

Author's response to reviews

Title: Centipede Grass exerts anti-adipogenic activity through inhibition of C/EBPbeta, C/EBPalpha, and PPARgamma expression and the AKT signaling pathway in 3T3-L1 adipocytes

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Author's response to reviews: see over

Dear Editor

BMC complementary and alternative medicine

Ms. No. : 1162177652731212

Title: "Centipede Grass exerts anti-adipogenic activity through inhibition of the AKT signaling pathway"

Dear Editor,

Thank you for sending us the reviewer's comments and your kind letter encouraging us to submit our revision. We would like to thank the reviewers for their efforts and time, particularly, for useful comments to make our paper better. We believe that our manuscript has been substantially improved by this revision.

Thank you very much for your time and consideration.

Your sincerely,

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Reviewer's report 1:

Major Compulsory Revisions

1. In order to exclude that the inhibitory effect of centipede grass extract on adipogenesis might be caused by its cytotoxicity, the authors first assessed that cytotoxic effects of CG in 3T3-L1 cells with MTT and LDH assay and concluded that CG extract did not cause cytotoxicity in both the undifferentiated and differentiated 3T3-L1 cells. However, there is a flaw in the experiment about cell viability. The cells were treated with CG extract, meanwhile the cells were induced with DMII or not. This finally resulted in different ratio of differentiated adipocytes and preadipocytes among the four groups when the MTT and LDH assay were performed. Owing to the possible different activity and

amount of the related enzymes between preadipocytes and adipocytes, the viability of cells could not be compared based on the reduced MTT and release of LDH. Additional experiments are needed to confirm the conclusion just in preadipocytes without induction or differentiated adipocytes.

Answer: We would like to thank the reviewer's comments in preparation of the revised manuscript. To assess whether CG inhibited the cell viability of 3T3-L1 cells, cells were treated with 0-300 ug/ml CG during differentiation and the cell viability was determined by using the MTT assay. Cell viability was decreased by 300 ug/ml CG, while not affected by 10, 100, and 200 ug/ml CG. Therefore, concentration range of 10-200 ug/ml was appropriate for treatment of cells in the subsequent experiments. 3T3-L1 preadipocytes were also treated at various concentrations (10, 100, 200, and 300 ug/ml) for 6 days without differentiation, and after treatment cell viability was not affected by 200 ug/ml CG treatments. In parallel to the results observed from cell viability, LDH activity in the 3T3-L1 cells was also measured to evaluate the influence of cell cytotoxicity on 3T3-L1 cells and CG did not show any cytotoxicity at up 200 ug/ml. It is well known that the differentiation of 3T3-L1 preadipocytes employ the adipogenic cocktail to treat postconfluent cultures of 3T3-L1 cells with a combination of insulin, 3-isobutyl-1-methylxanthine, dexamethasone, and indomethacin. During this differentiation process, the first stage of 3T3-L1 is growth arrest, which is achieved by contact inhibition at post confluence. Immediately after induction by the hormonal cocktail, growth-arrested postconfluent 3T3-L1 preadipocytes re-enter the cell cycle (called mitotic clonal expansion) and start the adipocyte differentiation program. Growth arrest is the first step for 3T3-L1 adipocyte differentiation, and cell cycle arrest of 3T3-L1 cells is associated with an increased risk and sensitive to external stimuli such as, cytotoxicity materials, nutrients, and chemical toxicity. In the present study, the 3T3-L1 cells were treated with different concentrations of CG (0, 10, or 100 µg/ml) with a DMII mixture for MTT and LDH assay. The text is revised in results section as 3T3-L1 preadipocytes were also treated at various concentrations (100, 200, and 300 ug/ml) for 6 days without differentiation, and CG did not show any cytotoxicity at up 200 ug/ml.

2. In the conclusion, the authors concluded affirmatively that CG inhibited adipocyte differentiation by attenuating the expressions of C/EBP α , C/EBP β and PPAR α mediated by suppressing the phosphorylations of AKT and GSK3 β . However, no direct evidence in this experiment supports above conclusion, although some previous reports demonstrated the link between AKT signaling and adipogenic transcription factors. It is not excluded other mechanism involved in the inhibitory effect of CG on adipogenic transcription factors. The author should interpret the data more stringently.

