Original Research Article

Anticancer effect and enhancement of therapeutic potential of Vincristine by extract from aerial parts of Juniperus excelsa on pre-B acute lymphoblastic leukemia cell lines

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ABSTRACT

The natural products and conventional chemotherapeutic drugs combination can increase the efficacy of anticancer treatment through their potential synergistic effects and reduce its toxicity. The current study investigates the effect of methanolic extract from aerial parts of Juniperus excelsa on cell death activities induced by vincristine in acute lymphoblastic leukemia cells. Cytotoxic activity of J. excelsa extract and vincristine in Nalm-6 and Reh cells was determined using MTT assay and synergism was evaluated using the CompuSyn software. Apoptosis was assessed by caspase 3 activity assay and flow cytometry. The expression levels of some apoptosis-related genes, Caspase 3, BAX and BCL-2 were determined by Real-time PCR. Statistical analysis was assessed by one-way ANOVA and Tukey’s tests.

The combined treatment of VCR and J. excelsa extract showed a synergistic cytotoxic effect on both Nalm-6 and Reh cells at low doses of vincristine (CI < 1). J. excelsa extract also significantly increased VCR-induced apoptosis (P < 0.001). Expression of CASP3 and BAX genes were upregulated, while BCL-2 gene was downregulated in both cells (P < 0.05).

Our study suggested the combined use of lower doses of VCR and J. excelsa extract promote its effects by apoptosis induction and this combination could potentially decrease the side effects of the drug.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most incident malignancy in children (Pui et al., 2002). The treatment of ALL usually consists of three phases: Induction of remission, consolidation (or intensification) and maintenance. Vincristine (VCR) and glucocorticoid (prednisone or dexamethasone) are two commonly used drugs to treat ALL that can be used in all three phases (Inaba et al., 2013). These agents have many side effects, such as diarrhea, nausea, vomiting, and gastrointestinal toxicities that these side effects are dose-dependent (Moudi et al., 2013). Vincristine also has peripheral neurological side effects. These problems can affect the quality of life of the patients (Lopez-Lopez et al., 2016). Natural products and conventional chemotherapeutic drug combinations are believed to increase the efficacy of anticancer treatment through their potential synergistic effects. Furthermore, combination therapy could potentially decrease the side effects of chemotherapeutic drugs (Hemalswarya and Doble, 2006). Juniperus excelsa is distributed in Europe, south-western Asia, and North America (Bais and Prashar, 2015). This plant is a species of juniper which belongs to the family of Cupressaceae (Nabi et al., 2012), known as a medicinal herb which has been traditionally used to treat cold, bronchitis, hemorrhoids, urinary infection, cough, jaundice, tuberculosis, digestive disorders, cardiac and nervous problems (Asili et al., 2008; Muhammad et al., 1992; Pirani et al., 2011; Sadeghi-Aliaabadi et al., 2009; Topçu et al., 2005). This plant has been reported to possess various pharmacological effects. For example antibacterial activity (Muhammad et al., 1992), Antileishmanial activity (Nabi et al., 2012) and also cytotoxic
effects of J. excelsa were reported. Accordingly, J. excelsa extracts were reported to show antiproliferative effects on leukemia, myeloma, breast, prostate, colon, cervix and lung cancer cells (Och et al., 2015; Saab et al., 2012; Sadeghi-Alia badi et al., 2009; Topçu et al., 2005). Regarding leukemia, only the antiproliferative activity of J. excelsa extract on T acute lymphoblastic leukemia CCRF/ADRS000, CCRF-CEM, CEM/C1, J45.01 cells and acute promyelocytic leukemia HL-60 cell were reported (Och et al., 2015; Saab et al., 2012). The main components of this plant are α-pinene, α-cedrol, sabinene and verbenone (Unlu et al., 2008). Apoptosis or programmed cell death is a natural process that plays a key role in the normal homeostasis of all multi-cellular organisms. Caspases are synthesized as pro-caspases that are activated by proteolytic cleavage and can cleave many cellular substrates in regulating apoptosis (Hengartner, 2000). There are two mechanisms for caspase-dependent apoptosis, intrinsic or mitochondrial pathway and extrinsic or death receptor pathways. Caspase 3 is one of the most important executioner caspases, which is activated by both extrinsic and intrinsic pathways (Elmore, 2007). The expression of caspase 3 is highly regulated by a set of proteins, for example, Bcl-2 family proteins. Bcl-2 and Bax are anti-apoptotic and pro-apoptotic members of Bcl-2 family, respectively (Cory and Adams, 2002). In this study, we tried to measure the synergistic cytotoxic effects of methanolic extract of aerial parts of J. excelsa and vincristine on acute lymphoblastic leukemia Nalm-6 and Reh cell lines.

