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The effect of Ganoderic Acid A on miR-17-5p and miR-181b expression level and apoptosis induction in human leukemia Nalm-6 cells

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Abstract

Background: In various cancers, Ganoderic Acid A (GAA), an active triterpenoid derived from Ganoderma lucidum, has been proved to show potent anti-tumor effects. However, the possible impacts of GAA on the human leukemia cell line (Nalm-6) are not fully elucidated. Therefore, this research aimed to study the antineoplastic effect of GAA on Nalm-6 cells.

Materials and Methods: In this laboratory trial study, Nalm6 cells were cultured in vitro and treated with different doses of GAA (25, 50, 100, 200, and 400 µg/mL) for 24, 48, and 72 hours. The optimal treatment concentration of GAA was determined by the MTT assay. Flow cytometry was used to determine the death of Nalm-6 cells caused by GAA treatment by utilizing FITC-conjugated propidium iodide (PI) and annexin V staining. After incubation, the expression levels of miR-17-5p and miR-181b were monitored using real-time polymerase chain reaction (PCR).

Results: Based on the half-maximal inhibitory concentration (IC50) measurements of the MTT assay, the optimal treatment concentration of GAA was 140 µg/mL (in a dose and time-dependent manner, $p<0.0001$). The GAA treatment was selectively toxic to the leukemia Nalm-6 cells and could remarkably induce cell apoptosis ($p<0.0001$). Besides, GAA downregulated the expression of miR-17-5p and miR-181b in the Nalm-6 cells compared with the untreated cells ($P=0.0067$ and $P=0.0014$, respectively).

Conclusions: Based on the present findings, GAA merits further investigation as a promising natural reagent for treating hematologic malignancies.

Keywords: Apoptosis, Ganoderic Acid A, MiR-17-5p, MiR-181b, Nalm-6 cells

Introduction

Acute lymphoblastic leukemia (ALL) is a hematologic neoplastic disorder and the most common pediatric cancer (0-14 years) worldwide (1). This hematologic malignancy accounts for approximately one-third of all childhood cancers (2). Despite various available treatments for ALL patients, these treatments are associated with a relatively high rate of side effects (3, 4). Moreover, resistance to conventional therapies and disease relapse can threaten ALL patients and lead to therapy delay or discontinuation (5). Therapy-related toxicity has become a major therapeutic challenge in ALL (6). Therefore, developing novel and effective natural agents with minimum adverse effects to increase therapeutic efficacy is fundamental in ALL therapy. Ganoderma lucidum (G. lucidum), also known as "Reishi" and "Lingzhi" mushroom in Japan and China, respectively, has been consumed broadly as a health tonic and ancient therapeutic in China and other oriental countries (7). This beneficial fungus has been utilized to treat various conditions, including cancers, due to its significant salutary effects (8). Although different bioactive molecules have been identified in this medicinal mushroom, its

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pharmacological activity is primarily attributed to polysaccharides and triterpenoids (9). Ganoderic Acid A (GAA) is the most promising candidate from the triterpenoid family, isolated from G. lucidum with a range of biological activities (10). Previous studies have shown that GAA exhibits various beneficial activities, such as antioxidative (11), anti-viral activity (12), hepatoprotective (13), and particularly anticancer activities (14). Currently, evidence suggests that GAA has remarkable cytotoxic effects against leukemia, lymphoma (15), hepatocellular carcinoma (16), osteosarcoma (17), and breast cancer cells (18) through suppressing cell growth and invasion and inducing apoptosis. Besides, GAA can be considered an adjuvant therapeutic intervention for various malignancies. MicroRNAs (miRNAs) are non-coding endogenous RNAs, ranging from 19 to 25 nucleotides in size, which modulate gene expression at the post-transcriptional level (19). Emerging evidence suggests that aberrant miRNA expression is correlated with multiple types of human diseases, including cancer (20). Considering the predominant function of miRNAs in cancers, they can act as either oncogenes or tumor suppressors by regulating their respective target genes (21). MicroRNA-17-5p belongs to the miR-17-92 cluster family, with an established oncogenic function (22). It is currently known that miR-17-5p is highly expressed in many human cancers, including ALL (23). It has been shown that miR-17-92 components lead to the progression of B-cell malignancies through downregulation of phosphatase and tensin homolog (PTEN) and enhancement of ACT/mTOR signaling pathway activation (24). As a member of the miR-181 family, miR-181b may have a unique role depending on tumor type and cellular environment (25-28). The literature suggests that the expression of miR-181b is directly regulated by signal transducer and activator of transcription (STAT3) pathway and high-mobility group AT-hook 1 (HMGA ((29). Notably, growing evidence shows that natural compounds can govern miRNAs, including the G. lucidum extract (30). Recently, it has been reported that G. lucidum polysaccharide extract can effectively inhibit the progression of hepatocellular carcinoma via downregulation of the accumulation of regulatory T cells by inducing miR-125b (31). Moreover, Qing-Ping Wu's study (2012) revealed that ergosterol peroxide obtained from G. lucidum could be used to counteract the drug resistance given by miR-378 (32). Additionally, Ganoderma lucidum polysaccharide extract inhibited Oral squamous cell carcinoma (OSCC) cell proliferation and migration by controlling the miR-188/BCL9/-catenin signaling system (30). According to the previous studies, Ganoderma lucidum extract could alter the expression of miRNAs in different cancer cell lines. Previous studies have shown that miR-17-5p and miR-181b act as incomers in the Nalm-6 cell line (33). Consequently, the present study was focused on miR-17-5p and miR-181b. This study investigated the impact of GAA content of the Chinese herb G. lucidum on apoptosis induction and alteration of miR-17-5p and miR-181b expression in Nalm-6 cells.