Answer: We appreciate reviewer comments. Akt is known to play a major role in glucose regulation and lipid metabolism in insulin signaling. A downstream component of insulin signaling, the serine/threonine kinase Akt plays a central role in the metabolic actions of insulin and is a marker for

insulin signaling. Overexpression of constitutively active Akt in 3T3-L1 adipocytes increased glucose uptake and adipocyte differentiation (Xu and Liao, 2004). A study of Akt-knockout mice showed that Akt is essential for adipocyte differentiation and for the induction of PPAR γ expression (Peng et al., 2003). Akt phosphorylates and regulates a large number of substrates that are involved in a diverse array of biological processes, many of which could contribute to the role of Akt in driving adipocyte differentiation. GSK3 β is a critical downstream signaling protein for the phosphoinositide 3-kinase (PI3K)/Akt pathway. Insulin signaling activates Akt through PI3K and induces serine/threonine phosphorylation of downstream targets such as GSK3 β . GSK3 β is a critically important protein kinase in adipocyte differentiation because GSK3 β phosphorylates a number of substrates, such as the transcription factor β -catenin, C/EBP β , and C/EBP α , and promotes glycogen synthesis, which are all adipogenesis-related genes. C/EBP β is expressed early in the adipocyte differentiation program, which initiates mitotic clonal expansion (MCE) (Tang et al., 2003). In response to an adipogenic induction, C/EBP β and δ are first activated to promote PPAR γ and C/EBP α expression (MacDougald et al., 1995). The PPAR γ transcription factor is a master regulator of adipocyte differentiation, and similar to Akt, its activation is both necessary and sufficient for adipocyte differentiation (Lehrke and Lazar, 2005). The activation of the C/EBP α and PPAR γ is involved in terminal differentiation by their subsequent transactivation of adipocyte-specific genes (Farmer, 2006). During adipogenesis, C/EBP α and PPAR γ expression activate the expression of lipid-metabolizing enzymes, which include aP2, LPL, and FAS. Text is revised as centipede grass exerts anti-adipogenic activity through inhibition of C/EBP β , C/EBP α , and PPAR γ expression and the AKT signaling pathway in 3T3-L1 adipocytes in title.

Minor Essential Revisions

1. In methods, the differentiation/induction medium (DMII) contains 100 mM insulin. The concentration of insulin was very high, contrasting to that of inducer commonly used in adipocyte differentiation. Is there a mistake in the writing? It is better to explain the provenance of this protocol.

Answer: We appreciate reviewer comments. Text is corrected as 167 nM Insulin.

2. The sources of antibodies used in Western blot should be mentioned.

Answer: Text is revised in material and method section as directed.

3. In Fig.3 A and B, the categorical label of each bar is obscure.

Answer: In the present study, 3T3-L1 cells were treated with various concentrations of CG (0, 10, or 100 μ g/ml) during differentiation, and the phosphorylation level of Akt was examined on day 4 or 6. The extent of phosphorylation was calculated by determining the amount of phosphorylated Akt relative to total Akt levels (DMII or DMII + 100 μ g/ml CG). Text is revised in Legend and

Figure section.

4. The labels of Y-axes are missing in Fig.2 C.

Answer: Text is corrected in figure section as directed.

Discretionary Revisions

It is better to move the data and interpretation of TG content assay to first part of the result, combined with the data of Oil red staining, both of which describe effect of CG on adipogenesis. So, Fig.3 C should be changed to Fig. 1 D.

Answer: Text is revised as directed.

Reviewer's report 2:

The authors described that Centipede grass extract has antiadipogenic activity by inhibiting the expression of regulators of adipogenesis and Akt signaling pathway.

The study design is sound.

Minor comments

1. The authors should discuss whether the anti-adipogenic effect of CG is depend on C-glycosyl flavones or phenolic constituents containing in CG.

Answer: We appreciate reviewer comments. In the present study, we examined the effects of CG extracts on the differentiation of preadipocytes to adipocytes, 3T3-L1 preadipocytes were treated with various concentrations of CG with a DMII mixture for 6 days. We found that CG extracts suppressed lipid droplet formation and adipocyte differentiation in 3T3-L1 cells in a dose-dependent manner. Treatment of the 3T3-L1 adipocytes with CG inhibited the expression of C/EBP α and PPAR γ , the central transcriptional regulators of adipogenesis. Moreover, the expression of genes involved in lipid metabolism, aP2 were significantly inhibited following the CG treatment. The CG treatment down-regulated the phosphorylation levels of Akt and GSK3 β . Several studies showed that C-glycosyl flavonoid and phenolic constituents affect the hypoglycemia activity and hypolipidaemic potentials in vitro or animal studies. We agree with reviewer comment a good point. Recently, we found to contain the C-glycosyl flavonoid and phenolic constituents in CG extract, more further studies are needed to establish the effects of each constituent of CG extracts in anti-adipogenic/anti-obesity/diabetes study.

