Author's response to reviews

Title: Evaluation of antioxidant and anticancer activity of extract and fractions of Nardostachys jatamansi DC in breast carcinoma

Authors:

Shilpee Chaudhary (shilcdy@gmail.com)
Kodangala S Chandrashekar (cksbhat@yahoo.co.in)
Karkala S R Pai (ksr.pai@manipal.edu)
Managanahalli M Setty (mm.setty@manipal.edu)
Raviraj A Devkar (ravirajdevkar@gmail.com)
Neetinkumar D Reddy (reddy.neetinkumar95@gmail.com)
Shoja M Haneefa (shojamh@gmail.com)

Version: 3 Date: 20 November 2014

Author's response to reviews: see over
November 20, 2014

From,
Dr. K S Chandrashekar,
Associate Professor,
Department of Pharmacognosy,
Manipal College of Pharmaceutical Sciences,
Manipal University,
Manipal-576104,
Karnataka, India.

To,
The Editor,
BMC Complementary and Alternative Medicine.

Dear Sir,

Subject: Submission of revised manuscript (ID: 4212233731394997) for evaluation.

I am sending you herewith our manuscript titled, “Evaluation of antioxidant and anticancer activity of extract and fractions of Nardostachys jatamansi DC in breast carcinoma”. As per the reviewers’ comments, we have made the required changes in the manuscript. Also, kindly note that we have changed the title of the manuscript as this title is more suited after revision. Below we have addressed the queries raised by the reviewers. Please note that the questions are shown in bold face and the responses are in normal blue font. We believe that the manuscript is suitable for further review.

Thank you.

Sincerely,

K. S. Chandrashekar
Response to first reviewer’s comments:

1. (Introduction)
- Authors should mention the below references as previous antioxidant and cytotoxicity work done on *Nardostachys jatamansi* roots extracts and draw the originality of their study compared to those previously published.

Response –
We have incorporated the references in the introduction and drawn the originality of our study compared to previously published data. Bhagat *et al.*, (2013) have reported the cytotoxicity of alcoholic extract and n-butanol fraction of *N. jatamansi* in lung, liver, ovary and prostate cancer cell lines. However, this is the first study comparing the cytotoxic activity of the whole methanol extract and subsequent fractions of *N. jatamansi* in ER-positive (MCF-7) and ER-negative breast cancer (MDA-MB-231) cells simultaneously. Also, to our knowledge, this is the first report of the effect of *N. jatamansi* extract/fractions on cell cycle progression, apoptosis and clonogenic capacity of breast cancer cells. Sharma and Singh (2012) have reported the antioxidant potential of crude hydroalcoholic extract of *N. jatamansi* by DPPH, superoxide, hydroxyl radical scavenging and total antioxidant capacity assays however, we report for the first time the antioxidant activity of extract and subsequent fractions of *N. jatamansi* to identify the bioactive fraction by various free radical scavenging assays and investigate a possible correlation between the antioxidant activity and total phenolic and flavonoid content of the plant extract/fractions.

2. (Introduction)
“The plant is described in the traditional systems of medicine for its use as sedative, antidepressant, antiepileptic, antihysterical, hypotensive, antispasmodic, anti-inflammatory, and cardiotonic”. This statement need to be referenced.

Response –
We have added the reference for the above statement.

3. (Results)
What is the rationale behind the presentation of the percentage of inhibition when the IC$_{50}$ values are also presented?

Response –
As per the reviewer’s suggestion, we have presented the IC$_{50}$ values of extract/fractions.
4. Figure 4; what is the contribution of these graphs in the study? Four chromatograms presented that might serve as a quality control for future reference. This seems to be unnecessary.
Response –
We have excluded the HPTLC fingerprinting of extract and fractions. As shown in Figure 4 in the revised manuscript, we have carried out a validated HPTLC method for detection and quantification of lupeol and β-sitosterol in the plant extract and fractions. Since, these marker compounds have already reported anticancer and antioxidant activities, the biological activities can partly be attributed to the presence of these compounds.

5. Table 2: (1) Please check for the significant difference between NJAQ and NJM since the SD for NJAQ is 0.94. (2) Please check for the meaning of NA as “Not active”, this might be ND for “Not determined”.
Response –
In table 2: (1) In Tukey’s multiple comparison test, the IC_{50} values for DPPH scavenging activity between NJAQ and NJM are not significantly different. This could be due to the higher SEM value (5.76) for NJM extract. The 95% CI of diff is -42.18 to 0.37. (2) NA stands for not active. NJPE fraction did not show nitric oxide scavenging activity even at the highest concentration tested (1 mg/mL) and no dose-response curve was obtained. Hence, it was written as not active.

6. In table 3: The meaning of the letter “a” in the correlation between total phenolic content and ABTS scavenging activity should be checked. If this represents a statistical analysis, what about the other values in the table?
Response -
The letter “a” stands for a statistically significant correlation (P < 0.05) between total phenolic content and ABTS scavenging activity. The correlation coefficients between other antioxidant activities and total phenolic and flavonoid content were not found to be significant, hence no alphabets are denoted.

