Autophagy or Apoptosis: Anticancer Molecular Mechanism of Epigallocatechin Gallate with Natural Polyphenol Effect on HepG2 Cells Viability

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Abstract

The anticancer impact of Epigallocatechin gallate (EGCG) the highly active polyphenol of green tea was abundantly studied. However, the exact mechanism of its cytotoxicity is still under investigation.

The current study was designed to investigate the effects of EGCG on thirteen autophagy- and/or apoptosis-related genes in HepG2 cells.

The apoptosis detection analyses such as flow cytometry and dual apoptosis assay were used on HepG2 cells. The genes expression profile of HepG2 cells was explored using the real-time quantitative-PCR. EGCG increases G0/G1 cell cycle arrest and the real-time apoptosis markers proteins leading to stimulate apoptosis in 70% of the treated HepG2 cells. The down-regulation was recorded in two of autophagy inhibitory genes (FOS, MAPK9) and the gene that implicated in protein biosynthesis and protein modification (ITGB1). The genes that have pro-apoptotic function in cells (CAPNS1, CFLAR, EIF4G, and RB1) were also showed down-regulation after treatment.

Thus, the results demonstrated a potential effect of EGCG to induce apoptosis rather than autophagy in the treated HepG2 cells that could play as good targeted therapy.

Keywords: EGCG; Apoptosis, Autophagy, HepG2, Cancer Cell.

Introduction

Epigallocatechin gallate (EGCG) is a flavonoid that proved its therapeutic effect in cancer (1). It is the most active polyphenol in green tea, which has anti-inflammatory, antioxidant (2), and protective for cardiac muscle (3,4). Many in vivo and clinical studies (Phase I/II) have acclaimed that EGCG is an efficient agent and quite safe in providing protection against cancer (5). The health benefits of green tea, particularly the positive role of EGCG in stimulating autophagy in the treatment and prevention of liver cancer was proved (6).

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Moreover, EGCG mediated cell death in glioblastoma cell line and cell line of the no small cell lung cancer by an induction to one of the apoptosis pathways (1,7).

In cancer cells, apoptosis and autophagy play a critical role in the biological process of cancer (8). Apoptosis (the programmed cell destruction) has a significant role in the treatment of cancer as it is a common target of many treatment strategies (9). Autophagy has dual effect on tumorigenesis by suppressing the survival of cancer-cell and stimulating cell death. In addition, it accelerates tumorigenesis by inducing cancer-cell proliferation and tumor growth (10).

Natural products-promoted autophagy is mostly causing death of tumor cells when apoptosis is totally defectve in cancer cells (11). The increment of apoptosis resistant has been proved among cancer cells together with a high rate of adverse events associated with chemotherapy/radiotherapy. Highlight the needs to consider the activity of the natural products in regulating the cancer development events with focus on autophagy.

The purpose of the current study is to explore the effect as anticancer agent on HepG2 cells by exploring certain genes that were not investigated before. It intended to investigate the role of EGCG as a tumor-suppressor in treating or preventing hepatocarcinoma. The aim of this study was conducted by using DNA analysis to explore the autophagy- and apoptosis-related genes’ expression namely (FOS-1, FOS-2, DDIT3, BNIP3, BNIP3L, BIRC5, MAPK9, ITGB1, CAPNS1, CFLAR, EIF4G, NBR1, and RB1).

Materials and Methods

The materials:

The following materials were purchased from Sigma, Germany: (-)-Epigallocatechin gallate (EGCG, minimum 95%, E4143-50MG), fetal bovine serum, dimethyl sulfoxide (DMSO), and Ribonuclease A. RPMI-1640 medium with L-glutamine, amphotericin B, and penicillin-streptomycin were from Biowest, Florida, USA. The Proliferation Assay of Cell Titer 96 Aqueous One Solution Cell was from Promega, USA. Propidium iodide was from MP Biomedicals, LLC, IIIKrick, France). Apoptosis Assay Kit (Dual), NucView™ 488 was from Biotium Inc, USA. Nucleic acid extraction kit GF-1 was from (Vivantis Technologies, Malaysia). The iScript cDNA synthesis kit was from (BIO-RAD, Hercules, Canada). RT-PCR master mixes were from Life Technologies, USA.

