

EFFECT OF CRUDE SAPONIN EXTRACTED FROM ALFALFA (*Medicago Sativa L*) ON NEOPLASTIC AND NORMAL CELL LINES

Rafid Abdul Wahed A.

Department of Biochemical Engineering, Al-Khwarizimi Collage Engineering,
Baghdad University.

Corresponding author: Rafid Abdulwahid, fax +9647901473844.

Email: rafid_sigma@yahoo.com.

Abstract

Aim of the present investigation was to assess by using (M.T.T) assay a (colorimetric assay) to measure the cytotoxic activity of crude saponin extracted from alfalfa *medicago sativa L*, Al-jjat, on two malignant tumor cell line (Rhabdomyosarcoma (RD) and Human epidermoid larynx carcinoma (HEP-2) cell line, and one type normal cell line Rat Embryo Fibroblast (R EF) cell line. through different exposure time (24, 48, and 72 hrs) and different concentration of alfalfa crude saponin extract (1000, 500, 250, 125, 62.5, and 31 mg/ml).

The Result showed that the crude saponin extracted from alfalfa have significant effects P value ≤ 0.05 on the Growth of the two malignant cell line (RD and HEP-2 cell line) in culture in dose and time- dependant manner. Viability of cell lines decreased with time and concentration reaching its lowest after 72 hrs of treatment with the highest concentration used (1000 mg/ml).

In addition, the growth of (RD) cell line was more sensitive to the alfalfa crude saponin in comparison with the growth of (HEP-2) cell line. From the other side the result showed that there is no significant effect P value ≥ 0.05 at these crude extract on the viability of normal cell line (MEF cell line). Only the Treatment with higher concentration (1000 μ g/ml) after 72 hrs caused significant reduction P value ≤ 0.05 in the (MEF) cell line growth.

Key word: Alfalfa, Saponin. Anticytotoxic Activity, Mtt Assay.

Introduction

Medicinal herbs have been widely used for treatment of diseases in traditional way for several generations and the interaction between traditional medicine and modern biotechnological tools is to be established towards new drug development. Although alfalfa is a food crop for animals, it also employed as an herbal medicine for at least 1500 years. Alfalfa used for thousands of years in many parts of the world, as a medicinal herb. The name "Alfalfa" is derived from the Arabic "al-fac-facah" which means "father of all foods". Alfalfa or jatt (*Medicago Sativa L*) is a nutritive herb from *legminacea* family Native to southwest Asia, Iraq and Iran (1). The primary properties of Alfalfa are considered to be anticancer, anti-inflammatory, diuretic, nutritive, stomach tonic, Historic medicinal uses of alfalfa include treatment of cancer, stomach upset, arthritis, bladder and kidney problems Alfalfa Contain saponin, Betacarotene, Bioflavinoids, Calcium, Carotene Chlorophyll, Copper, Folic acid, Iron, Magnesium, Phosphorous, Potassium, Protein, Silicon, Sodium, Vitamins (2).

Recent studies showed that twenty-four saponins have been identified in alfalfa, including: 13 medicagenic acids, 2 zanhic acids, 4 hederagenins, 1 soyasapogenol A, 2 soyasapogenol B's, 1 soyasapogenol E, and 1 bayogenin glycoside (3). Saponins are steroid or triterpenoid glycosides, common in a large number of plants and plant products that are important in human and animal health. Alfalfa saponin have shown anti tumor, antimutagenic and cytotoxic activities and can lower the risk of human cancer by preventing cancer cells from growing. (4), Saponin have the unique ability to stimulate the cell-mediated immune system, as well as to enhance antibody production (5). Various in vitro studies demonstrated that saponin from alfalfa can inhibit the growth of gram positive and gram negative bacteria by leakage the membrane (6).

Saponins also have shown to be capable of deactivating HIV-1 and Herps virus (7). saponin extracted from alfalfa have shown to be antifungal and have some use in fighting fungi. It has been suggested that alfalfa

saponin causes damage to the cell membranes of fungi (8).

