

Actinomycin- Production Assay: A simple Bacteriological Method For Antitumor Activity Screening *In Vitro*

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Abstract:

We present and evaluate the actinomycin-production assay, a bacterial test for detecting DNA-damaging agents as a primary step for detecting the antitumor activity of new synthesized, modified or isolated compounds. It's a simple test based on the activity of the agents against actinomycin antibiotic production of *Streptomyces antibioticus*, bacteria whose value is monitored by the inhibition activity of *E. coli* and *S. aureus* bacterial growth using two standard anticancer drugs cisplatin and flurouracil. In this assay, production of actinomycin antibiotic is measured as a specific indication of the ability of an agents to directly or indirectly damage DNA and that are of potential interest in carcinogenesis and cancer chemotherapy.

Because *in vivo* tumor test systems using mammalian cell cultures were slow and expensive, we tested a novel cytotoxicity test, simple, sensitive, inexpensive and amenable to large-scale screening using bacteria cultures, as well as, it will offer another practical application in addition to cytotoxicity screening, the assay is used for testing antibacterial of the test compounds..

Key words: *Streptomyces antibioticus*, Actinomycin, Cytotoxicity test, antitumor activity, antibacterial activity.

اختبار إنتاج الاكتينومايسين :
طريقة بيولوجية بسيطة لاختبار الفعالية المضادة للأورام خارج الجسم الحي

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الخلاصة:

تضمنت الدراسة الحالية تصميم اختبار بكتيري أطلق عليه اختبار إنتاج الاكتينومايسين وتقييمه للتحري عن قابلية المركبات للتأثير على الحامض النووي DNA كخطوة اولية للتحري عن الفعالية المضادة للأورام للمركبات الجديدة (المصنعة او المحورة او المعزولة) . الاختبار بسيط يعتمد على فعالية المركبات ضد إنتاج المضاد الحياتي الاكتينومايسين من قبل البكتريا الخيطية *Streptomyces antibioticus* واستخدمت البكتريا القياسية الموجبة لصبغة كرام (*Staphylococcus aureus* ATCC 25923) والسالبة لصبغة كرام (*Escherichia coli* NCTC 25922) كمؤشر لحساب

الفعالية باستخدام الدوائين القياسيين المضادين للسرطان Cisplatin و Flurouracil. اعتمد الاختبار على إنتاج المضاد الحيوي الاكتينومايسين واستخدم كدليل على قدرة المركبات على التأثير المباشر أو غير المباشر على DNA وهذا ذو أهمية بالغة في دراسة السرطان وعلاجه.

إن اختبارات السرطان داخل الجسم الحي *in vivo* التي تستخدم الخلايا حقيقية النواة بطيئة وباهظة الكلفة ولذلك صمم هذا الاختبار ليكون اختبارا بسيطا وفعالا وواظئ الكلفة باستخدام المزارع البكتيرية للتحري عن الفعالية المضادة للأورام ولأعداد كبيرة من العينات فضلا عن إن هذا الاختبار يمكن استخدامه في التحري الأولي عن الفعالية المضادة للبكتريا إضافة إلى استخدامه في اختبار السمية الخلوية خارج الجسم الحي *in vitro*.

Introduction :

Cancer is an ancient disease. It has afflicted our ancestors throughout history. Cancer may be defined as a disease or group of disease in which the cells divide and multiply without control, have the capacity to metastasis in the body , destroy healthy tissue, and endanger life (1,2).

Chemotherapy has been used in cancer treatment for more than 50 years, sometimes in combination with or parallel to surgery and radiotherapy, and has proven to be one of the most efficient strategies for treating cancer (3).

Cytotoxicity is the cell-killing property of a chemical compounds, terms such as cell survival, cell killing, and integrity are often arbitrarily employed (4).

Agents that interact with DNA have potential clinical utility for the treatment of cancer .Most toxic chemicals exert their action by interference with basic cellular functions, these functions are fundamental to the life of the cells, and are expressed in a similar way in all cells regardless of whether they are found *in vitro* or *in vivo*(5).

There are presently over one hundred protocols for measuring cell toxicity using mammalian cells including: membrane damage (permeability, active transport or surface chemistry changes); alteration in the synthesis degradation, conformation or availability of macromolecules or growth-related factors; modifications in intermediary metabolism; and changes in activity, growth, morphological (including organelle changes or behavioral characteristics), such as neutral red assay, MTT test, the succinate dehydrogenase assay, glutathione enzymes test, lactate dehydrogenase assay, and trypan blue dye exclusion assay (6,7,8).

