**Author’s response to reviews**

**Title:** Acute and sub-acute toxicity study of a Pakistani polyherbal formulation

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**Version:** 3 **Date:** 20 Jul 2017

**Author’s response to reviews:**

Respected Editor;

We would like to express our deepest gratitude for your response and reviewer’s valuable comments on our manuscript entitled “Acute and sub-acute toxicity study of a Pakistani polyherbal formulation” (Manuscript No. BCAM-D-16-01381R2). We appreciate the inputs given by the worthy reviewers. These inputs really helped us to improve the manuscript. We have modified the paper in response to the extensive and insightful reviewer comments and we hope that this will comply with the reviewer’s comments.

Editor’s Comments:

1) What is the dose and duration of treatment Soshiho-tang in people? It’s impossible to judge the applicability of the toxicity study without having this information.
The polyherbal product is Hab e Kabad Noshadri not Soshiho-tang as it was an example. The dose of the Hab e Kabad Noshadri as claimed by the label is 6 tablets a day for adults and 3 tablets a day for children. The exact duration of therapy of the product is not mentioned on the label and in this study we have identified the accurate dose based on the body weight using rat model. (http://qarshi.com/product/hab-e-kabad-noshadri/)

2) Why was a limit test selected for mice? Also why were males and females dosed? In standard practice, it’s usual to only dose the females unless one expects a different toxicity profile in males?

Answer: The limit test was scheduled for the mice because we had the information that 3g/ day dose of the polyherbal product is employed for adults clinically by the herbal practitioners, so limit test at one dose was carried out to find out the mortality in the animals.

The prevalence of liver dysfunctions is more in male population. Men are 2-fold more likely to die from chronic liver disease and cirrhosis than are women. Liver transplant occurs less commonly in women than in men. On average, there were twice as many liver cirrhosis male deaths as female deaths [1, 2]. Besides, the Animal ethical Committee of University and OECD guidelines for toxicity testing suggests the use of females, as they are more sensitive towards the toxic actions of the drug. Therefore, both genders were included to evaluate the safety profile of the polyherbal product.

References:


3) How were the doses for the rats determined?

Answers: The different doses selected for the toxicity study were chosen on the basis of the claim of the polyherbal formulation label (3g/day for adults). The 50mg/kg/day was selected according to the dose employed in adult human beings on daily basis. Higher doses (100 and 200 mg/kg/day) were selected to assess the toxicity profile for sub-acute toxicity study. The dose of the individual rats in all different groups was calculated based on the body weights before the start of the study.

4) Why were the clinical pathology parameters aimed at cholesterol and lipids?

Answer: Liver disorders generally results into impairment in the lipid metabolism [1]. Therefore, the lipid profile was assessed during the sub-acute study.
Reference:


5) Stats: The change in weight over time needs to evaluate by a more appropriate stats model, since the study has repeated measures.

Answer: The change in the statistics weight of animals have been done as per your requirement.

6) Stats: For the clinical pathology parameters mentioned. The study relies on a parametric statistic for significance. Was normality of the parameters shown?

Answer: During the study a control group has been designed, the parameters of which are mentioned in tables (Table 2, 3, 4, 5 & 6).

7) Data presentation: Why it is important to present the results by sex for the subacute study, the combined result independent of sex needs to be provided.

Answer: Either sex can be used for the toxicity study. However both genders are treated as separate subgroups due to significant size differences [1]. That is why we presented the results by sex difference in the results.

Reference:


8) In table 4: What is the female groups control value for bilirubin, is It meant to be 1.0, or 0.1

Answer: The value of bilirubin control for females is 1.0, it is corrected in Table 4.

9) Mention is made to normal parameters: Where did this range come from. Was genetic drift taken into consideration when choosing the range?

Answer: Yes the normal parameters range was taken from Toxicological handbook and the genetic difference was taken into consideration [1].

Reference:


10) The study makes the conclusion that the produce decrease liver enzymes: I have major concerns with this statement. The study made use of healthy animals. Why would the animals have raised enzymes levels? If the assertion is that the animals were not healthy, then the entire study is flawed and the results cannot be used.
Answer: Healthy animals were incorporated in this study. When the study was carried out in healthy subjects it was observed during the study that 50 mg/kg/day and 100 mg/kg/day resulted into decrease in the liver enzymes but not below the lower range. So the enzyme were within the normal range but lesser as compared to the enzymatic levels of the control group at the end of the study.

