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Cytotoxic Evaluation of Doxorubicin Combination with Baicalein and Resveratrol Against Hct116 and Hepg2 Cancer Cell Lines (Conference Paper)

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Abstract

The combination of natural polyphenolic compounds with chemotherapeutic agents is recently being a novel strategy in cancer therapy research owing to their potential antioxidant and anti-inflammatory properties that modulate several intracellular signaling pathways.

Baicalein and resveratrol are well known polyphenolic compounds that belong to flavone and stilbene subclasses, respectively. This study aims to investigate the possible enhancement effect of baicalein and resveratrol when combined with doxorubicin using a different combination ratio and applied on two cancer cell lines: HCT116 (colorectal cancer cells) and HepG2 (hepatocellular cancer cells). It also investigates the possibility of such natural compounds to provide a protection effect on cardiocyte (H9C2) when baicalein and resveratrol treatment followed by doxorubicin is used. The two cancer cell lines were treated with different combination groups, including the combination between doxorubicin and baicalein or resveratrol and the combination between the three compounds using a different combination ratio for both treatment groups (i.e., two drugs or three drugs combination). Treatment applied on cells, using cell density of 7000 cells /well and incubation time was 48 hrs. MTT test was performed to assay the cell viability. The results obtained showed that the cytotoxicity of doxorubicin in the two cancer cell lines has increased when combined with baicalein and resveratrol. Doxorubicin IC_{50} decreased from 4.99 µg/ml to 0.3657 µg/ml and from 7.3 µg/ml to 0.676 µg/ml on HCT116 and HepG2 cells, respectively, using constant combination ratio (1:1:1). The combination of doxorubicin, baicalein, and resveratrol has resulted in a less cardiotoxic effect compared to treatment with doxorubicin alone. This decrease was obviously seen when the three compounds were combined using a low concentration range and with a constant combination ratio.Conclusion: combinations of baicalein and resveratrol with doxorubicin chemotherapeutic drug *in-vitro* had enhanced the cytotoxic activity of such a chemotherapeutic drug, while simultaneously eliminating its cardiotoxicity side effect.

Key words: Doxorubicin, Baicalein, Resveratrol, IC_{50}, Combination index, Cardiotoxicity.

تقييم سميه الدوكسوروبسين في مزيج البايكلين والريزفيراترول ضد خطوط خلايا سرطانيه مختلفة(بحث مؤتمر)#

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الخلاصة

يعتبر الجمع بين مركبات البوليفينول الطبيعية وعوامل العلاج الكيميائي استراتيجية جديدة في أبحاث علاج السرطان نظرًا لخصائصها المحتملة المضادة للأكسدة والمضادة للالتهابات التي تعدل العديد من مسارات الإشارات داخل الخلايا.

Baicalein و Resveratrol هي مركبات بوليفينول معروفة جيدًا تنتمي إلى عائلتي Flavone و Stilbene الفرعية ، على التوالي.

تهدف هذه الدراسة إلى التحقيق في تأثير التعزيز المحتمل لـ Baicalein و Resveratrol عند دمجهما مع دوكسوروبيسين باستخدام نسبة توليفة مختلفة وتطبيقه على سطرين من الخلايا السرطانية: HCT116 (خلايا سرطان القولون والمستقيم) و HepG2 (الخلايا السرطانية الكبدية). كما تدرس إمكانية وجود مثل هذه المركبات الطبيعية لتوفير تأثير وقائي لخلايا القلب (H9C2) عند استخدام العلاج بالـBaicalein و Resveratrol متبوعًا بالDoxorubicin. تم علاج سطرين الخلايا السرطانية بمجموعات مختلطة مختلفة ، بما في ذلك الجمع بين Doxorubicin و Baicalein و Resveratrol والجمع بين المركبات الثلاثة باستخدام نسب مختلفة لكلا مجموعتي العلاج (أي دواءين أو ثلاثة أدوية). تم تطبيق العلاج على الخلايا باستخدام كثافة الخلايا ٧٠٠٠ خلية / بئر ومدة الحضانة ٤٨ ساعة. تم إجراء اختبار MTT لفحص صلاحية الخلية.

