Original Article

**R-(-)-carvone Attenuated Doxorubicin Induced Cardiotoxicity In Vivo and Potentiated Its Anticancer Toxicity In Vitro**

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**Background:** Doxorubicin (DOX) is one of the most potent broad-spectrum antitumor and chemotherapeutic agents. However, it produces cardiotoxicity.

**Aim:** To investigate whether (R)-(-)-carvone exerts cardioprotective effect against DOX toxicity in vivo and in vitro.

**Study design:** Tissue culture and animal study.

**Methods:** The synergistic effect of (R)-(-)-carvone with DOX was evaluated in MCF7 cancer cell line while its protective effect against doxorubicin (DOX) toxicity was evaluated in normal heart cell line (H9C2) and in vivo. Furthermore, the mechanism of its cardioprotective effect was studied.

**Results:** (R)-(-)-carvone exerted cytotoxic action on MCF7 cancer cell line with IC₅₀ value of 14.22 µM and potentiated the cytotoxic action of DOX while it decreased the toxicity of DOX on normal heart cell line (H9C2). In BALB/c mice, (R)-(-)-carvone protected the heart from the toxic action of DOX as was evident by biochemical and histological studies. The protective effect of (R)-(-)-carvone on H9C2 heart cell line and on heart in vivo was due to an increase in catalase activity.

**Conclusion:** (R)-(-)-carvone has synergistic anticancer action with DOX on MCF7 cell line while decreasing its cardiotoxicity.

**Keywords:** Doxorubicin, Cardiotoxicity, Catalase, R-(-)-carvone, H9C2, MCF7

**INTRODUCTION**

Doxorubicin (DOX), an anthracycline antibiotic, is one of the most potent broad-spectrum antitumor and chemotherapeutic agents used since late 1960s [1]. Unfortunately, the cytostatic action of DOX in therapeutic doses is insufficient in many cases, therefore higher doses of DOX are used for treatment. As a result, systemic toxicity, particularly cardiotoxicity develops [2]. Recent research aims at increasing DOX efficacy in tumor cells while minimizing its associated toxicities to non-cancerous tissues [3]. To minimize DOX effective chemotherapeutic dose and thereby its side effects, a variety of approaches have been examined. One of these approaches is the use of natural compounds with anticancer or chemo-preventive properties that can be used in combination with DOX [4]. Another approach is the use of natural antioxidants that can ameliorate DOX-induced cardiotoxicity by reducing oxidative stress [5].

Carvone belongs to volatile monocyclic terpenoid that is a constituent of many essential oils, but it is mostly concentrated in caraway (Carum carvi), dill (Anethum graveolens) and spearmint (Mentha spicata) seed oils [6]. This monoterpen is present as two enantiomers differing in biological properties. These enantiomers are (S)-(+)-carvone (D-carvone) present in caraway seeds and (R)-(-)-carvone (L-carvone) present in mint leaves [6]. R-(-)-Carvone was used as an effective chemopreventive agent for colon carcinogenesis [7]. Also, (4S)-(+)-carvone caused about 60% reduction in forestomach tumor and nearly 35% in pulmonary adenoma formation in female A/J mice by inhibiting N-nitrosodiethyamine-induced carcinogenesis [8].
The protective effect of carvone against paclitaxel-induced retinal and optic nerve cytotoxicity was reported recently [9]. However, its protective effect against DOX-induced cardiotoxicity was not studied yet. Therefore, this study was designed to investigate the cardioprotective effect of (R)-(−)-carvone in a normal heart cell line (H9C2) in vitro and BALB/c mice in vivo. Also, the synergistic cytotoxic effect of (R)-(−)-carvone with DOX on MCF 7 cell line was investigated.

