Studying the Optimum Conditions of Hygromycin B Production and Detect their Toxicity

Zahraa Abdulmunim
Biotechnology Division, Applied Sciences Department, University of Technology
Rabah N. Jabbar
Biotechnology research center, Al-Nahrain University
Abdulwahid B.Al-Shaibani
Department of Biotechnology, College of Science, Al-Nahrain University
zahraa_zoz82@yahoo.com

Abstract

Hygromycin B was extracted with ethyl acetate, which separates organic phase from aqueous phase in the broth culture filtrate, only the aqueous phase showed significant antimicrobial activity by using agar well diffusion technique. At a concentration of 25mg/ml (as crude extract), this phase excreted its activity against the test microorganisms which include; one G(+) bacteria (*Staphylococcus aureus*), five G(–) bacteria (*Pseudomonas aeruginosa , Proteus mirabilis, Escherichia coli , Klebsiella pneumoniae, Salmonella typhi*) and one yeast (*Saccharomyces cerevisiae*).

After detecting the aminoglycoside hygromycin B by the Thin Layer Chromatography (TLC) method to ensure presence of the antibiotic, same flow rate (R_f) value (0.357) as that of the standard hygromycin B was obtained.

Results of the optimization conditions showed that the highest antimicrobial activity of hygromycin B was obtained at a medium pH of 8 and incubation temperature of 35°C for 10 days. When the toxicity of hygromycin B crude extract under such conditions was examined on mice liver, a mild effects were appeared.

Key words: Thin Layer Chromatography, hygromycin B, aminoglycoside

الخلاصة

تم استخلاص ال hygromycin B بأستخدام خلات الاثيل, فُصل المحتوى العضوي عن المائي في راشح المزرعه البكتيرية السائلة, واعطى المحتوى المائي فقط فعالية حيوية بأستخدام تقنية الأنتشار في الحفر على سطح الأغر (Agar well diffusion technique) عند تركيز 25ملغم/مل (كمستخلص خام) , اعطى هذا الطور فعالية حيوية ضد مجموعة من الاحياء المجهرية اشتملت على بكتريا واحدة موجبة لغرام (*Staphylococcus aureus*) وخمس سالبة لغرام *Pseudomonas aeruginosa), Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae* , *Salmonella typhi*) و خميرة واحدة(*Saccharomyces cerevisiae*).

عند الكشف عن ال hygromycin B aminoglycoside بأستخدام كروموتوغرافيا الطبقه الرقيقه لتأكيد وجود المضاد الحيوي, تم الحصول على نفس معدل الجريان (R_f) 0.357 للـ hygromycin B القياسي .

اظهرت نتائج الظروف المثلى للوسط الزرعي البكتيري ان اعلى فعالية حيوية للـ hygromycin B تم الحصول عليها عند الرقم الهيدروجيني 8 والحضن بحرارة 35 م° لمدة 10 ايام.

تمت دراسة التأثير السمي للـ hygromycin B على كبد الفئران المختبرية وأظهرت تغيرات طفيفه على انسجة كبد الفأر.

الكلمات المفتاحية: كروموتوغرافيا الطبقه الرقيقه، hygromycin B، aminoglycoside.

Introduction

Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that active against bacteria, fungi and higher eukaryotic cells by inhibiting polypeptide synthesis , It stabilizes the tRNA-ribosomal acceptor site, thereby inhibiting translocation (McGuire and Pettinger, 1953)**.**

It has been reported to interfere with translocation1 and to cause mistranslation at the 70S ribosome (Gonzales, *et al*. 1978; Singh, 1979)**.**

Hygromycin B was originally developed in the 1950s for use with animals and is still added into chicken feed as an anthelmintic or anti-worming agent (product name: Hygromix).. Resistance genes were discovered in the early 1980s (Burgett *et al.*, 1983; Davies *et al.*, 1983). Hygromycin B show in figure (1) has a chemical formula $C_{20}H_{37}N_3O_{13}$ and molecular mass 275.53g/mol.

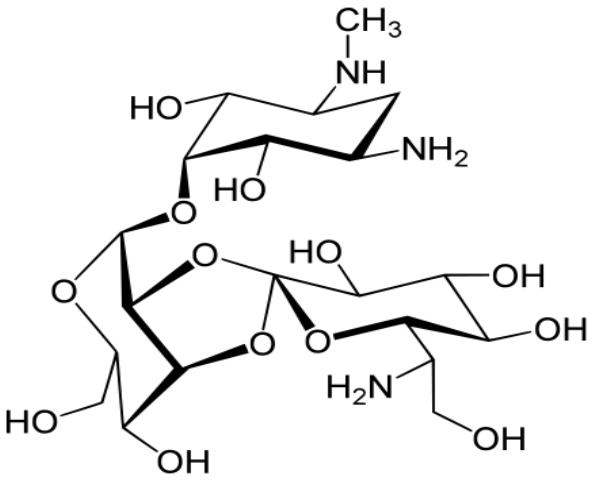


Figure 1 :- Chemical structure of Hygromycin B.(McGuire and Pettinger , 1953)

