Author's response to reviews

Title: Cytotoxicity effect of degraded and undegraded kappa and iota carrageenan in human intestine and liver cell lines

Authors:

Shahrul Hisham bin Zainal Ariffin Prof. (shahroy8@gmail.com)
Wong Woan Yeen Miss (wanyingyy@hotmail.com)
Intan Zarina binti Zainol Abidin Dr. (izzarina7@gmail.com)
Rohaya binti Megat Abdul Wahab Assoc. Prof. (shahroy7@gmail.com)
Zaidah binti Zainal Ariffin Assoc. Prof. (zaida55my@yahoo.co.uk)
Sahidan bin Senafi Assoc. Prof. (sahidan@ukm.edu.my)

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

Please find enclosed our manuscript, “Cytotoxicity effect of degraded and undegraded kappa and iota carrageenan in human intestine and liver cell lines” by Shahrul Hisham Zainal Ariffin et. al. which we would like to submit for publication as Research Article in BMC Complementary and Alternative Medicine. We have revised the manuscript according to the comments of Referee 2. The explanation of the revisions made is included in this covering letter.

The study was to compare in vitro cytotoxicity effect of acid degraded and water soluble undegraded carrageenan in human cancer and normal cell lines. The cell lines used were intestine and liver cell lines.

To our knowledge, this is the first report to compare food grade degraded and undegraded carrageenan in cell lines that involve the gastrointestinal system and also detoxify organ. In this manuscript, we show that acid degraded k-carrageenan have cytotoxic effect on human cancer intestine (Caco-2), normal intestine (FHs 74 Int), cancer liver (HepG2) and normal
liver (Fa2N-4) cells as assessed by cell viability, morphological and biochemical analyses. However, undegraded carrageenan was not toxic to the cells.

We believe our findings would appeal to the readership of BMC Complementary and Alternative Medicine because it is related to the toxicological studies of carrageenan, a food additive which used in variety of food such as jelly, chocolate milk and meat.

We declared that this article is original, not published nor under consideration simultaneously by any other publication and also free of conflicts of interests. We also affirm that all the authors stated in the manuscript have been given their contribution at all the way of this research.

We look forward to hearing from you at your earliest convenience.

Thank you.

Yours sincerely,

ASSOC. PROF. DR. SAHIDAN SENAFI
(Corresponding author and contacting author)
School of Biosciences and Biotechnology.
Faculty of Science and Technology,
Universiti Kebangsaan Malaysia
43600 Bangi Selangor,
Malaysia.
Tel: +603-89214361
Fax number: +603-89252698
Revisions made according to comments

