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Inhibition the Growth of Some Malignant Cells Lines by Alfalfa Aqueous Extract

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Abstract :

The aim of the present study was to assess the cytotoxic activity of aqueous extracts of Alfalfa (Medicago sativa) by using (MTT) (colorimetric assay) to measure the cytotoxic activity on two malignant tumor cell lines (AMN3) Ahmed Mohamed Nahi 2003 and (GB) Glioblastoma cell line ,and one type of normal cell line (MEF) Mouse Embryo Fibroblast cell line, through different exposure time (24, 48 ,and 72hrs) and different concentration of aqueous extract ; (1000,500,250,125 ,and 62.5, mg/ml), the study was carried out in the Iraqi Center for Cancer and Medical Genetics Research ,The result showed that the aqueous extracts from alfalfa have significant effects P value ≤ 0.05 on the growth of AMN3 cell lines and GB malignant cell line in culture in dose and time- dependant manner ,Viability of cell lines decreased with time and concentration reaching its lowest value after 72 hrs of treatment with the highest concentration used (1000 mg/ml). The results also indicated that GB cell lines were more sensitive to the Alfalfa aqueous extracts as compared with the growth of AMN3 cell lines .

From the other side the result showed that there is no significant effect P value ≥ 0.05 of these aqueous extracts on the viability of normal cell line (MEF3 cell line). Only the treatment with higher concentration (1000µg/ml) after 72 hrs caused significant reduction P value ≤ 0.05 in the (MEF) cell line growth.

Key Words:

alfalfa, aqueous extracts .anticytotoxic activity ,Mtt assay.

Introduction:

Al-jatt (Medicago Sativa) is a perennial flowering plant native to Iraq which lives from five to twelve years, it is also known as Lucerne, and Trefoil. Alfalfa is now grown throughout the world as forage for cattle but in sprouting form, it has many nutritional benefits to humans. The name “Alfalfa” is derived from the Arabic “al-fac-facah” which means “father of all foods (1). Alfalfa has been employed as a herbal medicine for at least 1500 years. And has been used for thousand of year in many parts of the world as a source of food for people and livestock and as a medicinal herb ,Alfalfa has been used by the Chinese since the sixth century to treat kidney stones cancer, bladder and prostate discords, arthritis, urinary tract infections, antidiabetic activity, for treatment of dyspepsia, and as an anti-asthmatic (2).

Alfalfa sprouts contain concentrated amounts of phytochemicals which can protect us against disease such as pancreatic, colon and leukaemia cancers, osteoporosis, fibrocystic breasts tumors, high cholesterol, arteriosclerosis and cardiovascular disease and symptoms associated with PMT and the menopause (3). Alfalfa contain hundreds of biological active compounds make it difficult to analyze, Alfalfa contained many important substance including several saponin,, many sterols ,alkaloid falvonoids, amino acids ,carotene .and tannin. The primary properties of Alfalfa are considered to be antitumor, antibacterial,intinflammatory,diuretic,stomac h tonic, cleans the liver and blood stream(4). Several recent studies reported the ability of alfalfa extracts to induce cell cycle arrest in mammalian cells, the mode of action in target cell appears to involve induction of apoptosis by mitochondrial perturbation (5) ,and able to alter membrane activity and have strong haemolytic activity(6). Studies have shown that Alfalfa extracts have antitumor and anti-mutagenic activities and can lower the risk of human cancers, by preventing cancer cells from growing. Alfalfa seem to react with the cholesterol rich membranes of cancer cells, thereby limiting their growth and viability (7).

