Response to Reviewers' Comments

Manuscript Title: Rhubarb extract partially improves mucosal integrity in chemotherapy-induced intestinal mucositis

Authors: Juliana E. Bajic, Georgina L. Eden, Lorrinne S. Lampton, Ker Y. Cheah, Kerry A. Lymn, Jinxin X. Pei, Andrea J. Yool and Gordon S. Howarth

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General comments:

In the present manuscript the authors aim at investigating the effects of orally gavaged aqueous rhubarb extract on 5-Fluorouracil-induced intestinal mucositis in rats. They suggest rhubarb extract (at a low dose) improves mucosal integrity and reduces ileal inflammation induced by 5-FU. The manuscript is well written and data are overall consistent.

> **Comments:** The authors would like to thank the reviewers for their comments and have made amendments to the manuscript and highlighted the changes in yellow to their easy location throughout the paper.

Reviewer's major comments:

Reviewer 1: Is the phytochemical composition of the RE used in the present study known (tannin's percentage in the present batch, etc.)? This information is important so as to compare present data to other studies. This is part of quality control on studies involving herbal products. Reviewer 2: Dear Authors Congratulations for your manuscripts. I gave some comments to the editor. My main question is about the composition of RE that you use, and the potentially active component.

> **Comment:** The authors have made the following amendment to the Materials and Methods, Rhubarb Extract Preparation section: "Based on fractionation of the extract, the active agent appears to be a water-soluble ethanol-insoluble glycopeptide. Lectin array profiling has indicated that mannose and N-acetylglucosamine are predominant components of the carbohydrate structure. The precise chemical structure and possible presence of more than one isoform with biological activity remain to be determined."
>
> Our method of preparation of rhubarb extract collected only highly water soluble constituents, and excluded previously characterised organic-solvent-extracted bioactive compounds. In separate studies, commercially available compounds known to be present in rhubarb (including aloe-emodin, rhein, oxalic acid, and others) were tested individually in oocyte swelling assays and were found to have no effect on AQP1-mediated water fluxes (data not shown).

Authors should introduce data on AQP first (it is Figure 1 here) or move figure on these data to the end on the manuscript. There's no problem to discuss these data before since it is a potential mechanism of action for RE very far from reality presented here (here data are mostly in rats), although very relevant to the literature.

Comment: The authors concur with the reviewer's comment and accordingly have amended the manuscript so that the AQP data is the final figure (Fig. 7) in the manuscript.

"Cloned mammalian AQP 4 water channels ". It would be correct to mention "clonned rat AQP4 water channels". Authors mention "mammalian" in the figure and in the text, but in the material and methods section they clearly specify the use of a rat AQP4 cRNA, which is pretty logical.

Comment: The authors concur with the reviewer's comment and accordingly have amended the manuscript to include "cloned rat AQP4 water channels" when mentioned in the Methods and Results section of the abstract, the core tip and in the Results section titled *" Dose-dependent blockade of AQP4 water channel activity by extracellular aqueous rhubarb extract"*.

Before the experiment per si, how do the authors evaluate adequate expression and function of cloned rat AQP4 in the oocytes? This information is important.

Comment: There's actually no positive control to evaluate viability and function of oocytes upon changes induced by osmotic gradients so as to be compared to RE in the present dataset.

Expression of functional rat AQP4 channels was confirmed by the consistent significant increase in osmotic water permeability as compared to control (non-AQP4 expressing) oocytes. Methods followed standard protocols established in prior work (Jung JS et al, 1994. PNAS 91:13052-60) which were verified by immunocytochemistry and western blot. The use of oocytes for assays of analyses of osmotically-driven water fluxes has been a principal tool for the analysis of mammalian aquaporin channels since the cloning and characterisation of the first water channel, AQP1, more than two decades ago (Preston GM et al. 1992, Science 256: 385-7).

The authors should have used a purified tannin or substance abundantly found in RE as a positive control in the AQP4 experiment. There's actually no positive control to evaluate vibility and function of oocytes upon changes induced by osmotic gradients so as to be compared to RE in the present dataset.

Comment: No rhubarb components other than the extract characterised here have to date been found to block aquaporins, and thus a positive control other than RE is not available. The key comparison with control oocytes serves as the essential negative control. Prior work has defined other small-molecule pharmacological blockers of aquaporins using the oocyte expression system (Pei, J.V., Burton, J.L., Kourghi, M., De Ieso, M.L., Yool, A.J.. 2016 Drug discovery and therapeutic targets for

pharmacological modulators of aquaporin channels. Ch 14 (pp 275-297) in *Aquaporins in Health and Disease: New Molecular Targets For Drug Discovery* (2015), eds Soveral, G., Casinin, A., Nielsen, S., CRC Press, Oxfordshire UK).

"It is therefore plausible that the caloric index of HDR may have been contributing to the reduced appetite, yet maintenance of bodyweight in the rats receiving high dose RE". Is this caloric index (numbers, percentage) known?

