Iraqi Journal of Cancer and Medical Genetics

Anti tumor potential of Local Aslerk *(Eremurus spectabilis)* Leaf Extracts by HPLC and applying on Cancer cell Lines *in vitro*

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Abstract:

Many scientists have focused on the treatment of cancer for along time by studying cytotoxic activities of plant extracts , the E.spectabilis have been used in anticancer studies due to their phytochemical content included (carvone , carvacrol, pentane , 2-methyl , 9-caryophyllene). In present study, we demonstrated the cytotoxic activity of aqueous and Hexan _ ethanolic extracts on cell viability of Rabdumyosarcoma cell (RD) , and transformed kidney epithelium of African green monkey (Vero). In dose and time dependent manner Aqueous and hexan_ ethanol extracts (10.000,1000,100,10,1,0.1,0.01,0.001)µg/ml at (24, 48, 72hrs.) were examined on RD and Vero cells. The result of current study showed that Aqueous and hexan_ ethanol extracts of E. spectabilis effectively inhibited the cell proliferation by decreasing the cell viability of both cell line at different concentration, in which (RD) cell line was sensitive to all concentrations of the plant extract in this period, while the (Vero) cell line was resistant at the two concentrations which was (100 and 10)µg/ml with the means (0.080,0.16).While Hexan_ethanolic extract of different concentrations for (72hrs.) of exposure on both cell lines (Vero and RD) on cell growth assay show that the RD cell line was not respond to all the concentrations that used for killing of RD cell line while Vero cell line was represent its sensitivity for all concentrations from (10.000_0.001)µg/ml.

Keywords- *Eremurus spectabilis, , cytotoxicity, Vero and RD cell,.*

Introduction:

Cancer is a term describing conditions characterized by uncontrolled proliferation of abnormal cells in our body. Because proliferation and growth of all cells are regulated by our genes, cancer is basically a genetic disease. Cancer always starts from genetic mutation which through multiple steps of changes may sometimes lead to formation of cancer(1). Stomach and skin cancers where amongst the most common in Kurdish males, while breast cancer followed by skin and stomach cancers where reported to be the most common in females. With haematological cancers (a cancer which effects blood or bone marrow, for example leukaemia or lymphoma) accounted for 21.3% of all cancers in males and 18.8% of females were reported with breast cancers, showing the highest figure of the type of cancer amongst the two genders(2). E. spectabilis is used as a vegetable in Kurdistan. In this study, E. spectabilis obtained from different growing areas have been analyzed for its nutrition value and medical uses. This plant was used in Kurdistan region for different purposes like removing colon's pain, as a treatment for the patients that have colon's inflammatory, for eyes inflammatory and for those who exposed poisoning by snake's or scorpion's biting with addition that this plant used for eating. The results of Tosun suggested that E. spectabilis could be a valuable source of antioxidants, phenolics and minerals(3). The main aims of the present project were to search for natural products that exhibit activity against tumor cells, including a cytotoxic effect, and that potential currently used anticancer drugs.

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Material and Methods:

Plant Collections

The vegetative part of E.spectabilis was collected from Doly akoyan / (Rwandwz) city-Kurdistan region/ Iraq in August 2012. The plant was washed then the leaves were separated and left to dry at room temperature in the shade for nearly three weeks. Dried leaves were ground to fine powder. Using blender then passed through fine pored mesh sieve to provide homogeneous powder.

Aqueous Extract Preparations

Fifty grams of macerated dried leaves were placed in 500ml

volumetric flask then 400 ml of deionized distilled water (DDW), added (4). The volumetric flask was incubated in Ultrasonic bath for two hours (5). Then the suspension was filtered through a clean gauze and then through a Bückner funnel several times. The residues re extracted for 48 and then 72 hours. The filtrations were performed daily, until the solution appeared to be colorless. The solution was collected in a clean bottle, and then dried in rotary evaporator at 40 °C. This final crude extract stored at -20°C until using in the experiment.

Hexane: Ethanol extract preparations

The Hexane_Ethanol extract of E. spectabilis leaf was prepared as the same way as aqueous extract , except of using hexane-ethanol mixture (82:18. v/v) instead of DDW (6).

