From

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To

Jing Yu,
Science Editor, Editorial Office
Baishideng Publishing Group Inc

Sub: Submission of revised article entitled "Investigation of events related to inhibitory effect of Gallic acid in HCT-15 colon cancer cells, Manuscript ID: 22558"

First and foremost, I would like to thank the editor for providing a wonderful opportunity to revise and resubmit our work. We believe that we had addressed the reviewer comments efficiently to seek a chance for publishing our work in the prestigious World Journal of Gastroenterology.

Further, I take this opportunity to thank the reviewers for the excellent comments which helped us to enrich our review. Now we feel our review is well organized and could be considered positively for publication.

The correction made in the original article as per the reviewer's comments is highlighted in red fonts in the main manuscript. Moreover, we have attached the document containing the list of responses to the reviewer comments for your reference.

Yours Sincerely,
Dr Saravana Kumar Jaganathan.

REFEREE REPORT(S):

Referee: 1 (reviewed by 03062384)

Phytochemicals modulate key cellular signaling pathways and have proven anticancer effects. In the past, a large number of substances derived from plants have been studied in antitumor research fields and many have proven to exhibit chemopreventative properties which could be used as adjuvant chemotherapy. The current study explored the role of Gallic Acid (GA) as a potential anti-cancer agent. The study is important and may advance the field of chemoprevention. There are however, deficiencies that lowered the enthusiasm to some extent:

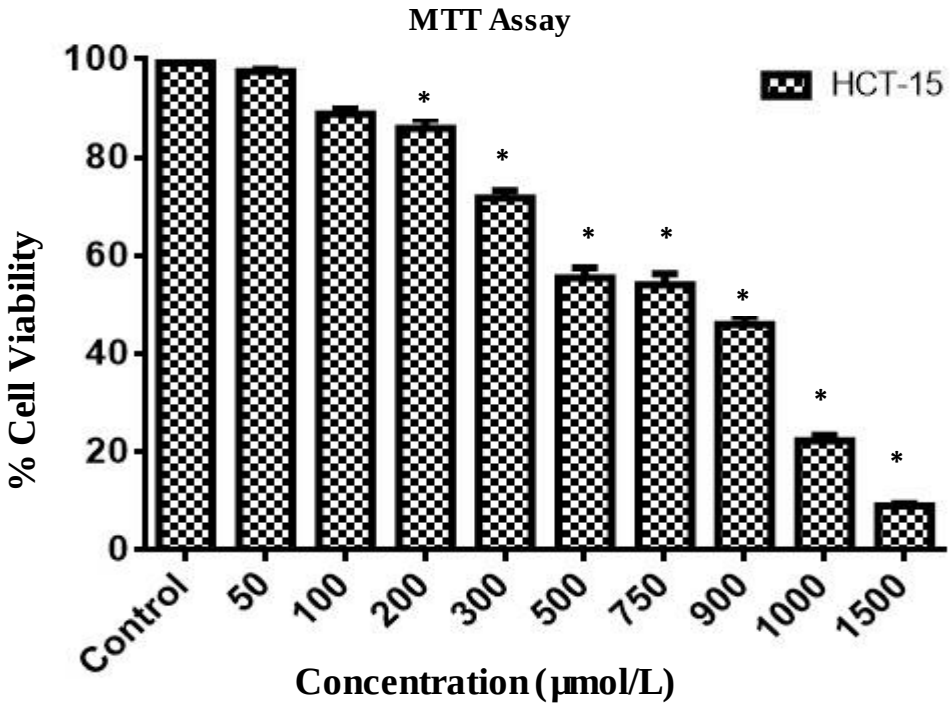
1. Since authors have only focused on one cell line, it is not clear how generic the response is. Do other colon cell lines follow similar mechanism?

The cell line of choice is HCT-15 colon cancer cells (Organism: *Homo sapiens*, human; Tissue: colon; Disease: Dukes' type C, colorectal adenocarcinoma; Gender: male). It belongs to Duke's C classification, where the cancer has spread to at least one lymph node in the area close to the bowel, at a beginning stage of metastasis. This cell line represents the most commonly occurring type of among the population, as 25% of people with colorectal cancer are diagnosed at this particular stage according to cancer research UK. It is also a cell type that has a metastasis characteristics. Hence, the assessment to this particular type of cell is more likely to be a reasonable evaluation for assessing the anticancer property of this particular compound against colon cancer. As Gallic acid has an excellent anticancer activity against HCT-15 colon cancer cells, it may be concluded that GA exhibits antiproliferative activity against the various colon cancer cells. Besides these points there are quite a few cell lines that come under the same Duke's classification of colon cancer cells. For instance, the commonly investigated cell lines HT 29, LoVo, Sw48 fall in the same classification and are expected to have a similar type of response when treated with GA.