Materials and Methods

Materials

The American Type Culture Collection provided us with the Nalm6 B-cell precursor leukemia cell line (ATCC; Gaithersburg, Maryland, USA. Gibco Life Technologies (Waltham, MA) and Sigma-Aldrich (Munich, Germany) provided all of the ingredients and reagents utilized in cell culture. The cells were regularly maintained at 37°C in a 5 percent CO2 and 95 percent air humidified environment in RPMI-1640 media supplemented with 10 percent fetal bovine serum (FBS), 2 mM l-glutamine, and 100 U/mL penicillin-streptomycin solution

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(Sigma-Aldrich). Cell integrity was ensured by modifying the growth medium for the Nalm-6 cell line using conventional cell culture methods. Cell viability was also assessed using the trypan blue exclusion technique. The GAA extract was purcased from Sigma-Aldrich Co. (St. Louis, MO, USA), and stock solution was made in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/mL and kept at -80°C.

MTT assay

The MTT test was used to determine the inhibitory effect of GAA on the metabolic activity of the cells. In a 96-well plate, 2×10^4 cells were added to 200 µL of growth media and cultured for 24, 48, and 72 hours, using various doses of GAA (25, 50, 100, 200, and 400 µg/mL). Next, 20 µL of freshly prepared MTT solution (5 mg/mL in PBS) was added to every well then incubated at 37°C in a 5 percent CO2 environment. The plate was incubated for 3-4 hours at 37°C before being centrifuged at 350 g for 10 minutes to remove the supernatant. Besides, 150 µL of DMSO was used to dissolve the formazan crystals, and a BioTek ELx800 microplate photometer was used to examine the spectrometric absorbance at 570 nm (SN211805; BioTek, Winooski, VT, USA). The medication concentration that prevented cell growth by 50% was known as the half-maximum inhibitory concentration (IC50). Each experiment was repeated three times to ensure accuracy.

RNA extraction

The TRIzol reagent (Thermo Fisher Scientific, MA, USA) was used for cellular RNA extraction in each group. An RNA spectrophotometer (NanoDrop 2000; Thermo Fisher Scientific, USA) was used to measure the amount and quality of RNA, which was then frozen at −80°C.

cDNA synthesis

The reverse transcriptase (RT) microRNA PCR kit bought from Pars Gene Company (Tehran, Iran) was used to synthesize cDNA using 2µg of total RNA for the study. The RT enzyme was inactivated in all samples by heating the samples to 85°C for one minute. All samples were then incubated at 42°C for 60 minutes. The miR-17 and miR-181b qRT-PCR assays were performed using an ABI 7500 real-time PCR equipment (Applied Biosystems, Foster City, CA, USA), SYBER Green Master Mix (Pars Gene Co., Iran), and particular primers (Pars Gene Co., Iran). Primers sequences for Real-time PCR are presented in Table1. To normalize the levels of expression of each miRNA, the U6 rRNA gene was utilized as an internal reference.