The compound preparation and cell culture

EGCG was dissolved in an absolute DMSO giving a stock concentration of 50mg/ml and incubated at -20°C for subsequent analyses. The cell line: Hepatocellular carcinoma cells (HepG2; ATCC HB-8065), were cultivated in RPMI-1640 medium with 10% fetal bovine serum, 2.5 μg/ml of amphotericin B, and 50 U/ml penicillin-streptomycin at 37°C with 5% CO₂.

Cell cytotoxicity assay

The cytotoxic effect of EGCG on HepG2 cells was measured. Freshly prepared EGCG working concentrations of (10, 20, 40, 80, and 160 μg/ml) were diluted in 10% RPMI-1640 medium. HepG2 cells (1 × 10⁵ cell/well) were treated with each concentration (200 μl/well) and incubated for 48 h in 5% CO₂ at 37°C. A negative control composed of DMSO in 10% RPMI-1640 medium was used. Later, a solution composed of 10% RPMI-1640 medium (100 μl) and MTS (20μl) was used to replace the wells’ contents. The absorbance at 490 nm, after 4 h was read by ELISA reader (Sunrise Basic Tecan, Grödig, Austria). The compound concentration that destroyed 50% of the cell line (IC50) was calculated using the following formula (12):

\[
\text{Cytotoxicity %} = \frac{1 - (\text{ODt}/\text{ODc})}{100}
\]

In which (ODt) represents the optical density of the tested compound and (ODc) represents the optical density of the negative control.

Treatment of HepG2 cells

Based on EGCG IC50 value obtained from the MTS assay, duplicates of (1 × 10⁵ HepG2 cell/well) was treated with10% RPMI-1640 medium with or without EGCG IC50 for 48 h. Both EGCG-treated HepG2 cells and untreated cells were harvested and washed by 10% RPMI-1640 medium, after the end of the incubation time. The resulting cells were subjected to the following assays.

Analysis by the flow cytometry

The treated and untreated cells were analyzed to explore the apoptosis level of cells and cell cycle arrest. The cells were fixed as (1:10 v/v) of ice-cold ethanol 70% with PBS. The fixed cells were incubated for 2h at −20°C and after that washed by PBS. Staining DNA solution (500 μl) in PBS was prepared, which composed of Ribonuclease A (50 μl) as 1 mg/ml and propidium iodide (25 μl) as 1 mg/ml (13). The flow cytometry CyAn ADP apparatus was used for measurement (BECKMAN COULTER, USA). Analysis was performed in triplicates of each sample and control (untreated cells) using the software Summit (V4.3).

A real-time detection of apoptosis

A Dual Apoptosis Assay Kit was used along with sulforhodamine 101 (Texas Red™) conjugated Annexin V and caspase-3 substrate. This kit provides two real-time apoptosis events in intact cells, phosphatidylserine translocation and caspase-3 activity. Based on the instructions of the manufacturer, an aspiration of the wells’ contents was done to wash the treated and untreated cells with Annexin V-binding buffer. Later, the following materials were added to each well and incubated for 45 minutes: sulforhodamine 101-annexin V (5 μL), Annexin-binding buffer (100 μL), and 0.2 mM...
NucView™ 488 caspase-3-substrate (5 μL). Fluorescence microscopy (Nikon Eclipse 80 i) using FITC and Texas-Red filters, was used to observe the apoptotic events in the stained cells which were performed in triplicates. The apoptotic index was determined as apoptotic cells’ number as proportion of the total number of cells.

