Research Square

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The evaluation of the anti-cancer effects of Anoectochilus roxburghii on hematological cancers in vitro

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Abstract

The cause of hematological cancers is the uncontrolled proliferation of hematopoietic and lymphoid tissues, and chemotherapy is used to treat cancer. However, adverse side effects of chemotherapy are common. Therefore, the use of plant extracts as a method for treating cancer is becoming increasingly popular. *Anoectochilus roxburghii* (wall.) Lindl. (*A. roxburghii*) is one of the original sources of the valuable medicinal plants known as the king medicine and the golden grass. This study investigated the potential anticancer effect of *A. roxburghi* (AR) on JURKAT, MM1S, THP1 and U266 cells. To test the cytotoxic and apoptotic effects of AR, hematological cancer cells were exposed to increasing doses of AR (0.1–0.5 μg/μl). The spectrophotometric MTT assay and the flow cytometric Annexin V staining were used to examine the viability and apoptosis of the cells, respectively. qRT-PCR was used to determine the expression levels of the apoptosis-related genes BAD, BAX, BIM and BCL-2. Our results show that AR treatment decreased cell viability and induced apoptosis in each cell line. Our RT-PCR data showed that AR significantly increased the expression levels of the pro-apoptotic BAX gene in JURKAT and MM1S cells, whereas it significantly increased the expression levels of both BAX and BIM in U266 cells. This is the first study to investigate how AR modulates apoptosis in hematological cancer cells. As a result, AR therapy may be a promising treatment modality for the treatment of cancer.

INTRODUCTION

Cancer is the second most common cause of death after cardiovascular disease, despite advances in diagnostic techniques and chemotherapy. Global statistics indicate that it will be responsible for 9.96 million deaths and 19.2 million new cases in 2020. Both incidence and mortality are expected to increase progressively. To overcome cancer, many new alternative methods are being investigated. Recent studies have shown that plant extracts not only improve the effectiveness of treatment, but also allow for reduced doses of chemotherapeutic drugs, thereby reducing the possibility of multidrug resistance and serious side effects of treatment. [1]. It is estimated that 80% of people worldwide still use herbal therapies to treat various illnesses, including cancer, according to the World Health Organization (WHO) [2]. The widely recognized medicinal orchid Anoectochilus species, which is grown in Asian countries, contains a wide variety of substances, including flavonoids, polysaccharides, glycosides and alkaloids, all of which have effective pharmacological uses [2–5].

Few studies have analyzed the *in vitro* biological activities of Anoectochilus species [2]. Owing to its antioxidant, antihyperglycemic, hepatoprotective, and vascular protective properties, *Anoectochilus roxburghii* and *Anoectochilus formosanus* are two species that are frequently used in traditional medicine in Vietnam, Taiwan, and China [2]. In previous studies, Shyur et al. (2004), Ho et al. (2018) demonstrated the anti-proliferative activity of *A. formosanus* or *A. roxburghii*, which is largely associated with the induction of apoptosis [6, 7].

Polysaccharides, flavonoids, amino acids, minerals, polyphenols, alkaloids, saponins, and essential oils are abundant in *A. roxburghii* (AR), according to the literature findings [8, 9]. Among these,

polysaccharides are the main active constituents of *A. roxburghii*. Polysaccharides have prominent therapeutic effects, including anti-inflammatory, antidiabetic, antioxidant, vascular protective, and hepatoprotective properties, according to many medical and clinical trials [4, 10, 11], among others. Extracts from synthetically grown *A. roxburghii* exhibit a growth-inhibitory activities in different cancer cell lines [2, 12].

To determine the efficacy of *A. roxburghii* in hematological cancers is the main objective of this study. The induction of apoptosis and the molecular mechanisms of cell death were investigated. We assessed whether *A. roxburghii* could induce apoptosis. Our data showed for the first time that *A. roxburghii* induced apoptosis in JURKAT, MM1S, THP1 and U266 cell lines through the modulation of apoptosis-related genes.

MATERIALS AND METHODS

Cell culture

Human multiple myeloma (MM1S, U266), human acute T-cell (Jurkat) and human acute monocytic leukemia (THP1) cell lines were grown in RPMI-1640 medium supplemented with L-glutamine (Bio-Ind, USA), 10% fetal bovine serum (Bio-Ind, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37^{o}C in 5% $CO_{2,}$ and 95% humidity. Three of these human cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Robert Z. Orlowski and his team provided the human MM1s cell line at the MD Anderson Cancer Center (Texas, USA).

