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Anti-neoplastic effect of epigallocatechin gallate on breast cancer cells through glucose metabolism

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Abstract. Breast cancer (BC) is the primary cause of women cancer death, which could be prevented by EGCG that has been recently shown several health properties included anti-cancer, however the mechanism underpinning still poorly understood. In this study, several biological activities of both MCF7 and MDA-MB-231 cells were evaluated in response to EGCG. Cell viability and the role of Akt and AMPK inhibitor molecules, and sodium pyruvate on this viability, apoptosis, metastasis, and interestingly regulation of glucose metabolism were assessed. EGCG promoted cytotoxicity in both BC cell lines after 24h but not less. Co-incubated cells with Akt and AMPK inhibitors alongside EGCG significantly caused more reduction in cell viability compared to the effect of EGCG alone with maximum effect referred to Akt inhibitor. While supplemented sodium pyruvate significantly restored the decreases in cell viability. Remarkably, EGCG induced apoptosis through increased caspase 3/7 activation associated with upregulated Bax gene, in addition to anti-metastatic effect through decreasing cellular migration. Importantly, lactate production was sharply reduced after 6h (no alteration of viable cells) and 24h (decreased viable cells) concomitant with significant blocked glucose uptake in response to EGCG. In conclusion, EGCG could be a potential anti-migration, the anti-cancerous therapeutic agent through targeting cancer cells glucose metabolism.

Keywords: EGCG, MCF7, MDA-MB-231, glucose metabolism, apoptosis, anti-metastasis
1. Introduction

Breast cancer (BC) is an invasive heterogeneous tumour of breast tissue which is frequently diagnosed in females. BC is a second leading cause of cancer death next to lung cancer worldwide, and first leading cause of female cancer death [1]. Accordingly, there were nearly 2.1 million new diagnosed BC cases which represent 11.6% from all diagnosed cancer types, off this number about 627 thousand death cases which represent 6.6% from all cancer death cases in 2018 [1].

Cellular metabolism is a complex fundamental mechanism that tightly depends on a signalling pathway, and gene and post-translation protein express to provide energy for normal cellular functions [2]. In cancer including BC, the cellular metabolism is altered to support cellular proliferation, growth, remodeling, and metastasis which regulated by tumour suppressor genes and microenvironment [3]. Therefore, this alteration is recognised hallmark of all cancer cells that metabolised glucose through aerobic glycolysis (Warburg effect) to produce energy and lactate [4]. This process is accelerated to produce sufficient energy required for survival, proliferation, and biosynthesis [5, 6].

Consumption of green tea has been recently increased worldwide due to several potential health benefits that assigned to catechins and specifically epigallocatechin gallate (EGCG) [7]. The potential health benefits of green tea or EGCG is to have anti-cancerous and anti-metastasis effects [8]. These impacts reported being mediating through decreasing viable cell and increasing apoptosis [9, 10], induced cell cycle arrest and inhibited cellular growth [11, 12] and inhibited cellular migration [11, 13]. Several molecular pathways thought to be involved in green tea mediated anti-cancer and anti-metastasis, however, the signalling pathway of these effects still not fully understood and require further investigation. Therefore, the current study investigated the effect of EGCG on BC and the potential signalling pathways of the effects focusing on the glucoregulatory role of EGCG.

2. Materials and methods

2.1 Cell culture and maintaining

Breast adenocarcinoma MCF7 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (ATCC® HTB-22™, HTB26™). MCF7 cells were proliferated and maintained in phenol red Dulbecco's Modified Eagle's Medium (DMEM) containing glucose (4.5 g/L), L-glutamine (LG) 4 mM, and without sodium pyruvate. MDA-MB-231 cells were grown and maintained in phenol red RPMI 1640 containing 4.5 g/L LG. Both cell lines were supplemented with Fetal Bovine Serum (FBS) 50:500ml (v/v) with 5:500ml (v/v) of 100 unit/ml penicillin and 0.1mg/ml streptomycin. The cells were incubated in a 5% CO2 humidified atmosphere at 37°C.

