Potentiate the anticancer effect of Methotrexate by *Zingiber officinale* Roscoe extract

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Abstract:

This project considered an explorer study for the effect of crude extracts of *Zingiber officinale* on two cell lines, one was cancer cell lines, and the other was normal cell lines as well as study its potentiating effect on anti-tumor activity of methotrexate.

The aqueous extract was prepared from dried rhizome of *Z. officinale* yields of extraction was 10%. Study the cytotoxic activity of the prepared extract on cancer cell lines mouse breast 4TI and normal cell line (human embryo kidney HEK293 in ten concentrations of two fold dilution begin with 4000 µg/ml until 7.81 µg/ml for extract and starting with 500 µg/ml until 0.976 µg/ml for methotrxate, at 48 hours of exposure. After the end of exposure of each cell lines to extract, the microtitration plates were washed and treated by crystal violet stain and the optical density of the plate wells were read by the ELISA reader at wave length of 495 nm used as parameter for the viability of cell line. The results showed that the aqueous extract exhibited significant concentration-dependent, specific inhibitory effects on 4TI malignant cell line in comparison with control and the cytotoxic effect increased according to the concentration. The extracts produced little cytotoxic effects on normal HEK293 cell line, when compared to mthotrexate which produced inhibitory effects on normal HEK293 cell line. In this study was showed that the cytotoxic effect of Methotrexat on 4TI cell line enhanced when given with *Z. officinale* extract together.

Keywords: Cancer, *Zingiber officinale*, Methotrexate, HEK293 cell line, 4TI cell line.

زيادة تأثير عقار الميثوتريكسيت المضاد للسرطان بمستخلص الزنجبيل

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الخلاصة:

يعد هذا البحث دراسة استكشافية لتاثير المستخلص الخام لنبات على خط خلوي سرطاني وخط خلوي طبيعي، بلاضافة الى دراسة تثير المستخلص في زيادة تاثير عقار الميثوتركسيت المضاد للسرطان .

حضر المستخلص المائي من الدرنات المجففة لنبات الزنجبيل وكانت نسبة الحاصل الناتج من عملية الاستخلاص 10%. حيث تمت دراسة التاثير السمي للمستخلص المحضر على نوعين من الخطوط الخلوية، خط خلايا الكلية البشري(HEK 293) هو خط خلية طبيعي لتقيم السمية الخلوية للمستخلص وخط خلايا سرطان الثدي الفاري (4TI) الخط الخلوي السرطاني، بعد معاملة الخطوط بعشر تراكيز مخففة تبدا من4000 µg/ml الى 7.81 µg/ml للمستخلص و من500 µg/ml الى 0.97µg/ml خلال 48 ساعة. بعد نهاية تعريض الخطوط الخلوية بالمستخلص يتم غسل الصفيحة ومعاملتها بصبغة ال crystal violate وتقرا الكثافة البصرية للآبار المجودة في الصفيحة بواسطة الELIZA عند طول موجي 495 nm بوصفه مقياس للخلية الحية. واظهرت النتائج تاثيرا مثبطا عاليا معتمدا على التركيز للمستخلص المائي على خط خلايا سرطان الثدي الفاري مقارنة بآبار السيطرة وتزداد نسبة التثبيط بزيادة التركيز، كما اظهر المستخلص تاثيرا قليلا على خط خلايا الكلية البشري الطبيعي مقارنة بعقار الMTX .وقد لوحظ في هذه الدراسة زيادة نسبة تثبيط عقار MTX على خط خلايا سرطان الثدي الفاري عندما يعطى مع مستخلص الزنجبيل سوية.

Introduction:

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. It is the most dangerous disease that threaten human life in various parts of the world, where it consider the second causes of death in the world after heart disease [1] and in Iraq the number of cases of illness of cancer was reached to more than 10,888 cases in the period between the year (1999 -2000) (Ministry of Health - Center for Cancer Registries in Iraq, 2000).

