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Antiproliferative Activity of Spironolactone: *In Vitro* Study

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Abstract :

Spironolactone SPL stock solution was studied against two cancer cell lines Hep-2, and AMN-3. These cell lines were exposed to different concentrations of SPL ranged from 0.0019 to 1000 µg/ml for 24, 48 and 72 hours durations. The optical density was measured under wavelength 492 nm with micro-ELISA reader after their staining with crystal violet dye.

The results revealed a clear cytotoxic activity of pure SPL with high significances in a two cancer cell lines during the three exposure time, suggesting that the cytotoxic effect of pure spironolactone is a dose and time dependant. The best cytotoxic activity was on AMN-3 and Hep-2 cell lines at the high concentrations 1000, 500, 250, 125µg/ml.

The results also suggest that AMN-3 cell line is the most sensitive cancer cell line to pure SPL than Hep-2 cell line after 24 hr. of exposure to drug.

Introduction:

Cancer is one of the major public health problems facing our world.(1) Several of the most common cancers occur in hormonally responsive tissues, and includ breast, endometrium, and ovary in women, and prostate in men. (2) Breast cancer is the most commonly diagnosed malignancy in women.(3) Cancerous growth may be

controlled by surgical removal, radiotherapy and chemotherapy(4,5) as well

as hormones and their analogues that themselves have inhibitory action on particular tissue and can be used in the treatment of these tumors.(6,7) Hormone antagonists that can be used effectively in several hormones sensitivity tumors include anti-estrogens (Tamoxifen)(8) and anti androgens (Spironolactone).(9)

The present study was designed to investigate the cytotoxic effect of spironolactone on cancer cell lines; and to evaluate the antiproliferative activity of this drug on tumor growth in cancer cell growth in vitro study.(10)

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Materials and Methods:

Stock solution of pure powder of spironolactone was prepared by dissolving 0.01g of pure powder of SPL in 0.1ml of dimethyl sulfoxide (DMSO) (USA-Sigma), and complete volume with 0.9 ml of serum free media (SFM) and filtered with sterile (0.22µm) Millipore filter, and 20 concentrations of drug were made in two folds, ranged from (0.0019 to 1000)µg/ml.

The cell lines were exposed to different concentrations of pure SPL ranged from 0.0019 to 1000µg/ml. The periods of exposure of cell lines were measured at 24, 48, and 72 hrs in a microtitration plate under complete sterile conditions. This was done in trireplicates for each concentration.

Cancer cell lines:

Human Epidermoid Larynx Carcinoma (Hep-2) (11) and Ahmed-Mohammed- Nahi-2003 (AMN-3) (12) were obtained for the Iraqi Center for Cancer and Medical Genetic Research. These cell lines were maintained in RPMI-1640 media with 10 % v/v bovine serum and incubated at 37° C in a humidified atmosphere containing 5% CO2 and 95% air.

Cytotoxic assay:

The cytotoxic assay was measured using the crystal violet stain was conducted as on(13).

Then tumor cells were seeded in 96-well microplates (Nunclon) at a concentration of 2x105 cell/well. After 24hr.

incubation at 37°C or confluent monolayer was achieved. Cells were treated with each concentration at 1000 µg/ml in two fold serial dilutions reaching 0.0019µg/ml.

The procedure of adding these therapeutic agents was made by adding the drug for 24, 48 and 72 hrs at 37°C for Hep-2 cell line and for AMN-3 cell line.

After the dedicated time, cells were washed with PBS. Cell viability was measured after removing the medium, followed adding 100 µl of solution of 5mg crystal violet (BDH-England) + 200ml methanol + 50ml formaldehyde 37% and incubating for 20 min at 37°C.

After removing the crystal violet solution, cells were washed with water three times. The absorbency was determined on a microplate reader (organon Teknika Reader 230S, Austria) at 492 nm (test wavelength); the assay was performed with modification in triplicate(14).

Endpoint parameters that were calculated for each individual cell line include cell proliferation rate, which is the percentage of control absorbance (15,16).

The inhibiting rate of cell growth (the percentage of cytotoxicity) was calculated as (A-B)/Ax100, where A is the mean optical density of untreated wells and B is the optical density of treated wells (17,18).

Statistical analysis:

Data were analyzed by 2-way analysis of variance with ANOVA. Data are presented as means ± SD. The level of significance $p<0.05$ was used for analysis of variance in all result presented this study(19).

