Author’s response to reviews

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

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Version: 2 Date: 06 Oct 2019

Author’s response to reviews:

Response to reviewer’s comments:

Ephrem Engidawork, PhD (Reviewer 1):

Thank you for providing us the opportunity to respond.

Q1 * The plant is a widely studied plant, reported to have antimicrobial, antioxidant, cytotoxic effects as well as effects on organ system, including the gastrointestinal system. It is demonstrated to have antidiarrheal activity against castor oil, arachidonic acid and prostaglandins induced diarrhea. Ex-vivo analysis also showed the plant to inhibit smooth muscle contraction of guinea-pig ileum. What value, if any, the present work would add to the existing body of knowledge?

Reply: We agree with the worthy reviewer that this plant has been studied for its multiple pharmacological actions including its use in gut disorders, like diarrhea which is actually highlighting the potential health benefits of this popular medicinal plant. Keeping all the data together which has been reported earlier support medicinal use of Euphorbia hirta in diarrhea and covers only its in-vivo antidiarrheal effects without providing insight into mechanism(s) likely to be involved in its antidiarrheal action. While no study is available to fill the existing gap of a preliminary report of Kumgang et al. (2009) who showed that it possesses gut stimulant property but couldn’t carried out its precise identification and characterization.

Current findings of our study clarify the paucity in the literature supporting the medicinal use of E. hirta in constipation and diarrhea. This is also the first study reporting possible mechanisms explaining the usefulness E. hirta in diarrhea and reporting pharmacological (insight into mechanisms and in-vivo laxative potential) basis to the effectiveness of E. hirta in constipation.
In earlier in the manuscript, we have already addressed this point highlighted by worthy Editor as

“In support of the dual medicinal utility of E. hirta in opposing gut disorders like diarrhea and constipation, previous reports have shown unclear findings like, Tona et al. (2000) has revealed non-specific antispasmodic action of E. hirta in the in vitro and Hore et al. (2006) demonstrated only gut inhibitory effects of E. hirta in naïve rats and castor oil administered mice. While, Galvez et al. (1993) reported its gastrointestinal transit delaying potential in only castor oil administered animals, while no such activity in naïve animals. On the other hand, Kumgang et al. (2009) showed its gut stimulant effects in the in vitro and antidiarrheal activity in the in vivo. Keeping in view the folk use of E. hirta in diarrhea and constipation, and to further explore the paucity in existing literature whether it possesses both laxative and antidiarrheal effects in the in vivo and/or in the in vitro, the primary objective of this study was to determine the antidiarrheal and laxative efficacy of E. hirta and the possible pharmacological basis of the identified effects. While the secondary objective was to estimate the distribution and comparative efficacy of gut stimulant and relaxant constituents in polarity-driven fractions of E. hirta.

Reference


Q2* I presume the authors used 70% methanol for extraction and the crude extract was subjected to fractionation using solvents of differing polarity. Why did the authors use 70% but not 80% methanol, which is considered as a universal solvent for extraction? If the authors used the crude extract (70% methanol extract) for fractionation, what is the rationale for using non-polar solvents like petroleum ether for fractionation. I would understand if the material used for extraction had been the plant not the extract.

Reply: We agree with the reviewer, either 70 or 80% methanol could have been used as solvents for components extraction, which needs proper pre-profiling of secondary metabolites of the plant and their maximum solubility. The mixture of 70 % methanol and water has been used to extract maximum constituents soluble both in methanol and water, further we carried out
polarity-driven fractionation of the crude extract using different solvents like petroleum ether, chloroform, ethyl acetate and water was carried out. This helps to isolate solvent specific constitutes isolation. The used proportion (70.30) of methanol and water has also been used in our lab and in other labs as well (Truong et al., 2019; Malik et al., 2017; Najeeb-ur-Rehman et al., 2012; Mehmood et al., 2011).

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


Q3* The authors stated that all experiments were performed in mornings. However, rodents are known to be nocturnal animals and experiments are recommended to be done after 4:00 pm or reverse the light/dark phase. Given this fact, how do the authors ruled out the effect of time of experiment on their findings?

Reply: Thank you for pointing out this important concern, we agree with reviewer, the dark-light cycle was reversed.

Q4* I am not sure whether there is a need to have a separate section for the chemicals. This is done usually in theses.

Reply: Keeping in consideration the comment of the worthy reviewer, we have excluded the section of chemicals from manuscript, however we added the source of chemicals along the chemical name where it was described to be used in revised manuscript.

