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Kertas Asli/*Original Article*s

Effects of Epigallocatechin-3-Gallate (EGCG) on Cell Cycle Distribution and DNA Integrity of K562 Cells, A Human Chronic Myeloid Leukemia

(Kesan Epigallocatechin-3-Gallate (EGCG) ke atas Distribusi Kitaran Sel dan Integriti DNA Sel K562, Leukemia Myeloid Kronik Manusia)

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ABSTRACT

Epigallocatechin-3-gallate (EGCG) is a naturally derived compound from green tea with high antioxidant activity and various anti-cancer properties. EGCG has been widely investigated worldwide. However, effects of EGCG on cell cycle of K562 have not been clearly stated elsewhere. This study was conducted with the aim to investigate the antiproliferative effect of EGCG on K562 human leukemic cells and its underlying mechanism of action on the cells. MTT assay was conducted to determine cytotoxicity effect of EGCG on the K562 cells. Meanwhile, cell cycle analysis and DNA damage on the cells were determined by Flow cytometry and Comet assay respectively. K562 cells were treated with EGCG at concentrations ranging from 0 to 100µg/ml for 48 hours. The results showed that EGCG effectively decreased the percentage of cell viability in a dose dependent manner. The IC_{10}, IC_{25} and IC_{50} of EGCG on K562 cell lines were 5 ± 2.44 µg/mL, 10 ± 5.93 µg/mL and 50 ± 1.93 µg/mL, respectively. In cell cycle assay, EGCG has shown no significant effect ($p>0.05$) on the cell cycle of K562 cell line as compared to negative control, whereas Imatinib mesylate as the positive control showed cell cycle arrest at S phase in this cell line. Hence, EGCG can be verified as a non-cell cycle specific compound. In addition, EGCG was found to cause a significant increase ($p<0.05$) in tail moment value and percentage of DNA tail in K562 cell line, suggesting DNA damage as an early signal of EGCG induced cell cytotoxicity. In conclusion, by decreasing the cell viability and inducing DNA damage, EGCG showed promising potential as an alternative treatment for leukemia through non-cell cycle specific pathway and further investigation on other mechanisms of action of EGCG on the cells is recommended.

Keywords: Anti-cancer; genotoxicity; Antileukemic effect; EGCG; K562; Cell cycle analysis

ABSTRAK

Epigallocatechin-3-gallate (EGCG) adalah sebatian yang berasal dari teh hijau yang kaya dengan aktiviti antioksidan dan pelbagai ciri anti-kanser. Kajian terhadap EGCG telah banyak dijalankan di seluruh dunia. Walau bagaimanapun, kesan EGCG pada kitar sel K562 masih belum dikaji dengan jelas. Kajian ini dijalankan bertujuan untuk mengenalpasti kesan antiproliferasi EGCG pada sel leukemia manusia K562 dan mekanisma tindakannya terhadap sel tersebut. Ujian MTT dijalankan untuk menentukan kesan sitotoksik EGCG terhadap sel K562, manakala analisis kitaran sel dan kerosakan DNA pada sel tersebut ditentukan menggunakan sitometri aliran dan asai Comet. Sel K562 dirawat EGCG pada kepekatan di antara 0 hingga 100 µg/mL selama 48 jam. Hasil kajian menunjukkan bahawa EGCG berkesan menurunkan peratusan viabiliti sel secara bergantung dos. Nilai IC_{10}, IC_{25} dan IC_{50} EGCG pada sel K562 adalah 5 ± 2.44 µg/mL, 10 ± 5.93 µg/mL dan 50 ± 1.93 µg/mL masing-masing. Dalam ujian kitaran sel, EGCG tidak menunjukkan kesan yang signifikan ($p> 0.05$) pada kitaran sel K562 sel berbanding dengan kawalan negatif. Manakala Imatinib mesylate sebagai kawalan positif menunjukkan pemerangkapan kitaran sel pada fasa S dalam sel kajian. Oleh demikian, EGCG boleh disahkan sebagai sebatian khusus tidak mengganggu kitaran sel. Tambahan lagi, EGCG didapati menyebabkan peningkatan signifikan ($p<0.05$) pada nilai momen ekor dan peratusan ekor DNA dalam sel K562, yang mencadangkan kerosakan DNA sebagai isyarat awal kesan sitotoksisiti EGCG terhadap sel. Kesimpulannya, dengan mengurangkan viabiliti sel dan mengaruh kerosakan DNA, EGCG berpotensi digunakan sebagai rawatan alternatif ke atas leukemia melalui tapak jalan selain kitaran sel dan kajian lanjutan mengenai mekanisma tindakan lain EGCG pada sel adalah disarankan.

