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Genistein elicits its anticancer effects through up-regulation of E-Cadherin in Acute Lymphoblastic Leukemia (ALL) cells: an *in vitro* experimental study

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Abstract

Background and aim: Genistein is an isoflavone that acts as a potent inhibitor of tyrosine kinase in several cancers. The aim of this study was to elucidate the impact of genistein on leukemia cell lines through E-cadherin signaling pathway.

Methods: This study was conducted in 2016 at the Molecular Medicine Research Center (Hormozgan University of Medical Sciences, Bandar Abbas, Iran). In this *in vitro* experimental study, the Acute leukemia lymphoma (ALL) cell lines MOLT4 (as mild acute lymphoblastic leukemia cell line) and JURKAT (as high aggressive acute lymphoblastic leukemia cell line) were cultured and then treated with different concentrations of genistein (10, 25, 40 and 55 μM) for 24, 48 and 72 hours. Its cytotoxicity and anti-cancer properties on ALL cell lines were evaluated by calculating the growth rate and MTT assay. Eventually, the effect of genistein on the expression level of E-cadherin was examined using a quantitative Real-Time PCR. In the present research, we used *in vitro* experimental study.

Results: The percentage of vital malignant cells treated with genistein in comparison with non-treated cells was significantly decreased ($p<0.03$). E-cadherin expression was significantly ($p<0.02$) up-regulated between 2 to 4 times as compared with non-treated ones. Even though it seems that genistein induces its anti-cancer properties on all doses, it was found that there is a negative correlation between these anti-cancer properties and increasing the concentration and exposure time of genistein.

Conclusion: The present findings suggest that genistein possesses a growth inhibitory effect on ALL similar to solid tumor cells. It can also be deduced that genistein could be considered as a natural agent to potentially control the invasion of malignant cells and expedition of disease, which is promising and fascinating. From increasing the expression of E-cadherin as a tumor suppressor gene, what it is pioneering in reporting is another mechanism of action of genistein.

Keywords: Cell lymphoblastic leukemia-lymphoma; Cadherins; Genistein

1. Introduction

Acute leukemia lymphoma (ALL) has been universally fatal for the past fifty years (1). ALL includes a heterogeneous group of malignant lymphoid disorders with different biological and clinical features (2). ALL prognosis and therapy is based on age, immunophenotype, karyotype, and predicted risk of central nervous system involvement (3). Substantial advances have been made in the past five decades in the treatment of ALL patients (4).

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Genistein is a potent inhibitor of tyrosine kinase, which is involved in proliferation signal cascades of cell growth (5) but with no effect on threonine and serine kinases (6). Moderate doses of genistein have been found to have inhibitory effects on the prostate, cervix, brain, breast, and colon cancers (7). Genistein has therapeutic effects on ALL (8), and has been suggested as an alternative compound. Several studies have been performed to elicit the anti-cancer mechanism against cancer cells over last decade, (9) particularly, association between dietary flavonoids such as genistein and infant leukemia (10). Hence, genistein could be a suggestive substance in the treatment of ALL. There are reports that genistein acts through inhibition of mTOR pathway and blocking of protein synthesis in ALL cell lines (9).

E-cadherin is a cell-cell adhesion molecule, transmembrane glycoprotein 120-kDa (11, 12) involved in facilitating cell-cell adhesion between nearby epithelial cells in different tissues (12, 13). This glycoprotein is thought to play a significant role in trophoblastic differentiation. Cellular adhesiveness may be a critical step in the ability of epithelial tumor cells to invade and metastasize both *in vitro* and in animal model systems (12). Due to the novelty of the present study and the lack of similar research in this subject, the aim of this study has been to investigate the effects of genistein on the invasive potential of the human carcinoma cell line ALL. Based on our best knowledge and findings from web research, there is no documented investigation to evaluate the effect of genistein on ALL or E-Cadherin. For exploring its underlying mechanism, this program examined the role of E-cadherin signaling pathway in the inhibitory effects of genistein on ALL cell invasion.