In research it is used for the selection and maintenance of prokaryotic and eukaryotic cells that contain the hygromycin resistance gene. A more detailed examination of the mechanism of action has suggested that Hygromycin B also interferes with the translocation step in cell-free systems from both bacteria and yeasts (Cabanas *et al.*, 1978). Most of the aminocyclitol antibiotics can be inactivated by at least one of three enzymatic mechanisms: (i) acetylation, (ii) adenylylation, or (iii) phosphorylation. Enzymes carrying out these reactions and the genes that code for them have been isolated from bacteria resistant to aminocyclitols (Davies and Smith, 1978.) and from *Streptomyces* species that produce these antibiotics (Leboul and Davies, 1982). Haas and Davies (1981) pointed out that the presence of these enzymes in bacteria usually makes the bacteria resistant to the appropriate antibiotic. Although resistance to Hm has been found in the Hm-producing organism *Streptomyces hygroscopicus* there has been no previous report of plasmid-encoded resistance to this antibiotic in enterobacteria. Moreover, none of the numerous aminoglycoside-modifying enzymes reported to date has been shown to use Hm as a substrate.

The aminocyclitol antibiotic hygromycin is produced by *Streptomyces hygroscopicus a*nd specifically blocks the translocation step on both 70s and 80s ribosomes (Cabanas *et al.*, 1978; Gonzilez *et al.*, 1978).The drug also induces misreading both in vivo and in vitro and therefore promotes phenotypic suppression (Singh., 1979) *S. hygroscopicus* contains a phosphotransferase (HPH) activity which phosphorylates hygromycin B, thus potentially providing the producing organism

with autoimmunity against the toxic effects of the drug (Leboul & Davies, 1982). This interpretation appears to be valid since the gene (hyg) encoding the HPH activity has now been cloned in *S. lividans* and cells containing it became resistant to hygromycin B (Malpartida *et al.,* 1983). Such an enzymic inactivation is one of several ways by which antibiotic-producing *Streptomyces* spp. become resistant to their own secondary metabolites.

Materials and Methods

Extraction of bioactive compounds:

Antibiotic production medium (500 ml)was inoculated with 1% of exponentially growing culture of the selected *Streptomyces isolate* and incubated at 35°C for 7 days. After that filtration was carried out through No.1 Whatman filter paper. The filtrate was centrifuged at5000 rpm for 15 min and antimicrobial activity was tested .

After pH was adjusted to 8 , the procedure proceed by extraction with ethyl acetate 1:1(v/v) in the rotary evaporator. The extract was separated into organic phase and aqueous phase; the organic phase was evaporated at room temperature for 4 days, while the aqueous phase evaporated in the oven at 40°C for 6 days. After the residual material was diluted with 5 ml of sterile distilled water, 1 ml was took from each phase and put in a beaker previously weighted then dry and weighted again to calculate the concentration of the active compounds in 1ml of D.W. each one was tested antimicrobial activity by using agar diffusion method. The extract of the phase that gave biological activity was used for further Hygromycin B detection .

- Detecting Hygromycin B by TLC technique:

A small spot from each of the aqueous extract and the Hygromycin B standard solution (Sigma /USA) was applied to an aluminum foil coated thin-layer chromatoplates (20x20 cm) that placed in a separation chamber containing sodium acetate solution (eluent) as a mobile phase (taking in the consideration that the spots of the sample shouldn't touch surface of the eluent in the chamber). After the chamber was closed, it was left at 40°C . The solvent was then moved up via a capillary action and the sample was eluted, and the run was ended before the solvent reached the end of the plate. The spots was visualized by the ninhydrin spray reagent and heaing at 110 until the reddish spots appear and R_f value was calculated. R_f Value = Distance from Baseline travelled by Solute/ Distance from Baseline travelled by Solvent (Solvent Front) (Judit, 1972).

Optimization of antibiotic production conditions:

- Growing at different pH:

Each of the four conical flasks, contained 25ml of hygromycin B production medium but with different pH values (4, 5, 6, 7, 8 and 9). They were prepared and inoculated with 1% of the exponentially growing culture of *Streptomyces* isolate, and incubated at 30˚C under for 7 days. By using the agar diffusion method, result was determined by exerting antimicrobial activity against *Staph aureus, Protius mirabilis,* and *Saccharomyces cereviciae* through measuring diameters (mm) of inhibition zones.