The scientific researches tended to study the disease in all its aspects, and found the most accurate mechanisms that cause and develop the disease, and thus try to cure disease and save the life of human [2, 3]. The radiation and the surgical therapy are using in case of topical tumor ,the chemotherapy use when the cancer cells are spreading in the body [4], although the benefits of these treatments, but has side effects back negatively on the health of the patient, especially chemotherapy and radiation, which destroy normal cells along with cancer cells as well as weakening the immune system[5], where the chemical treatments contain different types of compound which have anti-metabolic activities or anti-mitotic activities[6]. So came the current researches in order to discover the natural benefits of some plants in various parts of the world to detect the compounds that have medical benefits in the hope to be the possible treatments for group of cancers with least side effects as well as reduce the economic cost of treatment.

The *Zingiber officinale* extract was elected for this study due to the presence of signals in the scientific literature as an anti-genetic toxicity there for this study was designed to

test and evaluate its effect on the growth of cancer cells, hoping to help cancer treatment, the *Zingiber officinale*, is one of the most widely used species, is a common condiment for various foods and beverages. It has a long history of medicinal use dating back 2500 years.

The *Zingiber officinale* belongs to the herbs that have effective against tumors, as found in a study carried out *in vivo* , it have not any effective carcinogenic or mutagenic activity [7]. And also found that the treatment of alcoholic extraction of *Zingiber officinale* protects against the formation of skin tumors developed in experimental mice [8]. The Zingiber officinale plant also have pronounced antioxidant activity and effective in preventing the formation of free radicals that attack and cause damage of the cell membrane and nucleus that responsible for the cancers[9].

Materials and method:

One hundred grams of the powdered plant added to 500 ml of distilled water in a sterile glass beaker and left for 24 hours with continued jolt , then passed on the layers of sterile soft cloth for its candidacy and then dried the liquid in the incubator at 37 ºC for 3 days until dry. Then collected in a sterile bottle and preserved at room temperature. The cells of HeK293 were seeded into two 96-well plates and then incubated for 48hours,the first plate was exposed to mehotrexate and the second for *Zingiber officinale* extract. The cells of 4TI exposed were seeded into four 96-well plates and then incubated for 48hours, the first plate was exposed to various concentration of methotrexate, the second plate was exposed to various concentrations of *Zingiber officinale* extract and the third plate was exposed to *Zingiber officinale* and methotrexate together.

The following protocol [10] was performed on each plate:

a) After trypsinization, cell suspension seed in a micro titration plates at 50000cells/ml RPMI-1640 growth medium with serum 5% was used for seeding.

b) Plates then incubated for 48hours.

c) By using maintenance medium, two-fold serial dilution were prepared starting from 500µg/ml ending with 0.976µg/ml (for methotrexate), and from 4000µg/ml ending with 7.81µg/ml (for *Zingiber officinale* extract. Only 20 µl of each concentration added for each well (5- replicates for each tested concentration), 20µl of maintenance medium added to each well of control wells (24 wells were used as control). The time of exposure was 48hrs. The plates sealed with self adhesive film then returned to the incubator at 36.8 ºC.

d) After the end of the exposure period, the medium decanted off and cells in the wells gently washed by adding and removing 0.1ml sterile PBS two times. Finally 0.1ml of

maintenance medium added in each well and incubated for further 48hrs.

e) At the end recovery time, the maintenance medium, threw away, and replaced by 50µl of 0.01% crystal violet dye. After 20min. the stain was washed gently with tab water for three times. The plate was left until become dry. The optical density of each well was read by using a micro-ELISA reader at 492nm transmitting wavelength.

The percentage of inhibition was calculated as:

Inhibition % = 100- [(optical density of test wells/optical density of control wells) x 100]. [11]

The HEK293 and 4TI cell lines were exposed to ten concentrations, of twofold dilutions of *Zingiber officinale* extracts for 48 hours durations. The optical density for the cell lines were measured at 495nm with micro-ELISA reader after their staining with crystal violet dye.

