Journal of Associated Medical Sciences 2023; 56 (2): 68-78

JAMS
Journal of Associated Medical Sciences

Scopus indexed & Thai-Journal Citation Index Centre (TCI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Cordyceps militaris extracts inhibit FLT3 protein expression and induce cell cycle arrest in FLT3 overexpressing leukemic cell models

Win Lae Aung[1,2,3] Wantida Chaiyana[4] Sawitree Chiampanichayakul[2,5] Songyot Anuchapreeda[2,5,6] Singkome Tima[2,5,6*]

[1]Master's Degree Program in Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.
[2]Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.
[3]Histopathology Section, Department of Pathology, Yangon General Hospital, Ministry of Health, Myanmar.
[4]Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Chiang Mai Province, Thailand.
[5]Cancer Research Unit of Associated Medical Sciences (AMS-CRU), Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.
[6]Center for Research and Development of Natural Products for Health, Chiang Mai University, Chiang Mai Province, Thailand.

ARTICLE INFO

Article history:
Received 7 October 2022
Accepted as revised 6 February 2023
Available online 14 February 2023

Keywords:
Cordyceps militaris, cordycepin, FLT3, anti-leukemia

ABSTRACT

Background: Leukemia is a type of blood cancer, which is characterized by the clonal proliferation of malignant leukocytes resulting in production of abnormal blood cells. Mutations in the FMS-like tyrosine kinase 3 (FLT3) gene are one of the most frequently identified genetic alterations that affect downstream intracellular signaling pathways thereby enhancing leukemogenesis. Nowadays, drugs from herbal sources were selected as alternative drugs for studying their effectiveness on leukemia treatment.

Objectives: This study aimed to evaluate anti-leukemia activities of various *Cordyceps militaris* (*C. militaris*) extracts including crude extract (Crude) and fractional extracts from hexane (Hex), ethyl acetate (EtOAc), and ethanol (EtOH) on EoL-1 and MV4-11 cells.

Materials and methods: Cytotoxicity and effects on FLT3 protein expression and cell cycle progression were investigated and compared with standard cordycepin using MTT assay, Western blotting, and PI staining, respectively.

Results: The results demonstrated that EtOAc and Hex fractional extracts showed the strongest cytotoxic effects on EoL-1 cells with inhibitory concentration at 50% growth (IC_{50}) values of 11.0±2.9 and 11.7±0.6 µg/mL and 45.9±0.4 and 36.8±15.3 µg/mL for MV4-11 cells, respectively. Crude and EtOAc fractional extracts exhibited excellent inhibitory effects on FLT3 protein expression. Moreover, the cell cycle progression of both leukemic cells was arrested at S phase after treatment. The obtained results are in the same pattern with standard cordycepin.

Conclusion: Overall, this present study showed that anti-leukemia activities of *C. militaris* on EoL-1 and MV4-11 may involve the inhibition of cell proliferation by inhibiting FLT3 protein expression and inducing cell cycle arrest at S phase and cordycepin may plays a major role of these activities.

* **Corresponding author.**
Author's Address: *Division of Clinical Microscopy, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai Province, Thailand.*
** **E-mail address:** singkome.tima@cmu.ac.th
doi: 10.12982/JAMS.2023.019
E-ISSN: 2539-6056

Introduction

Acute myeloid leukemia (AML) is a heterogenous malignant disorders characterized by abnormal proliferation and differentiation of myeloid cells. It is the most prevalent type of leukemia in adults with an increased risk of relapse and poor prognosis. Current treatments for AML patients include induction therapy, which is a combination of cytotoxic agents such as cytarabine and anthracycline as a "7+3" regimen and consolidation therapy, which consists of high doses of cytarabine for eradication of residual leukemia.[1,2] However, the use of cytotoxic agents can cause toxicities to the patients, especially in older patients who are over 65 years of age.[3] Thus, treatment of AML remains challenging, particularly in patients over the age of 65 and those with refractory or relapsed AML.[4] Therefore, the development of new drugs with lower toxicities is necessary for the treatment of AML.[5]

FMS-like tyrosine kinase 3 (FLT3) is a member of the type III receptor tyrosine kinase (RTK) which normally expressed on the surface of many hematopoietic progenitor cells, and plays a critical role in controlling cell survival, proliferation, and differentiation of hematopoietic cells.[6-9] It is one of the main mutated genes which can be found in nearly 30% of newly diagnosed AML cases; about 25% of patients with internal tandem duplication (ITD) mutations within the juxtamembrane domain (JMD) and 7-10% with point mutations in the tyrosine kinase domain (TKD), leading to the constitutive activation of kinase activity.[10-13] FLT3-ITD gene is the most common mutation that exhibits a high leukemia burden and used as a prognostic marker. It was associated with poor prognosis, increased risk of relapse and decreased overall survival.[11] Consistently, FLT3-TKD point mutations result in ligand-independent activation, phosphorylation of the receptor and constitutively activate kinase activity, thereby enhancing uncontrolled proliferation.[14] According to the previous studies, drugs from herbal sources were selected to evaluate their anti-leukemia properties.[15-17] *Cordyceps militaris* (*C. militaris*) is a species of fungus in the family Clavicipitaceae, and the genus Cordyceps which was originally described by Carl Linnaeus in 1753 as *Clavaria militaris*. It has been widely used as an herbal remedy or crude drug in oriental medicine and folk tonic foods in East Asia.

Several studies have shown that Cordyceps species contain numerous bioactive compounds which play a critical role in anti-inflammation, antioxidant, anti-microbial activity, anti-viral, enhancing immunity, anti-aging, and anti-tumor.[18-20] Aqueous extract of *C. militaris* has anti-proliferative effects on human leukemia U937 cells by enhancing apoptotic cell death through regulation of several major growth regulatory gene products and caspase protease activity.[21] Cordycepin in cultivated *C. militaris* possesses a synergistic cytotoxic effect on human leukemia cells.[22] In addition, *C. militaris* extract exhibited anti-leukemia efficacy on a xenografted leukemia model.[23] Moreover, hot water extract of *C. militaris* strongly inhibited the growth of HL-60 cells by promoting cell apoptosis through sequential activations of caspase-3 and PARP.[24] According to previous studies of Cordyceps species, they were rich in bioactive compounds and solvent polarity affected the contents of bioactive compounds in each fractional extracts which may differ in their biological properties.[19,25] Cordycepin represents one of the major bioactive compounds in *C. militaris* which is associated with several biological activities and widely used for various pharmacological properties.[26,27] Previous study of our extracts showed that the cordycepin content was demonstrated in all fractions of *C. militaris* extracts with different percentage in each fraction.[25] Therefore, it was suggested that cordycepin might involve in anti-leukemia properties presented in all fractions of *C. militaris* extracts. The mechanism by which cordycepin inhibits leukemic cell proliferation is only partially understood. As overexpression of FLT3 protein play a critical role in leukemogenesis, if cordycepin can inhibit leukemic cell proliferation in FLT3 overexpressing leukemic cell model, it may involve in the inhibition of FLT3 protein expression.