Keywords: Anti-cancer; genotoxicity; Antileukemic effect; EGCG; K562; Cell cycle analysis

INTRODUCTION

Chronic myeloid leukemia (CML) is a hematological malignancy that occurs due to t(9;22)(q34;q11) translocation characterized by increased in proliferation of granulocytic cell lines with loss capacity to differentiate (Houshmand et al. 2019). The main cause of CML is due to the fusion of *bcr/abl* oncogene forming a Philadelphia chromosome which then encodes a chimeric Bcr/Abl protein with a constitutive activation of tyrosine kinase activity (Li et al. 2012; Cao et al. 2014; Goker et al. 2014). Bcr/Abl fusion protein could affect cell proliferation and malignant transformation by activating different signal pathways or inducing uncontrolled transition of cell cycle.

Chemotherapy has been the first choice of treatment for those patients with advanced CML and Imatinib was approved as frontline CML management in 2001 as it inhibits the activity of tyrosine kinase. Since approved, Imatinib has been proven to be effective in achieving high remission rates and improving prognosis (Bhamidipati et al. 2013). However, this treatment always comes with toxicity and several adverse effects such as cardiac toxicity, hepatotoxicity, cutaneous toxicity, gynecomastia hypothyroidism, abnormal bone and mineral metabolism (Mughal & Schrieber 2010). Therefore, it is of high importance to find an alternative treatment for leukemia, which does not produce these adverse effects.

EGCG, which is the most active catechin found in green tea has various biologic activities, including, apoptosis induction, cell cycle arrest, anti-proliferation and anti-oxidation. EGCG was proven possessing this various biologic activities against various human cancer cell lines in several studies suggesting that it may be a promising anti-cancer agent (Otsuka et al. 1998; Lazaro et al. 2011; Stearns et al. 2011; Zhou et al. 2013; Goker et al. 2014). To further explore its anti-tumor effect especially regarding cell cycle progression and explore its mechanism, human chronic myeloid leukemia K562 cell line served as a model in our study.

This study aimed to determine the cytotoxic and genotoxic effect of EGCG in K562 cell line. This study also aimed to detect alteration in cell cycle distribution after treatment with EGCG in K562 cell line.

MATERIALS & METHODS

CELL CULTURE

Human chronic myeloid leukemia K562 cells (ATCC) were grown and maintained in IMDM medium supplemented 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

CELL TREATMENT

Stock solution of EGCG (Sigma-Aldrich) at a concentration of 100 mg/mL was prepared in phosphate buffer saline (PBS) and stock solution of Imatinib mesylate (Nacalai Tesque) at a concentration of 5 mg/mL as a positive control was prepared in distilled water. Both of the stock solutions were stored at -4˚C until use. Required concentrations (1-100 µg/mL EGCG and 1-50 µg/mL Imatinib) were freshly prepared by diluting the stock solution in culture medium immediately before use. Twenty four hours after seeding, the cells were treated with appropriate concentration of EGCG and Imatinib for 48 hours in culture conditions (37°C, 5% CO_2, 98% humidity**).** Control cells were incubated under the same conditions without any treatment.