Results:

The results in table (1-1) revealed that the aqueous extract of *Zingiber officinale* exhibited significant concentration-dependent, specific inhibitory effects on 4TI malignant cell line in comparison with control and the cytotoxic effect increased according to the concentration.

The Figure (1-1) showed the comparison between inhibition% of different concentrations (starting from 500 µg/ml until 0.976 µg/ml) of *Zingiber officinale* extract on HEK293 and 4TI cell lines after 48 hours of exposure and these concentrations were the same seven concentrations of methotrexate to compare between these two agents on the same two cell lines.

Table (1-1):Inhibition% of different concentrations of zingiber officinale extract on HEK and 4TI cell line after 48 hours of exposure		
concentration of Zingiber officinal extact(µg/ml)	Inhibition% ± SD	
4000	HEK cell line	4TI cell line
	5.6 ± 0.36	28.1 ± 2.45
2000	4.63 ± 1.11	20.16 ± 1.47
1000	4.26 ± 0.72	20.48 ± 0.58
500	4.259 ± 1.51	19.05 ± 3.2
250	3.889 ± 0.18	18.10 ± 1.71
125	2.78 ± 0.54	13.44 ± 3.23
62.5	2.222 ± 0.73	11.43 ± 0.45
31.25	1.852 ± 0.37	10.96 ± 1.73
15.62	1.777 ± 0.17	10.96 ± 3.22

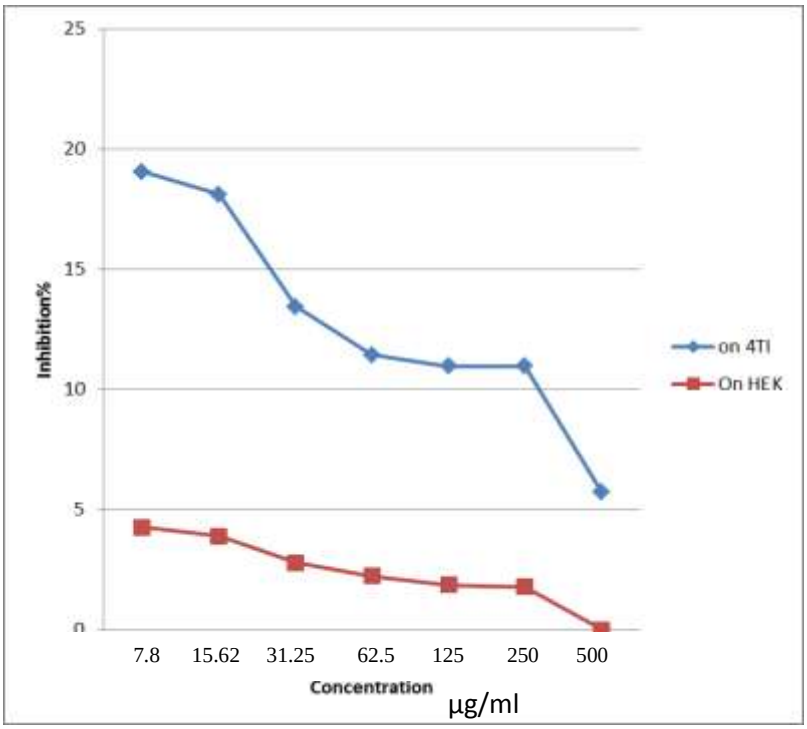

Figure (1-1): Comparison between inhibition% of different concentrations of *Z. officinale* extract on HEK293 and 4TI cell lines after 48 hours of exposure.

After application of inhibition% equation in the Table (1-2) the results revealed that methotrexate exhibited significant inhibitory effects on HEK293 and 4TI ell lines in comparison with control as well as the Figure (1-2) showed the comparison between inhibition% of different concentrations (starting from 500 µg/ml until 0.976 µg/ml) of methotrexate on HEK293 and 4TI cell lines after 48 hours of exposure.