According to various biological activities and anti-leukemia properties of *C. militaris* as mentioned above, the objective of this study was, therefore, to investigate the anti-leukemia proterties of *C. militaris* extracts on FLT3 overexpressing leukemic cell lines, including the wild-type FLT3 overexpressing leukemic cell (EoL-1) and FLT3-ITD mutant leukemic cell (MV4-11).

Materials and methods

Plant materials

Dried *C. militaris* powder was kindly contributed by Mushroom Research and Development Center (MRDC, Chiang Mai Province, Thailand).

Chemicals and reagents

Commercial grade cordycepin (purity≥98.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, Iscove's Modified Dulbecco's Medium (IMDM), fetal bovine serum (FBS), penicillin-streptomycin, and L-glutamine were purchased from GIBCO (Waltham, MA, USA). Anti-FLT3 antibody and rabbit polyclonal anti-GAPDH antibody were purchased from Abcam (Cambridge, UK). Luminata™ Forte Western HRP Substrate was purchased from Merck Millipore (Darmstadt, Germany).

C. militaris extraction

C. militaris extracts used in this study composed of crude extract (Crude), fractional extracts including hexane fractional extract (Hex), ethyl acetate fractional extract (EtOAc), and ethanolic fractional extract (EtOH). The extraction process was previously mentioned by Marsup P *et al*.[25] Briefly, crude extract was obtained from maceration of dried *C. militaris* powder in 95% ethanol, followed by filtration through Whatman No.1 paper and removed the solvent with a rotary evaporator (Buchi Labortechnik GmbH, Essen, Germany). On the other hand, sequential maceration was performed to obtain the Hex, EtOAc, and EtOH fractional extracts. First, hexane fractional extract (Hex) was obtained by maceration of dried *C. militaris* powder in hexane, followed by filtration through Whatman No.1 paper to obtain the solution of the extract. After that, the hexane was removed by using a rotary evaporator to get the hexane fractional extract (Hex). Subsequently, the

residue powder was macerated in ethyl acetate, filtrated through Whatman No.1 paper and the solution of the extract was evaporated out of the solvent using a rotary evaporator to get the ethyl acetate fractional extract (EtOAc). Afterwards, the residue power was macerated in 95% ethanol, using the same method as above and ethanolic fractional extract (EtOH) was obtained from this final step.

Leukemic cell lines and cell culture conditions

FLT3 overexpressing leukemic cell lines, including wild-type (EoL-1) and FLT3-ITD mutant (MV4-11) were used as leukemic cell models in this experiment. EoL-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. MV4-11 cells were cultured in IMDM medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. Both leukemic cell lines were cultured at 37 °C in a humidified incubator with 5% CO_2.

MTT cytotoxic assay

To check the cytotoxicity of *C. militaris* extracts on leukemic cell lines, MTT assay was performed by using EoL-1 and MV4-11 cells at 70-80% confluence in exponential growth phase. Briefly, EoL-1 cells at 3×10^4 cells/well and MV4-11 cells at 1×10^4 cells/well were plated in 96 well plates containing 100 µL medium prior to the treatment with the extracts at 37 °C in a humidified condition with 5% CO_2 for 24 hrs. Then, an equal volume of *C. militaris* extracts (including crude and three fractional extracts) and standard cordycepin at various concentrations (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) were added to each well, and incubated for another 48 h. After removal of 100 µL medium, 15 µL of MTT dye solution was added and the plate was incubated at 37 °C for 4 hrs. Then, 200 µL of DMSO was added to each well and mixed thoroughly to dissolve the formazan crystals. The absorbance of treated wells (test wells), vehicle control well (VC; 0.8% DMSO in culture medium), and untreated control or cell control well (CC) were measured using SpectraMax® M5 Multi-Mode Microplate Reader (Molecular Devices Crop, CA, USA) at 578 nm with a reference wavelength of 630 nm. Three independent assays were done. The average cell viability of the vehicle control was compared to untreated control. If there are no significant differences between CC and VC, the average cell viability of the treated well was calculated in respect to the vehicle control.

Western blotting

To evaluate the effect of *C. militaris* extracts on FLT3 protein expression in FLT3 overexpressing leukemic cell lines, Western blot analysis was done. At first, both EoL-1 cells (3×10^5 cells/mL) and MV4-11 cells at (1×10^5 cells/mL) were treated with the non-toxic doses (IC_{20}) of the extracts (including crude and three fractional extracts) and standard cordycepin, which was gained from MTT assay for 48 hrs. After that, the cells were harvested and washed 3 times with ice-cold PBS. The total cell number was counted for cell viability evaluation by using trypan blue dye solution. After that, the cell pellet was lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, and 0.1% SDS) containing protease inhibitors. Then, the whole protein lysate was separated, and protein concentration was measured by using Folin-Lowry method. Later, SDS-PAGE was performed using whole protein lysate (80 µg, 25 µL/well) of tested samples and the separated proteins on were transferred to the polyvinylidene difluoride (PVDF) membrane by using 32 volts of electricity overnight in blotting buffer. Then, the membrane was blocked with 5% skimmed milk in PBS and incubated with primary antibodies, the rabbit polyclonal anti-FLT3 (1:1,000) and the rabbit polyclonal anti-GAPDH (1:16,000). The membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:20,000) and proteins were visualized by means of chemiluminescent method using the Luminata™ Forte Western HRP Substrate. This experiment was performed in three independent experiments and the most effective C. militaris extract was selected and its effect on FLT3 protein expression was evaluated in term of dose dependent manner.

Cell cycle analysis

Cell cycle analysis was performed using DNA binding dye, propidium iodide (PI). In this study, various concentrations of crude extract (Crude) and ethyl acetate fractional extract (EtOAc) of *C. militaris* were used to treat EoL-1 and MV4-11 cells, respectively. The activities of extracts were compared to that of standard cordycepin. Briefly, EoL-1 cells at 3×10^5 cells/mL and MV4-11 cells at 1×10^5 cells/mL were starved for 24 hrs in 0.5% FBS RPMI-1640 and 0.5% FBS IMDM medium, respectively. Then, leukemic cells were treated with non-toxic dose (IC_{20}) value and 2 concentrations lower than IC_{20} value (IC_{10}, IC_5) of extract for 48 hrs. After that, the cells were harvested and washed with ice-cold PBS. Next, cells were resuspended in 300 µL PBS and then, 700 µL of ethanol was added and incubated for 30 min on ice. Thereafter, cells were centrifuged at 5,000 rpm for 10 sec and washed with ice-cold PBS. Finally, leukemic cells were stained with propidium iodide (PI) solution (1 mg/mL PI, 80 mM EDTA, 1% Triton X-100, 10 mg/100 µL RNAse A) in the dark at 4 °C and the DNA content was detected by flow cytometer. This experiment was performed in three independent experiments and the data were analyzed using the FlowJo Vx software.

