Author’s response to reviews

Title: Anti-proliferative and Cytotoxic activities of Allium autumnale P. H. Davis (Amaryllidaceae) on Human Breast Cancer Cell Lines MCF-7 and MDA-MB-231

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Author’s response to reviews:

Dear editor,

Thank you for the efficient way in which you handled our manuscript “Anti-proliferative and Cytotoxic activities of Allium autumnale P. H. Davis (Amaryllidaceae) on Human Breast Cancer Cell Lines MCF-7 and MDA-MB-231”.

We are grateful for the opportunity to submit a revised manuscript. We have addressed all of the points raised by the referees, including some experimental issues. Thus, we feel we have answered all the points one by one as follows:

Ephrem Engidawork, PhD (Reviewer 1):

* Include the authority (P. H. Davis) and family (Amaryllidaceae/ Liliaceae) in the title as well as when you first mention the plant in the text.

Response: We thank the reviewer for this oversight. These have now been included in the revised manuscript as ‘ Allium autumnale P.H. Davis (Amaryllidaceae)’ on lines 1-2 (Page 1), 25-26 (Page 2), 34-35 (Page 2), 77-78 (Page 4).

* The knowledge that other Allium species such as A. sativum, A. cepa, A. willeanum are demonstrated to have anticancer activity is said to be an impetus for the present investigation. What value, if any, could this work add to the existing body of knowledge?

Response: We thank the reviewer for raising this issue and giving us the opportunity to discuss this important point. There are around 850 Allium species known, with a number of them being traditionally used in various therapeutic approaches. However, different Allium species containing different therapeutic molecules are directed towards different diseases or different cancers. As our study also implicates, different Allium species or parts thereof can have different effects on different cancer types and cell lines. The island of Cyprus hosts 8 different kinds of
endemic Allium species, all of which are understudied, with unique molecular compositions. Learning about these Allium species that have evolved for years on the island and used by the local people for various therapeutic purposes, and understanding their potential will significantly improve botanical, medical, and pharmaceutical knowledge pools. In addition, the soil of Cyprus is rich in copper (in fact, the name “Cyprus” is derived from copper) and copper is known to have both pro- and anti-cancer properties, so the species we studied could be particularly interesting (D. Denoyer et al. Targetting Copper in cancer therapy: “Copper that Cancer”. Metallomics. 2015;11: 1459-76. doi: 10.1039/c5mt00149h)

* The rational for using 95% ethanol as an extracting solvent should be clearly spelt out.

Response: 95% ethanol is used routinely in studies, like ours, investigating the therapeutic aspects of Allium species as an extraction solvent. For example, Ponmurugan Karuppiah and Shyamkumar Rajaram (Asian Pac J Trop Biomed. 2012 Aug; 2(8): 597–601. doi: 10.1016/S2221-1691(12)60104-X) studying the anti-bacterial properties of an Allium species used 95% ethanol as the solvent. Similarly, Tsan-Chang Chang et al. (Food and Nutrition Sciences Vol.4 No.8A(2013), Article ID:35298,9 pages DOI:10.4236/fns.2013.48A022) studying antimicrobial and antioxidant properties of Allium species also opted for 95% ethanol as their extracting solvent. These and other studies used 95% ethanol as an extracting solvent so we felt confident doing the same also to make the findings more comparable especially as ours was the first time this particular Allium species was being studied. We do admit, however, that the nature of the solvent can affect the effectiveness of the extracts. In our future studies, we plan to use different extraction solvents for comparative studies.

* The Journal requires numerical citation. Please avoid the use of Author citation (Fraser et al., 2005) - Page 5, Line 111.

Response: We thank the reviewer for spotting this mistake. This has now been amended in the revised manuscript (line 110, Page 5).