Table (1-2): Inhibition% of different concentrations of MTX on HEK293 and 4TI cell lines after 48 hours of exposure

Methotrexate concentration (µg/ml)	**Inhibition% ± SD**	
	HEK293 cell line	**4TI cell line**
500	**20.23 ± 1.18**	**49.05 ±0.31**
250	**13.93 ±0.11**	**41.08 ±1.28**
125	**12.86 ±0.34**	**35.35 ±1.57**
62.5	**11.91 ±1.01**	**32.48 ± 0.64**
31.25	**9.29 ± 1.11**	**21.54 ± 0.94**
15.625	**6.91 ± 0.99**	**21.02 ± 2.72**
7.812	**5.6 ± 0.45**	**19.16 ± 1.60**
3.906	**4.77 ± 0.33**	**16.25 ± 0.92**
1.952	**1.91 ± 0.11**	**15.93 ± 0.62**
0.976	**0± 0**	**10.12 ± 0.32**

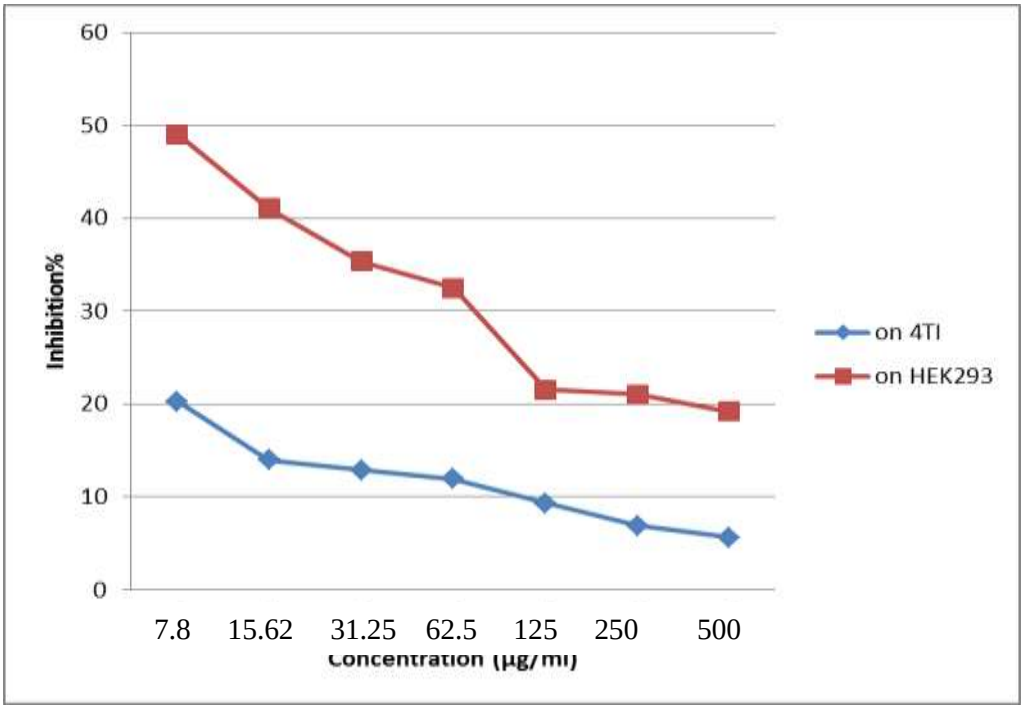

Figure (1-2): Comparison between inhibition% of different concentrations of Methotrexate on HEK293 and 4TI cell lines after 48 hours of exposure.

The Figure (1-3) showed the comparison between inhibition% of different seven concentrations (starting from 500 µg/ml until 0.976 µg/ml) of *Zingiber officinale* extract on HEK293 cell line after 48 hours of exposure and these concentrations were the same seven concentrations of methotrexate to compare between these two agents on the same two cell lines.