CYTOTOXICITY ASSAY

Cytotoxic effects of EGCG and Imatinib against K562 cells were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following method by Mosmann (1983). Cells were seeded onto 96-well microplates at a density of 1×10^5 cells per well and exposed to various concentrations of EGCG (3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) and Imatinib (1.5625, 3.125, 6.25, 12.5, 25 and 50 µg/mL) for 48 hours. MTT solution (5 mg/mL) was added following incubation at a final concentration of 0.5 mg/mL and further incubated for 4 hours. The supernatant was removed, and DMSO was added to dissolve the formed formazan crystal. The plate was further incubated for 15 minutes prior to measurement at 570 nm with an ELISA plate reader (Bio-Rad, USA). Cell viability was calculated as follows:

$$\% \text{ viability} = \frac{\text{optical density of sample}}{\text{optical density of control}} \times 100\%.$$

CELL CYCLE ANALYSIS

Cell cycle analysis was performed by PI staining at IC_{10}, IC_{25} and IC_{50} of EGCG following method from Zakaria et al. (2009). Imatinib was used as the positive control at IC_{25} and IC_{50} value. 1×10^5 K562 cells were treated for 48 hours. After 48 hours, the cells are collected, centrifuged (2500 rpm, 5 minutes, 4°C), fixed in 70% ethanol and then centrifuged again. Subsequently, cells were stained with 500 µL of PI solution (BD Bioscience, USA) for 15 minutes at room temperature. Stained cells were analyzed using FACSCanto II flow cytometer (BD Bioscience, USA) installed with ModFit LT (Verity Software House).

ALKALINE COMET ASSAY

Alkaline comet assay was used to detect DNA damage [15] at IC_{10}, IC_{25} and IC_{50} of EGCG treated cells. Imatinib was used as the positive control at IC_{25} and IC_{50} value. A total of 3 mL of K562 cells at 1×10^5 cells/mL was incubated for 24 hours and were then treated with EGCG and Imatinib. All cells were then incubated for 48 hours. Prior to incubation, cells were collected and centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and this process was repeated. The low melting point agar (LMPA) and normal melting point agar (NMPA) (Sigma Aldrich, USA) were preheated until the agar melted. When the NMPA reached a temperature of 37°C, a total of 100 µL of NMPA was pipetted onto a frosted slide and a coverslip (50 mm x 22 mm) was placed on top of the slide. The coverslip was then taken out slowly after the agar hardened. Next, about 80 µL of LMPA was added into each sample tube on top of the prepared NMPA slide and a coverslip was placed slowly onto the two layers of gel. The coverslip was placed in a coplin jar filled with the lysing solution and cooled at 4°C overnight before the slides were placed on the electrophoresis tank. Electrophoresis buffer was added into the tank to immerse the slide for 20 minutes. The electrophoresis process was carried out for 20 minutes at 25 V and 300 mA after which the slides were rinsed with neutralizing buffer three times for every 5 minutes. The slides were then stained with 50 µL of ethidium bromide (Sigma Aldrich, USA) at 20 µg/mL. The single and isolated cell for every slide were analysed for any DNA damage under a fluorescent microscope (Biochrom ASYS,UK).

STATISTICAL ANALYSIS

All data were analysed by using SPSS Software Version 22. All data were done in triplicate and were expressed as the mean ± S.E.M. from three different experiments. One-way ANOVA and Tukey post hoc test were used to measure statistical differences between the mean in all experiments. The statistical difference was indicated with value $p<0.05$.

RESULTS

EGCG AND IMATINIB EXERT CYTOTOXICITY AGAINST CHRONIC MYELOID LEUKEMIA K562 CELLS

As shown in Figure 1, a significant cytotoxic effect of EGCG and Imatinib in K562 human myeloid leukemia cells was noted. After exposed to EGCG for 48 hours, K562 cells showed a significant decreased ($p<0.05$) in viability with increasing concentration. Treatment with EGCG with the lowest dose at 3.125 µg/mL reduced the cell viability from 96.43 ± 2.44 % to 37.23 ± 3.88 % at the highest dose (100 µg/mL) and the IC_{50} of the treatment is 50 ± 1.93 µg/mL. Treatment with Imatinib as positive control showed a significant decreased ($p<0.05$) in viability with increasing concentration as well. The cell viability with Imatinib reduced from 55.29 ± 0.34 % to 3.12±1.46 %.

