterococcus* 820, *Staph. aureus* 284 and MRSA *Staph. aureus* ATCC 43300. Cultural filtrate of *Streptomyces pratensis* FHM572 showed a wider spectrum of antimicrobial activities against to *Enterococcus* 128 and *candida* 210 in addition to the aforementioned gram positive and Gram negative MDR clinical isolates. Their antimicrobial activity against clinical isolates was established by evaluating the MIC values of the crude extracts from ethyl acetate: chloroform (1:1), which ranged between 340 to 3110 µg/ ml comparing to 4.5 to 370 µg/ml for common target microorganisms (Rashad *et al*., 2015). The MIC value is not a constant for a given agent, because it is affected by the nature of the test organism used (Khajure and Rathod, 2011). According to Gibbons (2004) and Ríos and Recio (2005) extracts were classified as being good, MIC $\leq$ 1 mg/ml; moderately good, MIC > 1mg/ml or < 4 mg/ml; moderate, MIC = 4 mg/ml or < 6 mg/ml or poor, MIC $\geq$ 6 mg/ml.

The ethyl-acetate/chloroform (1:1) crude extracts from the fermentation media of *Streptomyces* strains were subjected to chromatographic analysis for separation and purification of bioactive molecules. Not all separated purified compounds from each crude extract showed antagonistic capacity, only the oily yellow color PF2 and PFH2 compounds that produced by *St. fulvissimus* FHM275 and *St. pratensis* FHM572, respectively, exhibiting antimicrobial activity as well as cytotoxic activity against human tumor cell lines. Based on the obtained patterns of color, R_f values and biological activities, it could be expected the close similarity between PF2 and PFH2. Built on the obtained spectral information (MS, UV, IR and NMR) along with the comparison to the data base library, these bioactive compounds were identified as methyl nonactate ($C_{11}H_{20}O_4$) and methyl homononactate ($C_{12}H_{22}O_4$), respectively. These pure compounds exhibited higher cytotoxic activities than the crude extract. Such effect might be due to synergism between compounds present in the extract (Sufian *et al*., 2013). These compounds also showed different range of cytotoxic activity against the tested human cancer cell lines, both of them showed activity towards both lung (A549) and colon (HCT116). However, only methyl homononactate exhibited cytotoxic activity against breast cancer cell line (MCF7). Together methyl nonactate and methyl homononactate compounds appeared much more potent with highest selectivity index comparing to doxorubicin which is used as positive control. Clinically, doxorubicin is used as active drug for the treatment of a great variety of cancer diseases (Gille *et al*., 2002; Wibowo *et al*., 2011), although cardiac toxicity is remaining a major side effect (Tokarska-Schlattner *et al*., 2006).

In the US National Cancer Institute (NCI) plant screening program, a pure compound is generally considered to be active if the IC_{50} value is 4 µg/ml or less following incubation between 48 and 72 h (Lee and Houghton, 2005). Hence, according to the NCI guidelines, the compounds methyl nonactate (PF2) and methyl homo nonactate (PFH2) could be considered as strongly active with highest selective index against lung carcinoma (A 549). Based on these criteria we suggest that methyl nonactate (PF2) is considered good active anti colon cancer as the product did not show any damage to the normal cell line up to 100 µg/ml. However, methyl homononactate (PFH2) showed moderate activity against breast and colon cancer (IC_{50} 11.30 and 14.4 µg/ml, respectively). Remarkably, it was selective towards breast and colon cancer cells (SI = 4.5 and 3.5, respectively).

Methyl nonactate is an outstanding natural product scaffold for library development; it is ideal for use in drug discovery. It is a stereochemically complex molecule with readily modifiable alcohol and ester groups. It is derived from fermentation broth of *Streptomyces griseus* that cotain high titer of macrotetrolide antibiotics by methanolysis process using *Rhodococcus erythropolis* as an efficient biocatalyst. However, this approach generates mixtures of homologues that are not economically separable (Nikodinovic *et al*., 2006; Luesse *et al*., 2008, Phillip *et al*., 2010).