- Growing at different temperatures:

Each of the four conical flasks, contained 25ml of hygromycin B production medium with an optimum pH determined from the previous step prepared and inoculated with 1% exponentially growing culture of *streptomyces* isolates then

incubated at different temperatures (25 , 30 , 35, 40 , 45˚C) under aerobic condition for 7 days, By using the agar diffusion method, result was determined by exerting antimicrobial activity against *Staph aureus, Protius mirabilis,* and *Saccharomyces cereviciae* through measuring diameters (mm) of inhibition zones.

- Growing for different incubation periods:

In each of the four conical flasks, a volume of 25 ml of Hygromycin B production mediium with the optimum pH was inoculated with 1% of the active culture of *Streptomyces* isolate before incubation at the optimum temperature for different incubation periods (5 , 6 ,7 , 8,10 days). By using agar diffusion method, results were read through measuring the diameters (mm) of inhibition zones formed the inhibitory activity of Hygromycin B against the three test microorganisms.

Detection of Hygromycin B toxicity:

The dose that prepared from the crude extract of Hygromycin B was in a concentration of (0.0002/gm body weight of mice, if we Know ,Practically, the recommended amount of Hygromycin B used as feed additive for chicken is 12g/ton and mean of chicken weight is 1500gm while the mean of mice weight is 15gm. The mice were divided into two groups; the first was daily treated by orally administration of 100µl of Hygromycin B for 14 day, while the second group was left without treatment as a control. A liver was taken and histologically analyzed

Results

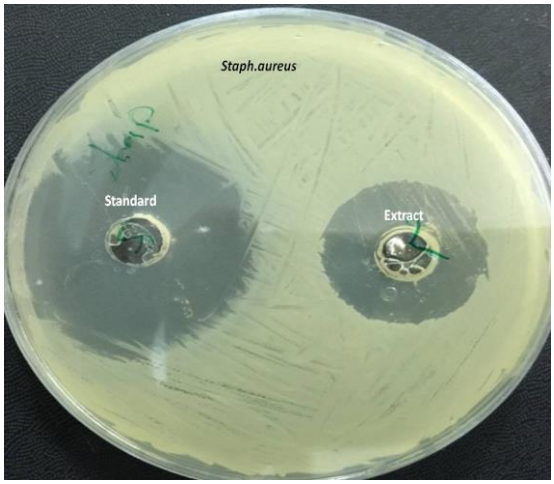
Production of Bioactive compounds:

Hygromycin B is an atypical aminoglycoside which is a large and diverse class of antibiotic has a unique structural and functional properties produced by *Streptomyces* isolates10cm/35pigment (Borovinskaya *et al*, 1996).

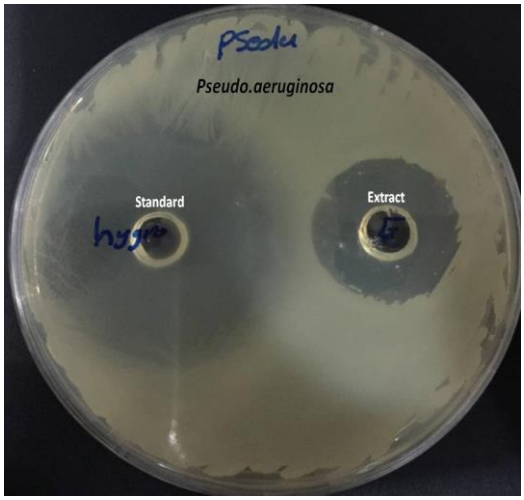
Clear filtrate which obtained after propagation of *Streptomyces* isolate 10cm/35pigment in cultural broth was tested for presence and activity of antimicrobial compounds against the seven test microorganisms. Separation of the filtrate by ethyl acetate resulted into two phases; organic and aqueous.

Results showed that when hygromycin B standard (100mg/ml) was used, no any antimicrobial activity was appeared in the organic phase at concentration of 3mg/ml, while in the aqueous phase, good antimicrobial activity was recorded at concentration of 25mg/ml of crude extract as illustrated in figure (2) and table (1).

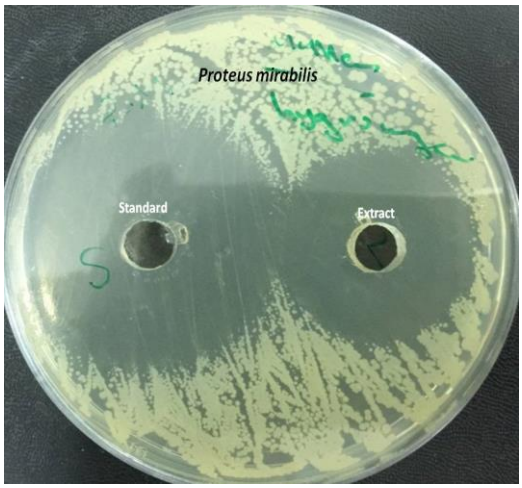
Hygromycin B is soluble in water, at concentrations >50 mg/ml, methanol, buffer solution or in ethanol, but it is practically insoluble in less polar solvents (Merck Index,1996). In the study of Plozza *et al.* (2011), the aminoglycosides antibiotics were extracted from meat tissue or milk using an aqueous buffer. also Slolte (1999) stated that the aminoglycoside antibiotics (aminoglycosides) are hydrophilic molecules consisting of an aminated cyclitol associated with an amino sugar. Then, aminoglycosides are readily soluble in water.