MATERIALS AND METHODS

In vivo study: Cardioprotective effect of R-(−)-carvone against DOX toxicity in mice

The study was conducted in the Experimental Animal Laboratory of the Faculty of Pharmacy, Al-Ahliyya Amman University. The ethical committee approved all submitted for this study (ethical approval number AAU-2/5/2018) at Al-ahlyyia Amman University. For acclimatization purpose mice were kept one week prior to the experiments under the standard environmental conditions (temperature 23±2 °C, dark/light period 12 h). Male BALB/c (Bagg albino) mice were divided into four groups each containing 8 animals weighing between 23–28 g. Group I; negative control group that received vehicle (2% tween 20) only. Group II; positive control that received vehicle and DOX (Sigma, USA) dissolved in sterile normal saline. Groups III and IV; treated groups that received R-(−)-carvone (Sigma, USA) (75 and 150 mg/kg suspended in 2% tween 20), respectively. Vehicle and R-(−)-carvone were given intraperitoneally (i.p.) once daily for 5 consecutive days (Figure 1). DOX (20 mg/kg) was given to groups II, III and IV as a single i.p. injection one hour after receiving treatment (vehicle or R-(−)-carvone) on the 4th day of the experiment. Animal euthanasia was on day 6 (48 h after DOX) under light ether anesthesia followed by cervical dislocation. The choice of R-(−)-carvone dose was based on previously conducted pilot studies while the choice of DOX dose was based on previous studies [10]. The DOX dose used produced 0% mortality with histological and biochemical changes evident for DOX cardiotoxicity.

Collection of blood samples and tissues preparation

After the designated period, blood samples were obtained from retro-orbital plexus using glass heparinized capillary tubes. Sera were separated and stored at −20 °C till biochemical analyses. Hearts were obtained immediately after scarification, washed several times with ice-cold saline, pieces of the heart were fixed in 10% buffered formalin for histopathological study and the remaining of heart was stored at −80 °C until homogenization.

Heart tissues homogenization

Using cell lysis buffer (RnD, Cat#895347, USA) about 100 mg of heart tissue was ice-cold homogenized (1:9 wt/vol) by Teflon homogenizer (Potter-Elvehjem). The homogenate was centrifuged at 12,000 g for 10 min at 4 °C. The collected supernatants were collected and kept at −80 °C until analyzed for catalase activity. Protein concentrations of supernatants were assayed by Lowry’s method [11].

Histopathological examination

The formalin-fixed tissues were embedded in paraffin, sectioned at 5 µm and the sections then stained with eosin/hematoxylin. Sections of the heart from each mouse in all groups were examined and photographed using light microscope MC 170 HD Leica Camera, Switzerland. All the qualitative histological analysis was performed by one of the authors (M.A.A.).

Biochemical tests

Total creatine kinase (CK, Biosystems Cat.#:11790) and lactate dehydrogenase (LDH, Biosystems, Cat.#:11580) activities were assayed in serum spectrophotometrically according to the manufacturer guidelines.

In vitro study: Cell viability assay

MCF 7 (an invasive breast ductal carcinoma) and H9C2 (normal cardiomyocyte) cell lines were obtained from The European Collection of Authenticated Cell Cultures (ECACC, UK). The cell lines were grown in a humidified 5% CO₂ atmosphere incubator at 37°C and in DMEM high glucose (Euroclone, S.p.A) containing 10% FBS (Fetal Bovine Serum), 10 g/L penicillin-streptomycin and 10 g/L L-glutamine. A stock solution of R-(−)-carvone and DOX (1:1 ratio) was freshly prepared in 2-fold serial dilutions. A stock solution of each agent was prepared in dimethyl sulfoxide (DMSO, 0.5%). Effect of R-(−)-carvone and DOX combination on MCF 7 and H9C2 was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Promega, USA). Cells were seeded into 96-well plate at a density of 7000 cells/well in a suitable medium. After 24 h incubation at 37 °C, cells were treated with R-(−)-carvone, DOX or their (1:1) combination in serial dilution ranging from 100 µM to 6.25 µM for 48 h. MTT was then carried out using MTT kit where 15 µl of the staining reagent was added to each well and incubated for 4 h at 37 °C followed by the addition of 100 µl of the solubilization stop/mix solution and incubation for 1 h before measuring the absorbance using microplate reader (Biotec, USA) at 590 nm.