Real-time PCR

During a typical PCR, denaturation, annealing, and extension cycles are repeated to achieve exponential amplification of the target sequence. Denaturation consists of robust heating of the samples to separate or denature the DNA strands, which provides a single-stranded template for the next step. Once the strands are separated, the temperature is decreased to the annealing temperature so that the primers may pair (or anneal) to complementary regions of the template. The polymerase extends the primer to form a nascent DNA strand during the extension step. The following conditions were used in the qRTPCR assay: first, denaturation at 95°C for 15 minutes, then 40 cycles of 95°C for 30 seconds followed by 62°C for 30 seconds, and 72 °C for 30 seconds. The length of Real-time PCR products for miR-181b and miR-17-5p were 25 and 22 nucleotides, respectively. The comparative CT technique was used to determine the relative fold alterations for each sample ($RQ=2^{-\Delta\Delta Ct}$). The current study utilized target Scan, Miranda, and miRTarBase databases to find miRNAs and their target genes.

Flow cytometry

A kit developed by BD Biosciences (San Jose, CA) was utilized to measure the percentage of apoptotic cells following GAA treatment. In brief, Nalm-6 cells were collected 48 hours after being treated

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with the specified dose of GAA isolated from G.lucidum. Phosphate buffered saline (PBS) was used to wash the cells two times before 5×10^5 of them were resuspended in 1 mL of 1X Annexin V binding buffer. The cells were then incubated in the dark for 20 minutes at room temperature with 5 μL of fluorochrome-conjugated Annexin V and 5 μL of PI staining solution. Next, 400 μL of binding buffer was added to the cell suspension, centrifuged for five minutes at 400 g. FACS Calibur flow cytometer identified the Annexin V-positive/PI-negative as early phase apoptotic cells, whereas those positive for both Annexin V and PI were believed to be in the late stages of apoptosis or necrosis (BD Biosciences, USA). FlowJo software was used to analyze the data for the apoptotic population (TreeStar LLC, USA).

Ethical Consideration

The local ethics committee approved this experimental study of medical experiments of Shiraz University of Medical Sciences with the ethics code of IR.SUMS.REC1399.838.

Statistical analysis

The results are presented as the mean and standard deviation (SD) of three separate studies. GraphPad Prism was used to perform all analyses (GraphPad Software Inc. La Jolla, CA, USA). One-way variance analysis (ANOVA) was used to compare various groups. $P<0.05$ was utilized as the statistical significance threshold.

Results

Analysis of the effect of GAA on cell viability in the Nalm-6 cell line

Chemical Structure of Ganoderic Acid A is shown in Figure 1. The findings showed that GAA significantly reduced the ability of Nalm-6 cells to proliferate. As shown in Figure.2, an MTT assay was used to determine the survival rate of Nalm-6 cells after 24, 48, and 72 hours of GAA treatment with deferent concentrations (25, 50, 100, 200, and 400 μg/mL). Results of cell viability determination exhibited that cell viability decreased in a dose-dependent and time-dependent manner. The IC50 was used to establish the optimum GAA concentration. Based on the IC_{50} measurements using MTT assay, the Nalm-6 cells could hardly grow in the presence of 140 μg/mL of GAA for 48 hours ($p<0.0001$). The present study's findings demonstrated the anti-proliferative effects of GAA content from *G. lucidum* on the human Nalm-6 cancer cells. Note that 140 g/mL of GAA was utilized in the subsequent tests after 48 hours.

Effect of GAA on cell apoptosis

The Annexin V-FITC Apoptosis Detection Kit was utilized to measure the amount of apoptosis based on the presence of phosphatidylserine in the outer membrane of Nalm-6 cells treated with G. lucidum GAA extract. Figure.3 illustrates the results of the experiment. Moreover, the Nalm-6 cells were exposed to GAA at a 140 g/mL concentration for 48 hours. The present findings f the present showed that the cells treated with Nalm-6 had a much higher proportion of apoptotic cells than the control groups. The present study's findings showed 40.5% of early and late apoptosis after 48 hours ($p<0.0001$).