Material and methods

Chemicals

Vincristine sulfate was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in sterile-filtered Dimethyl sulfoxide (DMSO) and aliquoted and stored at −20 °C. RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco (USA). MT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] and DMSO were obtained from Roth (Germany).

Plant extract

The aerial parts of Juniperus excelsa were collected from Kohgiluyeh va Boyer-ahmad province, southwestern Iran. A voucher specimen was deposited at the Herbarium of Traditional Medicine and Materia Medica Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (No. 3125). Total RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR

The Cells (3 × 10^5/ml) were treated with different concentrations of VCR and J. excelsa extract and were incubated for 48 h, and total RNA was extracted from each well using the Hybrid-R RNA purification kit (GeneAll, Korea) according to the manufacturer’s instructions. The quantity of the RNA samples was analyzed by UV-spectroscopy (NanoDrop TM 2000 Spectrophotometer, Thermo Scientific, USA) at 260 and 280 nm and the quality of extracted RNA was tested by the 1% agarose gel electrophoresis. RNA was reverse transcribed to first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) following the manufacturer’s instructions. The sequences of the primers are shown in Table 1. Real-time PCR analysis was performed using SYBR Premix Ex Taq (Tli RNase H Plus) kit (Takara Biomedical Technology, Japan) in Applied Rotor-Gene Q Real Time PCR System (Qiagen, Valencia, CA). The expression of ABL gene was utilized as an endogenous control to normalize the expression of the target genes. Melting curve analysis of caspase 3, Bcl-2, Bax and ABL showed a single peak. The mean Ct of the target genes was calculated from triplicate measurements and then normalized with the mean Ct of ABL gene. Data extraction was executed using the Rotor-Gene Q series software v. 2.0.2, and the Pfaffl method was used to calculate the relative expression of each gene (Pfaffl, 2001).

Caspase-3 activity assay

The activity of caspase-3 was analyzed using caspase-3 colorimetric assay kit (Abcam, USA) according to manufacturer’s protocol. Briefly, the Nalm-6 and Reh cells (1.2 × 10^5/ml/well) were treated with various concentration of VCR and J. excelsa

Table 1

<table>
<thead>
<tr>
<th>Genes and oligonucleotide primers for quantitative real-time RT-PCR</th>
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<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>ABL</td>
</tr>
<tr>
<td>CASP3</td>
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<td>BCL-2</td>
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<td>BAX</td>
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ABL (Abelson murine leukemia viral oncogene homolog), CASP3 (cysteine-aspartic proteases 3), BCL-2 (B-cell lymphoma 2), BAX (BCL-2 associated X protein).
extract in 12-well plates for 48 h. The untreated cells were used as a control. After 48 h incubation, cells were suspended in 50 μl of chilled cell lysis buffer and then incubated on ice for 10 min. The tubes containing cell lysates were centrifuged at 10000 g for 1 min. The concentration of proteins was measured by BCA protein assay. Then 50 μl of each sample were added to 96 well plate and 50 μl of 2 × reaction buffer containing 10 mM DTT and 5 μl of 4 mM caspase 3 substrate (DEVD-p-NA) were added to 200 μg protein from each sample and incubated at 37 °C for 4, 12 and 24 h. In background wells instead of sample, 50 μl of reaction buffer were used. After incubation, the plates were read at 405 nm by ELISA microplate reader. Comparison of the absorbance from an apoptotic sample with a control determines the fold increase in caspase-3 activity.

Statistical analysis

The IBM SPSS Statistics version 23 software was used for data analysis. The one-way ANOVA and post hoc Tukey multiple comparison tests were used in the comparison between more than 2 groups. Results are presented as means ± SD. \( P < 0.05 \) was considered significant.