2.2 cell viability assay

MCF7 and MDA-MB-231 cells were cultured in 96 well plates at a density of 5000/well in completed media for 24h. Cells were then exposed to 10, 50, 100, and 150μM of EGCG for 24h and subsequently, cell viability was measured using PrestoBlue® cell viability reagent (Life Technologies, UK). Briefly, 10μl of PrestoBlue was added to cells that cultured in 90μl completed media in addition to cell-free control wells. 4 hours incubation at 37°C and 5% CO2 incubator, followed by measuring fluorescence at 353/615nm (Ex/Em) by using fluorescence microplate reader SpectraMAX GeminiXS (Molecular Devices, UK) and SoftMaxPro software. Additionally, the assay was repeated after exposed cells to 100 and 150μM of EGCG for 6h using the same protocol. Furthermore, the assay was conducted again after exposed the cell to 100μM of EGCG in presence and absence 10μM of selective Akt inhibitor (10-(4'-(N, N-Diethyamino) butyl) 2-chlorophenoxazine hydrochloride, Tocris, UK), selective AMPK inhibitor (Dorsomorphin dihydrochloride, Tocris, UK), and 1mM of sodium pyruvate for 24h separately using the same procedure.
2.3 Apoptosis assay
Both MCF7 and MDA-MB-231 cells were cultured in 96 well-plates separately at a density of 5000/well for 24h and subsequently treated with 100 and 150µM of EGCG with and without 1mM of sodium pyruvate for 24h. Culture medium was discarded, and 100µl/well of fresh medium containing CellEvent reagent (CellEvent® Caspase-3/7 Green Ready Probes® Reagent, R 37111, Thermofisher Scientific, UK) (2 drops/1ml media) was added to cell culture and incubated for 60 minutes. Follow that; the fluorescence emission was measured at 502/530 Ex/Em using fluorescence microplate reader SpectraMAX GeminiXS (Molecular Devices, UK).

2.4 Lactate quantification assay
The amount of lactate that released in the medium in response to glucose metabolism was quantified by using Amplite™ Fluorimetric L-Lactate Assay Kit (AAT Bioquest®, Cat. 13814) following the manufacturer protocol. MCF7 and MDA-MB-231 cells at a density of 30000/well of 24 well-plates were cultured overnight and subsequently, serum starved for 2h followed by exposing to 100 and 150µM of EGCG with and without 1mM sodium pyruvate for 6 and 24h. The collected medium was deproteinization to inactivate all enzymes especially lactate dehydrogenase, and the obtained supernatant was assayed following manufacturer protocol. The amount of lactate was measured corresponding to fluorescence intensity that measured at 540/590 Ex/Em with 570 cut off point using fluorescence microplate reader SpectraMAX GeminiXS (Molecular Devices, UK) and SoftMaxPro software.

2.5 Glucose uptake assay
Fluorescent glucose analog 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino)-2-Deoxyglucose (2-NBDG, Cayman, Cambridge Bioscience, UK) was used to determine the amount of glucose uptake in response to EGCG treatment. MCF7 and MDA-MB-231 at a density of 10000/well of 96 well-plates were cultured and incubated overnight. Cells were serum starved for 2h in low glucose medium, and then treated 100 and 150µM of EGCG in addition to the 100µM final concentration of 2-NBDG in 100µl/well complete low glucose media for 4h. The 2-NBDG uptake was measured by washing cells 3x with pre-cold HBSS and leaving the last wash solution, and subsequently, fluorescence intensity was measured at 465/540 Excitation/Emission (Ex/Em) using fluorescence microplate reader SpectraMAX GeminiXS (Molecular Devices, UK) and SoftMaxPro software. The assay was performed again after exposed the cells to 100µM of EGCG without and with 10µM of selective Akt and AMPK inhibitors separately for 8h following the same previous protocol.

2.6 Cellular migration assay (cell IQ®)
Automated image capture system termed Cell IQ® (CM Technologies, Finland) was used to determine the cellular migration of breast cancer cell lines. The confluent culture MCF7 and MDA-MB-231 cells were exposed to 5µg/ml of mitomycin C in serum-free media for 2h. The scratched along the vertical axis of cell monolayer was introduced by using a sterile 20µl pipette tip, and any free cells were removed by washing with HBSS. The cells were then exposed to 50µM of EGCG, and plates were inserted into the Cell IQ® system, and two specific regions were selected. The captured images were selected at 0 and 24h of treatment. A percent of wound closure was analysed using Cell-IQ® analyse™ software according to the differences between initially scratched area and new closure area.

