Tea Constituent in Protecting Glyphosate Effect on Human Breast Cancers Cells (MCF-7) Growth

Irmanida Batubara¹, Rohayati Suprihatini², Silmi Mariya³, Suminar S Achmadi¹, Valentina Sokoastri², Agustin Sri Mulyatni², and Arif Rakhman Hakim²

¹Tropical Biopharma Research Center and Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Dramaga Campus, Bogor 16680, Indonesia
²PT Riset Perkebunan Nusantara, Jalan Salak No. 1A, Bogor 16128, Indonesia
³Primate Research Center, IPB University, Cilibende Campus, Jalan Lodaya II/5, Bogor 16151, Indonesia

Email: ime@apps.ipb.ac.id

Article Info
Received: June 23, 2023
Revised: August 15, 2023
Accepted: October 12, 2023
Online: November 15, 2023


Doi: 10.15408/jkv.v9i2.33229

Abstract
Glyphosate, which has been documented as a contaminant in tea, promotes the proliferation of human breast cancer cells (MCF-7). Tea, on the other hand, includes various antioxidants, including epigallocatechin gallate (EGCG), which may protect against cancer cell proliferation. The purpose of this research is to determine the preventative effect of concentrated brewed green tea on MCF-7 development caused by glyphosate. The glyphosate concentration that promotes MCF-7 development was determined using a serial concentration of glyphosate. Glyphosate concentrations of up to 64 mg/L were shown to have no effect on MCF-7 cell proliferation. Concentrated brewed tea and EGCG 200 mg/L have the potential to suppress MCF-7 cell proliferation in the presence of glyphosate up to 512 mg/L. The combination of glyphosate and concentrated brewed tea or EGCG protects against glyphosate toxicity via altering the expression of tumor suppressor protein (p53).

Keywords: glyphosate, EGCG, tea, human breast cancer cell, p53

1. INTRODUCTION
Glyphosate is the most weedkiller used globally ¹ ² and contributes to the economic importance for agriculture ³; it is known as a non-selective herbicide commonly used in croplands, including in tea plantation. Glyphosate, N-(phosphonomethyl)glycine, C₆H₁₇N₂O₅P, was then reclassified into Group 2A (probably carcinogenic to humans) by The International Agency for Research on Cancer (IARC) at the World Health Organization (WHO) ⁴ ⁵ and the more stringent regulations were implemented to set the maximum levels for glyphosate in agriculture products ⁵.

The carcinogenicity of glyphosate was reported by some researchers, such as toward human fibroblast ⁶, human fibrosarcoma (HT1080) ⁷, and Hep-2 cells ⁸. Glyphosate also exhibits cytotoxic and genotoxic effects in normal human cells (GM38) ⁷, hepatic (HepG2), embryonic (HEK293), and placental (JEG3) cell lines ⁹. On the other hand, an epidemiology study showed that the high glyphosate pollution in the Argentine agricultural village is not associated with increased cancer frequency ¹⁰. The same result is also found in the agricultural health study ¹¹ which showed no association between glyphosate and any solid tumors or lymphoid malignancies overall, including non-Hodgkin lymphoma (NHL) and its subtypes. Recently EFSA published its Reasoned Opinion Review of the existing maximum residue levels for glyphosate according to Article 12 of Regulation (EC) No 396/2005 ¹². The opinion foresees a decrease in the MRL for the herbicide glyphosate in tea (Camellia sinensis) from 2.0 mg/kg to 0.05 mg/kg, which will cause severe problems in practice.

Tea is the most consumed beverage due to its rich antioxidant content. Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS) ¹³. The polyphenols, including catechins, theaflavins, and thearubigins in tea, contribute to tea’s health
The high concentration of Camelia sinensis was then diluted three times with mL of drinking water following the SNI standards. A total of 45 g of green tea was brewed in 100 mL of water to get a high concentration of tea. The high concentration of brewed tea treats several health problems such as diarrhea and food poisoning. Tea is used for health problems due to its active compounds, including antioxidant compounds such as epigallocatechin gallate (EGCG). EGCG is also reported as the most cancer chemoprevention (Du et al., 2012) and anti-cancer, including those against breast cancer. Based on previous reports, EGCG can induce apoptosis in breast cancer (MCF-7) cells by downregulating the P53/Bcl-2 signaling pathway.

