Detection of bleomycin- like antitumor agent produced by *Streptomyces* spp. local isolates

تشخيص المضاد (بلومايسين) الشبيه المنتج بواسطة عزلات محلية لبكتريا *Streptomyces* spp.

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Abstract

Ethyl acetate extracts of fermentation cultures of five local *Streptomyces* isolates SRY-3, SRY-25, 5b, 10, NS-38 were identified as inhibitors of plasmacytoma cell line. Concentration at 0.016 µg/ml of ethyl acetate extract of (SRY-3,SRY-25,5b,10,NS-38) inhibited (46%,40%,21%,34%,34%) of mouse plasmacytoma cells respectively, where as 0.5 µg/ml inhibited (58%, 55%, 35%, 33%, 36%) of plasmacytoma cells .

Quantities analysis of crude extracts by using HPLC on the basis of their retention times showed that the values were (4.3,4.4,4.86,4.83&4.84) min. for SRY-3, SRY-25, 5b, 10,NS-38 respectively, while the retention time of standard antitumor compounds was (4.26) min. This suggests that unknown compound of SRY-3 extract contain bleomycin-like compound. Addition of standard bleomycin to crude extract of SRY-3 increased milliabsorbans unit (M.A.U.) from 0.49 to 1.4. The concentration of bleomycin-like antitumor in fermentation broth of SRY-3 isolate was 3.441 µg/ml.

The five *Streptomyces* isolates have moderate activity against gram-positive and gram-negative bacteria, while standard BLM have no activity.

المستخلص

تم تشخيص مثبطات خلايا البلازماسايتوما فى مستخلصات الاثيل اسيتيت لخمس عزلات محلية لبكتيريا الستربتومايسس ، اذ لوحظ ان تركيز 0.016 مايكروغرام/ مل من مستخلص الاثيل اسيتيت للنواتج التخمرية للعزلات SRY-3,SRY-25,5b,10,NS-3 قد ثبط خلايا البلازماسايتوما بنسبة مئوية تتراوح بين 34% و 46% على التوالى بينما ثبط تركيز 0.5 مايكروغرام/مل من المستخلص خلايا البلازماسايتوما بنسبة مئوية تتراوح بين 37% و 58% . تم التشخيص النوعى للمضادات المنتجة فى المستخلصات بواسطة HPLC بالاعتماد على قراءة وقت القدرة الامتصاصية لكل مستخلص مقارنة مع البليومايسين ، حيث كانت قراءة مستخلصات العزلات SRY-3,SRY-25,5b,10,NS-38 هى 4.3,4.4,4.86,4.83,4.84 دقيقة على التوالى ، بينما كانت قراءة وقت القدرة الامتصاصية لمضاد البليومايسين النقى هى 4.26 دقيقة . توضح هذه النتائج ان المادة الفعالة فى مستخلص عزلة SRY-3 هو شبيه للبليومايسين النقى .

وللتاكد من انتاجية العزلة SRY-3 لشبيه مضاد البليومايسين تم اضافة المضاد النقى الى المستخلص و مقارنة وحدة الامتصاصية المتناهية (M.A.U.) للمستخلص حيث كانت قبل الاضافة 0.4 و اصبحت 1.4 بعد اضافة البليومايسين النقى .

قدرت كمية المضاد الشبيه المنتج فى الوسط التخمرى بواسطة HPLC حيث كانت مساوية الى 3.4417 مايكروغرام/مل . أظهرت العزلات الخمس فعالية بايولوجية ضئيلة ضد كل من البكتريا الموجبة و السالبة لصبغة غرام بينما لم يظهر مضاد البليومايسين فعالية بايولوجية .

