Author's response to reviews

**Title:** Gallotannin-rich Caesalpinia spinosa fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model

**Authors:**

Claudia P Urueña CU (curuena@javeriana.edu.co)
Juan C Mancipe JM (mancipe.j@hotmail.com)
John F Hernandez JH (johnhernandezm@gmail.com)
Diana M Castañeda DC (dianauvi@gmail.com)
Luis M Pombo LP (miguel.pombo@juanncorpas.edu.co)
Alejandra Gomez AG (gomez_alejandra@javeriana.edu.co)
Alezxander Asea AA (aasea@msm.edu)
Susana Fiorentino SF (susana.fiorentino@javeriana.edu.co)

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Doctor
Tom Rowles PhD
Executive Editor
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Gallotannin-rich Caesalpinia spinosa fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model. Claudia Urueña¹, Juan Mancipe¹, John Hernandez¹, Diana Castañeda¹, Luis Pombo², Alejandra Gomez¹, Alexzander Asea³, Susana Fiorentino¹*

Dear Editor,

I would appreciate the comments and suggestions of the reviewers. We answer each one of the points; the corrections were included on the final version and revisited by a native. Additionally, as we added new date produced by one of the researcher of the group, he was added into the authors. “John Fredy Hernandez”.

As you suggested, we added the letter certifying the constitution of ethics committee and also the letter of approval of the project related with this publication. Please let me know if you need the document be translated.

Thanks for your interest in our work.

Best wishes

Susana Fiorentino G.
Reviewer's report
Title: Gallotannin-rich Caesalpinia spinosa fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model

Version: 2 Date: 5 October 2012

Reviewer: Kent Hunter

Reviewer's report:
This manuscript describes the in vitro and in vivo characterization of the P2Et extract, derived from Caesalpinia spinosa, on breast cancer progression. Using the 4T1 mouse mammary tumor model the investigators demonstrate that P2Et induces apoptosis in cultured cells, presumably through the mitochondrial apoptosis pathway. Furthermore they demonstrate that P2Et has effects on IL-6 secretion in both in vivo and in vitro systems. Finally they demonstrate that treatment of orthotopic tumors with the P2Et extract results in a decrease in primary tumor growth and dissemination of tumor cells into the spleen.

Concerns:
Major Compulsory Revisions

Figures 3&5: The investigators have demonstrated in figure 3 that treatment of 4T1 cells in vitro results in an up regulation of the pro-progression cytokine IL-6, but in vivo there is a decrease in IL-6 in the animal serum. How do the investigators account for this discrepancy? Some discussion of this opposing phenotype seems to be appropriate.

In the revised manuscript a discussion the opposing phenotype has been included on page 15; line 4 from bottom:

With regards to data demonstrating that treatment of tumor-bearing mice with P2Et fraction results in significant reduction in serum IL-6, we hypothesize that locally produced IL-6 may improve apoptosis mechanisms and are consumed immediately by tumor cells during its death process thus acting in an autocrine fashion as previously discussed. We concur that tumor reduction after P2Et treatment cannot simply explain IL-6 serum reduction and hypothesize that some other immune-regulatory mechanisms are mediated by the P2Et fraction cannot be excluded, including the inhibition of suppressor macrophages.

Figure 1 b: There are two sets of FACS plots for this panel. What do the two sets represent? 24 and 48 hour treatments? The figure legend states only 48 hours.

In the revised manuscript we included the time of treatments (24 and 48 hours) on the figure 1 legend. The top panel is a representative dot plot showing the various treatments and the bottom panel is a representative histogram showing percentage (%) of PS/PI cells after 24 and 48 hours of treatment.
Figure 3 legend: IL-6 should be labeled as panels a & b, not a & c, MCP-1 should be c & d, as indicated on the figure.

In the revised version of the manuscript, IL-6 is now labeled as panels a & b, and MCP-1 is now labeled as panels c & d.

Figure 5: Please be certain to provide high resolution photomicrographs for the manuscript.

The data presented in figure 5 also suggests that the in vivo anti-IL-6 activity of P2Et may not be responsible for the anti-metastatic effect, at least in the lung. 9.3 mg/kg of P2Et is sufficient induce all of the measured in vivo phenotypes, but does not appear to have an effect on pulmonary metastases. In fact, if these photomicrographs are representative, the opposite might be the case. Due to the quality of the images it is not possible to assess this possibility in the other organ tissues. Splenic metastasis is also relatively rare in breast cancer, so it is not clear how significant an effect this treatment might be in the clinical setting, particularly since it appears to be single cell dissemination rather than macroscopic metastasis that is effected.

In order to answer the reviewer suggestions, we took new pictures of the evaluated organs with two different magnifications (10X and 100X). Pictures taked at 10X are shown -in Figure 5, and pictures taked at 100X are included as a supplementary figure for the reviewers to evaluate tumor infiltration into various tissues (supplementary figure 3). Regarding splenic metastasis in this model, Heppner and colleagues (2000), described that 4T1 breast cancer model, is metastatic to different organs as lung, liver, bond, brain and spleen migrating by blood. We do not observed bond and brain metastasis, but in contrast other organs are clearly metastasied. Aditionally in the revised manuscript we included new data showing that P2Et fraction administered orally, diminishes the size of primary tumor and lung metastasis measured after lung India Ink staining (Figure 6). This figure was included and also a picture of the metastasis observed after this procedure.

Minor essential revisions

Page 9: clonogenic assays are routine assays used for tumorigenesis. However, no in vitro assay has been found to be particularly predictive of metastatic capacity. Therefore extending the results of the clonogenic assay to predict the terminal stages of cancer is probably questionable.

Page 11, last sentence of the first paragraph. Please clarify.

