Author's response to reviews

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

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Author's response to reviews: see over
Author’s response to reviews

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

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Version: 2 Date: 20 March 2014

Author’s response to reviews: see over
Reviewer's report

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7
Version: 1 Date: 20 January 2014
Reviewer: Heonyong Park

Reviewer's report:
In this manuscript, authors extracted four different medicinal plants used as folk medicine in Nigeria and then analyzed if they had anti-tumor activity. The cancer cell line they used in this manuscript was breast cancer cell, MCF-7. Authors determined various effects of four different extracts of plants (Picralima nitida, Pyrenacantha staudtii, Jatropha gossypifolia L, and Jatropha curcas L); cell proliferation, apoptosis, cell detachment and integrin beta 1 expression, and MTS assay. Authors concluded that those extracts had anti-tumor activities by unknown mechanisms.

Major comments:

A weak point of this manuscript is that four extracts have similar (sometimes higher) toxicity over a normal breast cancer cell line, MCF-10A, comparing to toxic effect over MCF-7. IC50 values of JCP1 and JCP2 for MCF-10A appeared much lower than those for MCF-5 based on Fig. 5. These data indicate that extracts may have (more) toxic to normal tissues, rather than anti-tumor activity. Accordingly, mechanistic understanding should be essential in this manuscript. If not, it is hard to scientifically explain traditional applications or usages of the extracts with only these in vitro results.

The breast cell line MCF-10A was used as a non-tumorigenic control cell line. But nevertheless, this cell line does not reflect the characteristics of a normal, primary breast cancer cell line, so we decided not to use the existing MTS results for this manuscript. In addition, the MTS method can only be an indicator of a possible influence on the cells. To validate the MTS results for the tumorigenic cell line MCF-7, the assay was repeated with new passaged MCF-7 cells (Figure 1). The comparison of the two measurements indicated that the viability results were reproducible.
However, to determine the influence on normal breast tissue, primary cells should be used and supplementary methods, for example to determine the rate of proliferation, induction of apoptosis and the influence on cell metabolism should be consulted. In further work, we will examine in detail the impact on normal breast cells. But this will be done only after the complete chemical analysis of the four extracts. Thus dispensing with the presentation of the MCF-10A MTS results in this manuscript.

Minor points

1. Fig. 1 & Fig.2 should be combined in one figure, because they are identical; Fig.1 is raw data and Fig. 2 is statistical.
   Done.

2. In Figure 2, authors have to execute additional experiments regarding cell proliferation (e.g., thymidine incorporation) and apoptosis (e.g., DNA laddering, caspases activation or annexin V flip-flop or etc) to clearly show or prove cell proliferative & apoptotic activities of extracts. For instance, when cells were
progressive in apoptosis, nuclear fragmentation usually occurs. However it is shown that no nuclear fragmentation was appeared in Hoechst staining (Fig 3). Accordingly, it would be better to show additional apoptotic data in addition to their FACS data.

Additionally, apoptosis induction was determined by using Alexa Fluor488 Annexin V/Dead cell apoptosis kit (Life technologies, Germany). Table 3 in the manuscript shows the results. To confirm the proliferation results, western blot analysis were performed with a PCNA (Proliferating Cell Nuclear Antigen) specific antibody (Fig. 4). The proliferation results of the cell cycle analysis are well comparable with the western blotting results. Furthermore, the Annexin V results confirm the apoptosis data obtained from the determination of the sub G1 phase in the cell cycle measurement. Ultimately, these results demonstrate that the cell cycle analyses were well performed and results were confirmed using other methods.

3. From Fig 4 and based on ‘Materials and Methods’ section, it is not clear cellular locations of integrin beta1; either in the cell surface or in whole cell. Authors have to show integrin beta1 by using Western blotting to know if extracts control gene expressions of integrin beta1 or alter its surface expression.

Done.

Preparations for the measurements of integrins and also immunofluorescence staining of integrin beta1 were performed with living cells, so that only integrins on the cell surface were labeled. Furthermore, western blot experiments were used to determine soluble integrin expression levels in comparison with membrane associated integrins (Fig. 4). Cells treated with 10 µg/ml Z1 extract showed a significant increased integrin ß1 level on the cell surface (immunofluorescence labeling; Fig. 3). The western blotting results confirmed the boosted expression in the membrane fraction while the soluble integrin content was significantly reduced.

4. In Fig 3 & 4, authors have to show quantification of cellular detachment and compare these quantification and expression level of integrin beta1. These comparisons may reveal the correlation between cellular detachment and expression of beta 1.

Completed.

Quantification of cell detachment is presented in Fig. 2 A. Association between cell detachment and integrin beta1 expression is discussed in the text (Page11).
5. In Fig 4A, y-axis may represent ‘% increase of control’, instead of ‘% of control’.

Done.

Level of interest: An article of limited interest

Quality of written English: Acceptable

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’s report

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

Version: 1
Date: 27 January 2014
Reviewer: Oliver Treeck

Reviewer’s report:

This is a well performed study using appropriate methods.

