Author's response to reviews

Title: The Selective Cytotoxic Anti Cancer Properties and Proteomic Analysis of Trigonella Foenum-Graecum

Authors:

Abdulaziz Alsemari (alsemari@kfshrc.edu.sa)
Fahad Alkhodairy (alhkodairy@kfshrc.edu.sa)
Ahmad Aldakan (Aldakan@kfshrc.edu.sa)
Mai Al-Mohanna (Al-Mohanna@kfshrc.edu.sa)
Eman Bahoush (Bahoush@kfshrc.edu.sa)
Zakia Shinwari (Shinwari@kfshrc.edu.sa)
Ayodele Alaiya (AAlaiya@kfshrc.edu.sa)

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Author's response to reviews: see over
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Dear

Tom Rowles PhD
Executive Editor
BMC Complementary and Alternative Medicine

We are submitting the revised manuscript and the reviewer’s comments responses. In response to the editor comments, we added a statement under patient’s material states that the patient consented for publication of the clinical report and the study was approved by the ethical committee of the research advisory council. The revised manuscript conforms to the journal style. Below is points by point responses to the concerns of the reviewers are attached. Thanks

Regards

Abdulaziz Alsemari

*Abdulaziz AlSemari, MD
Department of Neurosciences
King Faisal Specialist Hospital and Research Centre.
Riyadh 11211 PO box 3354. Saudi Arabia

Tel 0096614424804. Fax 0096614424763
alsemari@kfshrc.edu.sa
A-Response to Reviewer Sreemoyee Chaterjee comments

i. Background: what is the concentration (8mg/----ml or L??) that is given to the patient as it just mentions the amount? Why specifically 8mg? Is the dose enough to cure such a fatal disease?

Response

The concentration was approximately 8g/day based on estimation. The 8 mg was writing error and it was corrected to 8g.day.....

ii. Authors have mentioned selective cytotoxic effects in the title. What is the implication of the word Selective? Is it necessary to use the word or the title without it also renders the same meaning?

Response

We have used the word selective because the particular fenugreek extract that the patient took was used for in vitro treatment of cancer and normal cells. We observed that the fenugreek extract selectively killed cancer cells but not the normal cells. likewise, the MRI lymphoma lesions disappear without residual effects on the brain tissue.

iii. Some typing and spelling errors are there:

Methods Cell Viability Assay: 3-(4, 5-dimethylthazol-2yl)-2,5 diphenyletrazlium bromide (MMT)-assay (it is MTT assay) and 370C It should be 370C. The Scientific name i.e. Trigonella should be in Italics under the title background. In vitro should be in italics under the heading conclusion before the heading background.
Response: All typing spellings have now been corrected. (MMT)-assay has now been changed to MTT assay and 370C is now written as 370C, likewise Trigonella is now in italics Trigonella as well as in vitro.

liii: Provide the rationale for specifically studying the limited dose range of the fenugreek seed extract.

Response: Prior to the experiment we have titrated the LC 50 and observed cytotoxicity changes in the range of 20 – 100, therefore we have selected the treatment range between 25 mg/ml as minimum and 200mg/ml.

iv. Why in the present study lethal dose/ tolerance study for the fenugreek seed extracts were not carried out?

Response : We did not conduct ic50 titration but rather we have only adjusted the concentration of the treatment dose based on a previous report on Nigella sativa and a reference have now been cited Ref.( Al-Johar, D., Shinwari, N., Arif, J.M., Al-Sanea, N., Abdul Jabbar, A., El-Sayed, R., Mashhour, A., Billedo, G., El-Doush, I., and Al-Saleh, I. Role of Nigella sativa and a number of its antioxidant constituents towards azoxymethane-induced genotoxic effects and colon cancer in rats. Phytotherapy Res. 22:1311-1323 (2008)).

v. Has the author used any standard drug to compare the results and to plot the graph?? There is no standard graph and IC50 information.

Response : We did not conduct ic50 titration but rather we have only adjusted the concentration of the treatment dose based on a previous report on Nigella sativa and a reference have now been cited.
vi. There is no heading like Materials used wherein the author can mention plant material, cell lines used etc.

Response: we adjusted the title in the manuscript to material and methods

vii. Provide the rationale for specifically choosing the concentration of the fenugreek seed extract.

Response: This was based on our unpublished data that we have used different concentration and decided to use the range between 100 – 300 up/ml. The chosen range is in agreement with other published report on fenugreek, a reference has now been added

Viii Methods: Apoptosis analysis by Annexin-V staining: The applied fenugreek extract was reconstituted in sterile water to yield the desired concentration of 300ug/ml. Provide a reference if required.

