Author’s response to reviews

Title: The use of Euphorbia hirta L. (Euphorbiaceae) in diarrhea and constipation involves calcium antagonism and cholinergic mechanisms

Authors:

Muhammad Zeeshan Ali (zeeshan10feb@gmail.com)
Malik Hassan Mehmood (malikhassan.mehmood@gmail.com)
Muhammad Saleem (saleem2978@hotmail.com)
Anwar-ul-Hassan Gilani (anwarhgilani@yahoo.com)

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Response to reviewer’s comments:

CAM-D-19-00834R2

Underlying mechanisms explaining the use of Euphorbia hirta (Linn) in diarrhea and constipation

Reviewer reports:

Ephrem Engidawork, PhD (Reviewer 1): I thank the authors for providing a detailed response for the queries I raised. I have, however, some concerns that the authors should address before reaching a decision on the manuscript.

* The effort made to revisit the rationale for conducting this study is laudable. However, there is still a need for attending language issues.

Reply: Thank you sir for your kind and encouraging comment, we have given due consideration to our manuscript’s language issues and we hope that revised manuscript will be suitable for publication.

All changes are incorporated in the revised manuscript in green colored text

* You could modify the title as "The use of Euphorbia hirta L. (Euphorbiaceae) in diarrhea and constipation involves calcium antagonism and cholinergic mechanisms"

Reply: Thank you your kind suggestion, we have revised as suggested
Revised title has been incorporated in the revised manuscript in green colored text as

“The use of Euphorbia hirta L. (Euphorbiaceae) in diarrhea and constipation involves calcium antagonism and cholinergic mechanisms”

* Page 11, Preparation and extraction: plant collection and identification as well as specimen deposition should be included.

Reply: Thank you for your kind suggestion, initially we removed based on the suggestions of second reviewer, however, we have revised as suggested and incorporated changes in the revised manuscript in green colored text as;

“Whole plant was collected in November, 2017 from the surroundings of Faisalabad, Punjab, Pakistan. The plant was identified by the expert taxonomist Dr. Mansoor Hameed, Associate Professor, Department of Botany, University of Agriculture, Faisalabad. The specimen (voucher no. 415-1-2019) was kept at herbarium of University of Agriculture, Faisalabad.”

* I am not still convinced about the fractionation. The hydroalcholoic crude extract (70% methanol) was used for fractionation with solvents of differing polarity. The question here is what's the likelihood of getting constituents soluble in non-polar solvents (Pet. Ether) from a semi-polar-to polar crude extract?

Reply:

Thank you for pointing, we agreed with the worthy reviewer that there is a lesser possibility of getting non-polar constituents’ from semi-polar-to polar crude extract, this was also evident from our experiment that the yield of lower polar solvent led fraction, particularly Pet. Ether was lowest compared to relatively higher polar solvent led fractions. However, 70% methanol is considered to contain three hydrogen atoms and hydroxyl attached to carbon and supposed to solubilize the non-polar phytoconstituents as well. In previous studies this proportion (70.30) of methanol and water has also been used in our lab and in other labs as well (Zakaria et al., 2016; Truong et al., 2019; Malik et al., 2017; Najeeb-ur-Rehman et al., 2012; Mehmood et al., 2011) and further fraction was carried out as performed in our current study.

References


Najeeb-ur-Rehman, Mehmood, M.H., Adnan J Al-Rehaily, Ramzi AA Mothana, Anwar H Gilani Species and tissue-specificity of prokinetic, laxative and spasmodic effects of Fumaria parviflora, BMC Complementary and Alternative Medicine, December 2012, 12:16 | Cite as


* Laxative effect higher in physiological than pathological conditions (lopermiad-induced constipation), indicating diarrhea is a side effect rather than a therapeutic effect. What do the authors say about this assertion?

Reply:

Thank you for giving an opportunity to respond, in case of diseased model/pathological condition the loperamide-induced its effects predominantly involving opioid receptors which leads to reduce bowel movements, gastrointestinal secretions, water fecal contents and fecal output. The laxative effect of E. hirta in loperamide-induced constipation is mediated specifically through predominant involvement of opioid receptors while in naïve animals, E. hirta produced its effects also involving multiple pathways like muscarinic receptors activation and other unidentified mechanisms, hence we may not be able to conclude that laxative effect higher in physiological than pathological conditions (lopermiade-induced constipation), indicating diarrhea is a side effect rather than a therapeutic effect of E. hirta.

