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NATURAL DRUGS

MODULATION OF DOXORUBICIN CYTOTOXICITY BY ISOLIQUIRITIN AND CYNARIN COMBINATIONS ON DIFFERENT CANCER CELL LINES

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Abstract: Natural polyphenolic compounds produced in plants exhibit many pharmacological effects including antioxidant, chemopreventive, and anticancer properties. This study was conducted to investigate the effect of cynarin (from artichokes, *Cynara scolymus)* and isoliquiritin (from licorice*, Glycyrrhiza uralensis*) on doxorubicin (positive control) cytotoxicity in different cell lines including normal (MCR-5 fibroblasts and H9c2 myoblasts) and cancer (colorectal HCT-116 and hepatocellular HEP-G2) cell lines. The cytotoxic effect of doxorubicin, isoliquiritin, and cynarin alone or in different combinations was studied on cancer cell lines as well as normal cell lines. The results obtained indicated that both cynarin and isoliquiritin enhance the cytotoxicity of doxorubicin. Both cynarin and isoliquiritin also reduce the cardiotoxicity of doxorubicin on normal cardiac cell lines. The combination of the three compounds (cynarin, isoliquiritin, and doxorubicin) decreases the cytotoxicity of doxorubicin, which may indicate the presence of interaction and/or antagonism effect between cynarin and isoliquiritin. Cynarin was found to enhance the growth of HCT-116 and HEP-G2. This might suggest avoiding the use of artichokes in subjects susceptible to these cancers. All results were evaluated using a statistical path and showed significant findings. The mechanism of enhanced doxorubicin's cytotoxicity by cynarin or isoliquiritin also requires further investigation to explain the increasing and/or the decreasing effect of these polyphenolic compounds on the cytotoxicity of doxorubicin. The current findings can suggest safe minimum doses for the two or three-element combinations of compounds in the context of clinical trials and practice.

Keywords: cynarin, isoliquiritin, doxorubicin, different cell lines

Natural products (NPs) have a unique diversity of structures and complexity. They have played important roles in the discovery of drugs that are used to treat various diseases. Polyphenolic compounds are the most abundant group of all the phytochemicals (1). They are known for their potent antioxidant effect which represents the key property that contributes to their effects on cancer, chemopreventive and therapeutic (2). Currently, approximately half of the current anticancer agents are NPs or were inspired by NPs (3).

The combination of natural compounds with the conventional treatment of cancer such as chemotherapy and radiotherapy was found to enhance the efficacy and ameliorate the side effects of such treatment modalities (4). Liquiritin and isoliquiritin, the major components of licorice roots, are assigned to treat some types of tumors (5). The effect of isoliquiritigenin (ISL) on tumor-induced angiogenesis in ACC (adenoid cystic carcinoma) was investigated along with the effect of ISL on vascular endothelial growth factor (VEGF) and ACC cell-induced proliferation, migration, and tube formation of human umbilical vein endothelial cells (6). The cytoprotective effects of isoliquiritin on corticosterone-induced neurotoxicity in pheochromocytoma PC12 cells may be related to its antioxidant action, inhibition of [Ca^{2+}] overload, and inhibition of the mitochondrial apoptotic pathway. In addition, isoliquiritin increased the activity of dismutase and catalase and decreased the contents of reactive oxygen species and malondialdehyde. Also,

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the treatment with isoliquiritin reduced corticosterone-induced mitochondrial dysfunction by preventing mitochondrial membrane potential dissipation (7, 8).

Artichokes are also one of the most antioxidant-rich foods and thereby powerful cancer prevention. In fact, artichokes ranked seventh in antioxidant content out of 1,000 different types of food in a list compiled by the United States Department of Agriculture. Artichoke's antioxidant content comes primarily from potent cancer-fighting polyphenols. Artichokes also contain cynarin, which is considered a potent anticancer compound in the plant. Cynarin is mostly concentrated in the leaves and is the most studied component of artichoke (9).

Based on the previous studies with polyphenols and new approaches for the treatment of cancer with combination therapy, it was thought to be worthwhile to evaluate the cytotoxic effect of doxorubicin in combination with either isoliquiritin or cynarin or both, on a colorectal cancer cell line (HCT-116) and a liver cancer cell line (HEP-G2). In addition, this study also evaluated the cardio-protective effects of isoliquiritin and cynarin against doxorubicin-induced cardiotoxicity on a myoblast cell line (H9c2).