Nonactic and homononactic acids are the precursors of macrotetrolide antibiotics as building units of ionophoretic character. Nonactin is the parent compound of macrotetrolide class of antibiotics, it is consisting of enantiomer of nonactic acid joined head-to-tail in an alternating manner (+)(-)(+)(-).The higher macrotetrolide homologs of nonactin are monactin, dinactin, trinactin and tetranactin; which are made up of successive substitutions of nonactic acids with either homononactic acid or bishomononactic acid. Nonactate synthase was found to be the enzyme which catalyzes the formation of nonactic acid from an a cyclic precursor in nonactin biosynthesis (Bennett *et al*., 1962; Meyers *et al*., 1965; Woo *et al*., 1999, Kusche *et al*., 2009). Macrotetrolides exhibit a wide array of biological activities, ranging from antimicrobial, insecticidal, antiprotozoan, and immunosuppressive or antitumor agents (Meyers *et al*., 1965; Teunissen *et al*., 1992; Borrel *et al*., 1994; Zizka, 1998). The biological activities of the macrotetrolides are generally traced to their ionophoric properties, their activities depend upon its ability to form stable complexes with K^+, Na^+ or $NH4^+$ ions and to support the passive diffusion of these ions across cell membranes. The potencies of these activities appear to parallel the size of the alkyl substituents of the macrotetrolides. As nonactin shows selectivity for binding $NH4^+$ ions, it has found widespread use in ammonia-selective electrodes (Marrone *et al*., 1992; Buhlmann *et al*., 1998; Karakus *et al*., 2006).

CONCLUSION

Our results indicated that *St. fulvissimus* FHM275 and *St. pratensis* FHM572 represent promising sources for antimicrobial and antitumor bioactive agents. They showed broad spectrum of biological activities against human, agricultural pathogens, MDR clinical pathogens and different human tumor cell lines; the potency of *St. pratensis* was higher than that of *St. fulvissimus*. Isolation, purification and identification of the

bioactive compounds from *St. fulvissimus* and *St. pratensis* revealed that the antimicrobial and cytotoxic activities are ascribable to the active compounds methyl nonactate ($C_{11}H_{20}O_4$) and methyl homononactate ($C_{12}H_{22}O_4$), respectively; both are strong active with highly selective index against lung carcinoma (A 549), the IC_{50} value is $\leq$ 4 µg/ml was within the cutoff point of the guideline criteria of the United States National Cancer Institute. Methyl homononactate is more active than methyl nonactate, but the former is safer and meets the criteria needed to combat multi-drug resistance and the undesirable side effects of cancer drugs. It can be employed as lead compound for chemical synthesis of new analogues. In view of the potential of these compounds as anticancer agents, an additional study are necessary on the mechanism of cell death.

REFERENCES

[1] Abbas, A. S. and Edwards, C. (1990). Effects of metals on *Streptomyces coelicolor* growth and actinorhodin production. Appl. Environ. Microbiol., 56, 675-680.

[2] Augustine, S. K.; Bhavsar, S.P. and Kapadnis, B. P. (2005). Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39. Indian J. Med. Res., 121: 64-170

[3] Basak, K. and Majumdar, S. (1975). Mineral nutrition of *Streptomyces kanamyceticus* for kanamycin formation. Antimicrob. Agents Chemother., 8: 391-5.

[4] Bennett, R. E.; Brindle, S. A.; Giuffre, N. A. ; Jackson, P. W.; Kowald, J.; Pansy, F. E.; Perlman, D. and Trejo, W. H. (1962). Production of a novel cytotoxic agent, SQ 15,859, by *Streptomyces chrysomallus*. Antimicrob. Agents Chemother., 169-172.

[5] Bérdy, J. (2005). Bioactive microbial metabolites. J. Antibiot., 58: 1-26.

[6] Borrel, M. N.; Pereira, E.; Fiallo, M. and Garnier-Suillerot, A. (1994). Mobile ionophores are a novel class of P-glycoprotein inhibitors. The effects of ionophores on 49-O-tetrahydropyranyl-adriamycin incorporation in K562 drug-resistant cells. Eur. J. Biochem., 223:125-133.

[7] Buhlmann, P.; Pretsch, E. and Bakker, E. (1998). Carrier-based ion-selective electrodes and bulk optodes. 2. Ionophores for Potentiometric and Optical Sensors. Chem. Rev., 98, (4), 1593-1688.

[8] Bundale, S.; Begde, D.; Nashikkar, N.; Kadam, T. and Upadhyay, A. (2015). Optimization of culture conditions for production of bioactive metabolites by *Streptomyces* spp. isolated from soil. Adv. Microbiol., 5: 441-451.

[9] Büchs, J. (2001). Introduction to advantages and problems of shaken cultures. Bioch. Eng. J., 7: 91-98.

[10] Byrne, K. and Greenstein, M. (1986). Nitrogen repression of gilvocarcin V production in *Streptomyces arenae* 2064. J. Antibiot. (Tokyo), 39, 594-600.

