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Influence of amygdalin on genes expression of childhood B and T acute lymphoblastic leukemia cell lines

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Abstract

Introduction and Objective. B and T acute lymphoblastic leukemias (ALL) are the most common malignancies in children. In recent years, thanks to advances in the field of pharmacology and genetics, the 5-year survival rate for children and adolescents suffering from ALL has improved to more than 90%. However, because of toxicities and adverse effects of current treatment, the incorporations of novel therapeutic approaches are needed to enhance the quality of life of patients. Recent studies reveal that amygdalin is a potent inhibitor of cell growth in bladder, breast, pancreatic, prostate cancer, and renal cell carcinoma cell lines. The aim of the study is to assess the influence of amygdalin on the expression of different genes in B and T acute lymphoblastic leukemia cell lines.

Materials and Method. B-ALL CCRF-HSB-2 [CCRF HSB-2, HSB-2] CCL-102.1 cell line and T- ALL CCRF-SB [CCRF-SB] CCL120 cell line were cultured and treated with amygdalin in concentration of 5 mg/ml and 10 mg/ml. Clariom S human array was used to evaluate the expression of genes. Microarray data were analyzed using Transcriptome Analysis Console 4.0, following the TAC user guide.

Results. The results show that exposing tumour cells of T-ALL CCRF-SB cell line to amygdalin led to down-regulation in concentration-dependent manner a.o. of MYC, BCL2, ID1, HRAS, NOTCH1 pathway, PI3K/AKT pathway. In stimulated with amygdalin B-ALL CCRF-HSB-2 cell line downregulation of a.o. CRKL and EZH2 genes was observed.

Conclusions. The study provides the first insight into the potential influence of amygdalin on childhood acute lymphoblastic leukemia cell lines.

Key words

cancer, cell lines, amygdalin, childhood acute lymphoblastic leukemia

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in children, responsible for about 25 % of cancer diagnoses among children under the age of 15. Although the survival rate has improved from ~10 % in the 1960s to >90 % in recent years, it remains one of the leading causes of cancer-related deaths in children. The B immunophenotype of childhood ALL is diagnosed in 85% and T-ALL in 15% of cases. Expending of important genomic discoveries in ALL enable widening of the scope of precision medicine therapies for children with ALL, because the conventional therapies of chemotherapy and radiotherapy entail high toxicities [1, 2]. Adverse effects of glucocorticoids – prednisone and dexamethasone – which are used during induction of therapy include weight gain, infections, osteoporosis and bone fractures, myopathy, mood alterations and behaviour problems. Cytostatic agents used in consolidation of therapy, intensification, and remission can also cause severe complications. About 3.6–11% of children treated for acute lymphoblastic leukemia (ALL) have acute neurological complications such as: seizures and status epilepticus of unknown etiology, neurological deficits of unknown etiology, neuro-infections, posterior reversible encephalopathy syndrome (PRES), intracranial bleeding, systemic infections with neurological complication, hydrocephalus and aseptic meningitis. In 3–7% of patients, methotrexate is associated with neurotoxicity i.a.: leukoencephalopathy, transverse myelopathy, seizures, aphasia, mental status disorder, and stroke-like episodes. Neurotoxicity and symptoms of sensory, cranial, motor and autonomic neuropathy are the most serious side effects during therapy with vincristine. L-Asparaginase is also neurotoxic and may cause hyperammonaemia and thrombotic complications.

In recent years, many newly discovered immunotherapeutic drugs have been introduced into the treatment, but symptoms of significant toxicities have also been observed. In 15–50% of patients treated with blinatumomab, neurologic toxicities and hypogammaglobulinaemia have occurred [2, 3]. Hepatotoxicity, particularly sinusoidal obstruction syndrome (SOS), can be an

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adverse effect in patients treated with inotuzumab ozogamicin. Next generation treatment for ALL, such as chimeric antigen receptor (CAR) T cell therapy, which is used for patients with relapsed or refractory haematologic malignancies, is associated with mild to life-threatening intensity cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) [4]. Neurologic adverse effects were observed in 40–44% of children and young adults in studies using CAR constructs with a 4–1BB domain, and in 30% using CD19 CAR with a CD28 costimulatory domain [2, 3].

Thanks to advances in the field of pharmacology and genetics, the 5-year survival rate for children and adolescents with ALL has improved to more than 90% [2]. However, because of adverse effects, further improvements and the incorporation of novel therapeutic approaches in treatment are needed to further enhance the quality of life of children and adults suffering from ALL. The molecule of amygdalin was first isolated in 1830 by two French chemists, Robiquet and Boutron-Charlard [5]. D-Mandelonitrile 6-O-β-D-glucosido-β-D-glucoside (amygdalin) is composed of two molecules of glucose, benzaldehyde and hydrogen cyanide, and is highly concentrated in fruit kernels from bitter almonds, apricot and peach. The benzaldehyde component has analgesic action and hydrocyanic acid has anti-neoplastic activity [7]. The properties of amygdalin have been studied for decades and because of its' antibacterial, immunomodulatory and anti-inflammatory effects, it has been used in the treatment of i.a.: leprosy, asthma and bronchitis, emphysema, vitiligo, renal interstitial fibrosis, liver and pulmonary fibrosis [8]. Numerous studies have documented that amygdalin is a potent inhibitor of cell growth in bladder, breast, pancreatic, prostate cancer and renal cell carcinoma [9,10,11,12]. It was proved that amygdalin stimulates apoptosis by up-regulating expression of proapoptotic proteins (Bax and caspase-3) and down-regulating expression of anti-apoptotic Bcl-2, and promotes arrest of the cell cycle in G0/G1 phase, and decreases the number of cells entering S and G2/M phases, thereby blocking cell proliferation [13].

OBJECTIVE

The efficiency of the anticancer activity of amygdalin requires additional research. To gain insight into how amygdalin might function on the leukemic cells, an *in vitro* investigation was initiated to determine whether the molecules of amygdalin may have an influence on the progression of acute B and T acute lymphoblastic leukemias cell lines. The aim of the study is to evaluate the anticancer activity of amygdalin in acute lymphoblastic leukemia (ALL) cell lines of B and T lineage. To achieve this, B-ALL CCRF-HSB-2 and T-ALL CCRF-SB tumour cells were subjected to two different concentrations of amygdalin (5 mg/ml and 10 mg/ml), and the transcriptomic changes induced by the treatment subsequently analyzed.

MATERIALS AND METHOD

Cell cultures. B-ALL CCRF-HSB-2 [CCRF HSB-2, HSB-2] CCL-102.1 cell line and T- ALL CCRF-SB [CCRF-SB] CCL120 cell line form LGC Standards were grown and subcultured in RPMI 1640 (ATCC-30–2001), 10% foetal bovine serum (FBS) (ATCC 30–2020) and 1% penicillin/streptomycin 10000 U/ml (Sigma- Aldrich P4333) at 37 °C in a humidified cell incubator with 5% CO 2 and 95% O 2. The medium was changed every 2 days. The B-ALL CCRF-HSB-2 cells and T- ALL CCRF-SB were cultured to the amount of $10x10^3$/uL in the culture dish. The cells were treated with amygdalin (Amigdalina, Cytopharma de Mexico) at concentrations of 5 mg/ml and 10 mg/ml, for 24 h. The cells of the control group were left untreated. Each of the described experimental variants was carried out in 3 replicates. To assess the toxic effects of amygdalin, cell viability was determined by trypan blue (Gibco/Invitrogen). After incubation with amygdalin, about 90% of the counted cells were still alive.