We have also included in the discussion section of revised manuscript as;

In discussion section, page no 11, paragraph no. 1

“Carbachol, a cholinergic drug [38], is known to excite gut musculature through muscarinic receptor activation resulting in increased gut motility, augmented gastrointestinal secretions and enhanced total fecal output including wet feces [39]. Loperamide is known to induce spastic constipation, reduces fecal mass and delays fecal evacuation by inhibiting gut secretions and motility [40] mainly mediating its effect involving opioid receptors. Thus, these findings on the part of E. hirta in naïve and loperamide-fed constipated mice not only supports its folk use in
hypoactive gut disorders but also a further exploration of a preliminary report on E. hirta indicating the presence of only in vitro non-specific gut muscle stimulant effects [23].

The prominent laxative effect of E. hirta in naïve mice compared to its effect in loperamide-fed constipated mice signifies that the observed laxative effect may involve multiple mechanisms. In the isolated tissues, gut stimulant effect was found primarily mediated through activation of muscarinic receptors, while its effect in loperamide-fed constipated mice indicated the added involvement of opioid receptors, thus providing as evidence in part to the medicinal use of E. hirta in constipation.

Table 3, once you compare loperamide treated group with controls, you need to compare loperamide+extract group with loperamide only treated group to show the ability of the extract to reverse loperamide-induced constipation. Loperamide+extract group can also be compared with normal controls to see if the extract is able to return bowel movement to the basal level.

Reply:

Thank you for giving us an opportunity to revise table no.3, we have revised this table as advised.

Table 3 Laxative effect of E. hirta extract in loperamide–induced constipated mice

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Mean defecation /group</th>
<th>Mean amount of wet feces/group</th>
<th>% Wet feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Saline (10 mL/kg)</td>
<td>10</td>
<td>8.21 ± 0.45</td>
<td>0.79 ± 0.12</td>
<td>9.63</td>
</tr>
<tr>
<td>2.</td>
<td>Loperamide + saline</td>
<td>5 + 10</td>
<td>2.31 ± 0.14***</td>
<td>0.20 ± 0.09*</td>
<td>8.6</td>
</tr>
<tr>
<td>3.</td>
<td>Loperamide + EH.Cr (p.o)</td>
<td>5 + 50</td>
<td>4.16 ± 0.13@@/+</td>
<td>1.19 ± 0.38 ns/ns</td>
<td>28.6</td>
</tr>
<tr>
<td>4.</td>
<td>Loperamide + EH.Cr (p.o)</td>
<td>5 + 100</td>
<td>5.1 ± 0.35@@/+</td>
<td>1.80 ± 0.31 @@/ +</td>
<td>35.3</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM, n = 6 animals/group. *p < 0.05 and ***p < 0.001 show a comparison of group no. 2 vs group no. 1 (Students t-test). @ p < 0.05 and @@ p < 0.01 show a comparison of group no. 3 and 4 vs group no. 2. + p < 0.05 and ++ p < 0.01 show a comparison of group no. 3 and 4 vs group no. 1, ns represents non-significant (One-way ANOVA followed by Dunnett’s test).
Rabbit jejunum (Page 16, Line 41-51): The authors alluded that the chloroform, ethyl-acetate and aqueous fractions inhibited the spontaneous contractions and their effects were potentiated in the presence of atropine. However, the inhibitory effect of pet-ether fraction was remained unchanged when reproduced in the presence of atropine (Fig. 4). Similar to parent extract, the fractions of E. hirta also equally inhibited the high and low K+-induced contractions except aqueous fraction, which showed partial inhibitory effects (Fig. 4). I would say the chloroform fraction also behaved like the pet-ether fraction if one sees Figure 4, although the EC50 value given in Table 3 appeared to be a little bit different. In addition, no apparent change could be discerned among the crude extract and fractions in their effect in the presence of high and low K+ concentration, except the aqueous fraction, where the inhibitory effect was mild. I think the statement "The extract and fractions of E. hirta equally inhibited the high and low K+-induced contractions" is misleading. If one looks at Figure 3 & 4, the inhibition obtained at high and low concentration did not differ. Since high K+ is associated with Ca2+ influx and low K+ with K+ channel activation, the lack of difference between the two strongly suggests that Ca2+ antagonism rather than K+ out-flux to be the likely mechanism. Discussion should be rewritten in such a manner.