EXPERIMENTAL

Material and methods

Cell lines and cell proliferation assay

Cancer cell lines used in this work were human colon colorectal carcinoma HCT-116 (ATCC® CCL-247), human liver hepatocellular carcinoma HEP-G2 (ATCC® no. HB-8065), Rattus norvegicus (rat) myoblast heart myocardium H9c2 (ATCC® CRL-1446), human lung MCR-5 fibroblasts (ATCC® CCl-171). The Faculty of Pharmacy at the University of Jordan provided these cell lines, which were originally purchased from the European Collection of Authenticated Cell Cultures (ECACC), United Kingdom (UK). Cells reached the tissue culture laboratory in a frozen state where they were kept at a very low temperature (approximately -190°C) by means of liquid nitrogen. On the day of thawing, a culture flask, which contained the appropriate medium, was prepared; a vial of each type of frozen cell line was taken from the liquid nitrogen container and thawed as quickly as possible in a water bath (37°C). They were diluted using 10 mL of pre-warmed suitable medium and centrifuged for 10 min at 1,500 rounds per minute (RPM). The supernatants were then discarded and 15 mL of the suitable medium was added to the conical tube that contained the cells and mixed properly. Then, the vial contents were transferred into 25 cm^2 flasks and cultured in their corresponding suitable media. HCT-116, H9c2, and fibroblast cells were cultured in DMEM-high glucose medium supplemented with 10% fetal bovine serum FBS, 1% of L-glutamine, and 2 mL penicillin/streptomycin solution. While HEP-G2 cells were cultured in DMEM-low glucose medium supplemented with 10% fetal bovine serum FBS, 1% penicillin/streptomycin solution, and 2 mL L-glutamine. The flask stored in the incubator at 37°C in 5% CO_2 atmosphere with 95% relative humidity.

A hemocytometer was used to count the number of cells in a cell suspension. A mixture of 100 µL of trypan blue stain and 100 µL of cell suspension was placed in an Eppendorf tube. Then 100 µL of this mixture was removed and loaded carefully into the hemocytometer, and the number of cells per one mL of cell suspension was estimated. The cell suspension was then diluted to give the desired number of cells, i.e., 20,000- 25,000 cells/well (100 µL). This was then seeded into a 96-well tissue culture plate. The total number of cells /mL was determined using the following equation: Number of cells/mL = (number of cells in 64 square/4) × 2 × 10000.

The cells were seeded in a 96-well plate for all the experiments.

After the trypsinization and counting step, the suspended cells that were in the tube were transferred to a petri dish. Then a multichannel pipette handled 100 µL to be seeded in each well and for six replicates except for heart myocardium H9c2 cells that were triplicated to avoid any errors.

The 96-well plate was incubated at 37°C to allow cell attachment for 24 h before the desired standards were added. On the second day, 10 µL of each suitable treatment was added in each well. The final dilution used for treating the cells contained not more than 1% of the initial solvent DMSO. Reports indicated that at this concentration DMSO was not cytotoxic (10-12). Hence, 1% DMSO was used as a negative control solvent in the present experiments. However, 100 µL of the medium was added to cell-free wells for background control.

After the application of the treatments, the plates were incubated for 48 h, according to the preliminary studies indicating that the exponential growth phase of each cell line is 24-72 h (12). Then a micro-culture tetrazolium (MTT) assay was done.

Proliferation assays are used in cell biology to study growth factors, cytokines, nutrients, and cytotoxic agents. A Cell Titer 96® Non-

Radioactive Cell Proliferation Assay technical bulletin was used to determine a viable cell number in this bath. MTT, a yellow tetrazolium salt, was reduced into purple formazan in living cells, increasing the amounts of living cells resulting in increased purple coloring.

A prepared treatment was added and incubated for 48 h. Then, 15 µL of the dye solution was added to each well. The plate was incubated for 4 h according to a Promega, CellTiter 96® Non-Radioactive Cell Proliferation Assay. During a 4-hour incubation, living cells converted the tetrazolium component of the dye solution into a formazan product. A 100 µL from the solubilization solution/stop mix was added to each well; in order to solubilize the formazan product, the plate was incubated for 1 hour, and the absorbance of each well was read by an ELISA plate reader at 590 nm. Using Gen5 software, the percentage of viable cells and toxicity was calculated using the following equations:

Table 1. Cytotoxicity of doxorubicin, isoliquiritin and cynarin on cell line (HCT-116, HEP-G2, H9c2, and MCR-5).