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Methods and materials:

Preparation and extraction:

Fresh plant was obtained from the Garden of Baghdad University during June, 2008. Representative specimens were taken to the Baghdad university Herbarium, where

they were identified it as medicago sativa L. of the family Leguminosae . Fresh plant leaves were separated and placed in the shade inside a well-ventilated room in biochemical engineering laboratory, alkhwarizimi college as described by (8). Distilled water was added to powdered plant in a ratio of (1:5W/V) and boiled with continuous mixing for 10 minutes. Mixed for 15-20 Minutes away from heat. The mixture was then filtered through a piece of soft cloth and filter paper to remove all the residual materials. Further separation was done by centrifugation at 3000 rpm for 10 min to obtain clear solution of the extract. Then, it was dried at 45˚C by using hot air oven, with circulatory fan, and kept at 4˚C until the use. .Serial dilutions of aqueous crude extracts (1000, 500, 250, 125. and 62.5, mg/ml) were prepared in serum free media for cytotoxicity assay.

Chemical Tests:

Several Chemical Tests of general constitute were carried out on the aqueous extracts of alfalfa; Dragendorff Test for Alkaloids , Myer's Test for Tannins, Liebermann-Burchard Test for Triterpenoids ,Test for Flavonoids,Test for Saponins and Test for Glycosides .(9,10) .

Cell under investigate:

The cell lines under investigation were (AMN3) murine mammary adenocarcinoma cell line
Passage 48-51, (GB) -Glioblastoma-Multiforme- cell line Passage 10, and normal mouse Embryo fibroblast (MEF3) cell line Passage 4, kindly provided by the Iraqi Center for Cancer and Medical Genetics Research. These cell lines were grown and maintained using RPMI- 1640 supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) in 25 cc tissue culture flasks. Cells were incubated at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO2. Afterwards, 200 µl of cells in growth medium were added to each well of a sterile 96-well microtitrplate. The plates were sealed with a self adhesive film -and placed in an incubator at 37°C when the cells are in exponential growth at near confluence, the medium was removed and serial dilutions of alfalfa crude extracts in a serum free media (, 62.5, 125, 250, 500, and 1000 mg/ml) were added to the wells. Five replicates were used for each concentration of either extract. The middle two columns were used as a control (cells treated with SFM only). Afterwards, the plates were re-incubated at 37°C for the selected exposure times (24, 48 and 72 hrs).

MTT Assay:

Cell proliferation (Viability) was evaluated by MTT assay, After MTT addition [0,5 mg/mL], the plates were covered and returned to the 37C incubator for 2 hrs, the opti¬mal time for formazan product formation , follow¬ing incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml.(DMSO) DimethylSulfoxide, and the absorbance was measured at 570 nm in spectrometer. The OD750 of the DMSO solution in each well was considered to be proportional to the number of cells. The OD750nm, of the control (Treatment without supplement) was considered to be 100% (11).

Statistical Analysis:

Experimental data were analyzed using statistical software SPSS 10.0 for Windows. Significance between control and samples was determined using Student's t-test. P value ≤ 0.05 was considered statistically significant.

Results:

Extraction:

The aqueous extraction of alfalfa by using hot water has gave a Light brown color sticky extract with a strong odor, and strong foam which gives a dark-brown powder upon drying.

Chemical Tests:

Table (1) show results of the chemical tests for the general Constituents of the aqueous extracts of Alfalfa that's contained tannins, flavonoids, triterpenoids, saponins , glycosides and alkaloids.

Table (1) Phytochemicals detected in the water crude extracts of Alfalfa

Phytochemicals to Be Detected	aqueous Extract
Alkaloids	+
Tannins	+
Flavonoids	+
Triterpenoids	+
Saponins	+
Glycosides	+

+: The extract contains the designated phytochemical.
- : The extract does not contain the designated phytochemical.

Effect on (AMN3) murine mammary adenocarcinoma Cell Line:

The effects of treating (AMN3) cells with the aqueous extract of alfalfa are shown in Fig. (1) Alfalfa aqueous crude extract was showed dose and time dependent significant inhibitory effect on viability of (AMN3) cells lines P value ≤ 0.05. Viability decreased with time and concentration reaching its lowest value after 72 hrs of treatment with the highest concentration used (1000 mg/ml). The lowest percentage of cell viability was reached 20.43. %. This reflected a percentage of Inhibition of 79.57 %. while all the other concentrations of alfalfa aqueous crude extract caused no significant change (P value $\geq$ 0.05) in the cell viability after 24 , 48 and 72 hrs of treatment , as shown in fig(1).