2. It is difficult to believe that 200 micro-molar concentration has significant inhibition as is suggested by the authors. On a minor note, the authors need to show statistical significance on the bar graph.

As per reviewer's suggestion the statistical significance along with the bar graph as a foot note as follows:

Figure 2:



* Statistical analysis showed that GA treatment results in significant inhibition ($P < 0.05$) compared with untreated control cells starting at 200 µmol/L for HCT-15 cells. Data represents mean ± S.D.

It is evident that the growth inhibition of HCT-15 cells were significant starting from 200 µmol/L when treated with GA. However the half-maximal inhibitory effect of GA was witnessed at higher concentration.

3. There are several proof-reading errors in the text and in the figure legends. For example, Figure 5 in the legend should be Figure 4; Figure 5 is labeled as Figure 6 in the legend and finally, Figure 6 in the text is labeled as Figure 4 in the legend. Please clarify.

The order of the figures and their legends have been carefully corrected as per reviewer's suggestion:

Figure 4: Effects of GA on MMP ($\Delta\Psi m$) in HCT-15 cells. Human colorectal carcinoma (HCT-15) cells were treated with GA for specified time-periods and then mitochondrial membrane potential were determined using rhodamine-123 by flow cytometry. Mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 5: Lipid layer break provoked by GA; HCT-15 cells were treated with GA and evaluated using merocyanine-540 to quantify the lipid layer breaks (LLBs). The fluorescence intensity was estimated using flow cytometry. Data is representative of three independent experiments, mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 6: GA induced ROS generation; HCT-15 cells were cultured in the presence or absence of GA for the specified time points. DCFH-DA fluorescence intensity was detected by using flow cytometry. Data represented is the maximum of three independent experiments. Mean differences are significant at 24 h, 48 h and 72 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

4. While apoptotic assays are well documented, Western blots for checkpoint and anti- and pro-apoptotic proteins will increase the impact. Likewise, Western blots for changes, if any, in cyclins/CDKs at different time points after GA treatment, will be helpful.

As suggested by the reviewer the Western blots to find the apoptosis checkpoint would be quite interesting and add impact to our results.

We are planning this as our future research and the same is discussed in the section Discussion. The following has been added in the discussion part of the article:

Although apoptosis is confirmed by Yo-Pro-1 staining it would be more interesting to study the various pro-apoptotic and anti-apoptotic protein level in GA treated HCT-15 cells. The analysis of cyclin/CDK, p53, Bax, Bad, Bcl-2 and Bcl-xL protein levels at different time intervals would give more information regarding the apoptosis induced by GA. Hence in future research, this interesting points will be addressed.

Referee: 2 (Reviewed by 02510223**)**

In this manuscript, you found that diet-derived phenolic compound Gallic Acid (GA) inhibited the proliferation and induced the apoptosis of HCT-15 cancer cells through increased generation of ROS. This is an interesting finding, I still have some questions about this paper:

1. Initial in vitro data is presented for HCT-15 cell lines and the greatest effect was remarkable. Other cell lines including the normal human cells and colon cancer cell lines should be presented for further experiments.

The HCT-15 cells are derived from the Duke's C colorectal adenocarcinoma that is characterized as spread to the lymph nodes that are near to the bowel region. The particular Duke's stage has been given prominence as 25% of the patients are diagnosed at this stage (according to cancer research UK). As the cell type is frequently diagnosed and also has metastatic characteristics, the authors concentrated that evaluating the anticancer property of Gallic acid against HCT-15 alone would be sufficient. Apart from this the following data has been added in the discussion part.

Moreover, the effect of GA on intestinal epithelial cells (IEC) was investigated. It was seen that about 85% of cells were viable when treated with 3.5mM/L showing that the GA treatment was non-toxic to normal cells (results not shown). These results depict that the phenolic compound GA has insignificant inhibitory activity against colon cancer cells at even a very high concentration.