Drug	Concentration (µg/mL)	% Cytotoxicity on different cell line			
		HCT-116	HEP-G2	H9c2	MCR-5
Doxorubicin	100	90.491 ± 2.533	99.431 ± 1.391	97.070 ± 2.138	88.921 ± 2.592
	50	46.617 ± 4.896	97.788 ± 0.777	70.876 ± 10.913	48.028 ± 4.652
	25	39.172 ± 2.743	87.488 ± 1.698	79.411 ± 11.861	36.521 ± 5.122
	12.5	27.693 ± 7.115	74.375 ± 4.772	30.664 ± 18.201	39.308 ± 3.507
	6.25	31.393 ± 3.984	72.400 ± 4.318	22.114 ± 12.271	45.924 ± 3.643
	3.125	33.680 ± 4.731	58.027 ± 6.836	0.605 ± 24.440	37.126 ± 6.409
	1.56	36.575 ± 4.260	64.576 ± 4.568	70.057 ± 13.917	20.415 ± 6.803
	0.78	32.740 ± 4.349	55.140 ± 5.140	88.799 ± 7.020	4.369 ± 9.741
	0.39	19.241 ± 5.157	11.767 ± 3.702	42.677 ± 13.243	2.870 ± 7.323
	0.19	-1.574 ± 9.929	-0.139 ± 8.935	25.304 ± 7.497	2.515 ± 13.855
Isoliquiritin	400	78.975 ± 9.203	98.996 ± 0.137	11.569 ± 4.503	0.980 ± 8.512
	200	19.259 ± 7.118	67.403 ± 7.343	-13.611 ± 6.786	2.447 ± 7.222
	100	7.498 ± 7.741	11.443 ± 4.073	-4.054 ± 2.432	-9.411 ± 7.120
	50	14.487 ± 7.252	12.940 ± 6.640	-12.679 ± 5.162	-15.045 ± 9.669
	25	24.957 ± 4.712	11.591 ± 7.980	-6.321 ± 4.128	-18.349 ± 12.356
	12.5	25.722 ± 2.713	3.061 ± 7.840	-7.201 ± 6.616	-26.226 ± 13.846
	6.25	9.342 ± 7.190	7.352 ± 4.977	-8.874 ± 1.223	-31.240 ± 6.296
	3.125	8.936 ± 11.516	-3.828 ± 7.611	-15.770 ± 6.256	-54.276 ± 13.122
	1.56	-4.828 ± 7.788	3.661 ± 4.417	-5.320 ± 2.983	-34.301 ± 7.775
	0.78	-0.082 ± 7.342	4.929 ± 5.697	-9.828 ± 3.087	-11.418 ± 11.122
Cynarin	400	-168.193 ± 10.661	-215.071 ± 36.712	N.A	N.A
	200	-130.323 ± 8.994	-198.733 ± 34.492	N.A	N.A
	50	-110.812 ± 8.987	-51.082 ± 12.988	-62.024 ± 12.023	-51.686 ± 8.841
	25	-51.830 ± 10.357	-0.785 ± 7.144	-40.586 ± 14.595	-47.526 ± 16.881
	12.5	-20.329 ± 5.724	1.554 ± 5.104	-58.644 ± 9.993	-53.140 ± 8.681
	6.25	-4.737 ± 9.232	23.784 ± 6.664	-47.454 ± 15.396	-42.135 ± 42.614
	3.125	-8.701 ± 9.414	-4.527 ± 7.695	-31.931 ± 3.499	-70.041 ± 33.909
	1.56	5.187 ± 7.015	0.029 ± 7.343	-53.593 ± 11.425	-73.061 ± 16.089
	0.78	13.234 ± 5.740	-4.731 ± 4.538	-49.519 ± 7.219	-36.607 ± 38.685
	0.39	1.165 ± 5.816	-3.272 ± 9.034	-51.505 ± 11.003	-106.65 ± 35.279
	0.19	-12.290 ± 4.273	-5.834 ± 9.300	-43.024 ± 17.829	-73.418 ± 45.325
	0.09	-5.301 ± 7.304	-2.550 ± 3.714	-24.600 ± 15.639	-43.727 ± 16.895

NA: Denoted to samples were not performed/ analyzed at these concentrations.

Viability = (Absorbance of treated wells/Absorbance of the negative control) ×100%
Toxicity = 100 – viability

Calculation of inhibitory concentration (IC_{50})

The IC_{50} is the concentration of an inhibitor where its response (or binding) is reduced by half (18). By using Sigma Plot software (SYSTAT Software Inc.), the IC_{50} was determined, which indicated the concentration of each drug alone or in a combination that produced 50% toxicity. The IC_{50} was calculated according to the following equation:

$$y = \text{min.} + (\text{max.} - \text{min.}) / (1 + (x/IC_{50})^{(\text{-Hill slope})})$$

Statistics

The analysis was performed using Microsoft Office Excel (for MAC. 2011). A Gen5 microplate reader and imager software (version 3.0) were used to transform raw data and images into meaningful results. Graph Pad Prism software (version 5) was used for two-way ANOVA. Sigma Plot (version 12) was used for IC_{50} determinations.

RESULTS AND DISCUSSION

Cytotoxicity results for single compound treatments

As shown in Table 1, the cytotoxic effect of doxorubicin was clearly noticed on all cell lines used (HCT-116, HEPG2, H9c2, and MCR-5) especially at high concentrations as was reported in other studies (13, 14).

Table 1 also shows that the effect of isoliquiritin serial dilution on each cell line was concentration-dependent starting from high to low concentration (400 µg/mL to 0.78 µg/mL). Isoliquiritin showed a cytotoxic effect at a high concentration on cell lines (HCT-116 and HEP-G2), but it was not toxic on H9c2 and MCR-5 since they are normal non-immortal cells. Isoliquiritin at a high concentration (400 µg/mL) showed high cytotoxicity (78.9% in HCT-116 cell lines and 98% in HEP-G2) while there was little cytotoxicity using the same concentration on normal cells (H9c2 myoblasts and MCR-5 fibroblasts).

Table 1 indicates that cynarin on each cell line showed no cytotoxic effect and promoted the growth of both cancerous cells (HCT-116 and HEP-G2) and normal cell (H9c2 and MCR-5) lines. A previous study found that the extract of Cynara species may have some cytotoxic

Table 2a. Cytotoxicity of different combinations (doxorubicin, isoliquiritin and cynarin) on cell line (HCT-116, HEP-G2 and H9c2).