RNA isolation from cell culture. RNA was isolated by PureLink™ RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The integrity of the obtained RNA was analyzed with a 2100 Bioanalyzer (Agilent Technologies) using the RNA 6000 Nano Lab Chip Kit (Agilent Technologies).

Microarray analysis. 50 ng of total RNA that passed initial quality control screening was used for target preparation using the GeneChip™ WT PLUS Reagent Kit (ThermoFisher Scientific, Waltham, MA, USA). The samples prepared were hybridized with a single Clariom S human array and incubated for 16 h in an Affymetrix GeneChip Hybridization Oven 645 at 45 °C, 60 rpm. The arrays were stained with an Affymetrix GeneChip Fluidics Station 450, according to the specific fluidics protocol, and scanned with an Affymetrix GeneChip Scanner 3000 7G. The raw intensity CEL files generated by GeneChipTM Command ConsoleTM were imported into Transcriptome Analysis Console (TAC) 4.0 (Applied Biosystems). Microarray data were first normalized and analyzed using Transcriptome Analysis Console 4.0, following the TAC user guide. The analysis type employed was Expression (Gene + Exon) with the SST-RMA summarization method. To identify differentially expressed genes (DEGs), One-Way ANOVA was applied, with the eBayes empirical parameter Improvement. The fold change threshold was set at >1.5 or <-1.5, and p-value required to be <0.05.

Downstream analysis. Gene Ontology (GO) enrichment and pathway analysis were conducted separately for up-regulated and down-regulated genes using gProfiler. Statistical significance ($P < 0.05$) was determined using the default g: SCS method for multiple testing correction of p-values obtained from the GO and pathway enrichment analyses. Additionally, lists of differentially expressed genes (DEGs) were uploaded to the Ingenuity Pathway Analysis (IPA) software by Qiagen. For interpreting the DEG data, the focus was on canonical pathways and diseases using the 'core analysis' function within IPA.

RESULTS

Differentially expressed genes after amygdaline treatment. Exposing the tumour cells of B-ALL CCRF-HSB-2 and T-ALL CCRF-SB cell lines to amygdalin led to concentration-dependent reduction and increase of expression of different genes. Table 1 shows the number of differentially expressed genes under the conditions tested (p-val <0,05; fold change <–1.5 or >1.5).

Table 1. Number of differentially expressed genes in T-ALL CCRF-SB and B-ALL CCRF-HSB-2 cells treated with 5 mg/ml or 10 mg/ml of amygdalin versus control cells

Cell type	B-ALL		T – ALL	
Amygdaline concentration	5 mg/ml	10 mg/ml	5 mg/ml	10 mg/ml
Number of up-regulated genes	368	234	856	858
Number of down-regulated genes	382	361	904	1894
DEGs in total	750	595	1760	2752

Top 10 up- and down- regulated genes in T-ALL (Tab. 2) and B-ALL (Tab. 3) cells exposed to amygdalin. The levels of concentration of amygdalin were 5 mg/ml and 10 mg/ml.

Table 2. Top ten up- and down- regulated genes in T-ALL CCRF-SB cells treated with 5 mg/ml (left panel) or 10 mg/ml (right panel) of amygdalin versus control cells

T cell 5mg/ml vs non treated			T cell 10mg/ml vs non treated		
Gene Symbol	FC	P-val	Gene Symbol	FC	P-val
KRT3	7.19	3.6E-05	MTUS1	6.51	4.0E-04
MTUS1	6.19	4.0E-04	SEC14L6	4.8	9.0E-04
MMP9	5.22	2.8E-05	GSDMA	3.73	4.4E-05
SERHL2	4.92	3.4E-05	OR14I1	3.62	1.7E-03
TARM1	4.53	1.0E-02	CHI3L2	3.51	4.9E-05
GNLY	4.26	5.6E-06	KIF13A	3.49	1.7E-03
C15orf48; MIR147B	3.62	9.2E-05	TARM1	3.25	3.7E-02
PKHD1L1	3.6	4.0E-04	NKX2–6	3.24	8.9E-05
KRT79	3.55	1.9E-05	SPIN3	3.17	5.5E-03
COLEC10	3.32	5.7E-03	ALDH1A2	3.16	2.0E-04
LTB4R; LTB4R2	-3.11	6.8E-03	ID1	-3.4	3.0E-03
BCL2	-3.16	5.0E-04	TCEB2	-3.42	5.0E-04
USP9Y; TTTY15	-3.18	4.0E-04	URB1	-3.43	3.1E-02
ASB8	-3.21	4.2E-05	ULK1	-3.45	8.4E-03
PRDM11	-3.44	8.0E-04	CSRNP2	-3.67	1.0E-03
AFF2	-3.5	2.6E-03	DHRS13	-3.79	2.0E-04
ACP2	-3.51	3.9E-03	IER2	-3.79	2.0E-04
FAM179B	-3.52	3.9E-05	STRA6	-4.02	6.0E-04
CEP162	-3.52	3.4E-05	DNM2	-4.12	4.0E-06
FDXACB1	-3.62	1.3E-02	FDXACB1	-4.7	2.4E-03

Many of deregulated genes in the T-ALL cell line following amygdalin treatment, such as *BCL2* (which encodes an anti-apoptotic protein), *MMP9* (involved in extracellular matrix degradation and tissue invasion), and *ID1* (a transcriptional regulator that controls differentiation and cell proliferation), are well-known for their significant roles in cancer biology.

Treatment of B-ALL cells with amygdalin changed the transcription of many genes contributing to important processes, such as *EZH2* (epigenetic regulation), *CRKL* (signal transduction), *RAD54B* (DNA repair), *CCL4* (immune modulation), and *ITGAL* (cell adhesion), all of which are critical in the development and progression of B-ALL. Some genes, e.g. *EZH2* and *CRKL*, have been established as playing key roles in leukemogenesis and represent potential therapeutic targets, while others are less well characterized and may require further investigation to determine their roles in B-ALL.

