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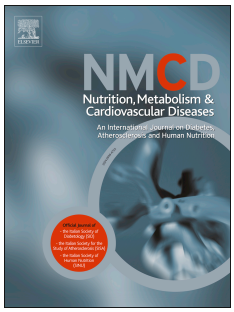

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PII: S0939-4753(17)30037-6

DOI: 10.1016/j.numecd.2017.02.002

Reference: NUMECD 1699

To appear in: *Nutrition, Metabolism and Cardiovascular Diseases*

Received Date: 22 September 2016

Revised Date: 1 February 2017

Accepted Date: 11 February 2017

Please cite this article as: Petroni K, Trinei M, Fornari M, Calvenzani V, Marinelli A, Micheli L, Pilu R, Matros A, Mock H-P, Tonelli C, Giorgio M, Dietary cyanidin 3-glucoside from purple corn ameliorates doxorubicin-induced cardiotoxicity in mice, *Nutrition, Metabolism and Cardiovascular Diseases* (2017), doi: 10.1016/j.numecd.2017.02.002.

Dietary cyanidin 3-glucoside from purple corn ameliorates doxorubicin-induced cardiotoxicity in mice

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Abstract word count: 234

Text word count 3649

Keywords: Doxorubicin; Anthocyanins; Purple corn; Cardioprotection

Number of references: 33

Number of figures: 5

Number of tables: 1

Number of supplementary material: 5

Abstract

Background and Aims: Anthracyclines are effective anticancer drugs that have improved prognosis of hundred thousand cancer patients worldwide and are currently the most common chemotherapeutic agents used for the treatment of blood, breast, ovarian and lung cancers. However, their use is limited because of a cumulative dose-dependent and irreversible cardiotoxicity that can cause progressive cardiomyopathy and congestive heart failure. Aim of the present study was to determine the cardioprotective activity of a dietary source of cyanidin 3-glucoside (C3G), such as purple corn, against doxorubicin (DOX) -induced cardiotoxicity in mice.

Methods and Results: In vitro studies on murine HL-1 cardiomyocytes showed that pretreatment with both pure C3G and purple corn extract improved survival upon DOX treatment. However, C3G and purple corn extract did not affect the cytotoxic effect of DOX on human cancer cell lines. We then validated *in vivo* the protective role of a C3G-enriched diet against DOX-induced cardiotoxicity by comparing the effect of dietary consumption of corn isogenic lines with high levels of anthocyanins (purple corn - Red diet - RD) or without anthocyanins (yellow corn - Yellow diet - YD) incorporated in standard rodent diets. Results showed that mice fed RD survived longer than mice fed YD upon injection of a toxic amount of DOX. In addition, ultrastructural analysis of hearts from mice fed RD showed reduced histopathological alterations.

Conclusion: Dietary intake of C3G from purple corn protects mice against DOX-induced cardiotoxicity.

Introduction

Anthracyclines such as Daunorubicin and Doxorubicin (DOX) are the most diffused anti-cancer drugs used against a variety of cancer types, including acute lymphoblastic and acute myeloid leukemia, Hodgkin lymphoma, multiple myeloma, breast and ovarian carcinoma, small cell lung carcinoma, sarcoma and neuroblastoma, epatocarcinoma and other gastric cancers. Anthracyclines block DNA and RNA synthesis by inhibiting topoisomerase-IIβ, thus preventing the replication of rapidly dividing cancer cells [1]. In addition, anthracyclines form oxygen radicals through the interaction with the intracellular iron pool. In turn, accumulation of reactive oxygen species (ROS) causes oxidative stress that leads to cell death particularly in the myocardium [2]. Indeed, anthracycline-induced cardiotoxicity occurs in almost 60% of treated patients and is the most important limitation of anthracycline chemotherapy [3]. Currently, dexrazoxane, an iron chelator, is the only approved drug for the prevention of anthracycline-induced cardiotoxicity in cancer patients, but its clinical use is controversial, due to haematological and hepatological toxicity [4, 5]. Another approach to prevent the onset of severe cardiomyopathy upon DOX chemotherapy is the treatment of patients with beta-blockers or angiotensin-converting enzyme (ACE) inhibitors [6, 7].

Supplementation of relatively high doses pro-anthocyanidins from cranberry and grape extracts [8, 9], anthocyanins from bilberry extracts [10, 11] or purified kaempferol [12] was found to protect against DOX-induced cardiotoxicity in murine models. Using isogenic anthocyanin-enriched plant material in rat models, we have previously demonstrated that dietary intake of cyanidin 3-glucoside (C3G) from blue corn reduced myocardial injury upon ischemia/reperfusion and increased omega-3 levels in blood and cardiac glutathione levels [13, 14].

Here we report the validation study of C3G supplementation against DOX-induced cardiotoxicity by using a novel isogenic plant material (*i.e.* purple corn) in mice and cell

culture models. Our results demonstrate that the protective effect of purple corn against DOX-induced cardiotoxicity *in vivo* may be mainly attributed to C3G and its derivatives and that a physiological dose of C3G was sufficient to prevent the typical DOX-induced cardiac alterations.

Methods

Maize production

Maize genotypes were originally in W22 background, homozygous dominant for the *a1*, *a2*, *c1*, *c2*, *bz1*, and *bz2* genes, homozygous recessive for the *r1* gene and different in *b1 pl1* constitution. To obtain anthocyanin-rich and anthocyanin-free corn with an isogenic background, a novel maize anthocyanin-rich hybrid was developed carrying the *B1* and *Pl1* alleles, which confer anthocyanin pigmentation in seed pericarp and all plant tissues [15-18]. Plant and seed tissues carrying the *b1 pl1* alleles are anthocyanin-free. To obtain ears with a high production of kernels, the homozygous inbred line *B1 Pl1* W22 and the *b1 pl1* W22 inbred line were crossed to a *b1 pl1* B73 inbred line and the F1 progeny seeds were used to produce two synthetic populations differing only in *b1 pl1* constitution.

