Author's response to reviews

Title: In vitro anticoagulant and antioxidant activities of Jatropha gossypiiifolia L. (Euphorbiaceae) leaves aiming therapeutical applications

Authors:

Juliana Félix-Silva (julianafelix_rn@outlook.com)
Thiago Souza (thiagoazuos@hotmail.com)
Rafael BG Camara (rafael_bgc@yahoo.com.br)
Bárbara Cabral (barbara_cabral01@hotmail.com)
Arnóbio A Silva-Júnior (arnobiosilva@ufrnet.br)
Ivanise MM Rebecchi (ivaniserebecchi@gmail.com)
Silvana M Zucolotto (silvanazucolotto@ufrnet.br)
Hugo AO Rocha (hugo@cb.ufrn.br)
Matheus F Fernandes-Pedrosa (mpedrosa@ufrnet.br)

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Author’s response to reviews: see over
Dear Editor,

Thank you for considering our manuscript MS 4760992181296929 “In vitro anticoagulant and antioxidant activities of Jatropha gossypiifolia L. (Euphorbiaceae) leaves aiming therapeutical applications” by Juliana Félix-Silva, Thiago Souza, Rafael B. G. Camara, Bárbara Cabral, Arnóbio A. Silva-Júnior, Ivanise M. M. Rebecchi, Silvana M. Zucolotto, Hugo A. O. Rocha and myself, for publication in BMC Complementary and Alternative Medicine Journal.

We have carefully read the comments of the reviewers and have answered all their questions in this letter and performed the required changes in the revised manuscript.

We hope this revised manuscript is now suitable for publication in BMC Complementary and Alternative Medicine Journal.

Yours sincerely,

Matheus F. Fernandes-Pedrosa, Ph.D
Department of Pharmacy
Federal University of Rio Grande do Norte
Phone: +55.84.3342-9820 Fax: +55.84.33429822
E-mail: mpedrosa@ufrnet.br
Some question should be addressed before publication:
Cremophor specifications are missing, e.g.:
- Cremophor EL/ Kolliphor EL: polyoxyl 35 castor oil
- Cremophor RH 40: polyoxyl 40 hydrogenate castor oil;
- Cremophor RH 60: polyoxyl 60 hydrogenate castor oil.

The Cremophor used in this work was de Cremophor EL/ Kolliphor EL: polyoxyl 35 castor oil. This specification was added to manuscript, as requested by the Referee.

The methodology for total phenolic content should be described or a primary citation must be included.

The total phenolic content was determined by the Folin-Ciocalteu method. A primary citation of the procedure was included in the manuscript, as requested by the Referee.
1- This manuscript reports the evaluation of the anticoagulant and antioxidant activities of *J. gossypiifolia* leaves, looking for new therapeutic uses for this plant. I enjoyed reading this analysis of the effects exerted by *Jatropha gossypiifolia* L. (Euphorbiaceae) leaves. The biochemical experiments were performed competently, but I believe that there are still many aspects that should be studied, now or in the future. This is understandable, as the MS does not deal with purified compounds, but with the semi-purified fractions. The main aspect is about the possible presence of peptides in CE and RA fractions, which was not addressed. The contents of sugars, proteins and phenolic compounds were measured, but the peptide content was overlooked. The Bradford method is not suitable for measuring peptide dosages. The use of fluorescamine may be useful for the measurement and detection of peptides. Also, on page 18, the test performed with ninhydrin indicated the presence of primary amines in the fraction called RA, but, again, were correlated only to proteins (and not to peptides). Several papers in the literature indicate the presence of protease inhibitors in plant extracts and could be cited as examples. Thus, it would be of utmost importance that the authors mention, in the discussion, the possible presence of protease inhibitor peptides in both CE and RA fractions.