Statistical analysis

The data were expressed as the mean±SD of three independent experiments. The statistical differences were determined using unpaired Student's t-test and statistical comparison was processed by one-way ANOVA using SPSS program (SPSS Statistics Base 17.0 for Windows (SPSS License No.7 (50)). The differences were considered significant when the probability value was less than 0.05 ($p<0.05$).

Results

Cytotoxic effects of *C. militaris* extracts

Cytotoxicity of crude and fractional extracts of Hex, EtOAc, and EtOH were determined by an inhibitory concentration at 50% growth (IC_{50}). All the test compounds demonstrated the cytotoxic effects on EoL-1 and MV4-11 cells with various IC_{50}. It may be due to the different constituents of bioactive compounds in each extract. During fractionation, solvents are added according to the order of increasing polarity for extraction of both polar and nonpolar compounds. The polarity of solvents in the extraction process influences the yield of bioactive compounds.[28] The higher polar solvents yielded higher bioactive compounds which may differ in their biological activities. In this study, it was found that different *C. militaris* extracts exhibited different cytotoxic effects on leukemic cell lines. By comparing the results from these two cell lines, all the extracts showed the strongest inhibitory effects on both EoL-1 and MV4-11 cells (Table 1). The IC_{50} values of standard cordycepin, crude, and fractional extracts of Hex, EtOAc, and EtOH on EoL-1 cells were 28.7±7.3, 43.5±9.1, 11.7±0.6, 11.0±2.9, and >100 µg/mL, respectively (Figure 1a). On the other hand, the IC_{50} values on MV4-11 cells were 50.0±13.0, >100, 36.8±15.3, 45.9±0.4, and >100 µg/mL, respectively (Figure 1b).

According to the obtained results, the Hex and EtOAc fractional extracts exhibited the greatest cytotoxic effects on leukemic cells. However, the cytotoxicity in these leukemic cells were different due to the difference in leukemic cell-type. In our study, EoL-1 cells were found to be more sensitive to the extracts than MV4-11 cells. This result was well in accordance with pervious study reported that EoL-1 cell lines were sensitive to sorafenib and cytarabine than MV4-11 cells due to the presence of activated FIPL1-PDGFRα fusion gene in EoL-1 cells.[29]

Table 1 Inhibitory concentration values of *C. militaris* extracts on EoL-1 and MV4-11 cell lines.

C. militaris extracts	EoL-1		MV4-11	
	IC_{20} (µg/mL)*	IC_{50} (µg/mL)*	IC_{20} (µg/mL)*	IC_{50} (µg/mL)*
Cordycepin	8.3±1.1	28.7±7.3	14.9±8.0	50.0±13.0
Crude	5.8±3.8	43.5±9.1	4.3±1.8	>100
Hex	1.5±0.2	11.7±0.6	2.2±1.0	36.8±15.3
EtOAc	1.5±0.2	11.0±2.9	2.9±1.8	45.9±0.4
EtOH	4.7±0.5	>100	5.4±15.5	>100

** Data are the mean±SD of three independent experiments.*

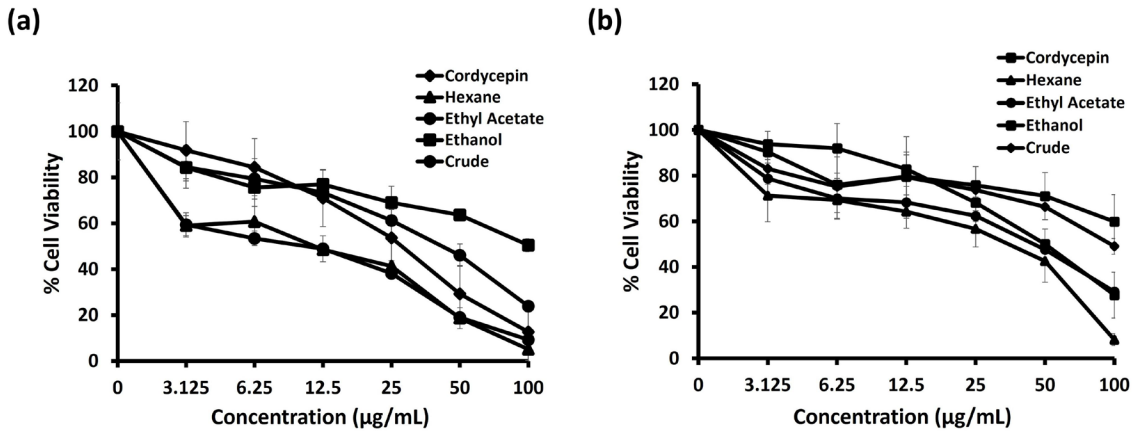

Figure 1 *Cytotoxicity of C. militaris extracts on (a) EoL-1 and (b) MV4-11 cell lines.*

Effects of *C. militaris* extracts on FLT3 protein expression

The effects of *C. militaris* extracts on FLT3 protein expression in EoL-1 and MV4-11 cells were evaluated using Western blot analysis. After treatment of leukemic cells with the non-toxic doses (IC_{20}) of the extracts for 48 hrs, it was found that crude extract exhibited the strongest inhibitory effects on FLT3 protein expression in EoL-1 cells followed by Hex fractional extract. The FLT3 protein level was decreased by 29.1 and 20.6%, respectively. Besides, standard cordycepin demonstrated a greatest inhibitory effect on FLT3 protein expression by decreasing the FLT3 protein level for 32.3%. In addition, the total cell number after treatment was decreased in the same pattern of the decreasing of FLT3 protein level (Figure 2). By contrast, EtOAc

fractional extract has the strongest inhibitory effects on MV4−11 cells (44.7%), followed by EtOH fractional extract and crude extract which were 38.0% and 23.6%, respectively (Figure 3).

Thereafter, crude extract and EtOAc fractional extract were selected as the most effective extracts for EoL-1 and MV4-11 cells, respectively. Their effects on FLT3 protein expression were evaluated in term of dose-dependent manner by comparing to standard cordycepin. After the treatment of leukemic cells for 48 h, crude extract exhibited the strongest inhibitory effects on FLT3 protein expression in EoL-1 cells by dose-dependent manner (Figure 4). Similarly, EtOAc fractional extract showed an excellent inhibitory effect on FLT3 protein expression in MV4-11 cells by means of dose-dependent manner (Figure 5).