Material and Methods

Preparation and Extraction of Saponin:

Fresh plant was obtained from the Garden of Baghdad University during May, 2007. 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 as described by Harborne(1984). The modern methods available for the separation and analysis of saponins was well reviewed by Marston (9). There were several strategies available for the extracting saponins. As a general rule, they begin with the extraction of the plant material with aqueous methanol or ethanol. Further processing of the extract was carried out after evaporation under reduced pressure, dissolution in a small amount of water and phase separation into n-butanol. Serial dilutions of saponin crude extracts (1000, 500, 250, 125, 62.5, and 31.2 mg/ml) were prepared in serum free media for cytotoxicity assay.

Cell Under Investigate:

The cell lines under investigation were Human larynx epidermoid carcinoma (HEp-2) cell line, Rhabdomyosarcoma (RD) cell line and Rat Embryo fibroblasts (REF3) cell line. This cell line were grown in 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 saponin crude extracts in Serum Free Media (31.2, 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 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, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-di Methyl Thiazol-2-yl) 2,5-diphenyl Tetra-zolium bromide] (MTT) to a blue formazan crystal by mitochondria! succinate dehydrogenase activity of viable cells. This test is a good index of mitochondria! Activity and, thus, of cell viability. After MTT addition [0,5 mg/mL], the plates were covered and returned to the 37 C incubator for 2 h, the optimal time for formazan product formation, following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml. (DMSO) Dimethyl Sulfoxide, 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% (10).

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

Effect on Human Larynx Epidermoid Carcinoma Cell Line:

The effects of treating HEp-2 cells with alfalfa saponin crude extract was illustrated in Fig.(1). The alfalfa saponin crude extract showed dose and time dependent significant inhibitory effect on viability of HEp-2 cells P value ≤ 0.05. Viability decreased with time and concentration reaching its lowest after 72 hrs of treatment with the high concentration used (1000 mg/ml). The lowest percentage of cell viability reaching 16.6 %. This reflected a percentage of Inhibition of 83.4%. While all the other concentrations of alfalfa saponin crude extract caused no significant change (P value ≥ 0.05) in the cell viability after 24, 48 and 72 hrs. of treatment.

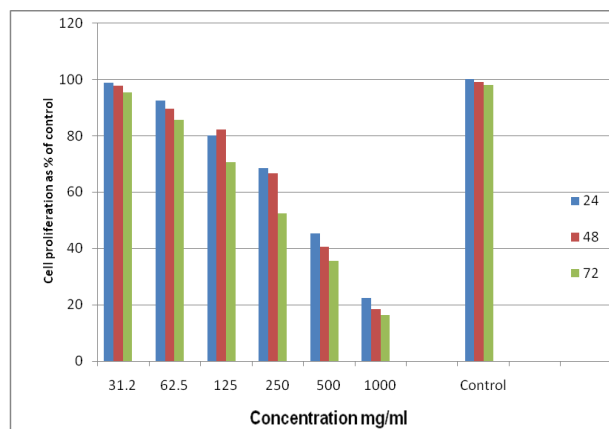


Fig.(1): The effects of treating HEP-2 cells with alfalfa saponin crude extracts.

Effect on Rhabdomyosarcoma Cell Line:

The Result of treating RD cell line with the saponin crude extract of alfalfa (*Medicago sativa L*) was presented in Fig.(2). Cell viability gradually reduced with time and Concentration reached its lowest percentage after 72 hrs of exposure with high concentration 1000 mg/ml, which was 9.3%. This reflected a percentage of inhibition 90.7% (P value ≤ 0.05), while all other concentrations showed no significant inhibitory effects P value ≥ 0.05 on RD cells during the 24, 48, and 72-hour of exposure period. Beside that RD cell line was more sensitive to the alfalfa saponin crude extracts in Comparison with the viability of HEP-2 cell line.