The autophagy - and apoptosis-related genes’ Expressions

**RNA extraction**

The expression of autophagy- and apoptosis-related genes in hepatocarcinoma cells was investigated by extracting total RNA from the collected cells, following instructions of GF-1 kit. RNA concentration of (546 ng/ul) was measured by the spectrophotometer Life Science UV/Vis (BECKMAN COULTER, USA). Total RNA concentration, quality, purity, and integrity were checked by the Bioanalyzer Agilent 2100 (Agilent, USA). Total RNA extraction was carried out using GF-1 kit.

**Semi-quantitative Real-Time PCR**

The isolated RNA from HepG2 cells (0.5 μg) was converted to cDNA by the Synthesis Kit. Following the manufacturer’s instructions, 5× reaction mix iScript (4 μl) was mixed with reverse transcriptase iScript (1 μl) and RNA template (15 μl) in (20 μl/reaction) as a final volume. By using Thermo Bath (FINEPCR, Seoul, Korea), the prepared mixture was incubated at 25°C for 5 min then at 42°C for 30 min. Following, the temperature was raised to 85°C. The efficacy for all the used primers was measured. The amplified PCR products were run on 2% agarose gel electrophoresis and compared with DNA standards run in parallel. The expression level of genes was measured in folds according to delta-delta Ct method, where E stands for the value of PCR efficiency calculated out of the efficiency curve. In general, all runs of PCR turned out efficiency >90% with R²=0.99. A comparison between the expression levels of untreated controls with treated cells was performed. After low-efficacy wells expelled to be zero, PCR efficacy for all the used primers was measured. The target genes differential expression was detected using Sybergreen RT-PCR master mixes on the detection system iQ5 real-time PCR (Bio-Rad, USA) between treated and untreated HepG2 cells. The housekeeping gene β-actin (ACTB) was used for normalization with forward and reverse primers’ sequence (R: 5-AGCACTGTGTGCGGTACAG-3, F: 5-AGAGCTACGAGCTGCTGAC-3). The thirteen primers used to specifically amplify target gene cDNA are shown in (Table 1). The sequence of primers and the followed cycling regimen for the designed primers was carried out using Primer3 and Blast, NIH, USA (the online Basic Local Alignment Search Tool) at https://blast.ncbi.nlm.nih.gov/Blast.cgi. According to the specifications entered to the designing utilities and software and after in-lab verification of the PCR amplification, the thermal cycling conducted was as follows: one cycle at 95°C for 180sec followed with 40 cycles at 95°C for 60sec, and at 60°C for 70sec. Quadruplicates of all samples were assayed. Preparation of the negative controls (none-template controls) was done. The differential expression of the housekeeping gene ACTB along with the target genes was measured in folds according to delta-delta Ct method, known as the E^ delta method, where E stands for the value of PCR efficiency calculated out of the efficiency curve. In general, all runs of PCR turned out efficiency >90% with R²=0.99. A comparison between the expression levels of untreated controls with treated cells was performed. After low-efficacy wells expelled to be zero, PCR efficacy for all the used primers was measured. The target genes differential expression was detected using Sybergreen RT-PCR master.

