

Cytotoxicity and genotoxicity evaluation of stevioside on CCD18Co and HCT 116 cell lines

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Abstract

Recent findings showed that stevioside can demonstrate anti-cancer property in selected cell lines. In this study, the cytotoxicity and genotoxicity of stevioside were examined on human colon carcinoma cell, HCT 116 (targeted cell) and human colon derived CCD18Co myofibroblast cell lines (non-targeted cell) using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and alkaline comet assay, respectively. Result demonstrated that stevioside induced cell death on both HCT 116 and CCD18Co cell lines only at the highest concentration, 200 μ M by causing not more than 20 and 30 percent of cell death on CCD18Co and HCT 116 cell lines, respectively ($p < 0.05$). The DNA strand break measured via alkaline comet assay showed that it did not cause DNA damage at the same concentration on CCD18Co as well as in HCT 116 cell lines ($p > 0.05$). In conclusion, stevioside did not exhibit cytotoxic and genotoxic effect on HCT 116 and CCD18Co cell lines respectively hence secured its uses as a non-caloric sweetener.

Keywords

Stevioside
Cytotoxicity
Genotoxicity
MTT assay
Alkaline comet assay

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Introduction

Cancer is the major cause of mortality throughout the world. As a multifaceted disease, it was predicted to increase up to 80% by 2030 especially in the low and middle income countries (Khazir *et al.*, 2014). As the third common cancer in United States, colorectal cancer is estimated to affect 136,830 individuals with 50,310 mortality cases due to unhealthy lifestyle (Siegel *et al.*, 2014). Meanwhile in Malaysia, colon cancer is affecting 21 person per 100 000 Malaysian (NCPR, 2014).

Despite a large number of potent chemotherapeutic agents being developed, cancer still remain the main cause of death due to the development of resistance and lack of selectivity towards the anticancer agent. As such, the main goal of chemotherapy is the development of anticancer drug which can target the cancerous cell without affecting the normal cells (Lu and Low, 2012).

As been pointed out in previous work, *Phyllanthus emblica* Linn (Mahata *et al.*, 2013), *Cyathula prostrate* (Priya *et al.*, 2013) and *Stevia Rebaudiana* Bertoni (Paul *et al.*, 2012) are some of the medicinal plant found to exhibit anticancer properties. Stevioside is widely used as non-caloric sugar substitute in Japan and Brazil (Matsuit *et al.*,

1996). It is also as one of the steviol glycoside isolated from stevia plant was found to exhibit valuable pharmacological properties such as antihypertensive (Jeppesen *et al.*, 2003), antioxidant (Tavarini and Angelini, 2013), antihyperglycaemia (Jeppesen *et al.*, 2002), antiinflammation (Boonkaewwan and Burodom, 2013), antidiarrheal (Chatsudthipong and Muanprasat, 2009), antigingivitis (de Slavutzky, 2010) and anticancer apart from its uses in food and beverages industry.

Stevioside at dose of 2.5% and 5% able to prevent carcinogenicity in Fischer 344 rats (Toyoda *et al.*, 1997). Moreover, it exhibit similar activities as triterpenoid against tetradecanoylphorbol acetate (TPA) to suppressed tumor promotion on skin cancer model in mice. Interestingly, inhibitory effect on tumor compare to anti-tumor promoting agent, quercetin was observed (Yasukawa *et al.*, 2002). Besides, high concentration of stevioside (2-5 mM) shows cytotoxicity effects as cell viability significantly decrease in T84, Caco-2 and HT29 cell lines (Chaiwat *et al.*, 2008).

A novel anticancer agent will act by targeting the cancerous cell without bringing excessive damage to the normal cell. Therefore in this study, the focus was given to stevioside to evaluate its cytotoxic and genotoxic effect on human colon derived

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myofibroblast cell, CCD18co as the non-targeted cell. Meanwhile, the cytotoxic effect of stevioside on human colon derived cancerous cell, HCT 116 was evaluated as well.