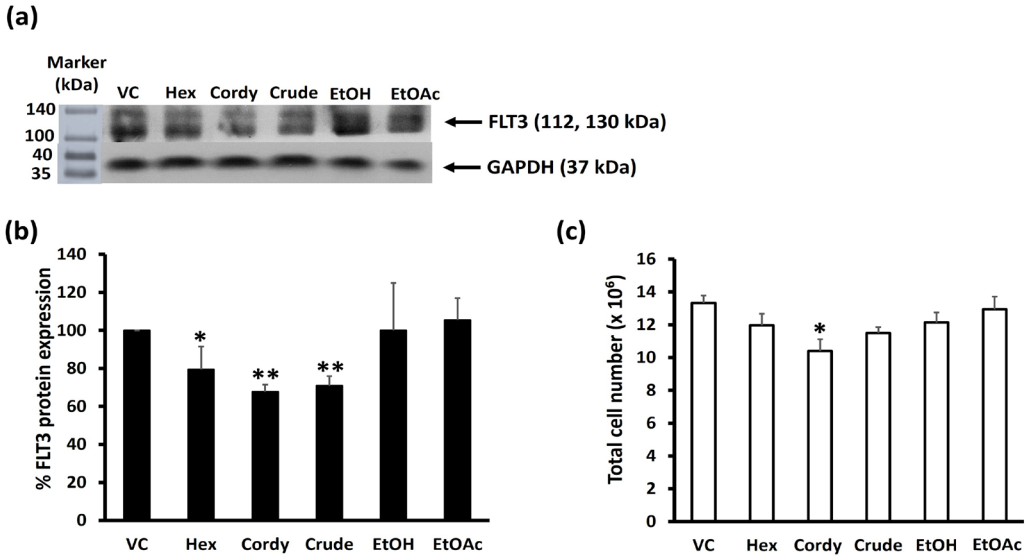

Figure 2 *Effects of C. militaris on FLT3 protein expression in EoL-1 cell line after 48 hrs of treatment. (a): The levels of FLT3 protein expression after treatments with standard cordycepin (Cordy), crude and fractional extracts of Hex, EtOAc, and EtOH were assessed by Western blotting. GAPDH was used as the loading control, (b): The protein levels were analyzed by a scan densitometer, (c): The total cell number after treatment was determined using trypan blue exclusion method. Data are the mean±SD of three independent experiments. The data were analyzed by unpaired, two tailed, Student's t test. Asterisks denote values that were significant difference from the vehicle control, *p<0.05; ** p<0.01.*

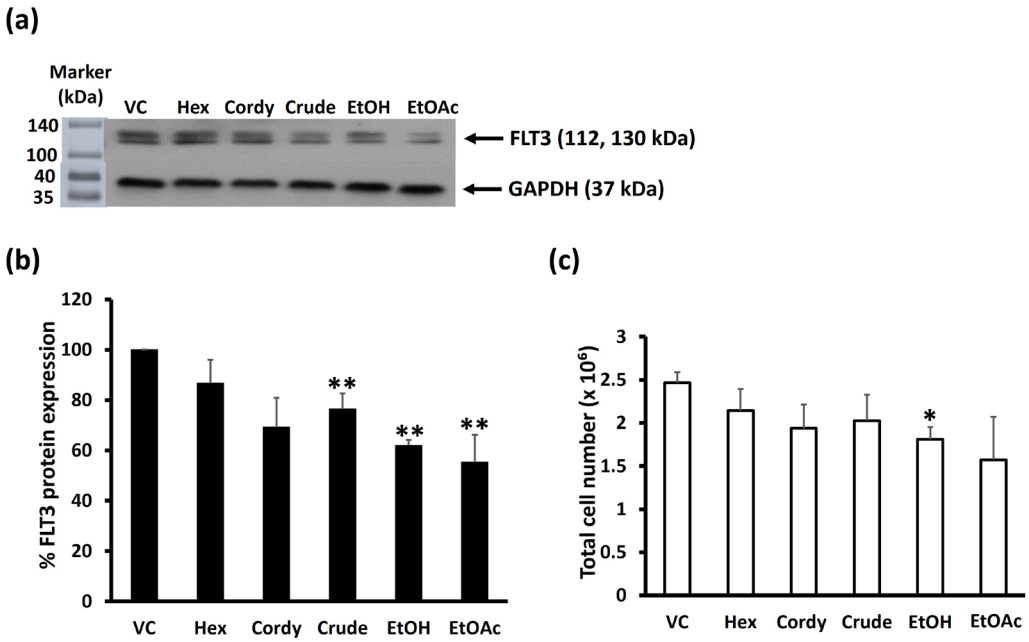

Figure 3 *Effects of C. militaris on FLT3 protein expression in MV4-11 cell line after 48 hrs of treatment. (a): The levels of FLT3 protein expression after treatments with standard cordycepin (Cordy), crude and fractional extracts of Hex, EtOAc, and EtOH were assessed by Western blotting. GAPDH was used as the loading control, (b): The protein levels were analyzed by a scan densitometer, (c): The total cell number after treatment was determined using trypan blue exclusion method. Data are the mean±SD of three independent experiments. The data were analyzed by unpaired, two tailed, Student's t test. Asterisks denote values that were significantly different from the vehicle control, *p<0.05; **p<0.01.*

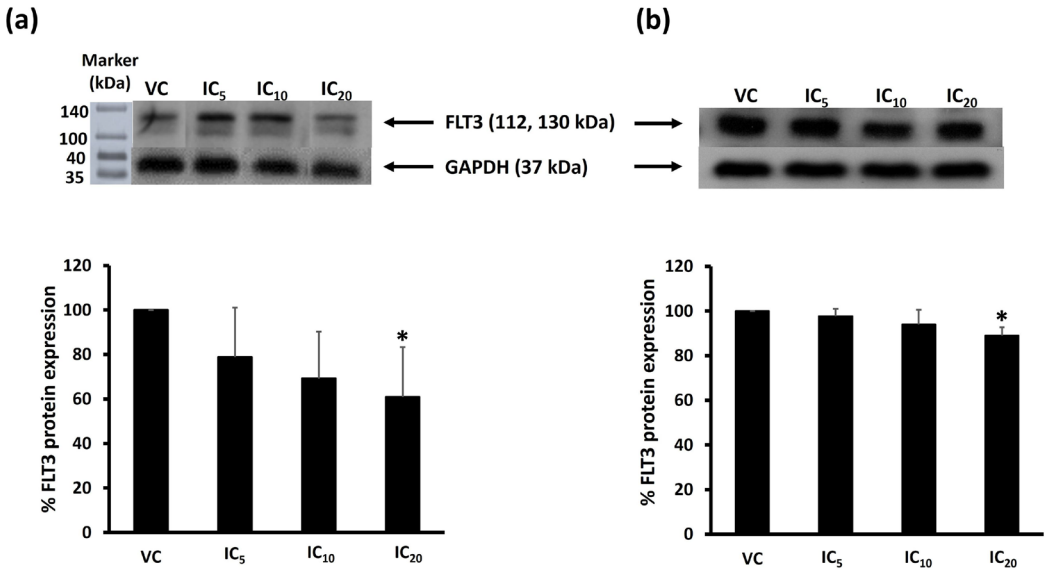

Figure 4 *Effects of concentration of (a) crude extract on FLT3 protein expression in EoL-1 cell lines compared with (b) standard cordycepin after 48 hrs of treatment. The levels of FLT3 protein expression after treatments with various concentrations (IC_5, IC_{10}, and IC_{20}) of the extracts were assessed by Western blotting. GAPDH was used as the loading control. The protein levels were analyzed by a scan densitometer. Data are the mean±SD of three independent experiments. The data were analyzed by unpaired, two tailed, Student's t test. Asterisk (*) denotes a significant difference from the vehicle control (VC) group ($p<0.05$).*