The presence of peptides could be an interesting hypothesis, but we don’t believe that high amounts of these molecules have been extracted in CE and RA fraction, due the extraction and fractionation methods used, that employs the boiling of the dried leaves with water for 15 min, and the partition with organic solvents, respectively. In fact, we have done the electrophoresis analysis of the CE and RA fraction (with Coomassie Brilliant Blue and silver nitrate stain) and the data did not show the presence of peptides and/or proteins, even in the low molecular weight region of the gel. Regarding the test with ninhydrin, we now added in the manuscript that the color developed may be due the presence of peptides too. The presence of proteins and peptides is very interesting and is a future perspective of our group investigates the presence of these molecules in *J. gossypiifolia*. In our laboratory, we already have standardized methods of selective extraction of peptides and proteins from other plants (See *Molecules* 2014;19(3):3552-3569; doi: 10.3390/molecules19033552), and have obtained interesting results. We believe that using the correct approach to extract specifically peptides and proteins, we could obtain interesting results in the future.

2- Do the authors know if the plant extract is able to inhibit the effects of the venom of *B. jararaca*? This venom, certainly, contains thrombin-like serine protease (which can be inhibited by the extract, inducing the anticoagulant action) and hemorrhagic metalloproteinases (which can be inhibited by compound(s) with chelating action presented by the extract (RA and CE) when the study of the antioxidant action). The authors can eliminate the presence of a protease with direct action on fibrinogen, but not a peptide inhibitor of serino- and metalloproteases. If the authors have more information...
about this, I think it would also be important to include them in the discussion of the MS.

Yes, we do. One of the focus of our research group is the antiophidic activities of medicinal plants and, recently, we published an article showing exactly the antiophidic activity of the same aqueous leaf extract of *J. gossypiifolia* against *B. jararaca* snake venom (See *Plos ONE* 2014;9(8): e104952; doi: 10.1371/journal.pone.0104952). Among others inhibitory activities, the extract was able to inhibit the procoagulant action (thrombin-like action, which could be related with serinoproteinase inhibition), proteolytic action (against fibrinogen and azocasein) and hemorrhagic activity (which could be related with metalloproteinase inhibition). Thus, one possible hypothesis for the anticoagulant action observed is the presence of inhibitors of proteases, since the coagulation cascade is constituted, basically, of proteases. This discussion was added in the text.
Major compulsory revision.
The manuscript deals on an interesting field of the phytochemistry, the ethnomedicine/pharmacognosy.
However, the manuscript needs a full revision concerning to language editing, the treatment of data (with inclusion of experimental controls) and the reorganization of the data presentation and consequently the discussion.
Moreover, the article is required to be assertive and not "suggestive".
Overall, the manuscript should be completely re-made before being accepted to any journal.
For instance,
Along the text (methods) the author describe "samples at different concentrations", this should be fixed by including the range of values.
The range concentrations used in the methods were added, as suggested by the Referee.

The legend of figure 1 should be improved to include how the extracts and organic fractions were used in the assay.
The information about the extract and organic fractions were carefully described in “Methods” section.

Also concerning to figure 1, in my view RA was able to prolong prothrombin time by 3-fold, not crude extract (CE).
The prothrombin time (PT) was not prolonged by any sample (extract or fraction) and it is not presented in the graph. In the Figure 1 is shown only the activated thromboplastin time (aPTT). In this graph, in the y-axis is showed the aPTT sample/aPTT control ratio, which measures directly how much times the sample prolongs the aPTT of the control sample (aPTT in absence of extract). So, the crude extract (CE) was able to prolong the aPTT in about 3 times (aPTT from control: 32.7 s; aPTT from CE at 2 µg/µL: 102.4 s; 102.4/32.7 = 3.13) and the residual aqueous fraction (RA) prolonged in about 6 times (aPTT from control: 32.3 s; aPTT from RA at 2 µg/µL: 196.4 s; 196.4/32.3 = 6.08).

The results concerning to the anticoagulant activity of the control heparin were not included in the figure 1 for comparison. This should be required for clarity of data interpretation.
The result of the anticoagulant activity of the heparin was not included in the figure 1, but it is described in the text:
“Heparin was used as positive control and as expected presented significant anticoagulant activity, with PT higher than 60 s (negative control: 16.27 ± 0.32 s) and aPTT higher than 240 s (negative control: 35.07 ± 0.03 s)”
These results were not included in the graph because the unit of the heparin (IU/mL) is different from that of the extract and fractions (µg/µL), so its inclusion in the graph would be difficult.