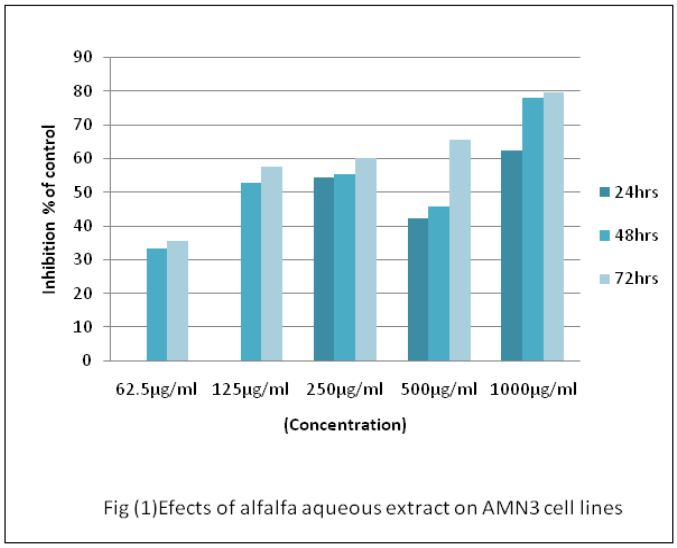

Fig (1)Efects of alfalfa aqueous extract on AMN3 cell lines

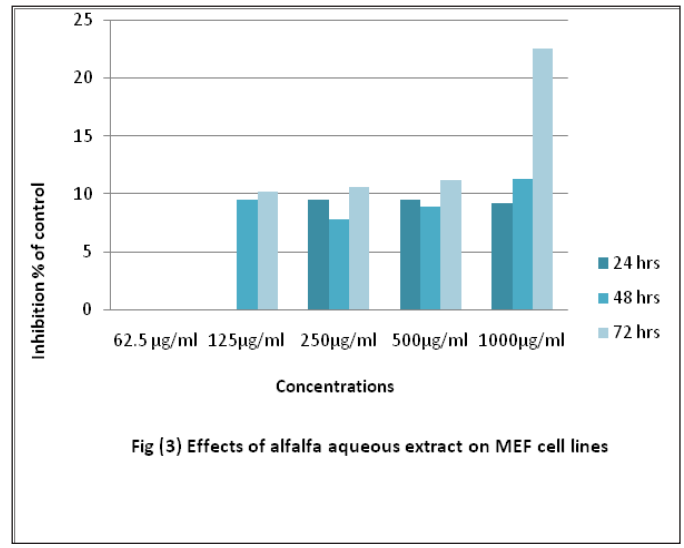

Fig (3) Effects of alfalfa aqueous extract on MEF cell lines

Effect on (GB) cell line Cell Line:

The Result of treating (GB) cell line with the aqueous extract of alfalfa was presented in Fig. (2). Cell viability gradually reduced with time and concentration reached its lowest percentage after 72 hrs of exposure with the highest concentration 1000 mg/ml, which was 14.68 %. This reflected a percentage of inhibition 85.32% (P value $\leq$ 0.05), while all other concentrations showed no significant inhibitory effects P value $\geq$ 0.05 on (GB) cells during the 24, 48, and 72-hrs of exposure period.