2. In Figure legends The authors describe that 3T3-L1 preadipocytes was (were) incubated with DMII media for 4 or 6 days and treated with different concentration of CG every day. Did the medium is changed every day? Then the authors should described on the medium using for experiment on cell viability in Methods.

Answer: Text is revised in legend section as directed.

Reviewer's report 3:

Major Compulsory Revisions

1. In title and Conclusions: From my point of view, suggesting that the word "AKT signaling pathway" is a very strong message and should be softened. Moreover, authors should provide more relevant experimental data.

Answer: We appreciate reviewer comments. Text title is revised as centipede grass exerts anti-adipogenic activity through inhibition of C/EBP β , C/EBP α , and PPAR γ expression and the AKT signaling pathway in 3T3-L1 adipocytes. Akt is known to play a major role in glucose regulation and lipid metabolism in insulin signaling. A downstream component of insulin signaling, the serine/threonine kinase Akt plays a central role in the metabolic actions of insulin and is a marker for insulin signaling. Overexpression of constitutively active Akt in 3T3-L1 adipocytes increased glucose uptake and adipocyte differentiation (Xu and Liao, 2004). A study of Akt-knockout mice showed that Akt is essential for adipocyte differentiation and for the induction of PPAR γ expression (Peng et al., 2003). Akt phosphorylates and regulates a large number of substrates that are involved in a diverse array of biological processes, many of which could contribute to the role of Akt in driving adipocyte differentiation. GSK3 β is a critical downstream signaling protein for the phosphoinositide 3-kinase (PI3K)/Akt pathway. Insulin signaling activates Akt through PI3K and induces serine/threonine phosphorylation of downstream targets such as GSK3 β . GSK3 β is a critically important protein kinase in adipocyte differentiation because GSK3 β phosphorylates a number of substrates, such as the transcription factor β -catenin, C/EBP β , and C/EBP α , and promotes glycogen synthesis, which are all adipogenesis-related genes. C/EBP β is expressed early in the adipocyte differentiation program, which initiates mitotic clonal expansion (MCE) (Tang et al., 2003). In response to an adipogenic induction, C/EBP β and - δ are first activated to promote PPAR γ and C/EBP α expression (MacDougald et al., 1995). The PPAR γ transcription factor is a master regulator of adipocyte differentiation, and similar to Akt, its activation is both necessary and sufficient for adipocyte differentiation (Lehrke and Lazar, 2005). The activation of the C/EBP α and PPAR γ is involved in terminal differentiation by their subsequent transactivation of adipocyte-specific genes (Farmer, 2006). During adipogenesis, C/EBP α and PPAR γ expression activate the expression of lipid-metabolizing enzymes, which include aP2, LPL, and FAS.

Minor Essential Revisions

2. Page 4, lines 116-117: differentiation/induction medium (DMII), please confirm the abbreviation.

Answer: In the present study, to investigate the anti-adipogenic effects of CG on differentiation of preadipocytes into adipocytes, postconfluent 3T3-L1 preadipocytes were induced to differentiate

using a cocktail of DMII mixtures (3-isobutyl-1-methylxanthine, **indomethacin**, dexamethasone and insulin) added to DMEM containing 10% FBS, although classical adipogenesis-inducing medium contains DMI mixtures (3-isobutyl-1-methylxanthine, dexamethasone and insulin). We found that DMII mixtures (3-isobutyl-1-methylxanthine, indomethacin, dexamethasone and insulin) in DMEM medium induced easily to differentiate the 3T3-L1 cell than that of using a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine, and insulin (DMI).

3. If more compounds (such as pure compounds from Centipede Grass) were tested, the results should be better.

Answer: We would like to thank the reviewer's comment. It is important to identify the content of the Centipede grass extracts. Maysin, a flavone C-glucoside, luteolin 6-C- β -D-boivinopyranoside found in Centipede grass, has been identified as an antibiotic resistant to fall armyworm. However, maysin cannot be obtained by chemical synthesis, γ -irradiation is a method for increasing the concentration of maysin and maysin derivatives. Further studies are needed to establish the variability of the bioactive content in CG extracts.