Materials and Methods

Materials

Eagle's Minimum Essential Medium (EMEM) powder and commercialized McCoy 5A (1x) medium were purchased from Invitrogen Cooperation, UK. Sodium pyruvate, non-essential amino acid (NEAA), fetal bovine serum (FBS) were obtained from PAA Laboratories, Australia. Ca^{2+} and Mg^{2+} free PBS, 3-[4,5-dimethylthiazol-2-yl]-2,5-difeniltetrazolium bromide (MTT salt), 0.4% trypan blue, sodium chloride (NaCl), potassium chloride (KCl), dinatrium hydrogen phosphate (Na_2HPO_4), potassium dihydrophosphate (KH_2PO_4), sodium hydroxide (NaOH), trizma base, Low Melting Point Agarose, Normal Melting Point Agarose and Triton X-100 were purchased from Sigma-Aldrich, United States. Sodium EDTA (HmbG Chemical, German), sodium hydrogen carbonate (NaHCO_3) (Bendosen Laboratory Chemicals, Britain), dimethyl sulfoxide (DMSO) (Fischer Scientific, UK) and ethidium bromide (EtBr) (BD Pharmingen, San Diego) were used in this study.

Cell culture

Human colon derived myofibroblast cell (CCD18Co) and human colon-derived colorectal cancerous cell (HCT 116) were obtained from American Type Culture Collection (ATCC) (Rockville, MD USA) with ATCC number ATCC: CRL-1459TM and ATCC: CCL-247TM, respectively. CCD18Co were cultured in EMEM supplemented with 10% of FBS, 2.2 g sodium bicarbonate (NaHCO_3), 1% of NEAA and 1% sodium pyruvate CCD18Co. As for HCT 116, the cells were cultured using commercially available McCoy 1x with the addition of 10% FBS.

MTT cytotoxic assay

The cytotoxic effect of stevioside on both CCD18Co and HCT 116 were determined by using MTT assay (Mosmann 1983). Cells cultured in monolayer were harvested and seeded in a 96 well plate (Jet Biofil, Canada) for 24 h at 37 °C and 5% CO_2 . Cells were then treated with stevioside at the concentration range from 0 μM to 200 μM for another 24 h. Following 24 h of incubation, 20 μL of MTT solution (0.5% w/v) was added into each well and allowed to incubate for another 4 hours before the medium was discarded. Then, 200 μL of DMSO was

added to dissolve the formazan crystal formed. The plate was shaken for 5 minutes using the microplate shaker (IKA, China) to produce a uniform mixture before the absorbance was read at 570nm with an I-Mark™ microplate reader (Bio-Rad Laboratories, USA)

Alkaline comet assay

Alkaline comet assay was used as a tool to evaluate the genotoxicity of stevioside on CCD18Co cell lines. Cells were seeded in a 6-well plate for 24 h before treated with 200 μM of stevioside for 24 hour. After the 24 h of treatment, cells pellet was collected, and alkaline comet assay was conducted as described previously (Tice *et al.*, 2000). Tail intensity (% DNA tail) and tail moment (tail length x DNA tail) were then quantified by using CometScore™ software (Tritek Corp. USA).

Statistical analysis

The test was performed three times which each time the plates for each concentration are in six replicates (n=6). One-way ANOVA were used to analyse the significant differences of tail moment and percentage of DNA in tail both cell lines for each concentration. Meanwhile, the percentage of cell viability was analysed by using Independent T-test for both cell lines. The level of confidence was set at 95% level and level of significant applied was $p < 0.05$.

Results

Cytotoxicity study on CCD-18Co and HCT 116 cells

Based on MTT assay, stevioside shows no cytotoxicity effect on both CCD18Co and HCT 116 cell lines (Figure 1). At 12.5 μM , stevioside showed a non-significant increase in cell viability and gradually decrease at following concentration (25 μM -100 μM). No significant difference was observed at 200 μM of stevioside on CCD18Co, $p=0.102$ ($89.33 \pm 6.24\%$) and HCT 116 cell lines $p=0.076$ ($77.66 \pm 8.59\%$).

Genotoxicity study on CCD18Co and HCT 116 cell lines

Alkaline comet assay was employed to determine the genotoxicity effect of stevioside at the highest concentration (200 μM) following 24 h of treatment. As indicated in Figure 2A, CCD18Co cells did not showed any significant changes ($p=0.798$) in tail moment (1.145 ± 0.179) as compared to control. However, HCT 116 cells showed significant increment in tail moment (2.445 ± 0.004) as compared to control group ($p < 0.05$). This result is parallel with