In the revised manuscript the sentence now reads “Doxorubicin increased IL-6 secretion at the IC50 concentration, while P2Et fraction increased IL-6 secretion at all tested concentrations.”

Level of interest: An article whose findings are important to those with closely related research interests
Major compulsory revisions:

1. On page 7, the authors state that toxicity studies were conducted with 1-8 mg/ml P2Et fraction. In the tumor model, the mice were injected with 9.3 or 18.7 mg/kg, how does this compare with the toxicity data? Please, show the LD50 data in a supplemental figure using the same units.

In the revised manuscript, we included the procedure in the Materials and Methods section and also the way in which therapeutic dose is determined. Additionally, we included supplementary figure 2, which explains the method in more detail.

2. The IC50 for cytotoxicity is given in µg/ml in Table 1. Please, show a dose response curve for how the IC50 was calculated for P2Et in 4T1 cells. Moreover, given that doxorubicin gave an IC50 of 0.5 µg/ml and the P2Et fraction gave an IC50 of 34.1 µg/ml, it is misleading to start the Results section with the heading “P2Et fraction is highly cytotoxic to 4T1 tumor cells” when the effects on cytotoxicity are modest.

In the revised manuscript the heading now reads “P2Et fraction is cytotoxic to 4T1 tumor cells in vitro”

Please, clarify the disparity in data from your previous study (reference 12) where you show IC50 of 64 µg/ml for fibroblasts and an IC50 >125 µg/ml for fibroblasts in Table 1.

The exact reason for this discrepancy is not known. However, differences of IC50 observed on fibroblasts could be due to the different stages of passages the cell lines were going through at the time of the experiments. This was a laboratory fibroblast cell line and for experiments done in reference 12 the cells were at an earlier passage number, and maybe were differentially susceptible. However, differences were only observed in one dilution.

3. Figure 5: The quality of the micrographs is poor and it is difficult to see what the authors call “metastatic infiltration”. The white arrows mask the cells stained with H&E. In this model, in 30 days the lungs should show clear metastatic foci not just infiltration (see Int J Exp Pathol. 2007, 88:351-560). Given that these are more than micrometastases in the lung, please, clarify how you counted individual cells. Also in the example in response to 9.3 mg/kg P2Et, there may be more metastatic cells in the lung compared to control.
In the revised manuscript the method used for the determination of metastatic infiltration has been included (page 8; second paragraph). In addition, we have provided new pictures. Analysis was made by a veterinarian pathologist.

Minor essential revisions:

1. Abstract: Methods
   In the sentence “The present study evaluated cytotoxic and antitumor activities of a gallotannin-rich fraction derived from *Caesalpinia spinosa* (P2Et).”, “tumor progression” should be changed to “anti-tumor progression”.

   This change has been included in the revised manuscript.

2. Abstract: Results, Define IL-6.

   IL-6 has been defined as interleukin-6 in the text and abbreviations section of the revised manuscript.

3. Introduction First paragraph, last sentence, “induces apoptosis in the murine and human breast cancer cells MCF-7 and S115, respectively [9].” Should be “induces apoptosis in the murine and human breast cancer cells S115 and MCF-7 respectively [9].”

   This change has been made in the revised manuscript.

4. Page 5, Heading, Please define MMP as mitochondrial membrane potential.

   This change has been made in the revised manuscript.

5. Ethanol is used as the negative control. Please, state the % of ethanol used throughout the manuscript or it will be assumed that you used 100% Ethanol.

   In the revised manuscript, we have included final Ethanol concentration (0.02%) in the Materials and Methods section (pages 5, 6 and 7).

6. Please, give the number of animals/group in Figures 4 and 5. The methods say that the N=7-8 but the experiments were conducted twice and the results are the mean. Therefore, is the N, 14 or 16?

   In the revised manuscript, the numbers of animals/group have now been included (Figure legends of figure 4 and 5).

7. Fig. 2A shows nuclear segmentation not DNA fragmentation which is usually shown by agarose gel electrophoresis.

   In the revised manuscript, this correction has been included.
8. The clonogenic assay as shown by cell clonogenicity is a measure of cell survival and proliferation. As the authors state, to use it as a “golden standard for antitumor activity and metastasis” the assay should be conducted with tumor cells recovered from metastatic foci, that can be grown in a clonogenic assay.

Clonogenic assay is a measure of cells ability for long term proliferation after drug treatment (Chen et al 2006). We are actually collecting metastatic tumor cells in order to compare this ability with primary tumor cells both after treatment in order to evaluate differences between both types of cells. In the revised manuscript, we have corrected the definition of this golden standard, to be more precise.

9. Please, edit typos and grammar mistakes throughout the document. For instance, “mice mammary fat pads should be “mouse mammary fat pads””. In Fig1 a, MM-Potencial should be “MMP” or mitochondrial membrane potential”. Fig. 4 caption, “tumor weight” should be “weight”, etc. Also in the Figures, decimal points designated by a “comma” (i.e. 3,4) should be designated by a “period” (3.4).

We changed “mice mammary fat pad” for “mouse mammary fat pad”. In figure 1 we changed MM-Potencial for MMP. In figure 4 we changed tumor weight por weight. We changed the comma, by a period in C and D of figure 5.

10. Please, give method for leukocyte counts in Methods section.

We included this part in Materials and Methods sections.

11. Figure 3, Please, change caption to indicate that a,b represents results for IL-6 and c,d for MCP-1.

In the revised manuscript these changes have been included

Discretionary revisions
1. Migration/invasion assays in response to P2Et are been better indicators of metastatic efficiency of cells in tissue culture than the clonogenic assay.

For this reason, in the revised manuscript both methodologies are used.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that have no competing interests