in vivo-radioprotective affects of a standardized tocotrienol rich extract of annatto seeds (*Bixa orellana* L.)

E. Nathalie Pineda [1,2], Francesca V. Lobianco[1], Yadira F. Ordonez[3], E. Santiago Ojeda[3], Kimberly J. Krager[1], Shraddha Thakkar[1], Darin E. Jones[1], Nukhet Aykin-Burns[1], Philip J. Breen[1], Omar Malagón[3] and Cesar M. Compadre[1]*

[1]Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR 72205,

[2]Bioinformatics Program, University of Arkansas at Little Rock. Little Rock AR 72204,

[3]Chemistry Department, Universidad Técnica Particular de Loja, Loja, Ecuador.

Abstract

For the past decades, the US Government Radiation and Nuclear Countermeasures Program has been seeking the development of radioprotectant and radiomitigator agents. Nevertheless, currently there is no ideal drug that can provide muti-organ protection against lethal doses of radiation, is affordable, does not show toxicity, can be stockpiled and be easily and rapidly administered. In this research we have prepared a standardized tocotrienol rich extract, from annatto seeds, Bixa orellana L. The extract contains 60% tocols, including: δ-tocotrienol and γ-tocotrienol, in a 10:1 ratio. The extract was prepared via solvent extraction and column chromatography. Radioprotective effects of the rich extract against total body radiation were determined, when administered, 24 hours before radiation, by subcutaneous injection in a dose of 300 mg/kg body weight on CD2F1 mice. Two experimental groups received different single radiation doses (8.5 Gy and 9.5 Gy). A 30-day survival response showed 100 % survival in all mice radiated comparing with 0 % survival in a control group. This study suggest that annatto standardized extract has the potential to protect humans against radiation and may be promising option to develop into an economical and efficacious radioprotectant product.

Introduction

Recently it has been shown that δ-tocotrienol (DT3) and γ-tocotrienol (GT3) have potent radiation protection activity. Unfortunately, the difficulty to separate and isolate tocotrienols from tocopherols makes them very expensive to produce. In this research we have explored the possibility of using a tocotrienol standardized extract obtained form the annatto plant (*Bixa orellana,* L), as cost effective alternative to the use of pure DT3 or GT3. In this study we use in vitro H9C2 cardiomyocyte cell model to evaluate the bioactivity, antioxidant properties, and radioprotective effects of DG3. Then, we conducted a radioprotection survival study using an in vivo mouse model of lethal total body irradiation. In addition, our extract procedure yields tocotrienols at a rate that is at least 1000-fold more cost-effective than purchasing purified tocotrienols from commercial vendors.

Methods

Figure 1. Annatto (*Bixa Orellana,* L) seeds

Annatto seeds were purchased from local growers in Ecuador and Mexico. A crude tocotrienol extract was prepared by percolation with hexane at 25ºC. The hexane was concentrated at reduced pressure to produce a clear red-orange oily extract with a 3.3 % yield. The crude extract contains 7.2 % of DT3 and 0.93 % of GT3. The crude was storage at 4ºC.

An enriched tocotrienol extract, was prepared from the crude extract using column chromatography in silica gel 70-230 mesh, eluting with hexane-ethyl acetate (9:1) The extract was standardized to contain 60% of a mixture of DT3 and GT3 (10:1). The bioactivity of the extract was evaluated by measuring their antioxidant potential, as well as by using clonogenic survival assays and by measuring the cellular bioenergetics of H2O2 treated cells.

Methods

Survival studies were performed using CD2F1 male mice. The mice were feed vitamin E deficient diets for seven days before irradiation, to minimize the effect of dietary tocols on the radioprotection study. Twenty-four hours before total body radiation, mice received subcutaneous injection of 0.1 mL of vehicle or extract (adjusted to contain 300 mg/kg body weight). The vehicle was 5% Tween in PEG 400 solution. Injections were administrated subcutaneously near the femur with a 23-gauge needle. Mice received total body irradiation in two different single doses (8.5 Gy and 9.5 Gy) using a Mark I Model 68A cesium-137 animal irradiator (Shepherd & Associates, San Fernando California, CA).

Results

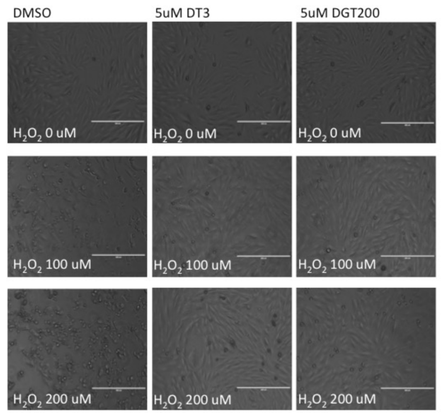

Figure 2. H9C2 heart cardiomyocytes were treated with 5 uM DT3 or 5 uM annatto extract or vehicle control (DMSO) overnight. Cells were then exposed to H_2O_2 for 4h. Morphological changes were recorded under an inverted microscope