Synergy
Synergy evaluation was performed for R-(-)-carvone in combination with anticancer drug (DOX). It was performed against MCF 7 and H9C2 cell line using MTT test as described above. The degree of synergism was determined by calculating the combination index (CI) using Compusyn software (version 1.0) based on the median-effect analysis of Chou and Talalay (1984) given that CI < 1, CI = 1, and CI > 1 indicates synergy, additive, and antagonism, respectively [12].

**Antioxidant test (catalase) assay**

For cultured cells, the cells were harvested and homogenized with cell lysis buffer (Cat. No: 895347, R&D Systems Inc. Minneapolis, USA) and sonicated in ice 3 cycles, 90 s/cycle using sonicator (TI-H-5 Elma, Schmidbauer GmbH, Germany). Catalase (Cayman Cat#707002, USA) test was performed according to manufacturer directions.

**Statistical analysis**

All data passed the normality test (Shapiro-Wilk). Values were presented as mean ± SD (standard deviation). Differences between group means in CK, LDH and catalase were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey’s test using GraphPad Prism version 6 (GraphPad Software, San Diego, USA). Results were considered statistically significant when \( p < 0.05 \).

**RESULTS**

**In vivo study**

The rat received dose of DOX (20 mg/kg) heart tissue histopathological analysis showed disorganization and degeneration of the myocardium. Concomitant treatment with R-(-)-carvone revealed normal muscle fiber architecture (Figure 2). DOX treatment increased CK and LDH activities in serum (Table 1). Only the high dose of R-(-)-carvone (150 mg/kg) was able to lower the activities of both enzymes significantly in animals that received R-(-)-carvone along with DOX.

**In vitro study**

The half maximal inhibitory concentration (IC\(_{50}\)) values of R-(-)-carvone, DOX and DOX and R-(-)-carvone combination (1:1) in H9C2 and MCF 7 cell lines are illustrated in Table 2. (R)-(-)-carvone was non-toxic up to 200 \( \mu \text{M} \) on H9C2 normal heart cells while it exerted cytotoxic action on MCF 7 cancer cell line with IC\(_{50}\) values 14.22 \( \mu \text{M} \). Using CompuSyn software, R-(-)-carvone/DOX exerted antagonistic effect on H9C2 cells but synergistic effect on MCF 7 cell line (Table 2). (R)-(-)-carvone potentiated the cytotoxic action of DOX on MCF 7 cell line while decreased the toxicity on normal heart cell line (H9C2) (Figure 3).

**Catalase assay**

Catalase activity was increased by R-(-)-carvone alone or by R-(-)-carvone/DOX combination in normal heart in vivo (Figure 4A). Similarly, both doses of R-(-)-carvone alone as well as the combination of R-(-)-carvone with DOX increased catalase activity in heart cell line (H9C2) (Figure 4B).

**DISCUSSION**

In the present investigation, the synergistic effect of R-(-)-carvone on DOX cytotoxicity was investigated in MCF 7 breast cancer cell line. MCF 7 cell line was chosen because DOX is used widely for treatment of breast cancer. However, the use of DOX is limited by its cardiotoxicity [13]. Therefore, the protective effect of R-(-)-carvone on normal heart cell line (H9C2) was studied. In the present study, R-(-)-carvone had cytotoxic effect on breast adenocarcinoma cell line (MCF 7) with IC\(_{50}\) value of 14.22 \( \mu \text{M} \). Other studies reported IC\(_{50}\) values of 166.2 \( \mu \text{M} \) [14], 1.2 \( \text{mM} \) [15] and 0.63 \( \mu \text{M} \) [16] on the same cell line. The difference in IC\(_{50}\) values could be due to the use of different carvone isomers in different studies, the use of different methods for assessing the cytotoxicity and/or different growth conditions.

The important finding of this study is that R-(-)-carvone exerted a synergistic cytotoxic effect when administered with DOX on MCF 7 while its effect was antagonistic on normal heart cell line (H9C2).