Table 3. Top 10 up- and down- regulated genes in B-ALL CCRF-HSB-2 cells treated with 5 mg/ml (left panel) or 10 mg/ml (right panel) of amygdalin versus control cells

B cell 5mg/ml vs non-treated			B cell 10mg/ml vs non-treated		
Gene Symbol	FC	P-val	Gene Symbol	FC	P-val
XKRY2; XKRY	3.04	5.1E-03	CCDC92	2.86	4.0E-04
TMEM255A	2.8	8.0E-04	TUBGCP3	2.85	4.7E-03
GLIS3	2.53	1.2E-02	WASF2	2.4	7.5E-03
ARHGEF28	2.52	2.9E-02	USHBP1	2.28	6.0E-04
FERMT1	2.44	4.2E-03	CCL4L2; CCL4; CCL4L1	2.22	5.2E-05
CYP4V2	2.43	3.0E-04	CCDC92	2.19	1.3E-03
SLCO4C1	2.33	9.6E-05	SYS1	2.18	4.0E-04
NLRP2	2.31	6.6E-03	CD1C	2.12	1.3E-02
TMEM2	2.3	4.3E-03	RP11–93O14.2; VPS35	2.05	2.5E-03
ITGAL	2.29	2.8E-03	C1orf204	2.04	1.2E-02
PHACTR3	-2.54	1.2E-03	EPB41L4A	-2.32	4.0E-02
SNX5	-2.54	6.5E-05	ZNF573	-2.32	2.6E-02
RAD54B	-2.55	1.2E-03	KRTAP12–2	-2.35	3.0E-04
USP34	-2.62	2.4E-02	PHACTR3	-2.35	1.2E-02
CRKL	-2.66	3.4E-03	MTRNR2L9	-2.41	6.0E-04
EZH2	-2.8	4.0E-04	LEKR1	-2.59	6.3E-03
TMEM14EP	-2.84	1.8E-03	PAPLN	-2.63	6.1E-03
NDUFAF2	-2.85	1.2E-03	TMEM14EP	-2.71	6.2E-03
LNPEP	-3.13	1.5E-03	EZH2	-3.08	4.0E-04
BBS4	-4.86	9.2E-03	CRKL	-3.13	2.6E-03

To elucidate the biological relevance of the observed alterations in coding transcript levels, Gene Ontology (GO) and pathway enrichment analyses was performed utilizing the gProfiler tool, and analysed independently for genes that were up-regulated and down-regulated across each cell type and amygdalin dosage condition. This approach enabled a more precise interpretation of the functional pathways and biological processes affected by amygdalin treatment, allowing for a comprehensive understanding of its impact on cellular mechanisms.

T- ALL. GO enrichment analysis of differentially expressed gene lists in T-ALL CCRF-SB cells treated with 5 mg/mL and 10 mg/mL concentrations of amygdalin, distinguishing between up-regulated and downregulated genes, revealed several over-represented gene ontology terms with a false discovery rate (FDR) < 0.05. The number of detected terms is summarized in Table 1S (supplementary tables).

Biological process (BP) enrichment revealed that, at both concentrations, the up-regulated genes were predominantly associated with developmental processes and cell differentiation. Additionally, pathway analysis using the REACTOME and KEGG databases revealed 2 enriched pathways at 5 mg/mL and 8 pathways at 10 mg/mL. At the lower concentration, the analysis highlighted significant enrichment in the 'ligand-receptor interaction' and 'cytochrome P450 pathways (arranged by substrate type)', according to REACTOME. At the higher concentration, treatment resulted in enrichment of the 'alpha-defensins pathway' in REACTOME and the 'neuronal system pathway' in KEGG. These findings suggest that amygdalin impacts processes linked to development and differentiation, with

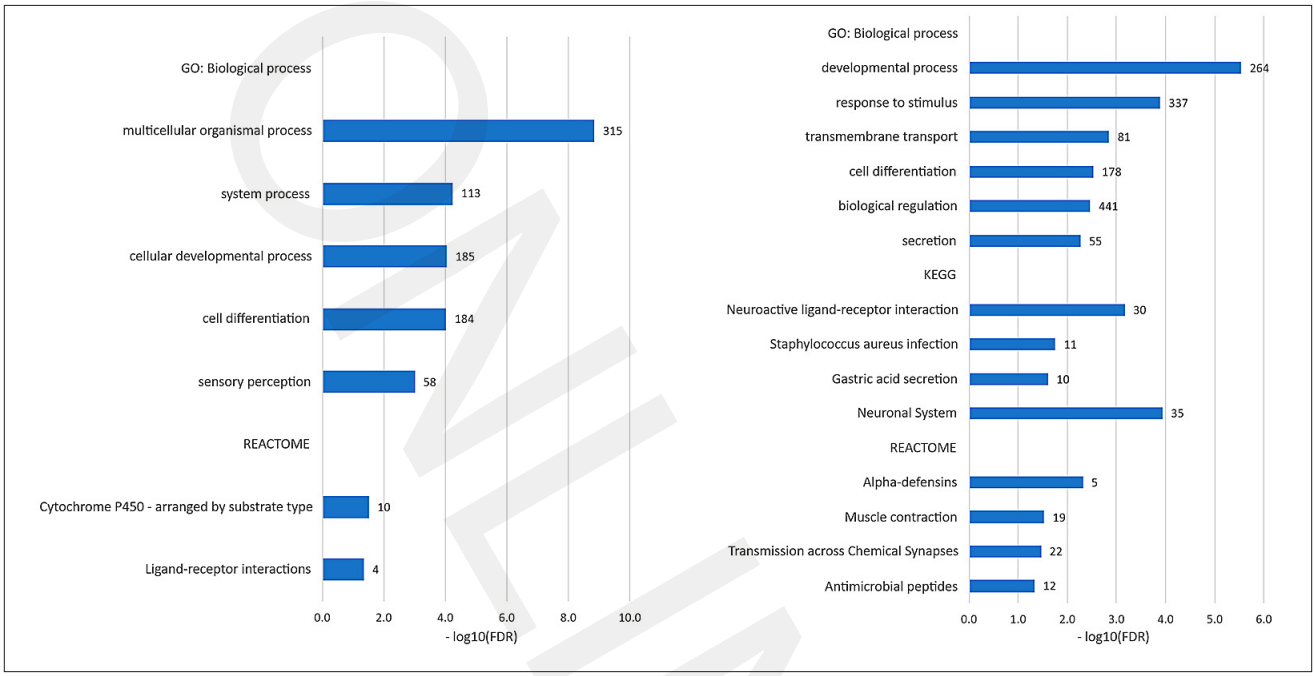


Figure 1. Bar chart displaying significantly enriched functional terms from the enrichment analysis of up-regulated genes following T- ALL CCRF-SB stimulation with 5 mg/ml (left panel) and 10 mg/ml (right panel) of amygdalin. Gene Ontology and pathway analysis were conducted using gProfiler, based on KEGG and REACTOME databases. The bar length represents –log10(FDR), and the number next to each bar indicates the number of genes involved in the pathway from the query list.

concentration-dependent modulation of pathways involved in immune defence and metabolic regulation. Selected top-ranked GO terms and pathways for upregulated genes are illustrated in Figure 1.

Subsequent Gene Ontology (GO) and pathway enrichment analyses were conducted on the group of down-regulated genes, revealing 14 and 49 molecular function (MF) terms, 50 and 107 cellular component (CC) terms, and 202 and 305 biological process (BP) terms, with a false discovery rate (FDR) < 0.05 at 5 mg/mL and 10 mg/mL amygdalin concentrations, respectively. Analysis of GO biological processes terms of down-regulated genes following low-dose amygdalin treatment in the T-ALL cell line, identified significant enrichment in terms related to cell cycle regulation, RNA metabolism, and other metabolic processes. Treatment with the higher concentration of amygdalin further impacted translation processes, in addition to its effects on the cell cycle.