Cell Lines

Vero is transformed cell line was initiated from kidney of a normal adult African green monkey (Cercopitecus aethiops) on 27th March 1962, by Yasummura and Kawakita at the Chiba University, Japan and RD human cancer cell line was derived from a biopsy specimen obtained from pelvic rhabdomyosarcoma. Both the cell lines were supplied by Tissue Culture Unit/ Iraqi Center for Cancer and Medical Genetic Research (ICC-MGR, Baghdad/ Iraq) then maintained on MEM, and RPMI media with 10% of fetal bovine serum culture, 1% penicillin-Streptomycin, cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

Cell Growths Assay

The dry crud extracts were dissolved in maintenance media were used to obtain serial dilutions of (10.000µg/ ml, 1000µg/ ml, 100µg/ ml, 10µg/ ml, 1µg/ ml, 0.1µg/ ml, 0.01µg/ ml) and 0 µg/ml as a control to study the effects of aqueous and ethanolic crude extracts on different cell lines. Cell monolayer were trypsinized washed with PBS buffer, Cells cultured (50000 cells/ ml for AMN3, 65000 cells/ ml for RD and 50000 cells/ ml for Vero) in the microtitration plate were exposed to a range of plant extract concentrations during the log phase of growth and the effect was determined of optical density of solution was measured at 492nm using a microplate reader (ELISA).

%Growth Inhibition =absorbance of sample – absorbance of control / absorbance of control×100

Statistical Analysis

The results were analyzed with a prepared program of SPSS, version 18 (7). Factorial experiment with a completely randomized design (CRD) were used for the reason of different factors.

Results and Discussion:

Cell Growth Assay

Optical densities of the different (concentrations, periods, extracts and cell lines) on a growth assay:

Table(1) shows the mean of optical densities of the different (concentrations, period, extract and cell line) of the growth assay which also shows the highest significance at($p<0.05$) of the cytotoxic effect of different extracts on a cell growth assay.

periods effected significantly at ($p<0.05$) the cell growth assay at the third period caused a highest cytotoxic effect on cell growth inhibition (0.1708±0.00759) L.S.D. (0.02207), while the high significant cytotoxic doses on cell growth inhibition were the (10µg/ml, 1µg/ml,0.1µg/ml) with mean optical densities (0.1673±0.01206) (0.1669±0.0132) (0.1658±0.01328) L.S.D. (0.0204922).

The more effective extract was aqueous (0.1582±0.00576) L.S.D.(0.05322) and the more sensitive cell line was (RD) cancer cell line with Mean±S.E which is (0.1876±0.00596) L.S.D.(0.053227029).

Table.1 Mean±S.E of optical densities of the different (concentrations, period, extracts and cell lines) on a growth assay in vitro

Treatment		Mean	±	S.E	L.S.D.
Extracts	**E1**	0.1582	±	0.00576	0.053227029
	E2	0.1485	±	0.0058	
Cell lines	**Vero**	0.1191	±	0.00477	0.053227029
	RD	0.1876	±	0.00596	
periods	**P1**	0.1584	±	0.00742	0.022077015
	P2	0.131	±	0.00581	
	P3	0.1708	±	0.00759	
concentrations	**C0**	0.163	±	0.01098	0.020492216
	10.000µg/ml	0.0989	±	0.00703	
	1000µg/ml	0.1526	±	0.01359	
	100µg/ml	0.1514	±	0.01185	
	10µg/ml	0.1673	±	0.01206	
	1µg/ml	0.1669	±	0.01324	
	0.1µg/ml	0.1658	±	0.01328	
	0.01µg/ml	0.1546	±	0.01082	
	0.001µg/ml	0.1598	±	0.01431	

Cytological Study

The typical morphological features of apoptosis, including cellular shrinkage and granulation in the nucleus, are major consequence of the apoptotic trigger the phenotypic characteristics of (Vero and RD) cell lines after exposed to aqueous and Hexan_ethanolic extracts of E. spectabilis were evaluated by using inverted microscope. Vero and RD cell lines treated with different concentrations of aqueous and Hexan_ethanol extracts exhibited apoptotic morphological changes including chromatin condensation, pyknosis, karyorrhexis, cytoplasmic vaculation, cell shrinkage and cytoplasmic disruption and these changes were depended on the different concentration of plant extracts and the periods of exposure.