أظهرت النتائج التي تم الحصول عليها أن السمية الخلوية لDoxorubicin في سطري الخلايا السرطانية قد زادت عند دمجها مع Baicalein و Resveratrol. انخفض Doxorubicin IC50 من ٤,٩٩ ميكروغرام / مل إلى ٠,٣٦٥٧ ميكروغرام / مل ومن ٧,٣ ميكروغرام / مل إلى .

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٦٧٦ ميكروغرام / مل على خلايا HCT116 و HepG2 ، على التوالي ، باستخدام نسبة تركيبة ثابتة (١: ١: ١).
أدى الجمع بين Doxorubicin و Baicalein و Resveratrol إلى تأثير أقل على القلب مقارنةً بالعلاج بالDoxorubicin وحده. لوحظ هذا النقص بشكل واضح عندما تم الجمع بين المركبات الثلاثة باستخدام نطاق تركيز منخفض وبنسبة تركيبة ثابتة. إن توليفات من Baicalein و Resveratrol مع عقار Doxorubicin للعلاج الكيميائي في المختبر قد عززت النشاط السام للخلايا لمثل هذا الدواء العلاجي الكيميائي ، مع التخلص في الوقت نفسه من الآثار الجانبية السامة للقلب.

الكلمات المفتاحية: Doxorubicin ، Baicalein ، Resveratrol، IC50 ، المؤشر المركب ، السمية القلبية .

Introduction

Colorectal cancer (CRC) is ranked in the third order of the most common cancers over the world and it becomes an emanating health problem and a leading cause of death among all types of cancer in both men and women [(1)]. Hepatocellular carcinoma (HCC) also is considered the third leading cause of death cases related to cancer worldwide. The number of cases diagnosed each year is more than half million. The HCC is characterized by its poor prognosis due to the lack of early observed symptoms and limited therapeutic options [(2)].

Doxorubicin (DOX) is one of the most effective anticancer drugs in treating various forms of cancers; however, DOX-induced cardiotoxicity (DIC) limited its therapeutic effectiveness. The exact molecular mechanisms of (DIC), include mechanisms dependent on mitochondrial dysfunction such as DOX influence on the mitochondrial electron transport chain, redox cycling, oxidative stress (OS), calcium dysregulation, and apoptosis pathways [(3,4)].

The resistance of cancerous cells to chemotherapeutic agents is one of the main challenges in cancer treatment [(5)]. Due to such challenges and due to drawbacks and severe toxicity of chemotherapeutic agents used in cancer therapy, several approaches are being explored in cancer research that aim to overcome such problems. Improving efficacy and providing less toxicity are the target of such investigations [(6)]. The use of natural products/herbal medicines is one of such attractive investigations in cancer research [(7)]. The combination of natural compounds with the conventional treatment of cancer such as chemotherapy and radiotherapy were found to enhance the efficacy and meliorate the side effects of such treatment modalities [(8)].

Polyphenolic compounds are the most abundant group of all phytochemicals [(9)]. The primary characteristic that leads to their chemo-preventive and therapeutic actions on cancer is their powerful antioxidant effect [(10)].

Baicalein (BA) and resveratrol (RSV) are polyphenolic compounds; where, BA is a flavone which is subclass of flavonoid [(11)] and is derived from Chinese herbal medicine *Scutellaria baicalensis* Georgi [(12)]; and RSV is a polyphenolic compound that is the most common example of stilbenes [(13, 14)] and is found in a variety of natural sources; it is highly abundant in red grapes; mainly in the skin, in berries, peanuts, red wine, pineapple and others. The present study aims to explore the possible enhancement effect of baicalein and resveratrol when combined with doxorubicin using a different combination ratio on two different cancer cell lines: HCT116 (colorectal cancer cells) and HepG2 (hepatocellular cancer cells). It also investigates the possibility of such natural compounds to provide a protection effect on cardiocyte (H9C2) when baicalein and resveratrol treatment followed by doxorubicin.