Extract preparation

In this study, both distilled water and ethanol were used as solvents to prepare 2 different plant extracts. In the trial with ethanol as the solvent, the alcohol content was fixed at 96%. For extraction, *A. roxburghii* powder (100 mg) was dissolved in 1 mL of solvent. This was homogenized with distilled water and ethanol using a clean pestle and stored in solvent at 4°C overnight. After 24 h, extracts were centrifuged at 15,000 rpm for 20 min at 4°C. The liquid supernatant was collected and the residue was extracted three times. Supernatants were combined, filtered through 0.22 µm sterile filters (Millipore, Turkey) and stored at 4°C.

Cell Viability Assay

Cell lines were incubated at a density of 4x10^{4} cells/well in 96-well plates with and without the plant extracts. The cells cultured with *A. roxburghii* at increasing concentrations (0.1-0.2-0.3-0.4-0.5 µg/µl) for 48 and 72 h were assayed for cell viability using the MTT assay. 10 µl (5 mg/ml) MTT reagent was added to each well for 2 h at 37°C. After 2 h, 100 µl detergent reagent was added. Using a spectrophotometric plate reader (Biotek, USA), each sample's optical density was measured at 550nm, using 690 nm as reference. Non-treated cells were considered 100% viable, and the percentage of viable cells for each treatment group was calculated accordingly. Each extract was dosed at a level not exceeding 0.5%.

Distilled water and ethanol were used separately in the control groups as solvents for *A. roxburgii*; these reagents were provided as negative controls in all of the groups. The percentage viability of the cells in each well compared with the control was calculated in Microsoft Excel and plotted using GraphPad Prism 9 (Fig. 1A-D). For each cell line, three independent experiments were performed.

Annexin V/7AAD staining

JURKAT, MM1S, THP1 and U266 cells (4x105 cells/well) were seeded in 24-well plates. The cells were treated with *A. roxburghii* for 96 h. After 96 h, the cells were collected, washed twice with PBS, centrifuged and suspended in binding buffer (1XBB) according to the manufacturer's protocol. Briefly, a cell suspension (100 µl) was treated with equal parts PE-Annexin V (5 µl) and 7-AAD (5 µl) and incubated for 15 min at room temperature (BioLegend, USA). After incubation, 1X BB (400 µl) was added. Apoptosis was analyzed by flow cytometry (Accuri C6) [13].

Real-time qRT-PCR assay

Real-time qRT-PCR was used to evaluate the expression levels of the apoptotic genes BAD, BAX, BCL-2 and BIM. Total RNA was extracted from the cell lines using Trizol (Invitrogen, USA). The quality of the RNAs was determined using a BioTek spectrophotometer (Epoch, BioTek, Germany). Reverse transcriptase was used for cDNA synthesis from RNA (iScript cDNA synthesis kit; Biorad, Hercules, CA). A CFX Connect Real-Time PCR Detection System (Biorad, CA) was used for real-time qRT-PCR. This system uses the SYBR Green PCR Master Mix (iTaq Universal SYBR Green Supermix, Biorad, CA). To normalize the calculations, the internal reference gene HPRT was used to measure the mRNA [13]. The primer sequences are listed in Table 1.

Table 1
Primer sequences for the real-time qRT-PCR assay

Gene	Primer Forward	Primer Reverse
HPRT	5’-TGACACTGGCAAAACAATGCA-3’	5’-GGTCCTTTTCACCAGCAAGCT-3’
BAD	5’-GATGAGTGACGAGTTTGTGGA-3’	5’-CAAGTTCCGATCCCACCAG-3’
BAX	5’- GACGGCAACTTCAACTGGG-3’	5’-AGGAGTCTCACCCAACCAC-3’
BCL-2	5’-CGCCCTGTGGATGACTGAGT-3’	5’-GGGCCGTACAGTTCCACAA-3’
BIM	5’-ATCTCAGTGCAATGGCTTCC-3’	5’-CATAGTAAGCGTTAAACTCGTCTCC-3’

Statistical analysis

Each experiment was performed in triplicate three times. All experimental data were averaged using standard deviation (SD). The statistical software GraphPad Prism was used to analyze the data. To compare the variations between the control and treated groups, an ANOVA test was performed. Statistical significance was calculated as $p < 0.05$ and $p < 0.001$.

RESULTS

Effects of A. roxburghii on the proliferation of human cell lines

To test the antiproliferative effects of *A. roxburgii*, cells were treated separately with *A. roxburghii* dissolved in distilled water or ethanol at different concentrations (0.1–0.5 µg/µl) for 48 and 72 h before the MTT assays. The viability of control cells incubated with only water or ethanol and without *A. roxburghii* was accepted as 100%.

Cell viability significantly reduced after 72 h in compared with the control group when water extract of *A. roxburghii* (0.1–0.5 µg/µl) was applied to the cell lines (JURKAT, MM1S, THP1 and U266) ($p < 0.05$). The proportion of viable cells is shown in Fig. 1A-D.