Diets and mice

Special diets were produced by replacing the maize content (29%) from a standard diet formula (4RF21, Mucedola srl, Settimo Milanese, Italy) with powder from seeds of the anthocyanin-rich *B1 Pl1* hybrid (Red diet, RD) or from the isogenic anthocyanin-free *b1 pl1* yellow hybrid (Yellow diet, YD). The RD contained about 0.21 ± 0.01 mg/g anthocyanin. Both diets were equal in energy with 53.50% carbohydrate, 18.50% protein, 3% lipids and 6% fiber (Mucedola srl).

Experiments were performed on wild-type C57BL/6J mice, bred and maintained in a specific-pathogen-free (SPF, FELASA) room in controlled temperature and light conditions (21 ± 1°C, 12 h light/12 h dark cycle) at IEO (Ministry of Health authorization: DM N°86/2005 - 17/06/2005). Food and tap water were supplied *ad libitum*. Body weight of mice and food consumption were controlled weekly. All procedures were in accordance with the Italian law (D. Lgs n°2014/26, implementation of 2010/63/UE) and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

DOX treatment in mice

To assess mice survival, 24 female C57BL/6J mice (8 weeks-old) were fed YD (12 mice) or RD (12 mice) for 3 weeks and then subjected to an intraperitoneal (i.p.) injection of 15 mg/kg body weight of DOX (Adriablastin clinical grade, Accord Healthcare Limited, North Harrow, UK) dissolved in sterile saline solution. After injection, mice continued to receive YD or RD and mortality and general conditions of the animals were daily scored for 74 days. Life span of mice was analysed using the Kaplan Meier estimator [19].

Ultrastructural analysis of myocardial damage

Eight female C57BL/6J wild-type mice (8 weeks-old) were fed YD (4 mice) or RD (4 mice) for 3 weeks and then i.p. injected with 25 mg/kg body weight of DOX dissolved in sterile saline solution. Hearts were isolated on day 3 after i.p. injection with DOX and placed into fixative buffer containing 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 16 h at 4°C. Samples were then placed in a secondary fixative buffer containing 2% osmium tetraoxide in 0.1 M cacodylate buffer (pH7.4), then embedded, sectioned and processed for analysis with TEM by the Imaging Service at IEO.

Cell culture

HL-1 cardiomyocytes were cultured in Claycomb Medium, supplemented with 10% fetal bovine serum, 100 µg/ml penicillin/streptomycin, 0.1 mM norepinephrine and 2 mM L-glutamine. All culture dishes were pre-coated with gelatin/fibronectin substrate: 0.02% gelatin and 1:200 fibronectin. MCF-7 and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine. Aforementioned chemicals for cell culture were obtained from Sigma-Aldrich (St. Louis, USA). All the cells were maintained in a humidified incubator containing 5% CO_2 at 37°C. Red extract was produced by SVEBA Srl (Appiano Gentile, Italy) from *B1 Pl1* purple corn cobs through extraction with ethanol:H_2O (30:70 v/v) at 55°C for 1 h, titrated to a concentration of 2.5% anthocyanins and spray-dried to a final concentration of 25 mg/g of anthocyanins. Yellow extract was produced from *b1 pl1* yellow corn cobs using identical protocols and volumes as Red extract. A comparable total amount of flavonoids of Red and Yellow extract, apart from anthocyanins, as in the original raw plant material was verified by HPLC at 280 nm, as described in supplementary methods. Cyanidin 3-glucoside (C3G) was purchased from Extrasynthèse (Genay Cedex, France).

Prior to the treatment, HL-1 cells were plated at a density of 50 000 cells/well in 96-well plates and grown for 16 h. The cells were treated for 48 h with different concentrations of DOX (0, 0.125, 0.25, 0.5, 1, 2, 4 µM), C3G or Yellow and Red extracts converted to concentrations 0, 5, 25, 125, 250 µM using the molecular weight of C3G (484.84 g/mol). Cell viability was determined using 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) test as described elsewhere [20]. Briefly, 10% of MTT solution per volume was added to each well and incubated for additional 4 h. Formazan crystals were dissolved in acidified

isopropanol (isopropanol, 0.1N HCl, 0.1% Tween). The optical density was measured at 570 nm wavelength, by using a microplate reader (Tecan Infinite F200PRO).

In all experiments, HL-1 cells were pretreated with the chosen dosage of C3G or Yellow and Red extract (125 μM) for 16 h and then 0.250 μM DOX was added for 48 h. Cell viability was evaluated as above. Cells were harvested at 4°C for molecular analyses. The effect of Yellow and Red extract on the antitumor ability of DOX was tested in MCF-7 and HeLa cells, treated with DOX (0.250 μM) in presence or absence of Red extract (125 μM) for 48 h. Cell viability was determined as above.