The presentation of TLC data is critical, because most of identification was based on 'suggestive of' identities of the compounds. Therefore, photographies showing the pattern of fractionated and revealed compounds are essential to be included as figure in the text or supplemental material.

Through TLC analysis was possible to observe the presence of orientin, isoorientin, luteolin, vitexin and isovitexin by comparison with the reference standards. In the TLC chromatogram was observed bands with the retention time and color similar to reference standards. However, to confirm this result, other techniques such as HPLC can be used. In this case, the increase of peak area may be seen by co-injection of reference standard + extract. Regarding the results of TLC, it is not common in Phytochemistry to add TLC chromatograms in manuscript, only chromatograms obtained by HPLC, but if editor agree, we can add these Figures.

The authors claim that "Polar compounds such as flavonoids, tannins and sugars are poorly described in the species so far". However, in the present work such compounds were not chemically characterized (at molecular level).

The polar compounds such as flavonoids, tannins and sugars are poorly described in the species so far. “Be described”, in Phytochemistry at least, means that the presence of a given compound or class of compound was already shown in some other work, and not necessarily that the compound was chemically characterized structurally. It is very common the use of qualitative screening reactions (such as TLC) to see which kind of compounds are detected; and for some plants, this qualitative phytochemical screening is sufficient to characterize it.

For J. gossypiifolia, its main characteristic is the presence of apolar compounds such as terpenoids and lignoids. Polar compounds such as flavonoids, tannins and sugar are not characteristic for this plant; only a few number of phytochemical works has showed the presence of these compounds by screening tests or has isolated and chemically characterized it. In our work, we have showed that, although the presence of these compounds is not very related in the J. gossypiifolia species, we observed the presence of them in the aqueous leaf extract of the plant.

The discussion is presented without of a coherent order (for instance, discussion about the extracts is after the data concerning to biological activity, then these are repeated again).

We tried to present the results in an order similar to that we followed in the experimental work, to turn possible to the reader understand our line of thought. So, we wrote the introduction according to the following line of thought:

- We first evaluated whether or not the aqueous leaf extract of J. gossypiifolia showed anticoagulant activity (if it was inactive, any other study would be unnecessary).
Since the extract was active, the next question was: which compounds are responsible for the activity? For this, we performed a phytochemical analysis of the extract and its fractions (the fractionation is necessary to view some compounds that has low concentration and are undetectable in the crude extract).

Besides the phytochemical analysis, to understand which compounds are necessary for the anticoagulant activity, the biological assessment of the fractions is necessary. We observed that the residual aqueous fraction (RA) was the most active one, even more active than the crude extract (CA). So, obviously, this fraction is that which contains the bioactive compounds in the extract. With this result we can also conclude that the flavonoids, tannins, terpenoids or alkaloids present in the extract are not the responsible for the anticoagulant activity (since the RA fraction did not show the presence of these compounds).

Having in mind that the RA fraction was the most active one, as well as considering that the anticoagulant activity associated with antioxidant properties could be beneficial for various cardiovascular diseases, the antioxidant of CE and the RA active fraction were also investigated, and the results of these analysis is presented.

However, another question is brought up: the extract and the RA active fraction are safe? Considering that the *Jatropha* genus is related with toxicity, we evaluated the *in vitro* cytotoxicity of CE and RA fraction.

Moreover the article needs language revision and editing for improvement in the text and for better comprehension of the findings. The whole manuscript was carefully revised for remove any inconsistence or mistake in the text and to improve the comprehension of the findings. Regarding the language revision, a revision by a native English speaker was performed, to ensure the correctness of the text, as showed in “acknowledgements” section.

Some phrases in the discussion declare what is obvious. For instance: "The RA fraction was the most active, prolonging the aPTT by up to 6 times, being 2 times more active than CE. Based on this, it is possible to conclude that this fraction contains the main compounds responsible for the anticoagulant action" Although the information is obvious, it is necessary, since the topic is “Results and Discussion”, so we need to explain which results we found, besides show them in the Figures.