* Figure 1: AAB at 625 µg/mL showed an effect on MCF-7 cells at 24 h but that effect worn out as time went by. This does need some sort of explanation. There is also an expression used to show the extract's concentration (not dose)- and time-dependent effect. However, this is not correct. In addition to the abovementioned lack of effect of the lower concentration with time, one does not see any difference in the effect of 1250 µg/kg of AAB on MCF-7 cells across the different time points. Even I do not see that much difference on the effect of the same concentration on MDA-MB-231, although a pound key was placed at 48 and 72h. The authors claimed doing One way ANOVA, probably it would be good if they also perform post hoc analysis.

Response: The reviewer brings up a valid point, i.e. AAB at 625µg/mL had an effect on MCF-7 cells which then disappears at later time points. This is an interesting result indicating that at this low concentration, the cells are able to compensate for / adapt to the prolonged application. At present, we do not know the exact mechanism(s) of this phenomenon. Nevertheless, cancer cells are well known to develop resistance to chemical influences, including drugs. This point has now been added to the text as ‘This is consistent with the cancer cells’ ability to compensate for/adapt
to the prolonged applications of chemical interventions and drugs’ in lines 166-167 on Page 8. As regards the anti-proliferative effect at 1250µg/mL being significant when compared to the control but not a drastic over time, the reason(s) are probably as outlined above for 625µg/mL. Overall, therefore, it seems, not surprisingly, that the observed effects are both concentration- and time-dependent. Thus the text has been modified as follows (lines 158-165, Page 8): ‘The reduction in proliferation was observed in a concentration and time dependent manner (Figure 1) which became more obvious especially at higher (5000 and 10000 µg/mL) concentrations. An initial proliferation decline was observed on MCF-7 cells upon addition of 625µg/mL AAB extract at 24 hours, however a recovery was observed at 48-72h time points which was followed by a consistent but slow decline at 1250-2500 µg/mL and a strong decline at 5000µg/mL onwards at all time points. This data suggests that at low concentrations, despite initially responding, the MCF-7 cells can recover from the anti-proliferative effects of the extract where a robust reduction is observed at higher concentrations’.

Thanks to the reviewer, we have now carried out Newman-Keuls post hoc analysis. This analysis has now revealed, as the reviewer suspected, a more significant level of anti-proliferative effects, especially at 1250µg/mL at later time points including at 72hrs (new Figure 1).

* Figure 2: AAS was consistently inactive up to 2500 µg/mL at all time-points. With increasing concentration, AAS did have effect in all but at 24 h. The interesting thing is that higher concentrations used produced a significant difference between the two cell lines at 24 h, this difference disappeared at 48 h, and reappeared at 72 h. What could be the source of these seemingly inconsistent results? The Analysis?? As the Analysis involves three variables Concentration, Effect and Time, it may be good to do ANOVA with repeated measure.

Response: We thank the reviewer for bringing up this point and would like to clarify that, as the reviewer implies, the trend shows the significance which disappears at the second time point. We do not believe that this could be due to the quality of the extract as we have purposefully used one extract to avoid variability. Instead, it could be due to the MCF-7 cells having the ability to transiently compensate the anti-proliferative effect of the AAS extract and then at a later time point leading to an eventual cell number reduction (Figure 2). In addition, we think this effect is likely also due to the decrease observed in MDA-MB-231 cell number along with a transient rebound of MCF-7 cells at 48hrs which has eliminated the initial cell line dependent variation that was observed at 24 hours. However, further continuous decline of MCF-7 at 72hr time point and stagnant reduced state of MDA-MB-231 cells allowed the cell line dependent differentiation to become significant again.

As the reviewer recommended, ANOVA with repeated measure analyses as well as Newman-Keuls post hoc analyses were carried out on these data points without any change in the trend suggesting the suspected abovementioned possibilities (Figure 2). The revised analysis representing this update is included in the text as Figure 2.

* Figure 3 &4: the effect produced by both AAB and AAS displayed a similar pattern, including the effect produced by 1250 µg/mL observed only at 48 h in both cases. This
observation is in stark contrast to the one mentioned under the Discussion section: Page 14: Line 293-295.