Pathway enrichment analysis based on the REACTOME and KEGG databases demonstrated 8 and 88 enriched pathways for the low and high concentrations, respectively. The pathway analysis results corroborated the findings from GO biological processes, highlighting pathways associated with cell proliferation, such as the cell cycle and mitotic prometaphase, following treatment with 5 mg/mL of amygdalin. In addition, higher concentrations of amygdalin (10 mg/mL) down-regulated genes were involved in pathways beyond the 'mitotic cycle', notably affecting those related to 'RNA metabolism', 'splicing', 'translation', and the 'proteasome'. Selected, significantly enriched GO terms and pathways are presented in Figure 2, illustrating the dose-dependent effects of amygdalin on key cellular processes of T-ALL cells.

B-ALL. Equivalent analyses, including Gene Ontology (GO) and pathway enrichment, were conducted for differentially expressed genes (DEGs) from amygdalin treated B-ALL cells. Table 2S (supplementary table) summarizes the number of enriched terms in the different GO categories

The results revealed that compared to T-ALL, significantly fewer GO terms and pathways were enriched in B-ALL cells following amygdalin treatment, and that distinct cellular functions were impacted. In B-ALL cells treated with 5 mg/mL amygdalin, GO biological process enrichment showed that upregulated genes were involved in processes such as cellular signalling, migration and apoptosis. At a higher concentration (10 mg/mL), amygdalin induced the transcription of genes involved in chemokine-mediated signalling and cytokine-cytokine receptor interactions, as indicated by both GO biological processes and pathway enrichment analyses using REACTOME and KEGG databases. These factors are critical for immune responses, highlighting the dose-dependent modulation of immune-related genes by amygdalin in B-ALL cells.

The comprehensive enrichment analysis of genes downregulated by 5 mg/mL amygdalin in B-ALL CCRF-HSB-2 cells revealed that it primarily affected broad biological processes. Pathway analysis identified only a single enriched pathway – Herpes simplex virus 1 infection, suggesting potential modulation of the immune response (Fig. 1S, supplementary data). Interestingly, GO analysis of genes down-regulated by 10 mg/mL amygdalin in B-ALL cells, highlighted a single significant molecular function term – cyclosporin A binding (–log10(FDR) = 1.39), which may also be linked to the regulation of immune responses.

In treated B-ALL cells, no significant enhancement of the cellular response was observed with the higher concentration of amygdalin. Not only was a smaller number of deregulated genes detected in B-ALL cells at 10 mg/ml amygdalin (Table 1), but fewer Gene Ontology (GO) categories and pathways were enriched in differentially expressed genes. This suggests that amygdalin may exert a broad, non-specific effect on gene

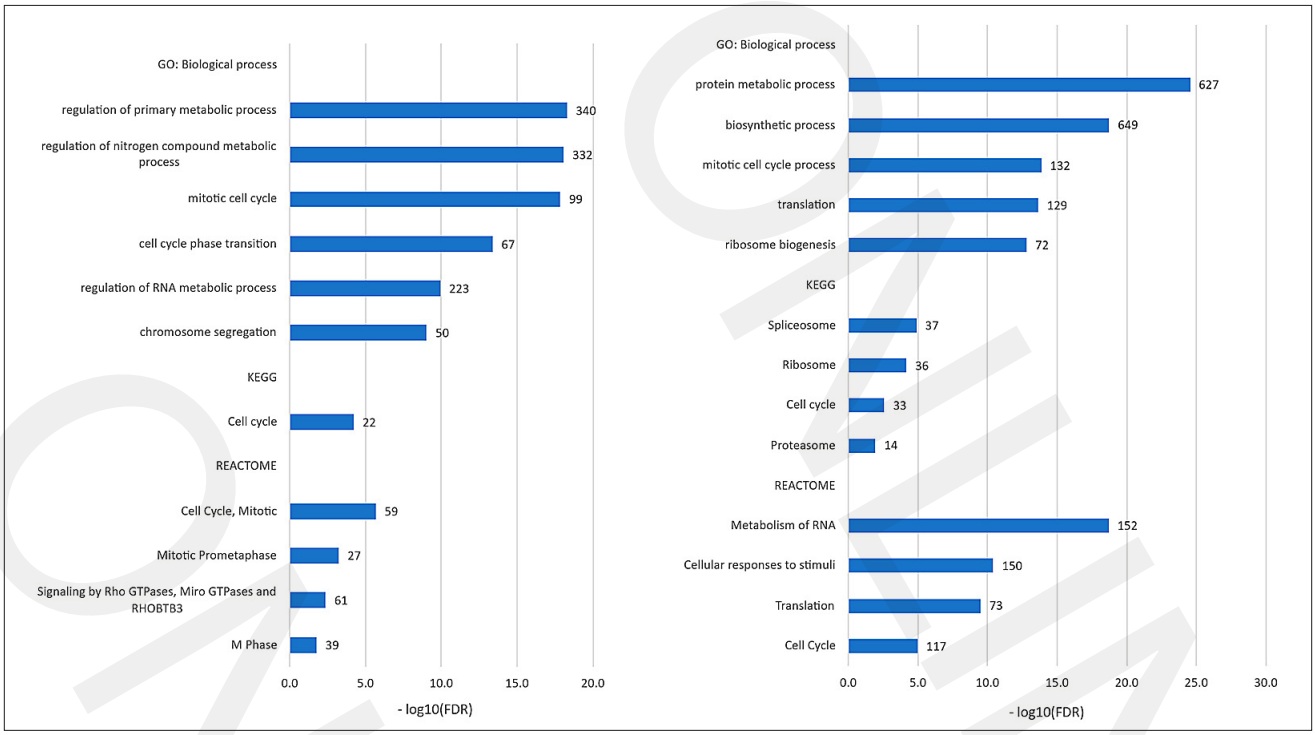


Figure 2. Bar chart displaying significantly enriched functional terms from the enrichment analysis of down-regulated genes following T- ALL CCRF-SB stimulation with 5 mg/ml (left panel) and 10 mg/ml (right panel) of amygdalin. Gene Ontology and pathway analysis were conducted using gProfiler, based on KEGG and REACTOME databases. The bar length represents –log10(FDR), and the number next to each bar indicates the number of genes involved in the pathway from the query list

expression in B-ALL cells, rather than targetting specific gene groups.

GO and pathway enrichment analysis suggests that although amygdalin influences key biological functions in both T-ALL and B-ALL cells, the specific biological processes and pathways impacted differ markedly between the 2 cell types. In T-ALL cells, the effects were highly concentration-dependent, whereas in B-ALL cells, this dependency was more difficult to define.