Introduction

Streptomycetes have considerable medical, biological and commercial importance [1], since they produce over 60% of known antibiotics and many other substances with valuable clinical and other applications [2]. Various antitumor compounds are produced by streptomycetes, which have activities against different types of tumor cells [3,4,5]. These drugs have been discovered as a result of screening protocols which should be rapid, reproducible, inexpensive and help to select compounds for advanced testing [6]. Natural products are screened for potential activity by determining the cytotoxicity of various dilutions of broths or extracts against tumor cell such as p388 mouse leukemia or B16 mouse melanoma cells [7]. The principle advantage of these methods is that they directly assay cytotoxicity against cancerous cell lines.

Bleomycin (BLM) is a glycopeptide antitumor drug produced by *S.verticillus* [8], has significant antitumor activity against several human malignancies [9, 10], and it has demonstrated a greater degree of

potency against B16 mouse melanoma cells [11]. BLM is used in treatment of several diseases such as: testicular cancer, sequamous cell carcinoma and lymphoma [12]. However, differing from other antineoplastic drugs, BLMs characteristic of virtually no apparent bone marrow toxicity makes it suitable for the treatment of patients with poor bone marrow status [9]. The mechanism of BLM action has been attributed to DNA scission and fragmentation with inhibition of the usual DNA repair mechanisms; RNA and protein synthesis appear inhibited as well [13].

In this study an investigation was carried out for possible production of leomycin-like antitumour drug by local *Streptomyces* isolates.

Materials and Methods

Microorganisms:

Local isolates of *Streptomyces* SRY-3, SRY-25, 5b, 10, NS-38 were used throughout this work. These isolates were obtained from Iraqi soil. *Staphylococcus aureus*, *Bacillus thurengensis* and *Escherichia coli* were used to ascertain antimicrobial activity of *Streptomyces* isolates. *Streptomyces* isolates and test microorganisms were obtained from the personal microbial collection of prof. Mohammed A.K.Ibrahim, Department of biotechnology; College of Science; AL - Nahrain University. Mouse plasmacytoma SU99 cell line was obtained from Biotechnology Research Center /AL-Nahrain University.

Fermentation and antitumor agents extraction:

Streptomyces isolates have been grown at 30ºC in liquid production medium (S-medium) containing per liter: 10gm glucose; 4gm peptone; 4gm yeast extract; 0.5gm MgSO4.7H2O; 2gm KH2PO4; 4gm K2HPO4 [14]. Broth cultures were filtered; and extracted with ethyl acetate. The aqueous layer was then concentrated to dryness. The residue was dissolved in a small volume of methanol and the solution was applied to silica gel TLC chromatography [15] using mobile solvents of 10% ammonium acetate: methanol (1:1) [16]. Outline of extraction and purification procedure

of the drug is shown in figure (1). The TLC spots, which represent standard bleomycin and crude eluents, were scarped and eluted with methanol and dried for further investigation.

Cytotoxic activity assay:

The assay was done by microtiter plates according to the method adopted by Abdul-Majeed, [17]. Ninety-six well tissue culture plates were used for this test to ensure the toxic effect of crude extract of fermentation culture broth of SRY-3, SRY-25, 5b, 10, NS-38 on plasmacytoma SU 99 cell line. A 0.05ml of extract was added in the first and second wells of the first four lines. Two folds serial dilutions were made from the second well for the four lines which contain 0.05 ml tissue culture medium (RPMI 199) till the twelfth well (in duplicate). The wells of the late four lines in the same plate were filled with 0.2ml tissue culture medium only considered as a control. A 0.15ml cell suspension of plasmacytoma cell culture (10^4 cells per ml) was added to all wells of the plate. The culture plates were incubated at 37°C in humidified incubation with 5% CO_2 in air. The culturing plate was removed after 72 hours, and 0.05ml of 0.01% neutral red solution was added to each well, re-incubated at 37°C for 2 hours, after incubation the medium was discarded, and the wells were washed with PBS. The results were recorded as following:

Viable cells will take the dye, while the dead cells will not, 0.1ml of phosphate; buffered-ethanol (0.1M NaH_2PO_4-ethanol; 1:1) was added to each well to elute the dye from the viable cells. The plate was read by micro ELISA reader at optical density of 492nm.