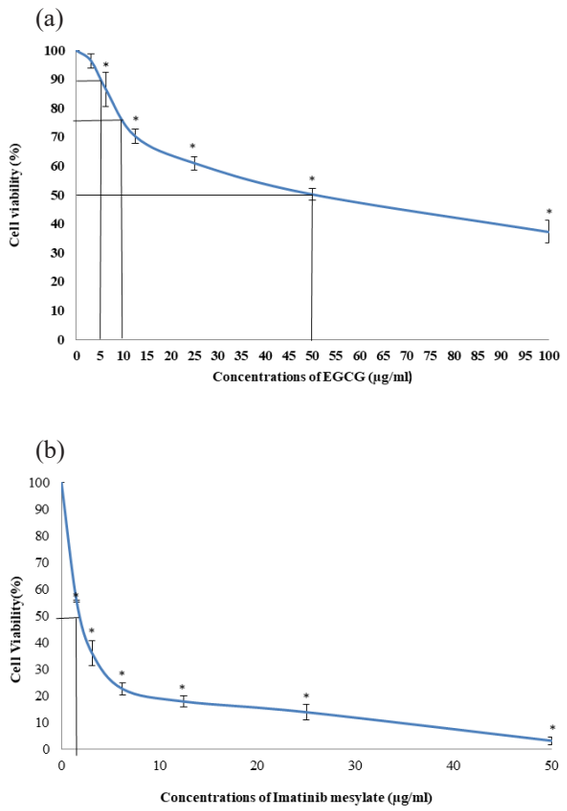
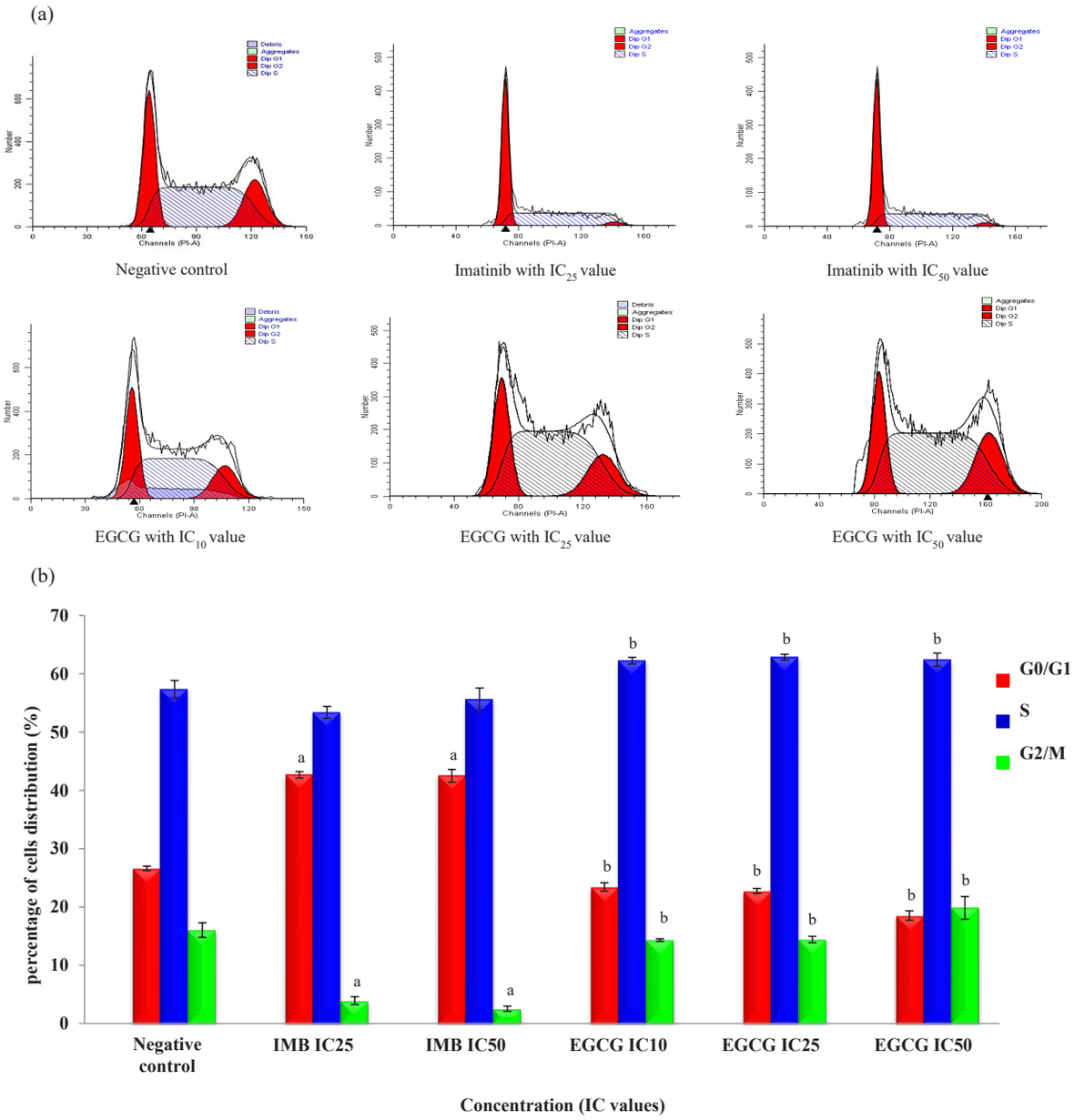