Materials and Method

Materials

Baicalein (BA), RSV and DOX were obtained as reference standards with purity > 98% from AdooQ BioScience (Canada). Dulbecco's Modified Eagle Medium (DMEM) (high glucose and low glucose) and other cell culture materials were obtained from EuroClone (Italy). Fetal bovine serum was obtained from Biowest (France). Dimethylsulfoxide (DMSO, analytical grade) was obtained from GCC (UK). The viability of cells treated with DOX, BA, and RSV was determined by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT kit) from Promega Corporation (USA).

Cell Culture

HCT116, HepG2 cells & H9C2 rat myoblast cells were obtained from ECACC (UK). Cells of HCT116 and H9C2 were maintained in DMEM (high glucose) medium supplied with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin whereas HepG2 was maintained in DMEM (low glucose) medium supplied with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. Cells were incubated in a CO_2 incubator at 37C°, with 5% CO_2 and 95% filtered air. All the studies were conducted at Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Amman, Jordan.

Drug combinations

Baicalein (BA), RSV & DOX were prepared in 10 serial dilutions starting from 100 µg/ml. A stock solution of each agent was prepared in DMSO with concentration not exceeding 0.5%, while the dilutions were obtained using a suitable medium.

HCT116 & HepG2 cells were treated with either fixed combination ratio (1:1:1) from the three agents over the concentration (conc) range 0.195 – 100 µg/ml or with non-fixed ratio using approximating values for the IC50 of BA & RSV and concentration range 0.0195 – 100 µg/ml of DOX. Agents were added in consecutive manner

starting always with BA followed by RSV and ends with DOX. The H9C2 cells were treated with only one combination of the three agents, which is 1:1:1 in the conc range 0.0195 – 100 µg/ml.

Cell viability assay

Effect of BA, RSV & DOX combination on HCT116, HepG2 and H9C2 was evaluated by MTT assay. Cells were seeded into 96-well plate at a density of 7000 cells/well in suitable medium. After 24hrs of incubation at 37 °C, cells were treated with these agents and their combination in various combination ratios and various conc for 48 hrs. The MTT was then carried out using MTT kit where 15µl of the reagent was added to each well and incubated for 4hrs at 37°C followed by the addition of 100µl of the solubilization stop/mix solution and incubation for 1hr before measuring the absorbance using microplate reader [Enzyme Linked Immunosorbent Assay (ELISA)] at 590nm.

Data Analysis

The toxicity versus concentration curves (dose-response curves) of compounds and their combinations were obtained. The half-maximal inhibitory concentration (IC50) was determined for each compound and the combination of the three agents in a fixed ratio on each type of cell using Systat software version (12.0). The effects of the combination were calculated for each experimental condition using the combination index (CI) method based on the median-effect analysis of Chou and Talalay,1984 (15) and by using Compusyn software (version 1.0). CI<1, CI=1, and CI>1 indicates synergy, additive, and antagonism, respectively.

Statistical analysis

All data were obtained from at least three independent experiments and expressed as mean±standard deviation (SD). Comparisons of the different groups were performed using Two-ways univariate analysis of variance (ANOVA), GraphPad prism software (version 7.0) was used. The $P<0.05$ was considered the minimal level of significance.

Results

The IC_{50} for each compound (BA, RSV & DOX) and for certain combinations of the three compounds and for each type of cells was determined (Table 1) after using a range of doses (concentration) & 48hrs incubation time followed by MTT assays that were performed to assess cell growth inhibition.

The IC_{50} then was used to generate fixed and non-fixed ratios for subsequent combination studies and for calculation of combination indices (CIs).Combination of the three compounds (BA, RSV & DOX) using fixed ratio (1:1:1) over the range 0.195–100 µg/ml or nonfixed ratio where IC_{50} of the two natural compounds was used with DOX that has concentration over the range 0.195–100 µg/ml. Dose-response curves were obtained for the combination of the three compounds (fixed and non-fixed ratio) on HepG2 & HCT116 respectively (Figures 1&2) and on cardiocyte (Figure 3). Combination indices were calculated also for these combinations on the three types of cells (HCT116, HepG2 and H9C2) (Tables 2, 3 & 4).