**Table 1. Gene symbols along with primer sequences applied in Real-Time PCR.**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sequence</th>
<th>Avg. Tm</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDIT3  DNA damage-inducible transcript 3 (human)</td>
<td>F: 5'-GAACGGCTCAAGCAGGAATC-3′ R: 5'-TTCACCATTCGGTCAATCAGAG-3′</td>
<td>59.85</td>
<td>80</td>
</tr>
<tr>
<td>FOS-1  Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (human)</td>
<td>F: 5'-CGTCTCCAGTGCCAACCTTCA-3′ R: 5'-GGTCCGGACTGTCGAGAT-3′</td>
<td>60</td>
<td>233</td>
</tr>
<tr>
<td>FOS-2  Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (human)</td>
<td>F: 5'-CCCAGACTGCACTTACTT-3′ R: 5'-ACTGATCTGTCTCGCTTCT-3′</td>
<td>59.72</td>
<td>553</td>
</tr>
<tr>
<td>BNIP3  BCL2 interacting protein 3 (human)</td>
<td>F: 5'-GGAGCTTAGTCAAGCCTGAGA-3′ R: 5'-CATCGTTGTGCAATGCG-3′</td>
<td>59</td>
<td>134</td>
</tr>
<tr>
<td>BNIP3L  BCL2 interacting protein 3 like (human)</td>
<td>F: 5'-CCTCGTCTCTCCACCAAT-3′ R: 5'-GTCCCTGCTGGTATGCATCT-3′</td>
<td>58</td>
<td>211</td>
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Continued table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>F-Sequence</th>
<th>R-Sequence</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBR1</td>
<td></td>
<td>5′-ATTCACCCACAGGGATAGC-3′</td>
<td>5′-GCAGGACCTTGTTAGTGCTCA-3′</td>
<td>59.9</td>
</tr>
<tr>
<td>BIRC5</td>
<td></td>
<td>5′-TGACGACCCCCATAGGGAACA-3′</td>
<td>5′-CGCACTTTTCTCCGCAGTTTC-3′</td>
<td>60.1</td>
</tr>
<tr>
<td>MAPK9</td>
<td></td>
<td>5′-AGTCATCTCGGTATTGGGGCT-3′</td>
<td>5′-AACCTTTACCCAGCTCTCCC-3′</td>
<td>60.0</td>
</tr>
<tr>
<td>ITGB1</td>
<td></td>
<td>5′-GAGCAGCCGCGGGAAAA-3′</td>
<td>5′-ACATCGTGCAAGTAGGGCA-3′</td>
<td>60.3</td>
</tr>
<tr>
<td>CAPNS1</td>
<td></td>
<td>5′-TGCGGCGGCTATTGGACTT-3′</td>
<td>5′-TTCATGAGTTCTGTCGCT-3′</td>
<td>59.9</td>
</tr>
<tr>
<td>CFLAR</td>
<td></td>
<td>5′-GAGTGCCGGCTATTGGACTT-3′</td>
<td>5′-ATAGCAACATCCGCAGCAA-3′</td>
<td>60.06</td>
</tr>
<tr>
<td>EIF4G</td>
<td></td>
<td>5′-GTCAGATCTAGGGCCGG-3′</td>
<td>5′-TCACGAGGTTCTCAAGAGC-3′</td>
<td>59.9</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma 1</td>
<td>F: 5′-ATCGACCTGCTGGTGTG-3′</td>
<td>R: 5′-ATCGAAGCTGCTGGTGTG-3′</td>
<td>59.7</td>
</tr>
</tbody>
</table>

Analysis of data

The data analysis was carried out using SPSS program version (16.0.0.2) and data were shown as (mean ± SD). The influence of the compound on cell growth inhibition was appraised by using 95% confidence intervals. Linear regression index equations were used to calculate IC50 value. The student t-test was used to compare the mean of each cell cycle phase, in triplicates, of treated vs untreated cells in flow cytometric analysis. In real-time qPCR, the comparative Ct method was used to build the used fold change calculation. The equation for comparative Ct method which is also known as the 2^ΔΔCt method was as follows: ΔΔCt = ΔCt treated – ΔCt untreated. Where: ΔCt treated = Ct treated – all mean treated and ΔCt untreated = ΔCt untreated – all mean untreated. Less than 0.05 of p values were reflected as significant.

Results

The cytotoxic effect of EGCG

In the current study HepG2 cell viability was affected by treatment with EGCG in a dose-dependent manner (Figure 1). EGCG concentration that destroyed 50% (IC50) of HepG2 cells was (10µg/ml). EGCG effect on the selected genes’ expressions was studied using this concentration.