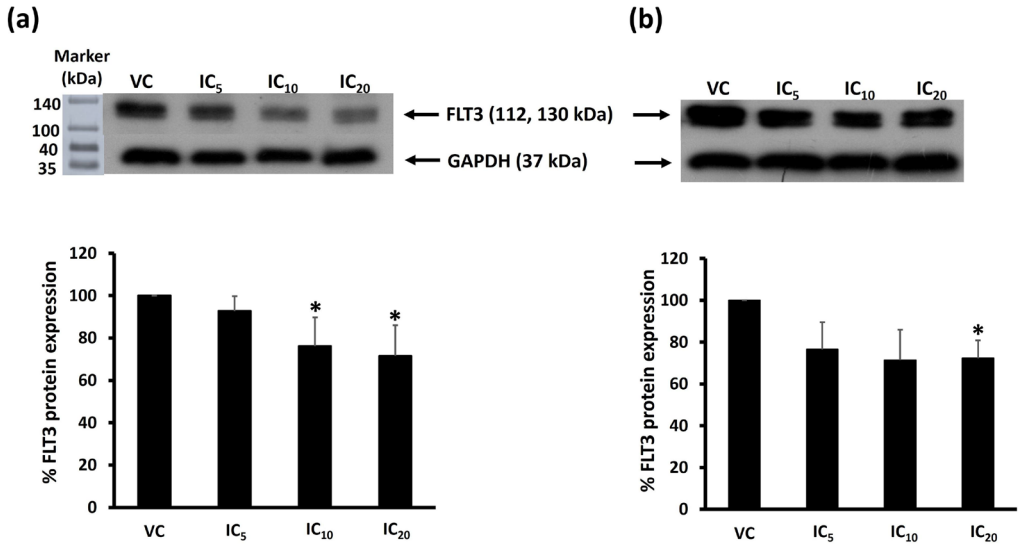

Figure 5 *Effects of concentration of (a) EtOAc fractional extract on FLT3 protein expression in MV4-11 cell line compared with (b) standard cordycepin after 48 hrs of treatment. The levels of FLT3 protein expression after treatments with various concentrations (IC_5, IC_{10}, and IC_{20}) of the extracts were assessed by Western blotting. GAPDH was used as the loading control. The protein levels were analyzed by a scan densitometer. Data are the mean ±SD of three independent experiments. The data were analyzed by unpaired, two tailed, Student's t test. Asterisk (*) denotes a significant difference from the vehicle control (VC) group at $p<0.05$.*

Effects of *C. militaris* extracts on cell cycle progression

In addition to the expression of FLT3 protein, the effect on cell cycle progression is also a critical parameter of the action of anti-leukemia drugs. Thus, crude extract and EtOAc fractional extract are selective as effective extracts for each leukemic cell line and cell cycle analysis was done in terms of dose dependent manner to check whether cell cycle progression is involved in decreased cell viability. Treatments of EoL-1 cells with crude extract (Figure 6a) and treatments of MV4-11 cells with EtOAc fractional extract (Figure 6b) significantly increased the cell populationat S phase by a concentration dependent manner, when compared to the vehicle control. In addition, standard cordycepin also arrested the cell cycle progression of both EoL-1 and MV4-11 at S phase (Figure 7). Moreover, total cell number of both EoL-1 and MV4-11 cells after treatment was decreased in a dose dependent manner (Table 2).

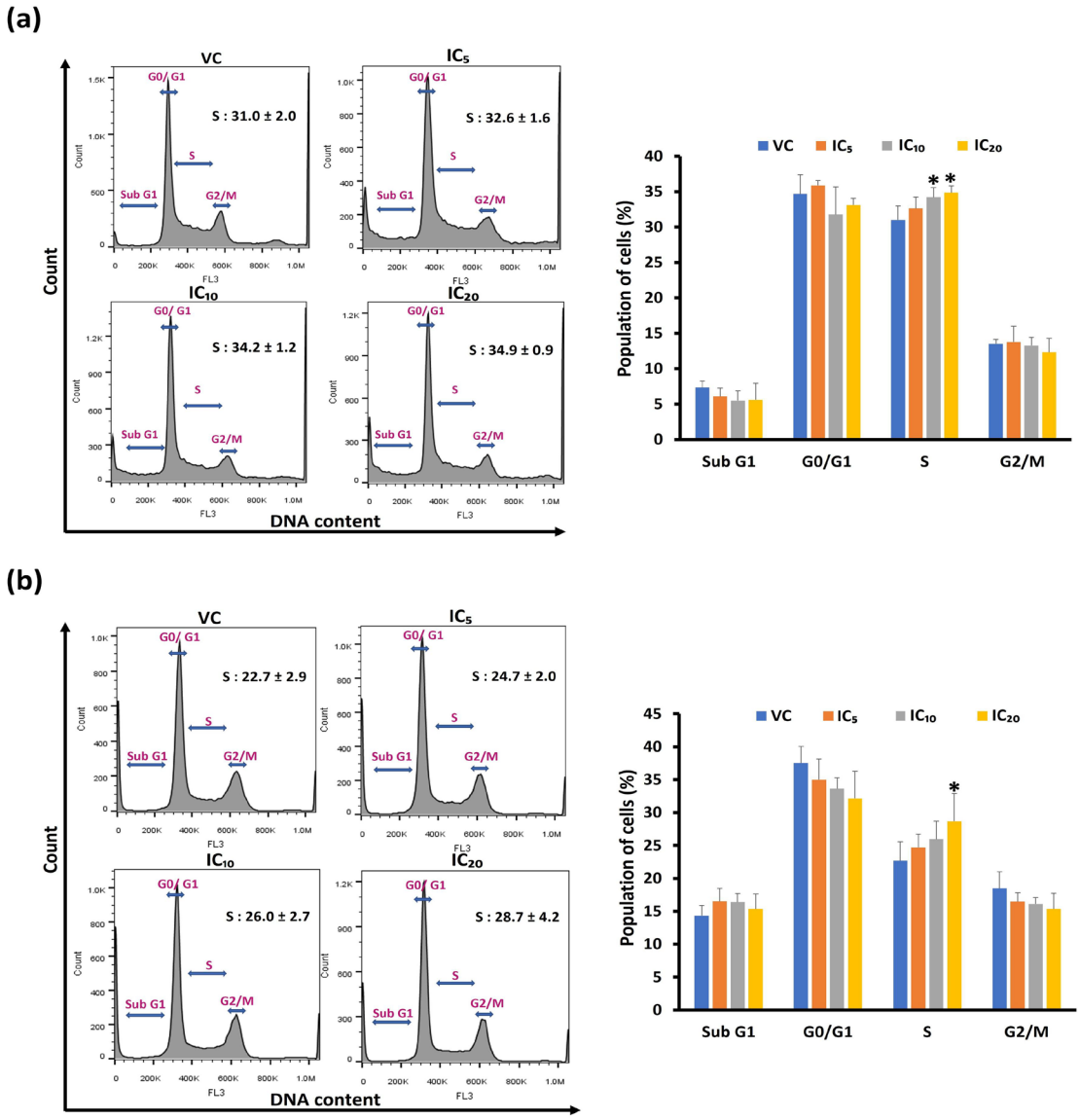

Figure 6 *Effects of concentration of (a) crude extract and (b) EtOAc fractional extract on cell cycle progression of EoL-1 and MV4-11 cells, respectively, was evaluated after 48 hrs of treatment. Cell population after treatments with various concentrations was assessed by flow cytometric quantitation of DNA contents using propidium iodide solution. The percent of cell population was analyzed using FlowJo Vx software. Data are the mean±SD of three independent experiments. The data were analyzed by One-Way-ANOVA test. Asterisk (*) denotes a significant difference from the vehicle control (VC) group at p<0.05.*