To deepen our understanding of the cellular changes induced by amygdalin treatment in T-ALL and B-ALL cells, Ingenuity Pathway Analysis (IPA) was used, with a

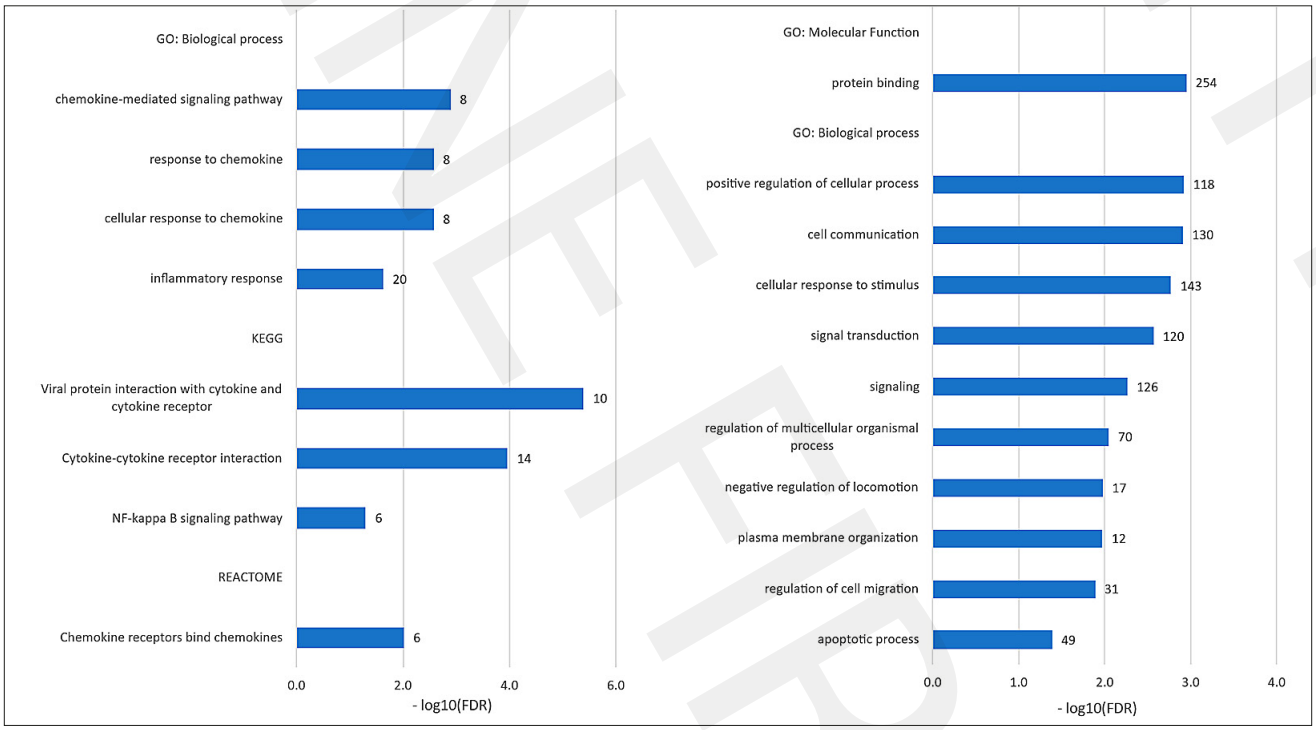


Figure 3. Bar chart displaying significantly enriched functional terms from the enrichment analysis of up-regulated genes following B-ALL CCRF-HSB-2 stimulation with 5 mg/ml (left panel) and 10 mg/ml (right panel) of amygdalin. Gene Ontology and pathway analysis were conducted using gProfiler, based on KEGG and REACTOME databases. The bar length represents –log10(FDR), and the number next to each bar indicates the number of genes involved in the pathway from the query list

particular focus on Canonical Pathways and Diseases and BioFunctions. IPA enabled the simultaneous analysis of multiple lists of differentially expressed genes (DEGs), and facilitated direct comparisons of the effects of varying amygdalin concentrations on leukemia cell function. In T-ALL cells, in both amygdalin concentration, the most significantly inhibited canonical pathways (z-score ≤ -2) were those associated with cell cycle regulation and RNA metabolism, highlighting a broad suppression of proliferative and transcriptional processes. In contrast, relatively fewer canonical pathways showed increased activity (z-scores ≥ 2), with granzyme signalling being the most prominently upregulated pathway. This suggests that while amygdalin treatment down-regulates critical regulatory pathways, it also activates specific immune-related pathways, such as granzyme signalling, which plays a role in cytotoxic T cell function and apoptosis induction.

Table 4 compares 20 of the most significantly altered canonical pathways in T cells under amygdalin stimulation at concentrations of 5 and 10 mg/ml.

Table 4. Comparison of the 20 most significantly altered canonical pathways in T- ALL CCRF-SB stimulated with 5 mg/ml and 10 mg/ml of amygdalin

	5 mg/ml	10 mg/ml
Canonical Pathways	**Z-score**	
Granzyme A Signalling	2.12	4.49
Cell Cycle Checkpoints	-4.6	-7.3
RHO GTPase cycle	-4.52	-6.27
Processing of Capped Intron-Containing Pre-mRNA	-4.49	-7.84
Mitotic Prometaphase	-4.13	-5.28
Intra-Golgi and retrograde Golgi-to-ER traffic	-3.8	-4.96
Mitotic Metaphase and Anaphase	-3.27	-6.18
DNA Methylation and Transcriptional Repression Signalling	-3.16	-4.36
Telomere Maintenance	-3.16	-4
RHO GTPases Activate Formins	-3.13	-4.7
Cilium Assembly	-2.92	-4.44
Mitotic G2-G2/M phases	-2.75	-5.52
Gene Silencing by RNA	-2.67	-4.26
DNA Replication Pre-Initiation	-2.53	-4.91
ESR-mediated signalling	-2.45	-4.32
RNA Polymerase II Transcription	-2.32	-5.57
Class I MHC mediated antigen processing and presentation	-2	-5.91
Synthesis of DNA	-1.89	-5.11
Regulation of mitotic cell cycle	-1.89	-4.71
Major pathway of rRNA processing in the nucleolus and cytosol	-1.5	-6.8

The results of the Ingenuity Pathway Analysis (IPA) are consistent with the findings from the enrichment analysis, further confirming the inactivation of essential cellular functions such as cell cycle, RNA transcription and processing. Notably, amygdalin treatment at both concentrations led to the inhibition of pathways critical for cell survival, including DNA methylation and telomere maintenance. Additionally, IPA highlighted the suppression of key signalling pathways, such as those involving RHO GTPases, which are small signalling proteins that regulate cytoskeletal dynamics, cell polarity, migration and cell division. The degree of inhibition of these pathways intensified with increasing amygdalin concentration, indicating a dose-dependent disruption of vital cellular mechanisms that are crucial for cancer cell survival and proliferation.

Subsequently, the results obtained in the IPA in the category of Diseases and Bio functions were analyzed. In stimulated T- ALL CCRF-SB (5 mg/ml) cells IPA analysis predicted decreased activity (z-score ≤ -2) of several pathways engaged in quantity and proliferation of lymphocytes, lymphopoiesis, T-cell development and T-cell homeostasis. Stimulation of T- ALL CCRF-SB with higher concentration of amygdalin (10 mg/ml) allowed the observation of a probable decrease in the activity of even more pathways connected to lymphocyte metabolism. Moreover, the analysis revealed decreased activity of pathways engaged in cell movement, migration and invasion of tumour cell lines, as well as adhesion of T lymphocytes, migration of lymphatic system cells and homing of lymphocytes, and an enhancement of organismal death.

Table 3S (supplementary data) presents a comparison of the 20 most significantly altered disease or function annotations in T-ALL cells stimulated with amygdalin at concentrations of 5 and 10 mg/ml. Similarly, lists of genes whose expression was altered by amygdalin's effect on B-ALL cells were used to perform functional analysis using IPA software. The enriched categories within the Canonical Pathways and Diseases and Functions differed significantly, depending on the concentration of amygdalin used.