FIGURE 1. EGCG and Imatinib exerted cytotoxic effect on K562 human myeloid leukemia cell line. Percentage viability of a) EGCG and b) Imatinib following 48 hours treatment. IC_{10}, IC_{25} and IC_{50} values for EGCG on K562 cells are 5 ± 2.44 µg/mL, 10 ± 5.93 µg/mL and 50 ± 1.93 µg/mL respectively. Meanwhile, IC_{10}, IC_{25} and Imatinib are 0.2 ± 0.0µg/mL, 1 ± 0.34µg/mL and 2 ± 0.0 µg/mL respectively. Results are expressed as the means ±SEM of three independent experiments. An asterisk * indicates statistically a significant different from untreated control ($p<0.05$)

EFFECT OF EGCG AND IMATINIB ON CELL CYCLE DISTRIBUTION

Cell cycle analysis was performed to elucidate the involvement of cell cycle arrest in the EGCG mechanism of action. Following 48-hour treatment with the IC_{10}, IC_{25} and IC_{50} values of EGCG, G0/G1 population of the K562 cells (23.44 ± 0.71 %, 22.74 ± 0.43 % and 18.53 ± 0.81 %) was lower than that of the negative control (26.61 ±

0.39 %). However, this reduction is insignificant ($p>0.05$) as compared to negative control. An insignificant ($p>0.05$) increase in the S phase population is noted as well. The cell number percentage of S phase increased from 57.35 ± 1.52 % to 62.25 ± 0.57 %, 62.85 ± 0.50 % and 62.42 ± 1.1 %, respectively. For G2/M population, the cells is lower in IC_{10}, IC_{25} (14.31 ± 0.21 % and 14.41 ± 0.55 %) and higher in IC_{50} values of EGCG (19.84 ± 1.95 %) as compared to negative control (16.03 ± 1.26 %). As for the Imatinib, our result showed a high distribution of cells in G0/G1 population and significant decreased ($p<0.05$) in G2/M population suggesting an arrest at S phase. Overall, this finding suggests that EGCG does not have a significant effect on the cell-cycle distribution of K562 cells (Figure 2).



a Significant differences in cells distribution as compared to negative control ($p<0.05$)
b Significant differences in cells distribution as compared to positive control ($p<0.05$)

FIGURE 2. Effect of EGCG and Imatinib on the cell cycle distribution of K562 cells. Histogram (a) represents cell cycle distribution by flow cytometry, and bar graph (b) represents the percentage of cells in every phase. Cells were exposed to IC_{10}, IC_{25} and IC_{50} values of EGCG for 48 hours and harvested. The cells were fixed and stained with propidium iodide and the DNA content was analyzed by flow cytometry. Results showed insignificant changes in cell cycle distribution with EGCG treatment as compared to negative control. Meanwhile, there is an arrest at S phase with Imatinib treatment. Results are expressed as the means ±SEM of three independent experiments