Results:

AMN-3 cells:

AMN-3

Figure (1) shows the effect of all concentration of SPL solution 0.0019 to 1000µg/ml on mammary adenocarcinoma cell line, at three exposure time 24, 48 and 72 hrs. spironolactone had the highest inhibitory growth on AMN-3 cell line at concentrations 1000, 500, 250, 125, 62.5 and 31.25 µg/ml, at the period of 24 hrs; where the higher growth inhibition rate were 94.5%, 92.5%, 89.6%, 87.8%, 84.4% and 83.8%, respectively.

However the best growth inhibition rate was, 94.5% at the concentration 1000µg/ml after 24hr. Lower concentration 0.0019 µg/ml gave the lowest growth inhibitory 47% after 24hr. exposures time. The antiproliferation effect of SPL on AMN-3 cell line showed at low concentration after 24hrs. 66%, 56%, 47%. However those growth inhibitory rate increased with time after 48 and 72 hrs. 61%, 57%, 52% and 64%, 61% and 57%, respectively.

The antiproliferative effect at high concentration (1000µg/ml) of pure (SPL) on AMN-3 cell line after 24hrs. exposure time, showed loss of confluent feature and large number of dead cells as seen in figure (3) compared with confluent monolayer and viability malignant cell of control seen in figure (2).

The cell line of AMN-3 showed a decrease in viability cell number with a presence of few dead cells after 24hrs. exposure to the lower concentrations of SPL figure (4) in compare with AMN-3 cell line exposure to higher concentration seen in figure (3).

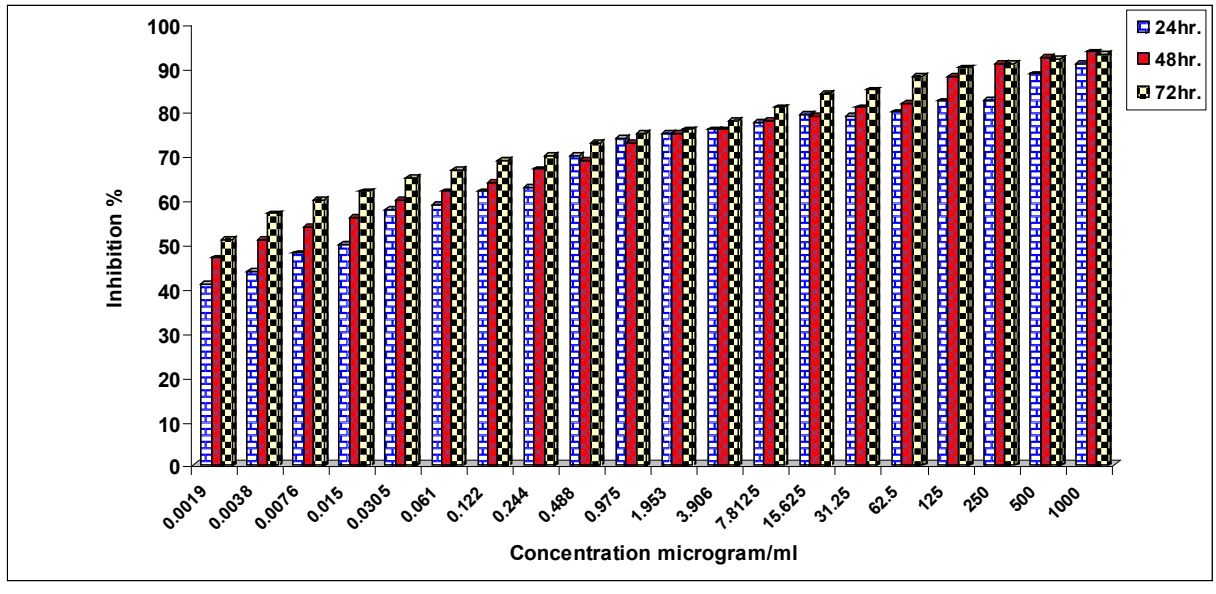


Figure (1): The growth inhibition percentage on AMN-3 cell line growth by different concentration of spironolactone solution at different exposure periods.