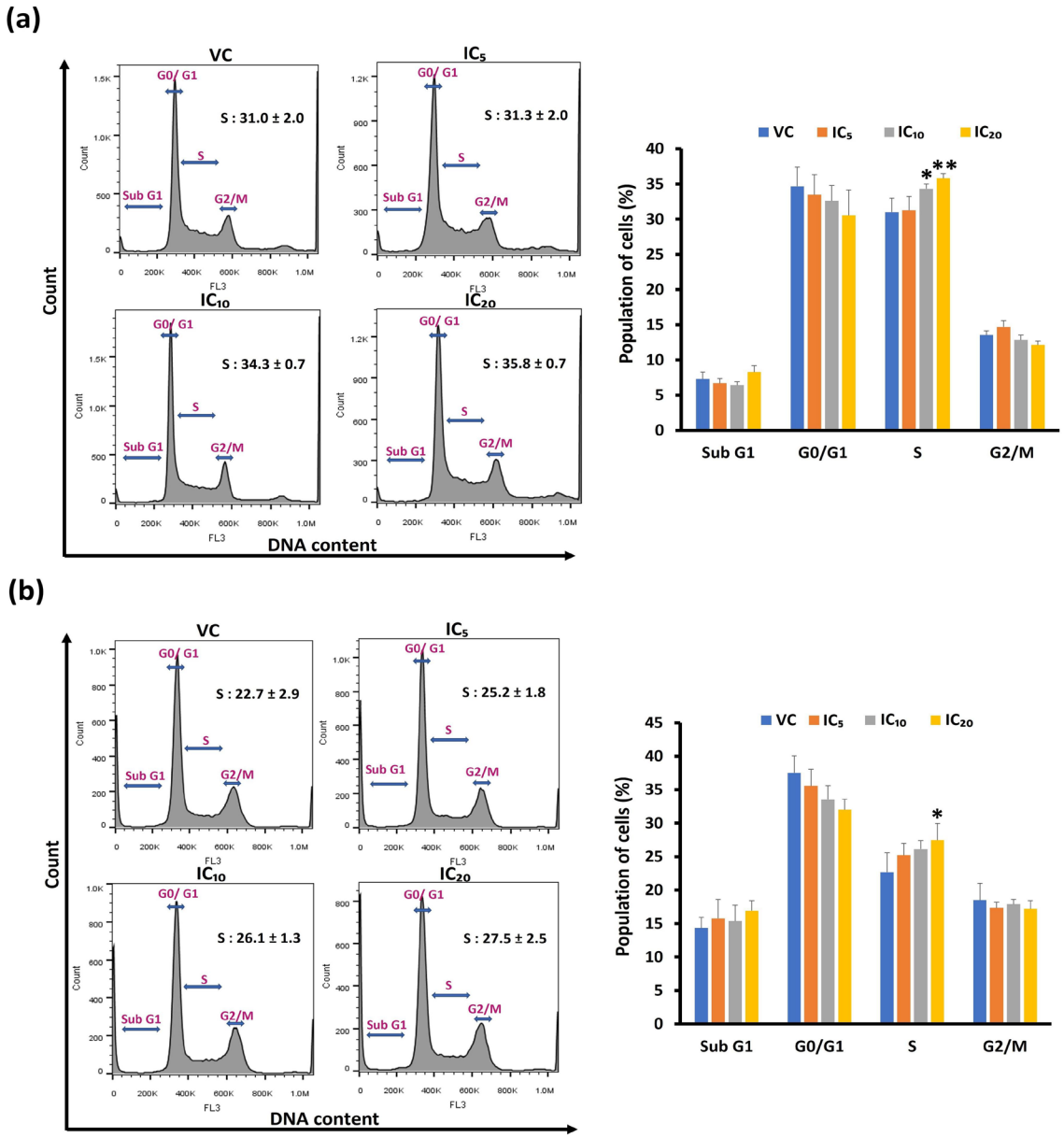

Figure 7 *Effects of concentration of standard cordycepin on cell cycle progression of (a) EoL-1 and (b) MV4-11 cells were evaluated after 48 hrs of treatment. Cell population after treatments with various concentrations was assessed by flow cytometric quantitation of DNA contents using propidium iodide solution. The percent of cell population was analyzed using FlowJo Vx software. Data are the mean±SD of three independent experiments. The data were analyzed by One-Way-ANOVA test. Asterisks denote values that were significantly different from the vehicle control, *p<0.05; **p<0.01.*

Table 2 Total cell number of EoL-1 and MV4-11 cells after 48 hrs of treatment with various concentrations of *C. militaris* extracts and standard cordycepin.

Treatment	EoL-1 (×10^6 cells)		MV4-11 (×10^6 cells)	
	Crude	Cordycepin	EtOAc	Cordycepin
VC	13.9±2.0	13.9±2.0	2.6±0.5	2.6±0.5
IC_5	13.4±1.9	12.9±1.6	2.5±0.6	2.3±0.8
IC_{10}	12.0±1.5	11.2±1.2	2.4±0.8	2.2±0.8
IC_{20}	9.3±0.6*	8.8±0.6*	1.9±0.3	2.1±0.7

Data are the mean±SD of three independent experiments. Asterisk (*) denotes a significant difference from the vehicle control (VC) group (p<0.05).

