Doxorubicin

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The antibiotic doxorubicin is often used as an anti-neoplastic drug; however, many patients showed very unpleasant side-effects. Previous studies have demonstrated that dietary substances such as *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* may have anti-oxidant, anti-proliferative, and anti-inflammatory effects. The purpose of this study was to investigate the protective effects of a mixture of these components in an experimental model of doxorubicin toxicity. Rats ($n = 30$) received doxorubicin (5 mg/kg/day) for 4 weeks and were randomized to receive the dietary mixture 2 hours following the first doxorubicin injection and until the end of the experiment. Animals were killed following 4 weeks, and blood, liver, and heart were collected for further analysis. The dietary supplement improved the depressed body weight and food consumption induced by DOX. In addition, the nutraceutical mixture reduced oxidative stress, ameliorated the morphological score, and preserved liver and heart structure, demonstrating a protective effect. These data show for the first time that the mixture of *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* may be useful to reduce the side effects following treatment with doxorubicin, and might ameliorate the quality of life of patients following chemotherapy.

quality of life | heart | rubra | dexorubicin | scutellaria | baicalensis | citrifolia | morinda | nutraceutical mixture

1. Introduction

Cancer represents one of the main health problems worldwide. The anthracycline antibiotic adriamycin, or doxorubicin (DOX), is a well-known anticancer drug, widely used for the treatment of various types of tumors [1]. DOX acts through two different mechanisms of action, i) binding within DNA basis blocking both acid nucleic synthesis and gene expression and ii) inhibiting topoisomerase II enzyme [2]. Cytotoxic or apoptotic effects induced by DOX administration are induced by membrane composition alterations and oxygen free radicals formation [3].

Since DOX administration causes undesirable multi-organ damages, Doxorubicin is often intravenously administered as lipidic particles in order to reduce drug toxicity and to prevent side effects on normal tissues. For this reason, its clinical use is limited. Despite these attentions, various side effects may occur such as appetite lacking with nausea and vomit, immunosuppression, alopecia, hepatotoxicity, and cardiotoxicity (i.e., heart arrhythmia). In particular, cardiovascular risk is often associated with high doses of DOX administration; in fact, anthracyclines are responsible for both short- and long-term cardiotoxic effects, such as changes in myocardial structure and function, severe cardiomyopathy and heart failure up to cardiac transplantation or death [4].

Cardiotoxicity is the consequence of DOX interaction with topoisomerases which corresponds with anthracyclines mechanism of action [5]. DOX toxicity is also ascribed to free radical formation that causes lipid peroxidation [1]. The reductive activation of DOX by reductases, including the reductase domain of endothelial nitric oxide synthase [6], produces superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which amplify oxidative stress in cardiomyocytes [4][7][8].

Moreover, a carbonyl group is located in DOX structure, and its reduction to alcohol changes DOX into DOXol. DOXol is more polar than DOX and it is responsible for chronic cardiotoxicity when is used at high doses and for long time [9].

About 40% of patients suffer from liver injury following DOX treatment because liver receives, accumulates, and metabolizes high concentrations of DOX [10]. These effects may be considered as a consequence of free radicals formation and oxidative stress activation [11].

In recent years, new potential adjuvant therapies have been proposed to reduce DOX side effects; however, polytherapy is not well accepted by patients and may cause other undesired side effects.