Response: We thank the referee for this insight. The statement has been modified to better reflect our findings. In order to clarify our discussion, now we have included the respective concentration ranges as follows (lines 317-321, Page 15): ‘Treatment with AAB extract demonstrated higher toxicity on MCF-7 cells at early and middle time points (24 and 48h) and concentrations (1250µg/mL-2500 µg/mL) whereas a more robust cytotoxic effect on MDA-MB-231 cells was observed at a later time point (72h). This is likely due to the faster growing, strongly metastatic nature of the MDA-MB-231 cell line.

* Figure 4: Blebbing, one of the features of apoptotic death, is said to be observed with AAS rather than AAB treated MDA-MB-231 cell lines. Interestingly, it was also mentioned that MCF-7 cell lines treated with the extract did not exhibit such feature. Comparison of the two extracts could reveal that AAB produced a consistently higher activity in both the antiproliferative and cytotoxicity assays. The phytochemical profiling also showed that the bulb to be endowed with more antiproliferative principles than the stem. And yet, the authors tended to focus on AAS's blebbing and increased activity on MDA-MB-231 than MCF-7. In fact, AAB produced a drastic effect on both cell lines, with the effect being better on MDA-MB-231 than MCF-7 over time. Blebs can be formed during apoptosis, necrosis and locomotion. What makes the author to make sure that blebs were as a result of apoptosis, as the study did not measure any apoptotic markers?

Response: We agree with the referee that AAB produced consistently higher cytotoxic and antiproliferative effect on both cell lines. We now have amended the text to focus on this observation by addition of the following text (lines 297-307, Page 14): ‘Our studies investigated both the bulb and the stem extracts of Allium autumnale. The bulb extract (AAB) gave rise to consistently higher cytotoxic and antiproliferative effects on both breast cancer cell lines, with the cytotoxic effects being generally more prominent on the strongly metastatic MDA-MB-231 cell line at higher concentrations than the weakly metastatic MCF-7 cell line over time. This feature is promising when potential therapeutic activity of this species is considered as inhibition of metastatic cancers is clinically more challenging than that of the weakly metastatic ones. The phytochemical profiling of the AAB extract also revealed molecules more consistent with cytotoxic and anti-proliferative activities (Table 1). While the AAB extract demonstrated significant anti-proliferative activity on both tested cell lines with a more robust effect on the MCF-7 cell line, the stem extract (AAS) exhibited lower anti-proliferative activity on both cell types'.

We agree that we have overstated the possibility that apoptosis is the main mechanism leading to blebbing. Now we highlight the comparative effects of the two extracts on the two cell lines and also emphasize other mechanisms which can cause blebbing in the text including necrosis and locomotion (lines 332-336, Page 15-16): ‘Several studies observed membrane blebbing occurring during locomotion, in instances like embryogenesis (Trinkaus JP (1980) Formation of protrusions of the cell surface during tissue cell movement. Prog. Clin. Biol. Res. 41: 887–906 ) and during necrosis especially after free radical exposure and metabolic poisoning (Jewell SA,
Bellomo G, Thor H, Orrenius S and Smith M (1982). Bleb formation in hepatocytes during drug metabolism is caused by disturbances in thiol and calcium ion homeostasis. Science 217: 1257–1259. While these are potential causes for bleb formation, the mechanism governing the bleb formation in our model is likely apoptosis mediated as a spike in Caspase-3 activation was observed upon incubation with the extracts (data not shown). As it is stated in the text, the main reason why we have initially focused on an apoptotic mechanism is the preliminary data we have received at the protein level revealing the apoptotic marker, Caspase-3 activation. Since these studies are still at a preliminary stage and, hopefully, will be published separately in the future. Basically, the microscopy showed that the apoptotic blebs caused by the AAS extract as they are much more prominent, consistent with the increased Caspase-3 levels. We shall be happy to share these preliminary data if it would help in any way at this stage.