[11] Crawford, D.; Lynch, J. M.; Whipps, L. and Ousley, M.A. (1993). Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Appl. Environ. Microbiol., 59: 3899 – 3905

[12] Demain, A. L. (1989). Carbon source regulation of idiolite biosynthesis. In: Regulation of Secondary Metabolism in Actinomycetes. (ed. Shapiro, S.), CRC Press, Boca Raton, FL. pp 127–134.

[13] Demain, A. L. and Sánchez, S. (2009). Microbial drug discovery: 80 years of progress. J Antibiot., 62:5–16.

[14] EL-Enshasy, H. A.; Farid, M. A. and EL-Sayed, A. E. (2000). Enfluence of inoculum type and cultivation conditions on natamycin production by *Streptomyces natalensis*. J. Basic Microbiol., 40(5/6): 333–342.

[15] Elibol, M.; Ulgen,K.; Kamaruddin, K. and Mavituna, F. (1995). Effect of inoculum type on actinorhodin by *Streptomyces coelicolor* A3(2). Biotechnol. Lett., 17(6): 579-582.

[16] Elmahdi, I.; Baganz, F.; Dixon, K.; Harrop, T.; Sugden, D. and Lye, G. J. (2003). pH control in microwell fermentations of S. erythraea CA340: influence on biomass growth kinetics and erythromycin biosynthesis Biochem. Eng. J. 16, 299–310.

[17] Fraústo da Silva, J. J. R. and Williams, R. J. P. (2001). The Inorganic Chemistry of Life in: The Biological Chemistry of the Elements. OUP Oxford, pp 253-254.

[18] El-Naggar, M. Y.; Hassan, M. A.; Said, W. Y. and El-Aassar, S.A. (2003). Effect of support materials on antibiotic MSW2000 production by immobilized *Streptomyces violates*. J. Gen.. Appl Microbiol., 49: 235-243.

[19] Gibbons, S. (2004). Anti-staphylococcal plant natural products. Nat. Prod. Rep., 21: 263–277.

[20] Gille, L.; Kleiter, M.; Willmann, M. and Nohl, H. (2002). Paramagnetic species in the plasma of dogs with lymphoma prior to and after treatment with doxorubicin: an ESR study. Biochem. Pharmacol., 64 (12): 1737–1744.

[21] Gross, E. and Morell, J. L. (1971). The structure of nisin. J. Am. Chem. Soc., 93:4634–4635.

[22] Hunt, G. R. and Stieber, R. W. (1986). Inoculum development. In: Manual of Industrial Microbiology and Biotechnology, (A. Solon, Ed.).Washington, pp 32-40.

