Author's response to reviews

Title: Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack.: Sinapinic acid acts as HDAC inhibitor

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Version: 3 Date: 2 August 2013

Author's response to reviews: see over
August 2, 2013

Dear Prof. Xiaolin Zi:

I am enclosing herewith a revised manuscript entitled “Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of *Hydnophytum formicarum* Jack.: Sinapinic acid acts as HDAC inhibitor” for consideration for possible publication as a research article in BMC Complementary and Alternative Medicine journal.

We have revised the manuscript to address all the concerns from the referees and highlighted all changes in blue letters for a revised manuscript.

The list of changes is accompanied with a point-by-point response to all the comments made by all the referees.

The response to all the referees is as following:

**Reviewer 1**

**Reviewer's report**

**Title:** Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of *Hydnophytum formicarum* Jack.: Sinapinic acid acts as HDAC inhibitor

**Version:** 2 **Date:** 15 June 2013

**Reviewer:** Chanin Nantasenamat

**Reviewer's report:**

The authors report an interesting finding on the Thai traditional medicinal plant from the rhizome of *H. formicarum* Jack and its histone deacetylase inhibitory properties against human cancer cell lines.

- Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore)

- Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Authors should compare the chemical structures of cinnamic acid and sinapinic acid in their manuscript and discuss this in light of the two methoxyl groups at C1 and C3 positions.

**Response:**
- In a revised manuscript, we have added the chemical structure of a cinnamic acid (Table 1, page 23).

- The two methoxyl groups are discussed as “The two methoxyl groups at C3 and C5 positions of sinapinic acid (Table 1) have little influence on its hydrophobicity while the hydroxyl group at C4 position contributes to a lesser extent of its hydrophobicity comparing to the prototype cinnamic acid (log \( P = 2.14 \); ChemAxon)” (page 14, lines 14-17).

Page 13, Lines 23-24, Discussion: Sodium butyrate and sinapinic acid was compared in terms of water soluble property. Authors should also include information on the LogP. For example, the LogP of sinapinic acid is 1.52 while sodium butyrate has 0.92 indicating that the former is more lipophilic than the latter. This physicochemical details support the author’s statement that sinapinic acid has difficulty of water solubility than sodium butyrate.

Response:
- In a revised manuscript, we have included information on the log \( P \) as “Indeed, sinapinic acid has a partition coefficient (log \( P \)) value (log \( P = 1.52 \); ChemAxon) greater than that of sodium butyrate (log \( P = 0.92 \); ChemAxon), indicating its difficulty of water solubility than sodium butyrate.” (page 14, lines 11-14).

- Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached)

None

1. Is the question posed by the authors well defined?

Yes.

2. Are the methods appropriate and well described?

Yes.

3. Are the data sound?

Yes.

4. Does the manuscript adhere to the relevant standards for reporting and data deposition?

5. Are the discussion and conclusions well balanced and adequately supported by the data?

6. Are limitations of the work clearly stated?

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished?

The authors should include in the manuscript the pharmacophore giving rise to
HDAC inhibitory activity. The following sentence was obtained from a recent article by Wu et al. The authors can paraphrase the following sentence in their article.

“HDACi can be categorized into four subtypes based on their chemical structures: (1) short chain fatty acid; (2) hydroxamic acid; (3) benzamides; and (4) cyclic peptides.”


Response:
- In a revised manuscript, we have included the sentences “In general, HDAC inhibitors that act on zinc-dependent HDAC isozymes have three structural characteristics: a zinc-binding moiety, an opposite capping group, and a straight chain alkyl, vinyl or aryl linker connecting the zinc-binding moiety and the capping group [9]. Based on their chemical structures, HDAC inhibitors can be classified into four subtypes: (1) short chain fatty acid; (2) hydroxamic acids; (3) benzamides; and (4) cyclic peptides [13]” (page 3, lines 11-16).
- Note that Ref. [13] was inserted (page 18, lines 46-48).

8. Do the title and abstract accurately convey what has been found?
Yes

9. Is the writing acceptable?
There are some grammatical errors present in the manuscript. The authors should have their manuscripts thoroughly proofread by a Native English language speaker. Examples of such grammatical errors are below.