This indicates that R-(-)-carvone can decrease the toxicity on normal heart cells while increasing it on the tested cancer cell line in vitro. Similarly, the protective effect of R-(-)-carvone in vivo was evident on heart by lowering the elevation in CK and LDH activities caused by DOX treatment. Also, the histological degenerative changes produced by DOX were less in R-(-)-carvone-treated groups. It should be noted that only males were used in this study because male sex hormone level is almost constant while female sex hormones vary along the estrous cycle. The results of the present work may differ if females were used [17]. So, it is interesting to investigate the effect of R-(-)-carvone in females in future studies.

The findings of this study raise a question: Why R-(-)-carvone exerted a synergistic effect with DOX on MCF 7 cell line while it produced antagonistic effect on normal heart cell line? The mechanism of R-(-)-carvone antagonistic effect was not investigated herein. However, previous studies have reported that R-(-)-carvone induces p53, caspase 3 mediated apoptosis in breast cancer cell line and arrested MCF 7 cells in S phase of the cell cycle [15]. Another proposed mechanism for R-(-)-carvone action in...
MCF 7 cells is that L-carvone (R-(-)-carvone) inhibits the enzyme poly ADP ribose polymerase (PARP) [15]. In fact, several forms of cancer are more dependent on PARP than regular cells making PARP an attractive target for cancer therapy.

Different mechanisms were proposed to explain DOX anticancer action. These include targeting the enzyme topoisomerase II, which is essential in DNA replication and iron chelation. High level of topoisomerase II is usually present in cancer cells especially solid tumors like breast cancer because they proliferate so quickly. Another proposed mechanism for DOX anticancer action is iron chelation. Iron is important in cell proliferation, DNA synthesis, mitochondrial electron transport, and oxygen sensing. Due to high rate of proliferation in cancer cells, more demand for iron exists. Therefore, by chelating iron DOX slows down cancer cell growth rate. Unfortunately, this process creates iron mediated reactive oxygen species (ROS), which may cause cardiomyopathy [15].

The ROS elevation is a therapeutic approach used in cancer treatment. Increasing the endogenous ROS threshold level in cancer cells might also put normal cells of some organs such as kidney liver and heart vulnerable to oxidative toxicity [18]. In fact, the most frequently proposed mechanism to explain the complex pathophysiology of DOX-induced cardiotoxicity is oxidative stress [19, 20]. Therefore, current research aims at identifying pharmacological agents which may enhance oxidative stress in cancer cells and protect normal cells from oxidative damage [18]. It has been recently demonstrated using microarray analysis that DOX changed the expression of genes involved in oxidative stress pathway [21].

In the present study, R-(-)-carvone exerted its protective effect by increasing catalase activity in normal heart cell line (H9C2) in vitro as well as in heart of mice in vivo. Catalase is considered one of the major enzymes involved in the detoxification of hydrogen peroxide (H₂O₂). Overexpression of catalase in the heart of transgenic mice suppressed DOX cardiotoxicity [22]. The antioxidant effect of R-(-)-carvone was demonstrated previously in vitro [14] [23] while D-(+)-carvone also increased catalase activity in vivo [7]. A recent study demonstrated that carvone exerted a protective role against paclitaxel-induced retinal and optic nerve cytotoxicity by counteracting oxidative stress [9].

The use of antioxidant in cancer therapy is controversial. On one hand, antioxidants may play important role in reduction of the severity of drug’s adverse effects but on the other hand antioxidants may interfere with DOX therapy effectiveness [3] since DOX cytotoxic action involved oxidative stress and the production of free radicals [5]. Clinical and experimental studies have shown that dietary antioxidants not only reduce the adverse effects of anticancer agents but also increase the efficacy of conventional cancer therapy. However, some reports supported that antioxidant supplements during the cancer treatments reduce the effectiveness of anticancer therapy [18].