* Although it is debatable whether the lateral motility assay predicts growth or motility, motility appears to be the main reason for doing this test as cancer cells invade normal cells and this test could give an idea about the potential for an agent to inhibit metastasis. An extract that is capable of producing antiproliferative and cytotoxic activity in breast cancer cell lines, including an invasive one, is expected to decrease motility. However, no difference was found between tests and controls. What does this mean?? Could it be related to the dose used for the study??

Response: In order to eliminate any impact that anti-proliferative or cytotoxic properties may play on motility, we selected an extract dose that exerts no anti-proliferative or cytotoxic effects. Lack of effect on motility, but the effect on cytotoxicity and proliferation are consistent with the well known notion that primary tumorigenesis (proliferation) and secondary tumorigenesis (invasion/metastasis) are controlled differently, even independently. This is also well known to occur in the clinic with metastasis with no identifiable primary tumor. Currently, we can argue that these two mechanisms are controlled and regulated differently via a mode of action that we presently do not understand.

* GC-MS analysis: compounds were identified using a data library. However, these days both Kovalt index (Retention index) and data library are used to make the identification more acceptable. In addition, the column is kept in the oven so it is the oven temperature that should be mentioned. Would be to merge the two statements (Page 139-140).

Response: Using Kovalt index analysis, despite being interesting, requires carrying out the GC-MS studies all over again. In order to do that, we need to obtain new batches of Allium autumnale from the nature. Unfortunately it is not possible to find this species in nature this time of the year and we would have to wait until September 2018. Our GC-MS studies have used peak area measurements, a method commonly used in publications (Devender et al., Journal of Pharmacognosy and Phytochemistry, 2017 or Gomathi et al., Journal of Food Science and Technology, 2015 etc.), which we believe demonstrates a valid representation of the contents of our extracts. We are in complete agreement with the reviewer that it is crucial to state the oven temperature so our statements at lines 139-140 have been amended in lines 138-139 on Page 7 as follows ‘The initial oven temperature was 50°C and was maintained at this temperature for 2 min and then gradually increased up to 280°C at the rate of 50 C/min and maintained for 9 min’.
Serhat Keser, Ph.D. (Reviewer 2): The work titled "Anti-proliferative and Cytotoxic activities of Allium autumnale on Human Breast Cancer Cell Lines MCF-7 and MDA-MB-231" is good and original study. According to my opinion, this manuscript can be accepted for publication in the BMC Complementary and Alternative Medicine. My corrections:

1. Throughout the Manuscript: "ml", "l", "µl" units should be change to "mL", "L", "µL".

Response: First, we thank the reviewer for complimenting and bringing up the originality of our work and would like to extend our thanks regarding the suggestion to amend the units. These are now corrected in the revised manuscript.

2. In Abstract, Page 2, Line 38: "…tryphan…" should be change to "…trypan…"

Response: We thank the reviewer for realizing and bringing up this typo. This is now corrected in the revised manuscript.

3. In Materials and Methods, Page 5, Line 111: "…Fraser, et al [10] (Fraser et al, 2005)…" should be change to "…Fraser et al [10]…"

Response: Thanks to the reviewer, this mistake is now corrected.

4. In Discussion, Page 14, Line 290: "…Tryphan blue…” should be change to "…Trypan blue…”

Response: Thanks to the reviewer, this mistake is now corrected.

5. In Discussion, Page 14, Line 309: "…MCF7 cells…” should be change to "…MCF-7 cells…”

Response: We thank the reviewer for pointing out these typos, this is now amended in the revised manuscript.

Guang Ji (Reviewer 3): The manuscript evaluate the anti-proliferative and cytotoxic activities of Allium autumnale on two human breast cancer cell lines, it proposed an interesting topic, but only the phenotypic data were provided. In addition, several issues should be considered:

1. The safety of the agents on normal cells were unclear;

Response: The reviewer brings up an interesting point. Unfortunately since we do not have access to normal breast epithelial cells, we have not been able to carry out these assays. However, since the used extracts are natural products that are routinely and historically consumed by the locals on the island, we do not believe there would be any detrimental effects associated with these extracts on normal cells regarding their safety.
2. In order to evaluate the effects of Allium species on lateral motility, the authors applied wound healing assay, so the representative images of the assay might be illustrated in certain Figure;

Response: We thank the reviewer for bringing up this issue. In order to address the reviewer’s question, we have now included a new figure on this topic which includes representative pictures as well as the quantification of the obtained data in lines 259-264 on page 12 (new Figure 6).