So, if it is decreasing the liver enzymes in healthy animals, then this could be interpreted that the product might also be given to population suffering from liver disorders.

11) An explanation needs to be provided for the clinical signs seen of nasal bleeding. Also an explanation needs to be provide why the clotting cascade was not evaluated after this clinical finding. Also a link needs to be drawn between the clinical pathology parameters and the clinical signs.

Answer: Liver damage results into the decreased synthesis of plasma proteins that are responsible for the clotting [1,2]. Liver damage results into portal hypertension which consequently shows the complication of bleeding due to raised pressure [3]. Compromising renal and ESRD, also results in hemostatic disorders mainly in the form of bleeding diatheses [4]. The clotting cascade was not noticed due to limited funds. The right upper paw paralysis may be due to the neurodegenerative action of the formulation in the specific part of brain controlling the right upper paw of the test subjects.

The decrease in the liver enzymes is due to the non-functional behavior of hepatocytes this happened due to the coalescence of the hepatocytes. The (atrophy) shrinkage and fusion of the hepatocytes can be observed in the histopathological slides of the liver as well.

The link among the clinical pathology parameters is simple. A drug after oral administration enters into liver through portal blood before it reaches the systemic circulation. The drug is first exposed to liver therefore the clinical biochemical parameters of liver were selected to be assessed for toxicity study. Also, the liver is the main site for the synthesis and metabolism of lipid, that’s why they were also assessed. Similarly, the kidney is the main organ for the excretion of drugs. Therefore, renal parameters were also included.

References:


12) An explanation needs to be provided as to why this product is not a hepatotoxin and nephrotoxin. The decrease in enzyme activity and lipid profiles is an indication of toxicity. Consideration must be given that this is a toxicity test and not a test of efficacy.

Answer: The reason for not suggesting the product as hepatotoxic and nephrotoxic because the decrease in the liver enzymes and lipid profile parameters was observed at higher dose i.e. 100 and 200 mg/kg/day. So, the product is said to be toxic if the daily recommended dose is above 7g for a 70 kg man.

The higher doses were selected in order to design the toxicity study and to find out the possible adverse effects that the drugs shows at higher doses.

13) The safety of the compound has not been properly discussed. The rodent NOEL needs to be converted to the human safe dose. The rodent toxicity study are just a basic indication of toxicity, but does not consider interspecies differences.

Answer: The product is already been used clinically in Pakistan community at the dose of 3g as claimed by the label. The study was designed to scientifically prove the safety of the claim dose and higher doses were chosen to assess the toxicity profile of the product.

14) The study mentions that nephrotoxicity is possible with chronic use. What is the extrapolated safe period that this product can be used, based upon the 28 days duration of treatment of the rodents.

Answer: The product label does not state any duration of therapy. In our toxicity study we have identified that the product is safe when used for 28 days at dose of 50mg/kg/day.

Reviewer Reports:

Reviewer 1

We are thankful to Meenakshi Bajpai for her appreciable comments regarding the MS.

Reviewer 2

We appreciate the comments of Ian Musgrave regarding our MS.

There is no explanation given for why mice are used for acute toxicity then rats for sub chronic, why not the same species throughout? Also, in the discussion it reads as if only rats were used.

Answer: The animal Ethical committee of our University emphasize on the use of smallest weight animals for the acute toxicity study. Therefore we used Swiss Albino mice for acute toxicity and Wistar rats for chronic toxicity study.
There is no citation to show that the preparation being studied is effective in liver disorders.

Answer: The citation is added (http://qarshi.com/product/hab-e-kabad-noshadri/) in the introduction.

Page 4, line 53: "After the arrival of 'medical science' the phytotherapy was given the grade of alternative therapy." This is not actually true. Some phytotherapies (fox glove, cinchona) were superseded because the pure compounds (digitalis, quinine) could be given reliable and accurately, others (salicylin in willow bark, yohimbine) were speeded by more effective compounds that were produced (acetylsalicylic acid, beta blockers). Some phytherapies are alternative medicines, but many became feedstocks for purified chemicals. Why is medical science in quotes?