2. Material and Methods

2.1. Cell line

This *in vitro* experimental study was carried out in 2016 at the Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran. The cell lines used in this study were MOLT4 and JURKAT purchased from the Pasteur Institute of Iran. ALL cell lines were cultured in RPMI and L-Glutamine medium supplemented with 10% fetal bovine serum (FBS) and 500µl antibiotics (penicillin) in a humidified incubator at 37 °C with 5% CO2. The number of cells counted after the second passage. Then cell viability was determined by using Neobar Lam and Trypan blue techniques, respectively. The plates that had cell viability of more than 95% were used. ALL cell lines were treated with 10 µM, 25 µM, 40 µM and 55 µM genistein (Sigma-Aldrich, USA). Treated cells were harvested after 24, 48 and 72 hours for further molecular assays.

2.2. MTT assay (Thiazolyl Blue Tetrazolium Bromide)

MTT assay was used to investigate the effects of genistein on cellular proliferation. To do this, $5x10^5$ cells were seeded in 96-well plates and incubation for 3-4 hours was done. Then, our cells were treated in various concentrations of genistein (10, 25, 40, and 55 µM/L). Furthermore, untreated cells were considered as a negative control. Then the MTT survival assay was done across the different time periods (24, 48, and 72 hours). According to the conversion of tetrazolium dye (MTT) to a blue formazan product, cell viability was predicted by a colorimetric analysis. The absorbance of cell lysates in DMSO solution was read by ELISA Reader (Anthos 2020; England) at 495 ηm.

2.3. RNA extraction

The total RNA was extracted from cells using an RNeasy Plus mini kit (QIAGEN, Germany) according to the manufacturer's protocol. The extracted RNA was quantified by running on 1.5% agarose gel electrophoresis. The 18S and 28S RNA bands should be visualized under a transillumiator (Figure 1). The yield was quantified spectrophotometrically at 260 and 280 ηm by NanoDrop 1000 (Thermo, USA). The proportion of 2.0 obtained from 260/280 indicates the quality of extracted RNA.

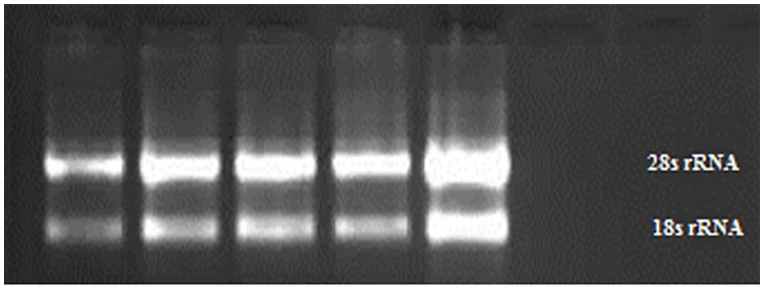

Figure 1. Extracted RNA electrophoresis.

2.4. cDNA synthesis and quantitative Real-Time PCR

The total RNA (2 µg) was reversely transcribed to cDNA using RevertAid TM First Strand cDNA Synthesis Kit (Cat. No.1632, Fermentas, Canada), by applying random hexamer primer following the manufacturer's protocol. Quantitative RT-PCR was conducted on a Real-time PCR (Corbett, Rotor-Gene 6000, Australia) and Sybr Green-Master Mix (Takara Cat No # RR820S #RR820Q, Japan) according to the manufacturer's instructions. All reactions were carried out in 20 µL of the mixture containing 2 µL of cDNA, Master mix 2X, ROX dye 50X and 10 pmol of each primer pair for E-cadherin and β-actin. The thermal cycling status was an initial denaturation at 95 °C for 30s followed by 4 cycles of denaturation at 94 °C for 5s, annealing at temperatures for each primer pair for 15s and an elongation at 60 °C for 30s. Expression levels of E-cadherin were normalized by β-actin expression as the housekeeping gene and calculated through the $2^{-\Delta\Delta CT}$ method. The sequence of specific primer sets for each gene, amplicon sizes and annealing temperature of the primers used for Real- time PCR time are displayed in Table 1.

Table 1. Primers designed for amplification of E-cadherin and β-actin

Gene	Primer (5′→3′)	Amplicon Size (bp)	Tm (°C)
E-cadherin	F: GAGAAGAGGACCAGGACTT R: CACGAGCAGAGAATCATAAG	216	59
β-actin	F: GCCTTTGCCGATCCGC R: GCCGTAGCCGTTGTCG	90	58

2.5. Statistical analysis

Statistical analysis was performed using IBM© SPSS© Statistics version 21 (IBM© Corp., Armonk, NY, USA), Microsoft Excel and Prism-5 software, and p-values less than 0.05 were considered statistically significant in our experiments. In addition, for investigating the expression pattern of target gene, Spearman's Rank-Order Correlation and Mann-Whitney U tests were used.