Results

Cytotoxic effects of vincristine and J. excelsa extract, alone on Nalm-6 and Reh cell lines

For examining the effect of J. excelsa extract in Nalm-6 and Reh cells, cell viability was analyzed by MTT assay after treatment with different concentrations of both agents for 48 and 72 h. As shown in Figs. 1 and 2, J. excelsa extract treatment of cells exhibited a time and a dose-dependent effect on cell viability. In Nalm-6 cells, 1, 2, 3 and 4 μg/ml doses of J. excelsa extract caused 94, 91, 78 and 53% cell viability following 48 h incubation and 82, 79, 57 and 36% cell viability after 72 h of treatment (Fig. 1A). Similar cytotoxic effects of J. excelsa extract were also apparent in Reh cells leading to 94, 93, 83 and 54% cell viability following 48 h incubation and 85, 80, 59 and 37% cell viability after 72 h of treatment (Fig. 2A). The cytotoxic effect of VCR on Nalm-6 and Reh cells also were assessed. In Nalm-
6 cells, 0.1, 1 and 3 nM doses of VCR resulted in 94, 83 and 55% cell viability after 48 h incubation and 88, 66 and 38% cell viability following 72 h incubation. Similar VCR treatments of Reh cells resulted in 94, 58 and 51% cell viability following 48 h and 87, 52 and 45% cell viability following 72 h incubation (Figs. 1B and 2B). The IC50 values for *J. excelsa* extract ranged from 3 μg/ml to 4 μg/ml and for VCR were around 3 nM in both Nalm-6 and Reh cells. All IC50 values were lower for VCR when compared to those for *J. excelsa* extract in both cell lines.

**Cytotoxic effect of *J. excelsa* extract–VCR combination on Nalm-6 and Reh cells**

Based on the data showing the efficacy of *J. excelsa* extract and VCR in ALL cells, next, we evaluated their effects in combination on Nalm-6 and Reh cell death and analyzed the viability of cells by MTT assay after treatment with different concentrations of *J. excelsa* extract plus VCR for 48 and 72 h. A decreased cell viability (Fig. 1C) in Nalm-6 cells were observed in two *J. excelsa* extract plus VCR combinations ranging from 1 and 2 μg/ml *J. excelsa* extract and 0.1 and 1 nM VCR, as compared to similar *J. excelsa* extract and VCR doses alone. As shown in Fig. 1C, in Nalm-6 cells, e.g. a combination of 2 μg/ml *J. excelsa* extract and 1 nM VCR resulted in 58% viability after 48 h incubation, whereas each agent alone showing 91% (extract) and 83% (VCR) viability (P < 0.001). A decreased cell viability (Fig. 2C) in Reh cells were also observed in three *J. excelsa* extract plus VCR combinations ranging from 1, 2 and 3 μg/ml *J. excelsa* extract and 0.1 nM VCR, as compared to similar *J. excelsa* extract and VCR doses alone. As shown in Fig. 2C, e.g. 3 μg/ml *J. excelsa* extract plus 0.1 nM VCR treatment resulted in 62% viability after 48 h incubation as compared to 83% (extract) and 94% (VCR) viability by each agent alone (P < 0.001). 48 h treatments were selected to quantify the synergistic effect of *J. excelsa* extract and VCR. Their certain combinations were assessed by combination Index (CI) analysis using the CompuSyn software program (ComboSyn, Inc, Paramus, NJ) that is based on the Chou-Talalay equation (Chou and Talalay, 1983). According to the Chou–Talalay method, the combination of both extract and VCR results in a synergistic effect exhibiting a CI value <1 in every combination assay (Table 2).

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**Fig. 2. Effects of combined treatment of VCR with *J. excelsa* extract on the growth of Reh cells.** The cells were treated with VCR or *J. excelsa* extract alone (A and B) or in combination (C) for 48 and 72 h. Cell growth was examined using MTT assay. In Reh cells, 0.1 nM VCR and its combination with 1, 2 and 3 μg/ml *J. excelsa* extract was assessed. Data are represented as means ± SD of at least 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control. *p < 0.05, **p < 0.01, ***p < 0.001 combination treatment compared to VCR.
Induction of apoptosis in Nalm-6 and Reh cells treated with *J.* excelsa extract and VCR

To determine whether the inhibitory effect of *J.* excelsa extract and chemotherapy drug VCR on Nalm-6 and Reh cells survival is relevant to the induction of apoptosis, cells were analyzed by Annexin-V/Propidium iodide staining and flow cytometry. First, the cells were incubated in the presence of some concentration of VCR and *J.* excelsa extract for 48 h. In Nalm-6 cells, the combination of 2 µg/ml *J.* excelsa extract and 0.1 nM VCR caused 53.9% total apoptosis and the combination of 2 µg/ml *J.* excelsa extract and 1 nM VCR resulted in 84.3% total apoptosis after 48 h treatment. On the other hand, only 15.6 and 25.4% apoptosis was seen with 0.1 and 1 nM VCR, respectively. About 25% apoptotic Reh cells were observed following treatment with 2 µg/ml *J.* excelsa extract plus 0.1 nM VCR and the combination of 3 µg/ml *J.* excelsa extract and

### Table 2

The combination indices (CI) for the growth inhibitory effects of combined treatments.