Staph.aureus
Standard
Extract

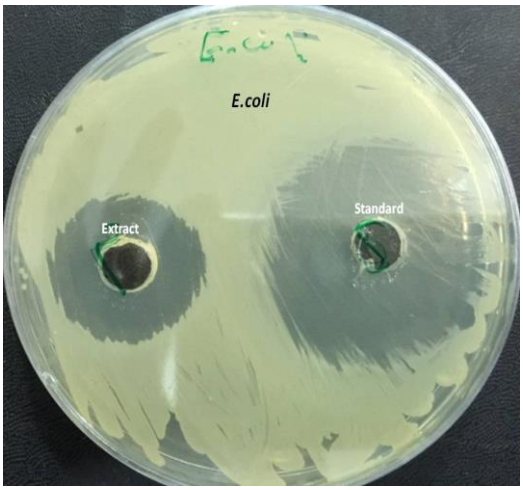
A


Pseudo.aeruginosa
Standard
Extract

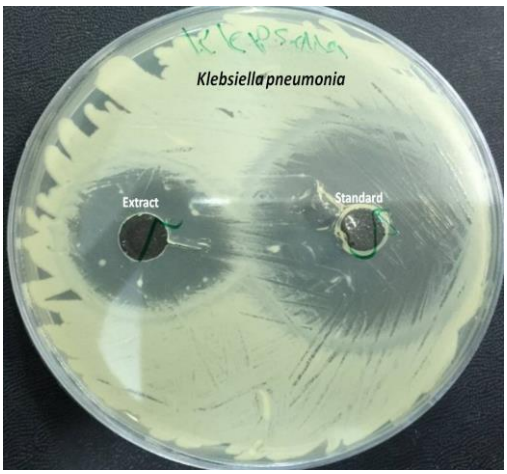
B


Proteus mirabilis
Standard
Extract

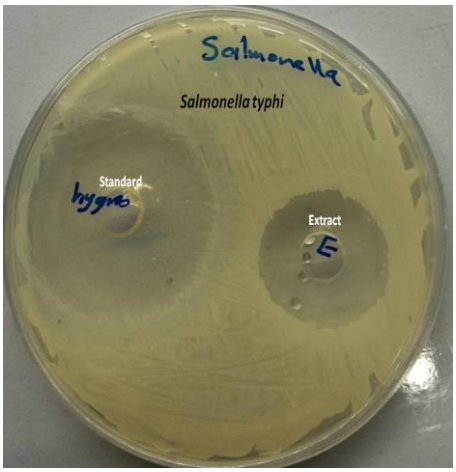
C


E.coli
Extract
Standard

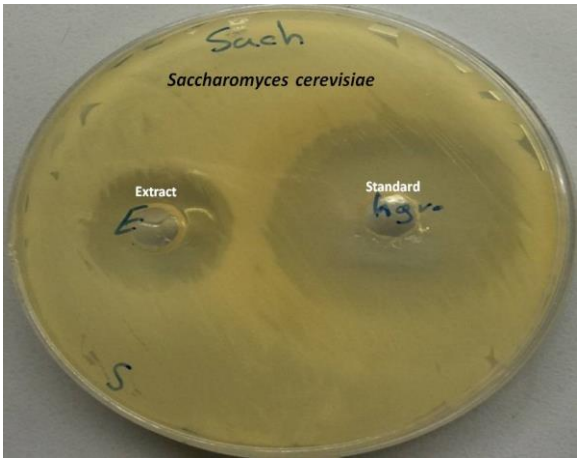
D


Klebsiella pneumonia
Extract
Standard

E


Salmonella typhi
Standard
Extract

F



G

Figure(2) :- Antimicrobial activity of hygromycin B (crude aqueous extract) and ((hygromycin B standard solution (100mg/ml)) aginst: A) *Staph aureus,* B) *Pseudomonas aeruginosa,* C) *Proteus mirabilis,* D) *E. coli,* E) *Klebsiella pneumoniae,* F) *Salmonella typhi and* G) *Saccharomyces cerevisiae.*

Table (1):- Inhibition zone diameters (mm) form by hygromycin B ((crude aqueous extract(25mg/ml)) and hygromycin B ((standard solution (100mg/ml)) against the test microorganisms .

