Author's response to reviews

Title: Ameliorating effects of aged garlic extracts against amyloid beta-induced neurotoxicity and cognitive impairment

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Author's response to reviews: see over
Dear BMC Complementary and Alternative Medicine Editorial Team:

We followed most part of the reviewers’ suggestions and revised accordingly. In addition, we responded some questions in detail to explain our points of view based on reported facts. I hope that the revised manuscript will be acceptable to you and thank you for the valuable suggestions.

Thank you!

Ho Jin Heo
Associate Professor
Major points

1. Aged garlic extract has already been reported to show antioxidative actions, even the physiological activities of a single active compound S-allyl-cysteine was proved, the effect of ethyl acetate fraction under the experimental settings is not very surprising. Authors conclude that this extract possess beneficial activities for Alzheimer's disease. Is there any advantage of this extract over other antioxidants with regard to anti-amnesic effects?

→ According to the reviewer’s question, we answered and added in ‘Discussion’.


→ Above recent references showed that thermally processed foods, especially fruits and vegetables, exhibited higher biological activities because of various chemical changes that occur during heat treatment. Major volatiles of raw and heated garlic were dimethyl disulfide, 2-propen-1-ol, allyl methyl disulfide, dimethyl trisulfide, diallyl disulfide, allyl methyl trisulfide, and diallyl trisulfide. However, a dimethyl disulfide (DMDS), which was not observed in raw garlic samples, was just observed in heated samples (autoclaved garlic clove and
autoclaved-crushed), indicating thermal reaction may cause the formation of this compound. Our study demonstrated that PC12 cell cytotoxicity through Aβ_{25-35}-induced neurotoxicity was suppressed by pretreatment with ethyl acetate fraction obtained from ethanolic extract of aged garlic. In our research, we showed that ethyl acetate fraction form ethanolic garlic extract had *in vitro* antioxidant activities, PC12 cell protections, and *in vivo* anti-amnesic effects. Consequently, DMDS was just identified as main compound through HPLC analysis. As the cumulative concentration of organosulfides consumed in aged garlic extracts is high, it is likely that the combined concentrations of ingested organosulfides including DMDS may reach levels high enough to bring about a cellular response and cognitive improvement in mice.

2. Comparative experiments with positive drug or with other compounds may be required *in vivo* test.

→ According to the reviewer’s question, we answered.

In general, there is no a positive drug on Aβ-induced cognitive deficit and the sample group is compared with the control group without Aβ. Just other recent cases of our study are as follows:

① Jeong HR *et al.*: Antiamnesic effects of ethyl acetate fraction from chestnut (Castanea crenata var. dulcis) inner skin on Aβ_{25-35}-induced cognitive deficits in mice. *Journal of Medicinal Food* 2012, 15: 1-6.
Pretreatment with the ethyl acetate fraction from chestnut inner skin increased spontaneous alternation behavior in the Aβ-injected mice (5 mg/kg of ethyl acetate fraction: E5, 90.80%; 10 mg/kg of ethyl acetate fraction: E10, 98.26%; 20 mg/kg of ethyl acetate fraction: E20, 103.98%).


The groups pretreated with kaempferol had an ameliorative effect on Aβ_{1-42}-induced decrease in alternation behavior (50 µM: 66.43%; 75 µM: 66.32%; 100 µM: 77.26%).

→ In comparing with that in our research, we showed that the groups pretreated with the sample increased spontaneous alternation behavior after Aβ injection (the ethyl acetate fraction 5 mg/kg: B5, 100.30%; 10 mg/kg: B10, 106.33%; 20 mg/kg: B20, 108.60%). The Aβ_{25-35}-induced cognitive deficits exerted via various cytotoxicities including oxidative stress and disruption of hippocampal network activity was suppressed by pretreatment with aged garlic.
Minor points

1. According to the reviewer’s recommendation, we complemented the results of statistical analysis.

   → Line 290 & 310-311: ‘Different superscripts indicate significant difference among groups at \( p < 0.05 \)’

   Line 339-340: ‘Significant difference \( (p<0.05 \text{ versus vitamin C}) \) was observed on the Aβ-induced cell death’

   Line 360-361: ‘\( p<0.05 \) versus control group, \( p<0.05 \) versus Aβ\(_{25-35}\) group. Values with the same letter are not significantly different.’