2.7 Real-time Polymerase Chain Reaction (Rt-qPCR)
MCF7 and MDA-MB-231 cells were treated with 100µM for 24h and then were lysed Trizol® reagent, and the total RNA was extracted following the manufacturer’s protocol, and subsequently quantified using NanoDrop1000 spectrophotometer (Thermofisher Scientific, UK). 1µg of total RNA was subjected to reverse transcription using cDNA synthesis kit (Primer design, UK) following manufacturer’s instruction including 20 minutes at 55°C for initiation reaction, 15 minutes at 75°C for heat activation using a programmed thermocycler. The obtained cDNAs were subjected to qPCR to
determine the amplification of human cancer apoptotic genes. 20µl of final volume reaction per well containing 5µl diluted cDNA with 15µl master mix SYBR® green with forward and reverse primers (B-cell lymphoma 2 (BCL2) and apoptosis regulator Bax (Bax), Invitrogen, UK) were placed into 96 PCR well-plates. The sealed plate was inserted in Stratagene MX3000P™ thermal cycler (Stratagene, UK) under the conditions including one activation cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for one minute, and one cycle of 95°C for 30 seconds followed by 60°C for one minute and the 95°C for 30 seconds. The cycle threshold (Ct) value was obtained for the housekeeping gene and interesting genes; the relative gene expression level was estimated using equation 2ˆ∆∆Ct.

2.8 Statistical analysis
All data were analysed using Graph Pad Prism version 7 software (Inc., USA), and expressed means ± standard error of the mean (SEM) of 3 independent experiments. One-way ANOVA analysis of variance followed by Tukey’s post-hoc test for multiple comparisons was used unless otherwise states. The statistical differences between the experiments were considered significant when p values< 0.05 (*), p values< 0.01 (**), p values< 0.001 (***) and p values <0.0001 (****).

3. Results

3.1 EGCG reduces breast cancer cell viability
The cytotoxic effect of various concentrations of EGCG including 10, 50, 100, and 150µM was initially investigated in MCF7 and MDA-MB-231 cells after 24h treatment. The result shows that 100 and 150µM of EGCG decreased MCF7 cell viability by 16.5% ± 5.2% (p= 0.0079) and 22.3% ± 7.8% (p= 0.0001), and MDA-MB-231 cell viability by 18% ± 6.8% (p= 0.0001) and 21.7% ± 1% (p<0.0001) compared to controls respectively (Fig 3.1 A, B). Whereas, the previous cytotoxic effects of EGCG are disappeared after 6h incubation (data not shown). Secondly, the role of Akt and AMPK, in addition to sodium pyruvate on cytotoxic effect of EGCG in both BC cell lines was determined by co-cultured cells with Akt and AMPK selective inhibitor molecules, and sodium pyruvate separately in presence and absence 100µM of EGCG for 24h. EGCG significantly reduced cell viability in both cell lines, and supplemented Akt and AMPK inhibitor molecules with EGCG caused further significant decreases of cell viability in MCF7 cells by 39.2% (p<0.0001) and 18.6% (p= 0.0147) and in MDA-MB-231 cells by 7.5% (p<0.0001) and 5.2% (p= 0.0041) compared to EGCG treated cells respectively with potent effect to Akt inhibitor (Fig 3.1 C, D). Whereas, additive sodium pyruvate in addition to EGCG significantly suppressed the cytotoxic effect of EGCG which caused increased cell viability by 40% (p<0.0001) in MCF7 cells and 7% (p<0.0001) in MDA-MB-231 cells compared to EGCG treated cells respectively (Fig 3.1 C, D).

3.2 EGCG induces apoptosis in breast cancer cell lines
Induction of apoptosis in both breast cancer cell line was measured through activation of caspase 3/7 in response to 100 and 150µM of EGCG in presence or absence sodium pyruvate substrate for 24h. The result appears that 100 and 150µM activated caspase 3/7 by 44.2% (p= 0.0085) and 52% (p= 0.0008) in MCF7 cells, and 55% (p= 0.0003) and 61% (p<0.0001) in MDA-MB-231 cells compared to controls respectively (Fig 3.2). Co-incubating EGCG with sodium pyruvate had no significant effect on the induction of apoptosis through increasing caspase 3/7 activation in response to EGCG.
Figure 3.1. The effect of inhibitor molecules Akt and AMPK, and sodium pyruvate on EGCG reduces BC cell viability. 