Q5* Suffice if you say grouping was done by randomization (Page 9, Method: castor oil induced diarrhea). What were the parameters used to determine antidiarrheal activities? Usually, onset of diarrhea, total number of feces, and number of wet feces are used. These should be described in the Method section.
Reply: - We have rectified indicated description in the revised manuscript both in methods and results, accordingly. Mean no. of wet feces and mean total fecal output were monitored and detailed in the results likewise. The onset of diarrhea was not monitored during the assay, hence it has been removed from the method section, we are sorry for this typo mistake and the inconvenience caused.

Q6* Rabbit jejunum was used for spasmolytic activity and rat ileum than guinea-pig ileum was used for spasmogenic activity. It would be good if the authors explain why they used these different preparations. Moreover, the way the data was presented is confusing. Naturally, one should start with acute toxicity study to show the plant is safe (If at all there is a need to do acute toxicity, as the literature is full of the activities of the plant). This should be followed by the effect of the extract and known spasmogens on isolated tissues to have an idea about how the effects are produced. This is then narrowed down by incubating the tissue with extract in the presence and absence of the respective antagonists. Once that is ascertained other mechanistic studies could be followed. Fractionation is performed to identify constituents for the observed activity. However, no effort has been exerted to compare the dose response curve of extract and different fractions, for example, on high K+ induced contraction. This would give an idea which one has a strong calcium antagonism. From the data presented in the manuscript, it appears that the petrol-ether fraction largely acts via calcium antagonism, the aqueous via cholinergic mechanism (see the next comment on this issue), and the others act by both ways, although I do not see such phrases in the conclusion, defeating the purpose of fractionation.

Reply: - Thank you for providing us opportunity to respond, rabbit jejunum is known to produce spontaneous pendular movements resulting in steady state of contraction and relaxation in the organ bath supplied with physiological salt solution at 37 oC and aerated with carbogen. It is considered a suitable preparation for the assessment of spasmolytic activity without using any agonist and antagonist (Mehmood et al., 2011).

We agree with the reviewer, guinea-pig ileum is suitable preparation and behaves like a quiescent preparations, devoid of any regular spontaneous movements to determine any pasmogenic activity. Similarly rat ileum also behaves better like semi-quiescent preparations and is known to respond better to the spasmogenic agents compared to rabbit ileum (Peddireddy, 2011; Najeeb-ur-Rehman et al., 2012), therefore we have used rat ileum in our experiments.

As worthy reviewer suggested, either of these approaches could have been used to assess and characterize spasmogenic effects of test material(s), in this study, we first determined presence of any spasmodic activity of the test material(s) and further narrowed down by reproducing same spasmodic effects in the presence of known antagonist like atropine (0.1 µM), a cholinergic antagonist, pyrilamine, a histaminergic receptor antagonist (1 µM), and methysergide, a serotonergic antagonist (1 µM) simultaneously to characterize nature of determined spasmodic effects of test materials (Najeeb-ur-Rehman et al., 2012; Mehmood et al., 2011).

As proposed by the worthy reviewer, we have already compared and discussed the distribution of the spasmolytic and spasmodic activities among fractions in the discussion section as
“The crude extract, its aqueous, ethyl acetate and chloroform fraction were found to possess both spasmolytic and spasmogenic components with varying degrees. The petroleum ether fraction was found to possess only relaxant effect. Further the distribution of spasmolytic effects, the pet-ether and chloroform fractions were found to possess prominent spasmolytic effects relative to ethyl acetate fraction while the aqueous fraction was found weaker in its spasmolytic activity. Thus, indicating the nonpolar plant components concentrated in organic solvent led fractions showed predominantly antispasmodic effects

In rat ileum EH.Cr and its fractions caused gut excitation mediated through the activation of cholinergic pathway with varying potency. The aqueous fraction was found the most potent followed by ethyl acetate and chloroform fractions while pet-ether was devoid of any excitatory effect, thus revealing the polar-nature of spasmogenic constituents”

Though we have earlier described the comparative spasmogenic and spasmolytic efficacy of the fractions in the discussion. As per suggestion of the worthy reviewer, we have added in the conclusion of revised manuscript as

“Fractionation revealed that petroleum ether fraction exclusively acts through calcium antagonist like mechanism, the aqueous fraction predominantly involves cholinergic agonist like pathway, while the others showed dual (spasmodic and antispasmodic) effects.”