[23] Jonsbu, E.; Ellingsen, T. E. and Nielsen, J. (2000). Effects of nitrogen sources on cell growth and production of nystatin by *Streptomyces noursei*. J. Antibiot. 53(12): 1354 -1362.
[24] Jonsbu, E.; Mclntyre, M. and Nielsen, J. (2002). The Influence of carbon sources and morphology on nystatin production by *Streptomyces nousei*. J. Biotechnol., 95(2): 133 -144.
[25] Juan, F. M. (2004). Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR–PhoP system: an unfinished story. J. Bacteriol., 186:5197–5201.
[26] Karakus, E.; Pekyardimci, S. and Kilic, E. (2006). A New potentiometric ammonium electrode for biosensor construction. Artif. Cells, Blood Substit. Biotechnol., 34: 523–534.
[27] Khajure, P. V. and Rathod, J. L. (2011). Antimicrobial and cytotoxic potential of the compound secreted by marine bacteria collected from the Karwar Coast, West Coast of India. Indonesian J. Biotechnol., 16(1): 60-65.
[28] Kusche, B. R.; Joshua, B.; Phillips, J. B. and Priestley, N. D. (2009). Nonactin biosynthesis: setting limits on what can be achieved with precursor-directed biosynthesis. Bioorg. Med. Chem. Lett., 19(4): 1233–1235.
[29] Lancini, G. and Demain, A. L. (2013). Bacterial Pharmaceutical Products In: "The Prokaryotes – Applied Bacteriology and Biotechnology. E. Rosenberg et al. (eds.). Springer-Verlag Berlin Heidelberg. P. 257-280.
[30] Lee, M., and Demain , A. (1997). Effects of nitrogen source on production of antibiotics J. Microbiol., 412-422.
[31] Lee, C. C. and Houghton, P. (2005). Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J. Ethnopharmacol., 100: 237–243.
[32] Ling, L. L.; Schneider ,T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V.A.; Cohen, D.R.; Felix, C.R.; Fetterman, K.A.; Millett, W.P.; Nitti, A.G.; Zullo, A.M.; Chen, C. and Lewis, K. (2015). A new antibiotic kills pathogens without detectable resistance. Nature, 571: 455-459.
[33] Luesse, S. B.; Wells, G.; Nayek, A.; Smith, A. E.; Kusche, B. R.; Bergmeier, S. C.;McMills, M. C.; Priestley, N. D. and Wright, D. L. (2008). Natural products in parallel synthesis: triazole libraries of nonactic acid. Bioorg. Med. Chem. Lett., 18: 3946-3949.
[34] Marrone, T. J. and Merz, K. M. (1992). Molecular recognition of potassium ion by the naturally occurring antibiotic ionophore nonactin. Biochem., 114: 7542–7549.
[35] Mahavorasirikul, W.; Viyanant, V.; Chaijaroenkul, W.; Arunporn Itharat, A. and Na-Bangchang, K. (2010). Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. BMC Complement. Altern. Med., 10: 55-.
[36] Menshawi, B .S.; Walid, F.; Khaled, M.; Salwa, M. H.; May, M.; Maria, H. and Stig, L. (2010). Screening of natural products for therapeutic activity against solid tumors" Indian J. Exp. Biol., 48: 258-264.
[37] Meyers, E.; Pansy, F. E.; Perlman, D.; Smith, D. A. and Weisenborn, F. L. (1965). The *in vitro* activity of nonactin and its homologs: monactin, dinactin, and trinactin. J. Antibiot., 18:128-129.
[38] Mosmann, T. (1983). Rapid colorimetric assays for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 55-63.
[39] MSTAT-C Version 4. (1987). Software program for the design and analysis of agronomic research experiments. Michigan University, East Lansing, Michigan, USA.
[40] Mukhtar, H.; Ijaz, S. and Ul-Haq, I. (2012). Production of antitumor antibiotic by *Streptomyces capoamus*. Pak. J. Bot., 44(1): 445-452.
[41] Newman, D. J. and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 201. J. Nat. Prod., 75(3): 311–335.
[42] Nikodinovic, J.; Dinges, J. M.; Bergmeier, S. C.; McMills, M. C.; Wright, D. L.;Priestley, N. D. (2006). Resolution of methyl nonactate by *Rhodococcus erythropolis* under aerobic and anaerobic conditions. Org. Lett., 8 (3): 443–445
[43] Omura, S.; Tanaka, Y.; Kitao, C.; Tanaka, H. and Iwai, Y. (1980). Stimulation of leucomycin production by magnesium phosphate and its relevance to nitrogen catabolite regulation. Antimicrob. Agents Chemother., 18, 691-695.
[44] Osborne, M.; Grossman, T.; August, P. and Macneil, I.(2000). Tapping into microbial diversity for natural products drug discovery. ASM News, 7:66.
[45] Oskay, M. (2011). Effects of Some Environmental Conditions on Biomass and antimicrobial metabolite production by *Streptomyces* sp., KGG32. Int. j. agric. biol., 13: 317-324.
[46] Pelczar, M. J.; Chan, E. C. S. and Krieg, N. R. (1993). Microbiology: Concepts and Applications. 5th ed. McGraw-Hill,USA.

[47] Perlman, D. and Langlykke, A. F. (1949). Methods for the extraction of streptomycin from fermentation media. Abstr. 116th Meeting Amer. Chem. Soc., pp. 18-19.

[48] Phillips, J. B.; Smith, A. E.; Kusche, B. R.; Bessette Jr., B. A.; Swain III, P. W.; Bergmeier, S. C.; Mark C. McMills, M. C.; Dennis L. Wright, D. L. and Priestley, N. D. (2010). Natural product derivatives with bactericidal activity against Gram-positive pathogens including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. Bioorg. Med. Chem. Lett., 20: 5936–5938.

[49] Rashad, F. M.; Abd El-Nasser, N. H.; Dawoud, I. E. and Motawe, F. H. (2015). Isolation and characterization of antibiotic / antitumor producing *Streptomyces*. RJPBCS, 6(2): 1917-1929.

[50] Reddy, N. G.; Ramakrishna, D. P. N. and Rajagopal, S. V. (2011). Optimization of culture conditions of *Streptomyces rochei* (MTCC 10109) for the production of antimicrobial metabolites. Egypt. J. Biol., 13: 21-29. http://dx.doi.org/10.4314/ejb.v13i1.4

[51] Ríos, J. L. and Recio, M. C. (2005). Medicinal plants and antimicrobial activity. J. Ethnopharmacol., 100: 80-84.

[52] Sánchez, S. and Demain, A. L. (2002). Metabolic regulation of fermentation processes. Enzyme Microb. Technol. 31, 895–906.