Answer: We agree with your comments. The part of introduction is reframed.

Page 4, line 54: "Herbal therapy encompasses … Homeopathic." Homeopathic medicine is not herbal medicine, as there is no trace of the original compounds in the homeopathic medicines.

Answer: The word Homeopathic is deleted.

Problems with references

Page 4, line 60: "a number of botanical drugs, have proved to be very efficient in curing sicknesses [4]." Reference 4, "Subacute toxicity and stability of Soshiho-tang, a traditional herbal formula, in Sprague-Dawley rats" does not support this statement, but is a toxicological study of one herbal therapy.

Page 4, line 64: "These combinations are employed for the betterment of various chronic disorders [6]" Reference 6, "Acute and subacute toxicity studies on the polyherbal antidiabetic formulation Diakyur in experimental animal models" does not support this (and should be reference 7, as it supports the following sentence).

Page 4, line 71: "In addition to this, conventional people and even still certain physicians invoke the usage of medicinal 72 and curative herbs to aid the medication therapy for better clinical outcomes [8]" is not supported by reference 8, "Evaluation of acute and sub-chronic oral toxicity study of baker cleansers bitters - a polyherbal drug on experimental rats".

Reference 23 is in the wrong format and has no journal, volume of page number.

Answer: All the problems with the references are addressed properly.

Statistics
The data are expressed as mean and standard error of the mean, not "Values are expressed as standard error of mean" as is used throughout.

Answer: Corrected

"** represents highly significant p<0.01" highly significant is not an appropriate statistical term "** represents significance of p<0.01" is more appropriate.

Answer: Corrected

Interpretation: Page 11, line 192 "This effect indicates that the formulation considerably decrease the raised liver enzymes" the control values for ALT and AST are within the reference ranges of normal rats, these values are not raised.

Answer: It was a typing error. The statement is reframed.

What is the normal dose of Hab e Kabad Noshadri for an adult human? How does this compare to the doses in this study? As the preparation is to be given to people with liver disease, the adverse liver effects must warrant more caution than suggested in this paper.

Answer: The study was designed based on the label claim of the product available in the Pakistani market and is recommended by the herbal practitioners to the population majorly suffering from hepatic disorders. The study was emphasized on the toxicity of the herbal product at different doses on different organs rather its hepatoprotective action.

Minor issues

Page 2, line 1: "Acute and sub-acute toxicity study of Pakistani polyherbal formulation" would be better as "Acute and sub-acute toxicity study of a Pakistani polyherbal formulation"

Answer: Corrected

Page 3 line 30: "methodology, has a wide spread to people at risk of contracting." Risk of contracting what?

Answer: “has a wide spread to people at risk of contracting the side effects of the herbal medicines.”

Page 3, line 31: "The aim of study was to assess the acute and sub-acute toxicity of polyherbal formulation" is better as "The aim of this study was to assess the acute and sub-acute toxicity of the polyherbal formulation"

Answer: Corrected

Page 3, line 32: "In acute study, single dose of 2000mg/kg was administered to the mice and were observed for physical symptoms and behavioral changes for 72 hrs” would be better as "In
the acute arm of the study, a single dose of 2000mg/kg was administered to Swiss Albino mice which were observed for physical symptoms and behavioral changes for 72 hrs".

Answer: Corrected

Page 3, line 33: "In sub-acute toxicity studies repeated doses of polyherbal preparation was administered in rats of both genders, separately." Is better as "In the sub-acute toxicity studies repeated doses of the polyherbal preparation was administered to rats of both genders, separately."

Answer: Corrected

Page 3, line 36: "On 28th day of experiment, blood sampling of animals were done" is better as "On the 28th day of experiment, blood sampling of animals was done"

Answer: Corrected

Page 4, line 50: "Herbal medicines are focused as the popular therapies to treat diseases by the largest group of world population" do the authors mean something like "For most of the world's population, herbal medicines are the most popular form of therapy"? For many, it is the only available or affordable therapy.