Effect on mouse Embryo fibroblasts (MEF) cell line:

All concentrations of alfalfa aqueous extracts had showed no significant effects on the growth of MEF cell line P Value $\geq$ 0.05. Only the treatment with the highest

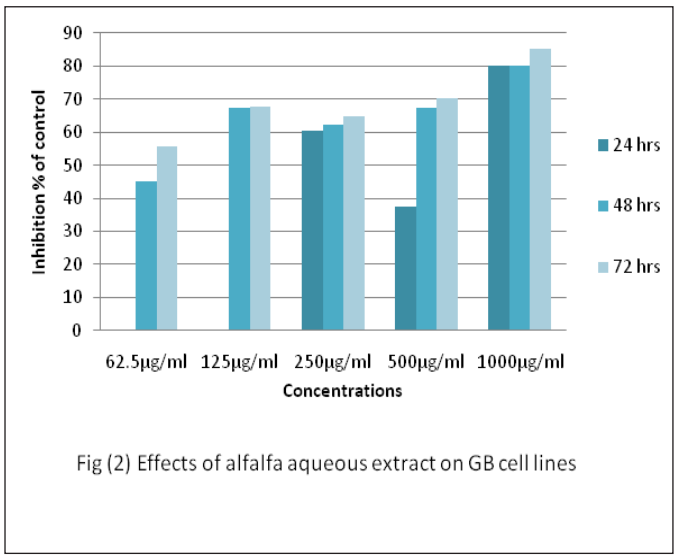

Fig (2) Effects of alfalfa aqueous extract on GB cell lines

concentration 1000µg/ml after 72 hrs caused significant reductions (P value $\leq$ 0.05) in cell growth. Highest reduction came due to treatment with a high concentration and gave 77.55 growth, this reflected percentages of inhibition 22.45%, fig (3).

The effects of alfalfa aqueous extracts treatment on the cells line that have been used in this study with different exposure times and different concentrations could be summarized in table (2).

Table (2) .The effects of alfalfa aqueous extracts on different cell lines treated With different concentrations and different exposure times.

Cell lines	Con mg/ml		% Inhibition	
		24hrs	48hrs	72hrs
AMN3	62.5	-	33.36	35.4
	125	-	52.76	57.53
	250	54.33	55.27	60.14
	500	42.34	45.62	65.62
	1000	62.35	78.14	79.57
GB	62.5	-	45.24	55.84
	125	-	67.31	67.65
	250	60.36	62.32	65.00
	500	37.4	67.44	70.45
	1000	80.22	80.28	85.32
MEF3	62.5	-	-	-
	125	-	9.5	10.21
	250	9.55	7.77	10.55
	500	9.51	8.86	11.16
	1000	9.25	11.25	22.45

Discussion:

Herbal extracts contain different phytochemicals with biological activity that can provide therapeutic effects. In this study, crude extracts were used because this is merely a Preliminary study of whether or not this plant species has an effect on cancer Cells. In addition, biological activity, if proven to exist, might be lost during the process of purification from the crude extract (12). The Preliminary chemical test on Alfalfa aqueous extract detected that this extract contains several phytochemicals such as Alkaloids ,Tannins, Flavonoids ,Triterpenoids ,Saponins and Glycosides and these phytochemicals with antitumor

or cytotoxic activity to cancer cells or to immortalized cells, but are less toxic to normal cells (13) , furthermore These active compounds in these extracts might have some specificity in their toxicity towards cancer cells rather than normal cells(14) . The aqueous extracts of Alfalfa showed cytotoxic activity against AMN3 and GB cell line ,viability decreased with time and concentration reaching its lowest value after 72 hrs of treatment with all concentrations used. While all the concentrations of alfalfa aqueous extracts caused no significant change in viability of MEF3 cell line after 72 hrs of treatment P value $\geq$ 0.05. Except the highest concentration which caused significant reduction P value $\leq$ 0.05 in MEF3 cell line viability in comparison with the control as illustrated in table (1).

