Author’s response to reviews

Title: The synergism of Clinacanthus nutans Lindau extracts with gemcitabine: downregulation of anti-apoptotic markers in squamous pancreatic ductal adenocarcinoma

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Authors’ response to reviewers’ comments on the paper

“The synergism of Clinacanthus nutans Lindau extracts with gemcitabine: downregulation of anti-apoptotic markers in squamous pancreatic ductal adenocarcinoma”

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Reviewer reports:

Reviewer #1: Eswar Shankar

Reviewer #1’s Comment: Hii et al in this manuscript have tried to assess the synergistic role of C. Nutans extracts with gemcitabine in pancreatic cancer cell lines. The work is indeed an interesting one. The authors also confirm synergism. Unfortunately the authors demonstrate a co-
relative response and do not actually demonstrate a mechanism. This becomes a huge caveat. Also, the authors have not included any invivo studies to actually show that the combination is a very efficacious one. The authors have in the introduction pointed out that these extracts were found to inhibit inflammatory mediators such as p65, p38, pERK, pJNK. Why have the authors not actually connected any of these mediators to apoptosis. The authors also have not indicated if this combination could overcome resistance to gemcitabine. Overall the work seem very incomplete as it does not address an actual mechanism.

Authors’ Response:

The authors wish to thank the reviewer#1 for your time and thoughtful comments that have led to a great improvement of the manuscript.

At present, the study was designed to answer two critical issues : (i) whether C. nutans extracts alone can exhibit potent anticancer effects, as claimed by folk medicine; (ii) would C. nutans extracts interfere (enhance or inhibit) conventional anticancer agents, since most cancer patients would likely to be receiving the chemotherapies and consuming the extracts concomitantly, without informing the clinicians. Thus, this study aimed to focused mostly on phenotypic response (such as cell viability and cell death) to establish the preliminary findings to support its traditional claim amongst the public. (Line 491-511).

The suggestion to incorporate In vivo study will be considered for a subsequent study in continuation of the current work in view the large amount of extracts needed. We acknowledge the shortcoming of this paper without in vivo finding and appreciate this being emphasised. We have highlighted the limitation in the discussion section of the manuscript to inform the readers of this limitation (line 491-511).

The crux of the study is to provide evidence-based data to support or refute traditional practices. Hence, we emphasised on phenotypic investigation, rather than mechanistic aspects because the phenotypic conclusion needs to be first established. To the best our knowledge, our results is the first to suggest that C. nutans extracts should not be used alone or as a replacement to chemotherapy since the extracts were not potent. Another study (reference 15) reported the extracts were effective against a very limited number of cancer cells with just IC50 in vitro despite the IC50 being recorded at a very high concentration (>30µg/mL). The criteria established by the American National Cancer Institute states that for a crude extract to be considered as a potential cytotoxic agent, it should achieve an IC50 less than 30µg/mL when
tested against a cell line. Therefore, we sought to study the extracts objectively to support evidence-based medicine. To our surprise, it enhanced the effect of gemcitabine in pancreatic cancer, which was unexpected. We repeated the experiment several times to ensure validity and the result remained unchanged. We also further investigated different combinatorial ratios of extracts to gemcitabine and the percentage of apoptotic cell death using ELISA and apoptotic array. We had added the results from Annexin V and immunoblotting on of bax, bcl-2, XIAP and GADPH, as suggested by the reviewer#1. We acknowledge our limitation in mechanistic study and it will be included in the upcoming in vivo study (line 491-511).

Indeed, our previous study focused on p65, p38, pERK, pJNK in view of the inhibited TLR-4 levels by the extracts in macrophages (non-cancerous cells). However, we didn’t expand this study in p65, p38, pERK, pJNK in pancreatic cancer cells because (i) TLR4 level was not affected when extracts were added alone or in combination with gemcitabine (revised Figure 3C); (ii) our preliminary unpublished immunoblotting data shown no change in TLR-4 level when extracts were added alone or in combination with gemcitabine. Since TLR-4 is the upstream activator to p65, p38, pERK, and pJNK, we did not focus on these pathways when TLR-4 level remained unchanged in (i) and (ii) above. Since the objective of the study was to identify the phenotypic hallmarks changes after treatment, we focused our study in established apoptotic hallmark markers. The RayBio® Human Apoptosis Antibody Array consists of 43 pro-, anti-apoptotic and death receptors proteins with picogram-per-millilitre (pg/mL) sensitivity. The assay is well established and thus we focused on these 43 apoptotic hallmark markers only. We will focus more on the mechanistic aspects of our study by including the effects of the extracts on p65, p38, pERK, pJNK as well as their correlation with apoptosis in future study (line 491-511).