Test organism	Inhibition zone diameter (mm)	
	Hygromycin B Standard(100mg/ml)	Aqueous Extract(25mg/ml)
Staph. aureus	39	27
Pseud.aeruginosa	38	25
Proteus mirabilis	45	33
Kleb.pneumoniae	37	27
Salmonella typhi.	33	23
E.coli	40	26
Sacch.cerevisiae	30	22

Hygromycin B Detection :

Thin layer chromatography (TLC) technique was used in detection of the aminoglycoside hygromycin B. The mobile phase carried the spot of the sample through a stable stationary phase, and each compound had a specific affinity for the mobile and stationary phases, therefore, migrates at a different speed. One spot of the sample mixture migrates at same speed of the hygromycin B standard and same Rf value that is constant under stable conditions of chromatography. It could be possible to say that the proposed antimicrobial agent is to be belonged to hygromycin B (Touchstone and Joseph, 1983). Medina MB and Unruh JJ.1996 also used thin chromatography method to separate and detect neomycin, gentamicin, spectinomycin, hygromycin B and streptomycin allowing multiresidue detection of these aminoglycosides. The respective RF values indicate the separation of these five compounds. This procedure provides a rapid and sensitive method for the semi-

quantitative estimation of aminoglycosides. Hubicka *et al.*,(2009) stated that a TLC for identification and determination of amikacin, gentamicin, kanamycin, neomycin, netilmicin, and tobramycin (aminoglycosidic antibiotcs).

Figure (3) shows the TLC plate of crude extract of hygromycin B in the aqueous .solution. It could be seen in this figure the movement of the extract and the standard where both of them reach the same distance from the bottom .

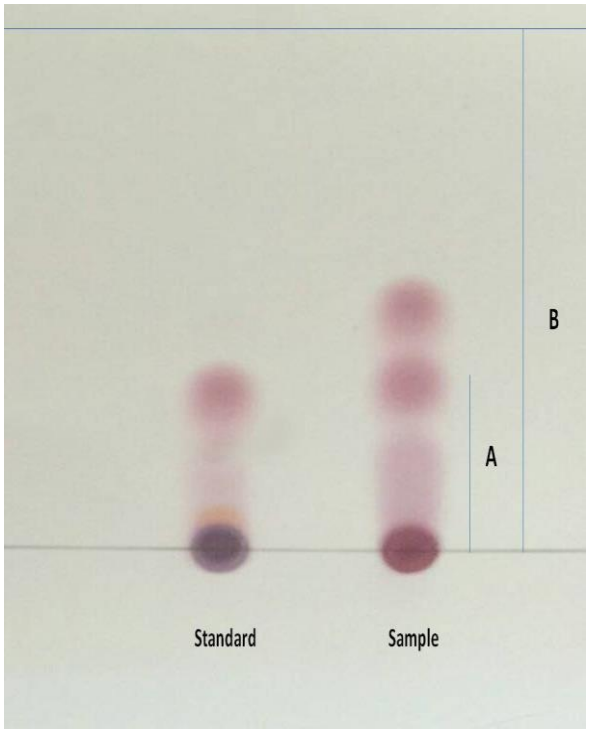


Figure (3) TLC analysis of crude extract hygromycin B produced by *Streptomyces* isolate 10cm/35pigment.

Rf = A/B

Rf for hygromycin B standard=2.5/7=0.357

Rf for sample =2.5/7=0.357

A= the distance from the starting point to the gravity center of the sample spot.
B = the distance from the starting point to the front of the developing solvent.

Optimization of hygromycin B production conditions:

-Optimization of pH:

To investigate the effects of the initial pH medium on hygromycin B production, the *Streptomyces* isolate was grown in hygromycin B production broth medium with different pH values (4 , 5 , 6 , 7 , 8 , 9) before incubated at 30˚C for 7 days .

Results in table (2) show that the isolate has recorded their highest activities when the pH of the medium was adjusted to 8. At this pH, Highest antimicrobial effect for hygromycin B was recorded with a (23) mm inhibition zone against the Gram negative bacteria *Proteus mirabilis.* Moreover, at this pH also, most efficient antimicrobial activity for hygromycin B against all three test organisms: *Staph aureus* (as Gram positive bacteria), *Proteus mirabilis* (as Gram negative bacteria) and *Saccharomyces cerevisiae* (as a yeast) was achieved when highest inhibition zone diameters (20, 23 and 18 mm, respectively) were recorded. Adversely, pH 4 led to the lowest antimicrobial activity for hygromycin B against all three test organisms with inhibition zones of (11, 11 and 9 mm, respectively).

Such results are closed to those obtained by Afifi *et al.,* (2012)when they found that the optimum pH for hygromycin B production was 8[16] and likely in agreement with result of Parthasarathi *et al.*,(2012) found that pH 7 showed maximum inhibition zone (26 mm) against *Kleb. pneumoniae* followed by *Ps. aeruginosa* (24 mm), *S. aureus* (20 mm), *B. subtilis* (19 mm) *and E. coli* (17 mm), in the substrate bombay rawa. Kavanag *et al.*,(1972) stated that , the assay of hygromycin B antimicrobial activity was more sensitive at pH 8.