Several studies have reported the effectiveness of natural products in decreasing the toxicity of DOX by enhancing antioxidant defenses. For example, the monoterpen D-limonene suppressed DOX-induced oxidative stress and inflammation in kidneys of Wistar rats [24]. Similarly, the monoterpen geraniol had protective effect on cardiomyocytes neonatal rat ventricular that was subjected to oxidative stress [25]. Also, hesperetin ameliorated doxorubicin-induced cardiotoxicity by reducing oxidative stress [5]. In our study, the monoterpen R-(+)-carvone protected the heart in vivo by increasing catalase activity. At the same time, it synergistically enhanced the cytotoxic action of DOX on MCF 7 cancer cell line.

In conclusion, R-(-)-Carvone exerted cardioprotective effect through increasing catalase activity while enhancing the cytotoxicity of DOX on breast cancer cell line. Future investigations are needed to explore the mechanism of synergistic effect of R-(+)-Carvone with DOX on breast cancer and other different cancer cell lines. Also, clinical studies are required to determine the efficacy of R-(+)-Carvone as an adjuvant in cancer therapy, its optimal dose and effectiveness in a specific cancer type [18].

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Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: No financial disclosure was declared by the authors.

REFERENCES
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TABLE 1. Results of biochemical tests (mean ± SEM) of mice treated with vehicle, DOX, R-(-)-carvone, or R-(-)-carvone with DOX.

<table>
<thead>
<tr>
<th></th>
<th>Control (20 mg/kg)</th>
<th>DOX (20 mg/kg)</th>
<th>R-(-)-carvone (75 mg/kg) + DOX</th>
<th>R-(-)-carvone (150 mg/kg) + DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (U/L)</td>
<td>57.2±13.41</td>
<td>160.9±12.07*</td>
<td>157.9±14.97*</td>
<td>70.22±15.90*</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>242.9±12.80</td>
<td>556.2±47.75*</td>
<td>388.8±43.89**</td>
<td>354.2±27.10*</td>
</tr>
</tbody>
</table>

* Significantly different from control group at p<0.05
* Significantly different from DOX group at p<0.05
* Significantly different from R-(-)-carvone (75 mg/kg) at p<0.05

TABLE 2. IC₅₀ (µM) of R-(-)-carvone, DOX, and R-(-)-carvone/DOX combination in studied cell lines and their combination indices according to CompuSyn software.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (µM)</th>
<th>R-(-)-carvone</th>
<th>DOX</th>
<th>R-(-)-carvone/DOX</th>
<th>CI* for R-(-)-carvone/DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9C2</td>
<td>Non-toxic up to 200 µM</td>
<td>6.518</td>
<td>8.066</td>
<td>2.836</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>14.22</td>
<td>4.390</td>
<td>7.585</td>
<td>0.341</td>
<td></td>
</tr>
</tbody>
</table>

* CI: Combination index. CI>1 indicate antagonistic effect, CI<1 synergistic effect, CI=1 additive effect

FIG. 1. Scheme for experimental design
FIG. 2. Histology of heart from mice receiving only vehicle (A), DOX (B), R-(-)-carvone (75 mg/kg) plus DOX (C) and R-(-)-carvone (150 mg/kg) plus DOX (D). Note myocardial fiber injury in B represented by vacuolation of the cytoplasm (circles). Also, congestion in blood vessels is obvious (black arrow). R-(-)-carvone 150 mg/kg in D protected the heart from the effects of DOX (H & E stain).

FIG. 3. Cytotoxic effect of R-(-)-carvone alone or in combination with Dox in H9C2, and MCF7 cell lines. Carvone decreased cytotoxicity of DOX in H9C2 cells (A) and enhanced the cytotoxicity of DOX in MCF7 cells (B). The IC50 value of all treatments was measured by the MTT assay.

FIG. 4. Results of catalase activity in normal heart in vivo. Catalase activity was increased by R-(-)-carvone alone or by R-(-)-carvone/DOX combination in normal heart in vivo (A). Catalase activity in heart cell line (H9C2) Both doses of R-(-)-carvone alone as well as the combination of R-(-)-carvone with DOX increased catalase activity in heart cell line (H9C2) (B).