Author's response to reviews

Title: Modulation of Heat Shock Proteins and Apoptosis by Flueggea leucopyrus (Willd) decoction: possible mechanisms mediating cytotoxicity to three breast cancer phenotypes.

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Author's response to reviews: see over
Executive Editor,
BMC Complementary and Alternative Medicine,

Dear Sir,

Our manuscript titled ‘Modulation of Heat Shock Proteins and Apoptosis by Flueggea leucopyrus (Willd) decoction: possible mechanisms mediating cytotoxicity to three breast cancer phenotypes’ has been submitted for publication as a research article in your highly esteemed journal, BMC Complementary and Alternative Medicine.

In view of the urgency of discovering natural agents that can target breast cancers based upon specific molecular subtypes, this article demonstrates the inhibitory effect of the decoction prepared from F. Leucopyrus (aerial parts) on the proliferation of specific phenotypes of breast cancer cells, via inhibition of Heat Shock Proteins and enhancement of apoptosis. Thus F. leucopyrus could be further exploited to develop cheaper and natural alternatives to existing drugs that would not only be useful for the treatment of breast cancer, but could also prevent disease relapse. F. leucopyrus can easily be cultivated without incurring much expenditure and, development of an anti-cancer agent from this plant would also be of economic benefit to breast cancer patients worldwide.

We declare that this manuscript is original, has not been published before and is not currently being considered elsewhere. We also wish to confirm that there is no competing interest and the manuscript has been read and approved by all the named authors.

We feel these findings will be of interest to the readers of your journal and we hope you find our manuscript appropriate for publication and look forward to hearing from you.

Yours Sincerely,

Anuka Mendis
Replies to the reviewers reports

I sincerely thank the reviewer’s for their comments and advice, and I have taken it in to consideration. I have made the necessary changes, and below I have replied to some of the comments.

Review 1

Major compulsory revisions:

1) It would be good if the author had shown the bioactive constituents in the decoction. Although the authors do feel that identifying/ displaying the bioactive constituents in the decoction would in fact be valuable information for this paper, this was a preliminary investigation to identify the effect of the decoction on HSP expression and apoptosis in breast cancer cells. Isolation of the bioactive constituents in the decoction are still underway.

2) There is no clear explanation for choosing 10 & 20 µg concentrations for HSP70 & 90 expression studies. How do you arrive at this concentration? These concentration were chosen as they are lower than the IC50 values obtained from the SRB assay. HSP expression occurs during the initial signaling events of apoptosis hence in order to detect HSP expression these doses were selected. This information has been included in line 151 of the manuscript. IC50 values indicates where 50% of the cells are dead, since expression of HSP 70 and 90 are early indicators of apoptosis concentrations below the IC50 value of the decoction was chosen. Line 153

3) MCF-10A cells should also be included for Apoptosis and HSP expression studies. MCF-10A cells was not included for apoptosis and HSP expression studies, as IC50 values from SRB assay were high (570.4 µg/mL ) and will not show effects on apoptosis and HSP expression with the doses used (10 & 20 µg/ml) to evaluate HSP expression and apoptosis. In addition, the main objective of the present study is to evaluate apoptotic and cytotoxic effects of the decoction on breast cancer cells (MCF-7, MAD-MB-231, SKBR-3). At the same time we evaluated cytotoxic effects of the decoction on normal breast cells (MCF-10A) just to confirm the less toxic effects of the decoction on normal cells compared to
cytotoxic effects on cancer cells. Therefore we feel that our objective has been achieved by present experiment.

4) To confirm apoptosis, Caspase-3/7 should be performed.

*Considering valuable comments from the reviewers, Caspase 3/7 assay was performed using Apotox- glo. Line 219 and 341, results in figure 6*

5) For DNA fragmentation analysis, there is no explanation for exposing the cells for 48 h while in other experiments, it is 24 h. In addition, no clear explanations for using high concentrations (400 and 600 µg).

*DNA fragmentation was done at higher doses with a longer incubation period as this event occurs at the execution phase of apoptosis. We used higher doses and longer incubation period to ensure immediate DNA fragmentation. Line 332*

6) Author should come up with a confirmatory experiment to show the relationship between HSP expression and Apoptosis. Results of SKBR cells contradict this hypothesis between HSP expression and Apoptosis. Also, there are no clear explanations for this. Authors need to include MCF-10A cells in their confirmatory experiments.

*IC50 value obtained in SRB assay for SKBR-3 cells is high. Results show that there is no HSP expression. At this point it is very difficult to carry out gene expression experiment s on MCF-10A due to some financial constrains regarding purchasing chemicals and reagents. However isolation of active compounds is in progress. Therefore we will consider these comments also when we will design experiments to check and confirm anti-cancer effects of isolated compounds from this decoction.*

7) Author should show the statistical values in the results.

*Statistical values have been shown.*

1) At line No. 166, is it crossing cycle number or threshold cycle number – clarify it.

*Definition of threshold cycle number has been replaced at the referred line. Line 168*

2) Line 285-286 states “same intensities of nuclear staining were maintained”, explain how this was measured?
We did not use any specific software to measure the intensity of the image and what we reported is the visual observation.

Sentenced referred to has been replaced by “On Visual observation the intensity of signal in cell cytoplasm appeared to decrease with increasing concentration of the decoction, though the nuclear staining remained relatively unchanged”.

However we have done a quantitative assay to show the reduction in the HSP expression.

Line 290

Minor essential revisions:

1) Authors need to state why there is a lack of uniformity in the number of experiments performed.

