Author’s response to reviews

Title: Subchronic toxicity, immunoregulation and anti-breast tumor effect of Nordamnacantal, an anthraquinone extracted from the stems of Morinda citrifolia L.

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Author’s response to reviews:

(Reviewer 1):

Comment: the major issues of this work is low number of independent experiments n=-3.

Response: Dear reviewer, for the in vitro study, sample size for each assay are n=3 and we have repeated the experiment three times. Thus, all in vitro results are based on n=9 in total. For in vivo study, we tested total of 6 mice with each mice as a biological replicates. Each of the mice were assayed for three technical replicates. We have improved the writing for the description of the statistic in the method section. In addition, authors agree with the comment by reviewer 1 and reviewer 2 that the total sample size for animal study is still low. However, this work was carried out based on the recommendation by ethical committee and availability of the resources. Thus, authors have rewrite the study as pilot study to test the potential of Nordamnacanthal against breast cancer in vitro and in vivo and authors put recommendation for future study to expand the current study. (Page 9, line 240-244)
Comment: MTT is not good test for compounds with phenolic groups because it can interact with them and give false results

Response: Author thanks the suggestion from reviewer. Thus, authors have performed trypan blue (Figure 2b) to validate the MTT results.

Comment: There is a several types of breast cancer authors should explain differences between these types in introduction section

Response: Classification of breast cancer was added to the introduction section (Page 3, line 56-59; 63-71).

Comment: More details about gating as well as compensation procedure in flow cytometry method should be given

Response: Gating details for AnnexinV/PI (Page 6, line 148) and immunophenotyping (Page 8, line 209) were stated in the method section.

Comment: Conditions of serum obtaining should be added

Response: Cat no and condition of serum used were added in the Method section. (Page 4, line 110)

Comment: Statistical analysis is not proper performed n=3 is to low to perform ANOVA analysis!!!! as well as to obtain clear results. S.E.M should not be used a SD is the proper parameter.

Response: For in vitro experiments, each of the samples have 3 replicates (biological) and we have repeated the experiment 3 times; thus total of n=9. For in vivo experiment, we tested on all 6 mice and each with 3 technical replicates. Authors have rewritten the statistic for method section. In addition, authors have changed the analysis to SD rather than SEM. (Page 9, line 240-244)
Comment: Some editorial bugs
Response: Authors apologize for the editorial mistake. Authors have corrected all the errors.

Comment: In figure 5 values are putted on the objects which make difficult to interpretive the results
Response: Figure 5 was edited accordingly.

(Reviewer 2):
Comment: Title: revise the title to specify that your study is on breast cancer only
Response: Title was revised as suggested by reviewer. (Page 1, line 1)

Comment: abstract: add the specified concentrations obtained in antitumor and immunomodulatory effects
Response: concentration for antitumor and immunomodulatory effects (50mg/kg body weight) was added in the abstract. (Page 2, line 41)

Comment: method: authors need to justify why they used the specified cell density for MTT and flow cytometry assays
Response: The cell density was selected based on the plating optimization to obtain ~95% confluence at 72 hours for 96 well plate. Conversion from 96 to 6 wells was based on conversion of growth area of 96 to 6 well, which are ~30 times (96 well plate: 0.32cm2; 6 well plate: 9.5cm2). (Page 5, line 117-119)

Comment: subchronic toxicity: why authors used male Balb/c mice in this assay although the antitumor effect was performed on female mice. Hormonal difference may alter the result. A justification is needed
Response: Authors apologize for the typo error in subchronic toxicity. All mice used in this study are female. Purpose for subchronic toxicity is to compare the different of feeding normal mice
with Nordamnacanthal as based line. Thus, result for IL-2 and IFN-gamma from the subchronic toxicity mice (collected on day 28) was added in the method section.(Page 7, line 188)

Comment: what was the weight of mice in the toxicity and antitumor assays?
Response: Results for the weight changes of the mice was added. (Table 1 and Figure 4C)

Comment: 6 mice is very low number for in vivo antitumor assay
Response: Authors agree that current use of 6 mice is comparatively low although it was the amount suggested by the ethical committee. In addition, authors have limited resources to perform this experiment. Thus, authors have indicated that this is a pilot study to show the potential of Nordamnacanthal immunodulatory and anti-breast cancer effects on 4T1 challenged mice and recommended for future study

Comment: why authors did not measure the level of IL-4. This is important to decide whether the treatment induce Th1 or Th2 immune response
Response: Results for IL-4 was added in Figure 7 and discussed accordingly at the last paragraph of Discussion section (Page 13, Line 350-357).

Comment: line 275: add the word (difference) after significant
Response: the word (difference) was added (Page 11, line 293).

Comment: figure 2: the quality of this figure is low. Please replace with figure of higher resolution
Response: Quality of Figure 2 (for both MTT and trypan blue) were improved.

Comment: what was the cure percentage in the antitumor assay?
Response: Dear reviewer 2, nordamnacanthal delayed the progression (based on the tumor volume and tumor weight) but not cure the 4T1 tumor in mice of this study.
Comment: figure 7: authors need to include values of IL2 and INF-gamma for healthy (tumor free) mice to make sure that the obtained results are not due to the presence of tumor

Response: Results for IL2, IL4 and IFN-gamma for healthy and tumor mice (control and treated) were presented in Figure 7. (Page 11, line 298-300)