This research focuses on breast cancer because breast cancer is the most diagnosed cancer for females. A recent report reveals that exposure to aminomethylphosphonic acid (AMPA), the primary metabolite of the herbicide glyphosate, may be associated with increased breast cancer risk. Previously, glyphosate was reported could induce human hormone-dependent breast cancer, T47D cells, but not induced hormone-independent breast cancer, MDA-MB23. This study aimed to determine the protective effect of EGCG and green tea brewing against glyphosate in various concentrations on MCF-7 cell growth. The possible mechanism was determined by the expression level of P53, PCNA, CASPASE-1, BAX, and BCL-2.

2. RESEARCH METHODS
Tea leaves collection and tea brewing preparation

The tea (Camelia sinensis) Assamica Variety used in this research was chosen deliberately from the most prominent green tea producer in Cianjur, West Java, Indonesia. The samples were taken from production in February 2019 following the Indonesian National Standard. A total of 45 g of green tea was brewed in 100 mL of drinking water following the SNI standards (heated at 80 °C for 3 minutes). This solution (45 g/100 mL w/v) was then diluted three times with the cell's growth medium to reach a so-called 15 times concentrated brewed tea (CGBT).

Determination of catechins in tea and brewed tea

The catechins in the tea and the EGCG content in the brewed tea were determined using high-performance liquid chromatography (HPLC). Hitachi LaChrom HPLC was used with column C18 (5 μm) and eluent A was 0.05% H3PO4 (v/v) and eluent B was CH3OH/CH3CN with ratio of 3:2, with gradient elution system of B 10-25% from 0 to 15 min, 25-60% from 15 to 25 min, and 60-100% from 25 to 40 min. The flow rate was 1.0 mL/min, column temperature of 40 °C, UV detection at 280 mm, and injection volume of 10 mL. All standards (gallic acid, catechin, EGCG, ECG, and EGC) were in analytical grade from Sigma Aldrich. The catechins content was determined by comparing the peak area of sample to the standard.

Determination of glyphosate concentration to give impact on MCF-7 cells viability based on the MTT assay

Glyphosate (CAS 1071-83-6) was purchased from TianfuChem. The effect of glyphosate concentration was determined on the breast cancer cell line, MCF-7, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), incubated in 5% CO2 humidified at 37°C. Viable cells (5×10^3 cells/well) were cultured in a 96-well plate and incubated for 24 hours. The following day, 100 μL various glyphosate concentrations (64, 32, 16, 8, 4, 2, and 1 ppm) were added, and the cells were allowed to grow. After 48 hours, 10 μL MTT was added to each well, and the mixture was incubated for 4 hours. After completing the incubation, the supernatant was discarded, and 100 μL of HCl 1N in isopropyl alcohol was added to the mixtures. Reading at 595 nm using a microplate reader to get the maximum concentration that impacts MCF-7 viability.

Cell counting on EGCG and CGBT treatment

The MCF-7 cells (1×10^4) were separately introduced in a 6-well plate and incubated for 24 hours in 5% CO2 humidified at 37°C. Glyphosate at the optimum concentration and were added to the cells. The protective effect was determined by adding CGBT and EGCG 200 ppm in separate wells. The mixture was then incubated for 48 hours in 5% CO2 humidified at 37°C. The supernatant was removed and resuspended in 5 mL phosphate-
buffered saline twice. Trypsin was added to each well and incubated for 5 minutes. The cells were again resuspended to ensure single-cell suspension was formed. Hemacytometer was used to confirm single-cell suspension; viable cells were calculated using trypan blue and prepared for molecular analysis.

**RNA Isolation and Real-Time PCR assay**

RNA was isolated from the treated and untreated cells using RNeasy Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was measured by spectrophotometer (SmartSpec-Plus, Bio rad) at \( \lambda = 260 \text{ nm} \). Real-time PCR (RT PCR) was performed on an IQ5 Multicolor Real-Time PCR Detection System (Bio rad) to analyze the expression level of P53, PCNA, CASPASE-1, Bax, and Bcl-2 relative to the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

The primer sets used for the amplification of the target gene are listed in Table 1. RT-PCR analysis was performed in a final volume of 25 μL containing 5 μL RNA template (equivalent to 400 mg of RNA), 1 μL of each forward/reverse primer, 12.5 μL of 2 × SYBR Green RT-PCR reaction mix, 0.5 μL of reverse transcriptase, and 5 μL of nuclease-free water. The cycling condition was: 50 °C, 10 min for activation of reverse transcriptase, 95℃, 5 min for inactivation of reverse transcriptase, and 40 cycles at 95℃ for 10 s, 52℃ for 10 s, and 72℃ for 10 s. The software automatically sets the baseline and the threshold. The crossing point of the amplification curve with the threshold represents the cycle threshold (Ct). Relative gene expression was calculated. All samples were normalized into the equivalent level of GAPDH.

