Author’s response to reviews

Title: Apigenin and hesperidin augment the toxic effect of doxorubicin against HepG2 cells

Authors:
Agnieszka Korga (agnieszka.korga@umlub.pl)
Marta Ostrowska (martaostrwska@umlub.pl)
Aleksandra Jozefczyk (ajozejfocyk@pharmacognosy.org)
Magdalena Iwan (magda.iwan@umlub.pl)
Rafal Wojcik (rafal.wojcik@umlub.pl)
Grazyna Zgorka (gzgorka@pharmacognosy.org)
Mariola Herbet (mariolaherbet@umlub.pl)
Gemma Vilarrubla (gemma5_5@hotmail.com)
Jaroslaw Dudka (jaroslaw.dudka@umlub.pl)

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Author’s response to reviews:

Response to the Reviewers

We would like to thank the Reviewers for their commitment, thoroughness and all remarks. We have not met such helpful comments for a long time which, we hope, helped us improve our work.

In the following we address their concerns point by point.

Josip Madunić, Ph.D. (Reviewer 1):

1. How do the authors explain that the combination of DOX with flavonoids other than apigenin or hesperidin, was not cytotoxic as DOX alone, as seen in Table 1? Could you please provide some theory behind this?

As Reviewer rightly pointed that other tested compound did not reveal synergistic toxic effect and what is more, they rather protect cancer cells in a presence of DOX. Protective activities are
mainly attributed to antioxidant properties of flavonoids that result from radical scavenging activity and the interaction with enzyme functions.

In present study only apigenin and hesperidin influenced DOX toxicity in tested cells and it seemed to be not related to oxidative stress. Often flavonoids activity is correlated with particular structure. In present study there is no simple structure-activity correlation. This two compound differ in glycosylation as well as hydroxylation pattern. What is more, they affected DOX cytotoxicity in a different way. Many biological effects of flavonoids are related to their ability to modulate enzyme activity, gene expression or DNA intercalation and even prooxidative properties. The present study confirmed this observation. This multidirectional activity causes that the effect of their action depends on many other than just the structure factors such as concentration, type of cells, possible interactions with other compounds.

We added a discussion part to the discussion section(the last 2 paragraphs before conclusions)

2. In the Results section on DNA oxidative damage determination, authors are claiming that they observed significant increase in AP sites in DOX-treated cells, and a drop in AP sites in DOX+A cells. These results (Figure 5) also show big SD deviation which brings in question the significance of authors’ claims, especially in the case of A vs DOX+A which (when included the SD) could possibly be 4.28 vs 2.9. The authors should be more careful when assigning the significance.

We agree with the reviewer’s statement. Thank you very much for your thorough analysis and attention. The error is the result of oversight caused by the person performing the graphs based on statistical analysis. We checked statistical data, and in fact, statistical significance is lower than 0.05 but not lower than 0.001. Therefore, the data on the chart were improved.

3. Authors state that the MTT cytotoxicity assay was done three times. It should also be noted if the data in Figure 2. is mean value of these three independent experiments or their representative assay? Please correct this.

Thank you for your attention. The figure’s caption was corrected.

4. In the Results section, authors are over-emphasizing the effect of DOX and flavonoids on HepG2 morphology; other than the lower number of cells in the treated cell-panels, there are not apparent differences in cell morphology. This is not helped by the poor quality of images due to the excess of white spots (present in control and treated cells) which could be a consequence of over-exposure in imaging software and not due to the "presence of cytoplasmic vacuoles" as the authors are stating. Please elaborate on this. If the authors insist on these morphological changes, the use of arrows within the Figure 3 indicating them is then suggested. Also, no magnification is mentioned and there are no scale-bars present in the Figure 3. Please provide. Figure 4 is also missing magnification and scale-bars.
We agree with the reviewer’s statement. The description of morphology in the Result section was corrected. The mentioned figures were improved and scale-bars were added.

5. In the Discussion section, authors state that the synergism of the cytotoxic effects of DOX and A is not related to the inhibition of glycolysis. Could you please elaborate on this in more detail?

After re-analyzing the text, we realized that this statement was poorly formulated and for this reason our assumption was too far-reaching. We meant that we did not observe any synergy regarding the effects of DOX and apigenin combination on expression of genes associated with glycolysis pathway. However, it is worth considering that DOX affected cells under conditions of decreased glycolytic gene expression. Moreover, it is unclear whether this is related to its cytotoxic activity.

We corrected that statement in Discussion section (as well as in Abstract).

6. Furthermore, authors suggest in the Discussion that combined administration of both agents - DOX and A leads to complete normalization of DNA oxidative damage and DSB. This is in contrast with the data presented in Figure 4. (panel DOX A), where one can observe plenty of CellROX signals indicating oxidative stress. The authors need to additionally clarify this before claiming that the oxidative damage by DOX is being abolished by apigenin treatment.