Concerning to antioxidant activity, it is not clear in the discussion why only CE and RA were the fractions used for this kind of evaluation. The RA fraction was the only fraction tested in antioxidant activity because it was the most active one on the anticoagulant activity, and the focus of the work is the potential application of the extract in cardiovascular diseases, as discussed in following text:
“Having in mind the potentiality of CE and, mainly the RA active fraction, as well as considering that the anticoagulant activity associated with antioxidant properties could be beneficial for various cardiovascular diseases, the antioxidant of CE and the RA active fraction was investigated.”

In addition, in practical way, what is the biological meaning of a compound that displays "an interesting AAE". Is the ascorbic acid an excellent standard? Why?

In the text: “… the detected values obtained in the present study were extremely interesting, which prompted us to conduct further antioxidant tests to determine the potential antioxidant mechanisms of the J. gossypifolia CE and RA fraction” the “interesting” refers to the result per si, not exactly to the AAE value. The result (a high value of AAE, especially when comparing with several other works, where the value is at most about 100 mg/g of AAE) is interesting because it shows that CE and RA possess good antioxidant activity. This test is very common to screen for compounds with antioxidant activity, and so, a high value of AAE is interesting because shows a good potentiality of have a very good antioxidant activity.

In all graphics presented as figures in the manuscript, controls (negative and positive) are not included.

As explained above, the positive control of anticoagulant test (heparin) was not presented in the graph, but was in the text (see above). The negative control (PBS or Cremophor 5% in PBS) is showed indirectly by the calculation of aPTT ratio.

In some other figures, the reason for the controls don’t be shown in the graphs is inherent to the methods, as shown below:

- In total antioxidant activity (Figure 2), the positive control is the ascorbic acid itself, used in the calculation of AAE. The negative control is the blank used for the spectrophotometric quantification (means zero).
- In the reducing power (Figure 3), the positive control is the ascorbic acid itself, used in the calculation of % activity of ascorbic acid (we can see clearly that CE and RA were more active than positive control, based on the values obtained). The negative control is the blank of the spectrophotometric quantification (means zero).
- In the copper and iron chelation tests and hydroxyl and superoxide ions scavenging tests (Figure 4, 5, 6 and 7), the negative controls are used to calculate % of activities (negative control, in chelation tests, means zero of chelation; and in radical scavenging tests means zero of scavenging ability). The positive controls are not showed on the graph because the range of concentrations used to see activity is different, and so it is difficult to put them all in the same graph. But, in turn, the values obtained with positive controls is showed in Table 3 in terms of IC50, where is possible clearly compare the potency of the samples between themselves and with the positive controls (standards) of each test.

In table 3, controls are again not included.
Positive controls are the standard. The negative control is not included because is illogical present IC50 of something that has not activity (IC50 = ∞).

The discussion is based on suggestion, it is necessary to be assertive and precise. The discussion is not based only on suggestion. We have the caution in make some affirmations, since we are dealing with biological activity, in special in vitro. For example, WE don’t suggest that the extract is active (even because for do this is not necessary any experiment); but we say that OUR RESULTS suggests or indicates or points to the biological activity of the extract. We believe that the term “confirm” is very strong for now.

About the cytotoxic assays, by calculating the concentration of RBC for the test the correct (calculated value is 0.2%) not 20%, as written. 20% is the concentration of RBC prepared. From this, was used 5 μL, which gives 0.2% of RBC in the reaction medium:
“5 μL of 20% (v/v) red blood cell (RBC) suspension were incubated at 37ºC for 60 min with 500 μL of samples at different concentrations”.