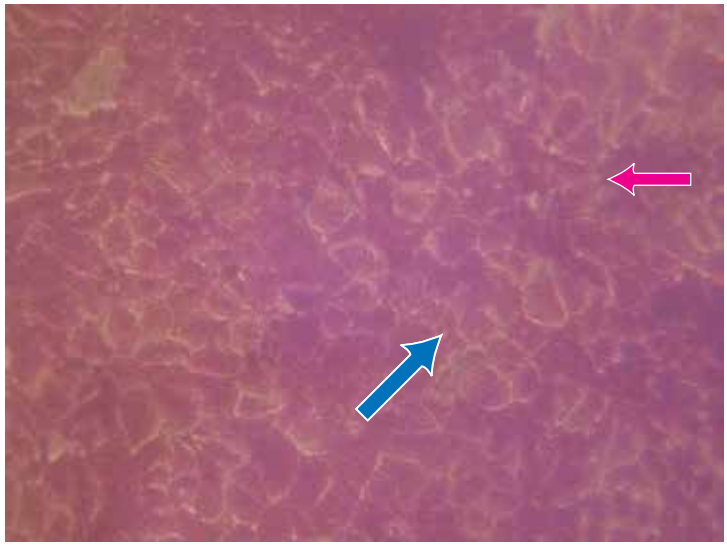
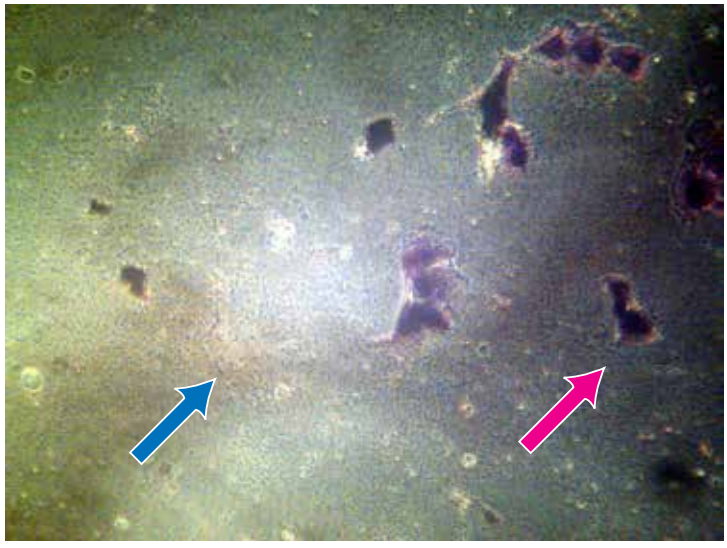


Figure (2): AMN-3 cell line shows confluent monolayer (→), no empty spaces (→), chohesive malignant cell control 100X, crystal violet.



Figure(3): AMN-3 cell line reveals great loss of cellular features(→), and large number of dead cells (→) after exposure to high concentrations (1000mg/ml) of spironolactone after 24 hr., 100X, crystal violet.

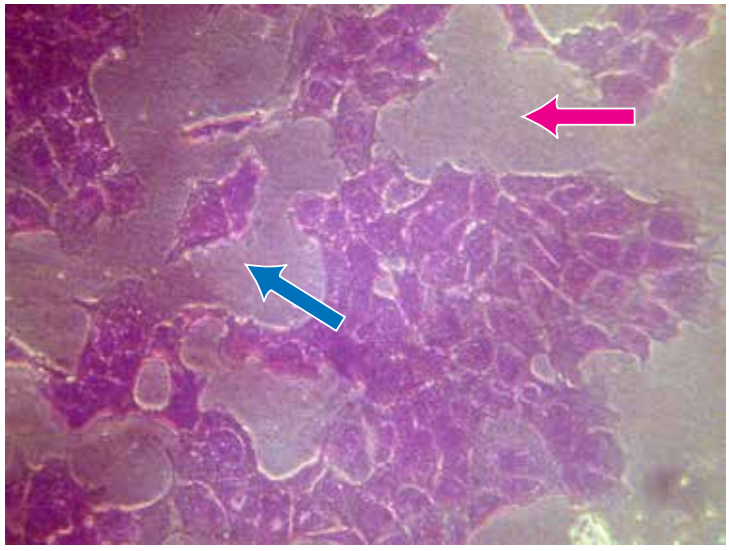


Figure (4):AMN-3 cell line shows loss cellular feature(→) with presence few of dead cells (→) after exposure to low concentration (0.0019 µg/ml) of spironolactone drug after 24 hr. 100X, crystal violet.

