Author's response to reviews

Title: Cytotoxic effects of Euterpe oleracea Mart. in malignant cell lines

Authors:

Dulcelena F Silva Dr (ddjonco@ig.com.br)
Flavia CB Vidal Dr (flavidal@hotmail.com)
Debora Santos Msc (debora.sqmc@live.com)
Maria Célia P Costa Dr (celiacosta@prof.elo.com.br)
José AM Díaz Dr (jmorgado.d@hotmail.com)
Maria do Desterro SB Nascimento Dr (cnsd_ma@uol.com.br)
Roberto S de Moura Dr (demoura@uerj.br)

Version: 2
Date: 7 April 2014

Author's response to reviews: see over
April 7, São Luís

Dr. Tom Rowles  
Editor-in-Chief  
BMC Complementary and Alternative Medicine  

Dear Dr. Tom Rowles,

Please find enclose a new version of our manuscript “Cytotoxic effects of *Euterpe oleracea* Mart. in malignant cell lines”, to be published in BMC Complementary and Alternative Medicine. In this new version, we have taken into consideration the comments of the Academic Editor and the Reviewer's, as described in the respective responses.

Sincerely yours,

Flávia Castello Branco Vidal  
Federal University of Maranhão  
Rua Coelho Neto nº 311  
São Luís, Maranhão, Brazil, 65020-140  
+55 98 2109-1273  
flavidal@hotmail.com
Revisions requested by the editor:

1. Source of the *Euterpe oleracea* Mart used in your study, and by whom it was formally identified. Please also confirm whether a voucher specimen of the plant material has been deposited in a publicly available herbarium, and include this information in your manuscript.

The informations were added to the first item of Methods by the name “Plant Material and Preparation of juçara hydroalcoholic extracts”.

Reviewer's report 1
Title: Cytotoxic effects of *Euterpe oleracea* Mart. in malignant cell lines
Version: 1 Date: 7 March 2014
Reviewer: Demetrios Kouretas

Major revision
In the present study, the authors investigated the cytotoxic effects of extracts from *Euterpe oleracea* Mart. plant against different malignant cell lines. The results showed that the extracts were especially effective against the growth of breast cancer cells MCF-7. Moreover, it was shown that the growth inhibitory effect on cancer cells was due to autophagy and not to apoptosis. The present study is well written and experimentally designed. Moreover, it provides new data about the possible beneficial effects of extracts from *Euterpe oleracea* Mart. plant on human health, since there are only few studies regarding its anticarcinogenic properties. The results are well presented and support adequately the conclusions. Of course, more research is needed so as to elucidate the molecular mechanisms involved in the antitumour effects of the extracts. thus, I would recommend the publication of the manuscript as long as the authors answer the following remarks:

Major compulsory revisions

1. Did the authors assess the polyphenolic composition of the tested extracts?

No, we only quantified the total polyphenolics contents of three different extracts. According to Ainsworth and Gillespie (2007) the Folin-Ciocalteu technique is a quantification method to total phenolic content in food products or biological samples and are based on the reaction of phenolic compounds with a colorimetric reagent, which allows measurement in the visible portion of the spectrum. The identification of the polyphenolic compounds is one of the main goals that we want to achieve in a current work in our laboratory.

2. A table or figure should be added that would show the total polyphenolic content of the extracts.
As recommended by the reviewer, a table showing the total polyphenolic content of the extract was added in the new version of the manuscript.

3. Quality of written English: Needs some language corrections before being published.

Our study was submitted to editing by Editage, a brand of Cactus Communications Inc. They gave us a certificate of English Editing and if you desire we can send it for you.

Reviewer’s report
Title: Cytotoxic effects of Euterpe oleracea Mart. in malignant cell lines
Version: 1 Date: 20 February 2014
Reviewer: Steve Talcott
Reviewer’s report: Abstract:

1. Common name for “açai” is also “juçara”. It was my understanding that juçara was the common name for Euterpe edulis, not Euterpe oleracea.

It is important to point that in the present study we used Euterpe oleracea Mart. which was authenticated by the Rosa Mochel Herbarium from School of Biological Studies of the Maranhão State of Brazil and the fruit of this plant is known as açai or juçara. On the base of the recommendation of the reviewer, we agree in to change the name of juçara by açai in all the manuscript.

2. Also a small point, but are açai fruits actually “berries” as stated on page 9? They more resemble a drupe than a true berry.