RNA extraction and qRT-PCR analysis

Total RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany). About 1 μg of total RNA was reverse-transcribed using the RT Superscript^{TM} II (Invitrogen, Carlsbad, CA) and, after first strand cDNA synthesis using an oligo dT primer, the samples were diluted 50 fold and used as templates for qRT-PCR analysis. Genes analysed and primer sequences are indicated in Supplementary Table 1.

qRT-PCR analysis was performed using SYBR Green with the Cfx96™BioRad Real Time system in a final volume of 20 μL containing 5 μL of 50 fold diluted cDNA, 0.2−0.4 μM of each primer, and 10 μL of 2X SOS Fast EVA-Green Supermix (BioRad Laboratories, Hercules, CA). The protocol used was: 95 °C for 2 min, 55 cycles of 95 °C for 15 s, and 60 °C for 30 s. As a reference for normalization, we used the *GAPDH* gene. Relative quantification was analyzed using Cfx Manager Software version 1.6 (BioRad Laboratories). Data are expressed as transcript level relative to a calibrator (control sample) and represent means ± SEM obtained from measurements performed in triplicate on two biological replicates.

Statistics

All values were expressed as means ± SEM. Data about food consumption and body weight were analysed using two-way ANOVA and the Fisher LSD (Least Significant Difference) test as *post hoc* comparison. To assess the significance of the survival times of mice on YD and RD, we used the Log-Rank Test of the χ^2 square values (Supplementary Table 2). Gene expression data were analysed by one-way ANOVA and followed by Tukey's multiple comparison test. Differences were considered significant when $p < 0.05$. All statistical analyses were performed with GraphPad Prism version 4.0 (GraphPad Software).

Results

DOX-induced cytotoxicity in HL-1 cardiomyocytes was reduced by purple corn extract

DOX addition to HL-1 cells is known to induce cell death [2, 3]. We treated HL-1 cells with different DOX concentrations for 48 h, revealing that 0.250 μM DOX determined about 40% reduction of cell viability, and this was the dosage chosen for further experiments (Fig. 1A). To determine the protective role of C3G against DOX-induced cardiotoxicity, we used pure C3G or extracts from cobs of the anthocyanin-rich *B1 Pl1* purple corn (Red extract; Red) and of the anthocyanin-free *b1 pl1* yellow corn (Yellow extract; Yellow). HPLC analysis of anthocyanin composition of *B1 Pl1* seeds and cobs showed mostly cyanidin 3-glucoside (C3G) and its malonyl derivatives, with small amounts of peonidin 3-glucoside and pelargonidin 3-glucoside, whereas *b1 pl1* seeds and cobs were devoid of anthocyanins (Supplementary Figure 1 and Table 1). Notably, none of the tested concentrations of C3G or Red and Yellow extracts had a statistically significant effect on HL-1 viability up to 250 μM for 48 h (Fig. 1B-D). Then, to assess whether pretreatment with C3G was able to repress the DOX-induced cytotoxicity, HL-1 cells were pretreated with 125 μM of C3G or Yellow and Red extract for 16 h followed by 0.250 μM DOX for 48 h (Fig. 1E,F). DOX reduced viability

at roughly 50% of the control, whereas the presence of 125 μM of C3G and Red extract maintained cell viability at roughly 80-88% (Fig. 1E-F), confirming the cytoprotective value of the C3G present in the purple corn extract. Yellow extract produced similar result as trend, though not reaching statistical significance (Fig. 1F), thus suggesting that the presence of generic corn extract may interfere with the effect of DOX in the HL-1 culture system.

Purple corn extract downregulated the DOX-induced transcriptional activation of the RAS system genes in HL-1 cells

DOX treatment leads to ROS accumulation and to the activation of a dysfunctional myocardial remodeling mediated by angiotensin II (Ang II) signaling that contributes to long lasting heart damage [21]. Cyanidin was shown to scavenge ROS upon DOX treatment [10, 11, 22, 23] and the purple corn diet was found to increase glutathione levels and reduce lipid peroxidation to the heart (13). Cyanidin was also shown to reduce the expression of *renin1* and of the angiotensin converting enzyme (*ACE)* mRNAs in kidney cells [24]. To determine the effect of purple corn on the cellular response to DOX treatment in cardiomyocytes, we measured the transcript level of *renin1* and *ACE* genes [25] in HL-1 cells treated with DOX and the Yellow or Red extract. HL-1 were pretreated with 125 μM of Yellow or Red extract for 16 h followed by 0.250 μM DOX for 48 h. Both extracts inhibited the DOX-induced transcriptional activation of *renin1* and *ACE* in HL-1 cells (Fig. 2A,B), indicating that components of corn other than anthocyanins block DOX effects in HL-1 cells.

Purple corn extract did not affect the effect of DOX in tumor cell line

We have investigated the activity of the corn extracts on DOX toxicity on the human tumor MCF-7 and HeLa cell lines. As the HL-1 cells, MCF-7 and HeLa cells were pretreated with 125 μM of Yellow or Red extract for 16 h and then challenged with 0.250 μM of DOX for 48

h. As shown in Fig. 3, DOX reduced viability at roughly 60% compared to control in all cell lines analysed. Some cytotoxicity was observed to MCF-7 (about 20-30%; Fig. 3A) when treated with Red extract. However, the presence of Yellow or Red extract in combination with 0.250 μM DOX did not suppress its antitumor activity against MCF-7 and HeLa (Fig. 3A-B). These results indicate that Yellow and particularly Red extracts reduce DOX-induced cell death in cardiomyocytes, but not in transformed cells.