The aqueous extract of E. spectabilis showed significant differences in morphological features of the (Vero) normal cell line in most concentrations (0.01µg/ml,0.001µg/ml, and 1µg/ml) respectively for (72)hrs of exposure, cause disruption of monolayer as shown in figures (3).

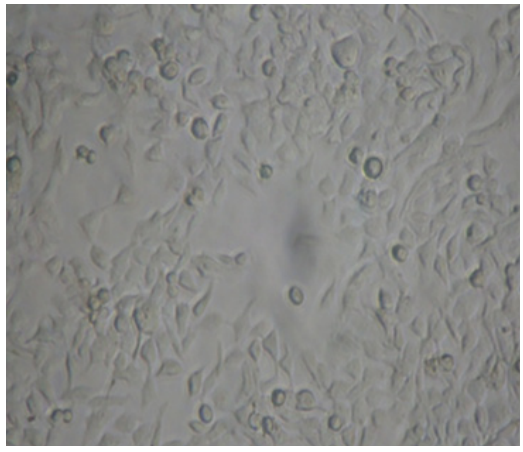


Fig 1. Control of Vero cell line

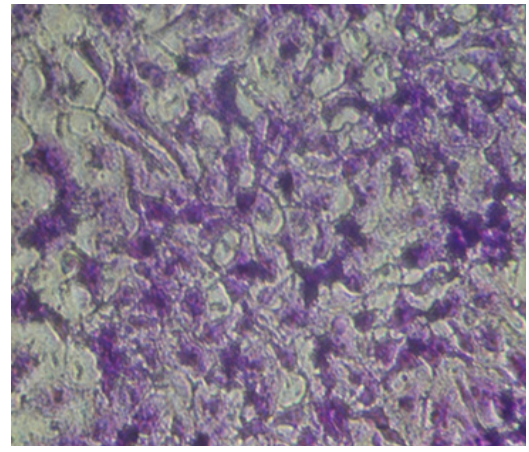
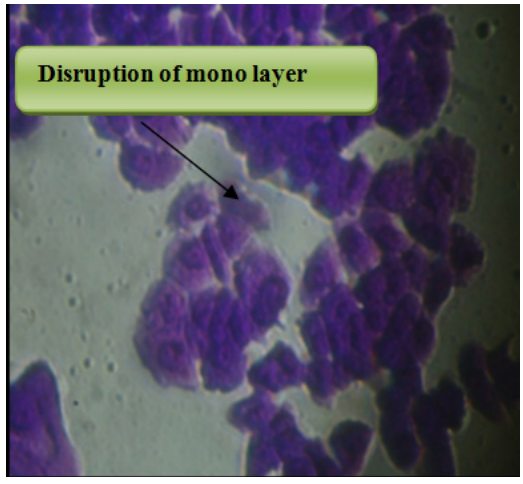


Fig 2. Control of RD cell line



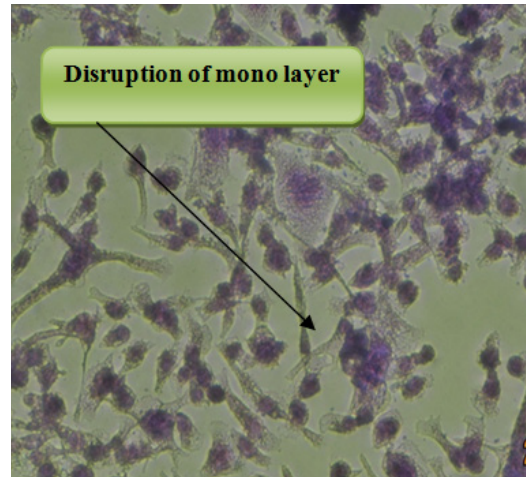


Fig 3. Vero cells treated by aqueous extract of E. spectabilis 10000µg/ml for 72hrs shows disruption of the monolayer (400X Crystal violet).Fig 4.RD cells treated by aqueous extract of E.spectabilis 10000µg/ml Shows , disruption of the monolayer (400X Crystal violet).

Inhibition concentration 50(IC50)

The cytotoxicity effect of the aqueous and hexan_ethanolic extract of E.spectabilis on RD and Vero cells was examined by exposing the cells to different concentration for 24,48 and 72h.when the two cell lines were exposed to plant extracts with concentration ranging from (10.000,1000,100,10,1,0.1,0.01,0.001)µg/ml the growth of the cell lines was found to be inhibited in a time dependent manner.