(A, B). MCF7 and MDA-MB-231 cells treated with EGCG for 24h, followed by measuring cell viability using PrestoBlue®. 100 and 150µM of EGCG significantly decreased viable cell compared to controls in both cell lines respectively. (C, D). MCF7 and MDA-MB-231 cells treated with EGCG without and with Akt and AMPK inhibitor molecules, and sodium pyruvate separately for 24h, followed by measuring cell viability. EGCG decreased viable cell, and Akt and AMPK inhibitors promoted further significant decreases in a viable cell in both cell lines compared to EGCG treated cells, while sodium pyruvate significantly increased cell viability compared to EGCG treated cells. Data presented mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, n=3.
EGCG induces apoptosis in MCF7 and MDA-MB-231 cells. MCF7 and MDA-MB-231 cells were exposed to EGCG in the presence and absence of 1mM of sodium pyruvate for 24h. CellEvent® Caspase 3/7 ready Probes® was used to measure activation of caspase 3/7 for 1h and therefore investigated the level of apoptosis. EGCG promoted apoptosis in both cell lines through significant increasing activation of caspase 3/7 compared to controls with no effect of supplemented sodium pyruvate alongside EGCG compared to EGCG treated cells. Data presented mean ± SEM, **p<0.01, ***p<0.001, and ****p<0.0001, n=3.

3.3 The effect of EGCG on Bcl2 and Bax gene expression

The impact of EGCG that activated caspase 3/7 and induced apoptosis on the expression level of apoptosis regulatory gene Bcl2 and Bax was assessed using qRT-PCR in both BC cells. EGCG significantly increased Bax gene expression by 1.5-fold (p=0.0018) in MCF7 cells and 2.7-fold (p=0.0037) in MDA-MB-231 cells compared to control respectively (Tab 3.1). No significant effect had been seen on Bcl2 gene expression in both BC cell lines in response to EGCG.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>mRNA</th>
<th>↑↓ Fold of control</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>BCL2</td>
<td>0.886 ± 0.7</td>
<td>0.526 (NS)</td>
</tr>
<tr>
<td></td>
<td>Bax</td>
<td>1.532 ± 0.087</td>
<td>0.0018 (**)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>BCL2</td>
<td>1.01 ± 0.632</td>
<td>0.9992 (NS)</td>
</tr>
<tr>
<td></td>
<td>Bax</td>
<td>2.753 ± 0.38</td>
<td>0.0037 (**)</td>
</tr>
</tbody>
</table>
3.4 **EGCG reduces lactate production from breast cancer cells**

The effect of EGCG on BC cells glycolysis was assessed in both MCF7 and MDA-MB-231 cells through measuring the amount of lactate released in the medium after 24 and 6h treatment with EGCG and co-incubated without or with sodium pyruvate substrate. 100 and 150µM of EGCG decreased lactate release by 43% (p=0.0002) and 56.5% (p<0.0001) from MCF7 cells compared to control respectively (Fig 3.3 A). These decreases in lactate were significantly restored by 68% (p=0.0042) and 95% (p=0.0004) compared to EGCG treated media respectively in response to sodium pyruvate. Similarly, EGCG reduced lactate production by 56% (p<0.0001) and 43% (p<0.0001) from MDA-MB-231 cells compared to control respectively (Fig 3.3 A). Additive sodium pyruvate has significantly reversed the levels of lactate by 43% (p=0.0051) and 36% (p=0.0030) compared to EGCG treated cells respectively. Furthermore, 6h treatment with 150µM of EGCG significantly reduced lactate released from both BC cell lines with no effect of supplemented sodium pyruvate (Fig 3.3 B).