Answer: The part of the introduction is reframed.

Page 4, line 51: They have accomplished widespread appropriateness as medicinal agents." I am uncertain as to what this sentence means.

Answer: The part of the introduction is rephrased.

Page 4, line 52: "Before 1800, when medicinal therapy was introduced in the scientific era, the herbal therapy was the only obvious choice" is better as "Before 1800, when science-based medicinal therapy began to be introduced, herbal therapy was the only available choice" (this is not actually true, mineral drugs such as mercury were in use since medieval times, and the use of sulphur as a medicine dates back to classical Greece).

Answer: We highly appreciate your comments. We comply with your statement about sulphur and mercury. The sentence was taken from the book as mentioned in the reference “In PDR for Herbal Medicines.” If you want us to remove and reframe, it will certainly be done.

Answers to Reviewer’s comments

Reviewer 2 – Ian Musgrave

Significant interpretation issues:

In regard to the herbal preparation reducing liver enzymes
"So, if it is decreasing the liver enzymes in healthy animals, then this could be interpreted that
the product might also be given to population suffering from liver disorders".

This is not an appropriate interpretation at all. There is no link to suggest that the reduction in
healthy enzyme levels will work in a pathological situation as you have no mechanistic link.
Especially as later on the authors say "The decrease in the liver enzymes is due to the non-
functional behavior of hepatocytes this happened due to the coalescence of the hepatocytes." If
anything this suggests that this preparation could exacerbate liver damage. Please be more
careful in your interpretations.

Answer: The interpretation was made on the basis of the product formulation. The label claims
the presence of Embelia and Senna as an ingredients in the formulation that have proved to be
effective in liver disorders [24, 25]. The formulation also contain chebulicmyrobalan yellow
(Terminalia chebula), that has phenolic content which inhibits phenotype change in HSC in liver
and reduces the infiltration of neutrophils in the liver and protecting liver from inflammational
damage [26]."

However your comment is appreciable as our sentence is not properly structured and presented,
the changes in the normal liver enzymes cannot interpret the effects of the medicine in diseased
state.

References:


[25]. Shanmugasundram R, Devi VK, Tresina PS, Maruthupandian A, Mohan VR.
hepatoprotective activity of ethanol extract of Clitoria ternatea L. and Cassia angustofolia vahl
leafs against CCl4 induced liver toxicity in rats. Int Res J Pharm. 2010; 201-205.

Inhibitory effect of yellow myrobalan (Terminalia chebula) extract on fibrosis induced by carbon

Page 10, Line 209: "The liver parameters of male and female rats of all treatment doses groups
showed that the levels of ALT and AST declined highly significantly as compared to control at
dose of 200 mg/kg/day". ALT and AST were significantly lower at the 50 mg/kg and 100 mg/kg
doses, even if they were still within the normal range. This is problematic.

The authors state in the response to the first reviewer "The decrease in the liver enzymes is due
to the non-functional behaviour of hepatocytes this happened due to the coalescence of the
hepatocytes. The (atrophy) shrinkage and fusion of the hepatocytes can be observed in the
histopathological slides of the liver as well." While this applies to the highest dose, that fact that
AST and ALT are falling at the lowest dose of herbal is concerning. While no frank
histopathology is seen, changes in enzymes may precede gross histopathological changes.
Answer: There is continuous oxidative stress naturally present inside the liver, as a result of which AST and ALT have a constant level inside the blood. The reason behind falling of enzymes at lower levels with lower doses (50 mg/kg and 100 mg/kg) is because the formulation ingredients contain flavonoids and phenolic contents. These species are reported to encounter the oxidative stress inside the body. As a result they are lowering the normally present oxidative stress due to which the enzymes levels fell but remained within the normal range. Therefore, no abnormal histopathology was observed at these doses.

From the first reviewer:

"The rodent NOEL needs to be converted to the human safe dose. ….[deletion]"

Answer: The product is already been used clinically in Pakistani community at the dose of 3g as claimed by the label. The study was designed to scientifically prove the safety of the claimed dose and higher doses were chosen to assess the toxicity profile of the product."

The question is "whether 3 g dose clinically used is safe or not" and converting the NOEL to human values is important to answer this question.