The GI50 of the aqueous and hexan_ethanolic extract after incubation the Vero and RD cells for 24hr,figure(5) showed low cytotoxic effect of aqueous and hexan_ethanolic extract at 24hr in most concentrations used in the study. The value of cell viability which reflect of aqueous extract were (10,12,11,10,10)% at (1000,100,10,and 1)µg/ml respectively on RD cells while the value of cell viability (10,10)% at (100,0.1µg/ml) on Vero cells. while the hexan_ethanolic extract had the ability for cell reduction at the concentration(1000,10,0.1) respectively with the inhibition percentage was (29,50,40)%.

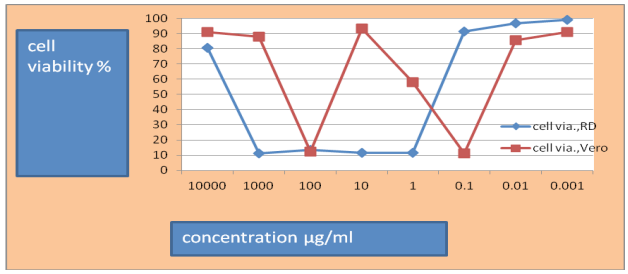


Fig 5. GI50 of (Vero&RD) cell lines after exposure to AE of E. spectabilis for 24hrs.

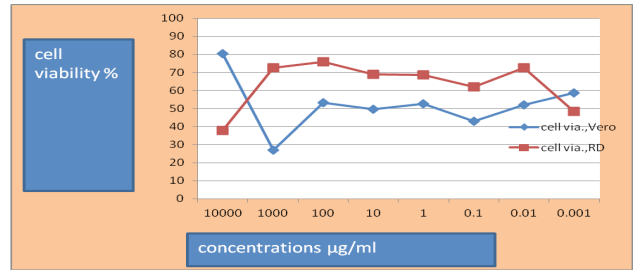


Fig 6. GI50 of (Vero&RD) cell lines after exposure to HE of E. spectabilis for 24hrs.

Figure (7) showed the values of cell viability of aqueous extract at 48hr on Vero and RD cells, the cytotoxicity effect progressed at three concentrations (10.000,1000 and 1)µg/ml was more active for killing the Vero cell lines the values of viability were (29,30,50%) respectively while the cytotoxic effect at 48hr of aqueous extract on RD cells only at first concentration (10.000)µg/ml were the viability value was (40%).

The figure(8) showed the result of Hexan_ethanolic extracts for both Vero& RD cell lines after exposure of 48 hrs The results are shown that the concentrations(10.000,100,0.1,0.01) µg/ml had the effects on Vero's cell viability value which was regarded as the best concentrations for killing of Vero's cell line at this times of exposure with their values of the cell viability which was (30,30,31,40)% in another hand the concentration (1000,1)µg/ml was represent the best concentration for RD cell line inhibition at 48hrs of exposure with its viability value which was (10,10)%.In general the HE extract was affect the Vero cell line more than RD cell line especially at (48hrs)of exposure. In general the hexan_ethanolic extract was more effective on Vero cell than RD cell especially at (48hrs) of exposure.

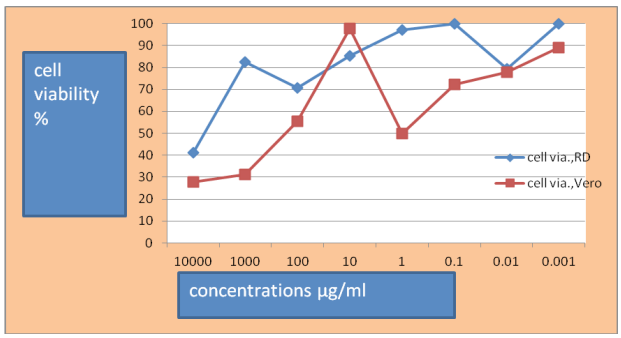


Fig 7. GI50 of (Vero&RD) cell lines after exposure AE of E. spectabilis for 48hrs.