In this concentration of RBC (0.2%), hemolytic activity is difficult to be measured. Moreover, water is not usually used to cause 100% of lysis, but triton X-100. This concentration (0.2% of RBC in the reaction medium) was chosen based on the literature. Many other works use concentrations of RBCs similar to us (see, for example, APCBEE Procedia 2013;7:103-8 doi: 10.1016/j.apcbee.2013.08.019; Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2014;127:434-8 doi: 10.1016/j.saa.2014.02.106; Asian Pacific Journal of Tropical Biomedicine 2012;S38-S39). In addition, a significant increase in absorbance in the 100% of lysis group was observed in relation to PBS control indicating that the hemolytic activity was perfectly measurable in our assay. In relation to the use of water as control, we have observed several works using it, instead of triton X-100. In fact, many works that we analyzed have used water as 100% of lysis control. Some examples can be seen at: Journal of Ethnopharmacology 2012;142:789-94 doi: 10.1016/j.jep.2012.05.065; Asian Pacific Journal of Tropical Medicine 2011;305-9; among others).

Discussion about the biological function of RBC is unnecessary to justify the use of this test for the evaluation described in the work. The text about the biological function of RBC was removed from the text, as suggested by the Referee.

The hemolytic test with standard conditions and controls is required in this work to adequately realized and included. The hemolytic tests developed in this work were performed in accordance with standards and criteria established and well accepted in the literature (examples: Journal of Ethnopharmacology 2012;142:789-94 doi: 10.1016/j.jep.2012.05.065; Asian Pacific
In cytotoxic assay using cells, the FBS concentration should be indicated and experimentally controlled, whenever samples are being tested. FBS concentration can influence on the activity of a drug/treatment. The concentration of FBS used in experiments with cell culture was 10%. This information was added in the Methods section, as showed bellow:

“Human embryonic kidney 293 cells (HEK-293) (ATCC® CRL-1573) were cultured under standard conditions in DMEM (Dulbecco’s modified Eagle's medium) supplemented with fetal bovine serum (FBS) at a final concentration of 10%. Cells were maintained in cell culture flasks at 37°C in a humidified atmosphere containing 5% CO2 and were collected by treatment with trypsin. Cells (1 × 104 cells per well) were seeded in medium supplemented with FBS (10%) and cultured for 24 h in 96-well microplates to promote adhesion. The following day, the medium was removed and replaced with fresh medium free of FBS. Serum deprivation was used for synchronizing cell cycle. The next day, the medium was replaced with fresh medium with FBS (10%) containing serial dilutions of the samples (3.9 – 500 µg/µL) previously sterilized in a 0.45 µm membrane. The negative control was exposed to the standard medium supplemented with FBS (10%) without samples. After 24 h, the MTT assay was performed as a marker of cell viability, as previously described in literature [36]. Briefly, medium containing extract was replaced with medium containing 5 mg/mL of MTT and incubated for 4 h at 37°C. After incubation, the supernatant was removed and the purple formazan crystal formed was solubilized in ethanol, stirred for 15 min and the absorbance was measured at 570 nm in a microplate reader (Epoch-Biotek®, Winooski, VT, USA). The absorbance of the negative control (no sample) was considered as 100% cell viability and the values of treated cells were calculated as a percentage of the negative control.”

Data from both cytotoxic assays (hemolysis and MTT) should be presented in the manuscript with controls included (at least in the suplemental materials).

The data from both cytotoxic assays (hemolysis and MTT) were not included in the text because this is not the main focus on this study. The objective of this work is to evaluate the anticoagulant and antioxidant activities of *Jatropha gossypiifolia* extract and fractions in vitro. The results about cytotoxic assays are important to show the safe of our samples on human cells. However, this is not the principal point of the study. As the result obtained was absence of cytotoxicity (absence of hemolytic activity: 0% of hemolysis, even at the highest concentration; absence of cytotoxic action against HEK-293 cells: 100% of cell viability in MTT assay, even at the highest concentration), we preferred to show the results of cytotoxicity only in the text (graphs not shown), to not to extend too much on this topic.
Many more weak points can be found in this research article that compromise the quality of the manuscript for publication in the actual version. As already stated above, the whole manuscript was carefully revised for remove any inconsistency or mistake in the text and to improve the comprehension of the findings and its quality, to make it perfectly suitable for publication.