It was not the intention of the manuscript to propose SN extracts can reverse gemcitabine resistance in pancreatic cancer. From the synergism cell viability study and apoptotic studies, we observed greater reduction in cell number and greater cell death in pancreatic cancer cells when the SN extracts combined with gemcitabine. We have edited the manuscript to ensure clarity of the message (line 74, and 509-511), and to avoid ambiguity. Thank you for highlighting it.

We agreed with the reviewer#1 and acknowledge our limitations in not incorporating in vivo data and mechanistic aspects. The authors will take note of these important points for continuation of this work. The manuscript has been edited (line 136, 137, 338, 491-511) to truly reflect the objective of the study on phenotypic cell death observation which may be beneficial to pancreatic cancer when combined with gemcitabine.
Reviewer #1’s Comment: The authors have not performed any annexin V to show the % of apoptosis.

Authors’ Response:

The authors wish to thank the reviewer#1 for the comments. We have included the percentage of apoptosis using Annexin V assay in line 326-329 and Figure 3B.

Reviewer #1’s Comment: The authors do not show the extent of protein changes of pro and any apoptotic proteins.

Authors’ Response:

The authors wish to thank the reviewer#1 for the comments. The protein levels of pro- and anti-apoptotic proteins were quantified using RayBio® Human Apoptosis Antibody Array (RayBiotech, USA), in Figure 3D. We have represented the data to ensure clarity of death receptors, pro-apoptotic proteins and anti-apoptotic proteins level, as established in previous study [Eur J Med Chem. 2014 Apr 22;77:378-87.] We have also included the immunoblotting of bax, bcl-2, XIAP and GADPH as confirmation of its pro-apoptotic and anti-apoptotic expression after treated with the combination of SN and gemcitabine.

The RayBio® Human Apoptosis Antibody Array can detect 43 pro- and anti-apoptotic proteins with picogram-per-milliliter (pg/mL) sensitivity. It is a much sensitive compared to other protein quantification method. Also since it is quantitative, it provides unbiased, and exact fold changes compared to immunoblotting, which is mostly qualitative. Other researchers studying the deregulation of apoptosis in diseases such as coronary artery disease, HIV infection and cancer were using the array to increase detection of apoptotic proteins within their samples. The RayBio® Human Apoptosis Antibody Array utilizes a fluorescent signal readout, allowing a wider dynamic detection range than can be achieved by chemiluminescence. The array utilizes the sandwich-ELISA design principle. In this assay, capture antibodies are printed in multiple identical arrays on a standard-sized histology slide. After a blocking step, samples are incubated with the arrays. Nonspecific proteins are then washed off, and the arrays are incubated with a cocktail of biotinylated detection antibodies, followed by a streptavidin-conjugated fluorescence. Signals are visualized using a fluorescence laser scanner. (Reference: http://www.genomax.com.my/index.php/hot-products/157-g-series-human-apoptosis-array-g1)
RayBio® Human Apoptosis Antibody Array has been cited as a sensitive pro and anti-apoptotic protein detection assay by other researchers across the world, and as evidenced in the following papers:


   Species: Human; Sample type: Cell Lysate, Protein Level


   Species: Human; Sample type: Cell Lysate, Protein Level


   Species: Human; Sample type: Cell Lysate, Protein Level


   Species: Human; Sample type: Cell Lysate, Protein Level


   Species: Human; Sample type: Cell Lysate, Protein Level

Species: Human; Sample type: Cell Lysate, Protein Level


Species: Human; Sample type: Cell Lysate, Protein Level


Species: Human; Sample type: Cell Lysate, Protein Level


Species: Human. Sample type: Cell Lysate, Protein Level

10. The Presence of HIV-1 Tat Second Exon Delays Fas-Mediated Apoptosis in CD4+ T lymphocytes: a Potential Mechanism for Persistent Viral Production