Figure 1. The mean percentages of EGCG cytotoxic effect on HepG2 cells after treatment for (48h). The viability reduced in a dose-dependent manner

Arrest in cell-cycle induced by EGCG

The treated HepG2 cells with EGCG IC50 underwent a cell-cycle arrest in G0/G1 phase when compared to untreated one (Figure 2). The treated cells were obstructed significantly in G0/G1 phase (68.35% ± 1.41) with (73.75% ± 2.21) in untreated cells, (p= 0.037). Non-significant results were manifested when HepG2 cells treated with EGCG after 48 h in comparison with untreated cells. In S phase the percentage was (7.10% ± 1.23) in treated cells with (8.82% ± 3.02) in untreated one, (p= 0.43). While, in apoptosis and G2/M phases the percentages were as follows: (16.40% ± 3.97) and (8.95% ± 3.96) comparing to untreated cells (9.62% ± 3.83) and (8.97% ± 1.71) with p - value of (0.1) and (0.99), respectively.
Epigallocatechin gallate anticancer molecular mechanism

Figure 2. Representative histogram of the flow cytometry analysis of HepG2 cells’ DNA contents after 48 h, treatment with EGCG IC50. Treatment with EGCG arrests cell-cycle in G0/G phase. The cells’ percentages with fractional DNA content was detected by CyAn ADP apparatus and Summit (Version 4.3) program: S (R4), apoptotic cells (R2), G0/G1 (R3), and G2/M (R5) phases. The p value of less than 0.05 was considered as significant.

Revealing of the real-time apoptosis in intact HepG2 cells

In order to detect the apoptosis induction by EGCG treatment, the morphological changes of apoptosis in live-cells were investigated by fluorescence microscopy (Figure 3). Two important apoptosis markers were monitored by the kit. The monitored markers were the translocation of phosphatidylserine on the cell membrane which was identified by sulforhodamine 101-annexin V. In addition, caspase-3 activation was identified by NucView™ 488 caspase-3 substrate activity within individual whole cells (14). The nucleus stained as a bright green after the cleavage of NucView™488 caspase-3 substrate by activated caspase-3 (Figure 3C). Besides, phosphatidylserine that had translocated from the inner to the outer leaflet of the plasma membrane, produced a red frame around the cell under a fluorescent microscope using a red filter (Figure 3D).

The morphological changes that could be detected after treatment like rounding of cells, detachment, and cell shrinkage reflected the apoptosis-related events (15). A comparison between the treated cells and untreated cells in the plates with negative control were done. After 48 h of treatment, EGCG IC50 (20 µg/ml) stimulated apoptosis in 70% of HepG2 cells.

Figure 3. The morphological alterations of EGCG treated HepG2 cells reveals apoptosis-related events. A) No morphological changes in the negative control HepG2 cell line were shown in the photomicrograph. B) The apoptosis-related events were shown in the treated HepG2 cell line with EGCG IC50 (20 µg/ml) after 48h. The cells viewed under a fluorescent microscope (Nikon eclipse 80 i). The morphological alterations were manifested after 48h of treatment with EGCG IC50. C) The photomicrograph of the treated HepG2 cell lines with EGCG IC50 (20 µg/ml) for 48h and stained with caspases-3 substrate. Caspase-3 activation was indicated by green nuclei in HepG2 cells. D) The photomicrograph of HepG2 cell lines treated with EGCG IC50 (20 µg/ml) for 48h and stained with annexin-V substrate. The translocation of the phosphatidylserine from the inner to the outer leaflet of the cell membrane was indicated as red borders around HepG2 cells. E) The photomicrograph of the stained and merged HepG2 cell lines treated with EGCG IC50 (20 µg/ml) for 48h. The apoptotic HepG2 cells were characterized by green nuclei surrounded by red frames.
Up-and Down-Regulated target genes after EGCG treatment

The profile of the gene expression of the HepG2 cells treated with 1C50 of EGCG for 48h was assessed by the semi-quantitative analysis of real-time qPCR.

Thirteen genes belong to different apoptosis or autophagy pathways that attributed to cancer progress in human normal cells were shown to be up- or down-regulated after treatment (Table 2).