2.6. Ethical approval

This article does not contain any studies with human participants or animals by any of the authors. For this type of study, formal consent is not required. The ethics committee of Hormozgan University of Medical Sciences approved the ethics of this research (ref. no.: HUMS.REC.1394.134).

3. Results

The growth rate of cell lines in effect of different concentrations of genistein in three periods of time 24, 48 and 72 hours are compared in Figure 2. The growth rate of treated MOLT4 cells declined with the increasing concentrations of genistein. With regard to the concentration of 10 µM, in comparison to non-treated cells, the growth rate of 100% was reduced to 75% on the first day. On the second day, it became 64.4% and on the third day, it reached 29.4 %. For the 25 µM concentration, the growth rate in the third day reached 26.47% (almost the same as 10 µM and no significant difference in growth inhibition was observed). Reducing the growth rate in effect of 40µM concentration compared to the non-treated state on the third day became 10.3%. A significant difference ($p>0.012$) was calculated in comparison with the growth rate of 10 and 25 µM. Finally, concentration of 55 µM compared to the non-treated state, the cell growth rate in the first, second and third days declined to 22.9, 10 and 5%, respectively (significant reduction as compared to the other three concentrations). Almost the same trend was observed for JURKAT cell line. In comparison with non-treated cells, no significant reduction in growth rate of JURKAT cells was observed in concentrations of 10 and 25 µM in the first day, but 35% and 27% reduction in rate of cell growth were observed in the first day of treatment with 40 and 50 µM genistein, respectively. Growth rate reached 12.5% and 8.8% in effect of 40 and 55 µM in the third day, respectively. To study the toxic effects of genistein, MTT assay was performed in three different above-mentioned concentrations in periods of 24, 48 and 72 hours. Totally, MOLT4 cells showed a decrease in the percentage of live cells along with increasing the concentration as well as time of exposure to genistein in comparison with non-treated ones. The same trend was also observed for JURKAT cell lines.