Comment: The compound, if a glycopeptide, would have a lower caloric content than pure sugar. However, estimating the amount as sugar only, the maximum estimated caloric content for a dose of 200 mg/kg would be approximately (~50 mg/animal) x (3.87 Cal/g) = 194 Cal which would be a significant proportion of the average rat total caloric intake in a day. However, in the case of LDR, the contribution would only about 10% of daily intake. Since there was not a substantial difference in body weights observed between the HDR and LDR groups, the differences in caloric content did not appear to be major factors in body mass responses to 5-FU treatment.

"These results are consistent with previous studies which have exploited plant polysaccharides for their anti-inflammatory and antioxidant properties". A plausible hypothesis although we do not know in the present study the phytochemical content of RE. Antioxidant effects might play a major role on the protection found by the authors, but this is greatly underestimated in the present manuscript. Neutrophil accumulation is likely to appear very quickly in response to alarmins (IL-33, IL-1-beta, chemokines) that will shortly follow ROS production within the mucosa. This inflammatory response is probably dumped by a rich antioxidant environment. However, we do not know how antioxidant RE is.

Comments: The authors addressed the phytochemical content of RE in the first major comment. Our method of preparation of rhubarb extract collected only highly water soluble constituents, and excluded previously characterised organic-solvent-extracted bioactive compounds. In separate studies, commercially available compounds known to be present in rhubarb (including aloe-emodin, rhein, oxalic acid, and others) were tested individually in oocyte swelling assays and were found to have no effect on AQP1-mediated water fluxes (data not shown). Future studies are warranted to target the antioxidant capacity of RE by analysis using appropriate assays; for example the 2,2-diphenyl-1-picryl hydracyl radical (DPPH) assay determines the radical scavenging ability of compounds and would be useful in this instance.

Reviewer's minor comments:

Introduction: Please provide an updated general reference on traditional herbal medicines on the treatment of a "wide variety of diseases and disorders", with a focus on cancer and cancer-chemotherapy side-effects.

Comment: The authors concur with the reviewer's comment and accordingly have amended the introduction as per below:

"Traditional herbal medicines have been used for centuries in the maintenance and improvement of health or the treatment of illnesses. Globally, ancient herbal remedies have been based on theories, beliefs and experiences representing various cultures at different times throughout history. Consequently, traditional herbal medicines are being investigated increasingly for their potential to treat and reduce the symptoms of a wide variety of diseases and disorders, specifically cancer and its treatment-related side-effects. Many cancer patients seek alternative medicines that will complement their standard-care treatments with the hope that they will improve symptoms associated with either the cancer or their anti-cancer treatments."

Introduction: How AQPs are impacted during inflammation, mostly on the TGI? How will they loose function: before, during, after tissue damage is established? Are they downregulated upon inflammation? Are they linked to barrier stability, tight junctions, etc? Please enrich this section.

Comments: The authors have enriched this point in the introduction to say:

"In the human gastrointestinal tract, AQPs 3, 7 and 8 are expressed throughout the mucosal epithelia, and AQP1 is present in endothelial cells of the vasculature. In early stage inflammatory bowel disease, tight junctions and transport systems are impaired, leading to a leaky epithelium. Clinical human biopsies showed that levels of expression of AQPs1 and 3 are reduced in Crohn's Disease and AQPs 7 and 8 are decreased in ulcerative colitis, based on quantitative PCR and immunolabelling assays (Ricanet P, et al., Clin Exp Gastroenterol 2015. 8:49-67). As well, the typical apical localisation of AQP8 in bowel was lost, and the appearance of a faint basolateral signal suggested intestinal epithelial cell polarity was disrupted."

How were RE and 5-FU doses defined (previous work?)?

Comments: LRE dose for gavage was based on the estimated dose needed to block aquaporin water channel activity in the oocyte expression system, and the dose HRE was selected as a 10 fold higher concentration for comparison. 5-FU dose was based on previous work from our lab inducing intestinal mucositis in an acute model (Whittaker *et al.*, Lab Anim 2016. 50(2):108-18; Whittaker *et al.*, Lab Anim 2016. 49(1):30-9; Cheah *et al.,* PLoS One. 2014 Jan 21;9(1): PMID 24465501; Mashtoub *et al.*, Exp Biol Med (Maywood). 2013 Nov 1;238(11):1305-17). Accordingly, the authors have included these details in the Materials and Methods section of the manuscript.

How do the authors macroscopically define GIT sections to collect tissues for further analysis? With lesions and contraction of the GIT upon 5-FU treatment this macroscopic perspective might change. Please clarify.

Comments: The authors concur with this comment as 5-FU exposure induces lesions and contraction in the GIT. Consequently, segments of the small intestine (SI) tract (2cm and 4cm) were calculated for each animal and collected at approximately 10% (jejunum) and 90% (ileum) of the total SI length and snap-frozen in liquid nitrogen for biochemical analysis or transferred to 10% buffered formalin.