The quantity of hygromycin B applied can be reduced by increasing the pH of the medium. At higher pH values, cells are more sensitive. The sensitivity of cells is pH dependent (i.e. the higher the pH of the culture medium the greater the sensitivity). Thus, the concentration of hygromycin B required for complete growth inhibition of given cells can be reduced by increasing the pH of the medium (Moazed, D. and Noller ,1987; Hemmi *et al.*, 1992)

Table (2) Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in production broth culture of different pH values for 7 days at 30˚C

pH / Test organism	Inhibition zone diameter (mm)					
	4	5	6	7	8	9
Staph. aureus	11	12	14	16	20	18
Proteus mirabilis	11	13	15	20	23	21
Sacch. cerevisiae	9	11	14	16	18	16

Optimization of growing temperature:

The *Streptomyces* isolate was grown in the production medium after adjusting its pH to the optimum (8), then incubated the shaker incubator at different temperatures (25 , 30 , 35 , 40 , 45˚C) for 7 days.

Results in table (3) show that the *Streptomyces* isolate excreted its maximum biological activity when incubated at 35˚C, while less activity was detected at 45˚C. In this regard, Afifi *et al.* (2012) recorded 35˚C as the optimum temperature for hygromycin B produced from *Streptomyces crystallinus* AZ151. Parthasarathi *et al.*,(2012) used (25°C, 28°C, 37°C and 50°C) as growth temperatures and found that 28°C was the optimum one to give maximum antimicrobial activity by producing zones of inhibition of; (24 mm) against *K. pneumoniae* followed by *P. aeruginosa* (17 mm), *E. coli* (16 mm), *S. aureus* (14 mm), *B. subtilis* (12 mm) in the substrate bombay rawa. In another study,Oskay (2009) the researcher found the maximum zone of inhibition (18 mm) against S. *aureus* after incubation at 30°C temperature .

Table (3) Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in pH 8 production broth culture at different temperatures for 7 days.

Temperature (°C) / Test organism	Inhibition zone diameter (mm)				
	25	30	35	40	45
Staph. aureus	17	20	23	18	17
Proteus mirabilis	20	23	28	25	19
Sacch. cerevisiae	13	15	18	16	12

Optimization of incubation period:

Optimum production of hygromycin B was also determined after incubation at different incubation periods (5, 7, 10, 12 days). Results in table (4) show that the maximum antimicrobial activty was obtained after 10 days of incubation at an optimum pH 8 and a temperature 35°C . Afifi *et al.* (2012) recorded the maximum production of hygromucin B also after 10 days of incubation while in the study of Parthasarathi *et al.*,(2012) the maximum zone of inhibition (24 mm) against *B. subtilis* after 10 days of incubation.

Table (4)Antimicrobial activity of hygromycin B produced by *Streptomyces* isolate 10cm/35pigment grown in production broth culture at different incubation periods with an optimum pH 8 and an optimum temperature of 35°C.

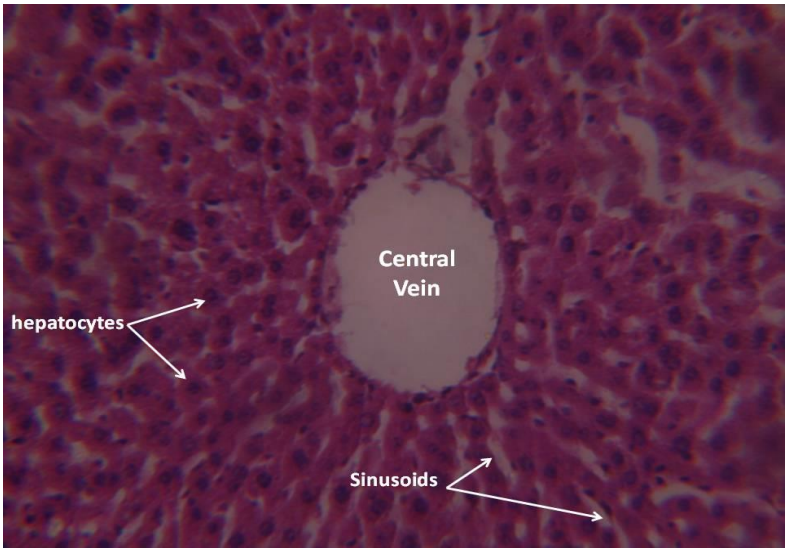
Period(days) / Test organism	Inhibition zone diameter (mm)			
	5	7	10	12
Staph. aureus	17	20	23	18
Proteus mirabilis	20	23	28	25
Sacch. cerviciae	13	15	18	16

Histological analysis of experimental mice:

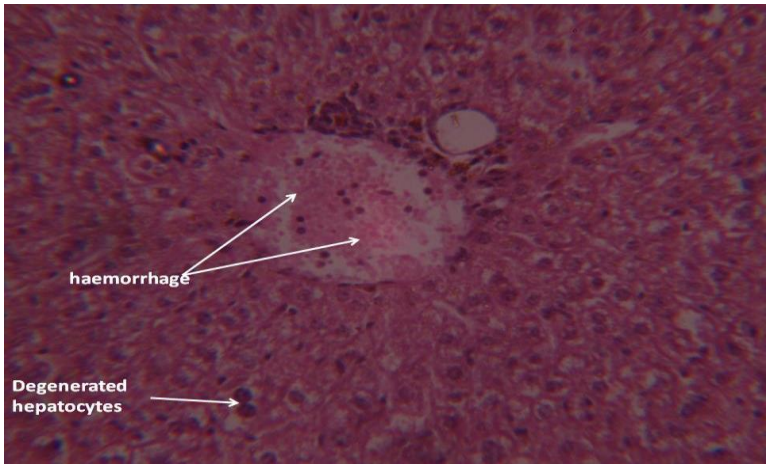
Since liver is the foremost organ that receives all the materials absorbed by intestine through the portal vein, and it is the organ that must neutralize venoms, the toxic effect of most medications on liver manifest itself quicker than other organs(Vahidieyerisofla *et al.,* 2014).

Oral addminstration with hygromycin B has been linked to mild effects which appeared after observing the microscopic slides of the liver tissue, of mice for both control and treated group . Microscopical examination of liver cross section slide of control mice showed normal structure, central vein,normal arrangement of hepatic cords, normal blood sinusoids and hepatocytes ,on the other hand the liver section of the treated mice with hygromycin B extract observed little hemorrhage with infiltration of PMN cell and degeneration of hepatocytes as show in figure (4).

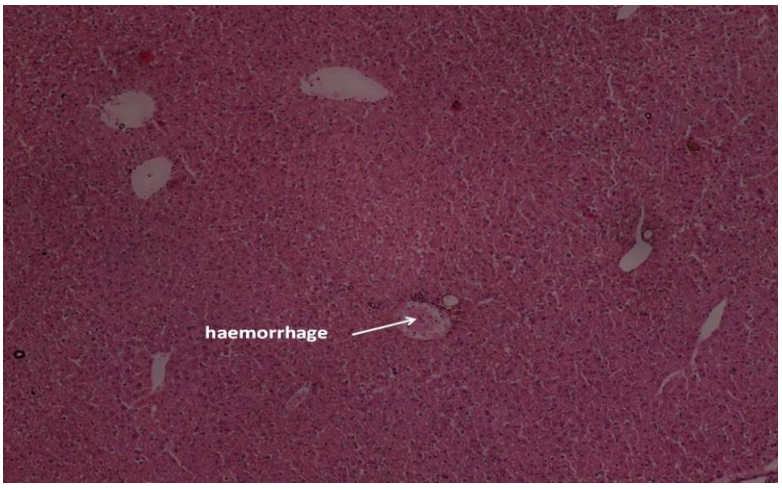
Khan *et al.,* (2011) stated that the administration with gentamicin belong the class of aminoglycosides had been also linked to mild and asymptomatic elevations in serum alkaline phosphatase levels, but rarely affects aminotransferase levels or bilirubin, and changes resolve rapidly once gentamicin is stopped. Only isolated case reports of acute liver injury with jaundice have been associated with aminoglycoside therapy including gentamicin, most of which are not very convincing. Recovery typically occurs within 1 to 2 months and chronic injury has not been described. Aminoglycosides are not listed or mentioned in large case series of drug induced liver disease and acute liver failure; thus, hepatic injury due to gentamicin and other aminoglycosides is rare if it occurs at all.



A



B



C

Figure (4) A- Liver tissue of control showing normal structure, central vein (C.V.), normal arrangement of hepatic cords (H.C.), normal blood sinusoids (S) and hepatocytes., X 40x10. B and C- Liver tissue of Hygromycin B extract treated mice showing haemorrhage in the central vein, infiltration of PMN cell and degeneration of hepatocytes . at X 40x10 and X 10 respectively .

References

Afifi**,** M.M.; Atta**,** H.M.; Elshanawany**,** A.A.; Abdoul-raouf, U.M. and El- Adly, A.M**.**(2012).Biosynthesis of Hygromycin-B Antibiotic by *Streptomyces crystallinus* AZ151 Isolated from Assuit, Egypt**..** Bacteriology Journal, 2: 46-65.

Borovinskaya M.A.; Shoji ,S.; Holton, J.M.; Fredrick, K.and Cate, J.H. (1996). A steric block in translation caused by the antibiotic spectinomycin. ACS Chem. Biol. 2007b;2:545–552Merck Index, 12th ed., 4900 .

