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Pharmacology

Cytotoxic Effect of *Silybum marianum* on Rhabdomyosarcoma Cell Line

Prof. Faruk H. AL-Jawad[1*], Prof. Shallal M. Hussein[2], Ridha R. Ardawe[3]
[1]Department of Pharmacology, Medical College, AL-Nahrain University Baghdad, Iraq
[2]College of pathological analysis, AL-Bayan University Baghdad, Iraq
[3]Master pharmacist, Medical College, AL-Nahrain University Baghdad, Iraq

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***Corresponding author**
Prof. Faruk H. AL-Jawad

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Abstract: The current study was designed to determine *in vitro* the cytotoxic effect of aqueous and alcoholic extracts of *Silybum marianum (*SM*) on* Rhabdomyosarcoma (RD) cell line and Rat embryo fibroblast (REF) as normal cell compared with pure silymarin. Both aqueous and alcoholic extracts of fruits and stems-leaves of SM were prepared by serial dilution to five concentrations (400, 200, 100, 50, 25) µg/ml. They were added to the cell culture using MTT assay three periods of incubation (24, 48, and 72) hrs. Show the cytotoxic effect of extracts (inhibition rate of growth) was in dose and time dependent. There were significant differences $P<0.05$ between concentration and time exposure. The highest inhibition rate on RD cell line for alcoholic fruit extract was 72.91% in concentration 400µg/ml, the lowest inhibition rate was 49.093% in concentration 25µg/ml for exposure time 48hrwith no effect on REF cell line. The alcoholic and aqueous extract of stem-leaves had less cytotoxic activity on RD cell line. Alcoholic SM extract had high inhibition to growth of RD cell line but with no effect on REF cell line for time of exposure 72hrs compared with silymarin which is less effective and the possibility to use alcoholic extract in cancer therapy.
Keywords: *Silybum marianum*, silymarin, Growth inhibition, MTT, RD cell line.

INTRODUCTION

Cancer is serious disease that predicted to be the second cause of morbidity and mortality and possibility to increase in the next few decades, in the world. Therefore, it is interested to search for newer agents with less toxicity to normal cell and in having possible cytotoxicity like folklore remedy *Silybum marianum*, Milk thistle (which is known locally called klighan).

It is a member of Carduus marianum family that is quite common cultivated in Iraq. It is nontoxic and had been used in liver disease and mushroom poisoning [1] Silymarin is polyphenolic flavonoids extracted from SM. It has antioxidant, antinflammatory, anti-fibrotic, antiproliferative, immunomodulation and antiviral effects [2, 3]. Silymarin is composed from isomers of silychristin, silydinin, silybin A, silybin B, isosilybin A, isosilybin B[4], but silybin in general is more active constituent of silymarin which has angiogenic effect through decrease the expansion HIF, IaTNF, INOS,PECAHM-1,VEGF[5].

All the constituents of silymarin are present in the SM plant after detection with HPLC method but with different proportions Table -1.

The aim of the study is to explore the action of aqueous and alcoholic extract of SM on RD and REF cell line *in vitro* in comparison with pure silymarin.

MATERIALS AND METHODS

Chemicals: Legalon (silymarin extracts) 70 mg tablet, is purchased by madaus Gmbh, Germany. All the reagents, RD and REF cell lines supplied by Iraqi Center for Cancer and Medical Genetic Research.

Silybum marianum was supplied from well-known bureau (Al-Madina) in Baghdad city Using fruits and stems with leaves. They were identified and authenticated by Iraqi National Institute for Herbs. It was cleaned and dried and then powdered with electrical grinder. The powder was stored in dark and air tight glass container at room temperature until to be use.

The weight end of aqueous extract of fruits and stems-leaves was more than alcoholic extract for both fruits and stems-leaves. In general the weight of fruits yielded more than stems-leaves.

The powder of both fruits (Fa) and stems-leave (Sa) were dissolved in 80c^{o} of hot water while the other parts (Fc) and (Sc) were dissolved in 70% alcohol. All these extracts were treated according to madaus procedure [6].

Table -1 Main active constituents detected in hplc for silymarin and other extracts of *silybum marainum* . Fa; aqueous extract of fruits, fc: alcoholic extract of fruits, sa: aqueous Extract of stems and leaves, sc: alcoholic extract of stems and leaves.