Analysis of bleomycin by HPLC:

The quantitative and qualitative assays of bleomycin were performed by high performance liquid chromatography (HPLC) the eluents were injected into the HPLC: column: shimi – pock MRS-ODS, mobile phase (methanol: sodium acetate buffer) (0.1M), flow rate: 0.4ml/min, temperature: 25 ^{o} C and detection at UV: 254-300 nm.

Antimicrobial activity:

Block assay method was used to determine the antimicrobial activity of *Streptomyces* isolates grown on Mueller – Hinton agar medium at 30 ^{o}C [18].

Antibiotic Susceptibility:

Antibiotic resistances of *Streptomyces* isolates were examined by using antibiotic sensitivity discs [19].

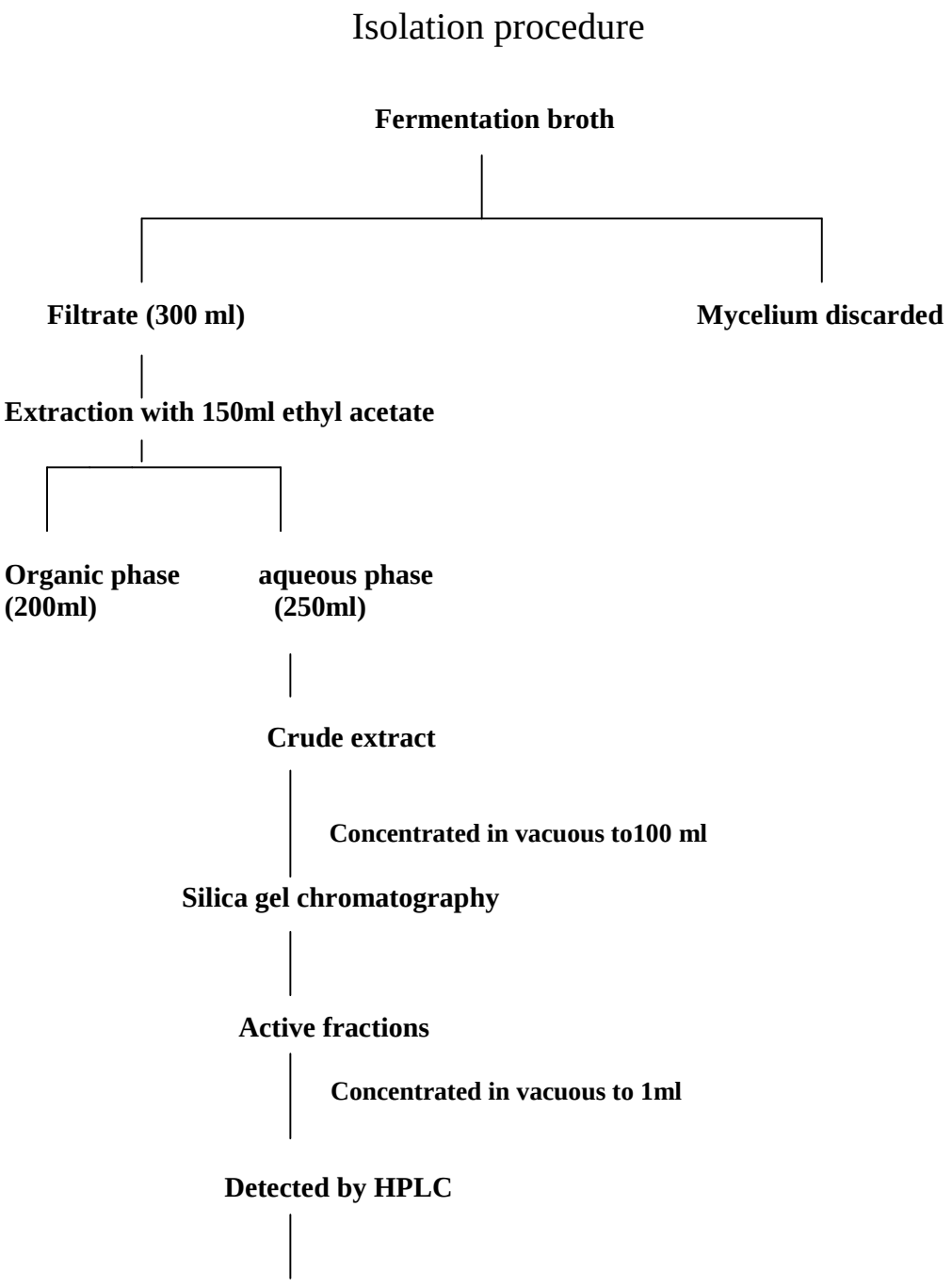