Concentration of Doxorubicin (µg/mL)	Doxorubicin and isoliquiritin (310 µg/mL)			Doxorubicin and cynarin 1.5 (µg/mL)			Doxorubicin, isoliquiritin (310 µg/mL) and cynarin (1.5 µg/mL)		
	HCT-116	HEP-G2	H9c2	HCT-116	HEP-G2	H9c2	HCT-116	HEP-G2	H9c2
100	95.16 ± 1.14	108.29 ± 1.02	99.25 ± 0.37	98.37 ± 2.19	100.21 ± 1.43	98.71 ± 0.97	37.75 ±7.60***	92.75 ± 4.12	93.74 ± 0.52
50	90.42 ± 1.26**	95.03 ± 1.05	78.45 ± 1.94	72.00 ± 3.66	96.02 ± 0.59	80.80 ± 3.35	37.41 ±3.05	69.66 ± 3.05**	71.06 ± 5.25
25	82.02 ± 1.65**	81.91 ± 4.79	54.43 ± 2.79	87.58 ± 2.23**	90.46 ± 2.07	48.60 ± 1.95*	21.07 ± 2.39	60.76 ± 5.32**	56.64 ± 4.29
12.5	79.34 ± 1.33***	91.61 ± 2.60	38.84 ± 0.75	23.56 ± 21.88	85.33 ± 2.26	37.18 ± 2.34	12.84 ± 5.94	56.80 ± 5.44	46.75 ± 3.36
6.25	76.15 ± 3.76**	75.17 ± 2.99	45.23 ± 1.16	1.14 ± 17.14	85.34 ± 1.01	34.69 ± 4.93	23.04 ± 1.99	49.38 ± 6.87*	39.83 ± 2.22
3.125	83.55 ± 2.86***	63.78 ± 7.91	30.70 ± 8.05	24.65 ± 10.87	72.11 ± 2.42	20.84 ± 4.99	28.81 ± 3.09	66.67 ± 3.38	42.49 ± 0.84
1.56	3.49 ± 1.91*	62.75 ± 6.22	41.85 ± 0.55*	-0.078 ± 25.06*	80.08 ± 4.25	34.99 ± 7.65**	24.44 ± 4.81	64.99 ± 5.29	45.52 ± 3.27
0.78	65.76 ±1.38	69.66 ± 2.80	47.03 ± 0.83***	-25.17 ± 23.33***	64.21 ± 5.12	32.68 ± 8.07***	6.06 ± 2.88	47.97 ± 4.52	48.48 ± 0.62***
0.39	47.49 ± 3.04	35.20 ± 9.36*	14.31 ± 1.71*	-21.34 ± 25.84*	57.32 ± 4.02***	7.67 ± 7.97**	2.16 ± 5.28	30.24 ± 8.36	25.80 ± 1.32
0.19	31.32 ± 3.57	7.26 ± 10.64	16.55 ± 1.20	-14.77 ± 16.25	50.78 ± 8.07***	1.84 ± 5.49	28.622 ± 5.66	29.39 ± 5.49***	13.03 ± 4.06

Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for significant values, compared to % Cytotoxicity of doxo. alone & combination with other drugs.

Table 2b. Cytotoxicity of isoliquiritin and cynarin on cell line (HCT-116 and HEP-G2).

	Isoliquiritin and cyanarin	
Concentration of Isoliquiritin (µg/mL)	HCT-116/ (Isoliquiritin + cynarin 1.5 µg/mL)	HEP-G2/ (Isoliquiritin + cynarin 1.5 µg/mL)
400	13.77 ± 4.02	95.90 ± 0.56
200	10.32 ± 4.09	24.74 ± 19.94
100	5.92 ± 4.17	15.87 ± 24.47
50	11.97 ± 2.25	6.59 ± 19.36
25	6.13 ± 3.45	0.82 ± 17.83
12.5	8.46 ± 2.75	4.42 ± 13.68
6.25	-1.76 ± 5.66	6.19 ± 18.51
3.125	-5.04 ± 4.27	-3.54 ± 19.58
1.56	3.42 ± 5.74	-12.08 ± 10.98
0.78	1.78 ± 4.33	-3.97 ± 8.09

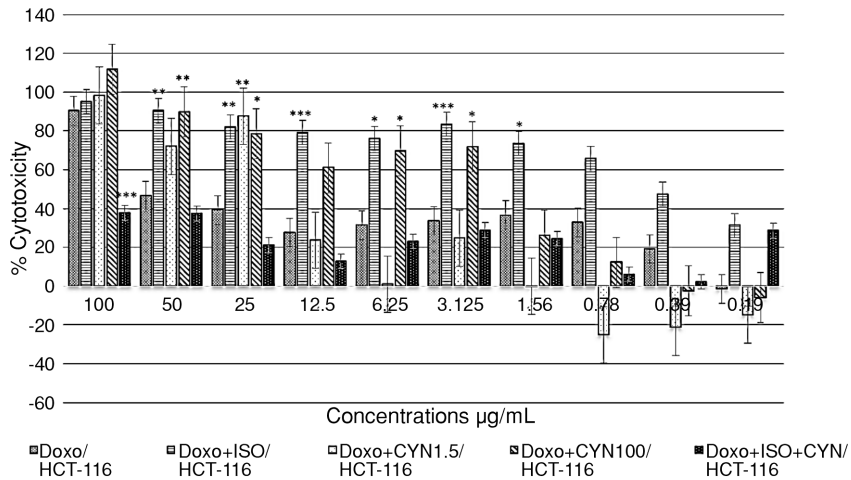

Figure 1. Comparison of doxorubicin cytotoxicity with different combination of doxorubicin (Doxo), isoliquiritin (ISO, 310 µg/mL) and cynarin (CYN, 1.5 µg/mL) on HCT-116 cell lines. (Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