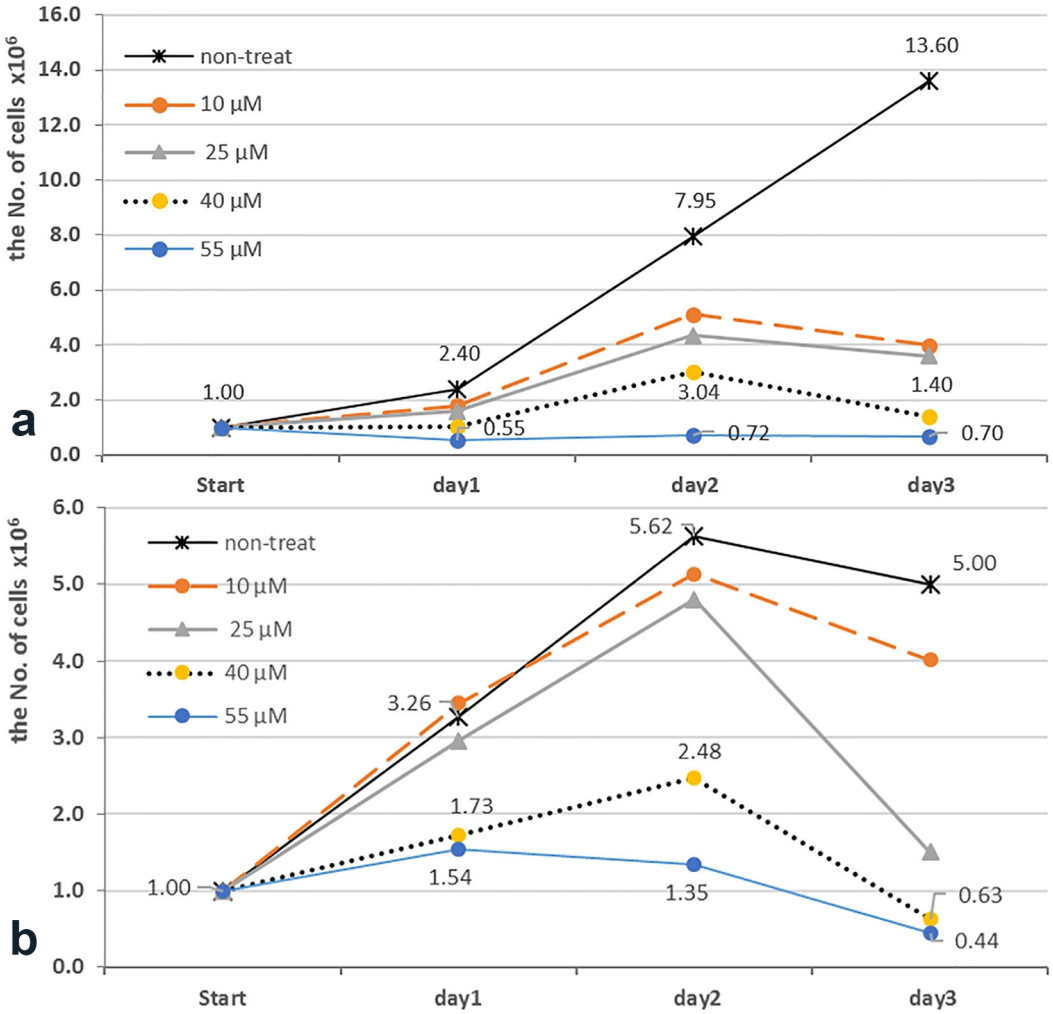

Figure 2. The comparison of growth rate in **(a)** MOLT4 and **(b)** JURKAT cell lines under the effect of different concentrations of genistein in three days.

In order to determine the effect of different concentrations of genistein on the expression levels of E-cadherin gene in MOLT4 cell lines, Real-time PCR analysis was performed after 24 and 48 hours. Totally, 10µM genistein could increase the expression level of E-cadherin as compared to the untreated cultures, but not significantly. In the other concentrations (25, 40 and 55µm), significant increase was relatively observed. After 48 hours, E-cadherin in treated cultures was significantly up-regulated in all four applied doses ($p=0.2$ for 2 4hr and $p=0.028$ for 48 hr) (Figure 3a). No significant modification in expression level of the E-cadherin gene was observed in JURKAT cells after 24 hours treatment. After 48 hours treatment, JURKAT cells presented a significant up-regulation in a dose-dependent manner ($p>0.4$ for 24hr and $p=0.0286$ for 48 hr) (Figure 3b). E-cadherin expression was significantly ($p<0.02$) up-regulated between 2 to 4 times in comparison with untreated cells. Overall, expression of E-cadherin after 48 hours treatment by various doses of genistein increased significantly in both MOLT4 and JURKAT cells (Figure 4).