Seq	Retention time	Area						
		Silymarin	Fc	Fa	Sc	Sa	Seq.	NAME
1	3.08	1249274	3265373	3216535	3420202	3207811	1	Silychristin
2	3.486	349712	66976	73848	405219	67919	2	Silydianin
3	3.761	902128	470621	464684	672818	457947	3	Silybin A
4	4.236	377653	885938	864879	761619	866344	4	Silybin B
5	5.028	1372196	682989	678582	1000158	679091	5	Isosilybin A
6	5.556	1513741	709903	709087	1216061	707109	6	Isosilybin B
7	6.755	548867	221208	224297	22153	225510		
8	7.299	840382	285988	287421	24958	307410		

The present study was performed on Rhabdomyosarcoma (RD) cell line, and Rat Embryo Fibroblast (REF) as normal cell line that were measured at three periods of incubation(24, 48, and 72) hours in a microtitration plate under complete sterile conditions. The Cells were grown in optimum condition at 37c° and 5% CO_2 [7]. Different concentrations of these extracts and silymarin were used starting from (400, 200,100,50,25) µg/ml of two fold dilution for each concentration were prepared and tested on each cell line, with three replicates for each concentration[8]. Add 200µ/l of each concentration to the cell culture (200µ/l of cell suspension in each well of microtitration plat of 96 well of flat bottom). MTT (3, 5-(Dimethylthioazol-2-yl)-2, 5-diphenyltetrazolium bromide) solution 28µl(2mg/ml) was added to calculate cell viability, read at 550nm by ELIZA reader. The percentage of inhibition rate for cell growth was calculated as (A-B)/A*100. A is the optical density for treated wells [9].

STATICALLY ANALYSIS

The descriptive data of these results was demonstrated as ranges, percentage, means, standard error as $p<0.05$ for comparison [10].

RESULTS

The aqueous and alcoholic extracts of fruits after drying showed a powder with light brown color while the both extracts of stems-leaves showed a dark brown sticky product converted to powder after drying. Table-1.

The aqueous extract had high end weight more than alcoholic extract. The detection of S.M constituents that performed by HPLC method showed similar constituents like silymarin but with different proportions. Alcoholic fruit extract is so near to pure silymarin Table -1.

The results obtained in Table-2- revealed significant cytotoxic effect at $p<0.05$ for all the extracts by inhibition the cell growth at highest and lowest concentrations. All the extracts are more effective than silymarin in all concentration for 24hrs exposure. The highest inhibition rate of alcoholic fruit extract on the RD cell line was 60.26% in concentration 400µl/ml the lowest rate was 39.10%. In concentration 25µl/ml for 24hrs exposure in comparison with silymarin that had 42.31% and 24.36% for highest and lowest inhibition rates. The results of table-4- showed significant cytotoxic effect of all the extracts but more than the cytotoxic effect of silymarin when used on the RD cell line up to $p<0.05$. The results of table-5- revealed significant inhibition rate of all the extracts and silymarin $p<0.05$ but there is increase in inhibition rate of silymarin and both aqueous and alcoholic stems-leaves extracts in the meantime. The alcoholic fruit extracts is slightly decrease in inhibition but still it has the maximum inhibitory effect for 72hr exposure. Really all the extracts and silymarin showed no significant cytotoxic effect on REF normal cell line for 72hr time exposure. Table-5-

Table -5- the vaibilty of REF normal cell when treated with silymarin and extracts of *silybaiw marinivw* in 72hrs of incubation