Experimental and clinical studies have demonstrated health benefits and protective action of several natural compounds to manage the side effects related to chemotherapy [12]. These natural products, such as catechin contained in green tea, have a potential for preventing and treating cancer, cardiovascular, inflammatory, and neurodegenerative diseases [13]. Aloe *arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* are natural compounds largely used in traditional medicine, able to enforce the immune system with also anti-degenerative and depurative effects. *In vitro* and *in vivo* studies have demonstrated the anti-cancer properties of aloe [14][15] which consist of anti-proliferative, immunostimulatory, anti-inflammatory, and anti-oxidant effects [16]. In particular, aloe anti-tumor and anti-proliferative effects are carried out by aloenin-like substances (aloe-emodine), which act against cancer cell lines [17]. Annona muricata, whose fruit is known as Graviola, is traditionally used for the treatment of cancer [18]. Also annona muricata, as aloe, has antioxidant properties, in particular, in its ethanolic leaf extract [19]. Scutellaria baicalensis is a traditional herbal medicine widely used for the treatment of inflammatory diseases and bacterial infection through NF-kB inhibition [20]. Bilberry (Vaccinium myrtillus) plays an important anti-inflammatory effect through its high anthocyanins contents. Moreover, anthocyanins contained in bilberry have anticancer, anti-proliferating, and anti-angiogenic effects. Morinda Citrifolia and Beta vulgaris rubra show important antioxidant and anti-inflammatory activity. Furthermore, Beta vulgaris has an anti-cancer potential probably due to flavonoid glycosides contents. However, these natural compounds currently are not directly used as drugs, but they might be useful for the development of potential anti-cancer agents [21] and to prevent chemotherapy-associated side-effects. Previous studies have demonstrated that *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* carried out i) detoxifying action, removing toxins from organs and tissues; ii) antioxidant action, protecting from free radicals production; iii) antidegenerative action, inducing apoptosis in neoplastic cells; iv) immune system modulation, reducing the risk of infections [13][14][15][16][17][18][19][22][23][24][25]. These dietary

substances may also exert anti-inflammatory, anti-allergy, and neuroprotective properties, thanks to all natural active principles.

[26][27][28][29][30]

The purpose of this study was to evaluate the effects of a dietary supplement composed by a mixture of *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis,* and *Vaccinium myrtillus* in an experimental model of doxorubicin (DOX) toxicity in order to reduce cytotoxic damage caused by DOX. The hypothesis of this study was that the use of the dietary mixture may reduce the side effects induced by DOX.

2. Materials and Methods

2.1. Experimental Protocol

87 Sprague–Dawley rats were used in this study and were obtained from Charles River (Calco, Italy). Rats were maintained in the Animal Facility of the Department of Clinical and Experimental Medicine, under controlled environmental conditions (12 h light/darkness cycle, at 24 °C), and provided with standard food and water *ad libitum*. All procedures were carried out according to the standards for care and use of animals as stated in the ARRIVE guidelines [26]. The procedures were evaluated and approved by the Ethic Committee of the University of Messina.

Doxorubicin toxicity was induced by intraperitoneal injection of DOX (5 mg/kg) for 4 weeks. After treatment with DOX, and every 24 hours, animals ($n = 15$) were treated with a mixture of *Aloe arborescens* (0.6 g), *Annona muricata* (2.5 g), *Morinda citrifolia* (0.6 g), *Beta rubra* (0.6 g), *Scutellaria baicalensis* (0.15 g), and *Vaccinium myrtillus* (0.5 g) (1 ml/os) or 1 ml of saline solution ($n = 15$) until the end of the experiment (4 weeks). Each single compound (*Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus*, 1 ml/os) was also tested in 42 rats (7 per group). During the experimental procedure, animals were monitored to evaluate food and water intake, alopecia onset, and other toxicity signs. At the end of the experiment, animals were killed, and blood, heart and liver were collected for further analysis.

Heart and liver were stored in 10% buffered formalin for histological analysis; blood was centrifuged at 3,000*g* for 15 minutes at 4 °C, and was used to assess the markers of both oxidative stress and hepatic and cardiac damage.

2.2. Evaluation of Body Weight and Food Intake

Body weight and food intake were monitored every week. Body weight results were expressed compared to food intake, by using the following formula: food intake (g)/body weight (g) × 100.

2.3. Measurement of MDA Levels

Malondialdehyde (MDA) levels were measured in liver and heart as a marker of lipid peroxidation. Equal amounts of tissue (heart left ventricle and liver median lobe) were homogenized with the MICCRA D-1 homogenizer (Miccra

Gmbh, Müllheim, Germany), in 1.15% KCl solution, a 0.1 ml aliquot of the homogenate was added to a reaction mixture containing 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% thiobarbituric acid, and 700 ml distilled water. Samples were boiled for 1 h at 95 °C and centrifuged at 3,000*g* for 10 min. The absorbance of the supernatant was measured by a spectrophotometer at 650 nm.

2.4. Evaluation of Cardiac and Hepatic Damage

Blood samples were centrifuged by a refrigerated centrifuge at 3,000*g* for 15 min, and serum was used to measure total creatine kinase (CPK), specific creatin kinase as a marker of heart damage (CPK-MB) and glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) to evaluate hepatic damage. Total CK, CPK-MB, GOT, and GPT in serum were measured using commercially available ELISA kits (Abcam, Cambridge, UK; Cusabio, P.R.China; MyBioSource, San Diego, CA). Samples were run in duplicate, and the absorbance was read at 450 nm.

