Reviewer's report

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

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Reviewer: Ephrem Engidawork

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* 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?

* 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.

* 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?

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

* 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.

* 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 spasmogenes 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 pet-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.

* 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.

* 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?

* 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).

* 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.

* 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.

Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.
Yes

**Does the work include the necessary controls?**
If not, please specify which controls are required in your comments to the authors.

Yes

**Are the conclusions drawn adequately supported by the data shown?**
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No

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I am able to assess the statistics

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