	conc.	mean	control	SD	viability	cytotoxicity	p-value
silymarin	400	0.38	0.35	0.01	107.52	-7.52	P<0.05
	200	0.40	0.35	0.01	113.33	-13.33	P<0.05
	100	0.38	0.35	0.01	109.81	-9.81	P<0.05
	50	0.37	0.35	0.01	106.38	-6.38	P<0.05
	25	0.40	0.35	0.01	113.71	-13.71	P<0.05
fa	400	0.32	0.35	0.01	92.29	7.71	P<0.05
	200	0.38	0.35	0.01	108.19	-8.19	P<0.05
	100	0.38	0.35	0.01	108.57	-8.57	P<0.05
	50	0.37	0.35	0.01	104.95	-4.95	P<0.05
	25	0.38	0.35	0.01	108.57	-8.57	P<0.05
fc	400	0.31	0.35	0.02	89.43	10.57	P<0.05
	200	0.30	0.35	0.02	86.76	13.24	P<0.05
	100	0.31	0.35	0.02	89.62	10.38	P<0.05
	50	0.31	0.35	0.01	87.81	12.19	P<0.05
	25	0.32	0.35	0.01	91.14	8.86	P<0.05
sa	400	0.31	0.35	0.02	87.71	12.29	P<0.05
	200	0.31	0.35	0.01	87.43	12.57	P<0.05
	100	0.33	0.35	0.00	92.95	7.05	P<0.05
	50	0.31	0.35	0.01	87.52	12.48	P<0.05
	25	0.32	0.35	0.01	91.43	8.57	P<0.05
sc	400	0.32	0.35	0.01	91.33	8.67	P<0.05
	200	0.31	0.35	0.02	87.33	12.67	P<0.05
	100	0.33	0.35	0.01	93.62	6.38	P<0.05
	50	0.32	0.35	0.00	91.71	8.29	P<0.05
	25	0.31	0.35	0.02	89.24	10.76	P<0.05

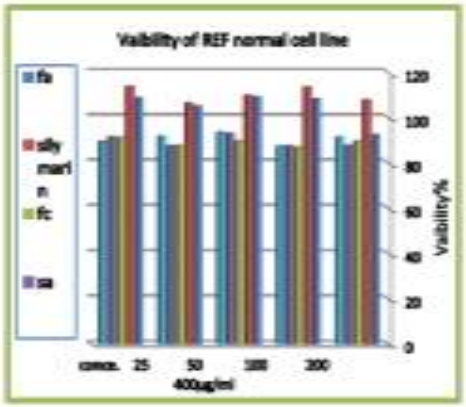

Figure -4- Vaibilty of REF normal cell when treated with silymarin and extracts of *silybaiw marinivw* in 72hrs of incubation Fa: aqueous extract of fruits, Fc: alcoholic extract of fruits, Sa; aqueous extract of stems and leaves, Sc: alcoholic extract of stems and leaves.

Table -4-: cytotoxic effect of extracts on RD in 72 hour.

	Concent. µg/ml	Mean	SD	Control	Viability%	Cytotoxicity%	p-value
conc. Of Fa	400	0.401	0.129	1.1	36.45	63.55	<0.05
	200	0.434	0.105	1.1	39.45	60.55	<0.05
	100	0.465	0.134	1.1	42.27	57.73	<0.05
	50	0.48	0.18	1.1	43.64	56.36	<0.05
	25	0.496	0.179	1.1	45.09	54.91	<0.05
conc. Of Fc	400	0.327	0.148	1.1	29.73	70.27	<0.05
	200	0.403	0.129	1.1	36.64	63.36	<0.05
	100	0.458	0.089	1.1	41.64	58.36	<0.05
	50	0.563	0.156	1.1	51.18	48.82	<0.05
	25	0.62	0.083	1.1	56.36	43.64	<0.05
conc. Of sc	400	0.396	0.056	1.1	36.00	64.00	<0.05
	200	0.447	0.208	1.1	40.64	59.36	<0.05
	100	0.492	0.082	1.1	44.73	55.27	<0.05
	50	0.515	0.207	1.1	46.82	53.18	<0.05
	25	0.55	0.105	1.1	50.00	50.00	<0.05
Ofconc. Of Sa	400	0.421	0.125	1.1	38.27	61.73	<0.05
	200	0.505	0.238	1.1	45.91	54.09	<0.05
	100	0.537	0.112	1.1	48.82	51.18	<0.05
	50	0.544	0.137	1.1	49.45	50.55	<0.05
	25	0.575	0.111	1.1	52.27	47.73	<0.05
conc. Silymarin	400	0.382	0.165	1.1	34.73	65.27	<0.05
	200	0.482	0.133	1.1	43.82	56.18	<0.05
	100	0.531	0.11	1.1	48.27	51.73	<0.05
	50	0.553	0.15	1.1	50.27	49.73	<0.05
	25	0.598	0.183	1.1	54.36	45.64	<0.05