Two of the FOS member family were up-regulated significantly, i.e., FOS-1 (32.97 ± 9.24 folds) and FOS-2 (10.31± 4.91 folds). The other gene that up-regulated in folds after treatment, was DDIT3 (18.26 ± 4.51). The ten rest genes were down-regulated after treatment with EGCG. Down-regulated genes was manifested with three of the mitochondrial autophagy marker proteins, BNIP3 (0.48 ± 0.09 folds), BNIP3L (0.42 ± 0.08 folds), and NBR1 (0.17 ± 0.002 folds). In addition, two autophagy regulator genes in cells were down-regulated, BIRC5 (0.44 ± 0.07 folds) and MAPK9 (0.43± 0.07 folds). Moreover, genes related to metabolism/modification and biosynthesis process found to be affected by EGCG treatment. Two of these genes were down-regulated, ITGB1 (0.38 ± 0.02 folds) and EIF4G (0.24 ± 0.01 folds). There were other three genes that have a role in apoptosis pathways down-regulated after EGCG treatment. They were CAPNS1 (0.36 ± 0.02 folds), CFLAR (0.32 ± 0.02 folds), and RB1 (0.11 ± 0.001 folds).

Table 2. The expression levels of apoptosis- and autophagy-related genes were shown in treated HepG2 cells after EGCG treatment for 48h.

<table>
<thead>
<tr>
<th>HepG2 cells Expressed Gene Name</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated genes</strong></td>
<td></td>
</tr>
<tr>
<td>FOS-2 (FOS Gene Family member)</td>
<td>32.97</td>
</tr>
<tr>
<td>DDIT3 (AP-1)</td>
<td>18.26</td>
</tr>
<tr>
<td>FOS-1 (FOS Gene Family member)</td>
<td>10.31</td>
</tr>
<tr>
<td><strong>Down-regulated genes</strong></td>
<td></td>
</tr>
<tr>
<td>BNIP3</td>
<td>0.48</td>
</tr>
<tr>
<td>BIRC5</td>
<td>0.44</td>
</tr>
<tr>
<td>MAPK9</td>
<td>0.43</td>
</tr>
<tr>
<td>BNIP3L</td>
<td>0.42</td>
</tr>
<tr>
<td>ITGB1</td>
<td>0.38</td>
</tr>
<tr>
<td>CAPNS1</td>
<td>0.36</td>
</tr>
<tr>
<td>CFLAR</td>
<td>0.32</td>
</tr>
<tr>
<td>EIF4G</td>
<td>0.24</td>
</tr>
<tr>
<td>NBR1</td>
<td>0.17</td>
</tr>
<tr>
<td>RB1</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Discussion

It has been proved that EGCG low concentrations didn’t significantly alter HepG2 cells viability. In contrast, a significant reduction in the number of viable cells was detected with its high concentration treatment, thus EGCG cytotoxicity is in a dose-dependent manner (6-10). The results of this study prompt this fact as the EGCG cytotoxic effect was in a dose-dependent manner, with high concentrations found to be cytotoxic to HepG2 cells after 48h. A study by Ahn et al. has shown that the high level of EGCG is associated with its cytotoxic effect. It is due to the expression of endoplasmic reticulum stress response proteins, such as ATF3, GADD34, and CHOP (DDIT3) (10). One of these endoplasmic reticulum stress response proteins; i.e. CHOP (DDIT3) showed significant up-regulation after treatment with EGCG in the current study. In 2019, Guan et al. discovered the important role of CHOP as an inducer to autophagy and apoptosis by the endoplasmic reticulum stress (17). The role of the stress caused by a cytotoxic agent treatment was proved by a study in 2015. In which such treatment may induce damage of organelles and proteins in cancer cells. Results in proapoptotic factors to be released due to a stress in mitochondria and endoplasmic reticulum and subsequent cancer cell death (11).