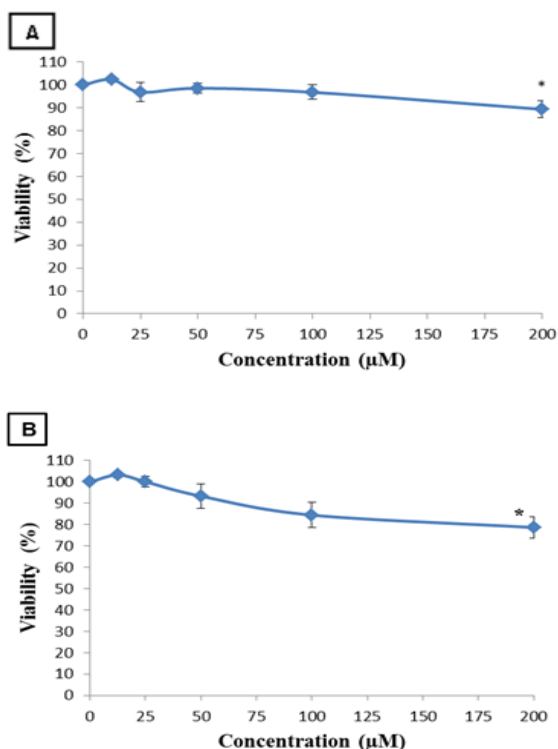


Figure 1. Percentage of cell viability (%) followed by 24 h treatment with stevioside at different concentration. (A) CCD18Co; (B) HCT 116. Each point represents the mean \pm SEM, (n=6). *p > 0.05 against vehicle control

MTT assay result whereby the cell viability of HCT 116 cells is much lower as compared to CCD18 Co cells. Meanwhile, the percentage of DNA in tail (Figure 2B) for both cell lines showed no significant differences as compared to the control group ($p > 0.05$). The microscopic appearance of both cells in three treatment conditions was shown in Figure 3. Based on Figure 3, vehicle control group demonstrated no DNA damage with intact rounded DNA as compared to positive control group treated using menadione (25 μM). The positive control group showed formation of fragmented DNA, forming comet tail. HCT 116 cells treated with stevioside showed slight DNA fragmentation as compared to CCD-18Co cells.

Discussion

Stevioside has gained considerable attention due to its valuable pharmacological properties. As a component isolated from a natural product, it has been used traditionally not only as a sweetener, but also a widely accepted remedy. Among all the medicinal properties, its effect on cancerous cell was found to be of utmost importance. In order to be developed as an anticancer agent, a compound must exhibit a potent cytotoxic effect to the targeted cell, by causing minimal damage to the nearby normal cell (non-targeted cell).

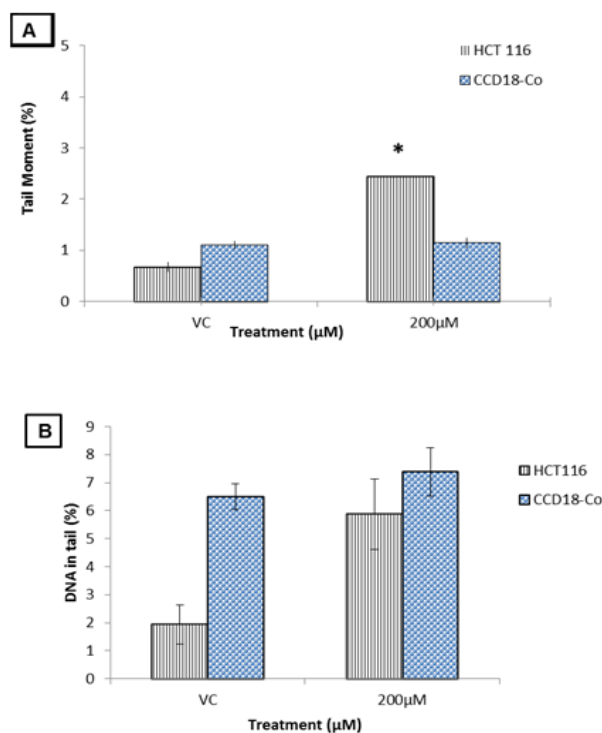


Figure 2. (A) Tail moment and (B) Tail intensity of CCD18Co and HCT 116 followed by 24 h treatment with stevioside. VC= vehicle control. The data represented the mean \pm SEM, (n=6). *p < 0.05 against vehicle control

Cytotoxicity of stevioside on CCD18Co and HCT 116 cell lines was assessed by using MTT assay. MTT assay is a type of colorimetric assay normally used to measure cell viability. As shown in Figure 1, neither of the cells showed value of IC_{50} indicating that no severe cytotoxicity effect was observed. Our current findings is in agreement with previous study which showed that high concentration of stevioside was required to give a cytotoxic effect to colorectal cancer cell T-48, Caco-2 and HT29 cell lines (Boonkaewwan *et al.*, 2008).