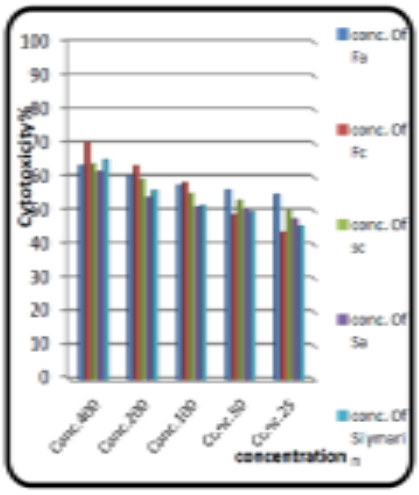

Figure 3: cytotoxic effect of extracts on RD in 72 hours, Fa=aqueous extract of fruits, Fc= alcoholic extract of fruits, Sa= aqueous extract of stems and leaves, Sc=alcoholic extract of stems and leaves.

Table -2-: cytotoxic effect of extracts on RD cancer cell in 24 hours

	Concentration µg/ml	Mean	SD	Control	Viability%	Cytotoxicity%	P-value
conc. of Silymarin	400	0.63	0.351	1.092	57.69	42.31	<0.05
	200	0.755	0.122	1.092	69.14	30.86	<0.05
	100	0.806	0.064	1.092	73.81	26.19	<0.05
	50	0.823	0.038	1.092	74.45	25.55	<0.05
	25	0.826	0.064	1.092	75.64	24.36	<0.05
conc. of Sc	400	0.496	0.348	1.092	45.60	54.40	<0.05
	200	0.528	0.358	1.092	48.35	51.65	<0.05
	100	0.571	0.051	1.092	52.29	47.71	<0.05
	50	0.625	0.093	1.092	57.23	42.77	<0.05
	25	0.665	0.064	1.092	60.90	39.10	<0.05
conc. Of Sa	400	0.587	0.002	1.092	46.43	53.57	<0.05
	200	0.566	0.284	1.092	51.83	48.17	<0.05
	100	0.612	0.042	1.092	56.04	43.96	<0.05
	50	0.622	0.063	1.092	56.96	43.04	<0.05
	25	0.669	0.032	1.092	61.26	38.74	<0.05
conc. Of fc	400	0.434	0.323	1.092	39.74	60.26	<0.05
	200	0.523	0.053	1.092	47.89	52.11	<0.05
	100	0.562	0.067	1.092	51.47	48.53	<0.05
	50	0.614	0.04	1.092	56.23	43.77	<0.05
	25	0.665	0.007	1.092	60.90	39.10	<0.05
conc. Of fa	400	0.472	0.345	1.092	43.22	56.78	<0.05
	200	0.56	0.028	1.092	51.28	48.72	<0.05
	100	0.602	0.035	1.092	55.13	44.87	<0.05
	50	0.693	0.045	1.092	63.46	36.54	<0.05
	25	0.739	0.374	1.092	65.84	34.16	<0.05