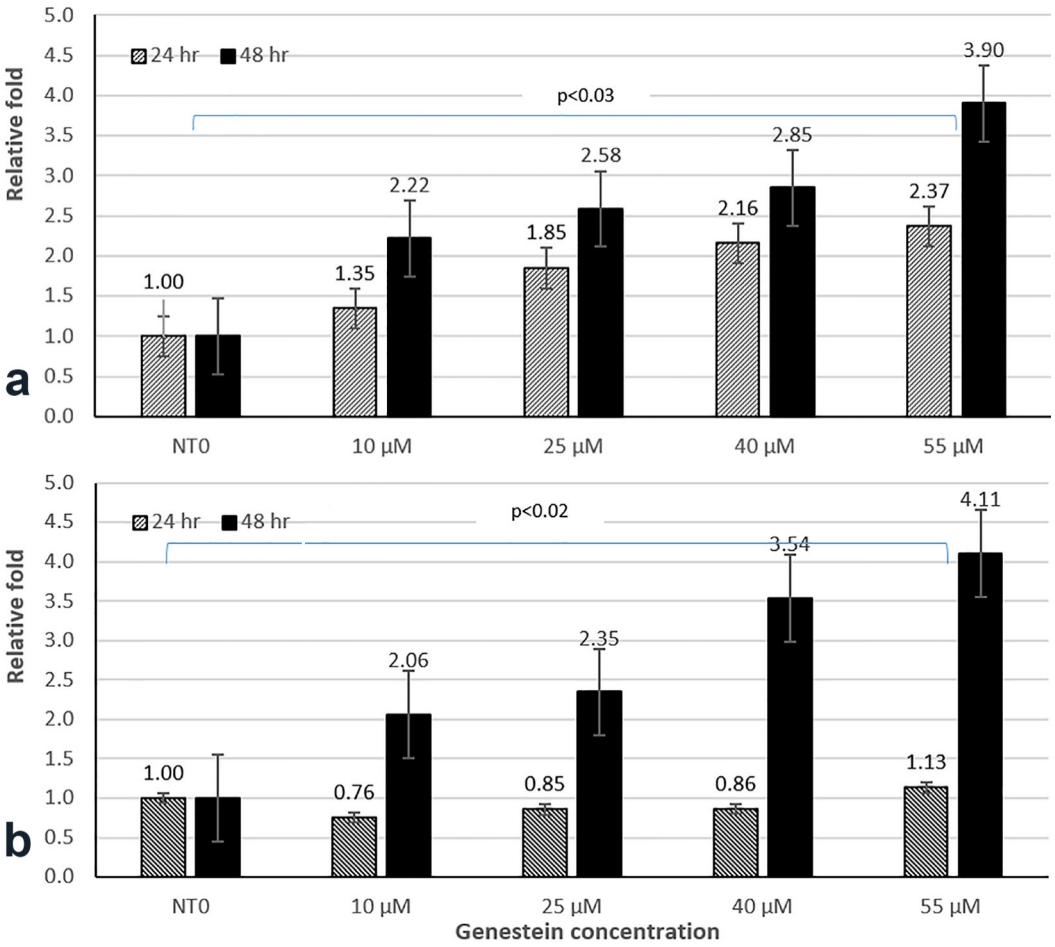

Figure 3. The Comparison the expression of E-cadherin after 24 and 48hrs treatment with different doses of genistein in **(a)** MOLT4 (p=0.2 for 24hr and p=0.028 for 48hr) and **(b)** JURKAT (p>0.4 for 24hr and p=0.0286 for 48hr) ALL cell lines.

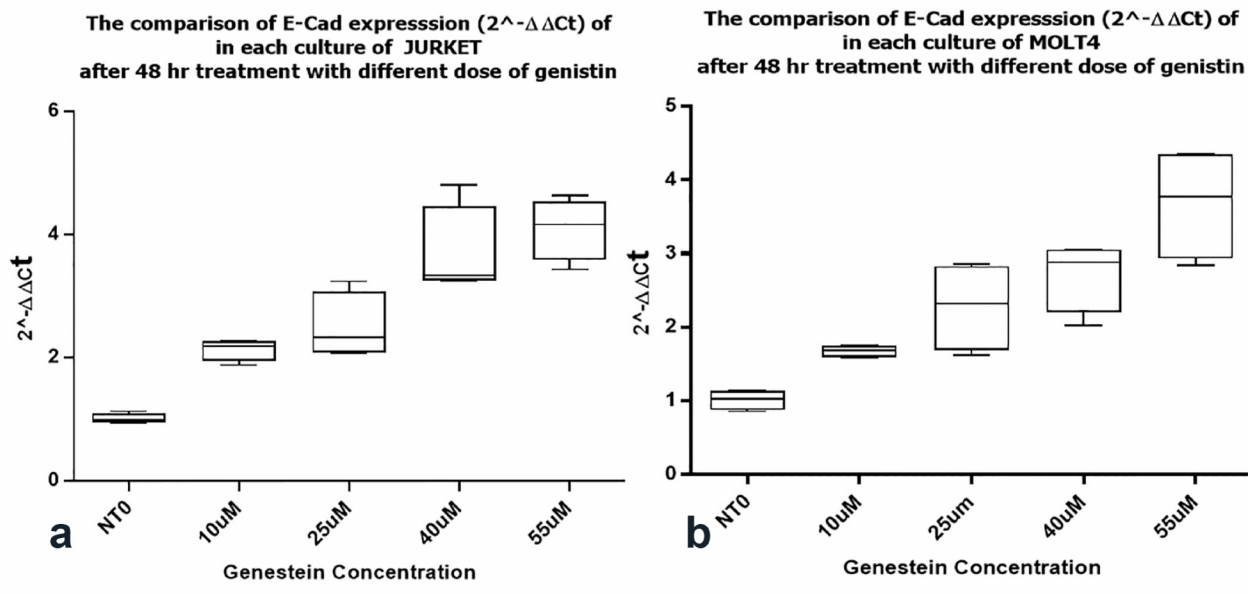

Figure 4. The comparison E-cadherin expression in **(a)** JURKAT and **(b)** MOLT4 cell lines after 48 hours' treatment with different doses of genistein.