Three independent experiments were done in triplicates for all experiments including SRB, Real time PCR, Immunofluorescence, and Caspase 3/7. Line 155

2) Paclitaxel should also be included for Apoptosis and HSP expression studies.

Paclitaxel was used as a positive control and cytotoxicity was obtained for each breast cancer cell line to indicate to the reader that the graph obtained by the Paclitaxel followed a similar pattern to that of the decoction. Concentration of Paclitaxel used for the experiment was obtained by previous studies done with MCF-7 cells. Paclitaxel is a pure compound. The decoction contains many compounds and some of them may interact with the most active compound in the plant therefore we need to use a higher dose of the decoction. It may be possible to use smaller doses once we have isolated the active compound which will give comparable results.

3) Line 316 is not complete.

This line is now completed. Line 320

4) Support AO/EB with some form of quantification.

AO/EB is a qualitative assay that enables the user to distinguish between viable cells and those that are undergoing apoptosis via visual observation.

5) Recent reference relating HSP and breast cancer may be included.

Recent references have been included

Review 2
1. Fig. 1.1 shows the cell survival of different breast cancer cell lines after exposure to different concentrations of *F. leucopyrus* decoction. It is confusing, especially for the MDA-MB-231 cells, that there is a dose-depending decrease of cell survival with up to 100 µg/ml, but with higher concentrations, cell survival increases again. This observation is not discussed or explained by the authors. Here, it’s the question, if it’s really necessary to include such high (probably unphysiologic) concentrations since there are already strong effects with low concentrations. Furthermore, the figure does not contain any standard deviations or information about significant changes of cell survival by the treatments.

*We have also noted this observation with your comment. With your advice we have removed the higher doses of the decoction. This range of doses were selected as this is a preliminary investigation, an overview of the cytotoxicity of the decoction. Standard deviations have now been added to the graph.*

2. It should be mentioned in general, how many independent experiments were performed to achieve the results in Fig. 1.1, 2.1 and 3.1 and the standard deviations. Performing one experiment in triplicates is not the same as performing at least three individual and independent experiments what should be the basis for the statistical analysis. Just one experiment in triplicates is not acceptable for a proven significance.

*Here we failed to mention that 3 independent experiments were done in triplicates. This correction has been made in the manuscript. Line 155*

3. In Fig. 1.2 it is obvious that already low concentrations of Paclitaxel reduce the tumor cell survival. The possible advantage of using *F. leucopyrus* decoction rather than established anti-cancer agents should be discussed since quite higher concentrations have to be used to achieve comparable results.

*Paclitaxel is a pure compound. The decoction contains many compounds and some of them may interact with the most active compound in the plant therefore we need to use a higher dose of the decoction. It may be possible to use smaller doses once we have isolated the active compound.*

4. It’s not mentioned by the authors, why Fig. 1 contains Paclitaxel as a positive control, but none of all the other experiments. The treatment with Paclitaxel should at least be included in
the assessment of DNA-fragmentation or Acridine orange/Ethidium bromide staining to compare the effectiveness of the decoction treatment.

*Paclitaxel was used as a positive control and cytotoxicity was obtained for each breast cancer cell line to indicate to the reader that the graph obtained by the positive control followed a similar pattern to that of the decoction. Concentration of Paclitaxel used for the experiment was obtained by previous studies done with MCF-7 cells.*

5. Fig. 5 doesn’t contain any legend which should be included since it is not explained which lane contains which treatment. Additionally, also this figure should contain at least one positive control (e.g. Paclitaxel). Furthermore the concentrations that were used for this experiment should be comparable to those that were used for Acridine orange/Ethidium bromide and HSP70/HSP90 staining. A DNA fragmentation with 400 µg/ml of decoction and more could also be a non-specific effect by overloading the cells with the isolated plant proteins. This is of importance to possibly link decreased HSP70/90 expression and

*Legend is stated in line 576 in the manuscript. DNA fragmentation was done at higher doses with a longer incubation period as this event occurs at the execution phase of apoptosis. We used higher doses and longer incubation period to ensure immediate DNA fragmentation.*

6. The author’s discussion may include, that the used concentrations of the decoction are possibly not achievable under physiological conditions and that the results warrant further investigations by using appropriate animal models.

*Thank you for this valuable comment. This has been added to the conclusion.*

Minor Essential Revisions

1. The passage “Evaluation of cytotoxicity…” in “Methods” contains a mistake in the concentration unit (“µg/µl” should be changed to “µg/ml)

*Correction has been made. Line 136*

2. The manuscript could hugely benefit from assessing the activation of caspases 3/7 (for example by Western Blot analysis of cleaved caspases or cleaved poly-ADP-ribose-polymerase) to further underline the anti-tumor effects elicited by *F. leucopyrus* decoction

*Caspase 3/7 assay has been performed.*
3. The immunofluorescence analysis could benefit from adding a staining with an isotype control antibody and could be supported by additional Western Blot analysis of HSP70 and HSP90 proteins isolated from treated breast cancer cells.

*Funds weren’t sufficient to purchase western blot kit.*

**Changes made to the manuscript**

1. Revised manuscript with line and page numbers.
2. Abstract has been revised with Caspase activity
3. 136 line – changed made advised by reviewer
4. 153 line – described why decoction doses were selected for HSP expression. Advised by reviewer
5. 154 – definition of IC50
6. 155 - change made advised by reviewer
7. 168 - Definition of CT real time PCR. Advised by reviewer
8. 219 - caspase assay protocol
9. 226 – statistical analysis of caspase
10. 244 – paclitaxel used as positive control
11. 290 – line replaced as advised by the reviewer
12. 320 – line completed
13. 332 – explanation of DNA fragmentation
14. 341 – caspase discussion