Given that there is very little margin between the 50 mg/kg dose, and the 100 mg/kg dose where problematic changes are seen, the margin of safety would appear to be very low in this preparation. This should be more clearly indicated.

Answer: The doses are converted according to the following chart [1].

Dose 1 = 50 mg/kg/day

Dose for 0.15 kg rat = 50 x 0.15 = 7.5 mg/ 0.15 kg

Conversion into Human equivalent dose (HED) = 7.5 x 0.162 = 1.215 mg/kg

Dose for 60 kg (Avg.) human = 1.215 x 60 = 72.9 mg/ 60 kg man ≈ 75 mg / 60 kg

Dose 2 = 100 mg/kg/day

Dose for 0.15 kg rat = 100 x 0.15 = 15 mg/ 0.15 kg

Conversion into Human equivalent dose (HED) = 15 x 0.162 = 2.43 mg/kg

Dose for 60 kg (Avg.) human = 2.43 x 60 = 145.8 mg/ 60 kg man ≈ 150 mg / 60 kg

Dose 3 = 200 mg/kg/day

Dose for 0.15 kg rat = 200 x 0.15 = 30 mg/ 0.15 kg

Conversion into Human equivalent dose (HED) = 30 x 0.162 = 4.86 mg/kg
Dose for 60 kg (Avg.) human = 4.86 x 60 = 291.6 mg/ 60 kg man ≈ 300 mg / 60 kg

According to product’s regimen = 2 Tab. TID (Wt. of Tab. is 500 mg), it means 6 tablets a day

So, 50 mg/kg = 75 x 6 = 450 mg ≈ 500 mg / 60 kg / day

100 mg/kg = 150 x 6 = 900 mg ≈ 1000 mg / 60 kg / day

200 mg/kg = 300 x 6 = 1800 mg ≈ 2000 mg / 60 kg / day

The clinically used dose i.e. 3 g doesn’t appear to be safe. As from our study it was seen that the margin of safety is narrow that’s why the conclusion was made to prescribe 100 mg/kg cautiously to the patients unless otherwise the therapy should be continued with 50 mg/kg.

Reference


"There is no citation to show that the preparation being studied is effective in liver disorders.

Answer: The citation is added (http://qarshi.com/product/hab-e-kabad-noshadri/) in the introduction." This is just the website of a provider, there is no citation to clinical studies showing efficacy.

Answer: There are no clinical studies available on internet but the formulation is still being prescribed by the herbal practitioners on the basis of the individual ingredient pre-clinical data available. The pre-clinical data is mentioned in the discussion. The references of individual ingredients activities are mentioned below;

References


"Page 4, line 52: "Before 1800, when medicinal therapy was introduced in the scientific era, the herbal therapy was the only obvious choice" is better as "Before 1800, when science-based medicinal therapy began to be introduced, herbal therapy was the only available choice" (this is not actually true, mineral drugs such as mercury were in use since medieval times, and the use of sulphur as a medicine dates back to classical Greece).
Answer: We highly appreciate your comments. We comply with your statement about sulphur and mercury. The sentence was taken from the book as mentioned in the reference "In PDR for Herbal Medicines." If you want us to remove or reframe, it will certainly be done." The sentence should be reframed, especially if it was taken directly from the book.

Answer: The sentence is reframed. “In the 18th century, when the medicinal therapy era was being introduced, herbal treatment was the most preferred and available therapy.”

Page 8 line 66: "have proved to be very efficient in curing various sicknesses. For example; Digitalis (Foxglove) as cardiotonic for heart failure," digitalis treats, but does not *cure* heart failure. This needs to be reworded. Also the most recent reviews on ginseng do not provide good evidence for cognitive support https://www.ncbi.nlm.nih.gov/pubmed/26268331 (let alone efficient "cure").

Answer: The sentence is reworded. The “curing,” is replaced with the word “management.” The line regarding, ‘Ginseng,’ is removed from the introduction.

Page 8 line 70. "Echinacea for the treatment of common cold etc.” The most recent Cochrane systematic review (2014) https://www.ncbi.nlm.nih.gov/pubmed/24554461 concludes "Echinacea products have not here been shown to provide benefits for treating colds”.