Hep-2 cells:

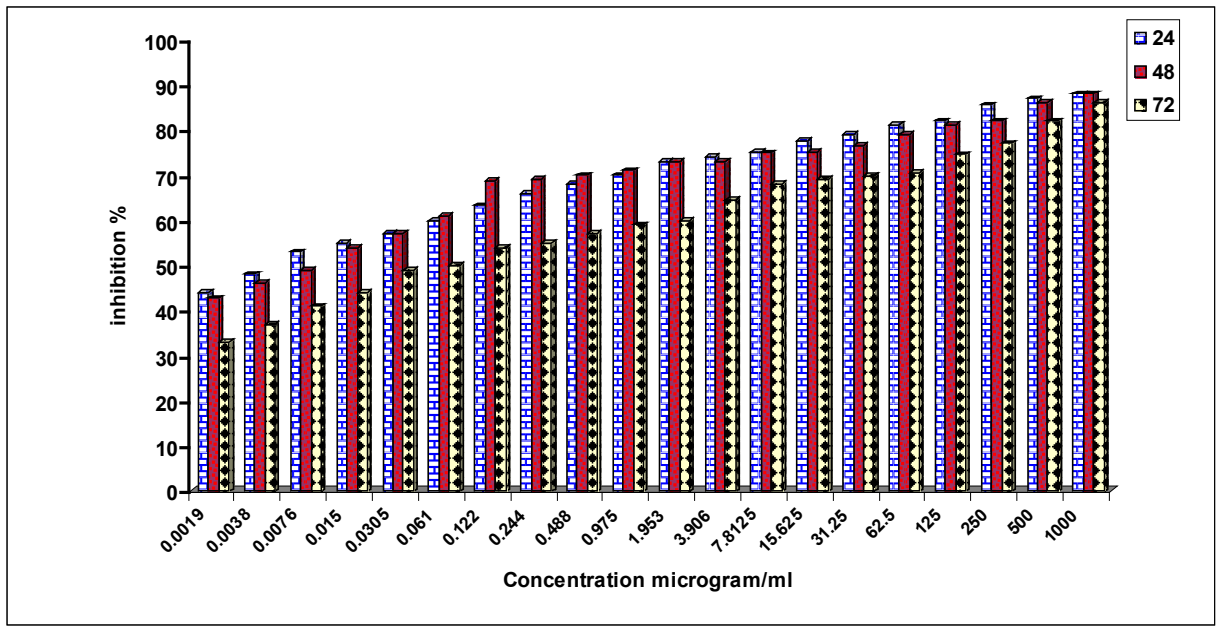
The result in table 5 reveals that the SPL stock solution has cytotoxic effect on Hep-2 cell line at all concentrations. It was found that significant ($p<0.05$) effectiveness was notice on the Hep-2 cell line by increasing the concentration and time exposure to 72hr. of exposure.

The antiproliferative effect of SPL on Hep-2 cell line revealed that spironolactone had the highest inhibitory growth on proliferation Hep-2 cell line at the high as concentrations 1000, 500, 250, 125µg/ml for the period of 48hrs, and ranged from 93.6%, 92.3%, 90.8% and 88% respectively, compared with control cells which have consider to give 0% rate.

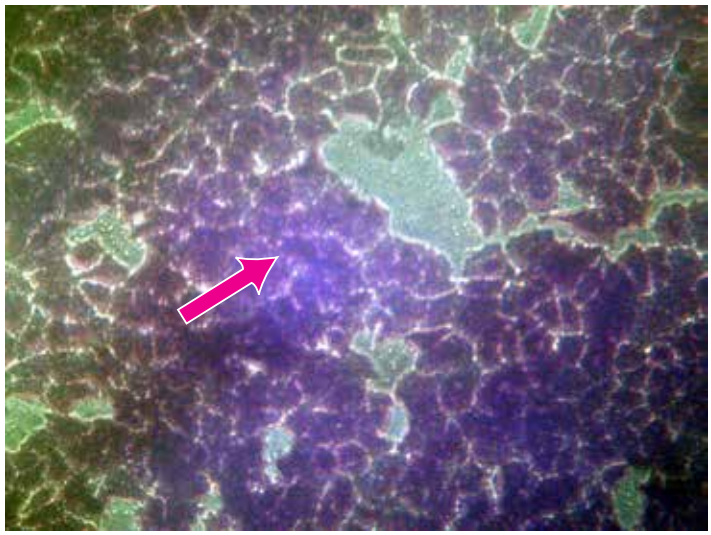
While the growth inhibition of treating Hep-2 cell line for 48hrs. of exposure with low concentrations (0.015, 0.0076, 0.0038 and 0.0019µg/ml) of pure (SPL) was reached to the (56,% 54%, 51% and %47%) respectively. However growth inhibition of (SPL) on Hep-2 cell line (50%, 48%, 44% and 41%) after 24hrs. exposure to stock solution of (SPL), at same concentration.