To determine acute toxicity test was not primary objective as plant has already been used in variety of ailments and for its toxicity studies (Ping et al., 2013), we have carried out this assay to further ascertain the safety of the plant material up to 10 folds of the maximum effective dose determined in our assays.

References


Q7* Doses used for evaluation of the antidiarrheal effect was larger (500 mg/kg & 1000 mg/kg) than the laxative (50 mg/kg and 100 mg/kg) one. What were the assumptions in selecting
these doses for the two opposite effects? The authors asserted that the plant has both gut stimulatory and inhibitory effects and suggested muscarinic and calcium antagonism, respectively, to be the underlying mechanisms. One has to take into account that muscarinic receptors (excitatory) are coupled to the phosphoinostide pathway that eventually culminates in increase in intracellular calcium concentration. Thus, this is in contrary with the calcium antagonism concept, unless otherwise the authors show that the source of calcium in the muscarinic case is solely from subcellular calcium stores. In addition, the gut stimulatory effect of the plant shown in Figure 6 and 7 is a bit confusing. Although the spasmogenic activity appears to increase with dose initially, it disappears with higher doses, raising doubt about the reproducibility of the results.

Reply: Thank you for highlighting this point, theoretically, there should be no co-existence of opposing nature of constituents in a single remedy, however, such combinations in natural test material(s) with opposing effects are in-built by the nature to either off-set the excessive effect on one end or the expression of desired dominant disease pathology oriented effect at dose-related approach. Thus, this needs further detailed studies to investigate the basis of opposing nature of effects to correlate with only different doses and/or with related disease pathology.

In this study, we selected doses for both antidiarrheal and laxative assays on the basis of available literature on this plant and our previous experience of experimentation on medicinal plants. Initially selected doses of this test material were also preliminary screened in the in vitro and in vivo assays (date not shown in the manuscript) before carrying out complete dose-dependent assays. We found dose-related in vitro findings and in vivo effects and the possibly explanation could be the dominant expression of antispasmodic components at relatively larger doses compared to spasmodic effects at relatively lower doses as described earlier in the manuscript in discussion section as

“The data showed that excitatory effect of E. hirta is mediated through the muscarinic involvement, which increase the gut motility directly effecting on gut musculature, however the muscarinic agonist could not be directly used to treat the constipation due to their non-specify in action and leads to undesirable effects as bradycardia, diarrhea, abdominal cramps, salivation, convulsions, respiratory increase and increased urination [50]. E. hirta possesses combination of gut stimulant and relaxant components with by nature are meant to antagonize their excessive effects when required. This dual nature of the plant is meant to overcome the harmful effects associated with excessive gut stimulant effect might be leading to abdominal cramps usually observed with the use of chemical drugs for the treatment of constipation. Therefore dual activity of E. hirta, gut inhibitory (Ca++ channel blockers) and excitatory (cholinergic agonist), has the merits on one hand for its use in the treatment of diarrhea and constipation and also meant by nature to off-set the excessive effect of either nature. This dual nature of herbs 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.

References
Q8* For evaluation of laxative activity, one has to induce constipation experimentally using for example loperamide. But, laxative effect was determined in the present study using normal animals. What was the purpose of using this paradigm?

Reply: As per worthy reviewer’s suggestion, we have further performed the laxative activity in loperamide-induced constipated mice and have modified accordingly the revised manuscript.

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</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 < 0.01 show comparison of group no. 2–4 vs group no. 1, (One-way ANOVA followed by Dunnett’s test).

Q9* Acute toxicity test is performed using a limit test (usually 2g/kg) and I do not understand why the authors used three dose levels, one of which is very high (10 g/kg).
Reply: The acute toxicity test was performed to ascertain safety of the test material up to as high as 10 folds (10 g/kg) of the determined maximum effective dose as seen in castor oil-induced diarrheal assay. Further, this also compelled us to include additional doses covering multiple times of lower and lowest effective doses, as seen in laxative activities, of our study. Hence, we selected three different doses covering multiple folds spectrum of determined effective doses in our experiments. Doses selection on such pattern have also been studied earlier in the literature (Mehmood et al., 2014).


Q10* Page 14, Line 47-53: the plant and quercetin caused equal inhibition of both high and low K+-induced contractions similar to the effect of nifedipine, a known Ca++ antagonist, indicating the ability to restrict calcium entry via blockage of calcium channels and ruling out the involvement of K+ channels activation in its spasmolytic effect. I do not think this is a right interpretation. If you look at Figure 3 & 4, the tested agents appeared to be relatively equipotent in inhibiting low and high K+-induced contraction, suggesting that both mechanisms (potassium channel activation and calcium channel inhibition) are operative in the inhibition process.