Answer: The line regarding, ‘Echinacea,’ is removed from the introduction.

Page 21 line 323: "During the sub-acute toxicity studies 200 mg/kg/day showed significant decrease in the liver enzymes in male rats.” Liver enzymes decreased significantly at ALL doses in both male and female, if you intend to say the levels only fell below the reference range at 200 mg/kg/day you need to say that as well.

Answer: The sentence is reframed as directed. “During the sub-acute toxicity studies, a significant decrease in the liver enzymes with 200 mg/kg/day was observed in both male and female rats and this decrease was below the normal reference range.”

Page 22 line 351. "The 28 days sub-acute toxicity study, revealed no significant changes with 50 mg/kg/day." AST and ALT were significantly reduced. In light of the histopathology at higher doses this is concerning. Increasing body weight is also a concern, given there is no nutritional benefit to this preparation. Did the authors check for oedema? (This would be consistent with alterations in serum proteins leading to coagulation dysfunction at higher doses)

Answer: There was a gradual increase in the body weights of the male and female rats of both untreated (control) and treated groups (50 mg/kg/day, 100 mg/kg/day, 200 mg/kg/day) as shown in the Figure 1 and 2. The comparison of weight was made with their respective body weights from day 1. The increase was there as the animals were on standard pelleted diet throughout the experiment. There was no nutritional value associated with the formulation. Besides, there wasn’t any oedema observed in the treated animals groups.
Page 22 line 353: "So, it is concluded that the formulation is safe to use at dose of 50 mg/kg/day for a period of 28 days" the 50 mg/kg dose and the 100 mg/kg dose is quite low, and should be commented on. "Whereas the 100 mg/kg/day should be cautiously employed" given the results, this recommendation is over optimistic. An explicit margin of safety should be calculated.

Answer: The recommended regimen of the product is 2 Tab. TID. The weight of the tablet is 500 mg. So, the prescribed daily dose is 3g/day. According to our calculation human daily dose is approx. 500 mg to 1 g maximum for avg. 60 kg BW human, in three divided doses per day but as this research was conducted in normal animals so further scientific analysis should be done in different liver disorders to calculate the dose depending upon the severity of the condition. Our data provides a safe therapeutic range to be suggested to avoid toxicity.

The duration of 28 days was suggested on the basis that if high dose i.e. approx. 1 g for avg. 60 kg BW is recommended than of the therapy should be cautiously continued for longer period of time along with the analysis of LFT’s.

Grammar and typographical:

Abstract: "has a wide spread to people at risk of contracting the side effects of the herbal medicines" may be better as "is widely used in the general population exposing them to the risk of the side effects of the herbal medicines"
Answer: The sentence is reframed.

Page 8 line 58: "Many compounds from herbal origin have accomplished widespread appropriateness as medicinal agents" is better as "Many compounds from herbal origin have achieved widespread use as medicinal agents"
Answer: The sentence is reframed.

Page 8, line 69. Garlic is Allium sativum, not Zingiber (that is ginger).
Answer: It is corrected.

Editorial comments:

Please include the email addresses of all manuscript authors on your Title Page.

Answer: The emails are added.

Please remove the ARRIVE checklist from your additional files. While we thank you for providing us with this, we do not consider it necessary for it to be published as an additional file alongside your manuscript.

Answer: It is removed.
Please ensure that you reference your 'chart' supplementary file, and it's contents, within your manuscript. For simplicity, you may wish to include a 'supplementary files' section in your manuscript.

Answer: We have added the section, "Supplementary material," in the MS. Kindly process it according to your journal's guidelines.

Reviewer's comments:

Please include all comments for the authors in this box rather than uploading your report as an attachment. Please only upload as attachments annotated versions of manuscripts, graphs, supporting materials or other aspects of your report which cannot be included in a text format.

Answer: All comments for authors are added in this box and supporting material is uploaded as suggested.

I wish to thank the authors for their extensive and detailed attention to my concerns. I am now satisfied with the manuscript.

Response: We are highly grateful to you for sharing your valuable comments regarding our MS. They certainly helped us a lot in improving the status and structure of the MS. Regards.