Figure 1. Effect of *A. roxburghii* dissolved in water and ethanol (0.1–0.5 µg/µl) on the viability of MM1S, U266, JURKAT and THP1 cells. **A.** Treatment of JURKAT; **B.** MM1S and; **C.** THP1 and **D.** U266 cells with *A. roxburghii* decreased cell viability.

*$p < 0.05$, **$p < 0.005$, *** $p < 0.0005$ and **** $p < 0.0001$ shows significant differences from the control group

According to MTT results, the most effective responses were achieved with treatment of all cell lines with 0.5 µg/µl dose of *A. roxburghii* dissolved in water at 72 h ($p < 0.0001$). Therefore, this incubation time, dose and solvent were chosen for further analyzes.

The role of A. roxburghii in inducing cell apoptosis

Cells were treated with 0.5 µg/µl *A. roxburghii* for 72 h to determine the type of cell death resulting from *A. roxburghii* treatment by flow cytometry.

Our findings showed that 91.3% of the JURKAT control cells (untreated) were viable (PE-/7AAD). On the other hand, it was discovered that in JURKAT cells treated with 0.5 µg/µl *A. roxburghii*, the rate of viable cells reduced significantly to 74.2% ($p < 0.005$) at 72h. The rate of early apoptotic (PE+/7AAD-) JURKAT cells treated with AR (0.5 µg/µl) was significantly higher (22.4%) than that of untreated control cells (7.5%) at 72h ($p < 0.05$) (Fig. 2, 3A).

When MM1S cells were treated with *A. roxburghii* (0.5 µg/µl), the rate of viable (PE-/7AAD-) cells was significantly reduced (68.2%) compared with the untreated control group (81.3%) at 72h ($p < 0.05$) (Fig. 2, 3B).

Moreover, the number of viable (PE-/7AAD-) THP1 cells was decreased (77.3%) after *A. Roxburghii* (0.5 µg/µl) treatment at 72h in compared with untreated cells (88.2%) significantly ($p < 0.001$) (Fig. 2, 3C).

The rate of viable (PE-/7AAD-) U266 cells were reduced (70.9%) significantly by treatment with *A. roxburghii* (0.5 µg/µl) in compared with their controls (85.6%) at 72 h ($p < 0.0001$). The rate of early

apoptotic (PE+/7AAD-) U266 cells significantly increased (12.9%) compared with that in controls (5.4%) when cells were treated with 0.5 µg/µl *A. roxburghii* ($p < 0.005$). On the other hand, the rate of late apoptotic (PE+/7AAD+) U266 cells treated with *A. roxburghii* (0.5 µg/µl) was significantly higher (13.1%) than that in the control group (6.7%) at 72h ($p < 0.05$) (Fig. 2, 3D).

Figure 2 *A. roxburghii* induces apoptosis in vitro. Flow cytometric analysis of JURKAT, MM1S, THP1 and U266 cells stained with Annexin V PE/7-AAD for 72 h. Flow cytometry results are represented as dot plots; Cells treated with/without 0.5 µg/µl *A. roxburgii*.

Figure 3 Flow cytometry analysis of cells with/without *A. roxburgii* for 72 h is shown as bar graph **A.** Bar graph view of flow cytometry in JURKAT; **B.** The bar graph view of flow cytometry in MM1S; **C.** The bar graph view of flow cytometry for THP1 and **D.** The bar graph view of flow cytometry of U266 cells

*$p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ and ****$p < 0.0001$ are significant differences from the control group

The effect of A. roxburghii on the expression profiling of apoptotic genes in human cell lines

In cell lines (JURKAT, MM1S, THP1 and U266), qRT-PCR was used to determine the gene expression levels of the BCL-2 family (BAD, BAX, BIM and BCL-2).

In comparison with untreated control cells, *A. roxburghii* (0.5µg/µl) was able to alter the expression levels of pro-apoptotic (BAD, BAX and BIM) and anti-apoptotic (BCL-2) genes at 72h in JURKAT, MM1S, THP1 and U266 cells.

A. roxburghii (0.5µg/µl) was able to significantly enhance the expression levels of the pro-apoptotic gene BAX in JURKAT cells compared with untreated cells ($p < 0.0001$) (Fig. 4A).

In MM1S cells treated with *A. roxburghii* (0.5µg/µl), the expression levels of the pro-apoptotic gene BAX increased compared with untreated cells. The overexpression of the BAX gene was found to be statistically significant ($p < 0.0005$), similar to the results in JURKAT cells (Fig. 4B).