Table 1. IC_{50} values of individual drugs and their combination treatment on HCT116 and HepG2 cell lines.

Treatment type	IC_{50} value (µg/ml)	
	HepG2 cells	HCT116 cells
Doxorubicin	7.3	4.99
Baicalein	59.4	50.87
Resveratrol	27.8	35.15
Doxorubicin + Baicalein	0.287	0.175
Doxorubicin + Baicalein IC_{50}	NA	NA
Doxorubicin + Resveratrol	0.49	3.34
Doxorubicin + Resveratrol IC_{50}	NA	NA
Doxorubicin+ Baicalein + Resveratrol	0.676	0.3657
Doxorubicin+ Baicalein IC_{50}+Resveratrol IC_{50}	NA	NA

NA: Not applicable

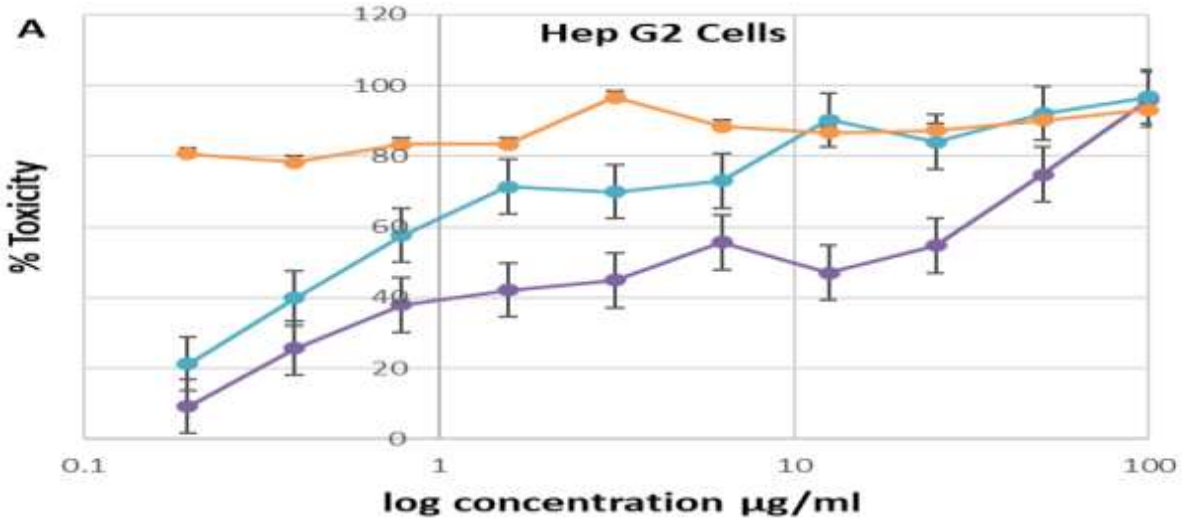

Figure 1. Log concentration versus toxicity of DOX, DOX+BA+RSV (1:1:1) and DOX+BA.IC_{50}+ RSV.IC_{50} (non-fixed ratio) against HepG2 cell line.