Discussion

AML accounts for 80% of acute leukemia in adults. The overexpression of FLT3-ITD was associated with poor prognosis and leukemia progression. Its expression promotes proliferation and inhibits apoptosis of leukemia cells. The role of FLT3-ITD in AML has been recently reviewed elsewhere.[12,14,30] FLT3 has been shown to be the most important protein marker in AML patients and its overexpression leads to the ligand-independent activation, phosphorylation of the receptor and constitutively activate kinase activity, thereby enhancing proliferation and survival of AML.[14] Although several FLT3 inhibitors have been identified and are in clinical trials, treatment of FLT3-mutated AML remains challenging. Thus, it is important to find new inhibitors to overcome the development of resistance and increase toxicities in AML patients with FLT3 mutations. Medicinal plants have been accepted that they contain a wide variety of chemical compounds which are used to perform important biological functions with low toxicity. Several researchers have been studied in their biological activities and accepted that they may be used as effective alternative drugs for treatment of a variety of malignancies. In this study, *C. militaris* is the plant of interest, which has been used for a long time as traditional medicine for many therapeutic purposes. Investigating the previous studies of plants in the genus Cordyceps, they were rich in bioactive compounds, cordycepin and other adenosine derivatives, polysaccharide, mannitol, peptides, etc.[19,31] Recently, ethanolic extract of *C. militaris* possess cytotoxic effects and inhibitory effects on WT1 protein expression and cell cycle progression in EoL-1 cells.[32] However, nothing was known concerning the effects of *C. militaris* extract on FLT3 protein expression, which is related to leukemic cell proliferation. Our previous studies demonstrated the activities of curcumin on the proliferation rate of wild-type FLT3 overexpressing leukemic cells, EoL-1.[16] The recent study demonstrated that both inhouse-*C. militaris* extracts and commercially standard cordycepin exhibited strong cytotoxic effects on both wildtype-FLT3 overexpressing EoL-1 cells and FLT3-ITD overexpressing MV4-11 cells with various IC_{50} values. The Hex and EtOAc extracts exhibited the greatest cytotoxic effects on leukemic cells. However, the cytotoxicity in these leukemic cells were different due to difference in leukemic cell-type. EoL-1 cells were found to be more sensitive to the extracts than MV4-11 cells. This result was well in accordance with previous study reported that EoL-1 cell line was more sensitive to sorafenib and cytarabine than MV4-11 cell line due to the presence of activated FIPL1-PDGFRα fusion gene in EoL-1 cells which is highly sensitive to the kinase inhibitors.[29] According to the study of FLT3 protein expression in leukemic cell lines, non-cytotoxic doses (IC_{20} values) of *C. militaris* extracts including crude extract, Hex fraction, EtOAc fraction, and EtOH fraction were used. After the treatment of leukemic cells for indicated time periods, crude extract exhibited the strongest inhibitory effects on FLT3 protein expression in EoL-1 cells by a dose dependent manner. Similarly, EtOAc fractional extract showed an excellent inhibitory effect on FLT3 protein expression in MV4-11 cells by means of a concentration dependent manner. Moreover, both extracts showed their ability to suppress leukemic cell proliferation when compared to the vehicle control. Thus, the results revealed that the active compounds involved in crude extract and EtOAc fractional extract have ability to inhibit cell proliferation, destroy leukemic cells at high doses and downregulate the target FLT3 protein level at non-cytotoxic doses. Previous study showed that crude extract of *C. militaris* contains the highest amount of cordycepin content and EtOAc fraction contains minimum amount of cordycepin.[25] The likely explanations were due to high polarity of cordycepin and polarity of solvents that used in extraction method which leads to differences in the yield of bioactive compounds. Higher polar solvents contained higher content of bioactive compounds due to their amine and hydroxyl groups.[25] Thus, the inhibitory effect of crude extract on FLT3 protein expression in EoL-1 cells might involve in the function of cordycepin. For EtOAc fraction, it can be explained that other compounds might exert a synergistic effect on inhibition of FLT3 protein expression in MV4-11 cells. Previous studies reported that cordycepin, adenosine, phenolic contents, flavonoid contents, polysaccharides, Ergosterol, cordycepic acid, isoflavonoid were identified in *C. militaris* extracts after extraction with polar solvent (ethanol or water) and semi-polar solvent (ethyl acetate). Not only cordycepin but also other active compounds, such as polysaccharides, cordycepic acid, flavonoids, that demonstrated anti-proliferation, antioxidant, and anti-cancer activities.[33-36] On the other hand, more than 80% of the compounds that extracted from non-polar solvent (hexane) are fatty acid, carboxylic acid and their esters. Only small amount of cordycepin was extracted by using hexane. In addition, the hexane extract demonstrated antioxidant and cytotoxic activity on HCT116 colon carcinoma cells.[37] According to the information mentioned above, various active constituents in each extract may exhibited different inhibitory effect on FLT3 protein expression. Another possible reason is the different between wild-type FLT3 in EoL-1 cells and mutant-FLT3 in MV4-11 may demonstrated the different pattern in response to the treatment and need to be investigated and described more in the future.

Crude extract and EtOAc fractional extract were selective as effective extracts on EoL-1 and MV4-11 leukemic cell lines, respectively. Cell cycle analysis were investigated whether cell cycle progression is involved in reduction of cell viability. In the previous study, ethanolic extract of *C. militaris* arrested cell cycle of K562 cells at S phase.[38] Moreover, cordycepin induced cell cycle arrest at S phase in U937 and NB-4 cells thereby inhibiting the growth of leukemic cells.[39] Consistently, our present study showed that both extracts arrested the cell cycle at S phase in leukemic cells in term of a concentration dependent manner. Previous study reported hat cordycepin-induced S phase cell cycle arrest involves inhibition of cyclin A2, cyclin E, and CDK2 expressions thereby accumulating cells in S phase.[39] In this study, the findings revealed that crude extract and EtOAc fractional extract of *C. militaris* have the ability to downregulate the target FLT3 protein level and inhibit cell cycle progression by the S phase cell cycle arrest at non-cytotoxic doses thereby inhibiting the proliferation of leukemic cells. According to our results, cordycepin may involve in S phase cell cycle

arrest of both EoL-1 and MV4-11 cells thereby decreasing the proliferations of those cell lines.

Conclusion

In this study, the growth of leukemic cells was inhibited by *C. militaris* extracts. This effect might be caused by the inhibition of FLT3 protein expression and the inhibition of cell cycle progression at S phase. Cordycepin might plays a crucial role of this inhibition. However, the inhibitory mechanisms of active compounds in crude and EtOAc extracts of *C. militaris* are still not clear and need further investigations. These findings may be used for an important guideline for further development of alternative drug for leukemia in the future.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the master's degree Program in Medical Technology, Faculty of Associated Medical Sciences (AMS), Chiang Mai University (CMU), under the CMU Presidential Scholarship and the Faculty of Associated Medical Sciences (AMS) research funding, Chiang Mai University.

References

[1] Kumar CC. Genetic abnormalities and challenges in the treatment of acute myeloid leukemia. Genes Cancer. 2011; 2(2): 95-107. doi: 10.1177/1947601911408076.

[2] Klepin HD, Balducci L. Acute myelogenous leukemia in older adults. Oncologist. 2009; 14(3): 222-32. doi: 10.1634/theoncologist.2008-0224.

[3] Chen KTJ, Gilabert-Oriol R, Bally MB, Leung AWY. Recent treatment advances and the role of nanotechnology, combination products, and immunotherapy in changing the therapeutic landscape of acute myeloid leukemia. Pharm Res. 2019; 36(9): 125. doi: 10.1007/s11095-019-2654-z.

[4] Ramos NR, Mo CC, Karp JE, Hourigan CS. Current approaches in the treatment of relapsed and refractory acute myeloid leukemia. J Clin Med. 2015; 4(4): 665-95. doi: 10.3390/jcm4040665.

[5] Dombret H, Gardin C. An update of current treatments for adult acute myeloid leukemia. Blood. 2016; 127(1): 53-61.doi:10.1182/blood-2015-08-604520.