The mRNA expression levels of the pro-apoptotic gene BAX and the anti-apoptotic gene BCL-2 were reduced in *A. roxburghii*-treated THP1 cells compared with untreated cells. The downregulation of these genes was found to be statistically significant ($p < 0.0001$ and $p < 0.05$ respectively) (Fig. 4C).

A. roxburghii was able to significantly increase the expression levels of apoptotic-related genes (BAX, BCL-2 and BIM) in U266 cells ($p < 0.05$, $p < 0.005$ and $p < 0.005$ respectively) compared with the untreated control cells (Fig. 4D).

Figure 4 m-RNA analysis of apoptosis-related genes BAD, BAX, BCL-2 and BIM after treatment with A. roxburghii (0.5 µg/µl) for 72h h by real-time PCR assay in cells **A.** JURKAT; **B.** MM1S; **C.** THP1 and **D.** U266

*$p < 0.05$, ** $p < 0.005$ and ***$p < 0.0005$ shows significant differences from the control group

DISCUSSION

Traditional chemotherapy is now the standard treatment for cancer and is often combined with other methods such as surgery and radiotherapy to be as effective as possible in targeting cancer cells. However, administering high doses of chemotherapy, which can have serious side effects in patients, may not be sufficient to eliminate the cancer cells. However, even if the side effects are reduced at lower doses, drug resistance can occur, which can ultimately lead to the tumour relapse. Several new and different approaches are being studied as an answer to this problem. One of these alternative methods, and a popular approach to cancer treatment, are plant extracts [1]. Of the many Chinese herbal products we have researched, *A. roxburghii* has a wide variety of pharmacological properties, including anti-diabetic, blood vessel protective, liver protective, antioxidant, antibacterial and anticancer activities [14].

There are few studies in the literature that have investigated the efficacy of treatment with *A. roxburghii* for cancer. In one of these studies, Yu et al. showed that an extract of *A. roxburghii* had a cytotoxic effect on several cancer cell lines (A549 lung cancer, 143B osteosarcoma, PC12 rat adrenal pheochromocytoma, MCF-7 breast cancer, HL60 acute leukaemia, K562 chronic leukemia, SW620 colorectal cancer, OE19 oesophageal cancer, HepG2 liver cancer and U251 neuroglioma cells). According to this study, *A. roxburghii* water extract had the strongest inhibitory effect on OE19 cells. Flow cytometric results indicated that *A. roxburghii* induced G2/M phase arrest and morphological apoptosis in OE19 cells in a dose-dependent manner. [12].

Another study has shown that cultured *A. roxburghii* can destroy cancer cells in vitro, including breast cancer MCF7 cells. In addition, the activation of the caspase-dependent intrinsic apoptotic pathway contributed to the majority of *A. roxburghii*-induced cytotoxicity in MCF7 cells [2].

In our study, we demonstrated for the first time the cytotoxic effect of *A. roxburghii* extract on hematological cancer cell lines (JURKAT, MM1S, THP1 and U266), in accordance with studies performed on various cancer cells in the literature. Our results showed that the most effective cytotoxic effect of *A. roxburghii* water extract was obtained at a dose of 0.5µg/µl and at 72 h in all cells. According to the MTT results, we observed that *A. roxburghii* statistically significantly inhibited cell proliferation in JURKAT, MM1S, THP1 and U266 cells compared with their control groups.

According to our Annexin V staining flow cytometry results, we showed for the first time that apoptosis was increased in hematological cell lines treated with *A.roxburgii* extract in compared with untreated cells. At a dose of 0.5µg/µl and at 72 h, *A.roxburgii* water extract induced apoptosis in all cell lines.

We found that *A.roxburghii* (0.5 µg/µl) significantly reduced the number of viable cells in the JURKAT, MM1S, THP1 and U266 cell lines at 72 h. Our study showed that treatment with *A. roxburghii* (0.5 µg/ml) significantly increased the number of early apoptotic cells in JURKAT and the number of both early and late apoptotic cells in U266 cell lines compared with untreated controls.

Induction of apoptosis in cancer cells is one of the most efficient cancer therapy strategies. To better understand the apoptotic pathways, we also analyzed the mRNA expression levels of pro (BAD, BAX and BIM) and anti-apoptotic (BCL-2) genes in the *A.roxburgii*-treated cells. BAX gene expression was significantly increased in *A.roxburgii* treated JURKAT and MM1S cells whereas BAX and BIM gene expression was significantly increased in *A.roxburgii* treated U266 cells compared with controls. The only study in the literature analyzing gene expression using this plant extract was Chung et al. In this study, BAX expression was increased in MCF7 cells treated with cultured *A. roxburghii* in vitro (iARE), which is consistent with our results [2].