effect in colorectal cancer cells (15). This variation in results may be related to some differences in experimental conditions such as different incubation periods, different cell densities/ wells, and the concentrations and purities of extracts.

The growth of cells was concentration-dependent starting from high to low concentrations (400 µg/mL, 200 µg/mL, and a serial dilution from 50 to 0.09 µg/mL). Cynarin at all concentrations studied was shown to enhance the growth of all different cell lines (HCT-116, HEP-G2, H9c2, and MCR-5). This promotion of growth due to cynarin has not been well studied so it requires further investigation. Also, this may explain the antagonism that may occur when combining cynarin and isoliquiritin on the toxicity of doxorubicin.

Cytotoxicity of two-combination treatments

As shown in Table 2, the cytotoxicity of doxorubicin (serial dilution from 100 µg/mL to 0.19 µg/mL) in combination with IC_{50} of isoliquiritin on each cell line (HCT-116, HEP-G2, and H9c2) was concentration-dependent starting from high to low concentrations (Fig. 1-3).

The combination of doxorubicin (serial dilution) and a fixed dose of isoliquiritin (310 mcg/mL proximate IC_{50}) on HCT-116 cell lines have significant results at concentrations (50, 25, 12.5, 6.25, 3.125, and 1.56 µg/mL) according to doxorubicin concentrations (Fig. 1). This indicates that the presence of isoliquiritin in combination with doxorubicin has increased the cytotoxicity of the cell line of doxorubicin especially at 50 µg/mL and lower

doses. This combination may also decrease the possible cardiotoxicity of doxorubicin.

Doxorubicin (serial dilution) and a fixed dose of isoliquiritin (310 µg/mL, approximate IC_{50}) on HEP-G2 cell lines showed a significant increase in the cytotoxicity of doxorubicin at concentration 0.39 µg/mL. This may allow using lower doses of doxorubicin to decrease its cardiotoxicity.

The result of the combination of doxorubicin (serial dilution) and a fixed dose of isoliquiritin (310 µg/mL) on H9c2 myoblasts showed a significant decrease in the cardiotoxicity of doxorubicin at certain concentrations (1.56, 0.78, and 0.39 µg/mL). This indicates the advantage of isoliquiritin in protecting normal cardiac cells from the damaging effect of doxorubicin (Fig. 3).

Table 2 shows the cytotoxicity of doxorubicin (serial dilution from 100 µg/mL to 0.19 µg/mL) in combination with cynarin. The lowest concentrations which did not promote growth on each cell line (HCT-116, HEP-G2, and H9c2) were concentration-dependent starting from high to low concentrations (Fig. 1-3).

The results of combining doxorubicin (serial dilution) and cynarin (100 µg/mL) on HCT-116 cell lines indicated a significant increase in doxorubicin cytotoxicity at specific concentrations (50, 25, 6.25, and 3.12 µg/mL).

The results of combining doxorubicin (serial dilution) and cynarin (1.5 µg/mL that produces minimum cell growth) on HCT-116 cell lines indicated significant increases in doxorubicin cytotoxicity at

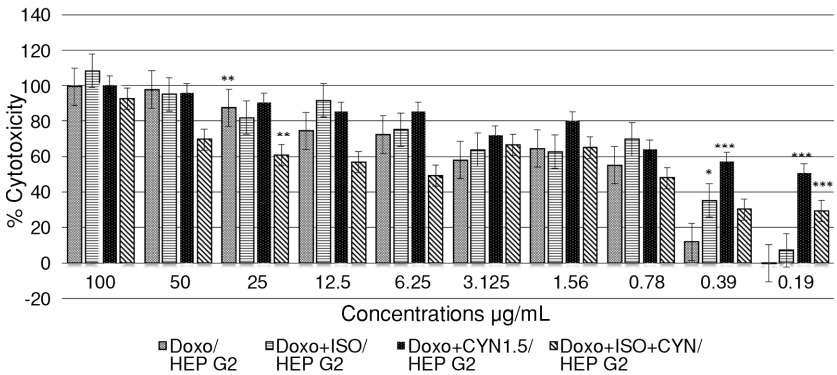

Figure 2. Comparison of doxorubicin cytotoxicity with different combinations of doxorubicin (Doxo), isoliquiritin (ISO, 310 µg/mL) and cynarin (CYN, 1.5 µg/mL) on HEP-G2 cell lines. (Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