Bacteria are widely used as indicator organisms in test systems for antitumor agents, they offer practical advantages and may provided insights into the basic mechanisms of cytotoxicity (9).

One of the best known system is the *Salmonella* microsome assay (mutatest). This reversion assay, performed on *Salmonella* strains, provided strong indications that "carcinogens were mutagens and vice versa . Many other bacterial

tests have been described, including phage induction assay (BIA assay) induce test (10,11). A biochemical (colorimetric) assay of bacteriophage lambda induction was utilized in the detection, identification, and purification of DNA-interacting products with potential antitumor activity using *Escherichia coli* that are lysogenic for a lambda-Lac Z fusion phage produced beta-galactosidase enzyme(12,13).

Streptomyces spp. are gram-positive soil-dwelling filamentous bacteria, members of the actinomycete group with a complex cycle of morphological differentiation (14). Members of genus *Streptomyces* are potential sources for secondary metabolites, its estimated this bacteria synthesizes more than 7000 metabolites (15). Actinomycin is a cyclic peptide antibiotic and also known as chromopeptide antibiotic isolated by Waksman in 1940 from *Streptomyces antibioticus* (16).

Actinomycin is an antibiotic obtained from species of *Streptomyces*, studies of the mechanism of action of this antibiotic showed that it acted by binding to double-stranded DNA and stopping the transcription of DNA into RNA, this compound inhibits the transcription of DNA to RNA, thus causing the death of cells (17). Actinomycin has used as a chemotherapy drug for many years, the power of this class of drugs is their specify in killing cells that are dividing, this makes it more likely that they will kill cancer cells, rather

than the cells of non-cancerous tissue (18).

In the present paper, we describe a simple biological assay for antitumor activity screening of cytotoxic agents which offers another simple, inexpensive, and sensitive with large-scale screening test for cytotoxicity, antibacterial activity, as well as further practical advantages.

Materials & Methods:

Bacterial cultures :

Bacterial cultures used in this study were provided by Dr. Kawther H. Mehdi/ Biotechnology Laboratory-Biology Department- College of Science/ Basrah University, these include: *Streptomyces antibioticus* bacteria isolated from soil, produce actinomycin antibiotic (19), as well as the reference strains of Gram positive bacteria (*Staphylococcus aureus* ATCC 25923) and Gram-negative bacteria (*Escherichia coli* ATCC 25922).

Growth media:

Actinomycin –isolation medium (22gm/L supplemented with 5ml glycerol) was used as growth medium for *S. antibioticus*. Whereas, nutrient agar (23 gm /L) was the growth medium of *E. coli* and *S. aureus* bacteria.

Standard antitumor drugs:

Antitumor drugs including Cisplatin[*Cis*-diamminedichloroplatinum(II) DDP] (Tamil Dallha, India) and 5-Flurouracil (Farnos, Finland) were used in this study at a concentration of (100µg/ml) using sterile normal saline for preparation.

Assay procedure:

The following steps were used to detect anticancer activity (cytotoxicity) *in vitro* (Figure 1):

[1] Actinomycin isolation medium was prepared, poured into glass test tubes (20ml/tube), and autoclaved at 121 °C for 30 minutes.

[2] Antitumor drugs including Cisplatin and 5-Flurouracil (100µg/ml) were prepared in 5ml sterile tubes.

[3] One ml of each test antitumor compounds were poured in sterile-marked Petri dishes (10x10 cm), then 20ml of sterile actinomycin medium was added to each dish (temperature of medium should be 25°C when it poured), mixed well with each test compound and left to solidified at room temperature.

[4] On the other hand, (1 ml) of sterile normal saline was used instead of test compounds to mixed with (20 ml) of medium to represent the positive control.

[5] All dishes were divided into two parts, the first part was cultured with *Streptomyces antibioticus* bacteria using streaking methods with a sterile loopful, while the other part left as negative control.

[6] All dishes were incubated at 28 °C for 3-days (period of bacterial growth with maximum actinomycin antibiotic production).

[7] Wells (15 mm in diameter) were cutted from each two parts using a stainless steel-cutting device, in which disc blocks from seeded parts referred to as block A, and the blocks from non-seeded parts were

referred to as block B, whereas, the block from positive control was referred to as block C.

Where:

Block A: contained cultured medium with S. antibioticus and test drug (either cisplatin or flurouracil)

Block B: represent non-seeded medium and anticancer drug (negative control).

Block C: contained seeded medium alone (positive control).

[8] Nutrient agar medium was prepared and poured in glass test tubes (20ml/tube) then autoclaved.

[9] 20 ml of nutrient agar was poured in sterile-marked Petri dishes and left to solidified at room temperature.