Species: Human; Sample type: Cell Lysate, Protein Level

Reviewer #1’s Comment: I recommend additional statistical review

Authors’ Response:

The authors wish to thank the reviewer#1 for the comments. We have consulted the International Medical University’s statistician – Dr Chong Chun Wie. He and the Institute for Research, Development and Innovation (IRDI) statistic team remarked no additional statistical review is needed for the manuscript. All the tests were appropriate.
Also, in the report from the Reviewer#2 Dr Heena Vatsalbhai, who also provided the same assurance that no additional statistical test is needed. Dr Heena’s reply was “Yes. All the statistical data was provided with the manuscript. Manuscript adheres to the relevant standards and data deposition. No need to add any additional statistical review for this manuscript.”

Reviewer #1’s Comment: Needs some language corrections before being published

Authors’ Response:

The authors wish to thank the reviewer#1 for the comments. The revised manuscript was also proof-read by a native English author, David Deegan, who is also the Executive Development Director and Director of Client Resourcing, Cranfield School of Management, United Kingdom.

Reviewer #2: Heena Vatsalbhai Dave, Ph.D.

Reviewer #2’s Comment: This study is an excellent work exhibiting synergistic effects of Clinacanthus nutans extracts with Gemcitabine and providing support that it may be useful for pancreatic cancer patients and this manuscript is acceptable for publication.

Authors’ Response:

The authors wish to thank the reviewer#2 for your time and thoughtful comments.

Reviewer #2’s Comment: Are the methods appropriate and well described?

The authors have investigated the effect of Clinacanthus nutans extracts as an alternative medicine for cancer patients. Moreover, they have demonstrated, the C. nutans has synergistic effects with Gemcitabine especially in non-polar stem extracts (SN extracts) and it enhanced chemosensitisation of pancreatic ductal adenocarcinoma. In vitro experimentation was done for the current manuscript. Cytotoxicity assay was performed by MTT assay. Drug combination analysis was done by the method developed by Chou and Talalay (Described in Reference #24)
and used Calcusyn 2.1 software. Sandwich ELISA was used to determine TLR-4 levels. Cell Death Detection sandwich ELISA was used to quantify the degree of cell death. Human Apoptosis Antibody Array kit was used to identify the cellular apoptotic markers. Overall, all the methods were appropriate and well described in the manuscript.

Authors’ Response:

The authors wish to thank the reviewer#2 for your time and thoughtful comments.

Reviewer #2’s Comment: Does the work include the necessary controls?

Yes. Total of 23 cancer cell lines were used in the current study and all the cell lines were compared with 4 non-cancer cells (MCF-10A, ARPE19, MRC5 and CCD84CON).

Authors’ Response:

The authors wish to thank the reviewer#2 for your time and thoughtful comments.

Reviewer #2’s Comment: Are the conclusions drawn adequately supported by the data shown?

Yes. In the current manuscript the authors have comprehensively studied the synergistic effects on various cancers like Breast, Colorectal, Lung, Endometrial, nasopharyngeal by using 23 different cancer cell lines. However, it showed effects of C. nutans with Gemcitabine in pancreatic ductal adenocarcinoma only. All the conclusions were drawn adequately and supported by data. The authors have explored and investigated all the aspects of the advantages as well as disadvantages of the study and mentioned in the discussion and conclusions. Moreover, the limitations and/or disadvantages of the work were clearly narrated.

Authors’ Response:

The authors wish to thank the reviewer#2 for your time and thoughtful comments.

Reviewer #2’s Comment: Are you able to assess any statistics in the manuscript or would you recommend an additional statistical review?
Yes. All the statistical data was provided with the manuscript. Manuscript adheres to the relevant standards and data deposition. No need to add any additional statistical review for this manuscript.

Authors’ Response:
The authors wish to thank the reviewer#2 for your time and thoughtful comments.

Reviewer #2’s Comment: Quality of written English?
Yes, Quality of the written English is good and acceptable for publication.

Authors’ Response:
The authors wish to thank the reviewer#2 for your time and thoughtful comments.

End of review.

Yours faithfully,

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