Author's response to reviews

Title: 3,4-Dihydroxytoluene, a Metabolite of Rutin, Inhibits Inflammatory Responses in Lipopolysaccharide-Activated Macrophages by Reducing the Activation of NF-kappaB Signaling

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Author's response to reviews: see over
Dear editor:

We very appreciate your kind reconsideration for publication. In the mention of reviewers' comments, we carefully checked these issues and responded point by point as followed. Thank you for your great help.

**Reviewer 1**

The authors have modified to a certain extent their manuscript taken into account the different suggestions of the reviewers. However, the manuscript still contains a myriad of errors and should be corrected by a native English speaker prior to be acceptable for publication. Increased attention should be paid to the abstract and the conclusion.

**Response:**

Thank the reviewer’s comment. We totally agree your suggestion. Please check the correction according to your kind help in following response.

Q4: DHT has an inhibitory effect on IkB phosphorylation. What about the effect of DHT alone on this phosphorylation?

The results of the study reported in J Agric Food Chem. 2010, 58, 10445-10451 concern the effect of osthole from the seeds of Cnidium monnieri and not DHT. Neither this study nor the MTT assay, allows the authors to tell that DHT alone has no effect on the phosphorylation of IkB.

**Response:**

Thank the reviewer’s comment. We are very sorry for our poor description leads to your misunderstanding. We also realize the consideration of the reviewer. In logically, to evaluate the effect of DHT alone on NF-kB as well as IkB can clarify the influence such as cytotoxicity or side effects of DHT on cell. But such extract like DHT or else\(^1\,\,^2\) usually had low effects on cell. Therefore, DHT alone will not alter NF-kB signaling except under the environmental NF-kB induced stress like LPS. However, to eliminate the reviewer’s misdoubting, we perform the western blot analysis for this issue. In the following figure, the result indicates that DHT only has not effects on IkB and NF-kB.
Below a no exhaustive list of sentences that should be corrected.

In the Methods section

-ELISA was performed according to the previous study[30]. Briefly, cells (2 × 10^6 in 2 mL medium) were seeded in 60 mm dishes and measured 6h after treatment.

**Response:**

Thank the reviewer’s comment. We have corrected it from “ELISA was performed according to the previous study[30]. Briefly, cells (2 × 10^6 in 2 mL medium) were seeded in 60 mm dishes and measured 6h after treatment.” to “ELISA was performed according to the previous study [30]. Briefly, 2 × 10^6 in 2 mL medium were seeded in 60 mm dishes for treatment. Six hours after treatment, 50 µL of biotinylated antibody…” (page 7, line 6-8)

**Page 7, line 6-8**

ELISA was performed according to the previous study[30]. Briefly, 2 × 10^6 in 2 mL medium were seeded in 60 mm dishes for treatment. Six hours after treatment, 50 µL of biotinylated antibody and 50 µL of supernatant were added to a stripwell plate precoated with antimouse IL-1β, IL-6, and TNF-α antibodies and incubated at room temperature…
- NO measurement Cell was cultured in 24-well plate (Nunc, USA) with $1 \times 10^5$ confluence at one day before experiment.

**Response:**

Thank the reviewer’s comment. We have corrected it from “NO measurement Cell was cultured in 24-well plate (Nunc, USA) with $1 \times 10^5$ confluence at one day before experiment.” to “$1 \times 10^5$ cells was seeded into 24-well plate (Nunc, USA) at the day before treatment. Twenty-four hours after treatment, the supernatant was collected and NO measurement was performed by Griess Reagent.” (page 7, line 19-21)

**Page 7, line 19-21**

$1 \times 10^5$ cells was seeded into 24-well plate (Nunc, USA) at the day before treatment. Twenty-four hours after treatment, the supernatant was collected and NO measurement was performed by Griess Reagent.

In the Results and discussion section

- To test the inhibitory effect of rutin metabolites on inflammation, we first measured the nitric oxide (NO) production, an inflammation marker, on LPS-simulated RAW 264.7 with rutin or its metabolites including DHT, DHPAA, HPAA and HVA. The result indicated DHT had a powerful inhibitory effect at 10mM on NO product when compared with other metabolites.

- “To test the inhibitory effect of rutin metabolites on inflammation, we first measured the nitric oxide (NO) production, an inflammation marker, on LPS-simulated RAW 264.7 with rutin or its metabolites including DHT, DHPAA, HPAA and HVA.......and a little further “We further tested whether DHT can reduce nitric oxide (NO) production after LPS treatment in RAW 264.7 cell.” As the effect of DHT on NO production has been already measured, the second sentence must be written differently.