In addition, recent studies have suggested that the use of plant extracts in combination with chemotherapeutic drugs may be promising in inhibiting of cancer development and progression. In the literature, Cheng et al. reported that extracts of *A.roxburghii* decreased breast cancer proliferation in mice treated with doxorubicin (DOX). In this study, BALB/c mice with breast cancer were treated with DOX. The results of the study showed that DOX treatment reduced body weight, blood pressure and heart rate, and induced myelosuppression, immunodeficiency, cardiotoxicity and osteoporosis. An oral administration of *A.roxburghii* to BALB/c mice with breast cancer increased the chemotherapeutic efficiency of DOX and reduced the side effects of chemotherapy [14]. Based on the study by Cheng et al, our study also suggests that the use of *A. roxburghii* alone, as well as in combination with chemotherapy drugs used in hematological cancers, may be a more effective treatment method.

In summary, *A. roxburghii* was identified for its apoptotic effects on hematological cancer cells. Because of the apoptotic activity of this extract, it can be concluded that this compound can be used as an anticancer candidate in hematological cancers.

CONCLUSION

This study has demonstrated that *A.roxburghii* is a cytotoxic and anticancer agent and that it induces apoptosis in hematological cancers. It may also enhance the efficacy of conventional therapies and suppress cancer recurrence after treatment. *A.roxburghii* along with chemotherapeutic drugs can alter the viability of cancer cells, thus demonstrating its more potent efficacy as a combinatorial therapeutic agent in cancer.

DECLARATIONS

AUTHORS CONTRIBUTION

Conceptualization, B.A.G., T.O.; methodology, B.A.G., T.O., N.G; investigation, B.A.G., T.O., N.G. and A.S.; formal analysis, B.A.G., T.O.; writing-original draft preparation, B.A.G., T.O.; writing, review, and editing, B.A.G., T.O., A.S. All authors participated in editing the paper. All authors approved the published version of the manuscript.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

ETHICS APPROVAL

Ethics committee approval is not applicable and not necessary for this study.

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Declarations of interest: None