Reply: Very respectfully, we would like to add that potassium channel openers only inhibit the low K+ induced contraction with no or very little effect against high K+-induced contractions as reported in the literature in case of cromakalim, a standard K+ potassium channel opener (Ali et al., 2015; Gilani et al., 2008). While the calcium antagonists are known to equally inhibit the both spasmogens, low and high K+ induced contraction (Khan et al., 2012). Similarly, we observed and described findings on the part of our test material in relation to nifedipine, a known calcium antagonist. And to further ascertain this effect, we have drawn the calcium concentration response curves of all test materials which showed a rightward non-parallel shift in the Ca++ CRCs with significant suppression in maximum response like nifedipine, while a only potassium channel opener is devoid of such effects.

References

Ali MZ, Janbaz KH, Mehmood HM, Gilani AH. Antidiarrheal and antispasmodic activities of Polygonum bistorta rhizomes are mediated predominantly through K+ channels activation. Bangladesh J Pharmacol. 2015;10:627-34.


Q11* Page 11, Line 34-36: Unpaired t-test/ or One-way analysis of variance (ANOVA) followed by Dunnet's test was applied for differentiation of data for laxative and antidiarrheal. Specify in the Table legend where the unpaired (For example, Table 3) test was used like you did for One way ANOVA.

Reply: - We are sorry for the typo mistake, it has been rectified as “One-way analysis of variance (ANOVA) followed by Dunnet's test and One-way ANOVA followed by Bonferroni test were applied for differentiation of data for laxative and antidiarrheal activities”

Dhan Prakash, Ph.D. (Reviewer2):

In most of the cases the conclusion drawn from experiments in results and discussion is mentioned for example The determined antidiarrheal effect of E. hirta is also in line with the earlier reports indicating its antidiarrheal efficacy in animal models.

In most of cases it is mentioned therefore, we are unable to understand the novelty and scientific merit of present studies.

Reply: We agree with the worthy reviewer that this plant has been studied for mostly for its in vivo antidiarrheal activities and a uncharacterized muscle relaxant effect, therefore when we assessed similar effects also referred earlier reports.

Keeping all the data together which has been reported earlier to support medicinal use of Euphorbia hirta in diarrhea covers only its in-vivo antidiarrheal effects and an uncharacterized muscle relaxant effect without providing insight into mechanism(s) likely to be involved in its antidiarrheal action. While no study is available to fill the existing gap of a preliminary report of Kumgang et al. (2009), who showed that it possesses gut stimulant property but couldn’t carried out its precise identification and characterization.

Current study clarifies the paucity in the literature supporting the medicinal use of E. hirta in constipation and diarrhea. This is the first study reporting possible mechanisms explaining the usefulness E. hirta in diarrhea and reporting pharmacological (insight into mechanisms and in-vivo laxative potential) basis to the effectiveness of E. hirta in constipation.

In earlier in the manuscript, we have already addressed this point highlighted by worthy Editor as “In support of the dual medicinal utility of E. hirta in opposing gut disorders like diarrhea and constipation, previous reports have shown unclear findings like, Tona et al. (2000) has revealed non-specific antispasmodic action of E. hirta in the in vitro and Hore et al. (2006) demonstrated only gut inhibitory effects of E. hirta in naive rats and castor oil administered mice. While, Galvez et al. (1993) reported its gastrointestinal transit delaying potential in only castor oil administered animals, while no such activity in naive animals. On the other hand, Kumgang et al. (2009) showed its gut stimulant effects in the in vitro and antidiarrheal activity in the in vivo.
Keeping in view the folk use of *E. hirta* in diarrhea and constipation, and to further explore the paucity in existing literature whether it possesses both laxative and antidiarrheal effects in the in vivo and/or in the in vitro, the primary objective of this study was to determine the antidiarrheal and laxative efficacy of *E. hirta* and the possible pharmacological basis of the identified effects. While the secondary objective was to estimate the distribution and comparative efficacy of gut stimulant and relaxant constituents in polarity-driven fractions of *E. hirta*.

Reference


Q 12. Suggestions marked on attached manuscript may also be taken into consideration.

Reply: All suggestion on manuscript file were modified/deleted as suggested by the worthy reviewer

All changes except deletions were made in red colored text in the revised manuscript. We hope now the manuscript in its revised form has been improved

Best regards,

Hassan