Response:

We have used water because it’s the simplest solvent and we achieved good solubility of the seeds. A Ref was added.

viii. Discussion part: median survival is 13.5, 22 months. What does the author mean by 13.5 and 22 months? The statement is in itself unclear.
Response:

This is the reported median survival in literature. The statement was changed to: (The reported median survival T-cell CNS lymphomas is between 13.5 and 22 months)

ix. What is the basis of choosing water as a vehicle/solvent for the drug extract preparation?  
Mention proper reference.

Response:

We have used water because it's the simplest solvent and we achieved good solubility of the seeds based on a previous paper. A reference has now been cited.

Ref

x. What is the relevance of conducting protein profile?? How is it related to the anticancer effect?

Response:

There are different types of fenugreek based on geographical location and this has well documented and we have cited references in the manuscript. The patient used a particular fenugreek from a particular geographical location. As mentioned in our discussion that other studies have confirmed the regional variations between fenugreek seeds across different geographical location. As we know most therapeutic agents acts as enzymes or proteins and the likelihood of discovery of proteins related to anti cancer effect of fenugreek was explored. Even though it is recognized that there are different types of fenugreek, this is the first study to characterized four different types based on their protein expression profiles. The protein expression in this study also support that the particular extract taken by the patient is different from other regional fenugreek. We recognized that the identification of implicated proteins were beyond the scope of this study and would be our future study to identify key implicating proteins as target for therapeutic or
treatment response monitoring. These are some of our rationales of the proteomics analysis of the four different fenugreek seeds.

**B-Response to Reviewer: Soundararajan Krishnaswamy Comments**

1. The second objective, the proteomic analysis of the fenugreek seeds, may not be very relevant to its anticancer properties unless the purpose of this part is sufficiently explained and related to anticancer function.

*Response*

_Under background_ It has been suggested that environmental and geographical regional differences may significantly affect the biologically active components in the extracts of different source of fenugreek harvests [24], but the extracts of different source of fenugreek has not been well characterized at protein level. One of the objectives of this study was to look at global protein expression pattern of the particular fenugreek type taken by the patient in comparison to other three different types of fenugreek. There are different types of fenugreek based on geographical location and this has well been documented and we have cited references in the manuscript. The patient used a particular fenugreek from a particular geographical location. As mentioned in our discussion that other studies have confirmed the regional variations between fenugreek seeds across different geographical location. As we know most therapeutic agents acts as enzymes or proteins and the likelihood of discovery of proteins related to anticancer effect of fenugreek was explored. Even though it is recognized that there are different types of fenugreek, extracts of different source of fenugreek has not been well characterized at protein level. To our knowledge, this is the first study to characterized four different types based on their protein expression profiles. The protein expression in this study also support that the particular extract taken by the patient is different from other regional fenugreek. We recognized that the identification of implicated proteins were beyond the scope of this study and would be our future study to identify key implicating proteins as target for therapeutic or treatment response monitoring. These are some of our rationales of the proteomics analysis of the four different fenugreek seeds.
2. Fenugreek extract from 8 mg of seeds per day for the patient is hard to believe. It must be more. This must be clarified. Also, additional treatment measures, which the patient underwent, should be mentioned. Or if the information is not available that should be mentioned in limitations of the study.

*Response:*

*The concentration was approximately 8g/day based on estimation. The 8 mg was writing error and it was corrected to 8g/day in the manuscript. No additional treatment was used during the palliative period.*

3. It is mentioned that fenugreek seeds were extracted in water (Methods section). Clarify if it was just cold water. Ground seeds or whole seeds? Also, mention the amount of seeds used for extraction; how they were dried and reconstituted?

*Response:*

*It as solubilized in water at room temperature, seeds were grounded, dissolved and solubilized. The solubilized extract was air–dried and reconstituted in sterile water. All procedures were done under sterile condition prior to in vitro treatment. This aspect has now been clarified under methods.*

4. Different varieties of fenugreek need more description as to how they differ from each other.

*Response:*

*The aim of the study was to demonstrate the effect of a particular fenugreek taken by the patients and we have attempted to describe the similarities and differences between fenugreek extracts from 4 different geographical regions using protein expression analysis. We showed marked quantitative and qualitative similarities in the protein expression pattern between all the four types of fenugreek. In addition, we showed that differential protein expression data revealed marked changes in the expression profiles between only one fenugreek type A and the rest fenugreek types B, C & D.*

*We belief more detailed descriptions of the differences between the fenugreek types is beyond the scope of this manuscript.*

5. Also, the goals of proteomic analysis of fenugreek varieties should be clarified;
are you looking for peptides with anticancer activities? Are there previous reports on similar peptides?