Anthocyanins from purple corn attenuate DOX-induced mortality

To validate the protective effect of a C3G-enriched diet against DOX-induced cardiotoxicity, mice were fed YD or RD for 3 weeks. Food consumption and body weight did not differ between the YD and RD groups (Supplementary Fig. 2) and both are comparable to the consumption of standard diet. Then, mice were injected with DOX (15 mg/kg body weight). Results revealed that mice fed RD showed an improved cumulative survival compared to YD fed mice, suggesting that dietary anthocyanins from purple corn have a protective effect against DOX-induced cardiotoxicity (Fig. 4). Notably, the mid-term cumulative survival (30 days) of mice fed RD was 100%, whereas for mice fed YD it was 75%. After 40 and 50 days, the cumulative survival of mice fed RD was still higher than for mice fed YD (Supplementary Table 2), whereas after 60 days mice under RD reached the same basic level of survival (41.7%) observed for mice under YD (Fig. 3 and Supplementary Table 2). At the end of the dietary treatment (74 days), the overall survival level was 25% for mice fed YD and 33% for mice fed RD (Supplementary Table 2).

Dietary anthocyanins from purple corn reduced the myofibril damage induced by DOX

To reveal effective cardioprotection in mice fed RD, we investigated by TEM, 3 days after DOX injection (25 mg/kg body weight), the ultrastructural morphology of the myocardium of

mice fed YD or RD for 3 weeks. As expected, hearts from mice fed YD and treated with DOX showed massive fiber fragmentation, lysis of myofibrils, interruptions in sarcolemma junctions and cytoplasmic vacuolization (Fig. 5A). Mitochondria appeared often swollen and irregularly distributed. Large lysosomes (autofagosomes) with mitochondria inside were also detectable, indicating the occurrence of extensive damage response (Fig. 5A). On the contrary, mice fed RD showed no significant alterations in the myocardium that maintained organized myofibril architecture. Mitochondria mostly retained a normal structure, with only rare fragmentation and no defects in sarcolemma junctions (Fig. 5B), indicating that dietary C3G from purple corn prevent the myocardial damage induced by DOX.

Discussion

The clinical use of DOX is restricted by its side effects that include cardiomyopathy and congestive heart failure [3]. Many studies reported that *in vivo* supplementation with flavonoids may reduce cardiac damages [8, 9, 12]. Here, we validated the protective effect of an anthocyanin-rich diet from purple corn (RD) compared to a diet without anthocyanins from yellow corn (YD) against DOX-induced cardiotoxicity by comparing isogenic corn lines. Previous studies showed that dietary supplementation of bilberry extracts significantly reduced DOX-induced cardiotoxicity in rats [10, 11], but daily intakes of anthocyanins used in these *in vivo* experiments were high (~ 244 or 500 mg/kg body weight/day) and could be achieved in humans only by assuming pharmacological doses of purified compounds [11]. However, we found that the consumption of RD, which provided about 36 mg/kg body weight/day of C3G was sufficient to prevent the typical DOX-induced cardiac alterations such as disorganized myofibrils, fragmentation and degradation of mitochondria, defects in sarcolemma junctions [26], which were instead observed after an acute dose of DOX in

cardiac tissues of mice fed YD. Nonetheless, it was also sufficient to obtain a significant mid-term attenuation of DOX-induced mortality, but not to reduce the long-term chronic effects after a sub-acute dose of DOX. Other *in vivo* studies using higher doses of anthocyanins [10, 11] or testing the preventive effects of other flavonoids [8, 9, 12, 27] were run in a shorter period of time (from 10 to 28 days) and have not performed a cumulative survival analysis. Furthermore, in previous studies it was not possible to determine which anthocyanin type was responsible for the protective effect, since several different glycosylated forms of cyanidin, delphinidin, peonidin, petunidin and malvidin were present in bilberry extracts together with a large amount of phenolic acids [28, 29]. In our study, we took advantage of isogenic corn lines, differing only in their capacity to synthesize anthocyanins in seeds and plant tissues. Purple corn mainly contains C3G and its acylated forms, whereas peonidin and pelargonidin are present only in small amounts. Since all other phenolic acids and flavonoids are identical between purple and yellow corn [16], the protective effect of purple corn against DOX-induced cardiotoxicity *in vivo* may be mainly attributed to C3G and its derivatives. However, pelargonidin may also contribute to reduce DOX-induced damages, as suggested by the protective effect of strawberry supplementation against DOX-induced hepatic oxidative stress in rats [30]. Surprisingly, our *in vitro* studies showed that a similar cytoprotective effect on HL-1 cardiomyocytes was obtained with both Yellow and Red extracts. A possible explanation is that the concentrations tested, despite within the range normally used in cell-based assays, were not physiological and differences between Yellow and Red extracts could not be observed as in the *in vivo* experiment. Apart from anthocyanins, other flavonoids present in purple corn are mainly quercetin derivatives and hydroxycinnamic acids, such as ferulic and p-coumaric acid [31]. It has been shown that the aglycone quercetin can decrease DOX-induced cytotoxicity and promote cell repair systems in rat H9C2 cardiomyocytes [32, 33].

Another issue considered was the effect of C3G-enriched extract on the antineoplastic activity of DOX. To this aim, our *in vitro* studies were performed on MCF-7 and HeLa transformed cell lines. Our results show that pretreatment with both Yellow and Red extracts did not compromise the antiproliferative effect of DOX, suggesting that not only anthocyanins, but also other flavonoids present in yellow corn did not interfere with the antitumoral activity of DOX.

Despite ROS generation during DOX intracellular metabolism represents the main mechanism underlying DOX-induced cardiotoxicity [2], other mediators, such as AngII, have been found to have a role in the development of DOX-induced cardiomyopathy [6]. Notably ACE inhibitors are used in clinic to prevent anthracycline-induced cardiotoxicity. We found that both Yellow and Red extract reduces the DOX-induced transcript expression of *renin1* and *ACE* in a cardiomyocyte cell culture as observed for kidney cells treated with C3G [24], again suggesting that other flavonoids, apart from anthocyanins, may contribute to reduce their expression Nonetheless, these data suggest that RD may counteract the abnormal intra-cardiac AngII signaling triggered by DOX treatment.