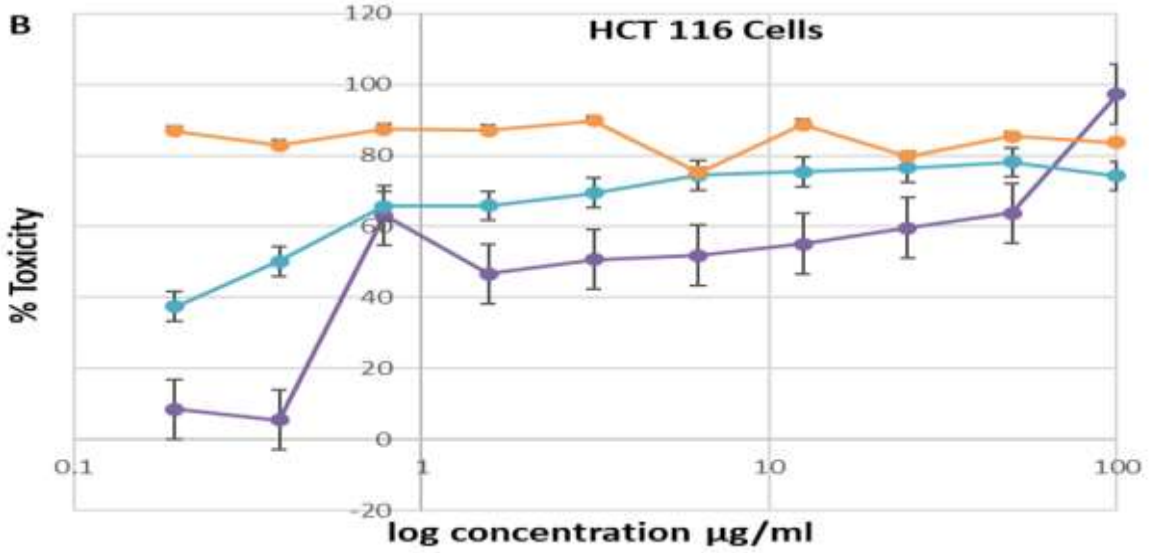

Figure 2. Log concentration versus toxicity of DOX, DOX+BA+RSV (1:1:1) and DOX+BA.IC_{50}+ RSV.IC_{50} (non-fixed ratio) against HCT116 cells.

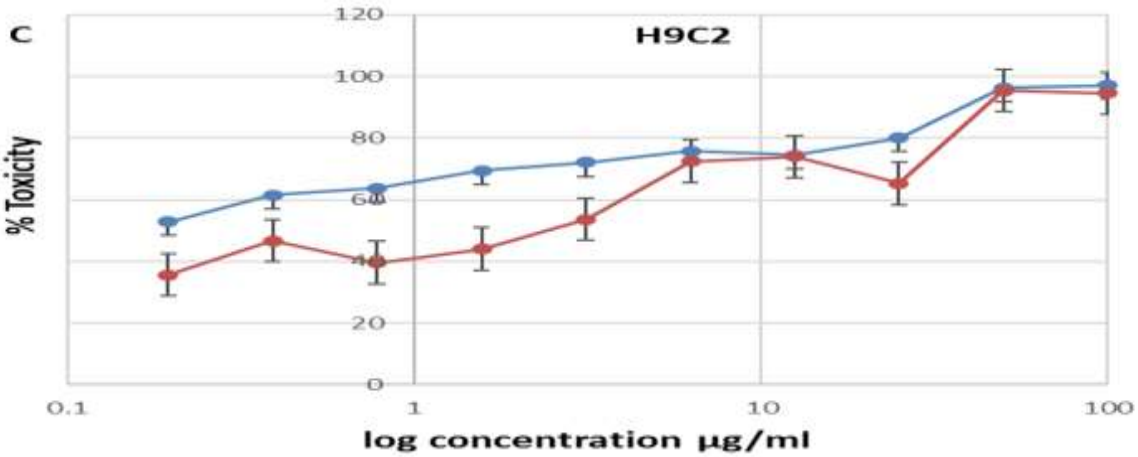

Figure 3. Log concentration versus toxicity of DOX and DOX+BA+RSV (1:1:1) combination against H9C2 cells.

Table 2. Combination index (CI) for the combination of doxorubicin (Dox), baicalein (BA) and Resveratrol (RSV) using a constant ratio (1:1:1) against HCT116 and HepG2 cell lines.

Doxorubicin + Baicalein + Resveratrol combination				
Concentration (µg/ml)			Combination Index (CI) at Constant ratio (1:1:1)	
Dox	BA	RSV	HCT116	HepG2
100	100	100	6.45071	0.80575 *
50	50	50	2.51902	0.66906 *
25	25	25	1.39832	0.67603 *
12.5	12.5	12.5	0.75036 *	0.19925 *
6.25	6.25	6.25	0.39897 *	0.37898 *
3.125	3.125	3.125	0.26753 *	0.23337 *
1.562	1.562	1.562	0.16359 *	0.10534 *
0.781	0.781	0.781	0.08182 *	0.12871 *
0.391	0.391	0.391	0.09682 *	0.19875 *
0.195	0.195	0.195	0.10021 *	0.42671 *

* Indicates synergism

Table 3. Combination index (CI) for the combination of doxorubicin (Dox), baicalein (BA) and resveratrol (RSV) using a non-constant ratio against HCT116 and HepG2 cell lines.