7. Discussion - In general the discussion is wordy. Authors should provide interpretation of the results obtained and discuss their relevance instead of unnecessary citation of published literature.
Response -
As per the reviewer’s suggestion, we have made the discussion more succinct and avoided extensive citation of published literature.

8. Check for language errors correction.
Response -
We have checked the manuscript thoroughly for language errors correction and made the required corrections. In addition, we have got the manuscript reviewed for language errors by an expert.
Response to second reviewer’s comments:

1. The authors examine the antioxidant activity of *N. jatamansi*, which has been previously studied by others. Although the methods used to evaluate the antioxidant activity are clear and well made, they are not sufficient to provide evidence of the antitumor activity of this plant and even less about the relationship between the oxidation and the antiproliferative effect they report.

   Response -

   Sharma and Singh (2012) have reported the antioxidant potential of crude hydroalcoholic extract of *N. jatamansi* by DPPH, superoxide, hydroxyl radical scavenging and total antioxidant capacity assays however, we report for the first time the antioxidant activity of extract and subsequent fractions of *N. jatamansi* to identify the bioactive fraction by various free radical scavenging assays and investigate a possible correlation between the antioxidant activity and total phenolic and flavonoid content of the plant extract/fractions. The methods used to evaluate the antioxidant activity have been chosen carefully to cover a wide range of antioxidant activities. Since generation of free radicals and oxidative stress are implicated in carcinogenesis, plants with significant antioxidant potential can act as chemopreventive agents. The rationale for carrying out antioxidant assays was to establish the use of *N. jatamansi* as an adjuvant in cancer prevention by mitigation of oxidative stress. The antitumor activity of the extract/fractions have been carried out subsequently.

2. It necessary to determine whether the cells do not proliferate because there are an cell cycle arrest, or because cells becomes quiescent or died, and in this case it is important to determine at least by the standard methods, whether apoptosis or other mechanisms participate, in order to present a clearest picture of the biological activity of the fraction. The gold standard of this anti-tumor activity is still the clonogenicity, so, it would be desirable to test the activity of the most active fractions, in the ability to inhibit long-term proliferation of tumor cells.

   Response -

   As suggested by the reviewer, we have established the mechanisms of action of antitumor activity by the following methods –

   (i) Effect of NJM/fractions on cell cycle of breast cancer cells by flow cytometry.

   (ii) Pro-apoptotic effect of NJM/fractions by Hoechst 33258 staining.
(iii) Effect of NJM/fractions on clonogenic capacity of breast cancer cells to determine the long-term proliferation of tumor cells.

3. The culture conditions are important for these determinations and in fact, for breast cancer, 2D culture is not always the most appropriate method to determine the antitumor activity of a plant. Additionally, the use of only two cell lines ER+ and ER is not enough for assuring that this plant acts on the more aggressive tumors. It is important to be more cautious with the conclusions given in the proliferation of tumor cells because many other mechanisms could be involved in control of tumor cells and the authors have not taken them into account for the discussion.
Response -
As per the reviewer’s suggestions, we have made the required modifications in the discussion and conclusion.

4. Moreover, although the different extraction methods allow them to have different fractions of the plant part, the chemical characterization is very preliminary. It is necessary; in case the authors want to deepen into the chemical nature of the fraction, improve the analytical evaluation of it.
Response -
We have carried out the phytochemical characterization of the extract and fractions using phytochemical tests for determination of major classes of phytoconstituents. Also, we have identified and quantified lupeol and β-sitosterol in the extract and fractions by a validated HPTLC method.

5. It is important to define what is the main question they want to solve and what is the main message of the article. Need to strengthen even more chemical analyzes and biological tests on the lines. The single study of proliferation is not enough.
Response -
The main question of the present study is to determine the anticancer activity of N. jatamansi extract in breast cancer cells, identify the most bioactive fraction and decipher the probable mechanisms of action. In addition, we have carried out in vitro antioxidant assays to establish the use of N. jatamansi as an adjuvant in cancer prevention by amelioration of oxidative stress which is implicated in cancer progression.
As mentioned earlier, we have carried out further tests to strengthen the chemical and biological analyses.

6. From the experimental point of view, there is a mishandling of the controls, which do not appear in the figures.

Response -
For in vitro antioxidant assays, we have used equal volumes of solvent vehicle used to dissolve the extracts as negative control and appropriate positive control for the assays. For MTT and SRB assay, 0.1% DMSO was used as negative control since, the extracts were dissolved in DMSO such that the final concentration of DMSO did not exceed 0.1% in the wells. In case of mechanistic studies also, 0.1% DMSO in media was used as negative control for cell cycle analysis, apoptosis and clonogenic assays.

7. The edition of the figures is not very professional, so should seek an advisory on this issue. The legends of the figures are not clear enough.

Response -
As suggested by the reviewer, we have taken advice on figures and artwork. The figures have been modified for better resolution and clarity.

8. It is important also that the authors determine whether the proliferation analysis by other methods allows them to get the same results, since some compounds could interfere with MTT.

Response -
We have carried out the sulforhodamine B assay to confirm the findings in MTT assay (Table 5). NJM/fractions showed comparable results in SRB and MTT assay thus confirming that constituents of extracts did not interfere with MTT.