Figure 1: Extraction and purification of bioactive compound from local isolate of *Streptomyces* fermentation broth culture.

Table 1: Cytotoxic effect of fermentation broth of *Streptomyces* local isolates on plasmacytoma cell line.

Dose (Mg/ml)	Inhibition %				
	SRY-3	SRY-25	5b	10	NS-38
0.001	49	27	17.2	32.8	32.4
0.002	45	20	17.9	33.1	32.8
0.004	44	34	18.6	33.5	33.4
0.008	49	32	19.3	33.5	33.5
0.016	46	40	21	33.8	34.1
0.031	44	46	20.7	33.1	34.8
0.063	44	32	24.1	34.2	35.2
0.125	46	40	24.5	34.2	35.2
0.25	50	34	27.6	34.2	35.9
0.5	58	55	34.5	32.4	36.2

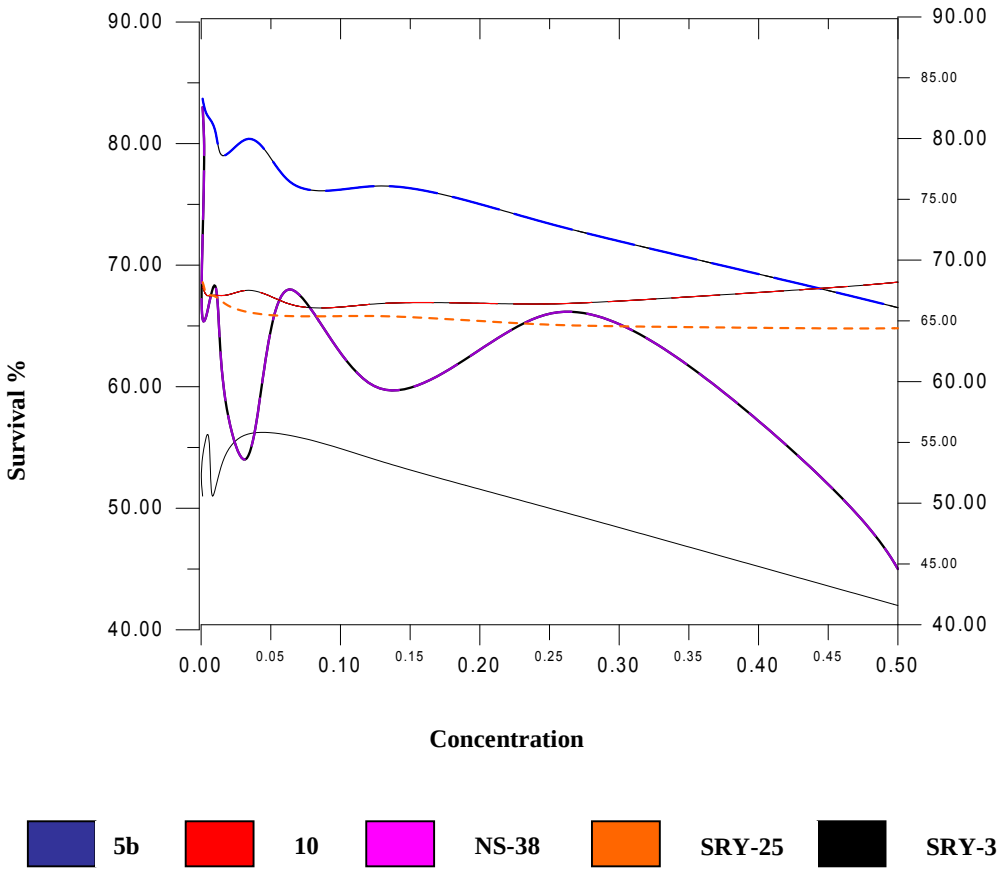


Figure 2: The inhibitory effect and survival of TLC fraction of *Streptomyces* local isolates on plasmacytoma cell line.