   Line 376-377: ‘\( p<0.05 \) versus control group, \( p<0.05 \) versus Aβ\(_{25-35}\) group. Values with the same letter are not significantly different.’

2. According to the reviewer’s request, we added description in figure legends.

   - Fig. 1 legend: ‘**ABTS radical scavenging activities (A) and Inhibition effect of ferric ion and vitaminC-induced lipid peroxidation on mouse brain homogenates (B) of various fractions from aged garlic extracts.** Each value represented the means±SD of triplicates. Different superscripts indicate significant difference among groups at \( p<0.05 \).’ (Line 287-290)

   Fig. 2 legend: ‘**Effect of ethyl acetate fraction from aged garlic extract on ROS production determined in the presence and absence of Aβ in PC12 cell.** PC12 cells were pretreated for 48 h with various concentrations. After 48 h, the cells were treated with 80 µM Aβ for 2 h. The increase of DCF-DA fluorescence was measured by fluorescence microplate reader.'
Each value represented the means±SD of triplicates. Different superscripts indicate significant difference among groups at $p<0.05$.

3. According to the reviewer’s suggestion, we checked and revised our manuscript.

4. According to the reviewer’s request, we partially removed or changed old one.
   - We were changing old references (2, 3, 9, 11, 15, 24, 27).

Reference 2 (Line 525)


Reference 3 (Line 528)


Reference 27 (Line 589)


**Thanks for valuable suggestions!**
[The replies on the review of ‘Reviewer 2’]

Major points

1. The effects of aged garlic extracts were limited to unphysiological form (Aβ25-35)-induced reactive oxygen species (ROS).

According to the reviewer’s question, we answered and added in ‘Discussion’.


→ Above recent references showed that extracellular aggregates of Aβ peptide, observed in AD patients, contain Aβ peptide in its most predominant sequences of Aβ1-40 or Aβ1-42; however, they also contain peptides with shorter sequences such as Aβ25-35. In addition, neurotoxic properties are characteristic not only of the whole Aβ peptide Aβ1-40(42), but also of a number of fragments, the most neurotoxic of which is fragment Aβ25-35. Peña et al. reported that Aβ25-35 is more rapidly toxic and causes more oxidative damage than the parent peptide Aβ1-42. Furthermore, administration of Aβ25-35 into the brains of rodents induces impairments of working memory characteristic of Alzheimer's disease, without
inducing the formation of amyloid deposits; thus, administration of Aβ_{25-35} is regarded as one potential model for the early stages of this disease. The neurotoxic activity of Aβ has been attributed to amino acids present in positions 25-35 of the full-length beta-amyloid. Therefore, Aβ_{25-35} is appropriate in this experiment.

2. I have a concern that the effect of aged garlic extracts is too restrictive in oxidative stress. If only Aβ-induced ROS are scavenged by the compounds, its toxicity and its induced cognitive impairment are completely overcome?

→ According to the reviewer’s question, we answered and added in ‘Discussion’.


② Woo KS et al.: Characteristics and antioxidative activity of volatile compounds in heated garlic (Allium sativum). Food Science and Biotechnology 2007, 16: 822-827

→ Aged garlic extract (AGE) contains several neuroprotective compounds, including S-allyl-L-cysteine (SAC) and allixin. In addition, various physiological compounds of raw and heated garlic were dimethyl disulfide, 2-propen-1-ol, allyl methyl disulfide, dimethyl trisulfide, diallyl disulfide, allyl methyl trisulfide, and diallyl trisulfide. Ito Y et al. also demonstrated that SAC also protected cultured hippocampal neurons against Aβ-induced neuronal
death. Therefore, in the study presented here, we have shown that aged garlic extract containing various compounds with DMDS is considered that may be helpful to overcome Aβ-induced cognitive impairment and cytotoxicity.

Minor points

1. According to the reviewer’s request, we checked and revised.

→ p. 28 (Figure 2).

Thanks for your valuable suggestions!
[The replies on the review of ‘Reviewer 3’]

1. On the background of the recognized antioxidant activities of garlic constituents, Jeong et al. further investigated the neuroprotective effects of aged garlic extract both in vitro, in PC12 cell cultures, and in vivo, in mice, particularly after amyloid beta-peptide challenge. The methods are appropriate, the data clearly presented and well discussed in correlation with existing data.

→ Thanks for your comment!

Thanks for your valuable suggestions!