<table>
<thead>
<tr>
<th></th>
<th>0.1 V + 1 J</th>
<th>0.1 V + 2 J</th>
<th>0.1 V + 3 J</th>
<th>1 V + 1 J</th>
<th>1 V + 2 J</th>
<th>1 V + 3 J</th>
</tr>
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<tbody>
<tr>
<td>Nalm-6</td>
<td>0.38 ± 0.04</td>
<td>0.20 ± 0.01</td>
<td>0.14 ± 0.02</td>
<td>0.50 ± 0.04</td>
<td>0.13 ± 0.05</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Reh</td>
<td>0.26 ± 0.05</td>
<td>0.17 ± 0.03</td>
<td>0.10 ± 0.06</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

Data are expressed as mean ± SD. A CI less than 0.9 indicates synergism; 0.9 to 1.10, additive; and more than 1.10, antagonism. V; vincristine (nM), J; *Juniperus excelsa* extract (µg/ml). ND: not determined.

**Fig. 3.** Promotion of VCR-induced apoptosis of Nalm-6 and Reh cells by *J.* excelsa extract. Cells were treated with 2 or 3 µg/ml *J.* excelsa extract and 0.1 or 1 nM VCR alone and in combination for 48 h the treated cells were incubated with annexin-V antibody and PI dye and analyzed by flow cytometry (A). Bottom left quadrant – annexin V-/PI- (living cells); upper left quadrant – annexin V+/PI- (early apoptosis); bottom right quadrant – Annexin V-/PI+ (necrosis), upper right quadrant – annexin V+/PI+ (late apoptosis). Data are represented as means ± SD of at least 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 significantly different from control; a *p < 0.05, b *p < 0.01,c *p < 0.001 combination treatment compared to VCR.
0.1 nM VCR induced up to 81.3% apoptosis in Reh cells. On the other hand, only 17.9% apoptosis was seen with 0.1 nM VCR (Fig. 3). Treatment with VCR plus *J. excelsa* extract substantially increased the annexin-V positive cells, indicating that *J. excelsa* extract promotes VCR-induced apoptosis of both cells, particularly in early-stage apoptosis (*p* < 0.001). The induction of apoptosis was dose-dependent in both cells. Moreover, increased percentage of annexin-positive cells after treating with *J. excelsa* extract plus VCR in flow cytometry analysis (Fig. 3) confirmed the induction of cell death of Nalm-6 and Reh cells.

**Effect of *J. excelsa* extract and VCR combination on the gene expression in Nalm-6 and Reh cells**

For confirming the synergistic cytotoxic effects of combined treatment of *J. excelsa* extract and VCR, caspase 3, Bax (proapoptotic) and Bcl-2 (antiapoptotic) gene expression was analyzed by real-time PCR. The regulation of gene expression was expressed as fold differences between treatment and control groups as shown in Fig. 4. The results showed that *J. excelsa* extract and VCR combination significantly upregulated the expression of caspase 3 and Bax, while downregulated the expression of Bcl-2 in both Nalm-6 and Reh cells. For example, Nalm-6 cells were treated with 3 μg/ml *J. excelsa* extract plus 1 nM VCR for 48 h, expression of Bcl-2 decreased by 5.5 folds (*P* < 0.001), in contrast, caspase 3 and Bax increased by 1.4 and 1.6 folds, respectively (*P* < 0.01) as compared to similar VCR doses alone. The expression of Bcl-2 decreased by 1.6 folds (*P* < 0.01) and caspase 3 and Bax increased by 1.3 and 2 folds in Reh cells treated with 3 μg/ml *J. excelsa* extract plus 0.1 nM VCR for 48 h (*P* < 0.001). The regulation of genes expression proposed their significant roles in the synergistic effects of combined treatment of *J. excelsa* extract and VCR in both cell lines.