[10] All dishes were cultured with *Escherichia coli* and *Staphylococcus aureus* bacteria using spreading method with glass-sterile L-shape spreader.

[11] With sterile forceps, each disc blocks (A,B, and C) were carefully transferred, and fixed in opposite to other onto seeded nutrient agar dishes, then incubated at 37 °C for 24 hours.

[12] After incubation period, the growth was monitored and the diameters (in millimeter) of inhibition zones around the discs (A, B, and C) were measured for each cisplatin and 5-fluriuracil compounds blocks as well as control blocks.

[13] Compound show antitumor activity when the diameter of inhibition zones of block A and

block B are equal as compared with control block C.

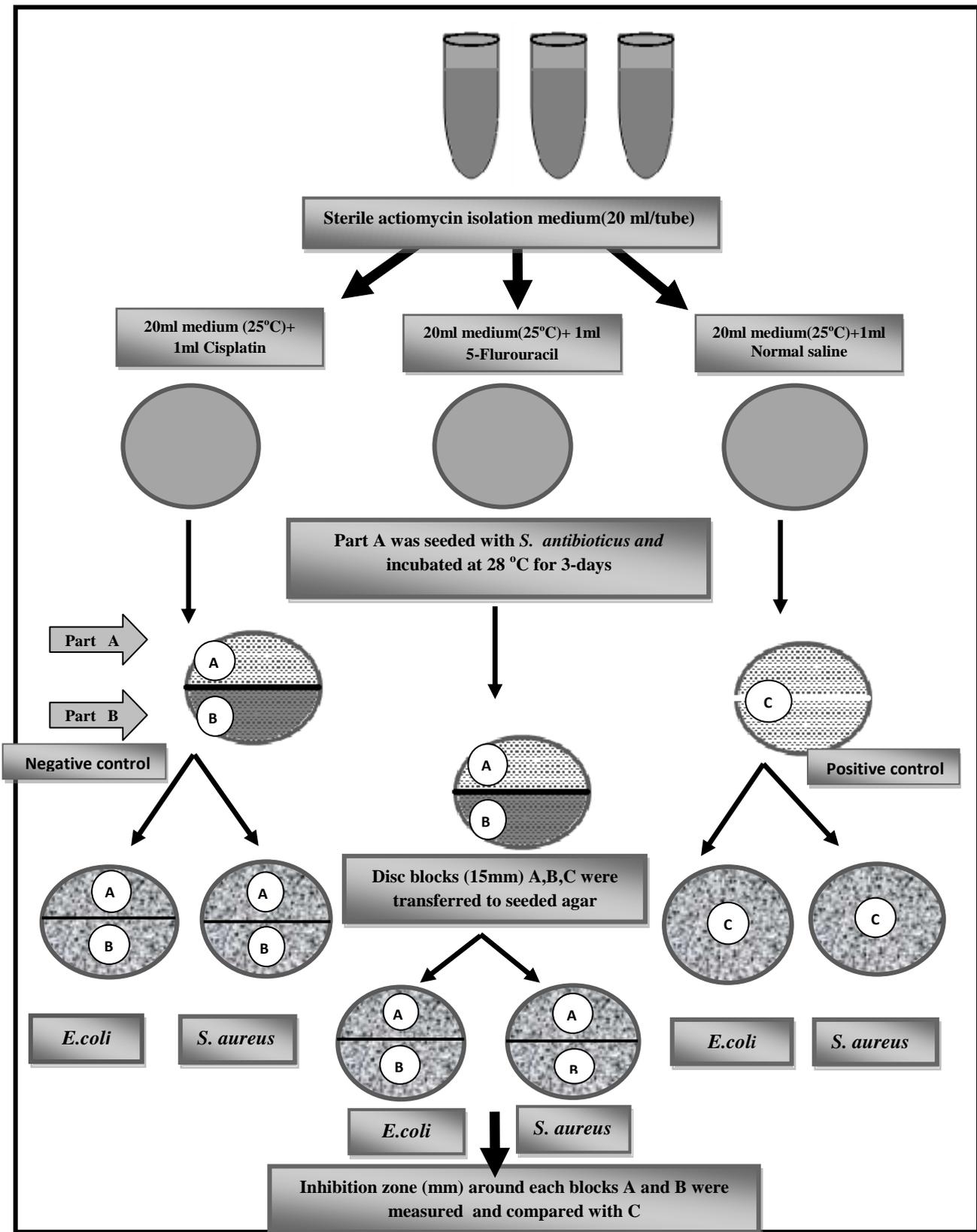


Figure (1): Procedure of Actinomycin- production assay .