2. Author should be pay attention on the analysis of data. Statistics analysis in many cases missing. (e.g. In Figure 7 there is no significant difference analysis). "*"or number ($p<0.05$) should be consistent.

We would like to thank the reviewer for pointing out the missing data which has now been added to the figure 7. The details related to statistical analysis has been given in the all the figure legends except the figure 1. This because the figure 1 shows the chemical structure of gallic acid. The figure legends are as follows:

Figure 1: Chemical structure of GA

Figure 2: Cell proliferation inhibition by GA of colon cancer cells**;** Human colorectal carcinoma HCT-15 cells grown in 96-well plate were treated with various concentration of GA (0-1500 µmol/L) for 72h. Percentage of mean cell viability along-with the SD is indicated (n=3). Mean differences are significant compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 3: Colony inhibitory activity of GA against colon cancer cells. After various incubation periods of GA treatment, colonies formed were stained with 0.5% crystal violet and counted, and percentage of survival was calculated by normalizing the values. Data reported is the mean ± SD from three different observations. Mean differences are significant at 12 h, 24 h and 48 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 4: Effects of GA on MMP ($\Delta\Psi m$) in HCT-15 cells. Human colorectal carcinoma (HCT-15) cells were treated with GA for specified time-periods and then mitochondrial membrane potential were determined using rhodamine-123 by flow cytometry. Mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 5: Lipid layer break provoked by GA; HCT-15 cells were treated with GA and evaluated using merocyanine-540 to quantify the lipid layer breaks (LLBs). The fluorescence intensity was estimated using flow cytometry. Data is representative of three independent experiments, mean differences are significant at 12 h and 24 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 6: GA induced ROS generation; HCT-15 cells were cultured in the presence or absence of GA for the specified time points. DCFH-DA fluorescence intensity was detected by using

flow cytometry. Data represented is the maximum of three independent experiments. Mean differences are significant at 24 h, 48 h and 72 h compared with untreated control cells ($P < 0.05$ vs untreated control cells).

Figure 7: A representative photomicrograph and scanning electron microscopic images of three independent experiments. A: Photomicrograph images of untreated and GA treated HCT-15 cells. The arrow mark indicates cell death after GA treatment; B: Scanning electron microscopic images of untreated and GA treated HCT-15 cells. The arrow mark represents the rounding up of HCT-15 cells after GA treatment.

Figure 8: Apoptosis assessment using Yo-Pro-1 dye by flow cytometry. HCT-15 cells was treated with GA for specified time points. The distribution of the cell population changed according to the exposure time as indicated by M1 and M2. Data represented is the maximum of three independent experiments and the differences in the values of M2 were significant at 48 h and 72 h compared to untreated control cells ($P < 0.05$ vs untreated control cells).

3. Discussion should be modified. you repeat results and methods in the disscussion part without discussing the significance.

The discussion has been modified in order to provide the significance of the results obtained as per reviewer's suggestion:

Diet is thought to have a major role in the etiology of colorectal cancer. Similar studies also proved that a phytochemical-rich diet, which is absorbed by the body from fruit and vegetable sources, could decrease the risk of developing colon cancer [15]. Previous work revealed various biological properties of phenolic content in the diet that we consume regularly. These phenolic compounds are commonly known for their anticancer property. The phenolic compound GA exhibited an antiproliferative effect on the HCT-15 colon cancer cell lines in a dose dependent manner which was similar to the effect of GA on the human hepatoma SMMC-7721 cell proliferation in *in vitro* condition [16]. Hence it can be inferred that GA treatment led to lysis of HCT-15 cells with increasing concentrations either by apoptosis or necrosis. Cell growth was inhibited significantly with an IC_{50} of around 740 µmol/L and this was parallel to the results obtained in the recently conducted study of the antiproliferative effect of GA on HCT-15 by Yumnam *et al* [18]. In a particular study, the oral consumption of six cups of black tea resulted in

344 mg GA [25]. However, about 25 mg of GA is enough to yield about 750 µmol/L in the colonic volume of 200 ml. This shows that the IC_{50} obtained in our study lies within the range of biological availability. Moreover, the effect of GA on intestinal epithelial cells (IEC) was investigated. It was seen that about 85% of cells were viable when treated with 3.5mM/L showing that the GA treatment was non-toxic to normal cells (results not shown). These results depict that the phenolic compound GA has insignificant inhibitory activity against colon cancer cells at even a very high concentration.