Doxorubicin + Baicalein + Resveratrol combination				
Concentration (µg/ml)			Combination Index (CI) at Non-constant ratio	
Dox	BA	RSV	HCT116	HepG2
100	50	30	3.18083	0.58628 *
50	50	30	1.91412	0.62880 *
25	50	30	1.96734	0.60467 *
12.5	50	30	1.11004	0.50938 *
6.25	50	30	1.60200	0.40431 *
3.125	50	30	0.96704 *	0.22792 *
1.562	50	30	1.04398	0.45588 *
0.781	50	30	1.02333	0.44342 *
0.391	50	30	1.14895	0.53132 *
0.195	50	30	1.03185	0.48068 *

* Indicates synergism

Table 4. Combination index (CI) for the combination of doxorubicin (Dox), baicalein (BA) and resveratrol (RSV) using a constant ratio (1:1:1) on H9C2 cells.

Doxorubicin + Baicalein + Resveratrol combination			
Concentration (µg/ml)			Combination Index (CI) at constant ratio (1:1:1)
Dox	BA	RSV	H9C2
100	100	100	0.94673
50	50	50	0.33128
25	25	25	23.8670 **
12.5	12.5	12.5	5.08621 **
6.25	6.25	6.25	2.96175 **
3.125	3.125	3.125	8.16311 **
1.562	1.562	1.562	8.89197 **
0.781	0.781	0.781	6.65993 **
0.391	0.391	0.391	1.82591 **
0.195	0.195	0.195	2.36383 **

** indicates antagonism.

Discussion

Natural products baicalein (BA) and resveratrol (RSV) combinations along with doxorubicin (DOX) at constant ratio (1:1:1) produced significant increase in DOX toxicity compared to treatment with DOX alone in both types of cancer cell line. The significant reduction in IC50 of DOX was yielded from this combination (from 4.99µg/ml to 0.3657µg/ml and from 7.3µg/ml to 0.676µg/ml DOX) on HCT116 & HepG2, respectively. The interaction between the three compounds produced a synergistic effect that was predominant at a lower concentration of DOX (12.5-0.195 µg/ml) in HCT116 cells, while the synergistic interaction between the three compounds in HepG2 was independent of dose and was predominant at all concentration levels indicating that diversity in mechanisms of action of the three agents yielded better antiproliferative and anticancer activity on such cancerous cells. The cytotoxic effect produced by this combination in HepG2 cells was greater compared to HCT116 cells, indicating higher sensitivity and better response of these cells to this combination at all concentration levels, however, the results conversely changed at lower concentration levels (0.78–0.195 µg/ml) where the response of HCT116 to this combination was become more than that of HepG2 cells.

The combination of non-constant ratios between the three compounds (DOX + BA.IC_{50} + RSV.IC_{50}) produced a greater enhancement in DOX toxicity compared to treatment with DOX alone in both cell lines where additive effects were obviously seen at a lower concentration of DOX on HCT116 cells and synergistic interaction at all concentration levels on HepG2 cells. The behavior or cell response to the cytotoxic action of this combination seems to be similar between the two cell lines (HCT116 & HepG2) with minor variation at certain concentration levels.

The results obtained from the combination of three drugs using the non-constant ratio (DOX+BA.IC50+RSV.IC50) showed a greater cytotoxic effect compared to the combination between the three compounds and using the combination of constant ratio (1: 1: 1) (DOX + BA + RSV) on both types of cell lines. Such combinations between the three compounds and the use of two combination criteria are being studied for the first time, revealing a better cytotoxicity profile than the use of DOX alone and on both types of cell line. Comparing all previous results obtained with two or three drugs' combinations revealed that three drugs combination and particularly the using of non-constant ratio produced the best toxicity and also the best enhancement in Dox toxicity in all cancerous cell line used in this study compared to all other drugs combinations, however combination between DOX and BA using non-constant ratio (DOX + BA.IC50) has the best cytotoxic activity among all combinations on HCT116 cells.