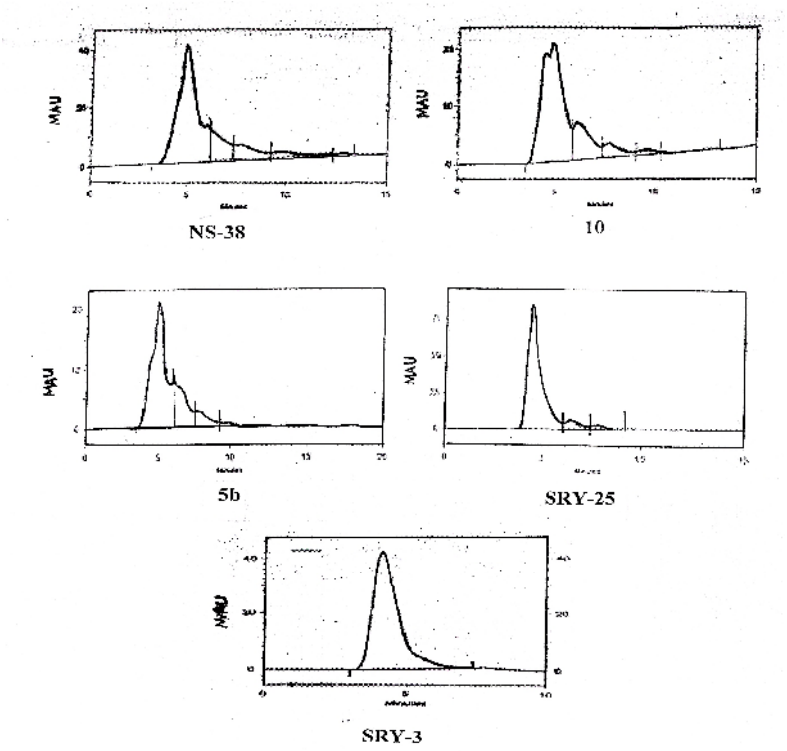


Figure 3: High performance liquid chromatography of local *Streptomyces* isolates.