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<th>No.</th>
<th>Comment</th>
<th>Explanation</th>
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<td>1.</td>
<td>Although the cells viability decreased significantly in response to degraded FGKC, DKC and CGKC, non-degraded FGKC, DKC and CGKC don’t affect the cell viability. Why the only degraded polysaccharides can induce apoptotic reaction in the cells?</td>
<td>The reason was discussed in p.21; line 2 to line 8. “Carrageenan is a large polysaccharide in which the undegraded form of carrageenan have molecular weight of 200-800 kDa, while the degraded carrageenan have low molecular weight of 10-20 kDa. Undegraded carrageenan did not show cytotoxic effect on cancer and normal cells in our study. It seems possible that this result is due to the difficulty of the cells to absorb or transport large polymers into cells. On the other hand, the degraded form of carrageenan can form interaction with the cells thus showing cytotoxicity activity.”</td>
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<td>2.</td>
<td>In Gene Expression analysis section (p.14;line 8), Figure 13,14,15 &quot; In HepG2 cells, the RT-PCR result showed that the gene expression of PCNA (lane 6,10,14: Figure 13), Ki-67 (lane 7,11,15: Figure 13) and survivin (lane 8,12,16: Figure 13) gene were suppressed by the treatments of degraded FGKC, DKC and CGKC, respectively&quot;. #But it’s difficult to discuss the mRNA levels obtained from the results of RT-PCR analysis. So I recommend that the author should perform additional experiments using quantitative methods, i.e. real-time RT-PCR.</td>
<td>Although real-time RT-PCR analysis was not performed, the mRNA levels of PCNA, Ki-67 and survivin genes were determined by using the same concentration of RNA, i.e. 1 µg from treated and untreated cells to synthesis first and second strand cDNA. Meanwhile, same cycle number (30 cycles) was applied during the PCR amplification steps for all genes and run at the same time. Therefore, the mRNA level of untreated and treated cells can be compared. This discussion had been added in p.24; line 18 to line 21. Besides, we changed the word of “suppressed” to “inactivated” in p.2; last line; p.17;line 24 and p.25;line 5. RT-PCR analysis used in cytotoxicity study was further supported by other studies which had cited in reference no 3.</td>
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| 3. | “The mod of cell death is suggested to be through apoptosis as the cells showed the characteristics of apoptosis”
|   | #But the evidence of apoptosis is poor in these experiments. So I recommend that the author should determine the caspace-3 activity, phosphatidylserine exposure on the cell surface, and other apoptotic reaction in the cells-treated with degraded carrageenan. |
| 4. | Gel electrophoresis analysis (p.14;line 6) In Figure 11B; “Figure 11b and 12b show DNA fragmentation of HepG2 and Caco-2 cells treated with TAM as positive control.”
|   | #This study used Tamoxifen as a positive control. Although the genomic DNA was prepared from Tamoxifen-induced apoptotic HepG2 cells, then why does not DNA ladder appear on gels of lane1 in Fig.11B ? (In figure 10f, The expression of nuclear fragmentation and apoptotic body were induced by Tamoxifen in HepG2 cells). So I recommend that the authors add the additional description in this points. |
|   | We were not only using morphological observation to determine apoptosis but also supported by the results of fluorescence staining (acridine orange/ ethidium bromide) and DNA fragmentation. Other studies using the same method i.e. May Grunwald Giemsa staining, acridine orange/ethidium bromide and DNA fragmentation to determine apoptosis as cited in references no. 61, 62, 63, 64 for morphological observation; no. 69 for acridine orange/ ethidium bromide staining and no.73,74,75 for DNA fragmentation. Therefore, we believe that the above analyses can be used to support the occurrence of apoptosis. |
|   | Actually the DNA ladder pattern do appear on gels of lane 1 in Fig. 7B and 7D (modified figures) following tamoxifen treatment. The occurrence of DNA ladder pattern in this study is same with others studies who used DNA fragmentation analysis to prove apoptosis in tamoxifen treated cells as cited in no. 59,76 and 77. |
5. p.8; line 8
"... cells was stained with 10 μL of a mixture (1:1) of acridine orange (100 mg mL\(^{-1}\)) and ethidium bromide (100 mg mL\(^{-1}\)) solution in 1x PBS."
#The concentration of acridine orange and ethidium bromide are 100 μg / mL.

p.8; line 10
"... cells was stained with 10 μL of a mixture (1:1) of acridine orange (100 mg mL\(^{-1}\)) and ethidium bromide (100 mg mL\(^{-1}\)) solution in 1x PBS."

**Changed to**
The concentration of AO/EtBr are 100 mg mL\(^{-1}\) in 1x PBS.

6. p.14; line 3
"genomes (lane 1: Figure 11a). However, DNA fragment ladder was not seen in degraded k-carrageenan treated Caco-2 cells (Figure 12a)."
#"genomes (lane 1: Figure 11A). However, DNA fragment ladder was not seen in degraded k-carrageenan treated Caco-2 cells (Figure 12A)

p.16; line 13
"genomes (lane 1: Figure 11a). However, DNA fragment ladder was not seen in degraded k-carrageenan treated Caco-2 cells (Figure 12a)."

**Changed to**
As shown in Figure 7A; lane 2, 3 & 4, DNA fragment ladder was not seen in degraded k-carrageenan treated Caco-2 cells.

7. Legends of figure 1-4
"and (d) tamoxifen at concentrations of 0.625- 20.000 μg mL\(^{-1}\) for 24 – 72 h."
#"and (f) tamoxifen at concentrations of 0.625- 20.000 μg mL\(^{-1}\) for 24 – 72 h."

Legends of figure 1-4
"and (d) tamoxifen at concentrations of 0.625- 20.000 μg mL\(^{-1}\) for 24 – 72 h."

**Changed to**
"and (f) tamoxifen at concentrations of 0.625- 20.000 μg mL\(^{-1}\) for 24 – 72 h."

8. Table 2
"IC50 values of FGKC, DKC, CGKC, FGIC, CGIC and Tamoxifen..."

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<th>Recommendation</th>
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<td>8</td>
<td>IC50 values of degraded FGKC, DKC, CGKC, FGIC, CGIC and Tamoxifen.</td>
<td>IC50 values of degraded FGKC, DKC, CGKC, FGIC, CGIC and Tamoxifen.</td>
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<td>9</td>
<td>Figure 5-8 I recommend that the authors merge the figure 5-8.</td>
<td>Figure 5-8 have been merged into Figure 5 A; control, Figure 5B; FGKC, Figure 5C; DKC, Figure 5D; CGKC and Figure 5E; TAM.</td>
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<td>10</td>
<td>Figure 11, 12 I recommend that the authors merge the figure 11 and 12.</td>
<td>Figure 11, 12 have been merged into Figure 7A, 7B, 7C and 7C.</td>
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