2.5. Histologic Evaluation

Histological examination was performed to assess heart and liver damage. Hearts and livers were collected and fixed in 10% neutral-buffered formalin. Fixed tissues were embedded in paraffin and cut into 5 µm serial sections. Sections were then stained with hematoxylin–eosin for light microscope examination. A score scale of values has been used to evaluate organ damage, assigning a score from 0 to 3, depending on the extent of the alterations observed in organs: 0, absent; 1, mild; 2, moderate; and 3, severe [27]. Infiltration of inflammatory cells, vascular congestion, and interstitial edema were the parameters considered for scoring heart and liver damage.

2.6. Statistical Analysis

All data are expressed as mean ± SEM for each group, and compared by using one-way ANOVA for non-parametric variables, with Tukey post-test for intergroup comparisons. A value of $P < 0.05$ was considered significant. Graphs were drawn using GraphPad Prism software version 5.0 for Windows (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Dietary Mixture Composed by Natural Compounds Increases Body Weight and Food Intake following DOX Administration

Animals treated with DOX showed a decrease of food and water consumption associated with weight loss, already following the first week of treatment. Food intake and body weight parameters were ameliorated following the administration of the dietary mixture, both during the first week and also during all 4 weeks (Figure 1A,B).

Figure 1. Food intake and body weight evaluated during the experimental period. Values were obtained from 15 animals per group and are expressed as means and SEM. Weight loss (A) #$P < 0.05$ Doxorubicin + vehicle vs

Control; *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle. Food intake (B) #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle.

Moreover, rats treated with the natural mixture showed a reduction of hair loss (alopecia), compared to untreated animals (data not shown).

3.2. Dietary Mixture Reduces Lipid Peroxidation

Determination of MDA levels was performed at the end of the experiment in order to estimate the extent of lipid peroxidation in the damaged tissues. Rats treated with DOX showed a remarkable increase of oxidative stress products. The administration of the dietary supplementation caused a significant reduction of MDA levels both in liver and heart ($P < 0.05$) (Figure 2A,B), decreasing tissue damage related to oxidative stress. In order to assess dietary mixture efficacy, MDA levels were also evaluated for every single compound. *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* alone reduced MDA levels compared to DOX-treated mice group. Interestingly, animals treated with the dietary mixture showed a greater reduction of MDA levels than animals treated with DOX and also compared to the single compounds alone (Figure 2C,D).

Figure 2. Malondialdehyde levels in liver tissue (A) #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle. Malondialdehyde levels in heart tissue (B) #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle. Malondialdehyde levels in liver tissue (C) #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, *Vaccinium myrtillus*, dietary mixture vs Doxorubicin + vehicle; §$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, *Vaccinium myrtillus*. Malondialdehyde levels in heart tissue (D) #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, *Vaccinium myrtillus*, dietary mixture vs Doxorubicin + vehicle; §$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, *Vaccinium myrtillus*.

Aloe arborescens, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* Mixture Decreases Biochemical Markers of Heart and Liver Damage

Doxorubicin administration induced both hepatic and myocardial damage in rats, as demonstrated by increased GOT, GPT, CK, and CPK-MB enzyme levels in serum, as markers of impaired tissues.

In fact, these enzymes are excessively released as a consequence of cell membrane damage in liver and heart. The administration of the mixture of *Aloe arborescens* (0.6 g), *Annona muricata* (2.5 g), *Morinda citrifolia* (0.6 g), *Beta rubra* (0.6 g), *Scutellaria baicalensis* (0.15 g), and *Vaccinium myrtillus* (0.5 g) caused the decrease of GOT, GPT, CK, and CPK-MB levels, confirming the protective role of the association of these natural substances in liver and heart, also preventing the side effects related to doxorubicin use (Figure 3(A–D)).

Figure 3. Total CK (A), CK-MB (B), ALT (C), AST (D), levels in serum of Control, Doxorubicin + vehicle, and Doxorubicin + dietary mixture. Values are expressed as the means and SEM of 15 animals. #$P < 0.05$ Doxorubicin + vehicle vs Control; *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle.