Reply: Thank you for pointing out this mistake, we have rectified accordingly in the revised manuscript as;

In results sections “Effect on Rabbit jejunum” as

“The ethyl-acetate (Et.Ac.EH) and aqueous (Aq.EH) fractions inhibited the spontaneous contractions, while the observed inhibitory effects were potentiated when studied in the presence of atropine. However, the inhibitory effect of the chloroform (CHCl3.EH) and pet-ether (Pet.EH) fractions remained unchanged when reproduced in the presence of atropine as seen in (Fig. 4). Similar to parent extract, the fractions of E. hirta inhibited the high and low K+-induced contractions without any specificity against high or low K+-challenged contractions except the aqueous fraction which showed mild inhibitory effect (Fig. 4).

In discussion section, page no.11 paragraph no.3

To investigate the possible mechanism of the observed inhibitory effect, the plant material was tested on sustained contraction induced by high [30] and low [43] concentration of K+ which involves the entry of Ca++ into muscle cell via voltage dependent Ca++ channels (VDCCs) and K+ channel opening, respectively. The plant and quercetin caused inhibition of both high and low K+ induced-contractions without any specificity against high and low K+- contractions, similar to the effect of nifedipine, a known Ca++ antagonist [32,33]. Since high K+ is associated with Ca++ influx and low K+ with K+ channel activation, the lack of difference between the two strongly suggests that Ca++ antagonism rather than K+ out-flux to be the likely mechanism.

In discussion section, page no.12 paragraph no.3
“In rabbit jejunum, the crude extract, aqueous and ethyl acetate fractions displayed dual (spasmolytic and spasmogenic) components with varying degrees, which was evident by the potentiation of the observed spasmolytic effect when reproduced in the presence of atropine, a cholinergic antagonist [38]. The chloroform and petroleum ether fractions exhibited prominent relaxant effects compared to ethyl acetate fraction while the aqueous fraction was found the weakest in its spasmolytic effect.

References


* Table 1 and 2: delete 10 from the dose column for the control and replace it with "-".

Reply:

It has been deleted as suggested in the revised manuscript

* Cite Tables consecutively in the text. For example, Table 3 is a new addition and cited in the text as EC50 values.

Reply:

It has been cited as suggested in the revised manuscript in green colored text as

“The comparative inhibitory effects in terms of EC50 values of the crude extract and its fractions are summarized in Table 4.”

Reply:

Dhan Prakash, Ph.D. (Reviewer 2): The confusing point is the use of Euphorbia hirta (Linn) in diarrhea and constipation which are antagonistic to each other
Reply: Thank you for providing us an opportunity to reflect on, we have provided details in discussion section along with evidence as detailed below;

In discussion section, page no.11 paragraph no.2

The co-presence of spasmodic and antispasmodic constituents is well known and has already been reported on the part of other medicinal herbs [1, 3, 41].

In discussion section, page no.12 paragraph no.4 and page no 13, paragraph no.1

The data showed that excitatory effect of E. hirta is predominantly mediated through muscarinic receptors activation, which causes an increase in the gut motility directly effecting gut musculature, however the muscarinic agonist(s) are not used to treat constipation due to their non-specificity in action which leads to undesirable effects like bradycardia, diarrhea, abdominal cramps, salivation, convulsions and increased urination [50]. E. hirta possesses combination of gut stimulant and relaxant components which are installed by nature to antagonize excessive gut stimulant and/or relaxant effects when required or specific expression of constituents (gut stimulant or relaxant) as per disease status (constipation or diarrhea). The co-existence of opposing nature of constituents is commonly observed in ispaghula [1], Carissa carandas [3], Hibiscus rosasinensis [30] and ginger [41] which indicates the possible synergistic and/or adverse effect overcoming combinations in same remedy.


All changes except deletions were made in green colored text in the revised manuscript. We hope that the revised manuscript has been improved.

Best regards,
Hassan