**Response:**

We thank the reviewer’s careful checking. We agree and re-write in different way according to the result. The sentence “We further tested whether DHT can reduce nitric oxide (NO) production after LPS treatment in RAW 264.7 cell.” had been changed to
“To evaluate the effect of DHT on NO production precisely, we performed NO measurement for RAW264.7 cell with different dose.” (page 9, line 3-5).

**Page 9, line 3-5**

To evaluate the effect of DHT on NO production precisely, we performed NO measurement for RAW264.7 cell with different dose. The result indicated that DHT can significantly…

- “The result indicated that DHT can significantly reduce NO generation after LPS treatment in a dose-dependent manner.” This result has been already mentioned. The dose dependent manner is important however, the authors should be more precise and write for example that this experiment shows that a significant inhibition is observed with 2.5mM of DHT.

**Response:**

We thank the reviewer’s suggestion. Therefore, we descript this phenomena precisely. Since the significant reduction of NO is occurred after 1.25µM DHT treatment and goes down gradually, we modify the sentence “The result indicated that DHT can significantly reduce NO generation after LPS treatment in a dose-dependent manner (Figure 2(a)).” To “The result indicated that DHT can significantly reduce NO generation after LPS treatment in a dose-dependent manner from 1.25 to 10µM (Figure 2(a)).”. (page 9, line 5-7)

**Page 9, line 5-7**

The result indicated that DHT can significantly reduce NO generation after LPS treatment in a dose-dependent manner from 1.25 to 10µM (Figure 2(a)).

The sentence : “Among these cytokines, DHT can significantly reduce the mRNA and protein expression levels of TNF-# (Figure 2(b)) at 5 and 10 mM while DHT only had relative low or limited effects on IL-6 and IL-1b mRNA and protein expression level (Figure 2(c) and (d)).” Can be replaced by: ”DHT at 5 and 10 mM can significantly reduce the mRNA and protein expression levels of TNF-# (Figure 2(b)). No significant effect was observed on IL-6 and IL-1b mRNA and protein expression.”
Response:

Yes. We agree the reviewer’s suggestion in order not to make the reader confused. Thank you. (page 9, line 8-11)

Page 9, line 8-11

DHT at 5 and 10 mM can significantly reduce the mRNA and protein expression levels of TNF-# (Figure 2(b)). No significant effect was observed on IL-6 and IL-1b mRNA and protein expression (Figure 2(c) and (d)).

The sentence “Since NO can be catalyzed by inducible NO synthase (iNOS) when stimulating, we further investigated the effect of DHT on iNOS expression. The result showed that DHT can significant reduce not only iNOS expression but also cyclooxygenase-2 (COX-2) (Figure 3) expression in RAW 264.6 cell after LPS treatment in a dose-dependent manner.” Can be replaced by: Since NO production can be catalyzed by inducible NO synthase (iNOS), we further....reduces iNOS expression in a dose dependent manner. Similar results were obtained for cyclooxygenase-2 (COX-2) (figure 3)

Response:

Yes. We agree the reviewer’s suggestion in order not to make the reader confused. Thank you. (page 9, line 14-16)

Page 9, line 14-16

Since NO production can be catalyzed by inducible NO synthase (iNOS), we further....reduces iNOS expression in a dose dependent manner. Similar results were obtained for cyclooxygenase-2 (COX-2) (figure 3).

This sentence “Since MAPK signaling was one of well-known pathways that LPS-activated through TLR4 followed by numerous responses including pro-inflammatory cytokines production,” must be reformulated.

Response:

We thank the reviewer’s comment and apologize our poor description. We have modified it from “Since MAPK signaling was one of well-known pathways that
LPS-activated through TLR4 followed by numerous responses including pro-inflammatory cytokines production,” to “MAPK signaling was one of the most important LPS-activated pathways, therefore,…”. (page 9, line 18-19)

**Page 9, line 18-19**

MAPK signaling was one of the most important LPS-activated pathways, therefore, we tested whether DHT can modulate LPS-stimulated MAPK related signaling.

This sentence “According to this signaling, in this study, we found that the phosphorylation level of IκB-a was significantly reduced in LPS-stimulating RAW 264.7 cell with DHT treatment (Figure 5(a)), subsequence the nuclear NF-κB (p65) was reduced whereas the cytoplasmic NF-κB was accumulated. (Figure 5(b)).” is too long and unclear.