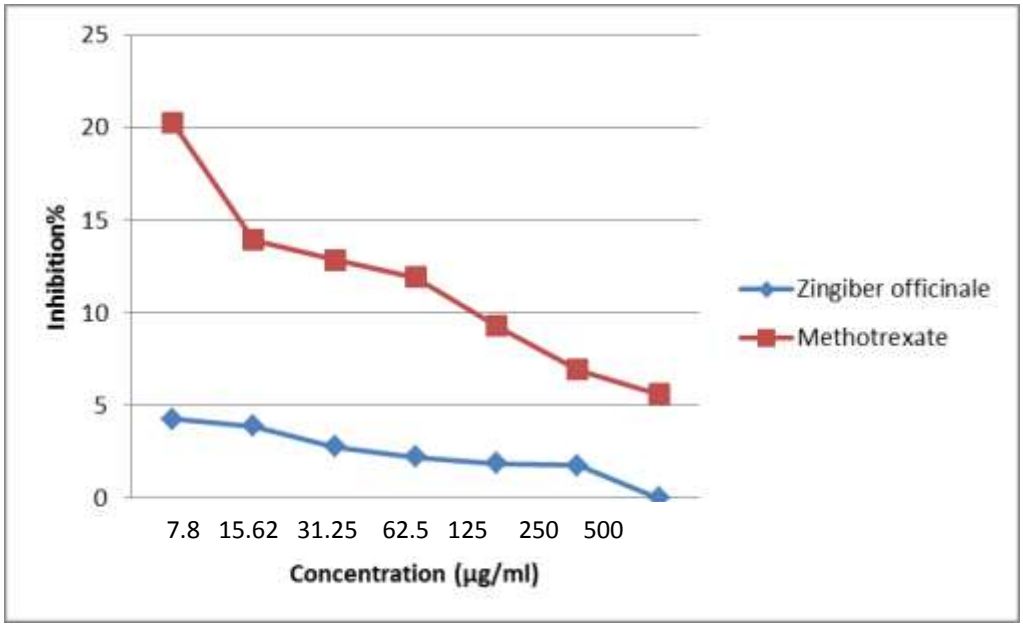

Figure (1-3): Comparison between inhibition% of different concentrations of MTX and *Zingiber officinale* extract on HEK293 cell line after 48 hours of exposure.

The Figure (1-4) showed the comparison between inhibition% of different seven concentrations (starting from 500 µg/ml until 0.976 µg/ml) of *Zingiber officinale* extract on 4TI cell line after 48 hours of exposure and these concentrations were the same seven concentrations of methotrexate to compare between these two agents on the same two cell lines.