EGCG INDUCED DNA DAMAGE IN K562 CELLS

Figure 3 presented tail moment of K562 cells after treatment with EGCG at IC_{10}, IC_{25} and IC_{50} values for 48 hours and Figure 4 presented percentage of DNA in tail after treatment with EGCG at IC_{10}, IC_{25} and IC_{50} values for 48 hours. One-way ANOVA analysis displayed a significant ($p<0.05$) increased of both K562 cell tail moment and percentage of DNA in tail when compared to the negative control (0.14 ± 0.02 A.U and 3.33 ± 0.38 %) which were 15.55 ± 1.55 A.U, 26.69 ± 2.45 A.U, and 63.16 ± 4.2 A.U, and 14.55 ± 1.10 %, 23.28 ± 1.51%, and 35.83 ± 1.75 %, respectively. The value of tail moment and percentage of DNA in tail increased as the concentration increased. The positive control, Imatinib at IC_{25} and IC_{50} exhibited significant increased ($p<0.05$) in the tail moment and the percentage of DNA in tail of K562 cells at 23.02 ± 2.23 A.U and 51.63 ± 4.31 A.U. , and 22.22 ± 1.56 % and 29.60 ± 1.90 %, respectively.

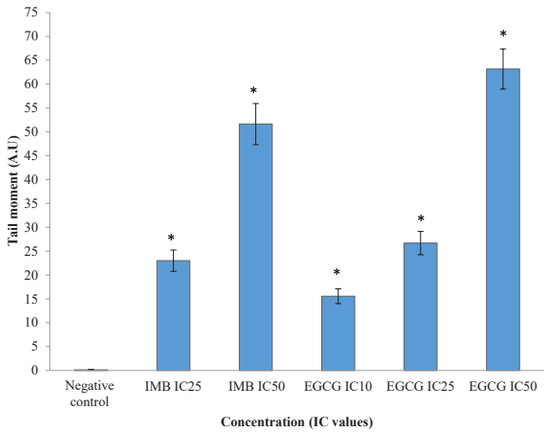


FIGURE 3. Tail moment of K562 cells after 48 hours of treatment with EGCG and Imatinib. There is significant ($p<0.05$) increased in the tail moment at IC_{10}, IC_{25} dan IC_{50} values as compared to the negative control (0.14 ± 0.02 A.U) at 15.55 ± 1.55 A.U, 26.69 ± 2.45 A.U, and 63.16 ± 4.2 A.U respectively. Treatment with Imatinib showed significant ($p < 0.05$) increased in tail moment at IC_{25} and IC_{50} values at 23.02 ± 2.23 A.U and 51.63 ± 4.31 A.U as compared to the negative control. Results are expressed as the means ±SEM of three independent experiments. An asterisk * indicates statistically significantly different from untreated control ($p<0.05$)

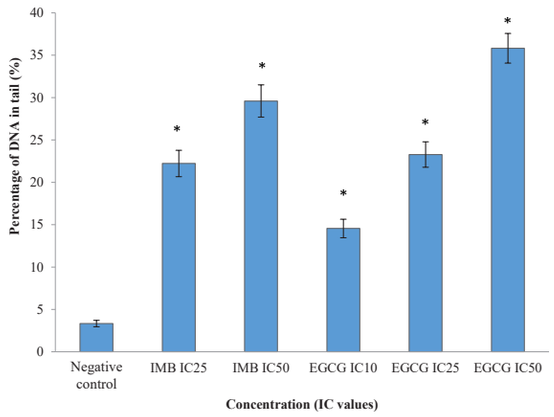


FIGURE 4. Percentage of DNA in tail of K562 cells after 48 hours of treatment with EGCG and Imatinib. There is significant ($p<0.05$) increased in percentage of DNA in tail at IC_{10}, IC_{25} dan IC_{50} values as compared to negative control (0.14 ± 0.02%) at 14.55 ± 1.10 %, 23.28 ± 1.51%, dan 35.83 ± 1.75 % respectively. Treatment with Imatinib showed significant ($p < 0.05$) increased in the tail moment at IC_{25} and IC_{50} values at 22.22 ± 1.56% dan 29.60 ± 1.90 U as compared to the negative control. Results are expressed as the means ±SEM of three independent experiments. An asterisk * indicates statistically significantly different from untreated control ($p<0.05$)