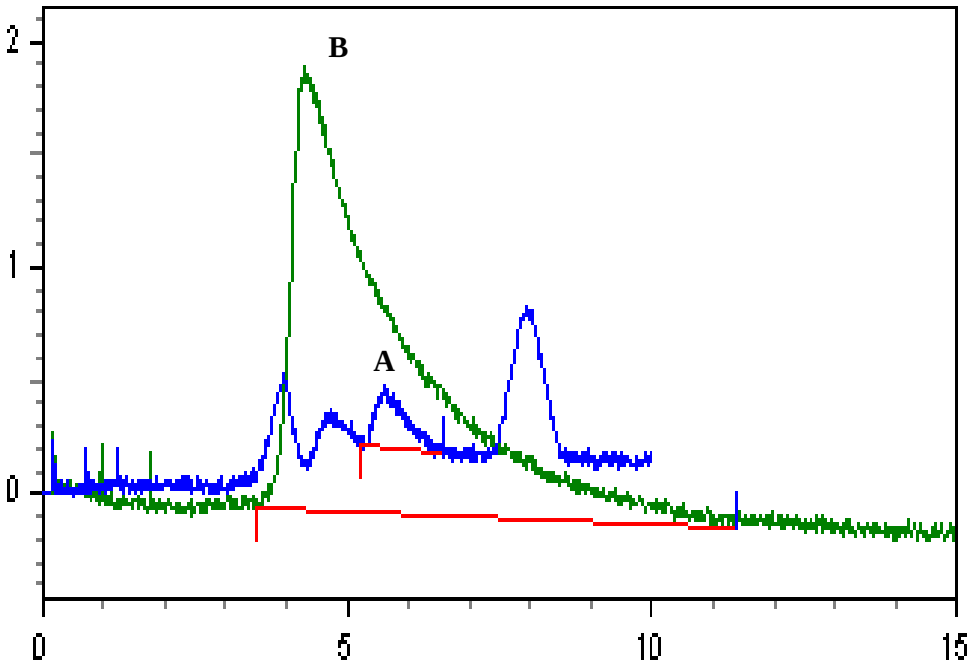
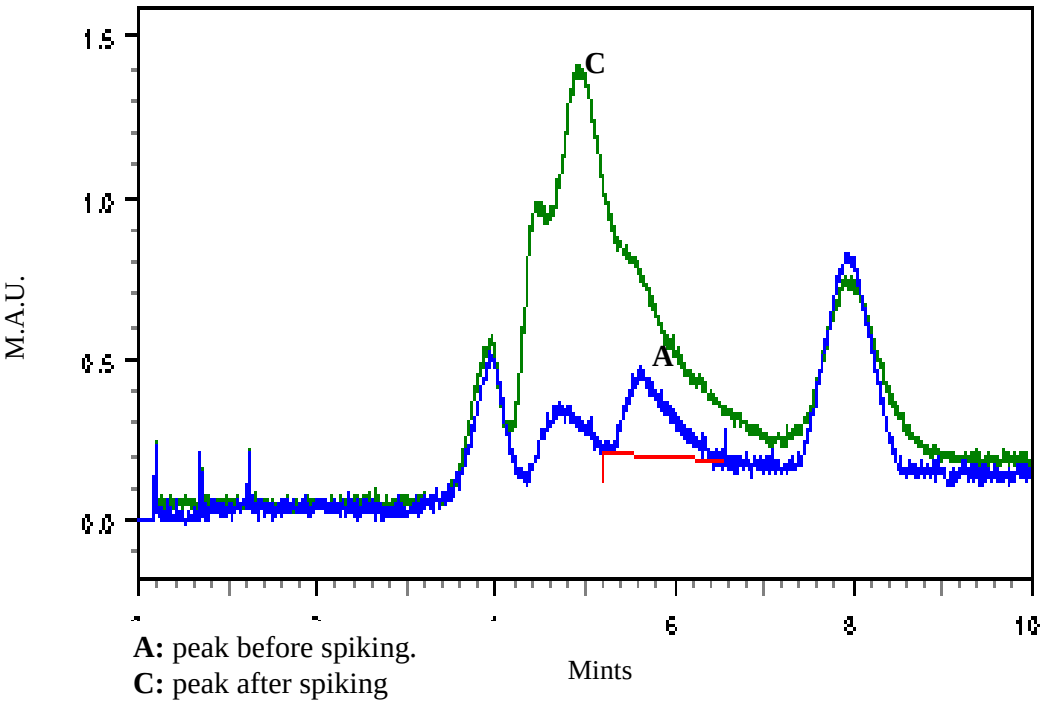


Figure 4: High performance liquid chromatography of TLC fraction of SRY-3 (A) and standard bleomycin (B).



A: peak before spiking.
C: peak after spiking

Figure 5: Spiking of TLC fraction of SRY-3 isolate with standard bleomycin.

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Table 2: Antibiotic susceptibility of *Streptomyces* isolates.