In conclusion, the present study established that purple corn-enriched diet protects against myocardial damages induced by DOX. The evidence that purple corn extract reduces the cytotoxic effects of DOX on cardiomyocytes, but not tumor cells, suggest purple corn as a beneficial functional food for patients subjected to anthracycline chemotherapy.

Acknowledgements

We received HL-1 cells as a gift from Dr. William Claycomb, (Louisiana State University Health Science Center, New Orleans, LA, USA), who first established and characterized this cell line. This work was supported by the Fondazione Umberto Veronesi, Milan, IT [Grant 2012 and Grant Fellowships to A.Marinelli and VC] and the EU-FP7 ATHENA collaborative

project [Grant Agreement 245121]. KP, CT, and MG designed research; RP provided corn; MF, A. Marinelli, VC, A. Matros, HPM and MT conducted research; LA Micheli performed statistical analysis of Kaplan Meier; KP and MG wrote the paper. The authors wish to thank Federica Tomay for critical reading of the manuscript. LA Micheli wish to thank Alberto Turco for his help and advice.

References

[1] Zhang S, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nature medicine. 2012;18:1639-42.
[2] Wallace KB. Doxorubicin-induced cardiac mitochondrionopathy. Pharmacol Toxicol. 2003;93:105-15.
[3] Zhang YW, Shi J, Li YJ, Wei L. Cardiomyocyte death in doxorubicin-induced cardiotoxicity. Archivum immunologiae et therapiae experimentalis. 2009;57:435-45.
[4] Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacological reviews. 2004;56:185-229.
[5] Hellmann K. Overview and historical development of dexrazoxane. Seminars in oncology. 1998;25:48-54.
[6] Ibrahim MA, Ashour OM, Ibrahim YF, El-Bitar HI, Gomaa W, Abdel-Rahim SR. Angiotensin-converting enzyme inhibition and angiotensin AT(1)-receptor antagonism equally improve doxorubicin-induced cardiotoxicity and nephrotoxicity. Pharmacological research. 2009;60:373-81.
[7] Colombo A, Meroni CA, Cipolla CM, Cardinale D. Managing cardiotoxicity of chemotherapy. Current treatment options in cardiovascular medicine. 2013;15:410-24.
[8] Elberry AA, Abdel-Naim AB, Abdel-Sattar EA, Nagy AA, Mosli HA, Mohamadin AM, et al. Cranberry (Vaccinium macrocarpon) protects against doxorubicin-induced cardiotoxicity in rats. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2010;48:1178-84.
[9] Li W, Xu B, Xu J, Wu XL. Procyanidins produce significant attenuation of doxorubicin-induced cardiotoxicity via suppression of oxidative stress. Basic & clinical pharmacology & toxicology. 2009;104:192-7.
[10] Choi EH, Park JH, Kim MK, Chun HS. Alleviation of doxorubicin-induced toxicities by anthocyanin-rich bilberry (Vaccinium myrtillus L.) extract in rats and mice. BioFactors. 2010;36:319-27.
[11] Ashour OM, Elberry AA, Alahdal A, Al Mohamadi AM, Nagy AA, Abdel-Naim AB, et al. Protective effect of bilberry (Vaccinium myrtillus) against doxorubicin-induced oxidative cardiotoxicity in rats. Medical science monitor : international medical journal of experimental and clinical research. 2011;17:BR110-5.
[12] Xiao J, Sun GB, Sun B, Wu Y, He L, Wang X, et al. Kaempferol protects against doxorubicin-induced cardiotoxicity in vivo and in vitro. Toxicology. 2012;292:53-62.
[13] Toufektsian MC, de Lorgeril M, Nagy N, Salen P, Donati MB, Giordano L, et al. Chronic dietary intake of plant-derived anthocyanins protects the rat heart against ischemia-reperfusion injury. J Nutr. 2008;138:747-52.
[14] Toufektsian MC, Salen P, Laporte F, Tonelli C, de Lorgeril M. Dietary flavonoids increase plasma very long-chain (n-3) fatty acids in rats. J Nutr. 2011;141:37-41.
[15] Petroni K, Pilu R, Tonelli C. Anthocyanins in corn: a wealth of genes for human health. Planta. 2014;240:901-11.
[16] Pilu R, Cassani E, Sirizzotti A, Petroni K, Tonelli C. Effect of flavonoid pigments on the accumulation of fumonisin B1 in the maize kernel. Journal of applied genetics. 2011;52:145-52.
[17] Radicella JP, Brown D, Tolar LA, Chandler VL. Allelic diversity of the maize B regulatory gene: different leader and promoter sequences of two B alleles determine distinct tissue specificities of anthocyanin production. Genes Dev. 1992;6:2152-64.