DISCUSSION

The search for an alternative treatment in order to have a good diagnosis and prognosis of leukemia with specific treatment and less adverse effect has attracted great interest recently, particularly from natural products. Anti-leukemic agent from natural products and its derivatives are considered as more effective in achieving good prognosis (Dirsch et al. 1998; Lin et al. 2005; Jia et al. 2009; Abdellatef et al. 2010; Khan et al. 2010' Qi et al. 2010; Choi et al. 2011). This study was using Epigallocatechin-3-gallate (EGCG), a derivative extracted from green tea, to determine cytotoxic, genotoxic effect and cell cycle analysis against human myeloid leukemia K562 cells.

In the present study, EGCG exerted a cytotoxic effect against K562 cells, as demonstrated by MTT assay (Figure 1). Our result showed that EGCG reduced cell viability in a dose-dependent manner as compared to Imatinib Mesylate, positive control used in this study. These findings demonstrated that EGCG exerted an antiproliferative effect in parallel with the primary treatment of CML which is Imatinib mesylate. It supports the facts that suggesting the usage of EGCG as one of the anti-cancer agents (Singh et al. 2011; Yang et al. 2011).

Cell cycle analysis was performed to investigate the possible events that occurred before cell death since our previous study concluded apoptosis as the primary mode of cell death in K562 after treated with EGCG. EGCG treatment decreased the G0/G1 and G2/M population in K562 cells. In addition, our result showed there is an increased in S phase population in K562 cells. However, these changes in these three phases are not significant as compared to negative control. This result suggests the possibility of a cell cycle arrest at S phase indicates there was a temporary arrest in the cell cycle due to DNA damage and with no specific mechanism the cell managed to progress through the next phases. In order to have further understanding of this occurrence, further research at the molecular level needs to be done particularly regarding the expression of protein regulators involved in modulation of the cell cycle. A study on the ethyl acetate extract of *Dillenia suffruticosa* reported a similar trend of cell cycle distribution in MCF-7, human breast cancer cells (Tor et al. 2014) and another study on the extract of *Clinacantus nutans* also showing similar trend in HeLa, cervical cancer cell line (Mohd Roslan et al. 2018).

Cytotoxic effect induced by EGCG towards K562 cells may be caused by DNA damage and the detected genotoxicity might be the early mechanism of cell death via apoptosis. Alkaline comet assay was used to detect the genotoxic effect of the tested compound by measuring its DNA damage at a single cell that can be observed under a fluorescent microscope with a comet head (nucleus) and its tail (DNA fragments). In this study, the parameters used to determine the DNA damage are tail moment and percentage of DNA in tail. Among the frequently used comet parameters, the percentage of DNA in tail and tail moment could offer the most precise result for the degree of damage (Olive & Banath 2006).

In this study, increased in tail moment and percentage of DNA in tail were observed indicating there was induction of DNA damage by EGCG against K562 cells. DNA is the key target by most cytotoxic anticancer drugs. The anti-cancer agent could act directly through reactive metabolites or indirectly through the incorporation into DNA nucleotide analogues or by blockade of DNA-metabolizing function such as DNA polymerase or topoisomerase (Roos & Kaina 2013). Nonetheless, the mechanism and type of DNA lesion of K562 cells induced by EGCG need further investigation to truly understand the mechanism of DNA damage that leads to apoptosis.

In summary, our data show that the mechanism of anti-cancer exerted by EGCG is by decreasing the cell viability and inducing DNA damage, which then lead to apoptosis through a non-cell cycle specific pathway. This mechanism of inducing apoptosis through a non-cell cycle specific pathway is similar to the finding from a study by Tor et al. (2014). This study suggested that apoptosis occurs due to the modulation of gene through oxidative stress pathway (Tor et al. 2014).

CONCLUSION

Our study emphasized the involvement of cytotoxic and genotoxic effect of EGCG through non-cell cycle specific in inducing apoptosis. This preliminary study needs further attention in order to gain a full understanding of possible mechanisms of action of EGCG to strengthen its therapeutic value as a leukemia treatment.

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CONFLICT OF INTEREST

The authors declared no potential conflict of interest with respect to the authorship and/or publication of this article.

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