[53] Sánchez, S.; Chávez, A.; Forero, A.; García-Huante, Y.; Romero, A.; Sánchez, M.; Rocha, D.; Sánchez, B.; Ávalos, M.; Guzmán-Trampe, S.; Rodríguez-Sanoja, R.; Langley, E. and Ruiz, B. (2010). Carbon source regulation of antibiotic production. J. Antibiot., 63: 442–459.

[54] Saudagar, P. S. and Singhal R. S. (2007). Optimization of nutritional requirements and feeding strategies for clavulanic acid production by *Streptomyces clavuligerus*. Bioresour. Technol., 98: 2010-2017.

[55] Shapiro, S. (1989). Nitrogen assimilation in actinomycetes and the influence of nitrogen nutrition on actinomycete secondary metabolism. In: Regulation of secondary metabolism in actinomycetes, (Shapiro S, editor). Boca Raton, FL: CRC Press, pp 135–211.

[56] Schwartsmann, G.; Ratain, M. J.; Cragg, G.M.; Wang, G. E.; Saijo, N. and Parkinson, D. R. (2002). Anticancer drug discovery and development throughout the world. J Clin Oncol;20:47S–59S.

[57] Song, Q.; Huang, Y. and Yang, H. (2012). Optimization of fermentation conditions for antibiotic production by actinomycetes yj1 strain against *Sclerotinia sclerotiorum*. J Agric. Sci., 4(7): 95- 102.

[58] Srinivasan, M. C.; Laxman, R. S. and Deshpande, M. V. (1991). Physiology and nutritional aspects of actinomycetes: an overview. World. J. Microbiol. Biotechnol., 7:171-184.

[59] Sujatha, P.; Bapi Raju, K.V.V.S.N. and Ramana, T. (2005). Studies on a new marine streptomycete BT-408 producing polyketide antibiotic SBR-22 effective against methicillin resistant Staphylococcus aureus. Microbiol. Res., 160: 119 -126.

[60] Sufian, A. S.; Ramasamy, K.; Norizan Ahmat, N.; Zakaria, Z. A.; M. Izwan M. Yusof, M. I. M. (2013). Isolation and identification of antibacterial and cytotoxic compounds from the leaves of *Muntingia calabura* L. Journal of Ethnopharmacology, 146: 198–204.

[61] Teunissen, M. B. M.; Pistoor, F. H. M.; Rongen, H. A. H.; Kapsenberg, M. L. and Bos, J. D. (1992). A comparison of the inhibitory effects of immunosuppressive agents cyclosporine, tetranactin, and didemnin B on human T cell responses in vitro. Transplantation, 53(4): 875-881.

[62] Thabrew, M. I.; Hughes, R. D. and McFarlane, I. G.(1997). Screening of hepatoprotective plant components using a HepG2 cell cytotoxicity assay. J. Pharm. Pharmacol., 49:1132-1135.

[63] Tokarska-Schlattner, M.; Zaugg, M.; Zuppinger, C.; Wallimann, T. and Schlattner, U. (2006) New insights into doxorubicin-induced cardiotoxicity: The critical role of cellular energetics. J Mol Cell Cardiol 41:389–405.

[64] Uddin, M.; Mahmud, N.; Anwar, N. and Abul Manchur, M. (2013). Bioactive metabolite production by *Streptomyces albolongus* in favourable environment. J. Microbiol. Infec. Dis., 3 (2): 75-82

[65] Valli, S., SuvathiSugasini, S.; Aysha, O. S.; Nirmala, P.; Vinoth-Kumar, P. and Reena, A. (2012). Antimicrobial potential of *Actinomycetes* species isolated from marine environment. Asian Pac J Trop Biomed, 2(6): 469-473.

[66] Waksman, S. A. (1961). The Actinomycetes. Classification, Identification and Descriptions of Genera and Species. The Williams and Wilkins Co., Baltimore, 363 p.

[67] Wibowo, A.; Ahmat, N.; Hamzah, A. S.; Sufian, A. S. and Ismail, N. H. (2011). Malaysianol A, a new trimer resveratrol oligomer from the stem bark of Dryobalanops aromatica. Fitoterapia 82, 676–681.

[68] Woo, A. J.; Strohl, W. R. and Priestley, N. D. (1999). Nonactin biosynthesis: the product of nonS catalyzes the formation of the furan ring of nonactic acid. Antimicrob. Agents Chemother., 43(7): 1662-1668.

[69] Zizka, Z. (1998). Biological effects of macrotetrolide antibiotics and nonactic acids. Folia Microbiol., 43(1): 7–14.