Effect of GAA on miR-17 and miR-181b expression in the Nalm-6 cell line

In the present study, to the best of our knowledge, qRT-PCR was performed for the first time for evaluating the expression of miR-17-5p and miR-181b in the Nalm6 cell line before and after GAA treatment. Figure.4 depicts the results of a miRNA RT-PCR experiment to see if GAA treatment (140 μg/mL) for 48 hours affected the miR-17-5p and miR-181-b in expression Nalm-6 cells. It was demonstrated that miR-17 (median fold change [FC]=0.23; P=0.0067) and mir-181b (FC=0.01; P=0.0014) expression was significantly reduced in treated Nalm-6 cells compared to untreated cells according to the findings of Real-time PCR.

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Also, results of the present study showed reduced miR-17-5p and miR-181b expression in the Nalm-6 cells treated with GAA compared to the group treated with L-asparaginase (P=0.042 and P=0.0053, respectively).

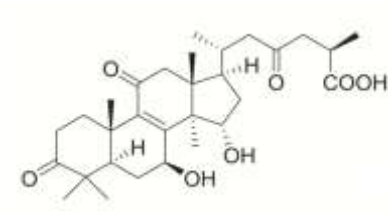

Figure1. Chemical Structure of Ganoderic Acid A

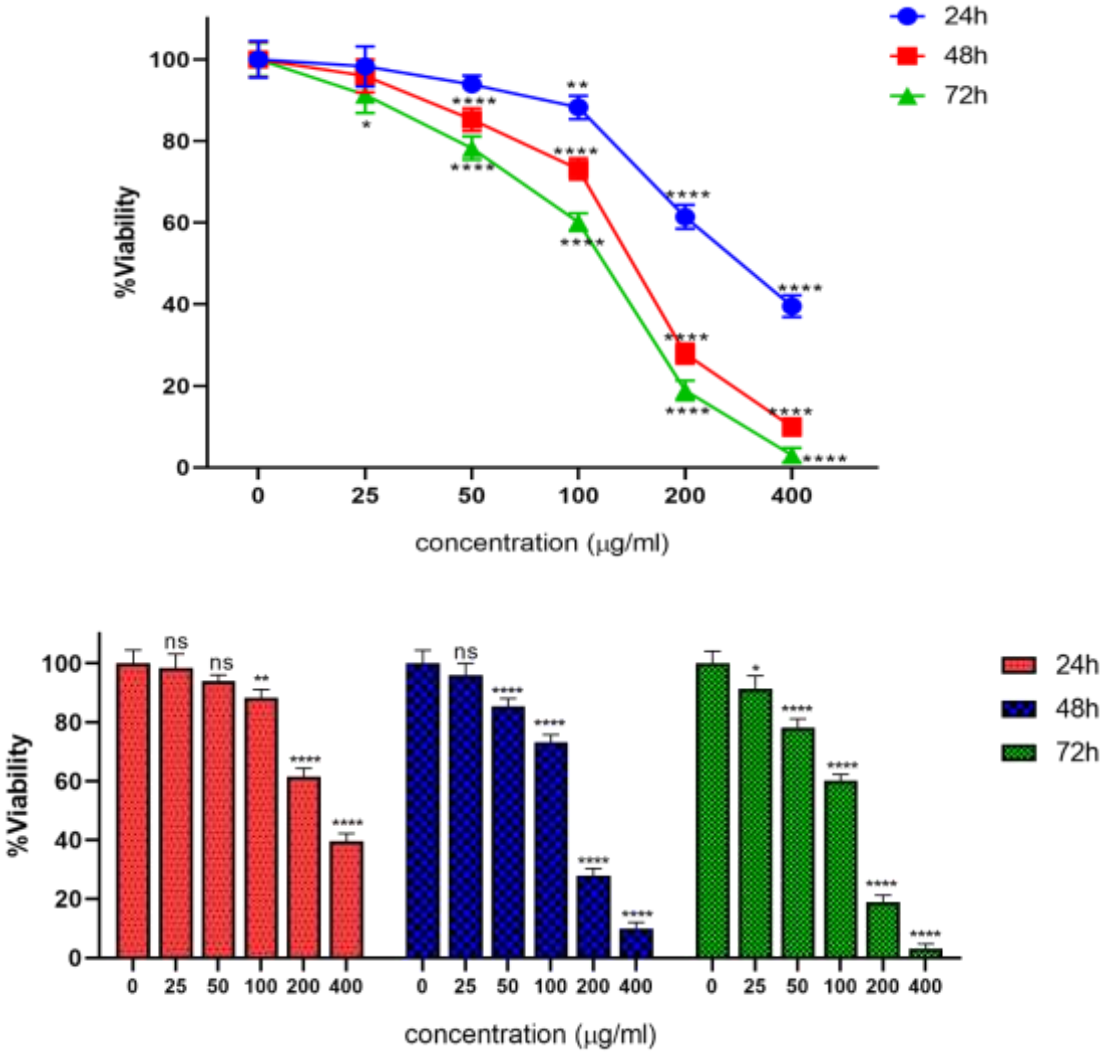

Figure 2. The IC_{50} of GAA treatment in the Nalm-6 cells was determined using MTT assay. The Nalm-6 cells (2×10^4 cells/well) were plated into 96-well plates and treated with increasing concentrations (25, 50, 100, 200, and 400 µg/mL) of GAA for 24, 48, and 72 hours. Data are shown as mean ± SD in triplicate. The cells treated without GAA were used as the controls.

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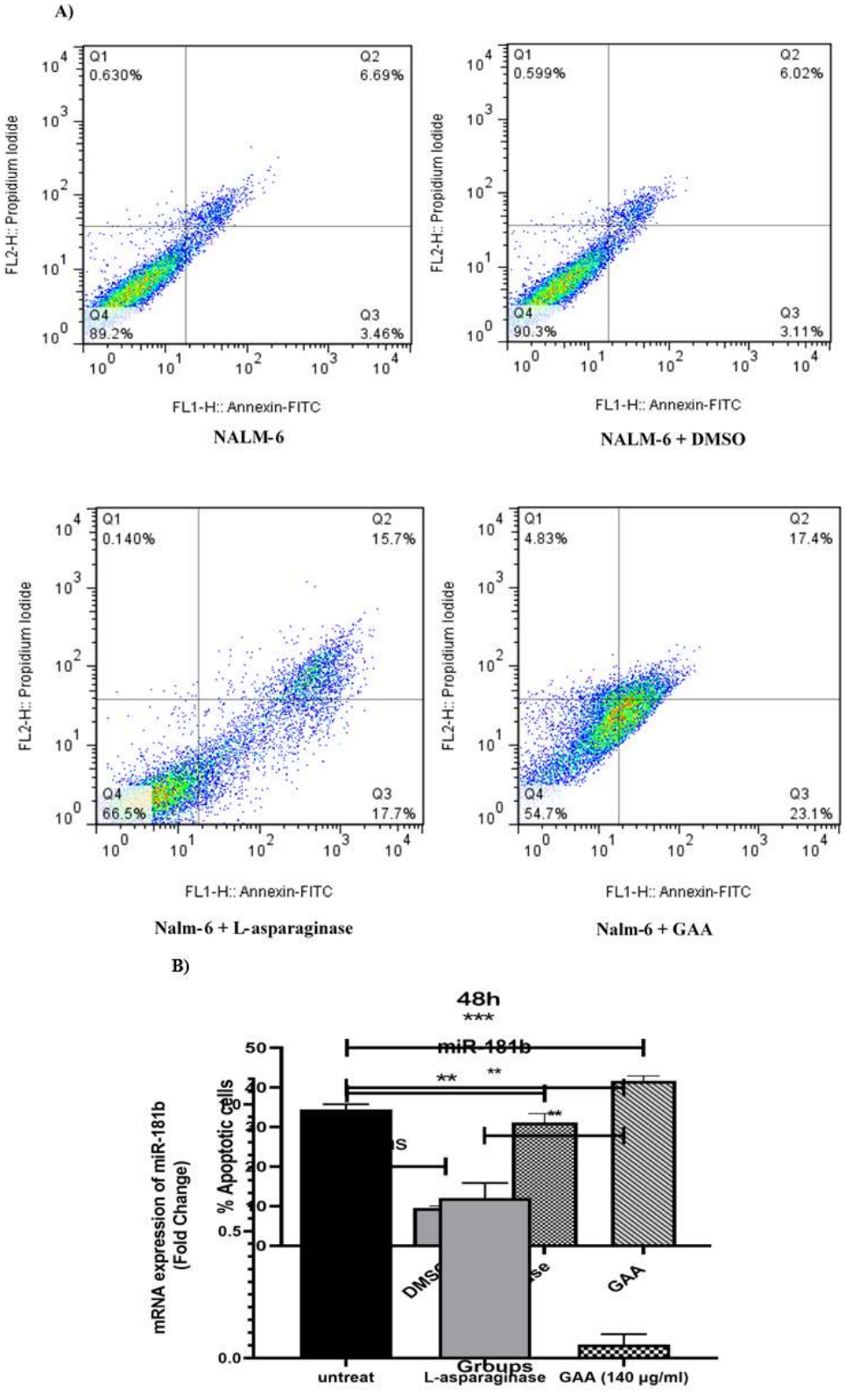