As with T-ALL cells, the IPA software was utilized to compare the effects of different concentrations of amygdalin on B-ALL cells. IPA applies a –log (p-value) cutoff of 1.3, equivalent to a p-value < 0.05, and considers Z-scores of ≥ 2 or ≤ -2 to be significant. Only 2 categories in B-ALL cells treated with 5 mg/ml of amygdalin meet both criteria, while only 1 category meets the criteria in cells stimulated with the higher concentration.

IPA analysis revealed significant inhibition of the generic transcription pathway and activation of the Toll-like receptor signalling pathway, which plays a crucial role in innate immunity. Although not significantly altered, several other pathways involved in immune regulation were identified, including NF-κB signalling, TREM1 signalling, and LPS/IL-1-mediated inhibition of RXR function. These findings are consistent with the results of the GO analysis. The response of B-ALL cells to amygdalin varies depending on its concentration; however, it is not merely an amplification of the same signalling pathways at higher doses; instead, it induces transcriptional modifications that do not specifically enhance biological processes or functions.

Regarding categories of Diseases or Functions, IPA showed probable changes in activation state of 1,000 annotations in B-ALL cells stimulated with a lower concentration of amygdalin (5mg/ml), but the predicted z-score was considered

Table 5. Comparison of selected differentially activated canonical pathways after amygdalin stimulation of B-ALL CCRF-HSB-2 cells predicted by IPA software. Bold categories reached the criteria for significance in at least one experimental condition

	5 mg/ml	10 mg/ml
Canonical Pathways	**Z-score**	
Generic Transcription Pathway	-2.858	-2.828
Toll-like Receptor Signalling	2	N/A

significant for only 5 of them. IPA analysis of differentially expressed genes after stimulation of B-ALL with higher concentration of amygdalin (10 mg/ml), predicted activity changes in 737 Diseases or Functions categories, although only 6 reached the Z-scores to be significant, among them 1 activity probably decreased and 5 increased.

IPA analysis of diseases and biofunctions categories indicated significant activation of the extravasation of leukocytes, which is essential for effective immune responses and inflammatory reactions. Additionally, there was activation of the pro-inflammatory response and the binding of endothelial cells, both of which are critical for the recruitment of immune cells to sites of inflammation (Tab. 4S, supplementary data). The results confirm the importance of amygdalin B-ALL treatment in immune response regulation.

DISCUSSION

To the best of the authors' knowledge, this is the first study on the influence of amygdalin on childhood acute lymphoblastic leukemia cell lines. There is no literature available on the effects of amygdalin on T-ALL and B-ALL in children. In the presented study, changes in the expression of genes involved in cell cycle checkpoints, apoptosis, lymphocyte proliferation and general transcriptional regulation were observed. These processes underlie the development of lymphoid malignancies, such as T-ALL, where signalling abnormalities lead to uncontrolled T-cell division and resistance to apoptosis. No functional testing was performed. Previous studies have highlighted the important role of signalling pathways, such as NOTCH1, PI3K-AKT-mTOR, JAK-STAT and MAPK/ERK, the over-activation of which is the molecular driver of T-cell leukemia [14,15,16]. Among these, the NOTCH1 signalling pathway remains particularly important, as its constitutive activation is observed in more than half of T-ALL patients. This pathway plays a key role in the control of T-cell survival, differentiation and proliferation [16]. Interestingly, the results obtained in this study indicate that the activity of the NOTCH1 pathway was significantly inhibited only by a higher dose of amygdalin, suggesting the existence of a threshold effect in the cellular response to therapeutic action. Among the genes whose expression was downregulated in this pathway, *HDAC1, HDAC10, MYC, NUMB, SKP1, TLE3, TLE5* and *UBA52* a.o., were identified. Reduced levels of HDAC1 and HDAC10, regulators of histone deacetylation, may indicate inhibition of transcription of *NOTCH1* target genes. The reduced expression of *MYC*, one of the major oncogenes activated by NOTCH1, confirms the functional silencing of this pathway. Importantly, reduced expression of *BCL2* (a gene that regulates survival) and *ID1* (a regulator of the cell cycle and differentiation) further confirmed the inhibition of signals that promote proliferation and survival.

The down-regulation of *URB1*, which is involved in ribosome biogenesis, indicates a reduction in the ability to synthesize proteins, which secondarily affects proliferation. In addition, the observed down-regulation of *USP9Y* – a gene that protects against apoptosis – may enhance the effect of inducing cell death. Interestingly, the up-regulation of *SPIN3*, involved in the regulation of mitosis, may indicate an attempt to arrest the cell cycle or to strengthen the mechanisms controlling cell division. All these changes indicate a coordinated inhibition of the function of the NOTCH1 pathway and the promotion of apoptotic processes in response to a higher dose of the test compound.

In contrast, the decrease in expression of *NUMB*, a NOTCH1 inhibitor, may reflect a complex regulatory mechanism – probably secondary to the general silencing of transcriptional activity. Reduced expression of other genes, such as *SKP1* (SCF complex), *TLE3*, *TLE5* (transcriptional co-repressors) and *UBA52* (ubiquitination component), supports the hypothesis that a higher dose of amygdalin leads to an overall inhibition of the pathway, involving both activating and regulatory components. Such an alteration may effectively disrupt the proliferative potential of T cells that is characteristic for T-ALL. It can therefore be concluded that only higher concentrations of amygdalin have a real therapeutic effect by down-regulating key components of the NOTCH1 pathway.

Another important pathway involved in the pathogenesis of T-ALL that was included in the present analyses was the PI3K/AKT/mTOR signalling axis, which is tightly regulated by the inhibitory activity of the PTEN pathway. Under physiological conditions, *PTEN* acts as a tumour suppressor by inhibiting the phosphorylation and activation of *AKT*, resulting in reduced cell proliferation, survival and growth. In T-ALL cells, loss of function of *PTEN* is often observed, leading to uncontrolled activation of the PI3K/AKT/mTOR pathway and contributing to transformation and resistance to treatment. In the current study, clear differences were observed in the response of T-ALL cells to different doses of amygdalin in terms of the regulation of these pathways. At the lower dose, the activity of the PI3K/AKT pathway was moderately inhibited (score -0.632), but a concomitant activation of the mTOR pathway was observed (score 0.816), which may indicate a partial activation of compensatory mechanisms to promote cell survival despite the attenuation of AKT signalling. The PIP3 which activates the AKT signalling pathway also showed a slight decrease in activity (score -1.155), confirming a partial effect of the drug at a lower dose.

Among the genes involved in these processes, alterations in the expression of *FGFR2, PIK3R2, PTEN, KL, CDKN1A* were observed, but these changes were not sufficient to effectively inhibit signalling at the mTOR level. With the higher dose of amygdalin, the effect was definitely stronger and more comprehensive. There was a profound inhibition of the activity of the PI3K/AKT pathway (score -1.342), the PI3K cascade (-2.236), which was also reflected in a marked suppression of the mTOR pathway (score -0.728). Compared to the lower dose, additional down-regulation of the expression of genes such as *GSK3A, HRAS, RELA, RELB, PIK3CD, RICTOR, MAPKAP1, PPP2R1A* and several ribosomal genes (*RPS14, RPS6KA1*) was observed, which may indicate inhibition of protein translation and inhibition of cell growth. Importantly, the *TP53* gene, whose activation can lead to apoptosis in response to cellular stress, was also identified as upregulated.