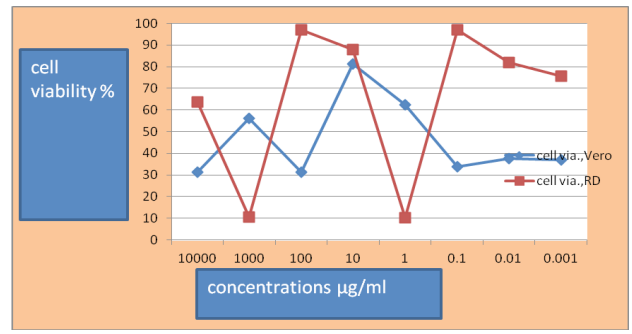


Fig 8. GI50 (Vero&RD) cell lines after exposure to HE of E. spectabilis for 48hrs.

Figure (9) showed high cytotoxic effect of the aqueous extract at 72 hours in most concentrations used in the study. The values of cell viability which reflect the cytotoxic effect of extract were (49,40,11,10,12,11%) at (10.000,1000,1,0.1,0.01,0.001)µg/ml. this effect progressed at 72 hours on Vero cell in all concentration used while the RD cell the cytotoxic effect progressed at all the concentrations (10.000,1000,100,10,1,0.1,0.01,0.001)µg/ml was to be effective as compared to the Vero cell line and at 72 hr exposure.

While figure (10) showed the low cytotoxic effect of the hexan_ethanolic extract at 72hr.in all concentration on Vero cell line used, while RD cell line at 72 hr exposure the cytotoxic effect progressed in all concentrations the values of viability were (45,9,10,10,11,12,10,13)% all the concentrations represent their inhibition percentage more than 50%.

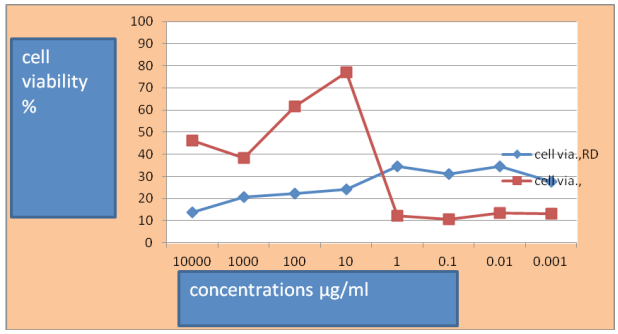


Fig 9. GI 50 of (Vero&RD) cell lines after exposure to AE of Eremurus spectabilis for 72hrs.

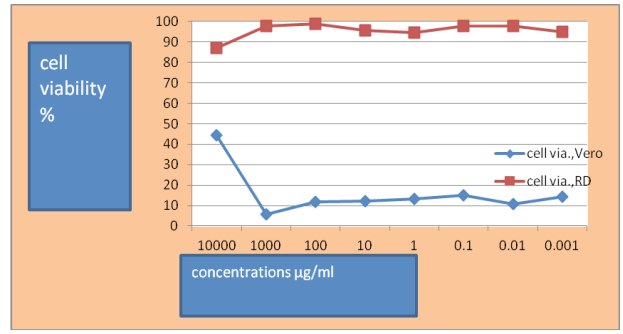


Fig 10. GI50 of (Vero&RD) cell lines after exposure to HE of E. spectabilis for 72hrs.

Finally both Vero and RD cell line were sensitive for both extract (Aqueous and Hexan_ethanolic) and in all concentrations and at all periods of time of exposure which was (24, 48 and 72) hrs. but some concentrations and extracts were more effective and had more inhibitory effect on these two cell lines than the other concentrations and extracts.

The percentages of active compounds of E.spectabilis

For identification of the major groups of compounds in the E. spectabilis we done HPLC for this purpose and the percentages were obtained as follow:

Table 2. Active compounds of *E. spectabilis* with their percentages.