Results & Discussion:

In this study, we used two types of anticancer drugs, which include Cisplatin and 5-Flurouraci (100 µg/ml). Both anticancer drugs were detected for their ability to damaged or modified the DNA molecule of *Streptomyces antibioticus* bacteria which used as indicator of test system in a simple bacteriological assay, this bacteria produced actinomycin antibiotic (a characters of specific gene activity within the bacterial DNA).

The results summarized in Table (1) and Figure (1) show the potent anticancer activity of these test compounds depending upon measuring the diameter of inhibition zone (against *Escherichia coli* and

Staphylococcus aureus bacterial growth) around the isolation medium blocks (A and B) in order to estimate T value using the formula: [A-B=T], then compared with positive control C where:

IZ(A): Antibacterial activity of *S. antibioticus* actinomycin antibiotic and anticancer test drug (either cisplatin or flurouracil).

IZ(B): Antibacterial activity of anticancer drug only (test agent) [Negative control].

IZ(C): Antibacterial activity of *S. antibioticus* actinomycin antibiotic alone before treated with test drug [Positive control]

IZ(T): Antibacterial activity of *S. antibioticus* actinomycin antibiotic after treated with anticancer drug.

Table (1): Antibacterial activity (mm) of disc blocks (A,B, and C) against *E. coli* and *S. aureus* bacteria.

Test agents 100µg/ml	<i>Escherichia coli</i> (ATCC 25922)				<i>Staphylococcus aureus</i> (ATCC 25923)			
	IZA	IZB	T	IZC	IZA	IZB	T	IZC
Cisplatin	30	30	0	30	25	25	0	40
Flurouracil	30	28	2		20	20	0	

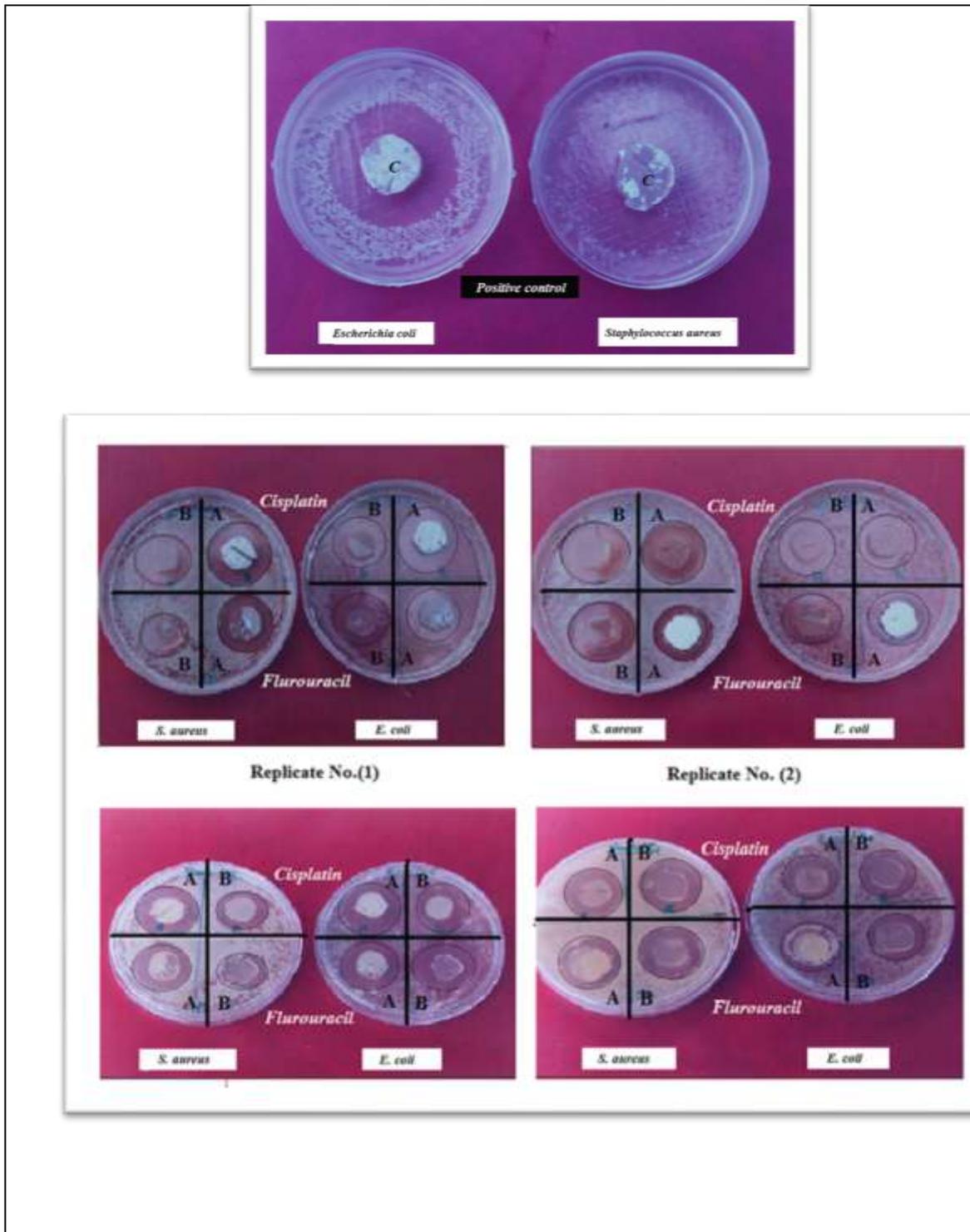
Where: IZA= inhibition zone around block A