Figure 3. The effects of GAA on the apoptosis of Nalm-6 compared with the control groups. This diagram shows the Nalm-6 cells treated with 140 µg/mL of GAA extract for 48 hours. Data are presented as mean ± SD of at least three independent experiments. The apoptotic index is the sum of the percentage of positive cells for Annexin-V-FITC alone and the positive cells for both Annexin-V-FITC and PI ($p<0.0001$).

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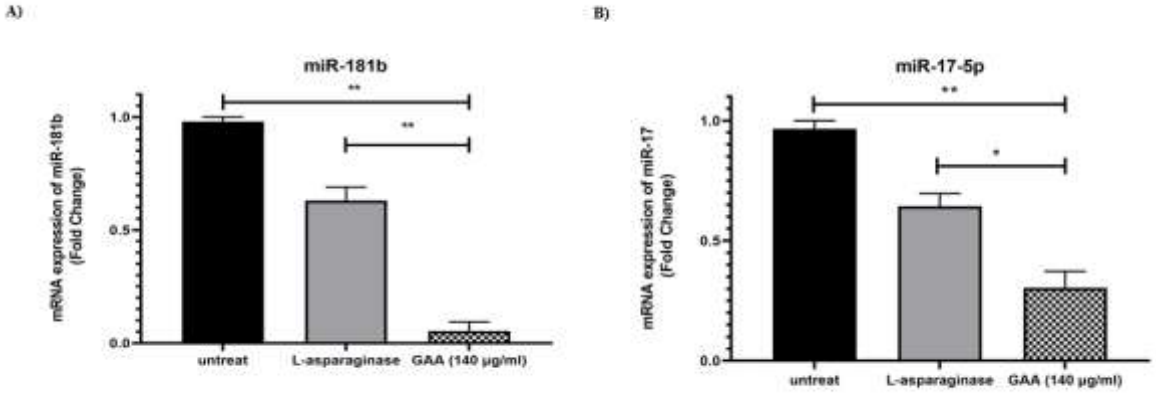

Figure 4. The expression levels of miR-17-5p and miR-181b in the Nalm-6 cells after treatment with GAA (140 µg/mL) for 48 hours. Significant differences in the expression levels of miR-181b (A) and miR-17-5p (B) were reported between the treated Nalm-6 cells and control groups by qRT-PCR assay. The U6 rRNA gene was used as the internal control (error bars represent standard deviations).

Discussion

GAA is a novel, highly oxidized triterpenoid extracted from the Chinese herbal medicine G. lucidum, which Asian physicians and naturopaths recommend preventing and treating various human diseases, including cancers (34). Substantial evidence suggests that GAA possesses significant anticancer properties against different human cancer cells in vitro (15-18, 35). Nevertheless, to the best of our knowledge, the molecular mechanism of GAA in the human cell line derived from B cell leukemia (Nalm-6 cell line) remains unknown, and there are only several pieces of literature of laboratory and preclinical studies on the anti-tumor activity of GAA in the Nalm-6 cells. Therefore, in the present study, the biological function of GAA was evaluated in the Nalm-6 cells in vitro and explored the potential underlying mechanisms associated with this activity. The present study showed that GAA treatment was cytotoxic and markedly suppressed cell proliferation and induced apoptosis in the Nalm-6 cells in vitro. Consistent with our findings, previous studies also established that GAA, as a component of GL extract, has cytotoxic effects on different cancer cell lines, such as leukemia cell lines (15). More importantly, the study results revealed that GAA significantly downregulated the expression levels of oncomirs 17-5p and 181b in the Nalm-6 cells compared to the control groups. Substantial evidence suggests that natural products, including G. lucidum extract, exert their anti-tumor activities via several mechanisms, including miRNA expression modulation (36). Recently, it has been suggested that G. lucidum polysaccharides (GLPs) markedly inhibit hepatocellular carcinoma growth in vitro and in vivo by decreasing Treg cell accumulation, which is correlated with the upregulation of miR-125b (31). Besides, another study reported that ergosterol peroxide obtained from G. Lucidum exhibits profound activities in inducing the apoptosis of miR-378-transfected cells, overcoming resistance to multiple drugs (32). In addition, it has been demonstrated that GLP increased miR-188 in HSC-3 cells expression, and miR-188 blocked the activation of the β-catenin signaling pathway via the binding to BCL9 (37). The present study appears to be the first to examine the impact of GAA on miRNA expression. Therefore, it was attempted to determine whether miRNAs could be involved in the response