Schauss et al (2006) and Mertens-Talcott et al (2008) cited in our paper, classified açai as a berry [7, 9]. Schreckinger et al (2010) in their study “Berries from South America: A Comprehensive Review on Chemistry, Health Potential, and Commercialization” described açai as a berry. On the other hand, Rogez et al (2011) in their work “Sigmoidal kinetics of anthocyanin accumulation during fruit ripening: A comparison between açai fruits (Euterpe oleracea) and other anthocyanin-rich fruits” described açai as a drupe. Rezaire et al (2014) also described açai as a drupe in their work “Amazonian palm Oenocarpus bataua (“patawa”): Chemical and biological antioxidant activity – Phytochemical composition”. Thus, technically, it seems that açai and others fruits called generally as berries such as cherry and plum are in fact a drupe. The observation of the reviewer is correct. The botanical classification of a drupe is that: “it is a fruit in which an outer fleshy part (exocarp, or skin; and mesocarp, or flesh) surrounds a shell (the pit, stone, or pyrene) of hardened endocarp with a seed (kernel) inside”. Therefore, it better describes the açai fruit and in the new version of the manuscript we changed the name berry by drupe on pages 9 and 10.

Methods:
3. Methods for extraction of (just the) seeds was no clear. Seeds boiled in water (water kept or tossed) and then extracted in MeOH (ground, particle sized, etc are not explained). How were seeds obtained. Why give these details for seeds and not for the bark and “total fruit”. Not sure what “total fruit” means. Was this a whole açai fruit, treated in a like-manner? A bit confusing, overall.

The observation is correct and we appreciate. Really, it was confusing but following the recommendation, in the new version of the manuscript we introduce a more detailed explanations about the extraction methods. Açaí fruits were collected and stored at -20ºC until use. After defrosting, fruits were separated into three portions: bark, seed and total fruit (bark + seed). Latter, all three portion were treated in the same manner.

4. Was a cytotoxicity test run on the cells first, to determine the dosing ranges to be used for additional assays? (As I read on, I see Figures 1 and 2; however, I am not seeing a dosing response from 0 control to a high concentration).

No, we not performed a cytotoxicity test on the cells. We understand the preoccupation of the reviewer, however in fact we used these concentrations based on experiments made on our laboratory with other substances and drugs using these cell lines. In addition, other studies used similar concentrations and time treatment with açai extract in MTT assay using others cell lines. For instance, Kim et al (2013) in their work entitled “Protein profiling of paraquat-exposed rat lungs following treatment with Acai (Euterpe oleracea Mart.) berry extract” evaluated the cytotoxic potential of açai on epithelial A549 cells by MTT assay using time-points of 12, 24, 48 and 72 h, and concentration of 30 µg/ml. Also, Schauss et al (2006) [7] demonstrated that açai extract inhibited lymphocyte proliferation at concentrations ranging from 5 – 1000 µg/ml.

5. Was any attempt to normalize among the plant extracts made, since seeds would logically contain low phenolic concentrations in açai compared to fruit pulp….meaning it may take 100 grams of seeds to get the same total phenolic content of 1 gram of “total fruit” extract. Many additional co-extractives could be present in such a situation.

No, we not made attempt to normalize among the plant extracts. We agree with the reviewer that seed extract should contain the highest concentration of polyphenols as compared to fruit and bark extract. Consequently, this fact is a reflex of the cytotoxic effect of the seed extract in MCF-7 cell line. The total fruit and bark extract was second and third respectively. It is important to emphasize that the present study is a first phase of the project in which the main goal was to determine if the açai extract were cytotoxic and which of them was more effective against human cancer cell lines. Thus, the next step will be the determination of the substances presented in the most effective extract, in this case, the seed extract.

6. Quantification of polyphenolics in the seed, “total fruit”, and bark are not clear. How was a concentration of 28, 25, and 15% obtained? 28% of what? By
weight? By final volume? Previous reports with açai seeds showed them to contain relatively low polyphenolics concentrations. Some basic indication of content or extract description could be given here (ie. colorless, brown, yellow, red) colored extracts, which will give some indication of what was present.

This observation is correct. In the new version of the manuscript we introduce more information at respect of polyphenolics quantification. Actually, determination of total polyphenols in açai extracts was performed by the Folin-Ciocalteau colorimetric method determined on a Spectrophotometer UV / VIS by monitoring the absorbance at 700nm using gallic acid as a reference standard (50, 100, 150, 250 and 1000 mg / mL). Values were evaluated as mg equivalent of gallic acid/g of extract. In addition, all three extracts after dissolved in Milli-Q water, presented a strong purple colour. This information was added in Methods section.

7. Figure 1 is a bit confusing, and I get the impression that a true dynamic dosage range was not obtained here. There is a narrow window from which efficacy is being tested, and there is illogical trends based on concentration (and between 24 and 48 hrs). Results on Page 7 are being “cherry picked” as to their importance/significance. I am also confused at the comparisons among the test samples. Without knowledge of the chemistry/composition of these extracts, the amount of biomass it took to attain these concentration, etc it makes the comparisons difficult.

As above explained, we chose these concentrations and time of treatment based on experiments made on our laboratory with other substances and on other works with açai extracts and cell lineages. We think that Figure 1 is clear. We are showing that none of the açai extracts were cytotoxic against Caco-2, HT-29 and MDA-MB-468 cell lines. In Figure 2A, we demonstrated that all extracts were cytotoxic against MCF-7 cell line but in a differential manner and in Figure 2B we compared the cytotoxicity of the three extracts at the same concentration. As previously mentioned, in the present study we are beginning to understand the anti-tumor activity of açai extract since we still don´t know the substances presented in each extract but it is for sure our next step. The studied extracts here are still crude and we will purified and study the different fractions separately.