IZB=Inhibition zone around block B

IZC= inhibition zone around block C

*Diameter of the inhibition zone including the diameter of *Streptomyces* medium block (15mm).

** Assay was performed in triplicate and mean value was used to represent the activity.



Figure(1): Antibacterial activity (mm) of disc blocks (A,B) against *E. coli* and *S. aureus* bacteria in compared with positive control (C) .

If the test drug has the ability to interact with DNA *S. antibioticus* bacteria (modified, damaged DNA or both), the actinomycin production will be affected (either, decreased or stopped), so the agent that show antibacterial activity (T) equal to zero or less than positive control antibacterial activity (C), the agent was considered as active antitumor agent because this mean there is no actinomycin production or the production was decreased and reflect the interaction with bacterial DNA whereas, if T value was equal or greater than control value (C) indicate that the bacterial actinomycin antibiotic was not affected and as a result the test agent has no ability to interact with DNA. Cisplatin drug exhibit its anticancer activity by direct damaging for DNA of tumor cells, and the second drug was 5-fluorouracil (5-FU) which classified as anti-metabolite drugs that interfere with DNA synthesis (20, 21, 22).

Depending on the results showed in Table(1) and Figure (1), T value which represent the antibacterial activity of *Streptomyces* actinomycin after treated with anticancer drugs (cisplatin and fluorouracil), we found the the actinomycin production was stopped (T=0) which indicate the potent antitumor activity of the tested drugs resulting from the direct interaction of the drugs with bacterial DNA in comparison with the antibacterial activity of bacterial actinomycin before treated with any

anticancer drugs (positive control C) which exhibit potent bacterial growth inhibition against both *E. coli* (30mm) and *S. aureus* (40 mm), the results were agreed with the literature in which actinomycin antibiotic was highly active against Gram positive bacteria (23).

Screening for antitumor agents requires assays that are rapid, inexpensive, and amendable to large-scale screening. *In vivo* tumor test systems meet none of these criteria, and mammalian cell culture assays are also slow and expensive. Therefore, *in vitro* microbiological assays systems are frequently used for the initial screening for antitumor compounds (24, 25).

The bacterial actinomycin production assay offers variable practical advantages such as the high sensitivity of the developed assay, it will designed for large-scale screening, simple and inexpensive equipments used to performed this assay beginning with bacterial cultures and ending with cultured media, on the other hand this assay added another practical advantage in addition to cytotoxicity detection, this assay used to study the antibacterial activity of new synthesized agents or extracted compounds.

The sensitivity of the assay could varied, depending on the length of incubation period, microbes, and nutrients usually interfere with the detection of cytotoxicity (12). So, in order to insure that all factors which may be interfere with the results, we

controlled on these factors as possible, these include: the thickness and the diameter of medium blocks used, incubation period, temperature (for medium and incubation), microbes, and solvents to prevent interference with the study factor.

Indeed, although bacteria are the simplest DNA-containing cells, they possess elaborate mechanisms to respond to DNA-damaging agents⁽¹⁰⁾. The physical nature of DNA is important to consider due to its fundamental importance in drug-DNA interactions. The low Pka value of the phosphate groups makes the DNA highly charged molecule in a large PH range. As a result, the charge density is high, and the DNA attracts positively charged ions and repels negatively charged ones. In this respect, the nucleobases act as ligands and thus coordinate to the drug of an already positively charged species (5,16,26).

In conclusion, the results of this study, confirmed the actinomycin-production assay to be a simple, cheap, sensitive and accurate bacteriological method suitable for screening programs designed to detect agents that damage DNA and that are of potential interest in carcinogenesis and cancer chemotherapy.

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