Author's response to reviews

Title: Anticancer activity of a dichloromethane sub-fraction of Strobilanthes crispus on human breast and prostate cancer cells in vitro

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Author's response to reviews: see over
Dear Dr. Norton,

Thank you for inviting us to re-submit our manuscript entitled “Anticancer activity of a dichloromethane sub-fraction of *Strobilanthes crispus* on human breast and prostate cancer cells *in vitro*” to be considered for publication in *BMC Complementary and Alternative Medicine*.

To our knowledge, this is the first ever report on the cytotoxic potential of various fractions of the *S. crispus* plant in breast and prostate cancer cell lines and the cell death activity of one of the most cytotoxic fractions, in comparison with a few common chemotherapeutic agents. We appreciate the comments made by the reviewers and hereby enclose a point-by-point response to their comments. All changes have been highlighted in the text in red. We hope that our submission now meets the high standard that you require.

We confirm that the manuscript has not been published before and is not under consideration by another journal, and that the submission is approved by all authors. We also declare that we do not have any actual or potential conflict of interest, except for financial support for the work, which is stated in the Acknowledgment section of the manuscript.

Thank you

Sincerely,

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Author’s response to reviews

Reviewer 1

1. Fig.6: The cytotoxicity of T15, P100, Dc10 and Dc100 on MCF10A cell line, all showed increased at 48 h and then decreased at 72 h. The reason must be provided in the section “Results and Discussion” in text.

This paper is regarding the action of SC/D-F9 and not about the action of the anti-cancer drugs. The main point of the graph is to show that SC/D-F9 (even at twice the EC50 value) did not affect normal cells for at least up to 72 h of exposure when it significantly killed the cancer cells. This desirable effect of SC/D-F9 was compared to those of the conventional anti-cancer drugs to highlight its potential advantage. Hence we feel that discussion about the observed action of these individual drugs is not appropriate since it is not the subject of this paper.

2. Page 10/24: The authors indicated that “Work is currently underway to determine the mechanism of apoptosis induced by the S. crispus fraction and to identify the specific signal transduction pathway(s) involved, as well as to identify the bioactive component(s) in SC/D-F9.”.

Reviewer’s comment: However, the true pharmacological action mechanism can only be achieved by using a purified compound rather than by the crude extract or fractionate like SC/D-F or any other crude preparations.

While it is desirable to identify purified compounds, the use of fractionated or crude extracts may also provide an initial indication of the mechanism of action. To avoid confusion, we have re-phrased the sentence “Work is currently underway to identify the bioactive component(s) in SC/D-F9 to further understand the mechanism of action of S. crispus”.

3. Please show the proximate composition of fraction SC/D-F9, indicating the total phenolic, total isoflavonoid, total organic nitrogen (or alkaloids), and total phytosteroidal content.

We have included the total phenolic and flavonoid contents of SC/D-F9 in “Results and Discussion” as suggested. Unfortunately, we are not able to determine the total alkaloid and phytosteroidal contents at this point. We hope that this does not jeopardise the positive consideration of our paper, given that the main thrust of the paper is to highlight the potential anticancer effect of this plant fraction for future studies.

4. The specific activity relevant to fraction SC/D-F9 should be correlated with Comment 3.

This comment has been addressed in the Results and Discussion section

5. A flowchart must be attached to show the whole extraction and fractionation process.

This has been included as Figure 2. Subsequent figures have been renumbered appropriately
6. You used “Gradient step elution” and stated that “Gradient step elution was carried out using a combination of hexane, DCM and MeOH with an initial ratio of hexane-DCM-MeOH, 9:1:0 (v/v/v), followed by 4:1:0, 3:2:0, 2:3:0, 1:4:0, 0:1:0, 0:95:5, 0:9:1, 0:4:1, 0:3:2 and 0:2:3 (v/v/v)”. I really don’t understand why used so many combined solvent to elute, especially the intervention of DCM, if this is right, then the title should be changed according to the true flowchart. Be aware of the fact that your title was “Anticancer activity of a dichloromethane fraction of Strobilanthes crispus on human breast and prostate cancer cells in vitro”

Gradient step elution was carried out using many combinations of solvents to obtain various eluents of differing polarity. As mentioned in the text, these different eluents were then subjected to TLC to obtain the 15 sub-fractions based on their chemical composition. We hope that the inclusion of a flow chart as suggested by the reviewer would clarify this point further. We also agree with the reviewer regarding the title and have changed it to “Anticancer activities of a sub-fraction of dichloromethane extract of Strobilanthes crispus on human breast and prostate cancer cells in vitro”

Reviewer 2

1- They have tested the cytotoxic effects of these compounds on the cancer cell lines. Result of non-malignant cell line should be added to the result part and discussed.

The cytotoxicity test on MCF-10A cell line has actually been discussed in the Result and Discussion section – please refer to paragraph 5

2- T test is not suitable for the experiment. ANOVA should be used.

A statistician has been consulted and the use of T-test is appropriate. ANOVA tests the associations/interactions between different test samples which is not relevant to the current study.

3- Figure 2 and 3 are complex and not informative. It should be presented differently such as column or ....

We do not agree with this comment as replacing the graphs with a column or any other formats would not provide additional information or clarification.

Figure 2 shows the different levels of cytotoxicity induced by various sub-fractions of S.crispus dichloromethane extract on four different cancer cell lines. It can clearly be seen that sub-fractions 8, 9 and 10 are consistently highly cytotoxic to all four cancer cells.

Figure 3 (A-D) shows that the cell killing activity of SC/D-F9 occurs in a dose- and time-dependent manner in all four cancer cell lines tested. Graphs E-H show how the constant/stable effective concentrations were derived.

4- Apoptosis has been shown only by one method and in a descriptive manner.
   a- They have to use more methods such as western blot for apoptotic proteins.
   b- Annexin V and PI stating data should be presented at least in semi-quantitative.. Apoptotic cells should be counted in each view and compared to each other.
a- We have now included the determination of caspase activity by fluorescence microscopy. The ability of SC/D-F9 to activate caspase 3/7 further confirms the apoptotic cell death mechanism in all four cancer cell lines. These results are presented in Figure 10. This test is a better test than Western blotting since it directly reflects the function of the caspases rather than just their expression.

b- Figure 9 (dot plot) has been included to show the percentage distribution of live, early and late apoptotic cells as well as very late stage apoptotic or necrotic cells as assessed by flow cytometry. This figure may replace the original Figure 7 showing the staining of apoptotic cells, if deemed necessary.

5- Discussion is so poor. The aim of study has not been strongly discussed. The authors have to explain why they used ER-dependent and ER-independent breast cancer cells as well as the androgen-insensitive prostate cancer cells ie they have to explain the correlation between different response of cells to SC/D-F9 and ER-dependent and ER-independent.

As mentioned in the Background, tamoxifen is only effective in ER-positive but not in ER-negative breast cancer patients. In contrast, we have shown that SC/D-F9 is effective against both ER-positive and ER-negative breast cancer cells. To stress this point, we have included the following sentence in the text: “Therefore, we can infer that the cancer cell killing activity of SC/D-F9 is independent of whether the cells express ER or not – which is an advantage over tamoxifen”

Additional discussion is included to expound on the use of androgen-insensitive prostate cancer cell lines. Nonetheless, we clearly demonstrate that SC/D-F9 is effectively killing all four cells at low doses and hence, the responses are very similar rather than different. However, as we have indicated, more work is definitely required to understand the specific mechanisms involved in these different cells.

Minor comments:
There are grammatical errors in text and #English should be improved.

We have made changes in the text as necessary.