Phosphatidylinerine (PS) exposure on the outer plasma membrane is considered as a unique characteristic of apoptotic cells that could be depended on AnnexinV staining of PS to distinguish apoptosis from other cell death pathways (18). This characteristic of PS externalization was investigated in this study to investigate the EGCG efficacy on HepG2 cells. The results came to the fact that about 70% of the cells underwent apoptosis by checking the morphological changes in the stained intact HepG2 cells after treatment. The real-time apoptosis markers proteins investigation, particularly, caspase activation was strengthened by the G0/G1 cell cycle arrest that recorded by the flow cytometry analysis. These results were supported by a study which found that EGCG increases caspase activity and the sub-G1 phase of the cell cycle leading to the stimulation of apoptosis in cancer cells (19). The effect of the autophagy related genes in cancer suppression or induction has been explored by many studies, in addition to their involvement in cancer development by different mechanisms (20, 21). Based on the fact that the cytotoxicity effect of EGCG on HepG2 cells is it mediated by autophagy or apoptosis, this study was designed.

The data of this study recorded up-regulation of two of FOS Family members Genes, namely, FOS-1 and FOS-2. These genes expressed as the activator protein-1 (AP-1) transcription factors in the host cells. These factors are participating in different aspects of cell survival or death, and cellular proliferation (22). Hence, a study in 2010 found that
the AP-1 proteins c-Jun and JunB, but not c-Fos, JunD, and Fra-1 can inhibit autophagy \(^{(23)}\). BCL2 interacting protein 3 (BNIP3) and BCL2 interacting protein 3like (BNIP3L) are mitochondrial autophagy marker proteins \(^{(24)}\). These two genes found to be down-regulated after EGCG treatment to HepG2 cells. Mitophagy is a mechanism which enhances cell protection in a healthy cell during mitochondrial damage \(^{(25)}\). One of the mechanistic pathways that lead to a mitophagy is mediated by BNIP3 and BNIP3L proteins. Each of these proteins provide joining of a mitochondrion to an autophagy apparatus via the interaction between receptor proteins like \((NBRI)\) \(^{(26)}\). NBRI autophagy cargo receptor was down-regulated after EGCG incubation with HepG2 cells. It is a specific receptor for selective autophagosomal degradation of ubiquitinated targets and pexophagy \(^{(27, 28)}\). Autophagy is mediated by the formation of double-membrane vesicles known as autophagosomes that fuses with lysosomes. Large cytoplasmic materials are selectively segregated and degraded in the lysosomes. Substrate collection is mediated by ubiquitylation and enrollment of ubiquitin-binding autophagic receptors NBR1 that engulfed by autophagosome \(^{(21, 28)}\). BIRC5 is verified as a straight autophagy regulator in cells \(^{(29)}\). A study has come to a result that BIRC5 can be a potential therapeutic target for renal carcinoma cell lines by using a drug targets BIRC5 \(^{(30)}\). The treated HepG2 cells with EGCG displayed a down-regulation in BIRC5 gene when compared to untreated cells.

MAPK9 regulates autophagy gene expression that may stimulate autophagy by inducing the expression of certain autophagy related genes \(^{(31)}\). Data of this study found that MAPK9 gene was significantly down-regulated which in turn may affect the mechanism of autophagic regulation. Unlike a previous study done by Ahn and his colleagues in 2009, they found that the expression of integrin subunit beta 1 gene \((ITGB1)\); a gene associated with protein modification and protein biosynthesis, was elevated after EGCG treatment \(^{(16)}\). The current study demonstrated down-regulated effect of EGCG on this gene after treatment. A recent study indicated that the treatment of breast cancer by a traditional Chinese medicine targeting ITGB1 could be a promising target to cease cancer progress and metastasis \(^{(32)}\). This result may enhance our findings that EGCG anticancer effect is associated with apoptosis induction rather than autophagy.

Translation initiation factors like EIF4G have complex functions in cells \(^{(33)}\). In 2010 a study carried by Fan et al., proved the anti-cancer activity of the tested drug by disrupting the eIF4E/eIF4G association through binding to eIF4E \(^{(34)}\). Studies display that the EIF-4E inhibition can lead to apoptosis-inducing and growth-inhibitory effects in cancer cells. Hence, could induce the potential to use such molecular target for therapy \(^{(34, 35)}\). These findings strengthen the current study results which found significant apoptosis induction by an inhibition to the selected genes, one of them EIF4G.