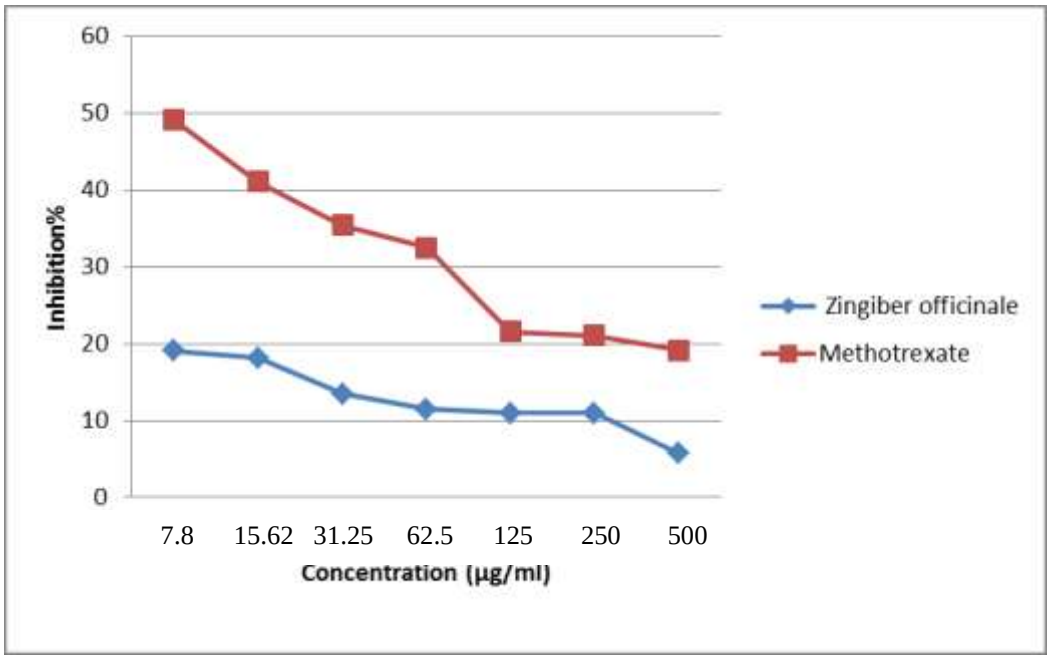

Figure (1-4): Comparison between inhibition% of different concentrations of MTX and *Zingiber officinale* extract on 4TI cell line after 48 hours of exposure.

The Table (1-3) showed the there is an increase in optical density of the effect of combination of Methotrexate with *Zingiber officinale* extract on 4TI cell line after 48 hour of exposure along with decreasing the concentration of both *Zingiber officinale* extract and Methotrexate ,whereas the inhibitory effect of the combination is decreased along with decreasing the concentration of both agent.

The Figure (1-4) showed the comparison of inhibition% of different concentrations of methotrexate, *Zingiber officinale* extract and their combination on 4TI cell line after 48 hours of exposure in Figure (1-5), revealed that the combination of methotrexate with *Zingiber officinale* extract increase the inhibitory effect of both agents.

Table (1-3): The inhibition% of *Z. officinale* plus Methotrexate on 4TI cell line

concentration of Zingiber officinal extact(µg/ml)	MTX	Zingiber officinale + Methotrexat	
		Optical density ± SD	Inhibition%± SD
4000	500	0.18 ± 0.001	58.14 ± 0.23
2000	250	0.195 ± 0.003	54.66 ± 0.69
1000	125	0.2 ± 0.006	53.49 ± 1.21
500	62.5	0.21 ± 0.002	52.32 ± 0.89
250	31.25	0.215 ± 0.003	51.17 ± 0.46
125	15.625	0.270 ± 0.005	50.00 ± 0.59
62.5	7.812	0.28 ± 0.004	37.91 ± 1.38
31.25	3.906	0.302 ± 0.001	34.89 ± 1.02
15.62	1.952	0.333 ± 0.005	25.59 ± 0.25
7.81	0.976	0.54 ± 0.002	22.56 ± 1.37