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of treated Nalm-6 cells. Notably, previous studies have reported that miR-181b in multiple types of cancers, such as pediatric ALL, acts as an oncogene and dramatically leads to the growth and development of malignancies (38-41). Previous studies have shown STAT3 and HMGA1 directly modulate the expression of miR-181b (42, 43). Recent studies have shown that STAT3 is an oncogenic protein (44). The STAT3 has been linked to the development, progression, and invasion of various malignancies, including lymphoma, multiple myeloma, and leukemia. Interestingly, it has been reported that GAA inhibits the STAT3 signaling pathway and is highly susceptible to cisplatin-induced cell death in HepG2 cells (45). Additionally, a study on a breast cancer cell line found that GAA exerted anticancer effects through JAK2/STAT3 signaling pathway, and the activities of JAK2 and STAT3 were directly inhibited by GAA treatment (46). Moreover, Balraj Singh Gill et al. investigated the effect of GAA extract on human prostate cancer cells and reported that GAA inhibited cell proliferation and viability and downregulated the expression of STAT3 in prostate cancer (PC) cells (47). However, this study did not investigate the direct relationship between GAA and STAT3 inhibition. It can be postulated that significant downregulation of miR-181b expression by GAA is probably linked to GAA's ability to suppress the STAT3 pathway, which ultimately resulted in the death of Nalm-6 cells; accordingly, further investigation is needed in this field. Upregulation of the miR-17-92 cluster is a major oncogenic event in various cancer types, including breast cancer, prostate cancer, pancreatic cancer, colon cancer, lung cancer, lymphoma, and leukemia (48). The literature suggests that the miR-17-92 cluster interferes with the tumor-suppressive pathway and spreads malignancies (49). Recent studies have reported that a principal target of miR-17-5p is the tumor suppressor, PTEN (24). It seems that the miR-17-92 components play a crucial role in the occurrence of B-cell malignancies by downregulation of PTEN potentiating the ACT/mTOR pathway (50). The PI3K/ACT/mTOR pathway is a substantial regulator of biological processes, including cell proliferation, survival, differentiation, death, cancer, and angiogenesis (51). The aberrant activation of at least one component of the PI3K/ACT/mTOR signaling network is correlated with the occurrence and development of tumors, including leukemia (52). Recent experimental findings suggested that human glioblastoma cells were strongly inhibited under GAA treatment via the PI3K/ACT signaling pathway inactivation (53). Also, they figured out that effects of GAA obtained from G.lucidum on apoptosis induction and proinflammatory cytokines release in hypoxia-treated neural stem cells (NSCs) were eliminated by suppression of PI3K/AKT pathway (54). In this regard, the previous study findings demonstrated that GAA could suppress phenotypic modulation of Pulmonary Artery Smooth Muscle Cells (PASMCs) via inactivation of the PI3K/mTOR signaling pathway (55). Previous research suggested that GAA may suppress miR-17-p activity by disabling the PI3K / ACT signaling pathway. Therefore, further experimental research is still needed to analyze the underlying regulatory mechanism or GAA on miR-17-5p expression. The current study exhibited that GAA, a triterpenoid isolated from G.lucidum, possesses the downregulated miR-181b and miR-17-5p in Nalm-6 cells. Therefore, it can be suggested as preliminary evidence on the underlying mechanism involving the anticancer activity of GAA.

Conclusion

The present study suggested that GAA, as a natural, non-toxic compound derived from G. lucidum mushroom, could inhibit

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the growth of human cancer Nalm-6 cells and drastically induce apoptosis. Moreover, the present study results suggested that GAA could decrease the expression of miR-17-5p and miR-181b. However, a further in-depth studies are required to elucidate the exact underlying mechanism of GAA as a novel clinical anti-cancer agent for the prevention or treatment of human leukemia.

Conflict of interest

The authors declare no conflict of interest.

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