Major Compulsory Revisions

However, the presented data are not sound, but even contradictory. The effects of the plant extracts on proliferation and apoptosis vs. MTS data are inconsistent. For example, with regard to JCP1 and PS, 10µg/ml reduce proliferation, but 25/µg/ml enhance it. With regard to JCP2, 1µg/ml activate proliferation, but 10µg/ml reduce it. Whereas 50 µg/ml JCP2 reduce viable cell (MTS) values by about 80%, proliferation is not affected by this dose and apoptosis is even reduced, which does not make any sense.

The presented data are sound. But to be sure, some experiments were repeated. And it makes sense to discuss the results profound. First, the MTS results cannot be compared with the proliferation data. MTS assay just reflects the metabolic activity as an indicator for the viability of the cells (see: McGowan EM, Alling N, Jackson EA, Yagoub D, Haass NK, et al. (2011) Evaluation of Cell Cycle Arrest in Estrogen Responsive MCF-7 Breast Cancer Cells: Pitfalls of the MTS Assay. PLoS ONE 6(6): e20623. doi:10.1371/journal.pone.0020623).

The proliferation measurement via cell cycle analysis determines the DNA content of the cells. As proliferative cells, cells in the S- and M-phase were counted. Second, it is possible that the cells behave differently depending on the concentration used. For instance, a variety of phytoestrogens e.g. genistein induce biphasic effects (see: Engel N, Lisec J, Piechulla B, Nebe B (2012) Metabolic Profiling Reveals Sphingosine-1-Phosphate Kinase 2 and Lyase as Key Targets of (Phyto-) Estrogen Action in the Breast Cancer Cell Line MCF-7 and Not in MCF-12A. PLoS ONE 7(10): e47833. doi:10.1371/journal.pone.0047833).

Third, an increase in apoptosis is often associated with an increase in proliferation. This is due to the self-preservation of the cell cluster or the tissue. This phenomenon explains the mentioned effect that both apoptosis and proliferation can raise at the same time. Fourth, a reduction in cell viability must not be associated with the proliferation rate. For example, paclitaxel induces a G2-arrest in the cell cycle but shows no inhibition of cell viability.

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's report

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

Version: 1 Date: 29 January 2014
Reviewer: Hsueh-Wei Chang

Reviewer’s report:

Major comments:

1. [material and method- Cell cycle analysis] “For statistical analysis, … the sub-G1-peak of the histogram as apoptotic ones.”# It is not suitable to use the subG1 as apoptotic detection. The “Annexin V”-based flow cytometry is more suitable.
Measurement of the sub-G1 phase is suitable to detect DNA fragmentation which is an indicator for apoptosis or dying cells. However, Annexin V-based flow cytometry was performed additionally.

2. [Fig. 1] The authors just provide the raw data without statistics and data in figure or table for the cell cycle distribution. I suggest the authors to add the mean+- SD for each data to show the possible cell arrest or not in addition to the flowJo figures.
FlowJo figures visualize the shift of the peaks (Fig. 1A). Statistics are given in the graphs below (Fig. 1B).

3. [Fig. 2] The subG1-based apoptotic detection cannot completely match to the proliferation. AnnexinV may be better for apoptosis detection. For example to JCP1, the 25 and 50 ug/ml have both higher apoptosis and proliferation in an inconsistent way. For PS, the 25 u/ml have both maximum apoptosis and proliferation.
An increase in apoptosis is often associated with an increase in proliferation. This is due to the self-preservation of the cell cluster or the tissue. This phenomenon explains the mentioned effect that both apoptosis and proliferation can raise at the same time.

4. [Table 2] The values for these measurements have to provide rather than the positive or negative symbol.
The detection of the substance classes was qualitative and not quantitative. Therefore, no specific values can be given.

5. The role of anti-adhesive effects on proliferatoin is not clear. It may be discussed further.
Completed.
Adhesion-relevant proteins like the family of integrins or cadherins are involved in the regulation of many cellular processes, even in proliferation. For example this renowned paper:

Minor comments:

1. [Background] “In this study, four plants were collected for evaluation of their antitumor activities with respect to anti-proliferative and pro-apoptotic properties.” But the title is “Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7”. Therefore, it may change to “anti-adhesive effects” in the background.
Completed.

2. [Result-Fig. 3] “The ZI extract exhibited the lowest rate of cell detachment and only a few dead cells, which correlated with the results of the cell cycle measurements where the ZI extract at a concentration of 10 µg/ml showed a significantly increased proliferative phase.”# Please add the values for them to show how it is correlated with the results.
Done. See the modified figure 2.

3. [Result-Fig. 5] Before this sentence “PS and ZI extracts displayed similar toxicity to both cell lines.”, I suggest the authors to the sentence “The IC50 values for JCP1, PS, ZI and JCP2 for MCF-10A cells were @, @, @ and @ µg/ml, respectively” where @ are the values.
Done.

4. [Result-Fig. 5] The JCP1 and JCP2 seem to more toxic to normal cell than cancer cells. It also needs to mention in this result for Fig. 5.
Done.
These results were canceled from the manuscript on the advice of another reviewer.

