Peer Review File

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Review Comments

Major points

Comment 1: Fig. 1. Proliferation assay, It would have been better to make proliferation curves with a control an treated cells. In this case, the cells were seeded an treated immediately with emodin. we only see a decrease of the treated cells, there is no comparison with the control.

Reply 1: The results shown in Fig1 came from the cell treated with Emodin with 24h after seeding and culturing cells for 24h, 0μmol/l emodin corresponds to the DMSO group. We have modified Fig 1 (including figure legend) as well as our text (see **Materials and methods** (*Cell proliferation assay*) part, the modified part is in red color). Because B16F10 is adherent cells, the drug is usually added after the cell adherence. Adding drugs at the time of inoculation may affect the detection effect.
Changes in the text: Fig 1 (including figure legend) and **Materials and methods** (*Cell proliferation assay*) part------- The seeded cells were cultured for 24h.

Comment 2: Fig. 4A. What is called Control in fact is a positive control given by FBS 10%, but it would be interesting also to know how much the cells migrate without any stimulus. I guess this value is given by the ratio of panel A. This representation is not easy to understand, at least it should be clearly specified that the positive control is given by serum 10%.

Reply 2: In Fig 4A, the control group in the scratch test is similar to the emodin-treated groups using serum-free (0.1%DMSO) medium in order to avoid the effect of serum on migration. We have modified our text as advised (see **Materials and methods** (*Cell scratch test*) part, the modified part is in red color)
Changes in the text: **Materials and methods** (*Cell scratch test*) part, the modified part is in red color---------- Serum-free (0.1% DMSO) medium was simultaneously added to a well as the control.

Comment 3: Fig. 4B. something is missing in the legend of the ordinate. In the Mat and Meth section, line 92, you wrote that the cell density was 2x105/ml (150 µl) whereas the volume at the bottom was 800 µl. Usually it is 100 µl at the top and 600 µl at the bottom for that type of porous membrane. Therefore, the number of cells counted should be as reported, but maybe a 10-2 or 10-3 is missing in the legend to the ordinate.

Reply 3: in Fig 4B, the number of migration cells was counted in different fields of view. Five visual field were randomly selected for statistics under microscope (×100).

We have modified Fig 4B (legend to the ordinate) and text (see **Materials and methods** (*Transwell test*.) part, the modified part is in red color).
Changes in the text: Fig 4(legend to the ordinate in Fig 4b) and the text (**Materials and methods** (*Transwell test*.) part, the modified part is in red color---------- The number of migrating cells was counted in different fields of view under a microscope. Five visual fields were randomly selected for statistical analysis, and the differences between the groups were statistically analysed.)

Comment 4: Fig. 6. This western blotting shows that emodin 20μM inhibits caspase-3, whereas the 40 and 60 increased it. This is a biphasic effect or as pharmacologists would say an hormetic effect U –shaped. I wonder why the authors decided to use so high concentrations of the emodin. It could be useful to use a wider concentration range starting from 10-7 – 10-8 M and going up.
The densitometry together with the blot should also be showed with the number of the exps, etc.
The Conclusion in the Discussion that emodin increase the caspase -3 is wrong, since at 20 μM there is a decrease and not an increase of the caspase-3. Again, the densitometry would show that potentially interesting result much better.
The conclusion is that emodin induces apoptosis through the mitochondrial pathway, but there are no experimental data supporting this conclusion.
The data show inhibition of proliferation, induction of apoptosis, decrease of transmigration and wound healing by emodin and involvement of caspase-3 in melanoma cells. Nothing support the involvement of mitochondrial pathway.

Reply 4: According to the reference (PMID: 31234244), emodin would have a significant proliferation inhibitory effect on mouse embryonic fibroblast at the concentration of 16μg/ml. To avoid the toxic effects on normal cells, we abandoned the 80μM. We have modified Fig 6 (changed figure legend and added the densitometry part as advised) and our text (see **Results/ Discussion part**, the modified part is in red color).
Changes in the text: Fig 6 (changed figure legend and added the densitometry part as advised) and our text (see **Results part**, the modified part is in red color--------- In our study, caspase-3 was detected and analysed by western blotting, which indicated that the expression levels of caspase-3 had increased in cells treated with 40 μM and 60 μM emodin (Fig. 6). These results confirmed that emodin may promote B16F10 cell apoptosis. **Discussion part**, the modified part is in red color-------- Apoptosis is mainly triggered by four pathways: the mitochondrial pathway, death receptor-mediated pathway, endoplasmic reticulum pathway and caspase-independent pathway. Caspase-3 is a protein shared in the first two pathways. It is a key factor that executes apoptosis, making it an important protein in the process of apoptosis. The activation of the mitochondrial pathway or death receptor-mediated pathway can cause an increase in caspase-3 expression. Its expression level also reflects the extent of apoptosis. The TUNEL assay showed an increased number of fluorescently stained cells, suggesting that emodin promoted the apoptosis of the melanoma cells. Moreover, the results of the western blot analysis showed that the expression of

caspase-3 increased in the cells treated with 40 and 60 μM emodin, supporting the hypothesis that emodin may cause the induction of apoptosis through the mitochondrial pathway or death receptor-mediated pathway.)

Minor points

Comment 1: instead of the brute formula it would be more useful to show an image of the chemical formula of the emodin, also because there is a comparison with aloe emodin in the Discussion Section.

Reply 1: We took the advice and added the picture of chemical formula comparison between emodin and aloe-emodin (see Fig 7)
Changes in the text: Fig 7(Chemical structural formulas of emodin and aloe-emodin)

Comment 2: The English language should be revised. A number of typos here and there

Reply 2:We took the advice and revised English language through AJE（medical

writing service）