[18] Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C. pl-bol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant Journal. 2003;36:510-21.
[19] Tripepi G, Catalano F. [Kaplan-Meier analysis]. Giornale italiano di nefrologia : organo ufficiale della Societa italiana di nefrologia. 2004;21:540-6.
[20] Xiao J, Sun B, Cai GP. Transient expression of interferon-inducible p204 in the early stage is required for adipogenesis in 3T3-L1 cells. Endocrinology. 2010;151:3141-53.
[21] Toko H, Oka T, Zou Y, Sakamoto M, Mizukami M, Sano M, et al. Angiotensin II type 1a receptor mediates doxorubicin-induced cardiomyopathy. Hypertension research : official journal of the Japanese Society of Hypertension. 2002;25:597-603.
[22] Choi EH, Chang HJ, Cho JY, Chun HS. Cytoprotective effect of anthocyanins against doxorubicin-induced toxicity in H9c2 cardiomyocytes in relation to their antioxidant activities. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 2007;45:1873-81.
[23] Amorini AM, Lazzarino G, Galvano F, Fazzina G, Tavazzi B, Galvano G. Cyanidin-3-O-beta-glucopyranoside protects myocardium and erythrocytes from oxygen radical-mediated damages. Free radical research. 2003;37:453-60.
[24] Parichatikanond W, Pinthong D, Mangmool S. Blockade of the renin-angiotensin system with delphinidin, cyanin, and quercetin. Planta Med. 2012;78:1626-32.
[25] Baker KM, Booz GW, Dostal DE. Cardiac actions of angiotensin II: Role of an intracardiac renin-angiotensin system. Annual review of physiology. 1992;54:227-41.
[26] Balli E, Mete UO, Tuli A, Tap O, Kaya M. Effect of melatonin on the cardiotoxicity of doxorubicin. Histology and histopathology. 2004;19:1101-8.
[27] Saeed NM, El-Naga RN, El-Bakly WM, Abdel-Rahman HM, Salah ElDin RA, El-Demerdash E. Epigallocatechin-3-gallate pretreatment attenuates doxorubicin-induced cardiotoxicity in rats: A mechanistic study. Biochem Pharmacol. 2015;95:145-55.
[28] Latti AK, Riihinen KR, Kainulainen PS. Analysis of anthocyanin variation in wild populations of bilberry (Vaccinium myrtillus L.) in Finland. J Agric Food Chem. 2008;56:190-6.
[29] Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaja K, Kujala TS, et al. Antioxidant activity of plant extracts containing phenolic compounds. J Agric Food Chem. 1999;47:3954-62.
[30] Diamanti J, Mezzetti B, Giampieri F, Alvarez-Suarez JM, Quiles JL, Gonzalez-Alonso A, et al. Doxorubicin-induced oxidative stress in rats is efficiently counteracted by dietary anthocyanin differently enriched strawberry (Fragaria x ananassa Duch.). J Agric Food Chem. 2014;62:3935-43.
[31] Pedreschi R, Cisneros-Zevallos L. Phenolic profiles of Andean purple corn (Zea mays L.). Food chemistry. 2007;100:956-63.
[32] Chen JY, Hu RY, Chou HC. Quercetin-induced cardioprotection against doxorubicin cytotoxicity. Journal of biomedical science. 2013;20:95.
[33] Dong Q, Chen L, Lu Q, Sharma S, Li L, Morimoto S, et al. Quercetin attenuates doxorubicin cardiotoxicity by modulating Bmi-1 expression. British journal of pharmacology. 2014;171:4440-54.

Table 1: Tentative annotation of the major anthocyanins detected in the *B1 Pl1* anthocyanin-rich corn[1]

Compound	**R_t**	**UV/Vis λmax**	**LC-ESI MS**	**LC-ESI MS fragments[2]**	**Structure**
	min	*nm*	*(m/z)* $[M+H^+]$		
1	2.48	281/515	449.10	287.13 $(Cy)^+$	Cyanidin-glucoside
2	3.12	280/510	433.11	271.06 $(Pg)^+$	Pelargonidin-glucoside
3	3.48	280/515	463.12	301.07 $(Peo)^+$	Peonidin-glucoside
4	4.11	281/520	535.10	287.13 $(Cy)^+$	Cyanidin-malonylglucoside
5	4.89	280/506	519,11	271.06 $(Pg)^+$	Pelargonidin-malonylglucoside
6	5.27	281/519	549.12	301.07 $(Peo)^+$	Peonidin-malonylglucoside
7	5.34	281/519	621.10	287.05 $(Cy)^+$	Cyanidin-dimalonylglucoside

[1]Peaks 1 to 7 (as indicated in Supplementary Figure 1C**)** were identified based on photo diode array absorbance and mass fragmentation patterns. The purified substances were analysed by HPLC and ESI-MS. Spectral characteristics, molecular ions and fragments of the different compounds are presented. [2]Fragments: Cyanidin (Cy); Peonidin (Peo); Pelargonidin (Pg).

Figure legends

Figure 1. Cell viability of HL-1 cells treated for 48 h with DOX (A), C3G (B), Red extract (Red) (C) or Yellow extract (Yellow) (D), was determined by MTT test and expressed as the relative percentage of controls (untreated samples). HL-1 cells were pretreated with 125 μM of C3G (D) or Yellow and Red extract (E) for 16 h followed by 0.250 μM DOX for 48 h. Cell viability was measured by MTT test and expressed as the relative percentage of controls (CNT). Results are means ± SEM of three independent experiments. **** $p<0.0001$; ***$p<0.001$; **$p<0.01$; *$p<0.05$ indicate significant differences (one-way ANOVA) versus CNT+DOX.

Figure 2. Expression levels of *renin1* and *ACE* transcripts in HL-1 cells pretreated with 125 μM of Yellow or Red extract for 16 h followed by 0.250 μM DOX for 48 h. Data represent means ± SEM of three replicates from two independent experiments. **$p<0.01$; *$p<0.05$ indicate significant differences (one-way ANOVA) versus CNT+DOX.

Figure 3. Effect of Yellow and Red extract on the antitumor activity of DOX. Cell viability of MCF-7 (A) and HeLa (B) cells treated with 0.250 μM DOX in presence or absence of Yellow or Red extract (125 μM for 16 h prior to DOX exposure) measured by MTT test. Values are expressed as the relative percentage of controls (CNT). Results are means ± SEM from three independent experiments. ***$p<0.001$; **$p<0.01$; *$p<0.05$ indicate significant differences (one-way ANOVA) versus CNT+DOX.