**MTT Assay on EGCG and tea brewing treatment at higher concentration of glyphosate**

Using the same MTT method as previously described, the protective effect of EGCG and CGBT caused by the higher concentration of glyphosate was determined. The glyphosate concentrations were 128, 256, and 512 ppm. In the presence of glyphosate at high concentration, EGCG at 200 ppm and CGBT were added, and the cell viability was determined.

**Data Analysis**

Each experiment was carried out in triplicate. The viability percentage of the treated and untreated cells was calculated according to the formula as follows:

\[
\text{Percentage of viability (\%) = } \frac{\text{Absorbance of the treated cells}}{\text{Absorbance of the control cells}} \times 100 \%
\]

The crossing point of the amplification curve with the threshold represents the cycle threshold (Ct). Relative gene expression was calculated. All samples were normalized into the equivalent level of GAPDH.

**3. RESULTS AND DISCUSSION**

The green tea utilized in this study is a typical tea plant clone developed in Indonesia, which has a high catechin content and won an Innovative Idea Award from The International Society of antioxidants in Nutrition and Health in 2009 for its high catechin content tea clones. Cross-pollination between local tea clones produced the Gambung (GMB) high catechin tea clones series. GMB1 (Gambung 1), GMB2, GMB4, GMB6, GMB9, and GMB10 high catechin tea clones were found in 1988 by the Indonesia Research Institute for Tea and Cinchona/IRITC. The concentration of catechins in green tea used in this research was determined, and the results are summarized in Table 2. The tea used in this research has a total catechin content of approximately 30% of the tea (dry-base). Among the five types of catechins, EGCG is the highest content, showing about 60% of the total catechins. These results showed that 45 g green tea leaves consist of 8 g EGCG. Based on HPLC data, EGCG content in CGBT was 1.07%. It means only 12.5% of EGCG in the tea leaves could transfer into concentrated tea brewing.

Under the glyphosate concentrations ranging from 1 to 64 ppm, MCF-7 cells still grow
well (cell viability from 85 to 100%). It means that glyphosate has no anti-proliferative activity against MCF-7 cell growth (Figure 1). Therefore, we used the maximum glyphosate concentration of 64 mg/L to determine the combined effect of EGCG and CGBT.

The cell morphology of all treatments is shown in Figure 2. The cells showed normal and epithelial-like morphology when untreated (control cells) and under glyphosate-treated at 64 mg/L. It clearly shows that glyphosate proliferates the breast cancer cells and has potency as a carcinogenic agent. However, when the glyphosate was combined with CGBT and EGCG, the cells were released from attaching to the plates, formed single cells, and shrunk. The abnormality of the cells was clearly observed.

### Table 2. Catechins content on green tea sample

<table>
<thead>
<tr>
<th>No</th>
<th>Catechins</th>
<th>Retention time (minutes)</th>
<th>Concentration (%) tea dry basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gallic acid</td>
<td>4.109</td>
<td>0.60</td>
</tr>
<tr>
<td>2</td>
<td>Catechin</td>
<td>13.204</td>
<td>8.82</td>
</tr>
<tr>
<td>3</td>
<td>Epigallocatechin gallate (EGCG)</td>
<td>18.142</td>
<td>17.78</td>
</tr>
<tr>
<td>4</td>
<td>Epigallocatechin (EGC)</td>
<td>10.658</td>
<td>1.69</td>
</tr>
<tr>
<td>5</td>
<td>Epicatechin gallate (ECG)</td>
<td>16.256</td>
<td>1.34</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>29.63</td>
</tr>
</tbody>
</table>

![Figure 1. MCF-7 cell viability on various concentrations of glyphosate](image)
Figure 2. Cell’s morphology showed epithelial-like when the glyphosate was added. A. Untreated cells, B. Glyphosate 64 mg/L, C. Glyphosate 64 ppm + CGBT, D. Glyphosate 64 ppm + EGCG 200 mg/L.

Figure 3. The level of viability of human breast cancer cells (MCF-7) by treating glyphosate 64 ppm, glyphosate 64 ppm + CGBT, and glyphosate 64 ppm + EGCG 200 ppm.