5. [Table 1 legend] “IC50 values for MCF-7 cells”# “IC50 values at 48 h for MCF-7 cells”
Done.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
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’ below
Reviewer's report

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

Version: 1 Date: 31 January 2014

Reviewer: Seda Vatansever

Reviewer's report:
The manuscript can be acceptable for publication and no necessary any revisions.

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

Quality of written English: Acceptable

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’s report

Title: Anti-proliferative and anti-adhesive effects of four plant extracts on the breast cancer cell line MCF-7

Version: 1 Date: 3 February 2014
Reviewer: Fang-Rong Chang

Reviewer’s report:

The authors described the results for in vitro anticancer assays of the ethanol extracts from African folkloric medicines, Jatropha curcas (JCP1), Pyrenacantha staudtii (PS), Picralima nitida (ZI) and Jatropha gossypifolia (JCP2) against human epithelial MCF-7 breast cancer cells in a dose-dependent manner (1 - 50 µg/ml) by using cell cycle analysis, vitality (MTS) and Live/Dead assay. The study also investigated in adhesion processes by monitoring #1-integrin expression and formation of the actin cytoskeleton. As described by authors, cancer cell growth inhibition and apoptosis induction was observed (IC50: 23 - 38 µg/ml). At a lower concentration of 10 µg/ml, all four plant extracts caused cell detachment accompanied by decreased #1-integrin expression except for that of ZI treatment.

In the bioassay part, it is clear in the initial stage for the anticancer agent discovery. However, I think the paper should be rejected or have a major revision.

1. Does the used part all the plants is the bark only?
   The part of the plants used in ethnomedicine is the stem bark. Hence, these were used in the assay procedure.

2. Why these four plants were selected? The authors state some reasons, it should be a long plant name list, and then, four of them are reported. I suggest the authors can provide the list for assay.
   The four plants were selected based on the frequency of traditional usage and ethnobotanical survey. As a matter of fact, in Nigeria, the traditional herbal practitioners have achieved huge success in the use of these plants in the management of breast cancer and related tumors.

3. The extraction rate 40% and 51% for ZI and JCP2, respectively. It seems not reasonable. It means every two gram bark of Jatropha gossypifolia can provide ca. 1 gram of JCP2. It is a very high extraction rate, and almost impossible.
   The extraction rate of 40% and 51% for ZI and JCP2 respectively are realistic and practicable because of the copious amount of secondary metabolites contained in the plants. For example, ZI which is Picralima nitida is a well-known plant rich in alkaloids. The alkaloidal constituent is about 95%. In addition, the extraction was maceration for 72 hours, ensuring exhaustive extraction.

4. In Table 1 legend,…a final concentration of 50 % µg/ml in percent of control… However, in the column of Table 1, Proliferation for 50 µg/ml (% of control). The text in the Table 1 legend should be wrong. Moreover, what is the meaning of
“Proliferation for 50 µg/ml (% of control)? I can’t understand the data. If the control is 100%, under 50 µg/ml Jatropha curcas (JCP1), Pyrenacantha staudtii (PS), and Jatropha gossypifolia (JCP2) treatments, the data will approach 0 %, e.g. +9…-7, -7… Except for the IC50 value of Picralima nitida (ZI) is 22.76; however, under 50 µg/ml ZI treatment, only 9 % cancer cells died (-91.14 ± 5.2, is that correct?). It is real unusual.

This column was deleted from this table. The statistical part of the proliferation and apoptosis measurement can be taken from Figure 1.

5. The crude material tests are not enough to tell the chemical compositions of four plant materials in the modern research. If the authors can’t determine the chemical profiles of the subjects, at least, the authors should describe the possible active components reported in the literature.

Chemical constituents: The identification, isolation and characterization of the chemical constituents in the plants extracts, responsible for the anticancer activity are currently in progress. As a follow up, the results of the chromatographic characterization, elucidation of the chemical structures and evaluation of the antiproliferative properties will be reported in future studies.

The chemical components previously reported in literature are as follows:
- Pyrenacantha staudtii: flavonoids, 3-carbomethoxylpyridine, cyclohexanone (Falodun and Usifoh, 2006; Falodun et al., 2009)
- Jatropha gossypifolia: chemical constituents include diterpenes, flavonoids (Falodun et al., 2010; Falodun et al., 2011; Falodun et al., 2012)
- Picralima nitida: Alkaloids, Phenols (Henry, 1972; Oliver et al., 1962)


Oliver L, Levy J, Le Men JJannot MM. Alkaloids of Picralima nitida, Isolation of a new

- Jatropha curcas: diterpenes (Kupchan et al, 1976; Taylor et al., 1983)

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Taylor MD, Smith AB, Furst GT, Gunasekara SP, Bevelle CA, Cordel GA, Fansworth
NR, Kupchan SM, Uchida H, Branfman AR, Daily RG, Sneden AT. Am Chem Soc. 1983,
105: 3177.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the
statistics.

**Declaration of competing interests:**
'I declare that I have no competing interests' below.