The cytotoxic effect may due to the presence of active components in the aqueous extract (Alkaloids ,Tannins, Flavonoids ,Triterpenoids ,Saponins and Glycosid) and these compound play a major role in arrest cell cycle at G1phase and stimulation of apoptosis at G0/G1 phase in mammalian cells (15). .and inhibited DNA topoisomerase I activity resulting in the cleavage of DNA and thus cytotoxicity towards cell culture of human mammary carcinoma.(16) .These compounds also have the ability to induce apoptosis in tumor cells of GB and AMN-3 ,beside that these compound are consider as an antioxidant scavenger and can reduce occurrence of reactive e oxygen species such as H2O2 and that might be the reason for the prevention of biomolecules damage (17) .

In spite of (GB) Glioblastoma is usually rapidly fatal and the most common and aggressive type of malignant glioma (18).but this malignant cell line as more sensitive to the Alfalfa aqueous extract than AMN3 cell line and this perhaps due to the physiological properties of each type of malignant cell line (19). From another side, all concentrations of alfalfa aqueous extract had no significant effects on the growth of MEF3 cell lines P value $\geq$ 0.05. But only the treatment with the highest concentration reduces the growth of MEF3 Cell lines that used in the study. This could be considered as further indication of the relative safety of aqueous extracts of Alfalfa towards normal cells.

Several studies have demonstrated that some plant extracts have been shown to be safe for normal cells. (20). The variation in cell viability between AMN3 and GB cell lines when they were treated with alfalfa aqueous extract was due to the difference in the receptors on the cell surface. In addition the biological differentiation properties between these cells was different (21). However, we hypothesized in more clarity prevention of entry of alfalfa aqueous extracts into normal cells, though it could be due to the difference in membrane properties between cancer and normal cells. And this extract may be posses some specificity in cytotoxcicity on cancer cells rather than normal cells because cancer cell have different membrane structure, with more cholesterol like compounds, and alfalfa extracts have a natural affinity for cancer cell membranes (22).

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دراسة تأثير المستخلص المائي لنبات الجت في تثبيط نمو بعض الخطوط الخلوية السرطانية

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الخلاصة:

يمثل هذا البحث المحاوله الأولى في البلد لتقييم تأثير الفعاليه السميه للمستخلص المائي الخام والمعزول من نبات الجت العراقي صنف Medicago sativa بأستخدام الطريقة اللونية (M.T.T) في اثنين من الخطوط السرطانية هما خط خلايا سرطا ن الغد ة اللبنية لأناث الفئران Ahmed mo-hamed nahi 2003) (AMN3) وخط خلايا سرطان الاورمة الدبقية البشري GB) Giloblastoma Multiforme) وخط واحد من الخلايا الطبيعية وهو الخط الطبيعي لجنين الفأر مولده الألياف Mouse embryo fibroblast(MEF) وبخمسة تراكيز مختلفة,وهي على التوالي (1000, 500 , 250 ,125, 62.5, مايكروغرام/ مل) وضمن مدد تعريض مختلفة (24 ,48, 72) ساعة , اضهرت النتائج وجود تأثير سمي و بمستوى معنوية واضح P value ≤ 0.05 لذلك المستخلص في نمو خطوط الخلايا السرطانية ,وخلال المدد الثلاثة من التعريض وكانت شدة السمية تزداد بزيادة التركيز ومدة التعرض ,كما اشارت النتائج الى ان خطوط الخلايا السرطانية (GB)كانت اكثر حساسية للمستخلص المائي الخام للجت عند المقارنة مع خطوط الخلابا السرطانية (AMN3) .في حين لم يكن هناك تأثير واضح وذو معنوية P value ≥0.05 لنفس المستخلص المائي الخام لنبات ا لجت في نمو خطوط الخلايا الطبيعية (EF M) فقط عند اعلى تركيز للمستخلص 1000 مايكروغرام /مل وعند اكبر فترة حضن والبالغة 72 ساعة ,لذا فأن هذه الدراسة كشفت عن امكانية استخدام المستخلص المائي الخام والمعزول من نبات الجت مستقبلا في التخصص في التأثير في نمو الخلايا السرطانية دون الطبيعية .