Page 13, Lines 19-21, Discussion:

“While many poor patients in these countries struggle to save their life with the traditional use of medicinal plants, most of the plant active ingredients remain to be investigated.” Should be changed to: “While many poor patients in these countries still struggle to save their lives with the use of traditional medicinal plants where most of the plant’s active ingredients remains to be investigated.”

Response: We have changed the sentence as suggested (page 14, lines 5-7).

Page 13, Lines 21-22, Discussion:

“To our knowledge, this is the first time that sinapinic acid, a derivative of cinnamic acids, is identified as HDAC inhibitor.” Should be changed to: “To our knowledge, this is the first time that sinapinic acid, a derivative of cinnamic acids, is identified as an HDAC inhibitor.”

Response: We have changed the sentence as suggested (page 14, lines 7-8).
Page 14, Line 1, Discussion:

“it has been reported that other two members” Should be changed to: “it has been reported that two other members”
Response: We have changed the words as suggested (page 14, line 18).

Page 14, Line 4, Discussion:

“investigation on a role” Should be changed to: “investigation on the role”
Response: We have changed the words as suggested (page 14, line 21).

Page 15, Line 12, Discussion:

“for cancer treatment in an alternative medicine” Should be changed to: “as an alternative medicine for cancer treatment”
Response: We have changed the words as suggested (page 16, line 5).

Page 16, Line 16, Conclusions:

“in vitro” should be changed to italic form.
Response: We have changed the word as suggested (page 16, line 9).

Page 16, Line 21-22, Conclusions:

“for cancer treatment in an alternative medicine” Should be changed to: “as an alternative medicine for cancer treatment”
Response: We have changed the words as suggested (page 16, lines 14-15).

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being Published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.
**Reviewer 2**

**Reviewer's report**

**Title:** Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack.: Sinapinic acid acts as HDAC inhibitor

**Version:** 2  **Date:** 24 June 2013

**Reviewer:** Raghu Sinha

**Reviewer's report:**

The manuscript by Senawong T et al., describes ethanolic and phenolic extractions of a Thai plant *H. formicarum* Jack, more specifically identification of sinapinic acid and its HDAC inhibitory activity in HeLa cells. This being an interesting finding on use of plant derived products for protection against cancer is worth publishing. Here are comments and suggestions to improve the manuscript.

1. Abstract, line 20: mention ethanolic extract as well in this sentence.
   **Response:**
   - In a revised manuscript, we have mentioned ethanolic extract in the sentence (page 2, line 21).

2. Background, page 3, lines 6-9: authors indicate that ethanolic crude extract from *H. formicarum* Jack. possesses HDAC inhibitory activity in vitro (unpublished by first author) and yet they are describing data on ethanolic extract in the current manuscript, this needs to be rectified.
   **Response:**
   - We have changed the sentence to “The screening for histone deacetylase (HDAC) inhibitors from Thai medicinal plants revealed that ethanolic crude extract from the rhizome of *H. formicarum* Jack. possessed HDAC inhibitory activity in vitro [8] (page 3, lines 6-9).
   - Note that Ref. [8] was inserted (page 18, lines 26-30).

3. Page 9, Line 19 and page 21, line 8: what is the function of using Alexa Fluor 488 Annexin V-FITC label? Typically, Annexin V-FITC is sufficient to capture early apoptosis in cells.
   **Response:**
   - It was a typing error. We have changed the words “Alexa Fluor 488 Annexin V-FITC” to “Alexa Fluor 488 Annexin V” (page 10, line 2, and page 22, line 11).

4. Figure 2. It would be informative to include the internal standard for sinapinic acid in the HPLC trace. What was % yield of sinapinic acid in this extraction?
Response:
- In a revised manuscript, we have provided a new version of Figure 2 as suggested. Note that the legend for Figure 2 has also been changed (page 21, lines 2-8).
- We have added two sentences “The yield of phenolic-rich extract from 10 g of *H. formicarum* Jack. rhizome was 67.5 mg. The amount of sinapinic acid was 3.4 µg/mg of phenolic-rich extract.” in the result section (page 11, line 19-20).