**Figure 3.3.** EGCG reduces MCF7 and MDA-MB-231 cells lactate production. (A). EGCG significantly decreased lactate released from both BC cells compared to controls after 24h, whereas sodium pyruvate significantly restored the level of lactate compared to EGCG treated cells. (B). EGCG significantly reduced lactate released from BC cell compared to controls after 6h with no effect seen to supplement sodium pyruvate. Data presented mean ± SEM, **p<0.01, ***p<0.001, and ****p<0.0001, n=3.
3.5 **EGCG decreases 2-NBDG uptake in breast cancer cells**

As EGCG showed a significant reduction in the levels of lactate released without alteration of viable cells, therefore the amount of cellular glucose uptake was investigated in both BC cell lines in response to 100 and 150µM of EGCG after 4h incubation. The result shows that the EGCG significantly reduced 2-NBDG uptake by 20% (p=0.0048) and 23% (p=0.0007) in MCF7 cells and by 20% (p=0.0031) and 24% (p=0.0003) in MDA-MB-231 respectively compared to controls (Fig 3.4 A). Furthermore, co-incubated cells with Akt and AMPK selective inhibitor molecules separately alongside 100µM of EGCG for 8h shows significant reduction of glucose uptake in response to EGCG compared to control in both cell lines with no significant effect of either Akt or AMPK inhibition to further suppressed 2-NBDG uptake (Fig 3.4 B).

![Figure 3.4](image)

**Figure 3.4. EGCG decreases BC cells 2-NBDG uptake.**

(A). EGCG significantly decreased cellular 2-NBDG uptake in both BC cell lines compared to control after 4h. (B). EGCG significantly reduces 2-NBDG uptake in BC cells compared to control after 8h, whereas no effect of supplemented Akt and AMPK inhibitor molecules separately alongside EGCG in both cell lines. Data presented mean ± SEM, **p<0.01, ***p<0.001, and ****p<0.0001, n=3.
3.6 EGCG reduces breast cancer cell migration

Cellular migration of BC cell in response to EGCG was assessed by using an automated cell lives imaging system (CellIQ™) based on wound healing to close an artificial scratch [14]. Cells with artificial scratches were treated with 50µM of EGCG, and the images were captured using automated live imaging (CellIQ™, Fig 3.5 A) at time 0 and 24h. The collected data were analysed and calculated as a percentage of wound closure of cellular migration. The result showed that EGCG reduced the percent of wound closure of MCF7 by 23.7% (p=0.0001) and MDA-MB-231 by 42% (p=0.0064) after 24h compared to controls (Fig 3.5 B).

Figure 3.5. EGCG decreases cellular migration of BC cells.
Artificial scratch was introduced in cultured MCF7 and MDA-MB-231 cells that treated with 5µg of mitomycin C in serum-free media for 2h. Cells then exposed 50µM of EGCG cellular migration was monitored and estimated as a % of wound closure. (A). CellIQ® images of wound healing analysis of both BC cells. (B). EGCG significantly reduces the percent of wound closure in both BC cells after 24h. CellIQ® data analysed using CellIQ® software. Data presented mean ± SEM, **p<0.01, and ***p<0.001, n=3.
4. Discussion

The whole object of this study is to determine the impact of various concentrations of EGCG on BC cell biology. The first experiments investigated the potential effect of EGCG to decrease viable cells and promote apoptosis of BC cells. Furthermore, the effect of this compound on BC cellular migration was determined due to the critical role of metastasis that is accountable for the rising mortality rate. However, the crucial purpose of this work is to explore the effect of EGCG on abnormal glucose metabolism of BC cells.

Initially, the results of this study showed that the specific doses of EGCG significantly reduced cell viability (Figure 3.1 A, B), and increased activation of caspase 3/7 which indicated cellular apoptosis (Figure 3.2) that associated with upregulation of pro-apoptotic gene Bax in both BC cell lines (Table 3.1). The cytotoxic data of EGCG would be expected as the previously published findings identified that the green tea and its abundant compound EGCG decreased cell viability in various cell lines ([15, 16]. These decreases of cell viability were associated with induction of apoptosis in BC cell lines which was reported by several studies through activation of caspase 3/9/7 with alteration of Bax/Bcl2 ratio [9, 17]. Since caspase three absent in MCF7 cells [18], therefore caspase 7 is responsible for apoptosis [19] with upregulated Bax gene expression. The present study data indicate that the reduction in cell viability detected is likely due to stimulating cellular apoptosis which fits with previously published results. Unlike the current study, the previous few studies focused on using low doses of treatment and depend on the Bax/Bcl2 ratio as an indicator for apoptosis after 48h in MCF7 and MDA-MB-231 cells. The data of the present study suggests that EGCG could be a potential chemotherapeutic agent which could promote its effect in a complicated method that required more investigation.