Based on the cells counted using a hemocytometer, the number of MCF-7 cell growth increased in the presence of glyphosate 64 mg/L compared to the control cell (Figure 3). This result is in accordance with a previous report that glyphosate, AMPA, and glyphosate-based formulations can induce oxidative stress. The IARC WG concluded that glyphosate is a ‘probable human carcinogen,’ putting it into IARC category 2A due to sufficient evidence of carcinogenicity in animals, limited evidence of carcinogenicity in humans, and substantial evidence for two carcinogenic mechanisms.

The antioxidant in tea has a potency to inhibit the growth of MCF-7 cells and can protect the effect of glyphosate. The cell viability demonstrated a higher value than cell control (135%) when the cells treated with glyphosate 64 ppm, but it decreased when green tea (8%) and EGCG 200 mg/L (5%) were added (Figure 3).

The presence of p53 shows that cell growth is being inhibited via the apoptotic pathway. To maintain genomic integrity, the tumor suppressor protein p53 activates cell cycle checkpoints, DNA repair pathways, and apoptotic responses. DNA-damaging agents boost p53 levels, which play an important role in directly activating the proapoptotic pathway. According to the RNA expression (Figure 4), cells treated with glyphosate, glyphosate + EGCG, and glyphosate +...
CGBT have higher p53 expression than the control. Glyphosate + CGBT cells had the greatest p53 expression of the three treatments. The expression suggests that proapoptotic proteins outnumber anti-apoptotic proteins, making cells more sensitive to apoptosis.

The ratio of proapoptotic protein (BAX) to anti-apoptotic protein (BCL2) is a regulator that regulates the ease with which cells undergo apoptosis. The BAX gene activates the apoptotic program, which is critical in the cell's response to the chemical. Apart from apoptotic events, the BAX/BCL2 ratio in the combination of glyphosate + CGBT and glyphosate + EGCG have a lower value than the BAX/BCL2 ratio in glyphosate alone. The degree of Caspase 1 expression, which is a hallmark of necrosis, suggests that there is necrosis in the treatment of samples on cells.

PCNA participates in the DNA repair process. Because cells spend a lengthy period in the G1/S phase of the cell cycle, gene expression is utilized as a measure of cell proliferation. Growth factors or DNA damage can cause an increase in expression level during the cell cycle. Because the cells were unable to repair DNA, the combination of glyphosate with EGCG and CGBT did not cause apoptosis in MCF-7 but did lower cell viability. The PCNA value is higher in combination (with CGBT and/or EGCG) than in glyphosate alone. These chemicals are hypothesized to be capable of suppressing ROS generation in response to oxidation induction, allowing for the antioxidant effect of the treatments.

Based on the effectiveness of EGCG and CGBT in inhibiting MCF-7 proliferation, the experiment on a higher concentration of glyphosate was performed. The results are shown in Figure 5. The 200 mg/L EGCG inhibits the MCF-7 growth by about 40-50% in the presence of glyphosate at high concentrations (128, 256, and 512 mg/L). By increasing the concentration of glyphosate, the inhibition activity is decreasing. The CGBT displays better inhibition activity compared to 200 mg/L EGCG. The CGBT inhibits more than 90% of MCF-7 proliferation in the presence of a higher glyphosate concentration. The CGBT is more potent in protecting the proliferation effect of the glyphosate compared to the EGCG alone, as the EGCG content in CGBT is 50 times higher. An antioxidant tea dose of 200 ppm EGCG which is a drinking dose of one cup of Indonesian green tea, can also neutralize and even inhibit the growth of MCF-7 breast cancer cells due to glyphosate contaminants.

Figure 4. mRNA expression on the cells treated by glyphosate, glyphosate plus EGCG, and glyphosate plus CGBT.
4. CONCLUSIONS

The presence of glyphosate (up to 64 mg/L) did not inhibit the MCF-7 cell growth. The concentrated brewed green tea and its bioactive compound (EGCG) could prevent the effect of glyphosate by modulating the expression of tumor suppressor protein (p53). The concentrated brewed tea and EGCG 200 mg/L inhibit the MCF-7 cell growth in the presence of glyphosate up to 512 mg/L. Therefore, there is no need to worry about the glyphosate present in tea as a cause of MCF-7 proliferation.

ACKNOWLEDGMENT

This study is a project funded by the Indonesia Endowment Fund for Education (LPDP) in 2018-2019 (Contract Number PRJ-35/LPDP/2018). Appreciation goes to Dewan Teh Indonesia (the Indonesia Tea Board) for the meaningful collaboration in implementing the study.

REFERENCES


Batubara et al. | 278