The cell line of Hep-2 showed total lyses of cell and loss their feature were noticed, the outline cellular feature has been lost and changes progressed with highly effect of high concentration (1000µg/ml) of (SPL) and this can be seen in figure (7).

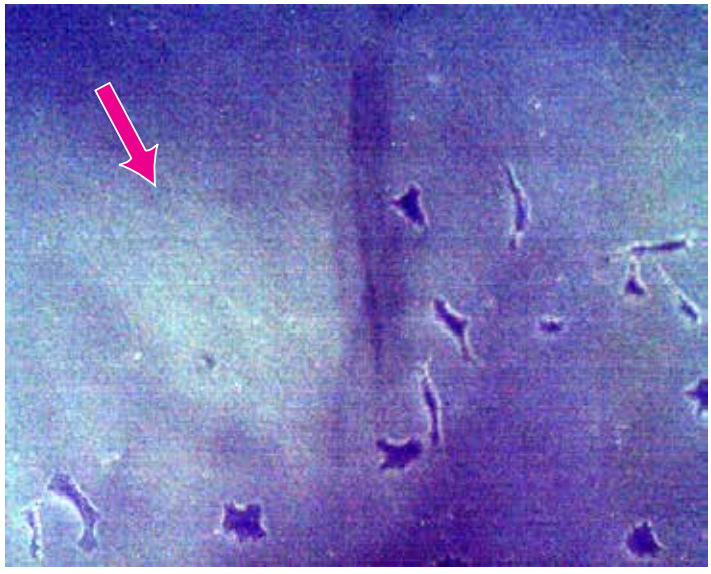
Cytotoxic effect of SPL for Hep-2 cell line exhibited loss cellular feature and few dead cells after exposure to the low concentration (0.00019µg/ml) of (SPL) for 48hrs. seen in figure(8), in compare with control cells in figure(6).



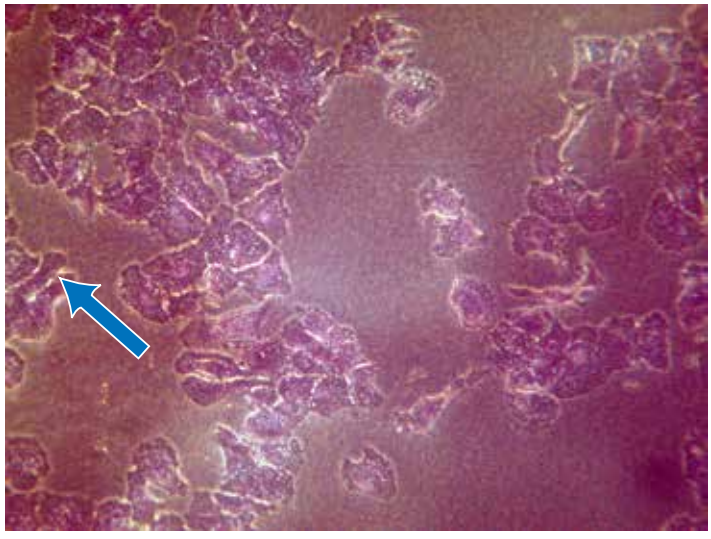
Figure(5): The growth inhibition percentage on Hep-2 cell line growth by different concentration of spironolactone solution at different exposure periods.



Figure(6): Hep-2 cell line shows confluent monolayer (→), cohesive malignant cell control 100X, crystal violet.



Figure(7):Hep-2 cell line shows highly reduce in viability cell number(→) after treatment with highest concentration (1000µg/ml), of spironolactone after 48 hr., 100X, crystal violet.



Figure(8): Hep-2 cell line loss cellular feature(→) after exposure to low concentration(0.0019µg/ml)of spironolactone drug for 48hr. 100X, crystal violet.

Discussion:

The results of in vitro study showed that SPL stock solution exhibited dose-dependent cytotoxic effect of all concentrations used in this study on cancer cell line.

Higher concentration 1000 µg/ml gave the highest inhibition rate for AMN-3 94.5% after 24hr of exposure time and the cytotoxic effect of the (SPL) at highest concentrations on HEP-2 cell line after 48hr of exposure reached which the best concentration 1000µg/ml gave the high growth inhibition rate (93.6%) after 48 hr of exposure time. The inhibitory effect of drug against AMN-3 cell growth observed during short period while their inhibitions against the growth of HEP-2 cell line were observed during long period. This can be attributed to sensitivity of AMN-3 cell line and or may be due to cytotoxic effect of drug against cancer cell line showed highly significant reduction in viability cell number at higher concentration (1000µg/ml) of (SPL) gave best inhibition rat 94.5% after 24hr and lowest inhibitory rate 47% at lower concentration (0.0019µg/ml), this result shows in figure(1).