Legend: Figure 6: Effects of AAB and AAS on lateral motility of MCF-7 and MDA-MB-231 cells from 24h incubation. Bar graph showing lateral motility data obtained for MCF-7(A) and MDA-MB-231(B) for 24h incubation. “x” represents no significant difference compared to the control experiments. Representative inverse light microscope images of AAB and AAS incubation of MCF-7 (C) cells and MDA-MB-231(D) which caused no significant effect on lateral motility. Scale bar 50µm.

3. The AAB and AAS extracts were analyzed by GC-MS and the ingredients contained were defined, since the cells were treated with compounds, so what is the significance and purpose of the GC-MS analysis?

Response: The GC-MS analysis for the first time defines the molecular contents of this never before studied Allium species. In addition to learning about the diverse molecular constituents of this species, the literature review our team has carried out also revealed the medical significance of the identified molecules. This has further provided us with an important body of knowledge regarding the molecular basis of the medical significance of this species. The observed cytotoxic and anti-proliferative properties of Allium autumnale can be linked to several molecules indentified by our GC-MS study.

4. The writing needs improved.

Response: Upon the reviewer’s and other reviewers’ recommendation, we have carried out several changes to the text to improve our writing. With the additions and edits recommended by other referees, our manuscript’s writing has been revised and updated. These updates, which we believe have significantly improved our paper, can now be seen in the revised manuscript as well as highlighted in this report.

Canan Eroğlu (Reviewer 4): Isbilen et al. investigated anti-proliferative and cytotoxic activities of Allium autumnale on Human Breast Cancer Cell Lines MCF-7 and MDA-MB-231. This manuscript is well presented. But, there are some major points.

1. Researchers showed anti-proliferative and cytotoxic activities of Allium autumnale. Moreover, they indicated formation of membrane blebs in BC cells under inverted microscope for apoptotic mechanisms. This is a very rough method. Therefore, changes of important genes in apoptosis can be demonstrated at protein and mRNA levels methods such as using qPCR, western blotting, elisa etc.

Response: We thank the reviewer for bringing up this important topic. We are in complete agreement with the reviewer that the dissection of the potential apoptotic mechanisms by
molecular methods would significantly aid in understanding the details of the mechanisms at work. That is why we are currently working on optimization of these and similar molecular methods to elucidate the mode of action of the extracts as a separate project on its own. However, while optimizing these molecular methods, to address the reviewer’s concern, we have carried out several preliminary studies to investigate the apoptotic mode at the protein and cellular level. As also noted above, we have investigated the Caspase-3 activity (Biovision, USA) of MDA-MB-231 breast cancer cell lines. Caspase 3, activated by the upstream Caspase-8 and Caspase-9 proteases is a crucial marker for the apoptotic signaling pathway. Therefore this protein serves as a convergence point for different signaling pathways and it is well suited as a read-out in apoptosis assays. The preliminary results revealed that when two extracts were compared, AAS extract demonstrated higher caspase-3 activity on MDA-MB-231, which support our microscopy studies. We shall be happy to share these preliminary data if it would help in any way at this stage.

2. GS-MS chromatograms should be given in figure or supplemented data. It is acceptable after these revisions

Response: To address the reviewer’s concern we have now prepared and included the GC-MS chromatograms as Figure 7 in lines 282-284 on Page 13.

Legend: Figure 7: GC-MS chromatogram of compounds from ethanol extract in (A) Allium autumnale bulb (AAB) and (B) Allium autumnale stem (AAS). The numbered peaks correspond to the numbers and molecules in Table 1.

This is complemented by Table 1 which shows the most prevailing compounds detected on GC-MS. We thank the reviewer for her valuable input.

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