Author's response to reviews

Title: Hexane fraction of Ardisia crispa Thunb A.DC root inhibits inflammation-induced angiogenesis

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Author's response to reviews: see over
Dear Editor,

I herewith attached the amendments (in bold) that have been done based on the comments from the reviewers.

Revisions made following review from Reviewer 1: Umesh K. K. Jain

**Major revision**

1. Methods: Plant material: the extraction is performed by Soxhlet method with 80% Ethanol at room temperature. How it was performed? Soxhlet method needs application of temperature. - *We have corrected the information regarding the Soxhlet temperature to 80°- 90°C.*
2. Compound AC-2: Publication reference required. No need to produce detailed data - *For compound AC-2, the spectroscopic data was omitted, and replaced with publication reference (27).*
3. Figure 1: Give title to fig 1. values should be self defined, give the subtitles to values. - *Title has already been given to figure 1. Subtitles to explain the process were also included below the figure.*
4. In RESULTS: Further separation of ACRH yielded about 2.3 g (38/38% w/w) of QRF. Here the % value seems incorrect. - *Correction has been made in the calculation of QRF percentage of yield. 38.38% w/w was changed into 38.33% w/w (2.3 g).*

**Minor revision**

1. ABSTRACT: Conclusion (.) should be removed from the beginning of sentence. And also (…) from last line of Results. - *Typing errors have been amended accordingly.*
2. In place of root, “roots’ maybe written. - *“Roots” was used to replace “root”.*
3. Separation of (QRF);hexane;ethyl acetate (9:1 5:5 (v/v), 500 ml): There should be “-“ between values. - *Addition of “-“ in hexane: ethyl acetate (9:1 – 5:5 (v/v), 500 ml).*
4. Figure 2, 3, 4: Title required. - *Titles have been given to Figure 2, 3 and 4, as per suggestion.*
5. No need to write “Graph not shown” in oral toxicity - *“Graph not shown” was omitted in oral toxicity result.*

**Discretionary Revisions:**

1. Number of references can be limited as it is research paper and not the review article – *The authors managed to omit a few references, as it is impossible to omit other references when most of the statements were cited.*
Some defences made following review from Reviewer 2: Denise Carmona Cara
Major compulsory revision

1. How the authors know that the results are connected with angiogenesis and not only increased vascular permeability of peripheral vessels? Did the authors perform histology analyses of the new vessels? The histology is also necessary to investigate the granuloma tissue which were not reduced by both treatments.

- There are two important findings found in the study. Firstly, through the permeability test, ACRH and QRF were shown to suppress permeability induced by VEGF, a potent permeable factor which is related closely with angiogenesis. Angiogenic cytokine such as VEGF is a strong chemoattractant that leads to recruitment of endothelial cells, followed by the formation tube-like structures (Pakhneshan et al., 2008). As hyperpermeability of blood vessel is one of the cardinal characteristic in the pathogenesis of many diseases (Pakhneshan et al., 2008), suppression of VEGF itself following the pre-treatment of ACRH and QRF compared to VEGF control is indeed an important finding. The second significant finding that could be obtained from this study is that ACRH and VEGF significantly reduced blood vessel surrounding the inflammation-induced air pouch. This is reflected by the reduction of Vascular index (VI), which determined the density of blood vessels surrounding air pouch area by measuring the content of carmine red per gram dry tissue. This model connected with the previous model as inflammatory processes such as infection is associated with increased vessel permeability and angiogenesis. Though it is still not proven in this study whether reduction of prostaglandin is involved in directly influence the decrement of either vascular permeability or inflammation-induced angiogenesis, it is clear that ACRH and QRF caused suppression of VEGF and attenuation of inflammation by reducing blood vessel formation, reflected in both studies. Taking the results together, since inflammation is also mediated by COX enzymes, which is in turn responsible for the production of different prostaglandins such as PGE2 that is shown to be associated with angiogenesis (Wang et al, 2005; Ghosh et al., 2000), the involvement of PGE2 is mentioned in discussion part as one of the possible mechanism, which is worthy to be explored in another experiment in future. Histological analysis was not performed in this experiment, as the air pouches harvested from the mice dried in an intact form to determine the dry weight, which was required in the calculation of Vascular Index (VI), the parameter in this study. Nevertheless, histological analysis is intended to be done as part of the histochemical analysis once the responsible bioactive constituents are isolated, which is now an ongoing study. We are also planning to determine the cytokines level i.e for TNF-α, IL-6 and VEGF for the study.

2. The Discussion section of the ms did not bring to the reader a mechanistic approach of the present results. This may confer to the text a merely descriptive nature. Conclusions are restricted to a few comparisons of the present results with other studies already reported. The authors deduce that ACRH and QRF are capable of halting of blood formation process by suppressing VEGF-induced vascular permeability once the blockade of PGs by indomethacin attenuates the vascular
permeability (literature). For this study dosage of PGs should be performed in the air pouch tissues in treated and non-treated groups.  
- The authors were actually tried to relate the link of anti-angiogenic potential of \textit{A.crispa} with the inflammation-induced angiogenesis models. The PG mentioned in the discussion is a postulate derived from previous literatures, thus it is included as one of the possible mechanism, knowing ACRH involved in the blockade of COX-2 (data not shown in the ms) in exhibiting its inflammatory effect, and PG involved in vascular permeability.

3. Since the lowest dose showed more efficacy, doses even lower of these components should be tested.
- The doses used were actually below the doses suggested in previous literatures, which utilised different model of inflammation. Previous study used 30, 100, 300 mg/kg of ACRH. We omitted 300 mg/kg of dose because through our pilot study, 300 mg/kg of ACRH was shown to cause a significant decrement in the body weight of mice. This explained the used of doses 10, 30 and 100 mg/kg in our study. Through the findings obtained from this study, we were enlightened that lower doses should be used in future, with this study served as the scientific basis of why lower doses should be implemented in angiogenic study concerning ACRH and TQRF the next time. In addition, in another study of chemoprevention, the authors found out that ACRH and TQRF exhibited its therapeutic effect at 30 mg/kg. Thus, explained the mentioned dosages used in the current study

\textbf{References}