**Effect of *J. excelsa* extract and VCR combination on activation of caspases 3 in Nalm-6 and Reh**

To confirm the apoptotic effects of *J. excelsa* extract and VCR combination, the activity of caspase 3 enzyme was also analyzed. After 48 h of incubation, the caspase activity in *J. excelsa* extract and VCR combination treated cells was increased as compared with an untreated and VCR-treated groups (Fig. 5). As for Real-time PCR analysis showed that expression of caspase 3 gene in both cells

![Fig. 4](image-url)
treated with VCR plus *J. excelsa* extract were significantly increased compared with the levels of expression in the cells treated with VCR or *J. excelsa* extract alone (Fig. 4).

**Discussion**

The central finding of the present study is that *J. excelsa* extract increases the efficacy of treatment with VCR in human acute lymphoblastic leukemia Nalm-6 and Reh cells. The significance of this finding lies in the fact that VCR is a well-known cancer therapeutic agent, but can cause neurotoxicity during cancer therapy (Lopez-Lopez et al., 2016). VCR is an agent that disrupts microtubule function by binding to β-tubulin and suppressing microtubule dynamics which inhibit the formation of mitotic spindle microtubules and leads to cell arrest in the G1, M and G2 phases and subsequently cell apoptosis (Blajeski et al., 2002; Groninger et al., 2002). *J. excelsa* extract has displayed antitumor activity against several cancer cells (Esmaeili et al., 2014; Saab et al., 2012; Sadeghi-Aliaabadi et al., 2009). Based on previous findings of apoptotic cell death induced by plant extract, the present study was carried out to examine the possible synergistic interaction between *J. excelsa* methanolic extract and VCR on inducing death of acute lymphoblastic leukemia cells. Combination therapy offers the advantage of possible dose reduction via the central phytochemical compounds and also, in vitro efficacy of *J. excelsa* extract, in comparison to VCR alone in both Nalm-6 and Reh cells.

In the current study, we assessed the expression of three apoptosis-related genes at the transcriptional level. Our gene expression study indicates upregulation of Bax and caspase-3, and downregulation of Bcl-2 in both cells. The relative activity of caspase 3, as the main caspase in common pathway in apoptosis, was increased in cells treated with VCR in combination with *J. excelsa* extract, in comparison to VCR alone in both Nalm-6 and Reh cells.

In Och et al. study (2015), *Juniperus* species was considered as an alternative source of podophyllotoxin (PDP) and deoxypodophyllotoxin (DO-PDP) and the cytotoxic activity of PDP and two ethanolic leaf extracts of *Juniperus scopulorum* and *Juniperus communis* against several leukemia cell lines were evaluated and the results show a possible correlation between the level of PDP and the cytotoxic effects of *Juniperus* leaf extracts. PDP also is a precursor for the synthesis of chemotherapeutic drugs such as etoposide, etopophos and teniposide (Zheljazkov et al., 2012). The extract from aerial parts of *J. excelsa* could also be a source of PDP and its significant cytotoxic effect may result from PDP.

In summary, our results clearly demonstrate a better effect of *J. excelsa* extract and VCR combination in acute lymphoblastic leukemia Nalm-6 and Reh cells than either agent alone, suggesting that further studies are warranted to assess molecular mechanisms in vitro and also, in vivo efficacy of *J. excelsa* extract and VCR combination. A positive outcome of such studies could be useful to increase the efficacy of VCR in the treatment of ALL.

**Conclusion**

An outcome from our study is the observation that *J. excelsa* extract synergized the cytotoxic action of VCR at sub-optimal doses...
of the drug in both Nalm-6 and Reh cells. The fact that we can use sub-optimal doses of VCR to achieve the appropriate cytotoxic effect suggests the potential for reduction in side effects or toxicity. The mechanism is likely to be dependent on transcriptional regulation of apoptotic signaling proteins. Investigation on the contribution of these signaling proteins and the potential of J. excelsa extract to overcome resistance to VCR can be the focus of future studies. The presence of various compounds in natural products such as J. excelsa extract can provide the advantage of acting on multiple pathways that regulate the process of cancer development and progression. The potential of the J. excelsa extract subfraction is thus to be exploited for improvement of therapeutic responses and perhaps the reduction in drug toxicity in cancer therapy.

Conflict of interests

The authors declare no conflict of interest in the present work.

Acknowledgements

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