5. Figure 4. What amount of sinapinic acid was obtained in phenolic extraction of 10 g of *H. formicarum* Jack.? What is the basis of choosing the concentrations for sinapinic acid for HDAC inhibition? Perhaps authors should calculate how much of phenolic extract (200µg/50µl; as shown in Figure 1) is sinapinic acid and use that dose to see if it has effective HDAC inhibitory activity.

Response:
- The amount of sinapinic acid obtained in phenolic extraction of 10 g of *H. formicarum* Jack. was 229.5 µg (3.4 x 67.5 = 229.5 µg).
- We used the same amount (in term of mg/ml) of sinapinic acid and sodium butyrate in order to compare their HDAC inhibitory activity in mammalian cells (Figure 4B). For the *in vitro* assay, we have varied the concentration of sinapinic acid in order to specify the IC\(_{50}\) value.
- The amount of sinapinic acid in 50 µL reaction of the *in vitro* assay was 0.68 µg which can be calculated to be a dose of 0.06 mM. With this dose, we found that sinapinic acid could inhibit enzyme activity only 10% *in vitro*, indicating that other active ingredients in the extract capable of inhibiting HDAC activity remains to be explored.

6. Figure 5. How was a dose of 0.7 mg/ml chosen for ethanolic and phenolic extracts for determining apoptosis in HeLa cells? The methods section indicates that several concentrations were used for treatment of cells for 6 hours, perhaps a table showing percent of apoptosis in a dose-dependent manner would be informative to provide in a table format. Why was NaB not included in this assay?

Response:
- The concentrations of 0.7 mg/ml and 1.4 mg/ml of both extracts were chosen according to their concentration used for HDAC inhibition assay in HeLa cells.
- The data were displayed in a table format as suggested (Table 2, page 24).
- We have included a result on NaB in a revised manuscript (Table 2, page 24).

7. Authors have treated the HeLa cells for 6 hr to examine the HDAC activity of extracts and sinapinic acid and from the apoptosis assay it is clear that 30-82.5% cells are undergoing cell death with these fractions--so how does one rule out that the inhibition in HDAC activity is not due to cells dying after 6 hr treatment. This point needs to be addressed in the discussion.

Response:
- We have used Camptothecin with no HDAC inhibitory activity as a positive control for apoptosis assay in order to rule out that the inhibition in HDAC activity is not due to cells dying after 6 hr treatment. Camptothecin-treated cells showed no hyperacetylation of histone H4 (Data not shown).

**Level of interest:** An article of outstanding merit and interest in its field
Quality of written English: Needs some language corrections before being Published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests.

Reviewer 3

Reviewer's report

Title: Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack.: Sinapinic acid acts as HDAC inhibitor

Version: 2 Date: 3 July 2013

Reviewer: Leah Cosgrove

Reviewer's report:

Review of Manuscript titled : Histone deacetylase (HDAC) inhibitory and Antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack.: Sinapinic acid acts as HDAC inhibitor
http://www.biomedcentral.com/imedia/2524370479290375_article.pdf

1. Is the question posed by the authors well defined?

The main premise that the authors in this manuscript propose is that the traditional medicinal herb (rhizome of Hydnophytum formicarum Jack) has anti cancer properties due to its histone deacetylase (HDAC) inhibitory activity. They isolated ethanolic and phenolic extracts from this herb and showed by HPLC that the main compound responsible for this activity is sinapinic acid. They then compared the extracts derived from this herb and sinapinic acid with butyrate a well known HDAC inhibitor with anti-proliferative activity on 5 cancer cell lines. They showed a differential response to inhibition of proliferation or induction of apoptosis was cell line dependent and did not appear to correlate with HDAC activity.

2. Are the methods appropriate and well described?
The methodology used by these authors is appropriate for this type of study.

3. Are the data sound?

The data appears robust but there are some specific issues that are not clear regarding the extracts. Did they compare DMSO, ethanol, methanol, ethyl acetate, and hexane to see the effects by the carrier alone or at different concentrations, as these solvents will effect viability of cells? As in the anti-proliferative assay they appeared to only compare with 0.05% DMSO or H2O alone. Also how did they determine the residual solvent concentration for their fractions so they could compare the effect of carrier solvent alone?