**Response:**

Yes. We agree the reviewer’s suggestion. We modified the statement into separated sentences in order to clearly illustrate. The original sentence “According to this signaling, in this study, we found that the phosphorylation level of IκB-a was significantly reduced in LPS-stimulating RAW 264.7 cell with DHT treatment (Figure 5(a)), subsequence the nuclear NF-κB (p65) was reduced whereas the cytoplasmic NF-κB was accumulated. (Figure 5(b)).” is changed to “According to this signaling, we found DHT can significantly reduce the phosphorylation level of IκB-a in LPS-stimulating RAW 264.7 cell (Figure 5(a)). This leaded to accumulation of NF-κB (p65) in the cytoplasm and nuclear NF-κB (p65) reduction (Figure 5(b)).” (page 10, line 4-7)

**Page 10, line 4-7**

According to this signaling, we found DHT can significantly reduce the phosphorylation level of IκB-a in LPS-stimulating RAW 264.7 cell (Figure 5(a)). This leaded to accumulation of NF-κB (p65) in the cytoplasm and nuclear NF-κB (p65) reduction (Figure 5(b)).

The sentences below should be reformulated.
On the other hand, increasing the expression of TNF-α and other cytokines has also demonstrated in metabolic diseases.

**Response:**

We have reformulated it to “Also, TNF-α was found in some metabolic diseases. [43, 44]”. (page 10, line 17-18)

DHT had potential in iNOS reduction through its upstream signaling and exhibited its therapeutic role. Furthermore, we found COX-2 also decreased in DHT treated LPS-stimulated macrophages.

**Response:**

We have reformulated it to “In this study, DHT can potentially inhibit iNOS as well as COX-2 expression through its upstream signaling in LPS-stimulated macrophages.”. (page 11, line 2-3)

Among involved transcription factors, NF-κB, which is a primary transcription factor and regulates various genes, is critical in the inflammation.

**Response:**

We have reformulated it to “Among transcription factors, NF-κB is a significant inflammation-related one for downstream gene activation including cytokines, chemokines, adhesion molecules, and acute phase proteins[47].”. (page 11, line 13-15)

Under basal conditions, NF-κB is inactive and prevented from binding with DNA and executing nuclear translocation because of the close association of the cytoplasm with inhibitory proteins.

**Response:**

We have reformulated it to “Under basal conditions, NF-κB is inactive by associated with inhibitory proteins in the cytoplasm to prevent DNA binding for transcription activity.”. (page 11, line 18-20)
- This phenomenon can be suggested that the following reduced iNOS

**Response:**

We have reformulated it to “It is suggested that the reduction of iNOS and COX-2 may be the consequence of NF-κB inactivation.”. (page 11, line 22-23)

- According to our finding, DHT may affect more upstream molecular in

**Response:**

IκB-NF-κB signaling.

We have reformulated it to “Therefore, DHT may affect the upstream molecular of IκB-NF-κB signaling, since the phosphorylation of IκB was attenuated by DHT.”. (page 11, line 25-27)

- This can be explained that DHT reduced TNF-α expression majorly through

**Response:**

PI3K/AKT followed by NF-κB activation

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We have reformulated it to “On the other hand, in this study, we found DHT had no influence on MAPK pathway (Figure 4) suggested DHT reduced TNF-α expression majorly through PI3K/AKT followed by NF-κB activation.”. (page 11, line 29 to page 12, line 2)

**Reviewer 2**

This manuscript describes the anti-inflammatory activity of rutin derivates from chinese Saussurea involucrate. The authors tested these effects with several in vitro tests. They showed that the component of rutin responsible of its anti-inflammatory activity.

Although the study is well conducted, this manuscript needs still a complete revision of English language by either a native English mother tong person or by a professional translator.
Response:
We thank the reviewer's suggestion and apologize for our careless. According to the first reviewer's suggestion, we not only response point by point for each comment but also check the language issue. We very appreciate your kind help for our publication.

Response to Editor
We very thank your great help and consideration for publication. We hope our significant finding can give this information to researchers in this field to development compounds for inflammation control. Following statement we wish to provide to you for reference including:

1. We are really sorry for the poor statement in language issue although we had been looking for editing (please see the follow proof).
2. We also really thank the first reviewer's careful correlation and we totally accepted his suggestion.