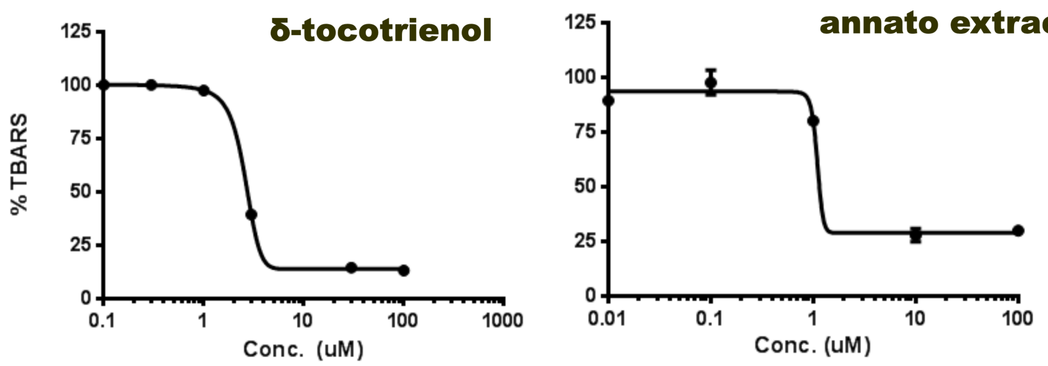

Figure 3. Inhibition of TBHP induced lipid peroxidation in Wistar rat liver microsomes by DT3 and enriched extract. Data are means ± SEM of triplicate experiments

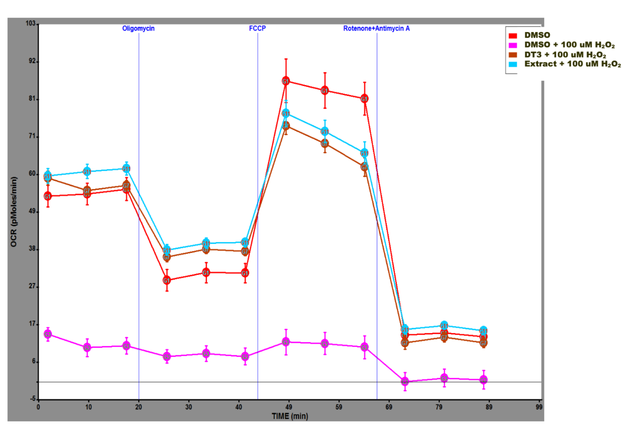

Figure 4. H9C2 heart cardiomyocytes were treated with 5 uM DT3 or 5 uM extract or vehicle control (DMSO) overnight. Cells were then exposed to 100 uM H_2O_2 for 4h in the presence or absence of tocotrienols. Cellular bioenergetic profiles were determined using XF96 Seahorse Extracellular Flux Analyzer

Results

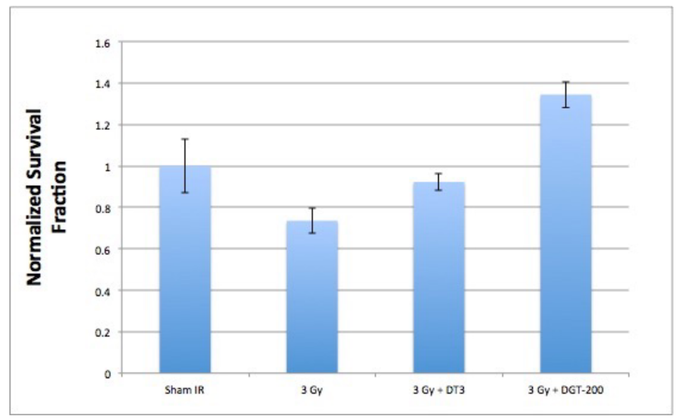

Figure 5. Clonogenic survival assay on H9C2 cells pretreated with tocols and received 3 Gy irradiation

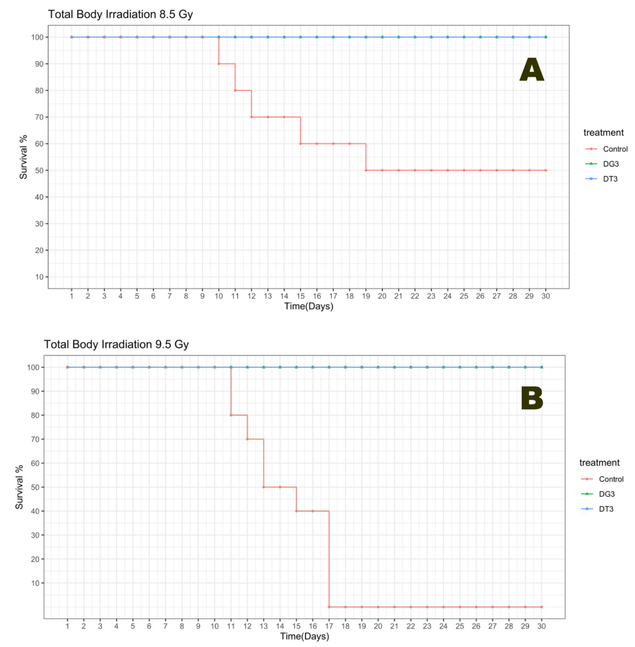

Figure 6. (A) Survival curve for CD2F1 irradiated mice at 8.5 Gy dose. (B) Survival curve for CD2F1 irradiated mice at 9.5 Gy dose.

Conclusions

An annatto extract, containing higher concentration of the natural tocotrienols, was prepared by extraction, purification, and standardization. In tests of its radioprotective efficacy, all mice treated with a single subcutaneous dose of annatto standardized extract survived a total body irradiation (TBI) at 8.5 and 9.5 Gy, which are otherwise lethal doses.

In summary, annatto standardized extract, an affordable source of tocotrienols, protected mice from lethal doses of total body radiation. This protection correlated with the total tocotrienol content. The contribution to the overall protective effect of annatto standardized extract from other, possibly radioprotective.

Acknowledgments

This research was supported in part by grants from the National Center for Research Resources award 1ULl RR029884, the IDeA Networks of Biomedical Research Excellence (INBRE), UAMS start-up funds, and NIH/NIAID grant AI67798 ENP was a recipient of a graduate scholarship from SENESCYT Ecuador.

The University of Arkansas has received patent protection for parts of this research. A potential royalty stream may occur consistent with the University of Arkansas policy.