Response

This is similar to our response above, briefly, that there are different types of fenugreek based on geographical location and this has well been documented and we have cited references in the manuscript. The patient used a particular fenugreek from a particular geographical location. As mentioned in our discussion that other studies have confirmed the regional variations between fenugreek seeds across different geographical location. Extracts of different source of fenugreek has not been well characterized at protein level. To our knowledge, no previous reports on particular proteins derived from fenugreek extracts with anti-cancer effects, this is the first study to characterized four different types based on their protein expression profiles. The aim of this study was not to identify the proteins in fenugreek with anti-cancer effect, rather it was to show similarities and differences of the different regional fenugreek extracts based on their protein expression patterns. We recognized that the identification of implicated proteins were beyond the scope of this study and would be our future study to identify key implicating proteins as target for therapeutic or treatment response monitoring.

6. Fenugreek proteomes analysis by 2D gel may be okey; however, what do the differently expressed proteins/peptides mean functionally? The reasoning that the different protein profiles may be the reason for their possible differences in medicinal properties is too speculative. To defend this section (the proteome part), you may have to introduce sufficient literature support, explaining how the proteome analysis by 2D gel can be useful in understanding the medicinal properties.

Response:

Under background Protein biomarkers are often defined as specific enzymes or proteins that can be quantitatively measured and evaluated as objective indicators of phenotypic similarities or differences between normal biological or pathological state. Successful completion of the human genome project has accelerated advancements in proteomic technologies that lead to huge interest as a biomarker discovery tool in translational research. Proteomics studies have resulted in identification of disease related or tissue specific proteins that could be potentially useful as disease biomarkers. (Alaiya et al 2005).


We have now indicated statement on potential of proteomics as biomarker discovery tool as relevant to this study and supported by a review article reference.
7. Also, cancer cell line specific anticancer activities of fenugreek has been reported earlier; so, please give reasons for your work.

Response:

We agreed that there has been some other studies on fenugreek, but this study is unique as to our knowledge this is the first reported case of effective use of fenugreek on human subject. Therefore we have combined clinical parameters, in vitro assay and proteomics to elucidate the anti-cancer properties of fenugreek in this study.

8. The limitations of this study (which are given below) are obvious and so should be discussed in detail:

a. Very low amount of fenugreek used in treatment (6 mg per day) and the lack of information on other measures the patient may have been subjected to.

Response:

The approximate dosage was corrected to 8 gm per day as mentioned above.

b. How is the proteome analysis related to fenugreek’s anticancer properties?

Please read responses 4 and 5

Minor Essential Revisions:

1. Use uniform style (capitals and small letters) in key words section. Corrected

2. Take care of numerous typos (many of them easy to spot). Done

3. Paragraphs need proper alignment. Done
C-Response to Reviewer: Abdul Khader Mohammed Comments

1. In the introduction part authors provided the brief details about the clinical profile of a previously reported case suffering with primary CNS T cell lymphoma with some references. And they have also mentioned that the treatment of the same case with fenugreek showed a significant improvement in the right intra cerebral lesion frontoparietal. However, it is unclear whether this is first time to report the study of this kind? If yes, this thing could have been phrased in the discussion.

Response:
the discussion was rephrased to demonstrate the reciprocal in vivo and in vitro anti cancer response to aqueous solution of fenugreek and to focus on the only one of its kind long duration remission of a patient with relapsed primary T-cell lymphoma after regular daily use of aqueous extract of a particular Trigonella foenum graecum.

2. The whole of introduction is centered on the clinical profile of a single subject rather than the primary objective of this study.

Response:
We were informed by the first review to summarise the previously published case report and to include the follow-up information regarding fenugreek administration under the background section.

3. For proteomics analysis authors have used four different types of fenugreek extracts. It needs to clarify out of four extract which is used for cell culture treatment.

Response:
We have conducted proteomics analysis on four types of fenugreek extracts derived from different locations based of previously report of geographical types of fenugreek as cited in the manuscript. The patient used a particular fenugreek from a particular geographical location (A). As mentioned in our discussion that other studies have confirmed the regional variations between fenugreek seeds across different geographical location. Therefore, we have compared extract that the patient took derived from location A with three different geographical locations B, C, and D. To our knowledge, the extracts of fenugreek from different source has not been well characterized at protein level. We did not divulge the different geographical location as that was not the primary aim of the study. However, we have demonstrated high degree of homogeneity as well as subtle difference between the four geographical sources of fenugreek extracts.

4. Results section, it is mentioned that "similar results were observed for Anaplastic Thyroid papillary carcinoma" this sentence is quite confusing and it not clear whether the effects are similar to normal or cancer cell line.
5. The authors need to rephrase the discussion and improve the clarity of message.

Response:

The discussion was rephrased and focused as possible on the message clarity.

6. The first paragraph in the discussion needs to be re-written. This paragraph should clearly state the novel findings of this study before subsequent paragraphs discuss this in more detail. Indeed, the whole discussion lack the flow and focus.

Response:

Changes in the discussion were done to improve the flow.

General points

1. The manuscript contains many grammatical and syntax errors. Corrected
2. Ensure capitals are not employed unless necessary. Done