Author's response to reviews

Title: Terminalia catappa attenuates urokinase-type plasminogen activator expression through Erk pathways in Hepatocellular carcinoma

Authors:

Ming-Ju Hsieh (kaross1006@hotmail.com)
Chiao-Wen Lin (cwlin@csmu.edu.tw)
Hui-Ling Chiou (hlchiou@csmu.edu.tw)
Shun-Fa Yang (ysf@csmu.edu.tw)
Chao-Bin Yeh (ycb@csmu.edu.tw)

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Author's response to reviews: see over
Dec 10, 2013

Dear editor:

Attached please find the revised manuscript (MS: 3097192699779014 “Terminalia catappa attenuates urokinase-type plasminogen activator expression through Erk pathways in Hepatocellular carcinoma”) with a point-by-point response listed in the following page to the reviewer’s criticism for resubmitting to BMC Complementary and Alternative Medicine. We hope that these changes and replies may meet your requirement for being published. Thank you very much for your kind assistance.

Yours Sincerely,

Shun-Fa Yang, Ph.D.,
Institute of Medicine,
Chung Shan Medical University,
Taichung 402, Taiwan
E-mail: ysf@csmu.edu.tw
Editorial comments:

Please provide details in your Methods section on who undertook the formal identification of the plant material used in your study. Please also confirm whether a voucher specimen of this material has been deposited in a publicly available herbarium, and include this information in your manuscript. A deposition number should be included, if available.

Answer: Thank you for this valuable suggestion. The information about who formally identified the plant material used in this study has been added in the Materials and methods section.
Reviewer's report (1):

The manuscript "Terminalia catappa attenuates urokinase-type plasminogen activator expression through Erk pathways in Hepatocellular carcinoma" by Hsieh et al, explores Terminalia catappa leaf extract (TCE) has an inhibitory effect on cell invasion and migration by regulating the activities and protein level of u-PA and their natural inhibitors involves the ERK1/2 signaling pathway. The findings provide new insight on the chemopreventive treatment on hepatoma. This submission is presented in a suitable way and shows a solid experimental data. In conclusion, this is a written manuscript in which the mechanism of the anti-metastatic activity of TCE could be convincingly demonstrated in a number of in vitro experiments. However, there are still a number of minor problems need to be addressed.

Suggestions:
1. The last paragraph of the “Introduction” describing NF-#B and AP-1 needs more clarification.

Answer: Thanks for your valuable comments. The sentences about NF-κB and SP-1 have been revised in the revised manuscript.

2. The authors should include information about whether the compound was tested at physiologically-relevant and attainable concentrations.

Answer: Thanks for your comments. For evaluation of the inhibitory effect on the invasiveness and migration of human Hepatocellular carcinoma Huh7 cells by TCE, we chose the concentration range up to 100 µg/mL which had no cytotoxic effect on Huh7 cells and it is consistent with in vitro studies from other laboratories (Ko et al., 2003). Moreover, the toxicity of TCE has been reported that an oral administration of 3,000 mg/kg TCE did not cause any lethality in the single-dose acute toxicity test and the treatment by 3,000 mg/kg/day for 30 continuous days did neither alter the body weights nor the hematological parameters in C57BL/6 mice in the study (Chu et al., 2007). Therefore, more animal studies and clinical trials using the concentration range of TCE are needed to further justify its clinical value and they are our plans.


Chu SC, Yang SF, Liu SJ, Kuo WH, Chang YZ, Hsieh YS: In vitro and in vivo

3. The authors should discuss in detail the next step in the investigation of the utility of TCE as a chemotherapeutic agent: its ability to inhibit breast cancer in vivo.

**Answer:** Thanks for your comments. The inhibitory effect of TCE on the growth and metastasis of Lewis lung carcinoma cells (LLC) in vivo was proven in our previously study (Chu et al., 2007). More animal studies in HCC cell of TCE are needed to further justify its clinical value and they are our plans. We also added the description in the discussion section of this revised manuscript.


4. The introduction gives a very nice background for the reader. I would like to see something about the bioavailability of TCE discussed in this section. I feel that this is an important aspect to be able to link an in vitro study to the feasibility of this compound as being functional in the body once consumed. Have the authors noted any similar effects of metabolites or breakdown products of TCE in their studies or others (these may also be important as they will be present in the body post consumption) ?

**Answer:** Thanks for your comments. Previous researchers have identified anticancer compounds in TCE extract, including some flavonoids and hydrolyzable tannins such as punicalagin, punicalin, terflavins A and B, tergallagin, tecatain, geraniin, granatin B, corilagin, etc. We have added the description in the Introduction section of this revised manuscript.

5. General, it is not clear where the authors included the statistical information gained from the analysis mentioned under M&M.