Active compounds	Percentages
1.Carvone	44.64%
2.Carvacrol	14.45%
3.Pentane, 2-methyl	7.34%
4.E-caryophyllene	5.57%
5.Valencene	5.11%
6.Cis-calamenene	2.01%
7.Cadalene	1.10%
8.Acetic acid	1.12%

Discussion:

The results of the present study showed that the growth inhibition aqueous and hexan- ethanolic extracts of Eremurus spectabilis which exhibited time and dose - undependent inhibitory effects on RD cancer cell line. E. spectabilis is used as a vegetable in Turkey, especially in Eastern Anatolia region. The antioxidant activity tests evaluated by using 2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging and β-carotene/linoleic acid assays indicated that the extracts of E. spectabilis samples had high antioxidant capacity. In the DPPH and β-carotene/linoleic acid systems, The average amount of total phenolics in samples was 223 mg. Protein, K, Ca, Mg, Fe and Cu contents of E. spectabilis species were found higher than in some other commonly used vegetables. The results suggest that E. spectabilis could be a valuable source of antioxidants, phenolics and minerals(8). Also E.spectabilis which contain carvacrol is believed to be disruption of the bacteria membrane(9). A study led by Supriya Bavadekar reports that carvacrol stimulates apoptosis in prostate cancer cells .[10] the plant contain Carvone This substance are believed to function principally in ecological roles, serving as herbivore- feeding deterrents, antifungal defenses, and attractants for pollinators .The commercial importance of monoterpenes as flavorings, fragrances, and pharmaceuticals has stimulated many efforts to increase their yield in plants. L-Carvone is also used as a spearmint flavor. this chemical has enantiomers that has different fragrances(11). Also this plant contain (valence and phenolics) The valence compound has an expression of the number of antigen-binding sites for one molecule of any given antibody or the number of antibody-binding sites for any given antigen(12).

Also the hexan ethanolic extract of Eremurus spectabilis were stronger affected the cancer cell lines than aqueous extract because the hexan_ethanolic extract which was non polar extracts were found to be strong inhibitors of at different concentrations, in comparison with polar extract (ethanol extract). the chloroform and hexane extracts exhibited the

radical scavenging activity(13) .

Conclusion

In the present study we found that the aqueous extract was less effective anti-proliferate agent as compared to hexane-ethanolic extract. Major components of E. spectabilis are volatile compounds were carvone , carvacrol , pentane, 2-methyl-,(E)-caryophyllene, valencene , cis-calamenene , cadalene , and acetic acid.

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فعاليات كامنة ضد الورم لمستخلص الاوراق لنبات (اريميوريس سبيكتابيليس) بواسطة طريقة (HPLC) على خط خلاية السرطانية خارج جسم

شاية ن رشيد ابوبكر، هازه جمال هدايت

قسم علةم الحياة/ كلية التربية/ جامعة صلاح الدين/ أربيل/ اقليم كردستان العراق

الخلاصه:

لقد ركز كثيرا من العلماء على معالجة السرطانات منذ فترة طويلة من خلال دراسة فعاليات السمية للاستخلاص النباتات, و استعمل نبات (اريميوريس سبيكتابيليس) لغرض الدراسة نظرا لاحتوائه على مواد كيميائية متضمنا (كارفون ,كارفاكرول,بينتان,-2 ميثايل,-9كاروفيلين.)

لقد بينت في الدراسة الحالية فعاليات السمية للخلية للاستخلاص المائي و هيكسان_الاثيلي على حيوية الخلية (RD) وخلاية المتحورة للقرد الاخضر الافريقي (Vero) معتمدا على الجرعة و الوقت و استخلاصات هيكسان_الاثيلي (10.000,1000,100,10,1,0.1,0.01,0.001) ملغم \ مل في (24,48 و 72) ساعة حيث اختبرت على خط الخلوي (RD)و خط الخلوي (Vero) .

لقد بينت نتيجة الدراسة الحالية بان استخلاص المائي و هيكسان_الاثيلي للنبات (اريميوريس سبيكتابيليس) قد ضبطت تماما نمو الخلاية بواسطة تقليل حيوية الخلية لكلا خطي الخلية لتراكيز مختلفة بحيث ان خط الخلية (RD) كانت حساسة لكل التراكيز المستخلصة للنبات في هذه الفترات , بينما خط خلية (Vero) مقاومة عند التركيزان هما (100 و 10) ملغم\مل في الوسط الحسابي (0.080 و 0.16) , في حين استخلاص هيكسان_الاثيلي لتراكيز مختلفة لمدة (72 ساعة) عند التعرض على كلا خطي الخلية (RD,Vero) في نمو الخلية, بينت بان خط الخلية (RD) كانت لا تستجب لكل التراكيز المستعملة لقتل خط الخلية (RD) بينما خط خلية (Vero) كانت تمثل حساسيتها لكافة التراكيز من (10.000_0.001) ملغم\مل.