A significant relationship was observed between the dose used and the degree of regulation of the activity of pathways related to *PTEN* function. At lower doses, moderate inhibition of the *PTEN* regulation pathway (score: -1.414) was observed, accompanied by a weak activation of *PTEN*-dependent signalling (score: 0.632). Such a pattern suggests partial impairment of *PTEN* suppressor functions, which

may not be sufficient to effectively block the pro-proliferative effects of the PI3K/AKT pathway. In contrast, at higher doses, a significant inhibition of activation of the *PTEN* regulation pathway was observed (score: -4.146), which was accompanied by a marked activation of *PTEN*-dependent signalling (score: 2.357). Such a profile may reflect a compensatory activation of anti-tumour mechanisms, including pro-apoptotic responses and inhibition of cell growth. A decrease in the expression of genes such as *BCL2, IKBKB, HRAS* and *RELA* supports this interpretation, indicating a more effective activation of the anti-proliferative pathway at a higher dose of amygdalin.

The data obtained indicate a significant relationship between the concentration of the compound studied and the degree of modulation of PTEN-dependent signalling. A notable activation of the PTEN signalling pathway, which plays a pivotal role in the regulation of proliferative and apoptotic processes, was observed only at a higher dose of amygdalin.

In the context of T-ALL, where deregulation of the PI3K/AKT/mTOR axis is a commonly described mechanism of pathogenesis, the results obtained may provide an important contribution to the optimizations of therapeutic strategies aimed at precise modulation of this signalling pathway.

In the analysis of the activity of JAK family kinase-related pathways in response to the application of different doses of amygdalin, a clear concentration-dependent effect was observed. At the lower dose, the activity of the JAK family kinases in the IL-6-type cytokine signaling pathway (z-score: -0.302) and the primary JAK/STAT signalling pathway (z-score: 0.378) remained virtually unchanged. Furthermore, additional pathways involving *JAK1*, *JAK2* and *TYK2* also demonstrated no significant functional changes (z-score 0), indicating limited modulation of JAK kinase-dependent signalling at low concentrations of the compound. Conversely, the higher dose instigated substantial alterations in the activity of the analyzed pathways. Strong signalling inhibition was observed in JAK family kinases in IL-6-type Cytokine Signalling (z-score: -2,138) and in the JAK/STAT signalling pathway (z-score: -1,155). Furthermore, a significant inhibition was observed in the *JAK2* in hormone-like cytokine signalling pathway (z-score: -0.707). This finding suggests that proliferative signal transduction, a hallmark of JAK kinase activity, is effectively blocked only at a higher dose of amygdalin.

Concurrently, expression analysis of the genes implicated in these pathways, including *SOCS3, STAT5A, RELA, NFKB2, RAF1, and PIK3CD*, revealed substantial alterations corresponding to the inhibition of JAK/STAT function. In particular, genes associated with the regulation of proliferation, such as *MYC* and *MCL1*, and genes involved in the inflammatory response, including *TGFB1, TGFB3*, and *IL6ST*, showed a more pronounced reduction in expression levels after a higher dose of amygdalin.

The findings of this study indicate that effective modulation of JAK/STAT signalling requires a higher dose of the compound, allowing inhibition of pathways responsible for proliferation, cell survival and inflammatory responses in T-ALL cells.

A detailed investigation into the activity of the ERK/MAPK pathway revealed a definitive correlation between the magnitude of the cellular response and the dosage of the test compound employed. At the lower dose, a moderate inhibition of the pathway was observed (z-score: -2.324), while the higher dose resulted in a much stronger inhibition of the pathway (z-score: -3.413).

In conditions involving lower doses, an alternation in the expression of genes, such as *DUSP6, EIF4EBP1, ETS1* and *PAK2,* was observed. *DUSP6* has been shown to function as a negative regulator of the ERK pathway, limiting *ERK1/2* phosphorylation and thereby inhibiting cell proliferation. In turn, *EIF4EBP1* is an important factor in the control of protein translation, while *ETS1* and *PAK2* are involved in the modulation of cell adhesion and migration. The observed increase in the expression of these genes may indicate the activation of compensatory mechanisms to limit excessive activation of the ERK/MAPK pathway. Substantial changes in the expression of genes essential for ERK/MAPK pathway activity, including *MYC, MAP2K2, RAF1, RAC1* and *RAPGEF3*, were evident in response to the higher dose of the drug. *MYC*, a pivotal oncogene that regulates cell proliferation, exhibited a substantial decrease in expression, indicating the efficacy of the inhibition of leukemic cell growth and division processes. Conversely, a decline in the expression of *MAP2K2* and *RAF1*, pivotal elements in activating the MAPK cascade, substantiates profound suppression of proliferative signal transduction. Furthermore, a reduction in the level of *RAC1*, which is associated with the control of cell migration and survival, may indicate a reduction in the invasive potential of the studied cells.

The results indicate that a higher dose of the test compound exerts a more intense inhibitory effect on the activity of the ERK/MAPK pathway compared to a lower dose. A more effective suppression of the activity of this signalling axis may contribute to a more effective reduction of leukemic cell proliferation and migration in T-ALL. Nevertheless, the potential side-effects associated with profound inhibition of this pathway should be considered in further studies.

Interestingly, in B-ALL CCRF-HSB-2 cells, no correlation was observed between the concentration of amygdalin and influence on JAK/STAT pathway. In B-ALL CCRF-HSB-2 cell line, down-regulation was observed of, a.o., *CRKL* and *EZH2* genes. CRKL is an adaptor protein which plays a role in diverse biological processes, including cell proliferation, migration, adhesion and differentiation. In multiple carcinomas the amplification of *CRKL* is observed and this over-expression nullifies the efficacy of anti-PD-1 treatment [17]. *EZH2* gene regulates gene expression, and its over-expression is associated with increased metastasis and poor prognosis in different types of cancer [18].

In comparing the results obtained in the presented study with the reaction to amygdalin of other cancer cell lines, some similarities were observed. Makarevic et al. [9] showed that amygdalin dose-dependently reduced growth and proliferation in bladder cancer cell lines: UMUC-3, RT112 and TCCSUP, causing a significant delay in cell cycle progression and G0/G1 arrest. Molecular evaluation revealed diminished phosphoAkt, phosphoRictor and loss of Cdk and cyclin components. It may block tumour growth by down-modulating cdk2 and cyclin. A. Li and Moon [19] proved that amygdalin down-regulated B-cell lymphoma 2 (Bcl-2), up-regulated Bcl-2-associated X protein (Bax) in Hs578T triple-negative breast cancer (TNBC) cells. It also inhibited adhesion of breast cancer cells. Chang et al. [7] assessed the effect of dose-dependent cytotoxicity of amygdalin on the DU145 and LNCaP cells (human prostate cancer cells). In

those cancer cell lines the concentration of *Bcl-2* mRNA significantly decreased, while *Bax* mRNA and Caspase-3 enzyme activity significantly increased.