**Answer:** Thanks for your comments. We have added more detailed description in the “Statistical analysis” section of this revised manuscript.
Reviewer's report (2):

Minor Essential Revisions. I appreciated the manuscript by Ming-Ju Hsieh et al. -Terminalia catappa attenuates urokinase-type plasminogen activator expression through Erk pathways in Hepatocellular carcinoma- The authors have raised interesting issues on the molecular mechanism underlying the antimetastatic effects of Terminalia catappa leaf extract (TCE). Since the overexpression of urokinase (at mRNA and protein level) in human hepatocellular carcinoma (HCC) is an unfavourable prognostic factor for HCC patients, the u-PA targeting by molecular technologies (AS-RNA, shRNA: Tavian D et al Cancer Gene Therapy, 2003; Salvi et al, Molecular Cancer Ther. 2004), also in human HCC xenografts (Salvi A et al Tumor Biology 2007) decreases the aggressive behaviour of HCC cells inhibiting their migration and invasion capabilities, the authors have decided to investigate the effects of TCE on uPA expression. Actually the authors in the text refer to antimetastatic effects of TCE (as I mentioned above); I think this term (antimetastatic) is inappropriate because the experimental design and the results shown have been obtained in cultured HCC cells and not in tumor animal models. I suggest to change this term. I appreciated the results obtained by the authors concerning the decrease of uPA expression, also those on the impairment of transcriptional uPA activity (uPA promoter assay, Chip IP data).

Answer: Thanks for your valuable comments. The term (antimetastatic) has been changed in the revised manuscript.

I would suggest a minor essential revision.
--Level of interest: an article of importance in its field, in HCC field, but probably also in other cancer types that might be examined in the future.

Answer: Thanks for your valuable comments. We will examine the anti-migration effects of Terminalia catappa leaf extract in other cancer types in the future.

--Background:
--line 6: I suggest to eliminate the information on the 2 alpha elices and 2 antiparalle beta strands; this information is not necessary. The list of uPA domain should refer to the position in the uPA molecule: from the N-terminus to the C-terminus (that is, GF domain, kringle domain and serine protease domain).
Answer: Thanks for your valuable comments. The sentences have been revised in the revised manuscript.
“The u-PA serine protease is secreted as a 53 KD zymogen (pro-urokinase), and has 3 structural domains: the growth factor domain, kringle domain and serine protease domain”

Further in the background the authors should summarize the information available on uPA as putative target for HCC therapy in animal model by siRNA.

Answer: Thanks for your valuable comments. The information available on uPA as putative target for HCC therapy in animal model has been added in the revised manuscript.
“Moreover, the u-PA targeting by molecular technologies (ex: antisense RNA or shRNA) [22,23], also in human HCC xenografts decreases the aggressive behavior of HCC cells inhibiting their migration and invasion capabilities [24].”

--Results:
-- title of the first paragraph: ….. and their endogenous inhibitors PAI-1. PAI1 is one inhibitor and the authors show results on this inhibitor; Therefore the sentence would be …. and its endogenous inhibitor PAI-1.

Answer: Thanks for your valuable comments. The sentence has been revised.
“Effects of TCE on the Protein Levels of u-PA and its Endogenous Inhibitor PAI-1”

--Effects of TCE on the protein levels of uPA and their endogenous inhibitor PAI1: the results shown in Fig 1 show uPA expression as enzymatic activity and at protein level as they were detected in the conditioned media. It is necessary to state if the conditioned medium did not contain serum or it contained a small l % of serum; the author refer to ref 24 ( materials and Methods p 8 ), but it is not easy to find this article. Further I would like to know the amount of conditioned media loaded on the SDS-PAGE (I think they were comparable amounts in the two experiments, enzymatic activity and Western blotting, but it seems that a major amount has been loaded on SDS gel for Western blotting,. It is necessary to give these sharp informations in the text. Further sine uPA and PAI1 are secreted proteins, beta actin is not an appropriate control. It is possible to add, as supplementary figure, the protein staining of the gels to ascertain the comparable amount loaded. It is not possible to adjust the secreted uPA and PAI1 protein levels with the beta actin protein level.
Since it is very difficult to have a control secreted protein, it is possible to consider the control sample as 100% and refer the tested samples to this.

**Answer:** Thanks for your valuable comments. The serum-free conditioned medium was used for casein zymography protease assays. The amount of conditioned media loaded on the SDS-PAGE was also mentioned. We have added the description in the Materials and Methods. Furthermore, the “cell lysates” were used for Western blotting to analyze the uPA and PAI-1 protein levels. We have added the more detailed description in the revised manuscript.

--Discussion:
P 16, line 2: …TCE has an inhibitory effect ( it lacks this term).

**Answer:** Thanks for your valuable comments. The term has been added.