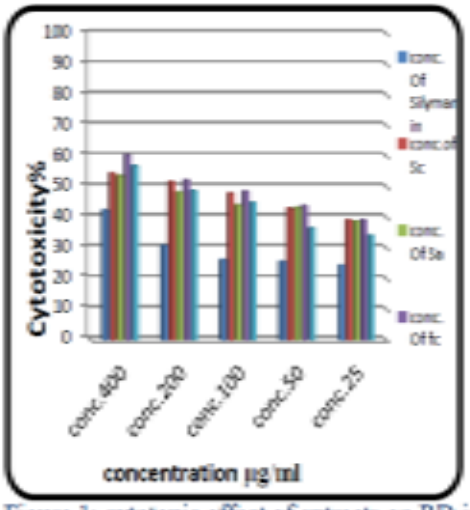

Figure 1: cytotoxic effect of extracts on RD in 24 hours

Table -3-: cytotoxic effect of extracts on RD cancer cell in 48 hours

Concentration µg/ml		Mean	SD	Control	Viability%	Cytotoxicity%	P-value
conc. Of fa	400	0.444	0.069	1.200	37.000	63.000	<0.05
	200	0.467	0.072	1.200	38.917	61.083	<0.05
	100	0.498	0.076	1.200	41.500	58.500	<0.05
	50	0.546	0.113	1.200	45.500	54.500	<0.05
	25	0.588	0.044	1.200	46.250	53.750	<0.05
conc. Of fc	400	0.515	0.103	1.200	27.083	72.917	<0.05
	200	0.488	0.089	1.200	38.167	61.833	<0.05
	100	0.529	0.109	1.200	44.083	55.917	<0.05
	50	0.503	0.154	1.200	46.917	53.083	<0.05
	25	0.623	0.083	1.200	51.917	48.083	<0.05
conc. Of sc	400	0.445	0.068	1.200	37.083	62.917	<0.05
	200	0.492	0.208	1.200	41.000	59.000	<0.05
	100	0.523	0.082	1.200	43.583	56.417	<0.05
	50	0.585	0.207	1.200	48.750	51.250	<0.05
	25	0.601	0.065	1.200	50.083	49.917	<0.05
conc. Of Sa	400	0.482	0.067	1.200	40.167	59.833	<0.05
	200	0.515	0.228	1.200	42.917	57.083	<0.05
	100	0.544	0.113	1.200	45.333	54.667	<0.05
	50	0.624	0.237	1.200	52.000	48.000	<0.05
	25	0.676	0.111	1.200	56.333	43.667	<0.05
conc. Silymarin	400	0.537	0.092	1.200	44.750	55.250	<0.05
	200	0.569	0.133	1.200	47.417	52.583	<0.05
	100	0.605	0.124	1.200	50.250	49.750	<0.05
	50	0.645	0.150	1.200	53.750	46.250	<0.05
	25	0.677	0.183	1.200	56.417	43.583	<0.05

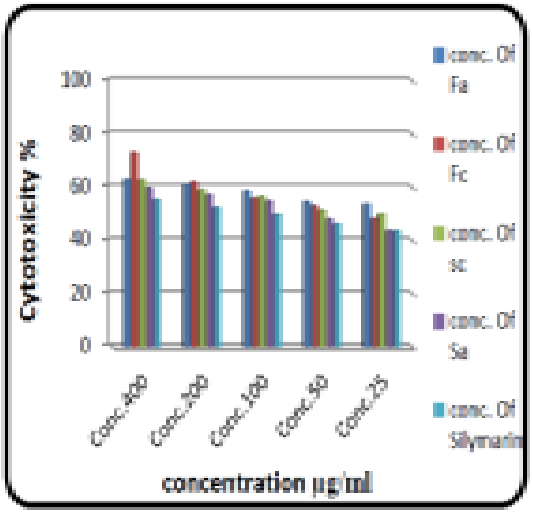

Figure 2: cytotoxic effect of extracts on RD in 48 hours

DISCUSSION

The detection for constituents of *Silybum marianum* was performed by using HPLC method, the results proved in having the same constituents of silymarin isomers but with different proportions table-1. This finding is similar to the finding of others [11].

The results of aqueous and alcoholic extracts of SM *in vitro* on RD cell line revealed significant cytotoxic effect through inhibition the cell growth in the highest and lowest concentration Tables 2, 3, 4. The alcoholic extracts had highest effect on the cancer cell line *in vitro* also the alcoholic fruit extract had more cytotoxic effect than alcoholic stem-leaves extract on the cancer cell line.

The cytotoxicity of SM extracts and silymarin is increase when the concentration and time of incubation increased figures 1, 2, 3, these effects are related to antiproliferative activity and apoptotic death of cancer cell line [12], which was similar to our results.

The absent cytotoxic activity of SM and silymarin on the REF normal cell line that used in the present study Table-6- is compatible to the other studies [13]. In using silymarin for treatment and protection of alcohol induce liver cirrhosis.

Now it's clear that the bioavailability of aqueous extract of SM is less than that of alcoholic extract due to less solubility of silymarin in water though may affect the cytotoxicity of aqueous extract [14]. Both compounds SM and silymarin had cytotoxic activity on cancer cell but silymarin potentiate the cytotoxic effect of 5-fluro uracil the anticancer drug [15].

CONCLUSION

SM extract and silymarin showed a promising anticancer effect as well as increase the activity of 5-flurouracil when used in combination, therefore both compounds can be used as adjuvant in cancer therapy.

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