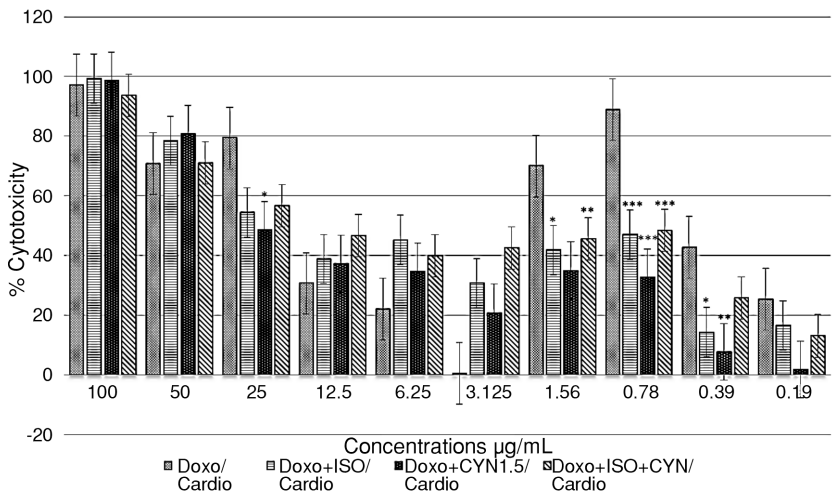

Figure 3. Comparison of doxorubicin cytotoxicity with different combinations of doxorubicin (Doxo), isoliquiritin (ISO, 310 µg/mL) and cynarin (CYN, 1.5 µg/mL) on H9c2 cell lines. (Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

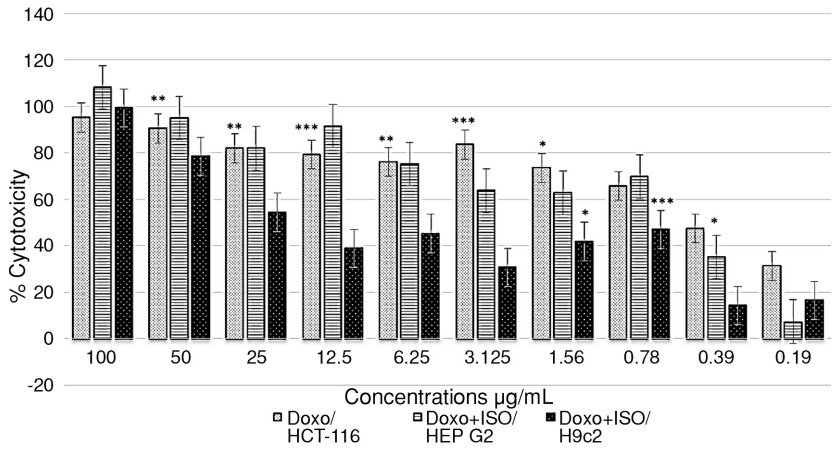

Figure 4. Percentage cytotoxicity of doxorubicin (Doxo) and isoliquiritin (ISO, 310 µg/mL) on different cell lines (HCT-116, HEP-G2 and H9c2). (Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

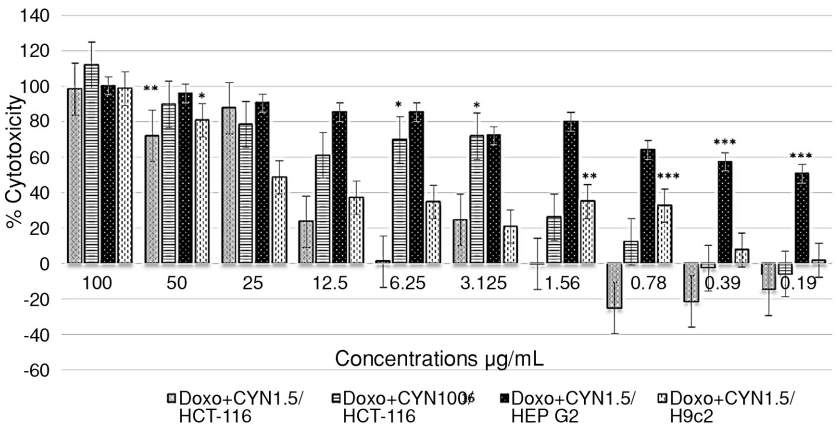

Figure 5. Percentage cytotoxicity of doxorubicin (Doxo) and cynarin (CYN, 1.5 µg/mL) on different cell lines (HCT-116, HEP-G2 and H9c2). (Symbols (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

specific concentrations (25, 1.56, 0.78, and 0.39 µg/mL). These results indicated that cynarin at doses 100 µg/mL and 1.5 mg/mL enhanced the cytotoxicity of doxorubicin on this cell line. At the same time, cynarin alone increased the growth of these cell lines. Therefore, lower doses of cynarin were chosen, which did not increase growth but enhanced the cytotoxicity of doxorubicin (Fig. 1). It was reported that cynarin has dose and cell type-dependent effects (16).