Burgett; Kaster; Burgett, SG.; Rao, RN.and Ingolia, TD. (1983). Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. Nucleic Acids Res. 11 (19): 6895–911.

Cabanas, M. J.; Vazquez, D. and Modolell, J.(1978). Dual interference of hygromycin B with ribosomal translocation and with aminoacyl-tRNA recognition. Eur. J. Biochem. 87:21-27.

Davies, J.; and Smith, D. I. (1978). Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32:469-518.

Davies; Gritz and Davies, J. (1983). Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*.", *Gene* 25 (2-3): 179–88.

Gonzales, A.; Jimenez, A.; Vasquez, D.; Davies, J.E. and Schindler, D.. (1978) Biochem. Biophys. Acta 521, 459-469.

Haas, M. J. and J. Davies. (1981). Aspects of enzymemediated aminoglycoside antibiotic resistance, In T. P. Singer and R. N. Ondarza (ed.), Molecular basis of drug action. Elsevier/North-Holland Publishing Co., Amsterdam. p. 387- 403.

Hemmi, S. ; Merlint,G. and Aguet,M. (1992). Functional characterization of a hybrid human-mouse interferon Y receptor: Evidence for species-specific interaction of the extracellular receptor domain with a putative signal transducer Proc. Natl. Acad. Sci. USA 89, 2737-2741.

Hubicka,U.; Krzek, J. ; Woltyńska,H. and Stachacz, B.(2009). *Simultaneous identification and quantitative determination of selected aminoglycoside antibiotics by thin layer chromatography and densitometry.* J AOAC Int;92(4):1068-75.

Judit ,J.K.(1972).Thin layer chromatography of basic water- soluble antibiotics on resin coated chromatoplates. The Journal of antibiotics Vol. XXV No.II.

Kavanagh,F.;lilly,E. and Indiana,I.(1972).Analytical microbiology. volume II , Academic Press.New York and London.

Khan, MR.; Badar, I. and Siddiquah, A.(2011). Prevention of hepatorenal toxicity with Sonchus asper in gentamicin treated rats.BMC Complement Altern Med. 15;111-113.

Leboul, J. and J. Davies. (1982). Enzymatic modification of hygromycin B in *Streptomyces hygroscopicus*. J. Antibiot. 35:527-528.

Malpartid,A, F. Zalacain, M.; Jimenez, J.and DAVIES, J. (1983). Molecular cloning and expression in Streptomyces lividans of a hygromycin B phosphotransferase gene from Streptomyces hygroscopicus. Biochemical and Biophysical Research Communications 117, 6-12.

McGuire and Pettinger (1953).Hygromycin I. Preliminary studies on the production and biological activity of a new antibiotic. Antibiot. Chemother. 3: 1268–1278.

Medina MB and Unruh JJ.(1996). Solid-phase clean-up and thin-layer chromatographic detection of veterinary aminoglycosides. J Chromatogr B Biomed Appl. 663(1):127-35.

Medina MB and Unruh JJ.(1996). Solid-phase clean-up and thin-layer chromatographic detection of veterinary aminoglycosides. J Chromatogr B Biomed Appl. 663(1):127-35.

Merck Index, 12th ed., 4900 (1996).

Moazed, D. and Noller, H.F. (1987). Interaction of antibiotics with functional sites in the 16S rRNA. *Nature* 327, 389-394.

Oskay,M.(2009). Antifungal and antibacterial compounds from Streptomyces strains.African Journal of Biotechnology. 8: (13) ;3007-3017.

Parthasarathi, S. ;. Sathya, S.;. Bupesh, G.; Durai R.; Mohan,R.;Kumar, S.; Manikandan, M.;. Kim, J. and Balakrishnan, K.(2012). Isolation and Characterization of Antimicrobial Compound from Marine *Streptomyces hygroscopicus* BDUS 49. World Journal of Fish and Marine Sciences 4; (3): 268-277.

Plozza, T.; Trenerry, V.C.; Zeglinski, P.;Nguyen, H.and Johnstone, P.(2011). The Confirmation and Quantification of Selected Aminoglycoside Residues in Animal Tissue and Bovine Milk by Liquid Chromatography Tandem Mass Spectrometry. International Food and Research Journal.; 18(3): 1077-1084.

Singh, A. (1979) . Use of a bacterial Hygromycin B resistance gene as a dominant selectable marker in Neurospora crassa transformation.
Nature 277, 146-148.

SÏolte , L. (1999). Aminoglycoside antibiotics Ð Two decades of their HPLC bioanalysis. Biomed. Chromatogr. 13: 3–10

Touchstone and , Joseph, C.(1983). *Practice of thin layer chromatography*. 2nd ed. New York: Wiley.

Vahidieyerisofla, N. ; Ahmadi, F. ; Golmakani ,R. and Eini,M. (2014). The Study of Levofloxacin Effects on Liver Tissue in Wistar Rat . International Journal of Pediatrics .2;(2):1.