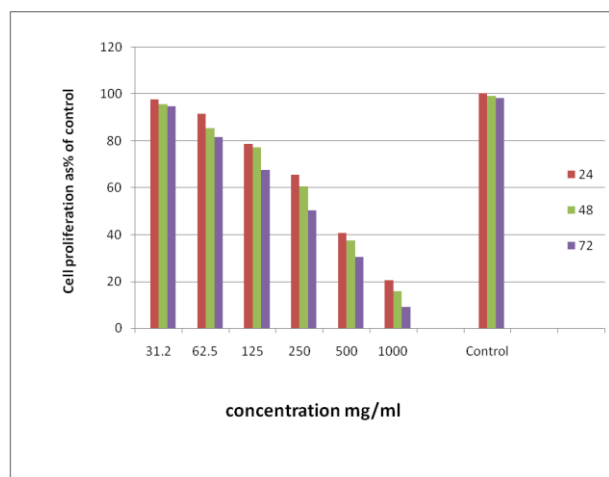


Fig.(2): The effect of treating RD cell line with saponin crude extract.

Effect on Rat Embryo fibroblasts Cell Line:

The effect of alfalfa saponin crude extracts on REF3 cell line was demonstrated in (Fig.3), all concentrations of alfalfa saponin crude

extracts had no significant effects on the growth of REF cell line (Pvalue ≥ 0.05). Only the treatment with highest concentration 1000 $\mu\text{g/ml}$ after 72 hrs. caused significant reductions (P value ≤ 0.05) in cell growth. Highest reduction came due to treatment with the high concentration and gave 85.5 growth, this reflected percentages of inhibition 14.5.

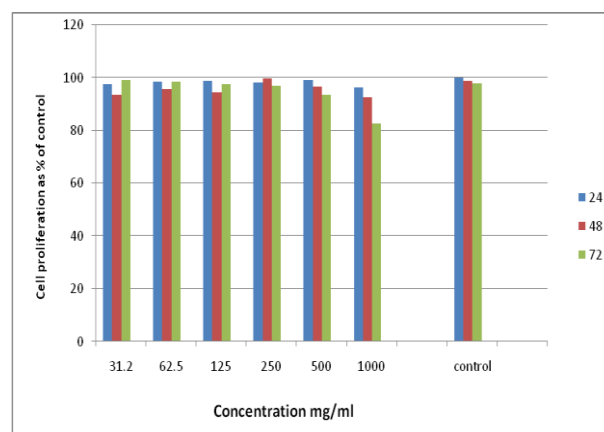


Fig.(3): The Effect of alfalfa saponin crude extracts on REF3 cell line.

The effects of alfalfa crude saponin treatment on the cell line that used in this study in different exposure time and different concentration could be summarized in table (1). Hep-2 and Rd cell line viability decreased with time and concentration reaching its lowest after 72 hrs of treatment with all concentrations used. While all the concentrations of alfalfa saponin extract caused no significant change in viability of REF cell line after 72 hrs of treatment P value ≥ 0.05 . Except the highest concentration which caused significant reduction P value ≤ 0.05 in Ref cell line viability in comparison with the control as illustrated in table (1).

Table (1)
The effects of alfalfa crude saponin on different cell lines treated with different concentration and different exposure time.

Cell lines	Con. (mg/ml)	% Inhibition		
		24 hrs.	48 hrs.	72 hrs.
HEP-2	31.2	98.7	97.5	95.3
	62.5	92.4	89.6	85.4
	125	80	80	70.5
	250	68.5	66.7	52.4
	500	45.3	40.6	35.6
	1000	22.5	18.4	16.3
RD	31.2	95.6	95.6	94.5
	62.5	85.4	85.4	81.4
	125	77.1	77.1	67.5
	250	60.3	60.3	50.1
	500	37.4	37.4	30.5
	1000	15.8	15.8	9.0
MEF	31.2	97.6	93.7	99.1
	62.5	98.4	95.6	98.7
	125	98.9	94.5	97.6
	250	98.2	99.7	97
	500	99.1	96.6	93.6
	1000	96.5	92.5	82.6

Discussion

There have been several reviews in recent years of published reports about various properties of saponins. Most of them, however, deal with either specific properties or biological activities of saponins (11).

Several phytochemicals were detected in the crude extract prepared from alfalfa such as saponin, triterpenoids, glycosides, flavonoids, isoflavonoid, carotene, tannins, and alkaloid.

Saponin extracted from alfalfa has the ability to induce cell cycle arrest in mammalian cells. Their mode of action in target cells appears to involve induction of apoptosis by mitochondrial perturbation, and alter membrane activity also has strong hemolytic activity (12).