In the T-ALL CCRF-SB cell lines investigated in the current study, stimulation by amygdalin also down-regulated *Bcl-2* and up-regulated *BCL2L14* in a concentration-dependent manner. In the study, significant down-regulation of genes involved in DNA synthesis and replication pathways in the T-ALL CCRF-SB cell line was observed following amygdalin treatment. Specifically, genes associated with the DNA synthesis pathway, including *MCM5, ORC6, PRIM2, PSMB5, PSMC4, PSMD10,* and *SKP2*, as well as genes involved in DNA replication, such as *H2AC14, H2AC7, H2AX, H2BC12, H2BC14, MCM3, MCM5, ORC6, PSMB5, PSMC4,* and *PSMD10*, were markedly down-regulated.

The results obtained also show that in the amygdalin-stimulated T-ALL CCRF-SB cell line, the expression of genes involved in DNA synthesis and replication was down-regulated at both tested concentrations – 5 mg/mL and 10 mg/mL. These findings are consistent with those reported by Albogami et al. [21], who demonstrated that amygdalin exerts antiproliferative effects in the human breast cancer MCF-7 cell line by down-regulating key genes critical for DNA replication. The study showed reduced expression of *POLA1, POLA2, PRIM1*; DNA polymerase δ component *POLD3*; DNA polymerase ε components *POLE* and *POLE2*; MCM complex members *MCM2, MCM3, MCM4, MCM5, MCM6*, and *MCM7*; as well as other replication-related factors, including *PCNA, RFC2, RFC3, RNASEH2A, DNA2, FEN1,* and *LIG1*. Notably, both studies identified consistent down-regulation of the MCM family genes, suggesting that disruption of the MCM helicase complex may represent a common mechanism by which amygdalin inhibits DNA replication and, consequently, cellular proliferation.

Mosayyebi et al. [22] investigated the effects of amygdalin on breast cancer cell lines (MCF-7 and MDA-MB-231) and a normal human skin fibroblast line. They demonstrated that amygdalin affects cancer cell adhesion and migration by altering the levels of α and β integrins in both cancer cell lines. Additionally, amygdalin reduced the viability of these cancer cells in a time- and dose-dependent manner, while showing no cytotoxicity in the human skin fibroblast line at the same concentrations. Correspondingly, results obtained in the current study demonstrate that stimulation of the T-ALL CCRF-SB cell line with amygdalin resulted in a concentration-dependent down-regulation of genes associated with cell migration and adhesion, including *ACKR3, CCL14, CCL21, CD99, CLDN24, CXCR2, CXCR3, FPR3, GNAI1, IL1B, ITGA4, MMP1, MMP2, MMP9, MSN*, and *SELE*. Moreover, multiple genes involved in integrin signalling pathways, such as *ACTA1, ACTN1, ACTN4, ARF1, ARPC1A, ARPC1B, ARPC4, ARPC5, CAPN1, CAPN11, CAPNS1, CTTN, GIT1, HRAS, ITGA4, ITGAL, MAP2K2, MYL12A, MYL9, MYLK, PAK4, PFN3, PIK3CD, RAC1, RAC2, RAF1, RHOA, RHOU, RND1, RRAS2, SHC1, SRC, TLN1, TNK2, VASP*, and *WIPF1*, were also significantly down-regulated. These findings suggest that amygdalin may exert its anti-migratory and anti-adhesive effects through the suppression of integrin-related signalling cascades in T-ALL cells.

Tsaur et al. [12] showed that amygdalin dose-dependently inhibited tumour growth and reduced tumour clones in taxane-resistant prostate cancer cells. A moderate influence of amygdalin on tumour cell adhesion and chemotaxis was also observed as well. Mani et al. [23] investigated prostate cancer cell lines DU-145 and PC3 cancer cells and induced down-regulation of α6 integrin in DU-145, but not in PC3 cells, suggesting that exposing certain prostate cancer cells to amygdalin might inhibit metastatic spread promoted by this particular integrin. Lin et al. [24] showed that amygdalin activates mitochondria-mediated apoptosis of lung cancer A549 and PC9 cells via enhancing the expression of NF-kappaB-1 and inactivating NF-kappa-B signaling cascade.

Results obtained in the current study show that in T-ALL CCRF-SB after stimulation with amygdalin, the expression of *RELA*, *RELB* genes which are subunits of NF-kappa-B transcription factor, was lower in comparison with unstimulated cells. In renal cell carcinoma (Caki-1, KTC-26 and A498) investigated by Juengel et al. [10], amygdalin caused significant reductions in chemotactic activity, invasion and adhesion to endothelium, collagen and fibronectin of those cancer lines, inhibited metastatic spread, and was associated with the down-regulation of α5 and α6 integrins. In the T- ALL CCRF-SB cell line, concentration dependent lower expression of *ITGA4* gene which encodes integrin subunit alpha 4, was observed. Cell adhesion plays a crucial role in cancer progression and metastasis [19].

Barakat et al. [20] observed inhibited cell adhesion in UMUC3, RT112, and TCCSUP bladder cancer cell lines following stimulation with amygdalin. General collagen binding and immobilization were reduced across all 3 cell lines. It was found that amygdalin directly and significantly influences the regulation of PI3K-mTOR activity, potentially blocking a phosphorylation cascade involving the mTOR/Rictor complex.

Liczbiński and Bukowska [25] highlight that decreased expression of integrins and catenins, as well as inhibition of the Akt-mTOR pathway in breast cancer, lung cancer, and bladder cancer cells after amygdalin stimulation, may subsequently lead to the inhibition of metastasis. Inhibition of the mTOR pathway was also observed In our the current study; however, this effect became evident only at higher doses of amygdalin.

CONCLUSIONS

Currently, no research has been carried out on the effects of amygdalin on paediatric B-cell acute lymphoblastic leukemia (B-ALL) and T-cell acute lymphoblastic leukemia (T-ALL). The presented study aimed to fill this gap and contribute to understanding the potential impact of amygdalin on the molecular mechanisms involved in the development of this disease. The study is of a preliminary nature, and no functional experiments were performed. The conclusions were drawn based on the results of gene expression analysis, without validating protein expression. The study shows that stimulation with amygdalin influenced the level of expression of different genes in B-ALL CCRF-HSB-2 and T- ALL CCRF-SB cell lines, compared with unstimulated cells. It also influences key biological functions in both T-ALL and B-ALL cells; however, in T-ALL cells, the effects are highly concentration-dependent, whereas in B-ALL cells, this dependency is more difficult to define. In T- ALL CCRF-SB a concentration dependent decrease was observed of the expression of genes particularly involved in cell viability,

leukopoiesis proliferation, quantity of lymphocytes, as well as the migration of lymphocytes.

In the B-ALL CCRF-HSB-2 cell line, the expression of genes involved in Toll- like receptor signalling and pro-inflammatory response were up-regulated, and genes implicated in the transcription pathway were down-regulated. Despite the use of steroids, chemotherapy and allogeneic transplantation, relapse is still the main problem in children with T-ALL. For that reason, the results of the current study, especially the response of T- ALL CCRF-SB cells to amygdalin, are highly significant. Further scientific efforts are needed to define the clinical relevance of the results as a potential component of anticancer treatment in B and T acute lymphoblastic leukemias in children. Future research projects by the authors will focus on checking the influence of amygdalin on T-ALL leukemia cell lines isolated from patients, and on B-ALL cells with which contribute to drug resistance.

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