The results of combining doxorubicin (serial dilution) and cynarin (1.5 mg/mL, which produces minimum cell growth) on HEP-G2 cell lines indicated a significant increase in doxorubicin cytotoxicity especially at lower concentrations (0.39 and 0.19 µg/mL) (Fig. 2). This result indicated that cynarin at lower doses enhances the effect of doxorubicin and may decrease its cardiotoxicity. For all concentrations of this combination, there was no cell growth of the cell lines, which is like the case of the HCT-116 cell lines that confirmed that cynarin has dose and cell type-dependent effects.

The combination of doxorubicin (serial dilution) and cynarin (1.5 µg/mL) on H9c2 myoblast cell lines led to a decrease in the cardiotoxicity of doxorubicin significantly at specific concentrations (25, 1.56, 0.78, and 0.39 µg/mL) (Fig. 3).

Cytotoxicity results of combination isoliquiritin and cynarin treatment

According to Table 2 and Figures 4-5, the combination of isoliquiritin (serial dilution) and a cynarin fixed concentration (1.5 µg/mL) on both cell lines (HCT-116 and HEP-G2) showed a sharp decrease in the toxicity of isoliquiritin starting from a high dose (400 mg/mL). This may suggest the presence of some antagonist interaction between cynarin and isoliquiritin, as was reported previously due to the counteracting effect of GSK-3beta, Glycogen Synthase Kinase 3-beta (17).

Cytotoxicity results for three-combination treatments

Table 2 and Fig. 6 shows that the three combinations of doxorubicin (serial dilution) with a fixed concentration of isoliquiritin (310 µg/mL) and cynarin (1.5 µg/mL) on HCT-116 cell lines decreased the cytotoxic effect of doxorubicin on the cell lines significantly at a concentration of 100 mcg/mL. While the two combinations of doxorubicin with cynarin and doxorubicin with isoliquiritin on HCT-116 cell lines showed higher cytotoxicity. It seems there may be some drug interaction between these drugs, which leads to a reduction of cytotoxicity. On the other hand, the result of the combinations among doxorubicin (serial dilution) and fixed concentrations of isoliquiritin and cynarin (310 and 1.5 µg/mL respectively) on HEP-G2 cell lines showed a reduction in the cytotoxicity of doxorubicin on the cell lines significantly at specific concentrations (50, 25, 6.25, and 0.19 µg/mL). Therefore, the combination of doxorubicin with isoliquiritin presented better results.

In addition, a doxorubicin combination (serial dilution) with a fixed concentration of isoliquiritin (310 µg/mL) and cynarin (1.5 µg/mL) on H9c2 myoblast cell lines presented a significantly lower cytotoxicity effect of doxorubicin at concentration (0.78 µg/mL).

Comparative cytotoxic effects of different combinations on cell lines

The cytotoxic effect of doxorubicin, isoliquiritin, cynarin, and combinations of them on HCT-116 cell lines showed that the two combinations have a better cytotoxic effect than the doxorubicin alone. Additionally, the cytotoxic effect of doxorubicin, isoliquiritin, cynarin, and combinations on HEP-G2 cells lines showed that the two and/or three combinations have a better cytotoxic effect than the doxorubicin alone.

The cytotoxic effect of doxorubicin, isoliquiritin, cynarin, and combinations on H9c2 cell lines showed that the two and/or three combinations have a better cytotoxic effect than the doxorubicin alone. The cytotoxic effect of doxorubicin, isoliquiritin, and cynarin on MCR-5 fibroblasts cell lines showed that the isoliquiritin and cynarin have no cytotoxic effect on the cell line compared to doxorubicin.

Table 3 shows the results of IC_{50} on normal cell lines and cancer cell lines.

Table 3. IC_{50} of different treatments on normal (fibroblasts MCR-5 and myoblasts H9c2) and cancer cell lines (colorectal HCT-116 and hepatocellular HEP-G2).

Drug combinations	Cell Type (IC_{50}, µg/mL)			
	Fibroblast MCR-5	Myoblasts H9c2	Colorectal HCT-116	Hepatocellular HEP-G2
Doxorubicin	27.38	22.54	46.63	1.36
Isoliquiritin	NA.	NA.	227.62	170.32
Cynarin	NA.	NA.	NA.	NA.
Doxorubicin + isoliquiritin 165 µg/mL	-	-	-	0.86
Doxorubicin + isoliquiritin 310 µg/mL	-	14.91	0.41	-
Doxorubicin + cynarin 1.5 µg/mL	-	18.61	13.15	0.16
Doxorubicin + cynarin 100 µg/mL	-	-	2.72	-
Doxorubicin + isoliquiritin 165 µg/mL + cynarin 1.5 µg/mL	-	-	-	2.07
Doxorubicin + isoliquiritin 310 µg/mL + cynarin 1.5 µg/mL	-	11.32	NA.	-
Isoliquiritin + cynarin 1.5 µg/mL				271.66

NA: indicated that there is no IC_{50}.