In contrast, another study conducted using MCF-7 breast cancer cell line (Paul *et al.*, 2012) showed that lower concentration of stevioside (2.5-10 μM) can lead to the cell death of MCF-7 cells. Results from this present study showed that CCD-18Co and HCT 116 cells were more resistance towards stevioside as compared to MCF-7 cell lines.

Response of a cell towards a compound is believe to depend on a few factors. The main factor is the molecular size of the compound involved. Stevioside is a hydrophilic compound with big molecular size. All of these characteristics present a barrier to limit the cellular uptake (Pluen *et al.*, 2001). Besides that, every cancer cell shows a wide variation in genetic composition depending on the origin of tissue, activation of oncogene and inactivation of tumour suppressor (Gottesman, 2002). The third factor is

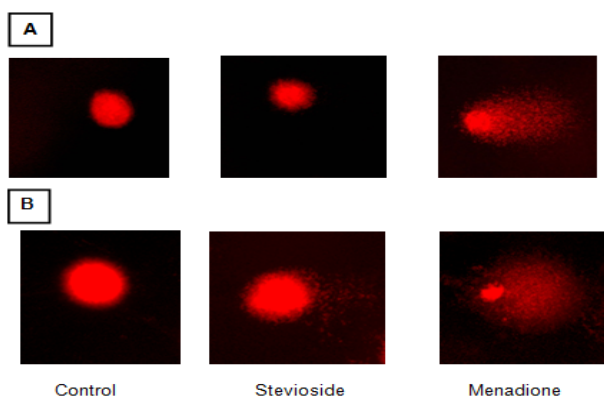


Figure 3. Microscopic fluorescent photo of vehicle control cell (CON) and stevioside subjected to electrophoresis showed a round nucleus. Cells treated with menadione exhibited migration of DNA as shown by the comet tail. (A) CCD18Co; (B) HCT 116

the changes that occur in the cell which may limit the accumulation of a substance inside the cell. For instance, by limiting the cellular uptake, stimulate reflux or by affecting the lipid membrane on the cell where the changes occurred might be able to block the apoptosis triggered by an anticancer drug or repair the DNA damage (Synold *et al.*, 2001).

Besides that, the mechanisms involved in different type of cancer were believed to play a role. In breast cancer, estrogen hormone and the associated receptor (Madeira *et al.*, 2014) was found to be a contributing factor. Estrogen can contribute to cancer formation by stimulate the division of the breast cell apart from its role in supporting an estrogen responsive tumour. On the other hand, as for colorectal cancer, lifestyle and nutrition factor was found to be the main factor (Touvier *et al.*, 2014).

Alkaline comet assay is useful in detecting single strand break and alkaline labile site in addition to double strand break (Tice *et al.*, 2000). It was carried out based on the formation of comet as the damaged DNA become free to migrate from the nuclear matrix to the agarose gel. The intensity of DNA damage was measured by using 2 components, namely the tail moment and percentage of DNA in tail. Tail moment was chosen as it showed the smallest variability in the extent of DNA damaged as compared to other parameters (Collins *et al.*, 2008).

In this study, cells treated with stevioside for 24 h did not exhibit genotoxic effect. The result was supported by previous study whereby stevioside did not induce DNA damage either in the presence or absence of metabolic activation factor (s9) which contained cytochrome p450 (Matsui *et al.*, 1996). The possible mechanism involved could be due to the role of stevioside which can act as an antioxidant to protect the cells from oxidative damage (Stoyanova *et al.*, 2011). Besides that, DNA repair could have

occurred after 24 h of treatment since it has been reported that increased in treatment time point (0.5-24 h) can decreased DNA damage (tail moment) (Sasaki *et al.*, 2007).

Conclusion

Our result revealed that stevioside did not exhibit a potent cytotoxic and genotoxic effect to both CCD18Co and HCT 116 cell lines and is safe to be use as natural sweetener. Further studies could emphasize on the optimal dose for the beneficial impact of stevioside in both *in vitro* and *in vivo* models.

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