Author's response to reviews

Title: Ethanol extract of Gleditsia sinensis thorn suppresses angiogenesis in vitro and in vivo

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Author's response to reviews: see over
Oct 4th, 2012

Dear Dr. Tom Rowles, Executive Editor of BMC Complementary & Alternative Medicine.

We are now submitting the revised version of original manuscript (MS# 6609144627736582) entitled; "Ethanol extract of Gleditsia sinensis thorn suppresses angiogenesis in vitro and in vivo" by Yi, Park, Oh, Lee, Kim, Oh, Bang, and myself. We are also providing point-by-point responses to the reviewer’s comments in this Cover Letter. We tried to answer all issues and expect our revised manuscript to be accepted for publication in the "BMC Complementary & Alternative Medicine" as a "Research Articles."

We really appreciate your time for reviewing our revised manuscript.

Sincerely,

No Soo Kim, Ph.D.

Principal Researcher.
Response to reviewers’ comments on the manuscript #6609144627736582 entitled “Ethanol extract of Gleditsia sinensis thorn suppresses angiogenesis in vitro and in vivo”.

Response to the comments issued by Dr. David Heber.

Comment #1. The background on the use of Gleditsia sinensis thorn

Reply to comment #1. The background of medicinal uses of Gleditsia sinensis thorn is now reinforced in the “Background” of the revised manuscript as followings; “…Traditional oriental medicine has used various parts of Gleditsia sinensis such as thorns, fruits, and anomalous fruits (fruits without seeds) to treat diverse diseases including thrombosis, obesity, and tumor-related disease [8-10]. In oncologic aspect the extract of Gleditsia sinensis thorn could prevent colon cancer in vitro and vivo through the induction of G2/M cell cycle arrest and extracellular signal-regulated kinase 1/2 (ERK1/2) activation [10], and cervical cancer in vivo through down-regulation of proliferating cell nuclear antigen (PCNA) and mutant p53 [11]. The extract of anomalous fruits of Gleditsia sinensis induced apoptotic cell death in primary leukemic cells of cancer patients [12]. In addition the extract of Gleditsia sinensis fruits showed anticancer effects in esophageal squamous cell carcinoma cell lines by inhibiting cyclooxygenase 2 (COX2) expression and telomerase activity [13]. The extract of Gleditsia sinensis thorn was also known to have antiatherogenic effect in vascular smooth muscle cells by inhibiting cell proliferation and TNFα-induced matrix metallopeptidase 9 (MMP9) expression [14]. However, the effect of EEGS on angiogenesis and its underlying mechanism are still in question in primary endothelial cells that form blood vessels. In this study, we demonstrated that the EEGS has antiangiogenic potential both in vitro and in vivo…”

Comment #2. The phytochemistry of Gleditsia sinensis thorn

Reply to comment #2. During preparation of this manuscript we could isolate one antiangiogenic active compound from the extract of Gleditsia sinensis thorn using in vitro activity-guided
fractionation. We are now in the process of identifying its structure and evaluating its antiangiogenic potential using in vivo angiogram. So, it is hard to disclose its identity in this manuscript. We would like the reviewers to take this situation into consideration. Instead, the previous other research groups’ phytochemical scientific findings of Gleditsia sinensis are now included in the “Discussion” of the revised manuscript as followings; “…Most previous phytochemical studies on Gleditsia sinensis were carried out using its fruit and anomalous fruit parts. The single compounds from the fruits or anomalous fruits of Gleditsia sinensis have been isolated as triterpene (echinocystic acid), flavonoid (aromadendrin), polyphenol (ellagic acid glycosides), and triterpenoid saponins (gleditsioside A-K, N-Q, and Z) [31-40]. Their identified pharmacological activities were antagonistic against dopamine D1 receptor (gleditsioside F) [33], protective against acute myocardial ischemia (echinocystic acid ) [32] or type 2 diabetes mellitus (aromadendrin) [35], antiallergic in mast cells (saponins) [41], and cytotoxic to leukemic cells (gleditsioside E) [38]. The single compounds from the Gleditsia sinensis thorns were isolated as a lupane acid with anti-HIV activity [42], and triterpenoid (D:C-friedous-7-en-3-one) and sterols with antimutagenic activity [43]. To our knowledge, there was no study reporting antiangiogenic active compound(s) from the Gleditsia sinensis. We are trying to identify antiangiogenic active single compound(s) from the extract of Gleditsia sinensis thorn using in vitro activity-guided fractionation…”

Comment #3. Protocol used for in vivo angiogenesis assay

Reply to comment #3. In general, to our knowledge, the protocols to evaluate the in vivo antiangiogenic potential of test drug(s) using a matrigel plug assay recommend mixing test drug(s) with matrigels containing proangiogenic factors. The manufacturer’s instruction of the directed in vivo angiogenesis assay (DIVAA) kit we followed (Trevigen) suggests also that the test drug (EEGS in this study) should be premixed with matrigels before implantation into mouse.

Comments #4. Statistics

Reply to comment #4. We were assisted for statistical analysis of data by an expertise. So, we are
Response to the comments issued by Dr. Komal Raina.

Comments #1. Higher concentration of DMSO vehicle than usual in overall assays
Reply to comment #1. As Dr. Komal Raina raised the question, we used a higher maximum concentration of DMSO vehicle (1%, v/v) than usual (0.1-0.5%) in assays because the solubility of EEGS in 100% DMSO was not sufficient to make a stock solution (1,000X). So, we evaluated the 1% concentration of DMSO in HUVEC primary cells for its effects on cell viability and in vitro angiogenesis assays such as cell migration and tube formation. We found that neither cell viability nor in vitro angiogenesis of HUVEC cells was significantly affected by DMSO up to 1%. So, we decided to use 1% of DMSO as a maximum dose of vehicle control in the present study. This issue is now addressed in the “Cell viability” section of “Methods” in the revised manuscript as followings:

“...The higher maximum concentration of vehicle (1%) than usual (0.1-0.5%) was used in this study due to low solubility of EEGS. However, we found that neither cell viability nor in vitro angiogenesis of HUVEC cells was significantly affected by DMSO up to 1%...”

Comments #2. Insertion of legend key in Figure 1A and 1B
Reply to comment #2. As suggest by reviewer, we labeled Figure 1 separately such as Figure 1A, 1B, and 1C in the revised manuscript. This change was reflected in the “Figure legends”. We also inserted the legend keys of Figure 1B in the revised manuscript.

Comments #3. Including densitometric values for the protein dots in proteome analysis
Reply to comment #3. As suggest by reviewer, we included the relative expression levels of EDN1 and FGF2 along with statistical significance compared with vehicle treatment in the “EEGS down-regulates the expression of proangiogenic proteins” section of “Result” as followings;

“...Treatment of 100 µg/mL EEGS decreased the EDN1 expression by 34.8% (p<0.001) in the cultured media, and by 55.2% (p<0.001) in the cell lysate compared with the negative vehicle control.
Because the expression of FGF2, which was supplemented in EGM-2 endothelial growth medium, was decreased by EEGS only in cultured media (41.7%, $p<0.01$) but not in the cell lysate (122.5%, $p=0.210$), it was excluded from our further studies.”