The calpain (CAPNS1) is a family member of calcium activated cysteine proteases which implicates in both pro- or anti-apoptotic functions \(^{(36)}\). CAPNS1 depletion increases sensitivity to apoptosis \(^{(37)}\) which support our results of CAPNS1 gene down-regulation after treatment and increased the chance of apoptosis pathway for cytotoxic effect on treated cells. CAPNS1 represents an encouraging target for cancer therapy since its action is tightly linked to tumor progress. It is required for autophagy and survival of cancer cells \(^{(37)}\). Thus, its down-regulation affects negatively on cancer cells survival. The anti-apoptotic protein CFLAR (CASP8 and FADD-like apoptosis regulator) has a significant attention as an anticancer therapeutic target in solid and haematological cancers where it is frequently overexpressed \(^{(38)}\). CFLAR is a main apoptosis-regulatory protein that inhibits necroptosis and autophagy \(^{(39)}\). This gene was affected negatively by EGCG, thus increased the chance to enhance apoptosis in treated HepG2 cells.

Additionally, the down-regulation to Retinoblastoma 1 gene was in harmony with other findings which investigated its role in the apoptosis pathways. The retinoblastoma protein (pRB) bounds to pro-apoptotic promoters that is transcriptionally active in the Rb-dependent apoptotic pathways \(^{(40, 41)}\). Other study revealed the straight role for pRB in the stimulation of apoptosis as response to genotoxic or oncogenic environment \(^{(42)}\).

The results of the current study agree with other findings which found that natural product treatment to cancer cells may induce signaling transduction but not in a sufficient way to induce autophagy \(^{(17)}\). Furthermore, a study on hepatocellular carcinoma in 2015 suggested that EGCG prevents the development of cancer through cytidical activity giving significant decrease in NF-kappaB and Bcl-2 expression, and increased the expression of Bax, p53, caspase-9, and caspase-3 with the release of cytochrome c \(^{(43)}\). Moreover, another study in 2018 has confirmed that EGCG selectively targets hepatic de novo lipogenesis (DNL) pathway in HepG2 cells and thus inducing apoptosis by inhibiting certain key enzymes in this pathway, namely, ATP citrate lyase, acetyl-CoA carboxylase, and fatty acid synthase (FASN) \(^{(44)}\). Unlike, other mechanism displayed by EGCG cytotoxic effects on HepG2 cells was investigated by Zhao et al., in 2017. They found that EGCG inhibits alpha-fetal protein (AFP) secretion and promotes the intracellular aggregation of AFP, the protein that enhances tumor cell proliferation, which
induces autophagy to degrade AFP and in turn inhibit the growth of HepG2 cells (6).

Additional studies are critically in need to explore the exact pathway of cancer cell death after a natural product treatment. Such studies need to focus on the regulation of multiple signaling pathways and the expression of molecules that are directly involved in autophagosome formation in cancer cells, particularly, HepG2 cells that could mediate the onset of autophagy and couldn’t be done by this study.

Conclusion
With the increase incidence of apoptosis-resistant cancer cells, the needs to investigate the cytotoxic effect of certain natural polyphenols products are increase. To indicate if their cytotoxicity is due to inducing the apoptosis or autophagy, the current study was conducted to investigate the effect of EGCG on thirteen genes related to apoptosis and/or autophagy in cancer cells, particularly, HepG2 cells. Some of these genes were investigated for the first time, i.e. (NBR1, ITGB1, CAPNS1, EIF4G, and RB1). The flow-cytometry analysis, Dual apoptosis assay and qRT-PCR all came to the conclusion that EGCG as a natural polyphenol enhanced apoptosis-related genes but not autophagy-related genes and its mechanism in inducing the cytotoxicity is apoptosis. Further studies are required to validate these results by using protein analysis for proteins involved in apoptosis or autophagy process.

Conflict of Interests
The authors have declared that no conflict of interest exists.

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