4. Discussion

Due to increased prevalence of ALL, efforts for finding new and novel therapeutic applications have increased worldwide in recent years. Genistein, which is abundantly found in soybean, is anti-proliferative cancer cell. Genistein is a potent inhibitor of tyrosine kinase, which is involved in proliferation signal cascades of cell growth (5), and moderate doses of genistein have been found to have inhibitory effects on prostate, cervix, brain, breast and colon cancers (7). The inhibitory effects of genistein on carcinogenesis and cancer progression have been known for years. Experimental evidence has revealed that the inhibition of cancer cell growth by genistein is mediated by the modulation of genes associated to the control of cell cycle and apoptosis, invasion, and metastasis. This compound is bioactive, exhibiting anti-inflammatory properties and playing roles in tyrosine kinase inhibition and phytoestrogen and anti-cancer effects (14). The present research examined whether genistein has an inhibitory impact in growth of leukemia cell lines or not. The second question that we tried to answer was whether E-cadherin pathway would be involved in the inhibitory effects of genistein on ALL cell invasion. Li et al. reported the role of genistein in cell growth and apoptosis-related gene expression in breast cancer cells MDA-MB-231. They found up-regulation in the expression level of Bax and $p21^{WAF1}$ and down-regulation of Bcl2 and p53 in treated cells with genistein. They showed that apoptotic cell deaths occur by treating cells with genistein, and flow cytometry demonstrated that with longer treatment of genistein, the number of apoptotic cells increased. They conclude that genistein has an inhibitory effect on the growth of MDA-MB-231 breast cancer cells, regulation of the expression of apoptosis-related genes, and induction of apoptosis through a p53-independent pathway. These findings suggest that genistein could be an effective chemopreventive and therapeutic agent against breast cancer (15). Zhang et al. demonstrated that treatment of prostate cancer cells with low-dose genistein could be a strategy for suppressing cell invasion through the reversal of epithelial mesenchymal transition (EMT), which clearly shows the potential use of genistein as a chemopreventive agent for patients with prostate cancer (16). Zhang et al and Parnes et al. showed that genistein displays several biological activities that lead to prostate cancer prevention and it could be considered as a cancer chemopreventive agent (16, 17). In addition, some studies suggested genistein with an anti-metastatic activity for prostate cancer and consumption of genistein leads to lower incidence of clinical prostate cancer metastasis (16, 18). Russo et al. and Spagnuolo et al. demonstrated the role of genistein in inhibition of cell growth (in both hormone-dependent and -independent cancer cells) in a dose-dependent manner (19, 20). Russo et al. demonstrated that genistein has an anti-proliferative activity in pharmacological doses (higher than 10 μM) and this suggests that genistein might have in vivo anticancer effects (21). Russo et al and Da Silva et al. revealed that genistein directly inhibits Akt and NF-κB pathways (two important pathways in activation of apoptosis) in prostate malignant cells (21, 22). Dai et al. investigated the mechanism of action of genistein on hepatocellular carcinoma (HCC) cells. They showed that genistein exhibits antitumor activity through modulating cellular motility and migration (23).

From the available literature on the web, there is no documented study regarding the effect of genistein as a potent growth inhibitor against ALL and it seems that is the first study accomplished. Two ALL cell lines including MOLT4 and JURKAT (respectively as mild and high aggressive acute lymphoblastic leukemia cell lines) were investigated by treatment with different concentrations of genistein (10, 25, 40 and 55 μM) for 24 and 48 hours in the present study. MTT assay was conducted to obtain the cytotoxic dose of genistein. It found that 40 to 55 μM can effectively inhibit the growth of ALL cell lines. Based on MTT assay results, direct proportion between increasing the percentage of apoptosis and increasing dose and exposure time to genistein was obtained. The percentage of vital malignant cells treated with genistein in comparison with non-treated cells was significantly decreased ($p<0.03$).
High correlation was seen between genistein and reduction of the growth rate in time- and dose-dependent manner, higher concentrations of genistein and longer treatment periods demonstrated more reduction in growth rate. Increasing the time of exposure to a higher non-toxic dose of genistein could effectively induce its anti-proliferative effect against acute lymphoblastic leukemia (5). Of course, reduction in growth rate in 72 hours could be due to the lack of nutrients in the cell. Even though it seems that genistein induces its anti-cancer properties on all doses, strong association found that these anti-cancer properties had a negative correlation with increasing the concentration and exposure time of genistein. Similar to other studies above-mentioned, in the present study, we showed that genistein exhibits growth inhibitory property against leukemia cell lines. In fact, our findings were in line with the findings of other studies in solid tumors.