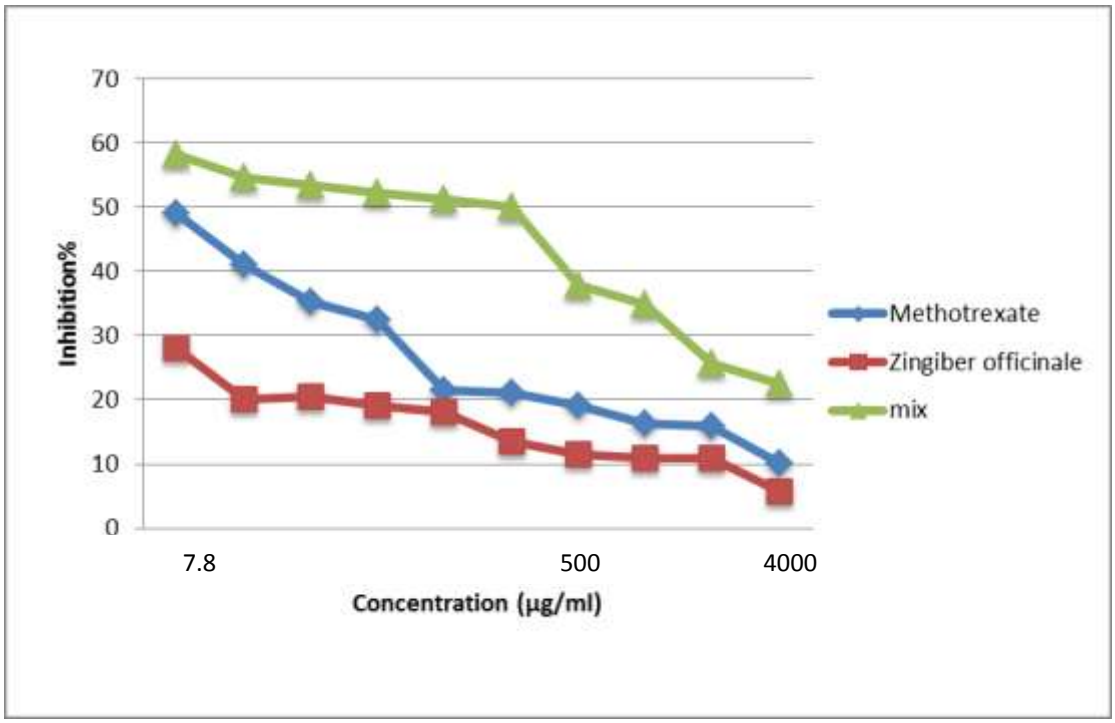

Figure (1-5): Comparison among the inhibition% of different concentration of MTX, *Z. officinale* and MTX plus *Z. officinale* extract on 4TI cell line after 48 hours of exposure.

The Table (1-4) resulted that the addition of *Z. officinale* extract increases the inhibitory effect of Methotrexte as well as the addition of insulin on 4TI after 48 hour of exposure.

Table (1-4): Comparison between inhibition% of MTX, MTX + Insulin, *Z. officinal* extracts and MTX +*Z. officinale* extract on4TI cell line after 48 hours of exposure

Agent	concentration(µg/ml)	inhibition%
MTX	500	49.05 ± 0.31
MTX+ insulin	500+ 1 IU	59.11 ± 4.45
Z. officinale extract	500	19.05 ± 3.21
MTX+*Z. officinale* extract	500+4000	58.14 ± 0.23

Discussion:

The extract of *Zingiber officinal*e plant in this study posses many of effective compounds that exhibit cancer preventive activity experimental carcinogenesis, with the relative differences between them. And these compound include potent antioxidant, alkaloids, monoterpene, phenols, and flavonoid such as [6] gingerol and [6]-paradol, as well as some other constituents like shogaols, zingerone [12]. To ensure the use of *Zingiber officinale* be safe in this study we test the toxicity of the extract in vitro by using normal cell line prior to the cancer cell lines. The test of safety in this study was carried out by using HEK293 cell line which is normal cell line and the result was positive thus the crud extract of *Zingiber officinale* considered safe when use as treatment. The cancer cell line was selected in this study, due to that it is available and can be used easily, to find out the antitumor efficiency of extraction, and carried out on 4TI cell line.

In this study the result of inhibition percentage of growth of *Zingiber officinale* extract on 4TI cell line was positive and this may be due to that the extraction contains high concentration of antioxidant compound that scavenge the free radical and reduce the DNA damage [13]. And the results showed that the high concentrations of extract affected the growth of cancer cells compared to different concentrations of low-lying, this variation in the readings as possible be attributed to

the density of major active compounds in the extract of Zingiber *officinale*, especially at concentration prepared from the original stock solution, and for this reason the device gives a high reading. The inhibition that occurred in cancer cells could be due to that the plant extracts contain the chemical compounds are toxic in nature, especially at high concentrations which affect either in the process of polymerization of mitotic spindle and this leads to a reduction in the proportion of division, or proteins that stimulate the process of division[14]. The inhibitory action of *Zingiber officinale* extract on cancer cells may be due to that its rhizomes are very rich in main flavonoid and terpenes which have inhibitory action outside the body of the organism in the cancer cell [15], the major oils, flavonoid and bilateral terpenes have the ability to scavenge the free radicals and therefore considerer effective antioxidant [16].