Regarding H9C2 cells, DOX showed sever toxic effect on such cells whereas BA showed a true safety profile at all concentration levels, when used on rat cardiomyocytes H9C2, similar behavior was reported with RSV except the moderate toxicity produced at the highest concentration (100µg/ml). Significant cardio-protection and decrease in the DOX toxicity achieved when the two naturally occurring compounds were combined with DOX. Best decrease in DOX toxicity on this type of cells was obviously-noticed at lower concentration levels of the three compounds, which is being evaluated for the first time. This is truly-explained by the CI between the three compounds that was >1 indicating antagonistic interaction leading to decrease efficacy (toxicity) of DOX on such cells. Such finding agreed with previous studies that evaluate each compound (BA or RSV) separately from the other on the toxicity of DOX. The BA when combined with DOX produces a significant reduction in DOX cardiotoxicity. The underlying mechanism was through the antioxidant mechanisms in the heart of mice that were initially reduced by DOX. Owing to its potent antioxidant properties, BA decreases reactive oxygen species (ROSs) generation and the apoptosis induced by DOX in chick cardiomyocytes [16, 17]. The RSV also decreases DOX toxicity as previously revealed using the same cells of the H9C2, where activation of SIRT-1 pathway leads to protection from the upregulation of P53 induced by Dox along with weakening of the cascade of events such as cytochrome c release and over expression of Bax induced by Dox in such cells [18, 19]. Combination of both BA and RSV with DOX provides additional advantage over the combination of either BA or RSV with DOX by giving a variety of molecular mechanisms in cardio-protection and enhancing the safety profile of DOX.

Compared to another study, which indicated that the use of cynarine, a natural polyphenolic compound, was found to improve HCT-116 and HEP-G2 and it was found to decrease the cardiotoxicity of DOX [20].

The development of these combinations between polyphenolic compounds with a wide range of anticancer activities (BA & RSV) and the well-known, potent chemotherapeutic drug, DOX, which resulted in a significant improvement in the cytotoxicity profile of DOX, may also offer an additional benefit by reducing the side effects of DOX. This is explained by the fact that the negative effects of DOX, particularly cardiotoxicity, a well-known and dose-dependent side effect, will be diminished when combined with such natural chemicals due to the considerable reduction of DOX concentration (IC50) [22]. Similar findings were

obtained from previous studies, which indicated that BA or RSV enhanced the cytotoxicity of DOX [23, 24].

Additionally, the addition of DOX to those two compounds improves the safety profile of the chemotherapeutic agent in the rat heart cells and offers additional protection to the cardiocytes. Low concentrations of these natural polyphenols' anticancer effects may hasten the nonmutagenic repair of DNA damage, and their therapeutic potential may interfere with DNA repair mechanisms [25].

The findings of this study also lend credence to a revolutionary method of treating cancer that involves combining natural substances with chemotherapy and other standard cancer treatments to increase their efficacy and lessen their adverse effects (8). However, the detailed information on the mechanisms of action at molecular levels of such combinations is required and needs further studies on both types of cell lines (HCT116 & HepG2) to investigate the actual mechanisms that stand behind the synergetic, additive and all interactions between those compounds.

Several combinations using BA, RSV, and DOX were tested on two cell lines (HCT116 & HepG2 cells) in an attempt to evaluate their effect on the enhancement of the anticancer activity of doxorubicin. Promising results were obtained from the combination of DOX with IC_{50} of BA and IC_{50} of RSV (Dox + BA.IC50 + RSV.IC50) that produced the best toxicity and the best enhancement in DOX toxicity in both cancerous cell lines at lower concentration of DOX. In conclusion, this study tried to highlight the use of combinations between natural polyphenolic compounds and chemotherapeutic drugs to improve the cytotoxic activity of chemotherapeutic drug and to eliminate their side effects, which is considered a novel approach in cancer therapy [21].

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Conflict of interest

The authors declare no conflict of interest.

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