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Figures

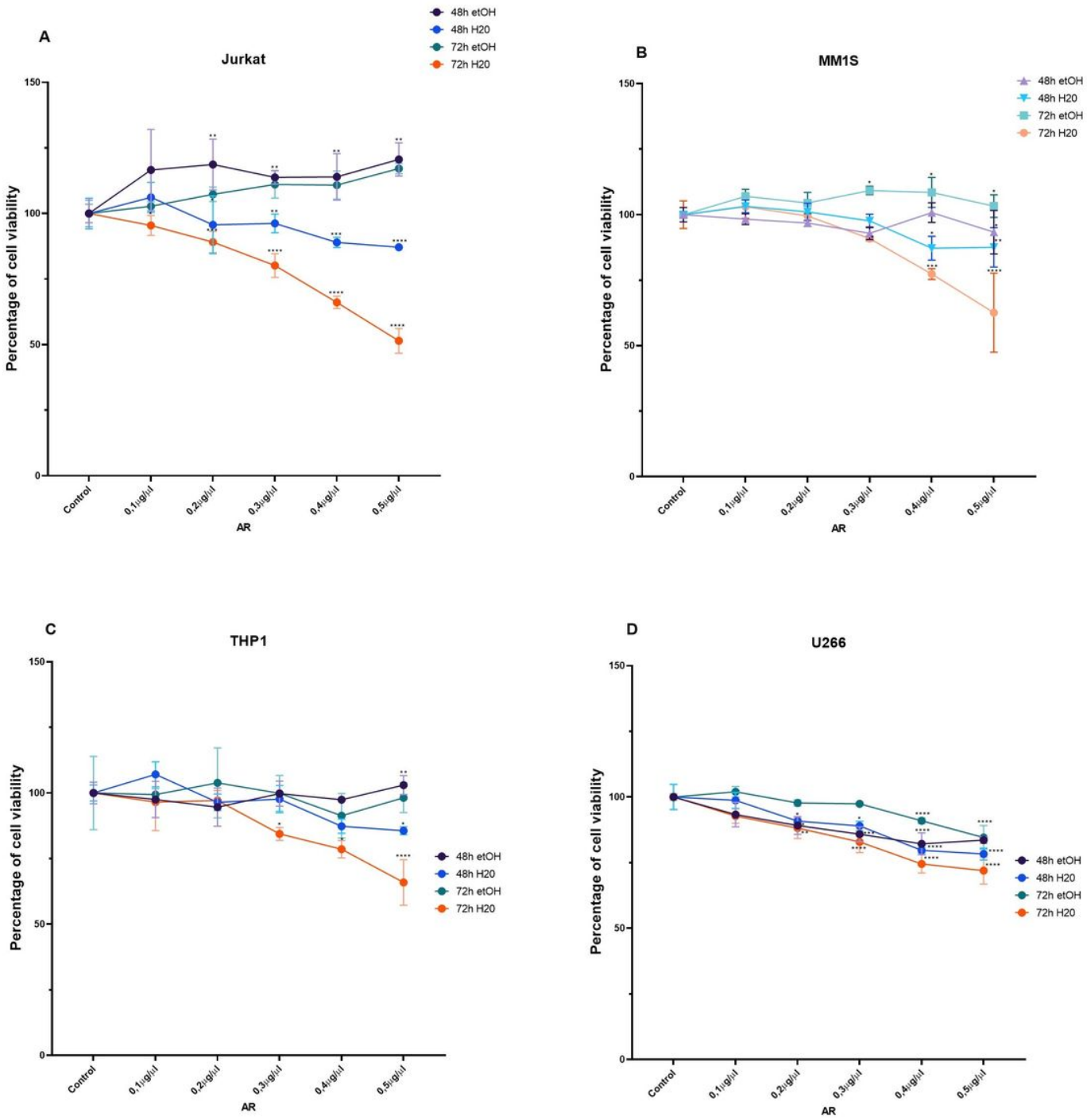


Figure 1

Effect of *A. roxburghii* dissolved in water and ethanol (0.1-0.5 µg/µl) on the viability of MM1S, U266, JURKAT and THP1 cells. **A.** Treatment of JURKAT; **B.**MM1S and; **C.** THP1 and **D.** U266 cells with *A. roxburghii* decreased cell viability.

*p < 0.05, **p < 0.005, *** p <0.0005 and **** p < 0.0001 shows significant differences from the control group

