Author’s response to reviews

Title: The flavonoid beverage Haelan 951 induces growth arrest and apoptosis in pancreatic carcinoma cell lines in vitro

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Author’s response to reviews: see over
Referee PP Tripathi

1. WST-1 Proliferation Assay Kit provides an easy to use tool for studying the induction and inhibition of cell proliferation in *in vitro* models and is widely accepted for these studies. Because we have measured WST-1 in numerous experiments (n=3-12), we are sure that our results are right.

   But we agree with the reviewer that methods such as BrdU and Ki67 are more comfortable. We will use these methods in further studies.

2. In reply to point 2, the following sentence was included in the “Discussion” section: compare page 15, lines 14-16

   “This concentration is approximately 100-fold higher than that found in the plasma samples obtained from a patient 1 h after ingesting 4-oz of Hael [16].”

3. According to our opinion, TUNEL assay cannot be the gold standard for measuring apoptosis, because some necrotic cells were also detected. We assume that electron-microscopic evaluation is more precisely.

   But as recommended, TUNEL assay was performed and the results were included in the “Results” section: compare page 11, lines 20-24

   “Using the TUNEL POD assay for apoptosis detection, the results also revealed that 8% Hael induced apoptosis in all cell lines investigated. Thus, in the CAPAN-1 cell population, only a small number of apoptotic cells (stained dark brown) was visible compared with the control, whereas in all other cell lines, the number of apoptotic cells was of greater degree (Fig. 5 A-H).”

   Using the FLICA reagent SR-VAD-FMK, we detected the activation of most caspases including caspase-3. According to the manufacture, FLICA measures the intracellular process of apoptosis instead of side-effects such as the turn-over of phosphatidyl serine.

   Thus, using the FLICA assay, we confirmed our results obtained by using the Annexin V assay.

   We also performed Western blots of active caspase-3. But unfortunately, we have a problem with the fibroblast cell line. We were not able to harvest sufficient cells for Western blots.

   However, we include the following sentence in the “Discussion” section: page 16, lines 3 and 4
“Investigating the effect of calpain inhibition on Hael-induced apoptotic activity, our results reveal an increase in Annexin V-positive cells in all PC cell populations investigated. Western blot analyses of active caspase-3 confirmed these findings (data not shown.”)

4. In reply to point 4, the following sentences were included in the “Discussion” section: compare page 14, lines 10-17

“Both compounds have been reported to increase the proliferation of prostate epithelial and estrogen-sensitive human breast cancer (MCF-7) cells, but not of estrogen-insensitive human breast cancer (MDA-MB-231) cells [20-22]. In MCF-7 cells, this effect has been attributed to an increase in DNA synthesis [23]. CAPAN-1 cells and fibroblasts are estrogen receptor-expressing cells as well [24, 25]. Consequently, Hael-induced increase in the proliferation rate of these cell lines may also be explained with an increase in DNA synthesis.”

Two additional references 23-25 were cited.

5. Because Haelan induced only a small increase in the number of Annexin V-positive cells in CAPAN-1 cell line compared to the control (Fig. 4A), we believed that these results are not accurately. But Western blot analysis of activated caspase-3 demonstrates that Haelan also triggers apoptosis in CAPAN-1 cells.
Referee H. Akrami

The space between a digit and a percent unit was removed in all cases.

The quality of written English was improved.
1. The authors include the description of the fibroblasts including the source into the manuscript: compare page 6, lines 17-19.

2. a), b) and c): The “Experimental Design” section was replaced by the following section:

   **Exposure of PC cell lines and fibroblast to increasing concentrations of Hael**

   The three PC cell lines and human fibroblasts (pro well: \(4 \times 10^4\) cells / 2 ml cell culture medium) were added to 24-well plates including the control and blank. After reaching subconfluence, the medium was removed and the cells were incubated with increasing concentrations of Hael (2% - 32%) containing in 2 ml of the corresponding cell culture medium for 24 and 48 h. The control cells were incubated with medium only.”

   (compare page 7, lines 3-8)

3. As recommended, the term “toxicity” was replaced by “cell damage”: compare page 10, line 12

4. The Trypan Blue assay has been removed previously, but in the manuscript, this was forgotten. This mistake was corrected.

5. The sentence “a similar increase” was revised. We changed it into “a small increase”

   (compare page 10, lines 19-23).

6. 16% and 32% Haelan cause cytotoxic effects as shown by the dramatic increase in LDH release.

7. We do not change labeling of y-axis for Fig. 2 and Fig. 3, because we think the labeling is o.k.

8. The reference list was revised.