The phenols and aromatic terpenes have toxic and strong effect on some types of cancer cells such as Islet cell carcinoma and NcIs 60 - human cell line [16]. And because the rhizome of *Zingiber officinale* contain high proportion of aromatic terpenes very, so it can be argued that it can be a factor in anti-cancer. It is possible to say that the inhibitory activity of *Zingiber officinal* extract may be due to that it has certain compounds work to modify or disable the expression of tumor genes and affect the products of protien. The major active compounds in *Zingiber officinal* also affect the cell cycle by accelerating the crossing of the G2 + M phase, as the cells in this phase can correct the damage that occurs in the DNA , thus the cells cannot repair the damage from a toxic reaction and this called cellular cytotoxicity effects[17].

References:

1- **Jemal, A.; siegel,R.; Ward, E.; Murray, T.; Xu, J. and Thun, M.J.(2007).**Cancer statistics. *Cancer J. clin., 57: 43-66.*

2- **Bourgaize,D.;Jewell,T.andBuiser,R.(2000).**Biotechnology,demytifing theconcepts,(4th ed.)McGraw Hill Press. New York, PP: 313-335.

3- **Leopold, L.; Berger, M.; Cheng, S.; Giles, E. and Estey, E. (2003).**Comparative efficacy and safety of Gemtuzamab Ozogamicin monotherapyand high-dose cytarabine combination therapy in patients with acutemyeloid leukemia in first relapse. *Clin. Adv. Hematol. Oncol., 14: 220-5.*

4- **Lopez-Lazaro, M. (2002).** Flavonoids as anticancer agents: Structure-activityrelation ship. *Curr. Med. Chem. Anti-cancer Agents, 2: 691-714.*

5- **De-vita, V.T.(1997).** Principle of cancer Management chemotherapy.In Devita,V.T.,Helman,S., and

Rosenberg,S.A.(eds) cancer: principles and practice of oncology. Lippincott-Ravin 5It ed.

6- -Maher, K. E. (2000). Principles of radiation therapy. In: B. M. Neuidjon andK. W. sowers(ed.). A nurse's guide to cancer care. Lippincott,Philadelphia.pp 215.

***7*- Nakamura , H . and Yamamoto ,T.(1982).**Mutagen and anti mutagen in ginger (*Zingiber officinale*). Res.; 103:119-135.

***8*- Katiyar ,S.K.;Agarwal .R.and Mukhtar . H. (1996).** Inhibition of tumor promotion in sencar mouse skin by ethanol extract of ginger .Cancer Res.;56:1023-1053.

9- Ali, B. ; Blunden ,G.;Tanira, M.andNemmar,A.(2008). Somephyt ochemical,Pharmacological Roscoe .Review research. Oman;46(2):409-229**.**

10- Freshney, R.I. (1994). Culture of animal cells. A manual of basic technique. New York. pp. 440.

11- Chiang, W. Chang, MY. and Lin, CC. (2003). *In vitro* cytotoxic antiviral and immunomodulatory effects of *Plantago major* and *Plantago asiatica*. American Journal of Chinese Medicine. 31(2): 225-234.

12- Bisset, N.G.(1994). Herbal drugs and phytopharmaceuticals: Ahandbook for practice on ascientific basis .Boca Raton ,F.L.:CRC press.

13- Watson, R.R. (2001). Vegetables, fruits and herbs in health promotion .CRC press .Chapter (12).pp.180.

14- Shiraishi, Y. (1978). Chromosome aberration induced germ cells of mice mutate.Res.57:313-324.

15- Ulubelon , A.; Topc , G. Chai ,H.B. and Pezzuto,J.M. (1999). Cytotoxic activity of diterpenoids isolated from saliva hyporgeia pharmaceutical. Biology.37:148-51.

16-Dennis, R.A .; Adraina, B.;darocha, Gilberdo schwats-mann (2000). Anti-cancer drug discovery and development in Brazil:Targeted plant collection as rational strategy to Aequire candidate anticancer compounds The oncologist 5:185-198.

17-Ross, W.; Rowe, T.,Glisson,;Yalowich, J.; Lin, L. (1984): Role of topoisomerase11 in mediating epipodo phylotoxin-induced DNA cleavage. Cancer Res. 44:5857-5860.