PI3K/Akt and AMPK are believed to be essential markers in the development of several pathological conditions including initiation and progression of a tumour. Therefore, the cancer cells required excessive activation of PI3K/Akt [20] and AMPK [21, 22] to provide energy for maintaining cellular proliferation, differentiation, growth, and survival. The result of the current study showed that suppressed Akt and AMPK in BC cells that treated with EGCG provoked further reduced in cell viability in both MCF7 and MDA-MB-231 cells with potent effect to Akt inhibition (Figure 3.1 C, D). The first data to appear fits with several studies findings that showed the EGCG exert its anti-cancer effect through inhibition of PI3K/Akt in various cancer cells [23, 24], which make the current study data interesting as it is firstly presented in BC cells. The second data is unique as it showed more decreases of cell viability in EGCG treated cells that supplemented with AMPK inhibitor molecule. This result is contradicted with most published data in this field which showed that the green tea and its compounds mediated their anti-cancer effect by activation of AMPK [8, 25]. Based on the previously published and the present data, the study suggests that EGCG could interact with the PI3K/Akt and AMPK pathways to induce the cytotoxic effect in BC cells and therefore could be a therapeutic solution for various types of a tumour. However, more quantitative studies to measure PI3K/Akt and AMPK protein expression levels is needed to confirm the anti-cancer effect of EGCG in BC cells by inhibiting these markers.

Fundamentally, the cancer cell possesses alteration of natural glucose metabolism. In line with this aspect, few studies assessed the glucoregulatory role of EGCG as anti-cancer which could be a key of chemotherapeutic promise [26]. Therefore, the present study assessed this role by measuring the amount of lactate released and glucose uptake in MCF7 and MDA-MB-231 cells in response to EGCG. The data showed a significant reduction in lactate production from both cell lines after 24h treatment with EGCG (Figure 3.3 A, B), while supplemented sodium pyruvate reversed the decreases level of lactate compared to cells treated with EGCG. This result is suggesting that the EGCG can interfere with glucose metabolism which successfully manipulated by adding lactate precursor. The decreases in lactate are associated with a significant reduction of cell viability which could be the reason for fall lactate. Short time analysis showed a significant decline in glycolysis (Figure 3.3 C, D) without alteration of viable cells, which indicated the proper effect of EGCG. This result is associated with significant reduction in cellular glucose uptake in both BC cell line (Figure 3.4 A, B) which is likely mechanism that responsible for declining glycolysis. The current study data is attractive due to few previous studies assessed the metabolic effect of EGCG in BC cells that showed a significant reduction in glycolysis after 4 and 24h
concomitant with reduced glucose uptake [27-29], however these results were associated with significant reduction in cell viability which unlike the present study data.

Identifying the impact of EGCG on BC cellular migration is crucial due to invasive and cellular migration increasing female mortality rate. The present study result revealed that the EGCG significantly decreased BC cells migration after 24h treatment (Figure 3.5 A, B). This result is expected in line with the anti-migration effect of EGCG in BC cells [30, 31]. However, the presented data is authenticated as the erroneous result is blocked by suppressing interference of cellular proliferation. This result suggests a potential anti-migratory role of EGCG in BC cells in addition to antiproliferative and apoptotic impacts, however signalling pathway of the anti-migration effect of EGCG in BC need to be investigated.

To conclude, EGCG shows promising chemotherapeutic role against two different BC cell lines, these including cytotoxic effect and induction apoptosis with the anti-migratory role. The cytotoxicity could promote through suppression of PI3K and interestingly AMPK which is a unique data in BC cell. Furthermore, this compound can modulate glucose metabolism through shortage of cellular glucose uptake and modulate glycolytic activity which is likely the anti-cancer effect. Therefore, EGCG could be a critical chemotherapeutic agent for different types of cancer, however, further research is required to clarify the precise mechanisms.

5. References


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