Figure 4. Cumulative survival of C57BL/6J mice fed YD or RD and injected with DOX (15 mg/kg body weight); n=12/group. Kaplan-Meier survival plot; for statistical analysis of survival of dietary treatments see Supplementary Table 2.

Figure 5. Electron micrographs showing preventive effect of dietary C3G from purple corn on DOX-induced alterations in cardiac tissues from mice fed YD (A) or RD (B) for 3 weeks and injected with DOX (25 mg/kg body weight). S, sarcolemma; Mf, myofibrils; L, lysosomes; arrows, fragmented mitochondria. Bars, 1 μm.

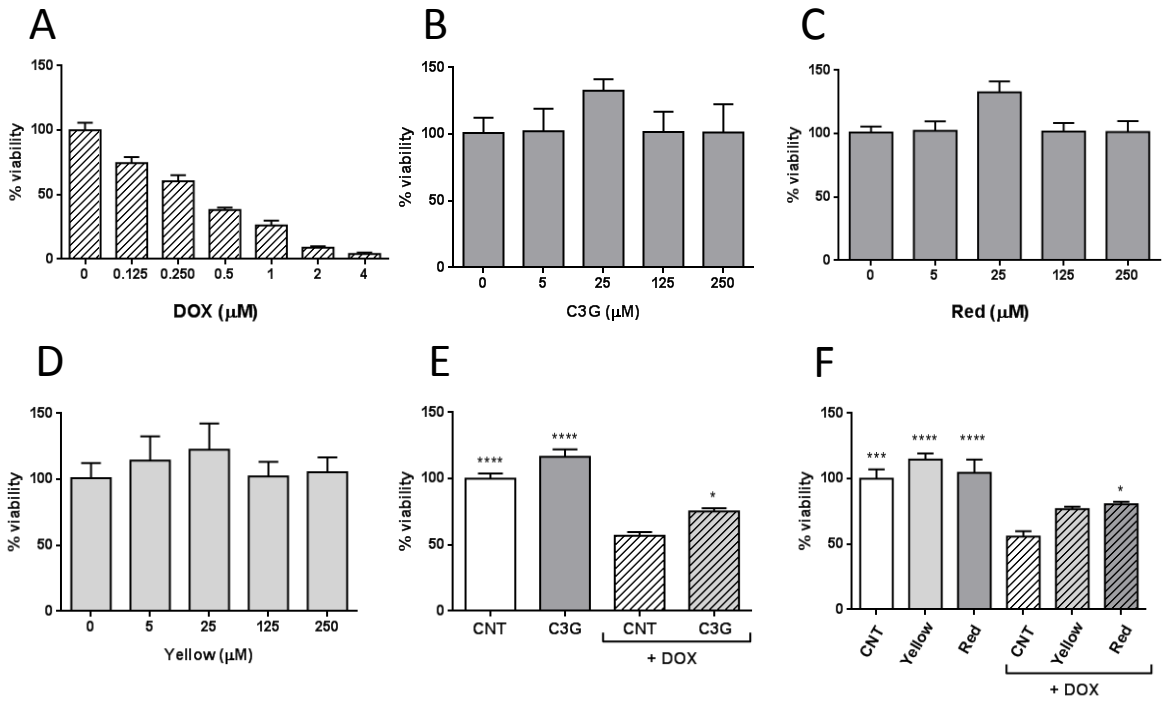
A
% viability
150
100
50
0
0
0.125
0.250
0.5
1
2
4
DOX (μM)
B
% viability
150
100
50
0
0
5
25
125
250
C3G (μM)
C
% viability
150
100
50
0
0
5
25
125
250
Red (μM)
D
% viability
150
100
50
0
0
5
25
125
250
Yellow (μM)
E
% viability
150
100
50
0
****
****
*
CNT
C3G
CNT
C3G
+ DOX
F
% viability
150
100
50
0
***
****
****
*
CNT
Yellow
Red
CNT
Yellow
Red
+ DOX

Fig. 1

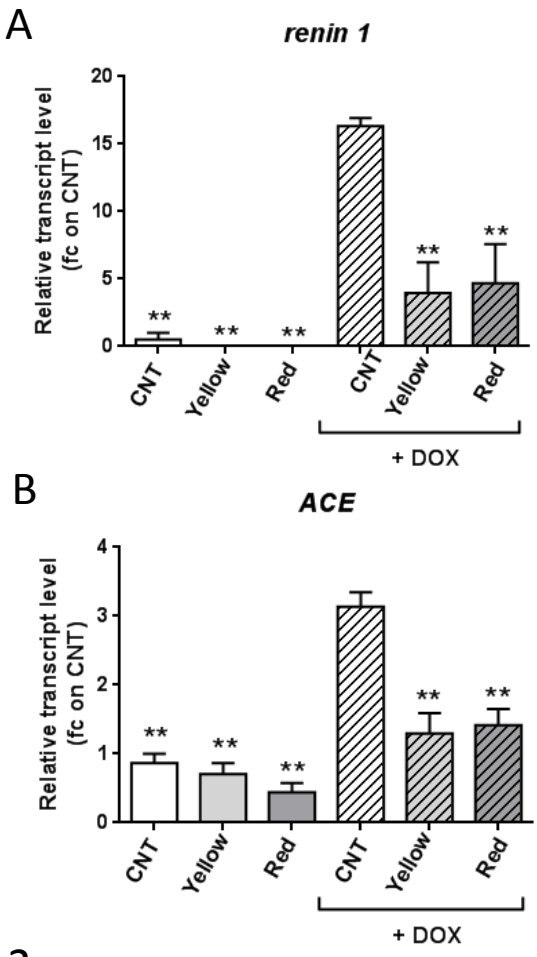
A
renin 1
Relative transcript level (fc on CNT)
20
15
10
5
0
**
CNT
Yellow
Red
+ DOX
B
ACE
4
3
2
1
0