This is important remark. We wrote about CellROX signal as oxidative stress marker, however we should clearly identify the CellROX signal with the presence of reactive oxygen species, not oxidative damage. Summing up, in our study, the presence of ROS in the cell nucleus was observed, while DNA damage was minimized (in cultures treated with DOX and apigenin in the same time). There is a report which perhaps explains this interesting phenomenon. Rusak et al. studied the influence of selected flavonoids on lymphocyte’s DNA. They observed that flavonoids can cause DNA damage as well as act in a protective way in the presence of an oxidative stress factor.

We improved manuscript in Methods section and changed heading “Oxidative stress detection” to “Reactive oxygen species detection”. Additionally we corrected the first sentence: “The CellROX Green Reagent (Invitrogen, USA) was used as the ROS indicator” instead of “used for oxidative stress detection”. Analogical changes was made in Discussion section.

7. There are many misspelled words (i.e. Nicon/Nikon, StaftSoft/StatSoft, chemotherapeutic/chemotherapeutic, etc), inconsistencies in verb tense usage, compound names (i.e. cosmosin/cosmosin), missing period and comma signs, as well as space between words, poor sentence constructs, etc.. Poor quality of writing does not seem to be a result of poor English, but more a consequence of negligence in manuscript
checking. The authors need to check manuscript more carefully before submitting and the manuscript should be language-edited by a native speaker.

Thank you very much for your thorough analysis and attention. All remarks noted in the attached pdf file as well as other errors were corrected.

Minor revisions:

Title: „Apigenin and hesperidin augments the toxic effect” should be changed to „Apigenin and hesperidin augment the toxic effect"

We agree with the reviewer’s remark. Therefore, the title was corrected.

Page 2, line 42: Please insert abbreviations for the hexokinase 2 and lactate dehydrogenase A

The remark refers to a part of the text that has been removed from the manuscript after first round of revision.

Page 3, line 4: Please change „of the couple of doxorubicin and apigenin” to more appropriate „of the combined (or synergistic) effect of doxorubicin and apigenin"

The sentence was corrected.

Page 4, line 35: Abbreviations for HK2 and LDHA should be given in full at the first mentioning in the text

The full names were given.

Page 4, line 44: "higher than normal cells" should be changed to "higher than in normal cells"

The rest of the grammatical corrections is noted in the attached pdf of revised manuscript.

The sentence was corrected. Thank you very much for your thorough analysis and attention. All remarks noted in the attached pdf file were corrected.

1. Last paragraph in the Introduction section is too long and not clear. I would suggest separating it in two sentences with clear emphasis on what was the aim of the research.

We agree with the reviewer’s comment. Therefore, the last paragraph of the Introduction section was rewritten.
2. Authors are constantly using the term "inoculation" in the Methods section. I would like to point out that cells in cell culture are not "inoculated", they are "seeded". "Inoculation" in layman terms means introduction of something (i.e. tumor cells in xenografts experiments or pathogen/antigen in antibody production) into a living organism to stimulate something. Please correct this.

The mistakenly used term was improved.

3. Many of the Figures are missing the description or just notion of the assay/method used for obtaining presented data. Please correct this where applicable.

The figures and captions were improved.

4. Proper format of cited reference in the Discussion on Page 13, line 42 is "Vrhovac Madunić et al" (two last names). Same in the list of references.

Thank you for your attention. The cited reference was corrected.

Juan Li (Reviewer 2):

1. In P8: the author mentioned "viability respectively 35.61±0.72…… of viability." However, the results are inconsistent with those shown in table 1. Check it carefully.

Thank you for your attention. The data were carefully checked and corrected.

2. "Usually, glycolysis is activated when an oxygen deficiency occurs. and is observed in the growth of soild tumours." should be modified in P12?

The statement was corrected.

3. I suggest the author to provide the clearer picture of figure 3. In fact, I can't find the morphological changes as the results indicated.

We agree with the reviewer’s statement. The mentioned figures were improved and scale-bars were added. Also, the figure’s description was rewritten.

4. Apigen and hesperidin have synergistic effect on the doxorubicin, however other flavonoids have no effect. These should be discussed.
We added a discussion part to the discussion section.

Please see point 1 in Reviewer 1 response.

5. This research only used one cell line of HCC, I think the normal cell or more cell lines are needed to verify the results.

Thank you, we agree with your statement. However, the subject of our study was the preliminary understanding of the mechanisms of substances in HCC. We will try to extend the preliminary observations with research on following cell lines, including normal ones. We are particularly interested in cardiomyocytes and the influence of flavonoids on anthracycline cardiotoxicity.