Colony formation is one of the characteristic features of cancer cells, which was inhibited by GA treatment in a time dependent manner which is alike to the results obtained by the clonogenic assay on the GA treated A549 human lung adenocarcinoma cells [17]. The cancer cells grow rapidly and multiply uncontrollably therefore, one of the fundamental features expected to be present in the anticancer drug, is the ability of the drug to affect the cell proliferation. Nevertheless, GA is found to evidently affect the colony formation of HCT-15 cells. The morphological changes such as cell shrinkage and membrane blebbing was visible in the colon cancer cells exposed to GA, which agrees to the earlier experimentation of Yumnam *et al* on the GA treated HCT-15 cells [18]. These cellular changes witnessed in GA treated cells are similar to characteristic changes in the cellular organelles during apoptosis [19]. Thus, the microscopic examinations show GA induces apoptosis in HCT-15 cells.

In normal biological systems, ROS is continuously generated and eliminated as well as plays an important role in driving various regulatory pathways. The cell balances the generation of ROS thereby controlling it. However, abundant generation of ROS during oxidative stress may affect the lipids, cellular proteins. In our study, GA promoted the generation of ROS in the colon cancer cell lines depending on the duration of exposure which is analogous to the antiproliferative effect of GA against MiaPaCa-2 human pancreatic cancer cells [21]. As the raise in ROS generation is said to cause apoptosis through extrinsic or intrinsic pathways in the cancer cells, the promotion of ROS generation in GA treated HCT-15 cells supports the antiproliferative effect of GA [20]. Other significant pro-apoptotic event is lipid layer breakage, which is said to favor the interaction between the drug tested and the other cell organelles Lipid layer breakage was enhanced by GA treatment in the colon cancer cell lines. This finding is supported by GA-

induced lipid layer breaks in the HSC-2 human oral cancer cells [22]. The lipid layer breakage is an optimistic event that favor the interaction between the drug tested and the other cell organelles [26]. Hence, the effect of GA on the lipid layer of HCT-15 cells may be related to the apoptosis-inducing ability of GA. A great increase in ROS has been associated with reduced cancer cell proliferation by induction of cell cycle arrest. The GA treatment caused a time-dependent cell cycle arrest at the sub-G_1 phase in HCT-15 cells which was similar to activity of GA on HL-60 human leukemia cells [23]. However, the Sub-G_1 phase is related to measurement of apoptosis or programmed cell death [13]. During apoptosis, the DNA is degraded and the content becomes less than that the DNA content in healthy cells undergoing cell cycle [27]. The increase in the amount of cells at Sub-G1 phase infers that GA treatment of HCT-15 cells may be ascribed to programmed cell death in a time dependent manner.

Mitochondrial malfunction is another key event that occurs during apoptosis. Mitochondrial membrane potential of GA treated cells showed decreasing intensity, with increase in the exposure time that is similar to the result acquired during the investigation of effect of GA on A375S2 human melanoma cells by Lo *et al* [24]. Various anticancer drugs cause mitochondrial membrane potential fluctuations and induce death of cancer cells [26]. Furthermore, the changes identified in the level of mitochondrial membrane potential in may be related to its inhibitory effect of GA against HCT-15 cells. Some recently concluded researches utilized Yo-Pro-1 as an effective agent in confirming apoptosis. The Yo-Pro-1 staining used to detect apoptosis induced by anticancer agents as it analyses the apoptotic cells without interfering cell viability [14]. Apart from the early and later events indicating the occurrence of apoptosis, Yo-pro-1 staining confirmed the apoptosis after GA treatment.

As apoptosis is confirmed it would be more interesting to study the various pro-apoptotic and anti-apoptotic protein level in GA treated HCT-15 cells. The analysis of cyclin/CDK, p53, Bax, Bad, Bcl-2 and Bcl-xL protein levels at different time intervals would give more information regarding the apoptosis induced by GA. However, further development of this research work would be *in vivo* experimentation with GA. This would need a proper understanding of the degree to which GA is absorbed or becomes available at the site of physiological activity after administration. As the half-maximal inhibitory concentration of GA obtained in our studies lies

within the range of biological availability, the *in vivo* experimentation can be preceded. Nevertheless, proper experimentation using humans with risk of colon cancer in a larger group may validate the anticancer activity of GA more precisely.