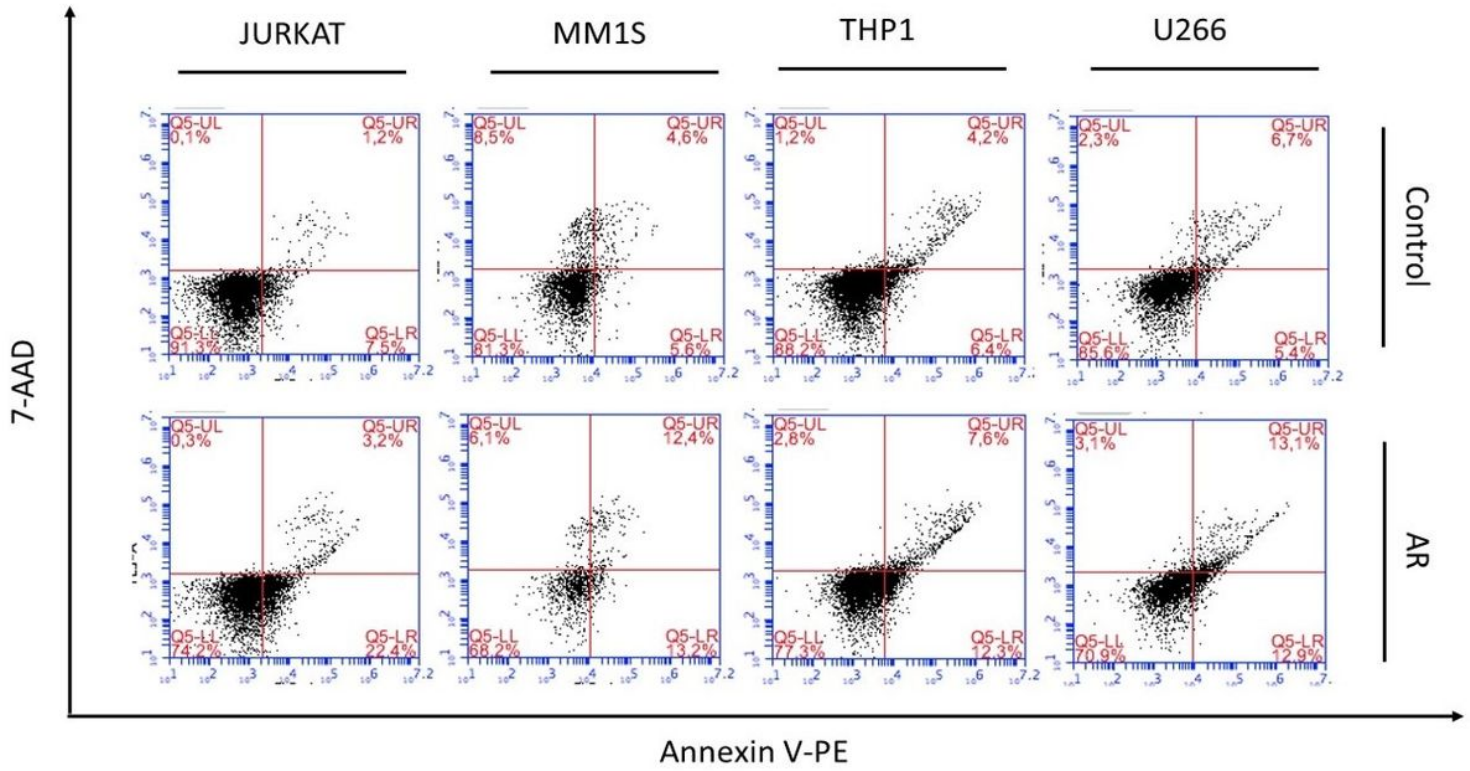


Figure 2

A. roxburghii induces apoptosis in vitro. Flow cytometric analysis of JURKAT, MM1S, THP1 and U266 cells stained with Annexin V PE/7-AAD for 72 h. Flow cytometry results are represented as dot plots; Cells treated with/without 0.5 µg/µl *A. roxburgii*.

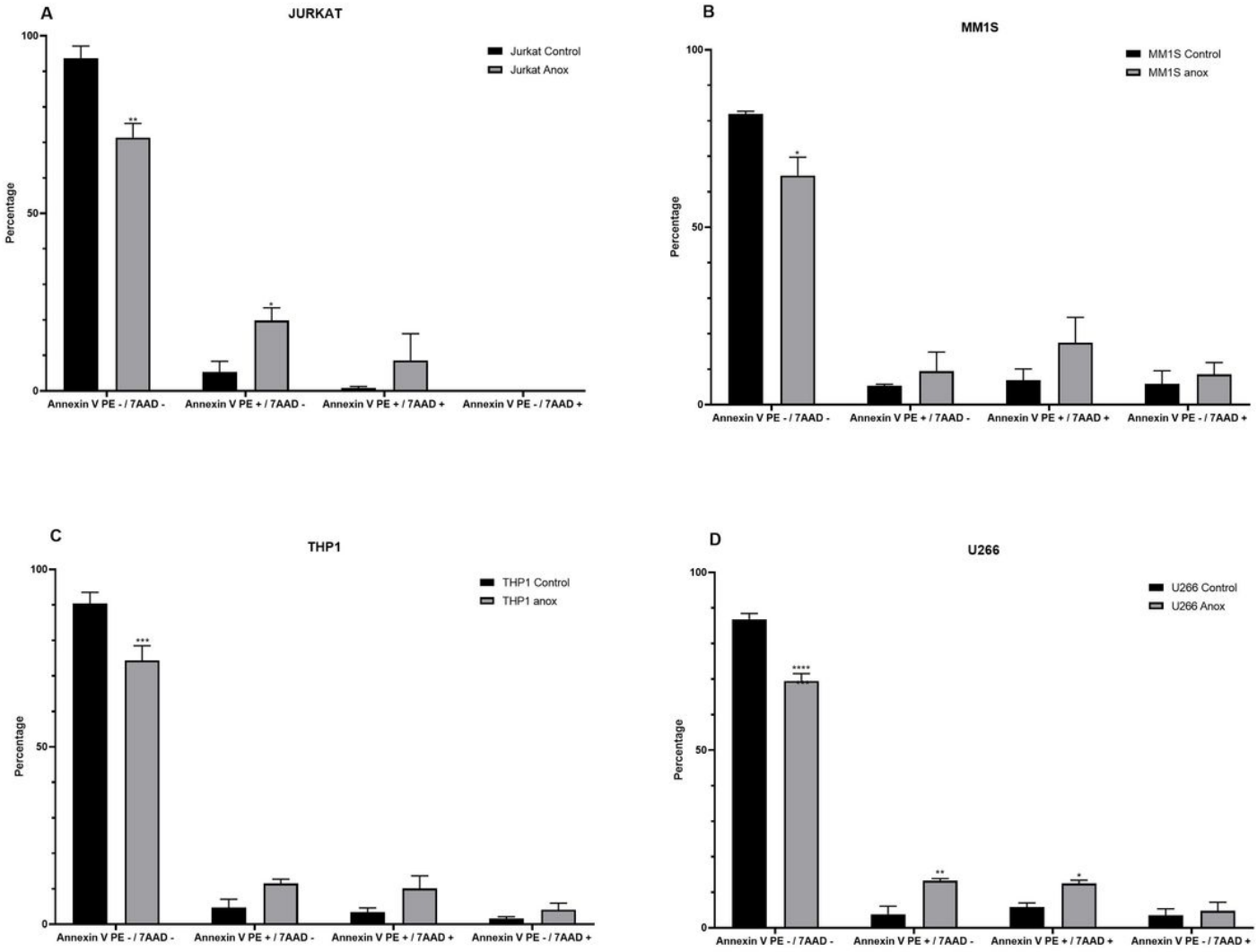


Figure 3

Flow cytometry analysis of cells with/without *A. roxburgii* for 72 h is shown as bar graph **A.** Bar graph view of flow cytometry in JURKAT; **B.** The bar graph view of flow cytometry in MM1S; **C.**The bar graph view of flow cytometry for THP1 and **D.** The bar graph view of flow cytometry of U266 cells