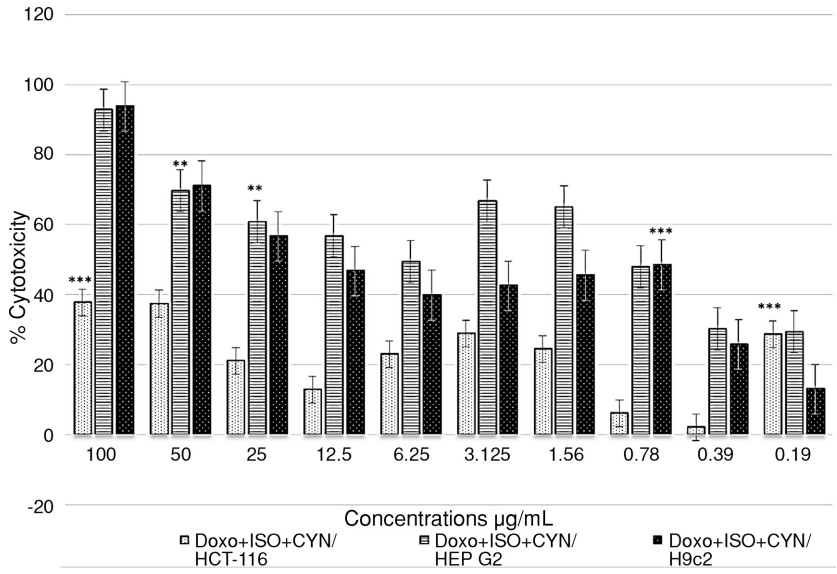

Figure 6. Percentage cytotoxicity of doxorubicin (Doxo), isoliquiritin (ISO, 310 µg/mL) and cynarin (CYN, 1.5 µg/mL) on different cell lines (HCT-116, HEP-G2 and H9c2). (Symbols (** $p < 0.01$, *** $p < 0.001$) is for statistically significant values).

Inhibitory concentration (IC_{50})

The effect of cynarin and isoliquiritin on normal cell lines (MCR-5 fibroblasts and H9c2 myoblasts) did not produce toxicity to these normal cell lines, so the IC_{50} cannot be calculated.

As shown in Table 3, both cynarin and isoliquiritin decrease the IC_{50} of doxorubicin in H9c2 myoblasts. The combination of cynarin with doxorubicin reduced the IC_{50} of doxorubicin from 22.54 to 18.6 µg/mL, which represents about a 17.40% decrease in the IC_{50} of doxorubicin alone.

The combination of isoliquiritin with doxorubicin reduced the IC_{50} of doxorubicin from 22.54 to 14.9 µg/mL, which represents about a 33.89% decrease in the IC_{50} of doxorubicin alone. The combination of cynarin, isoliquiritin, and doxorubicin reduced the IC_{50} of doxorubicin from 22.54 to 11.32 µg/mL, which represents about a 49.77% decrease in the IC_{50} of doxorubicin alone.

The effect of cynarin and isoliquiritin on the IC_{50} of doxorubicin for hepatocellular HEP-G2 cell lines is shown in Table 3. The combination of cynarin with doxorubicin reduced the IC_{50} of doxorubicin from 1.36 to 0.16 µg/mL, which represents about an 88.23% decrease in the IC_{50} of doxorubicin alone. Also, the combination of isoliquiritin with doxorubicin reduced the $IC5_{50}$ of doxorubicin from 1.36 to 0.86 µg/mL, which represents about a 36.76% decrease in the IC_{50} of doxorubicin alone.

The combination of cynarin, isoliquiritin, and doxorubicin increased the IC_{50} of doxorubicin from 1.36 to 2.07 µg/mL. This may indicate the presence of some antagonism in this combination in this cell line, as it was noticed that cynarin increased the IC_{50} of isoliquiritin from 170.32 to 271.66 µg/mL.

The effect of cynarin and isoliquiritin on the IC_{50} of doxorubicin in colorectal HCT-116 cell lines is shown in Table 3. The combination of cynarin with doxorubicin reduced the IC_{50} of doxorubicin from 46.63 to 13.15 µg/mL, which represents about a 71.99% decrease in the IC_{50} of doxorubicin alone. The combination of isoliquiritin with doxorubicin reduced the IC_{50} of doxorubicin from 46.63 to 0.41 µg/mL, which represents about a 97.76% decrease in the IC_{50} of doxorubicin alone. The combination of cynarin, isoliquiritin, and doxorubicin did not produce toxicity and the IC_{50} cannot be calculated. This may suggest the presence of antagonism between cynarin and isoliquiritin as it was noticed that cynarin almost inhibited the toxicity of isoliquiritin when combined.

CONCLUSION

This study highlights the use of combinations between natural polyphenolic compounds and chemotherapeutic drugs to enhance the cytotoxic activity of such chemotherapeutic drugs and eliminate their side effects, which is considered a novel approach in cancer therapy (19). Results of this study also support the novel approach of cancer treatment, which is the combining of natural com-

pounds with the conventional treatment of cancer such as chemotherapy that enhance the efficacy and ameliorate the side effects of such treatment modality. However, the detailed information on the mechanisms of action at molecular levels of such combinations is required and needs further studies to investigate the actual mechanisms that stand behind the synergetic, additive, and all interactions between those compounds.

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Conflict of interests

The authors declare no conflict of interest.

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