The results showed that AMN-3 cell line may be responsive to androgens and estrogen(20).

Estrogen stimulate AMN-3 cell proliferation and their effect are mediated by the (ER). In contrast (SPL) suppressed the effect of androstenedion-induced cell growth by antiadrogene affect (20) through inhibition of 5α-dihydrotesterone binding to cytosolic androgen receptor(21) and exert their anti-proliferative effect by interacting with the (AR)(22,23).

Our results were consistent with the results of the in vitro study suggest that a change in the balance between androgenic and estrogenic influences could modify the overall growth rate of breast tumors because these hormones have opposite effects on breast cancer cell growth(24). The proliferation of MCF-7human breast cancer cell line not only by a mechanism involving reduction of estrogens biosynthesis but, additionally, by permitting endogenous androgens to exert their antiproliferative effects the AR in a low-estrogen milieu(25,22).

This study show cytotoxic effect of (SPL) drug against cancer cell AMN-3 cell line may be due to ability of this drug to increase the activation of glutathione-S- transferase enzymes (GSTS)(26). The (GSTS) acted as an anti oxidant causing cellular detoxification by enhancing their combination with reduce glutathione leading the cancer cell toward programmed cell death apoptosis(13).

The action of (SPL) on Hep-2 cell line was appeared as inhibitory effect in all periods of exposure and at all concentration. The best concentration 1000µg/ml O.D (0.048±0.004) gave the highest cytotoxic effect and inhibitory cell growth (93.6%) after 48 hr. and lowest growth inhibition rate 47% with lower concentration (0.0019µg/ml) of (SPL) figure (3-2). The result presents in this study showed that the cytotoxic effect of (SPL) on Hep-2 cell line may be due to directly inhibited both bFGF-and VEGF-stimulated endothelial cell proliferation in vitro.

Spironolactone inhibition angio- genesis directly through blockade of vascular endothelial cell from responding to a wide spectrum of angiogenic stimulators, including VEGF, bFGF. As a result, the abilities of endothelial cells on proliferation cells are suppressed, and angiogenss is inhibited(27). This drug might be useful in the treatment of a variety of diseases dependent on angiogenesis, such as solid tumor growth and macular degeneration(28).

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فعالية مثبط التكاثر للسبايرونولاكتون: في تثبيط نمو الخطوط الخلوية السرطانية

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الخلاصة:

يعد هذا البحث دراسة أولية لاستكشاف فعالية مركبات الايض الثانوية لعقار السبايرونولاكتون بشكله الخام في التاثير في نمو الخلايا السرطانية خارج الجسم الحي، اذ تم تحضير الدواء بشكل مستخلص باستعمال المذيب (Dimethyl sulphoxide).

اختبرت الفعالية السمية للمستخلص الخام لعقار السبيرونولاكتون في الخطوط الخلوية السرطانية (AMN-3, Hep-2) بتعريضها لعشرين تركيز (0.0019-1000 مايكروغرام/مليلتر) وضمن مدد تعريض مختلفة (24،48،72) ساعة.

كانت النتيجة وجد تاثير سمي واضح، وبمعنوية عالية في نمو الخلايا السرطانية بنسبة 95.5% علماً ان شدة السمية تزداد بزيادة التركيز ومدة التعريض، لذا فان التاثير السمي لعقار سبايرونولاكتون الخام يعتمد على التركيز ومدة التعريض، وكان التركيز السمي الاكثر كفاءة في تثبيط نمو خط الخلايا السرطانية نوع (AMN-3, Hep-2) بتركيز 1000 مايكروغرام/مليلتر، علماً ان عقار سبايرونولاكتون يمتلك تاثير تثبيطي على نمو الخلايا السرطانية في كل التراكيز المستخدمة ولكنه يعطي اعلى نسبة تثبيط في التراكيز العالية.

وقد تبين من خلال هذه الدراسة ان الخط السرطاني (AMN-3) اكثر حساسية لعقار سبايرونولاكتون الخام من الخط السرطاني الثاني (Hep-2)فقد اعطى تثبيط بنسبة عالية وبمدة قصيرة خلال 24 ساعة من المعالجة بعقار سبايرنولاكتون. وهذه النتائج تشير الى وجود الفعالية التثبيطية لعقار سبايرونولاكتون الخام لنمو الاورام.