Response:
- Only ethanolic and phenolic-rich extracts were used for antiproliferation assay. Both extracts were dissolved with DMSO, therefore, DMSO was used as a solvent control. H2O was used to dissolve NaB, therefore, it was used as a solvent control for NaB treatment. The concentration of solvent used did not affect the viability of the cell lines tested.

The other issue is with the HDAC inhibition. The authors show gel photos but how did they quantitate this? It would have been better to scan the actual gels and compare with total histone. Also figure 4 shows a representative gel, how many biological replicates were done? The other issue with figure 4B it is difficult to draw conclusions to how histones are being effected and to also what cell line they are looking at. Are the results the same for the other 4 cell lines?

Response:
- We did not quantitate intensity of the protein bands because the increased intensity of acetylated histone H4 molecules was clearly demonstrated comparing to that of control treatment.
- The data shown are representative of two independent experiments performed in duplicate.
- In Figure 4B, the intensity of triacetylated histone H4, at the highest concentration of sinapinic acid tested (0.5 mg/ml), was clearly greater than that of the control treatment.
- We have done HDAC inhibition only in HeLa cells because of its high expression level of HDAC enzymes. We have not done this experiment in the other 4 cell lines.

Identification of one of the active components of phenolic-rich extract of *H. formicarum* Jack. rhizome was determined to be sinapinic acid based on identical retention time and spectra of known phenolic standards under the same chromatographic conditions. Are the authors sure that no other components could run at the same retention time or spectra?

Response:
- In a revised manuscript, we have provided a new version of Figure 2. The confirmation of peak was obtained by the addition of sinapinic acid standard into the sample for HPLC analysis (Figure 2C).

4. Does the manuscript adhere to the relevant standards for reporting and data deposition? Yes, it does.
5. Are the discussion and conclusions well balanced and adequately supported by the data? Yes.

6. Are limitations of the work clearly stated? No, not really. With the different extracts how did the authors account for how much phenolic activity was being derived and was this reproducible on different extractions? It is unclear if they did the extraction only once. Also there is the issue regarding the gels as discussed in question 2 in that the gels were not scanned so no statistics could be done to see how reproducible their findings were.
   **Response:**
   - With the different extracts, we have determined the phenolic acid profile by HPLC analysis to make sure that extraction procedure is reproducible.

7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? Yes

8. Do the title and abstract accurately convey what has been found? Yes

9. Is the writing acceptable? Yes, I have noted grammatical errors and they are minor.

   • Discretionary Revisions (which are recommendations for improvement but which the author can choose to ignore): Nil

   • Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

   • Page 2, Line 15 & 16: effective should be effectively
     **Response:** We have changed the words as suggested (page 2, lines 15&16).

   • Page 12, line 6: “acid inhibited HDAC activity not only in vitro but also in the cells” Cells are in vitro. This should be corrected.
     **Response:** We think that determination of an enzyme activity in vitro (96-well plate) is different from that in a cellular context.

Page 14: Line 21,22 The authors say “Based on our findings that sinapinic acid possesses antiproliferative activity more effective than a well-known HDAC inhibitor sodium butyrate”. In their figure 1 this is not the case for all of the 5 cells they examined. For Hela and HT29 cells this is true but not for HCT116 and Jurkat cell lines were they see the reverse. This should be corrected.
   **Response:** We have added the words “..against HeLa and HT29 cells..” in the sentence (page 15, line 15).

   • Major Compulsory Revisions (which the author must respond to before a decision on publication can be reached) Nil
**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

'I declare that I have no competing interests'

With the submission of this revised manuscript I would like to undertake that the above mentioned manuscript has not been published elsewhere, accepted for publication elsewhere or under editorial review for publication elsewhere, and that none of authors have any conflict of interest.

Thank you for your consideration of this manuscript. Please address all correspondence concerning this manuscript to me and feel free to correspond with me by e-mail.

Sincerely,

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