Antibiotics	Concentration (µg/dsc)	Diameter of inhibition zone (mm)				
		SRY-3	SRY-25	10	5b	NS-38
Tetracycline	30	R	R	R	R	R
Erythromycin	15	4	10	17	R	R
Streptomycin	10	30	24	27	32	25
Gentamycin	10	35	33	30	35	30
Chloramphenicol	30	19	R	R	R	R
Ampicillin	10	R	R	R	R	R
Rifampicin	30	16	15	R	R	R
Lincomycin	15	R	R	20	R	8
Carbenicillin	100	R	R	R	R	R
Cephalosporin	30	R	R	R	R	R
Bleomycin	1500	R	R	R	R	R

Result:

Mammalian cell cytotoxicity assay have been used to detect antitumor activity of natural products [20]. In this study plasmacytoma cell line technique was found as rapid and useful in screening for *Streptomyces* fermentation broths and their extracts.The cytotoxic activity of *Streptomyces* isolates SRY-3, SRY-25,5b,10&NS-38 crude extract was assayed on mouse plasmacytoma cell line in microtiter plate.

Table (1), shows the inhibitory effect of various concentrations of the SRY-3, SRY-25, 5b, 10&NS-38 TLC fractions on the growth of plasmacytoma cells as compared with the control.0.016 µg/ml of the above mentioned crude extracts, showed (46%, 40%, 21%, 34%, 34%) inhibition of mouse plasmacytoma cells respectively, whereas 0.5 µg/ml showed 58%, 55%,34.5%, 32.4% & 36.2% inhibition of cells. These results suggest that the potency of 5b, 10 and NS-38 was lower than the potency of SRY-3 and SRY-25. Figure (2), shows the survival of mouse plasmacytoma cell line against TLC fraction of the five *Streptomyces* isolates.

Thus the results indicated that fermentation culture of SRY-3 isolate was superior and had showed greater degree of activity against tumor cell line. It is possible to suggest that the crude extract contained unknown active cytotoxic compound. In this regard, cytotoxic effects of *Streptomyces* fermentation broth was shown against human tumor cell lines [21].

The observed variation in the percentage of inhibition might due to difference in number of colonies in duplicate plates [22].

Fermentation broth cultures of SRY-3, SRY-25, 5b, 10&NS-38 *Streptomyces* isolates were purified by following procedure shown in figure (1). Aqueous layer of each isolate and standard BLM were chromatographed on silica gel TLC plate. The objective of TLC method is for possible identification of bleomycin-like active antitumor compound produced in fermentation broth [23]. The UV exposure of standard BLM on TLC shows a spot with 0.68 Rf value; the major spots of *Streptomyces* isolates extracts when exposed to UV lamp (286) showed violet color with Rf values of 0.67,0.67,0.64,0.66 and 0.67)

for SRY-3,SRY-25, 5b, 10&NS-38 respectively.

In order to identify the active compound produced by *Streptomyces* isolates. TLC extracts of five *Streptomyces* isolates were subjected to HPLC techniques. The peaks were assigned on the basis of their retention time. Figure (3) shows the peaks of HPLC analysis of five TLC fractions with different retention times (4.3 min, 4.4 min, 4.86 min, 4.83 min and 4.84 min) for TLC fractions of SRY-3, SRY-25, 5b, 10& NS-38 respectively; on the other hand retention time of standard bleomycin was 4.26 min. figure (4).

The above results demonstrated that the reading of retention time of SRY-3 TLC extract was almost compatible with retention time of bleomycin drug, for more confirmation, TLC fraction of SRY-3 isolate spiked with standard bleomycin lead to an increase in milli absorbance unit of desired peak from 0.49 to 1.4, figure (5). This result might further suggest that the produced antibiotic is bleomycin-like compound, with concentration about 3.4417µg/ml, in fermentation culture.

The susceptibility of *Streptomyces* isolates to various antibiotics was examined (table 2). The observed results indicated that all *Streptomyces* isolates were resistance to tetracycline, ampicillin, carbenicillin, cephalosporin and bleomycin; on the other hand all isolates was sensitive to streptomycin and gentamycin.

Antimicrobial activity of the filtrate of *Streptomyces* isolates were investigated; all *Streptomyces* isolates have moderate activity against gram-positive and gram-negative bacteria While, standard BLM have no activity.

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