3.3. The Nutraceutical Mixture Reduces Cytotoxic Damage Induced by DOX

Animals treated with doxorubicin showed clear signs of damage with edema and degeneration both in heart and liver. Furthermore, myocytes vacuolization, myofibrils loss, and interstitial edema were detected (Figures 4A and 5A). The administration of the dietary supplementation ameliorated morphological and structural alterations both in heart and in liver, as shown in Figures 4B and 5B; heart and liver edema and degeneration were less evident in treated rats than in untreated DOX animals. These results demonstrated that the nutraceutical mixture reduced cytotoxic damage induced by DOX.

Figure 4. Heart H&E staining (original magnification ×10) of Doxorubicin + dietary mixture (A), Doxorubicin + vehicle (B). The graph represents the microscopic damage score (C). Values are expressed as the means and SEM of 15 animals. *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle.

Figure 5. Liver H&E staining (original magnification ×10) of Doxorubicin + dietary mixture (A), Doxorubicin + vehicle (B). The graph represents the microscopic damage score (C). Values are expressed as the means and SEM of 15 animals. *$P < 0.05$ Doxorubicin + dietary mixture vs Doxorubicin + vehicle.

4. Discussion

Doxorubicin is an anthracycline-group antibiotic commonly used for the treatment of a large number of tumors; however, its use is often related to several side effects such as lack of appetite, nausea, vomit,

immunosuppression, alopecia. Anthracyclines were detected to increase the superoxide anion and hydrogen peroxide formation; consequently, free radical formation and oxidative stress are responsible for epatotoxicity and cardiotoxicity in a drug dose-dependent manner [1][28].

Thus, the rationale of this study was to study whether the use of a mixture of *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* could ameliorate the side effects related to the use of doxorubicin. DOX administration caused a decrease of food and water intake, inducing weight loss, which is one of the main side effects due to chemotherapy. Moreover, animals treated with DOX showed hair loss, demonstrating that doxorubicin is also responsible for alopecia. Side effects related to chemotherapy are often due to oxidative stress; in fact, rats showed a significant increase of oxidative stress markers following doxorubicin injection.

The mixture of *Aloe arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* ameliorated body weight of treated animals, restoring their food and water consumption.

A previous study demonstrated that a single high dose of DOX (20 mg/kg) and increasing daily doses of DOX (5-25 mg/kg) enhanced cardiac NO level in rats [29]. Pacher et al. [30] showed that MDA products, evaluated as lipid peroxidation products, were augmented in the hearts of mice treated with DOX and were responsible for heart injury in left ventricle. Also in this experimental setting, DOX administration caused an increase of MDA levels, whereas the treatment with the single compounds decreased MDA levels compared to DOX-treated animals. Fascinatingly, the dietary mixture caused a greater reduction of lipid peroxidation both in liver and heart than the single compounds alone.

Moreover, heart and liver were preserved following treatment with the dietary supplementation, in fact, GOT, GPT, CPK-MB, and CPK were reduced in treated rats compared to animals that only received DOX, thus demonstrating that the nutraceutical formulation might prevent side effects DOX-related in heart and liver.

All these data were confirmed by histological analysis. Heart and liver sections showed that DOX induced organ failure, in fact edema, vacuolization, and degeneration were observed in rats treated with DOX. The nutraceutical formulation ameliorated organ structure, decreasing areas of edema and vacuolization, indicating that this dietary mixture may reduce cytotoxic damage induced by doxorubicin. These results led us to hypothesize that the efficacy of the dietary mixture is related to the antioxidant and anticancer activity of Aloe *arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus*: the combination strengthens the efficacy of each compound, as also demonstrated by MDA results. However, since DOX anticancer effects involve ROS production, a possible interference was considered between dietary mixture antioxidant effect and DOX antitumor activity. This possibility was ruled out thanks to previous experiments carried out in breast cancer cells which demonstrated that the dietary mixture does not interfere with DOX anticancer effect (unpublished data).

In conclusion, our results demonstrated, for the first time, that the mixture of Aloe *arborescens*, *Annona muricata*, *Morinda citrifolia*, *Beta rubra*, *Scutellaria baicalensis*, and *Vaccinium myrtillus* is useful to reduce the side effects following treatment with doxorubicin, and could be used in patients exposed to chemotherapy, ameliorating their quality of life.

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