8. Morphology. What caused the physical changes? Were there tannins present in any extracts?

We still don´t know what substance or substances caused the physical changes and what is the target in the cell. A study to identify and characterize the chemical composition of these extracts are in course, however it is matter of a next study.

Discussion:
9. Page 10 indicates that there are phytoestrogens in açai….however, the reference cited has no data in reference to such estrogens in açai. However, açai has been reported to contain lignans, but then it would be up to the authors to show that lignans were first present in their extracts and second that these compounds were absorbed or acted on surface receptors in the breast cells.

We agree with the observation of the reviewer. Actually, it was a misunderstanding. We tried explain that açai presents substances (like lignans) that may act like phytoestrogens. In the new version of the manuscript we changed reference 21, Chiang and Pan (2013) by Heinrich et al, (2011). Our extract is still crude and we don’t know yet what substances and in what amount they are on each extract as well as the molecular target(s) in the cell.

10. Absorption issues are also problematic for breast vs colon cells, since there should be some indication of phytochemical absorption/penetration by the compounds as opposed to the colon cells. Something to think about.

We agree with this issue. But it is important to remind that the MDA-MB-468 cell lineage is also a breast cancer lineage and it did not respond to açai hydroalcoholic extracts. Therefore, we think that maybe the phytochemical absorption/penetration is not the issue in our case.

11. It is unclear why caspase 3 or 7 was not activated. Most classes of polyphenolics will readily kill cancer cells by apoptosis, and this a widely used assay for confirming cell death mechanisms by phenolics. Why this research did not show such a response is puzzling.

We think that our negative results on apoptosis monitoring caspase 3 and 7 is not puzzling. For instance, other studies reported that plant extracts caused autophagy but not apoptosis in different cell lines (see: Tsuyuki et al. [29],Huang et al. [30] and Pietrocola [31]). In addition, Mohamed et al (2011) in their work “Acetonic Extract of Buxus sempervirens Induces Cell Cycle Arrest, Apoptosis and Autophagy in Breast Cancer Cells” demonstrated that MCF-7 cells died by an autophagic mechanism. Another study by Harum et al (2012) entitled “Autophagic Cell Death Is Induced by Acetone and Ethyl Acetate Extracts from Eupatorium odoratum In Vitro: Effects on MCF-7 and Vero Cell Lines” also demonstrated absence of apoptosis and occurrence of autophagy in MCF-7 cells. We added in the new version of the manuscript these informations and the two references to discussion on page 11.

The mechanism whereby cell death of MCF-7 was by autophagy and not apoptosis in our study needs to be determined.

Overall, this paper seems a little pre-mature for publication in its current form. The authors generated more questions than answers, which will obviously lead to many future elucidations of the mechanism of action. The chemical
characterizations need to be completed, since much of the discussion is based on supposition based on composition. It would be nice to see a standard polyphenolics run in the assays, ie. something with a known dose-response in cytotoxicity and induction of capases. This ‘positive control’ helps to further elucidate mechanisms. Please double-check your references, since the one paper I did look up did not have the correct content/data cited in this work.

We established previously that this is a first study that first characterize the effects of crude extract of açai. In subsequent studies that are in course we will do the chemical characterization of these extract and thus elucidate their mechanisms of action. Anyway, we thanks the suggestion of the reviewer, which will be taken into consideration in our next study.

In the new version of the manuscript we check the references as suggested.

Reviewer’s report
Title: Cytotoxic effects of Euterpe oleracea Mart. in malignant cell lines
Version: 1 Date: 6 March 2014
Reviewer: Ayse Nalbantsoy
Reviewer’s report:
1. The manuscript described the cytotoxicity effect of Euterpe oleracea Mart. The authors used limited number of cell line instead of using a panel of breast or colon cancer cell line since there are soe study on this plant species. On the other hand, they show interesting results which they should be detailed in future study such as pure compound tretment results. My opinion paper could be accepted after some revision such as comparing with the results with previous studies in discussion part.

These two cells lineages were chosen because they are derived from two high prevalent cancers in Brazil (breast and colon cancer). In addition, according to the latest world cancer statistics by the WHO (2012) breast and colon cancer are the second and third most commonly diagnosed cancers worldwide, respectively. The two colon cancer cell lines had different behaviors. HT-29 is a more aggressive lineage with a high proliferation rate comparing to Caco-2. The main difference about the breast cancer cell lines was hormone receptor status.

We added some new information on pages 11 and 12 on discussion and added three new references. We speculate more about the autophagyc effects of açai extract in MCF-7 cell lines and possible molecular targets. Two of these new references corroborate with ours findings were plant extracts caused also autophagy in MCF-7. The other reference we used to speculate what would be the possible target on the cell.