E-cadherin is a cell-cell adhesion molecule, transmembrane glycoprotein 120-kDa (11, 12) involved in mediating cell–cell adhesion between adjacent epithelial cells in various tissues (12, 13). This glycoprotein is thought to play a significant role in trophoblastic differentiation. Cellular adhesiveness may be a critical step in the ability of epithelial tumor cells to invade and metastasize both *in vitro* and in animal model systems (12). This gene is located on chromosome 16 (16q22.1) encodes a protein product important in the maintenance of the epithelial phenotype

mediated by a Ca-dependent, homotypic cell-cell adhesion (24). Qian Li et al. discovered that lack of E-cadherin gene expression in cancer cells leads to dysfunction of the cell–cell junction system activating cancer invasion and metastasis, therefore, E-cadherin could be an important tumor-suppressor gene (25). Many papers have reported regulation of E-cadherin expression by epigenetic modifications. Aberrant hypermethylation of CpG islands is a particular mechanism in cancer biology because of a strong association with gene inactivation (18). Although most CpG dinucleotides in a normal cell have undergone methylation, the CpG dinucleotides including CpG islands are essentially unmethylated in all tissues (19), but seem to undergo excessive methylation (hypermethylated), in particular tumor suppressor genes in most malignancies including leukemia. Hypermethylation is a main cause of decreased E-cadherin expression (26). While E-cadherin protein is mostly present in the cell surface of epithelial cells, it has also been accepted that colony-forming units–erythrocytes (CFU-E), normoblasts, and erythroblasts express E-cadherin (6, 7). It is definite that erythroid progenitor cells are reliant on E-cadherin gene expression for maturation (24).

Jalali et al. showed that E-cadherin could be a prognostic biomarker for breast cancer. They demonstrated through immunohistochemistry with monoclonal antibodies that increasing the expression levels of E-cadherin has a negative correlation with tumor size, malignancy stage and lymphatic metastasis level (27). Also, Jalali et al. concerning the role of the E-cadherin in prognosis of papillary thyroid carcinoma, revealed that as proportion of E-cadherin decreases in tumor cells, the probability of lymph nodes involvement increases (28). Tsao et al. showed that loss of E-cadherin is associated with progression and poor survival in nasopharyngeal carcinoma (NPC). In this study, the role of methylation on E-cadherin inactivation in NPC cell lines and NPC tissue samples was investigated. Results indicate that 5'CpG island methylation of the E-cadherin gene are important in the inactivation of E-cadherin in NPC and propose that decreasing the methylation of the E-cadherin gene may be an essential therapeutic strategy for NPC (12). Nishimura et al. indicated that 5'CpG island methylation leads to loss of E-cadherin expression, and could indirectly cause the suppression of gap junctional intercellular communication (GJIC) through aberrant localization of connexins (Cxs) in endometrial carcinoma cells (29). Corn et al., showed that 5'CpG island methylation caused silencing the expression of E-cadherin in acute leukemia. They found that methylation was associated with loss of E-cadherin RNA and protein in leukemia cell lines and primary leukemia. Their results revealed the methylation of E-cadherin in acute leukemia and provided a hypothesis for E-cadherin down-regulation in leukemogenesis (30).

5. Limitations

In this study, we have some limitations such as lack of normal cells, lack of access to antibodies for determination level of gene expression at the protein level due to the project's financial constraints, and no access to a flow cytometry device for determining the rate of induction of apoptosis in different genistein concentrations.

6. Conclusions

In conclusion, this study can be viewed as a pioneering research of its kind to raise another mechanism of action of genistein against ALL cancer cells. The obtained findings could suggest that genistein possesses certain anti-cancer properties in ALL similar to solid tumor cells. From increasing the expression of E-cadherin as a tumor suppressor gene, what it is pioneering in reporting is another mechanism of action of genistein and it can be deduced that this natural isoflavone could be considered as an agent to potentially control the invasion of leukemia cells and expedition of disease, which is promising and fascinating. Our findings suggest that further studies be carried out on effects of genistein on other cell lines and ALL patients. For genistein effects and epigenetic changes including cadherin hypermethylation and evaluation of the combined effect of genistein and other anticancer drugs on cell culture, in vivo and *in vitro* studies are suggested.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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