Fig. 2

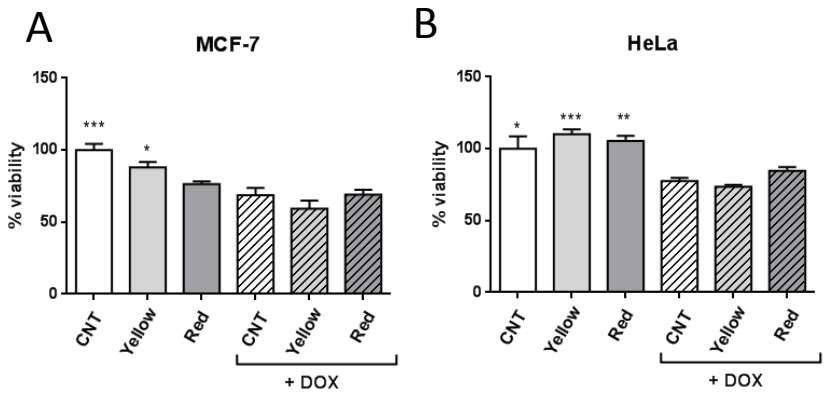
A
MCF-7
% viability
150
100
50
0
***
*
CNT
Yellow
Red
CNT
Yellow
Red
+ DOX
B
HeLa
% viability
150
100
50
0
*
***
**
CNT
Yellow
Red
CNT
Yellow
Red
+ DOX

Fig. 3

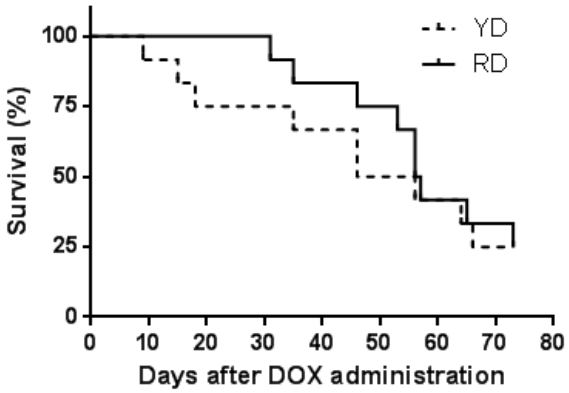
YD
RD
Survival (%)
100
75
50
25
0
0
10
20
30
40
50
60
70
80
Days after DOX administration

Fig. 4

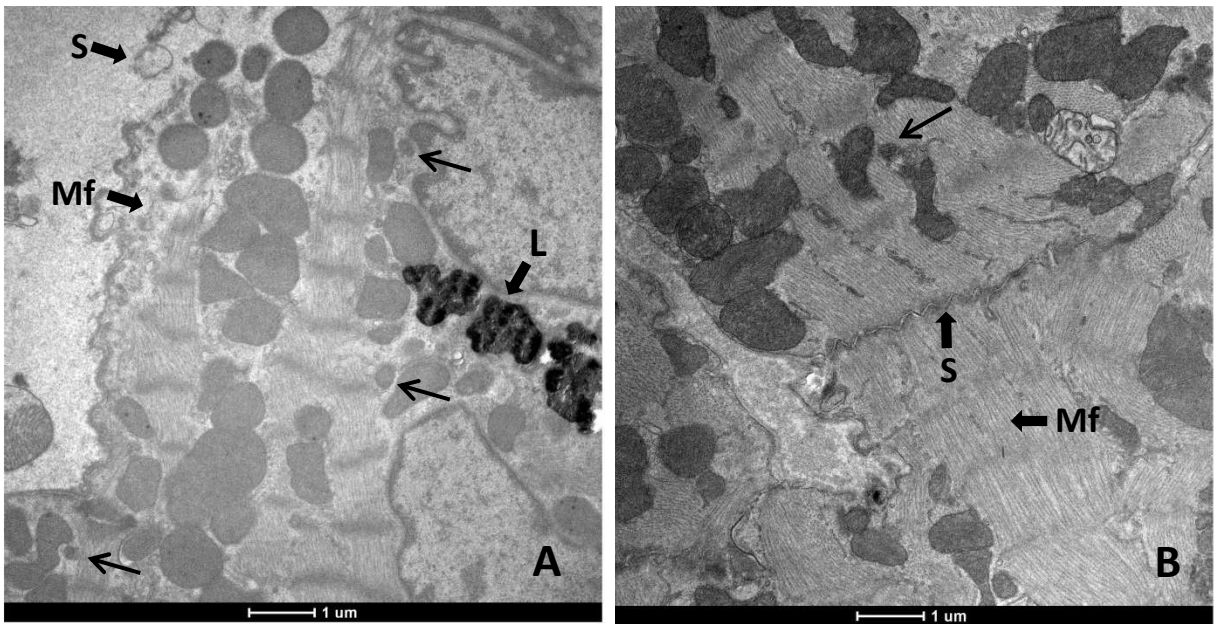

Fig. 5

Highlights -Petroni et al 2016-NMCD

- DOX-induced cytotoxicity in HL-1 cells was reduced by C3G and purple corn extract
- Dietary C3G from purple corn protected mice against DOX-induced cardiotoxicity
- Dietary C3G from purple corn reduced the histopathological alterations induced by DOX