Alfalfa saponin can arrest cell cycle at G1 phase and stimulation of apoptosis at G0/G1 phase and can inhibit the synthesis of DNA and RNA and protein synthesis in a dose and time dependant manner (13).

Alfalfa saponin may select cancer cell, because cancer cells have different membrane structure, with more cholesterol-like compounds, and saponin binds cholesterol, they

have a natural affinity for cancer cell membranes (14).

Saponins reduce occurrence of reactive oxygen species such as H₂O₂ by enhancing its breakdown by activation of peroxiredoxins and catalase, and glutathione peroxidase as well as by suppressing its production by inhibiting the phosphatidylinositol-3-kinase signaling pathway and that might be the reason for the prevention of chromosomal damage reported in (15).

In conclusion, alfalfa saponin extracts showed time- and dose-dependent inhibitory effects. On HEP-2 and RD cells respectively. Cell viability reached its lowest with extended exposure to time and dose beside that RD cell was more sensitive than Hep-2 cell to the saponin crude extracts.

Effects on Rat Embryo Fibroblasts Cell Line:

Rat embryo fibroblasts were used in this study to examine whether the extracts of alfalfa used in this study had adverse effects on normal cells. Compared with malignant cell lines, normal cell line of mouse embryo fibroblasts showed little reduction in viability after 72 hrs. of exposure to high concentration of alfalfa saponin crude extracts. This could be indicative of the safety of alfalfa extracts against normal cells by selectively effecting malignant cells.

A large number of the biological effects of saponins have been ascribed to their action on membranes. In fact their specific ability to form pores in membranes has contributed to their common use in physiological research (16).

The hemolytic action of saponins is believed to be the result of the affinity of the aglycone moiety for membrane sterols, particularly cholesterol, and cancer cells have different membrane structure with more cholesterol-like compounds, and saponin binds cholesterol they have a natural affinity for cancer cell membranes, with which they form insoluble complexes. The amount of glycosides required for permeabilisation is much lower for cholesterol-rich lipid layers than cholesterol-free membranes (17).

The variation in cell viability between Hep-2 and Rd cell line when they were treated with

alfalfa saponin extracts was due to the difference in the receptors on the cell surface. In addition to the biological and differentiation different between these cells (18).

However, we hypothesized in more clarity prevention of entry of alfalfa crude saponin into normal cells, though it could be due to the difference in membrane properties between cancer and normal cells. And may be that the alfalfa saponin crud extract posses some specificity in cytotoxicity on cancer cells rather than normal cells.

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الخلاصة

يمثل هذا البحث المحاولة الأولى في البلد لتقييم تأثير الفعالية السمية لمستخلص الصابونين الخام المستخلص من نبات الجت *Alfalfa* صنف *Medicago sativa* باستخدام الطريقة اللونية (M.T.T) في اثنين من خطوط السرطانية هما خط خلايا سرطان ن لحنجرة البشري (Human epidermoid larynx carcinoma HEP-2 cell) وخط خلايا سرطان العضلات المخططة البشري Rhabdomyosarcoma (RD) وخط واحد من الخلايا الطبيعية وهو الخط الطبيعي لجنين الجرذ مولده الألياف Rat embryo fibroblast(RE) وبسنة تراكيز مختلفة (1000,500,250,125,62.5,31.25 مايكروغرام/ مل) وضمن مدد تعريض مختلفة (72, 24, 48) ساعة، اضررت النتائج وجود تأثير سمي و بمستوى معنوية واضح $P \text{ value} \leq 0.05$ 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 HEP-2 كانت اكثر مقاومة للمستخلص الخام للجت عند المقارنة مع خطوط الخلايا السرطانية RD. في حين لم يكن هناك تأثير واضح وذو معنوية $P \text{ value} \geq 0.05$ لنفس المستخلص الخام للجت في نمو خطوط الخلايا الطبيعية REF فقط عند اعلى تركيز للمستخلص 1000 مايكروغرام/مل وعند اكبر فترة حضن والبالغة 72 ساعة، لذا فمن المحتمل ان يكون لمستخلص الصابونين الخام والمعزول من نبات الجت التخصص في التأثير في نمو الخلايا السرطانية دون الطبيعية.