4. In Figure 2C: the lack of Y axis.

Answer: We appreciate reviewer's comments. Text is corrected in figure section as directed.

5. In Figure 3A and 3B: the word "DMII DMII+100 ug/ml CG" (X axis) should be corrected.

Answer: Text is revised in Figure as directed.

6. Figure 3C should be combined to Figure 1C.

Answer: Text is revised as directed.

Reviewer's report 4:

1. Is the question posed by the authors well defined? yes. The study investigates the anti-obesity of Centipede grass GG and the molecular mechanisms in adipocyte, more particularly during its differentiation. The authors find that CG suppressed lipid droplet formation and adipocyte differentiation in 3T3-L1 cells in a dose-dependent manner, with a decrease of C/EBP β and PPAR α , and a down-regulation of the phosphorylation levels of Akt and GSK3 β .

2. Are the methods appropriate and well described? not at all. The content (flavonoid and other compounds) of the extracts tested is poorly described in terms of quantity of bioactive molecules. What is the percentage of flavonoids? What are the other molecules present in the extracts? What is the variability of the bioactive content?

Answer: We agree with reviewer's comments. It is important to identify the content of the Centipede grass extracts. Maysin, a flavone C-glucoside, luteolin 6-C- β -D-boivinopyranoside found in Centipede grass. Further studies are needed to establish the variability of the bioactive content in CG extracts.

3. Are the data sound?

Antiadipogenic activity by CG through the inhibition the expression of C/EBP β , C/EBP α , and PPAR γ and the Akt signaling pathway in 3T3-L1 adipocytes remains speculative. In fact, the bioavailability of bioactive flavonoids was not taken into account, and the dose appeared high even the absence of toxicity. If orally taken, it is known that flavonoids are poorly absorbed and extensively metabolized leading to conjugates with sulfates and glucuronides. Thus to get a right evaluation of CG extract, it would be relevant to test the circulating conjugates.

Answer: We would like to thank the reviewer's comments. In the present study, to investigate whether CG inhibited the cell viability, 3T3-L1 cells were treated with various concentrations of CG extracts during differentiation and the cell growth was determined by using the MTT assay. Cell viability was not affected by 10, 100, and 200 ug/ml CG, and those concentrations do not induce cell cytotoxicity. Therefore, concentration range of 10-200 ug/ml was appropriate for treatment of cells in the subsequent experiments. Treatment of the 3T3-L1 adipocytes with 100 ug/ml CG inhibited the expression of C/EBP β , C/EBP α and PPAR γ , the central transcriptional regulators of adipogenesis. Moreover, the expression of genes involved in lipid metabolism, aP2 were significantly inhibited following the 100 ug/ml CG treatment. The CG treatment down-regulated the phosphorylation levels of Akt and GSK3 β .

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

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

6. Are limitations of the work clearly stated? No, it is difficult to discriminate the role of the flavonoid structures reported in material and methods. To evaluate their role, the authors must test the same extract after removing flavonoids

Answer: We appreciate reviewer's comments. We would like to investigate the anti-adipogenic effect of the CG after removing flavonoids in further study.

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. Figure 1 part A and B must be removed, because of non significant results (the results in the text would be sufficient).

Answer: We would like to thank the reviewer's comments. In the present study, we determine whether CG inhibit the cell viability or induce cytotoxicity, the 3T3-L1 cells were treated with various concentration of CG extracts for 6 days. Following 4 or 6 days of incubation, cell viability/cytotoxicity was measured by the MTT/LDH assay. As shown in Figure 1, CG over a dose range from 10-100 ug/ml does not influence the metabolism of MTT assay by 3T3-L1 cells. This result indicates that none of this CG in the concentration range tested exhibit cell cytotoxicity, and moreover they do not influence the cell viability of 3T3-L1 cells. However, the higher concentration of CG than 300 ug/ml significantly induced the cell cytotoxicity, and this dose could not use the 3T3-L1 differentiation experiment since its concentration obviously inhibit the differentiation of 3T3-L1 preadipocyte into adipocyte. Therefore, concentration range of 10-200 ug/ml was appropriate for treatment of cells in the subsequent experiments and we have focused on concentration of 100 ug/ml CG in the study.