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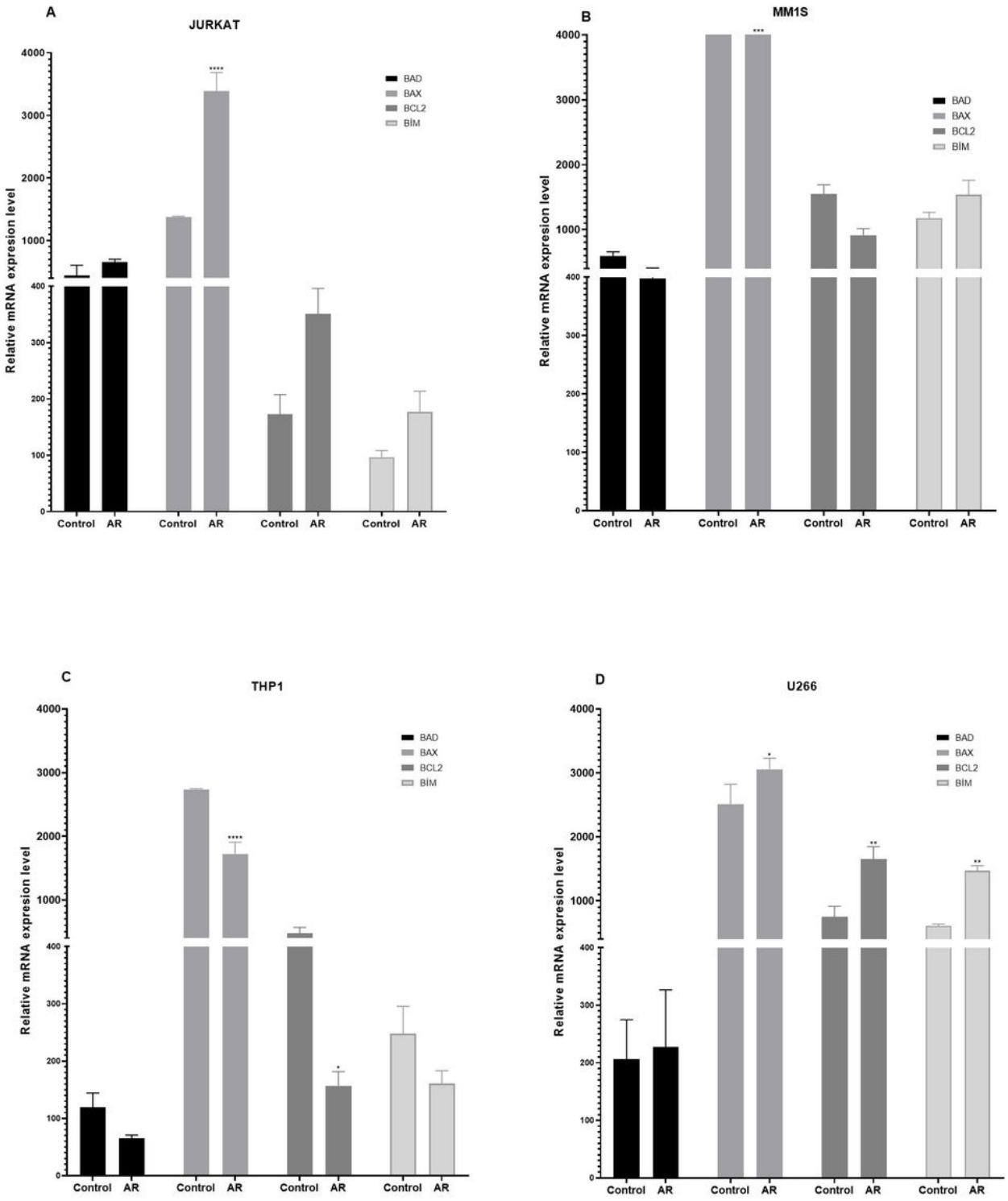


Figure 4

m-RNA analysis of apoptosis-related genes BAD, BAX, BCL-2 and BIM after treatment with A. roxburghii (0.5 µg/µl) for 72h h by real-time PCR assay in cells **A.** JURKAT; **B.** MM1S; **C.** THP1 and **D.** U266

*p < 0.05, ** p <0.005 and ***p<0.0005 shows significant differences from the control group