[6] Stirewalt DL, Radich JP. The role of FLT3 in haematopoietic malignancies. Nat Rev Cancer. 2003; 3(9): 650-65. doi: 10.1038/nrc1169.

[7] Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010; 141(7): 1117-34. doi: 10.1016/j.cell.2010.06.011.

[8] Tsapogas P, Mooney CJ, Brown G, Rolink A. The cytokine Flt3-ligand in normal and malignant hematopoiesis. Int J Mol Sci. 2017; 18(6): 1115. doi: 10.3390/ijms18061115.

[9] Kazi JU, Rönnstrand L. FMS-like Tyrosine Kinase 3/FLT3: From Basic Science to Clinical Implications. Physiol Rev. 2019; 99(3): 1433-66. doi: 10.1152/physrev.00029.2018.

[10] O'Donnell MR, Tallman MS, Abboud CN, Altman JK, Appelbaum FR, Arber DA, et al. Acute Myeloid Leukemia, Version 3.2017, NCCN Clinical Practice Guidelines in Oncology. J Natl Compr Canc Netw. 2017; 15(7): 926-57. doi: 10.6004/jnccn.2017.0116.

[11] Levis M. FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? Hematology Am Soc Hematol Educ Program. 2013; 2013: 220-6. doi: 10.1182/asheducation-2013.1.220.

[12] Kottaridis PD, Gale RE, Linch DC. Flt3 mutations and leukaemia. Br J Haematol. 2003; 122(4): 523-38. doi: 10.1046/j.1365-2141.2003.04500.x.

[13] Nagel G, Weber D, Fromm E, Erhardt S, Lübbert M, Fiedler W, et al. Epidemiological, genetic, and clinical characterization by age of newly diagnosed acute myeloid leukemia based on an academic population-based registry study (AMLSG BiO). Ann Hematol. 2017; 96(12): 1993-2003. doi: 10.1007/s00277-017-3150-3.

[14] Grafone T, Palmisano M, Nicci C, Storti S. An overview on the role of FLT3-tyrosine kinase receptor in acute myeloid leukemia: biology and treatment. Oncol Rev. 2012; 6(1): e8. doi: 10.4081/oncol.2012.e8.

[15] Cao ZX, Wen Y, He JL, Huang SZ, Gao F, Guo CJ, et al. Isoliquiritigenin, an orally available natural FLT3 inhibitor from licorice, exhibits selective anti-acute myeloid leukemia efficacy in vitro and in vivo. Mol Pharmacol. 2019; 96(5): 589-99. doi: 10.1124/mol.119.116129.

[16] Tima S, Ichikawa H, Ampasavate C, Okonogi S, Anuchapreeda S. Inhibitory effect of turmeric curcuminoids on FLT3 expression and cell cycle arrest in the FLT3-overexpressing EoL-1 leukemic cell line. J Nat Prod. 2014; 77(4): 948-54. doi: 10.1021/np401028h.

[17] Sangkaruk R, Rungrojsakul M, Tima S, Anuchapreeda S. Effect of Thai saraphi flower extracts on WT1 and BCR/ABL protein expression in leukemic cell lines. Afr J Tradit Complement Altern Med. 2017; 14(2): 16-24. doi: 10.21010/ajtcam.v14i2.3.

[18] Cui JD. Biotechnological production and applications of Cordyceps militaris, a valued traditional Chinese medicine. Crit Rev Biotechnol. 2015; 35(4): 475-84. doi: 10.3109/07388551.2014.900604.

[19] Das SK, Masuda M, Sakurai A, Sakakibara M. Medicinal uses of the mushroom Cordyceps militaris: current state and prospects. Fitoterapia. 2010; 81(8): 961-8. doi: 10.1016/j.fitote.2010.07.010.

[20] Hur H. Chemical Ingredients of Cordyceps militaris. Mycobiology. 2008; 36(4): 233-5. doi: 10.4489/MYCO.2008.36.4.233.

[21] Park C, Hong SH, Lee JY, Kim GY, Choi BT, Lee YT, et al. Growth inhibition of U937 leukemia cells by aqueous extract of Cordyceps militaris through induction of apoptosis. Oncol Rep [Internet]. 2005; 13(6): 1211-6. Available from: doi.org/10.3892/or.13.6.1211.

[22] Chou SM, Lai WJ, Hong TW, Lai JY, Tsai SH, Chen YH, et al. Synergistic property of cordycepin in cultivated Cordyceps militaris-mediated apoptosis in human leukemia cells. Phytomedicine. 2014; 21(12): 1516-24. doi: 10.1016/j.phymed.2014.07.014.

[23] Park JG, Son Y-J, Lee TH, Baek NJ, Yoon DH, Kim TW, et al. Anticancer efficacy of Cordyceps militaris ethanol extract in a xenografted leukemia model. Evid Based Complement Alternat Med. 2017; 2017: 8474703. doi: 10.1155/2017/8474703.

[24] Lee H, Kim YJ, Kim HW, Lee DH, Sung MK, Park T. Induction of apoptosis by Cordyceps militaris through activation of caspase-3 in leukemia HL-60 cells. Biol Pharm Bull. 2006; 29(4): 670-4. doi: 10.1248/bpb.29.670.

[25] Marsup P, Yeerong K, Neimkhum W, Sirithunyalug J, Anuchapreeda S, To-Anun C, et al. Enhancement of chemical stability and dermal delivery of Cordyceps militaris extracts by nanoemulsion. Nanomaterials (Basel). 2020; 10(8): 1565. doi: 10.3390/nano10081565.

[26] Tuli HS, Sandhu SS, Sharma AK. Pharmacological therapeutic potential of Cordyceps with special reference to Cordycepin. 3 Biotech. 2014; 4(1): 1-12. doi: 10.1007/s13205-013-0121-9.

[27] Ashraf SA, Elkhalifa AEO, Siddiqui AJ, Patel M, Awadelkareem AM, Snoussi M, et al. Cordycepin for Health and Wellbeing: A Potent Bioactive Metabolite of an Entomopathogenic Cordyceps Medicinal Fungus and Its Nutraceutical and Therapeutic Potential. Molecules. 2020; 25(12): 5347718. doi: 10.1155/2022/5347718.

[28] Abubabar AR, Haque M. Preparation of Medicinal Plants: Basic Extraction and Fractionation Procedures for Experimental Purposese. J Pharm Bioallied Sci. 2020; 12(1): 1-10. doi: 10.4103/jpbs.JPBS_175_19.

[29] Elmeliegy M, Den Haese J, Talati C, Wetzler M, Jusko WJ. Towards better combination regimens of cytarabine and FLT3 inhibitors in acute myeloid leukemia. Cancer Chemother Pharmacol. 2020; 86(3): 325-37.doi: 10.1007/s00280-020-04114-z.

[30] Marhäll A, Heidel F, Fischer T, Rönnstrand L. Internal tandem duplication mutations in the tyrosine kinase domain of FLT3 display a higher oncogenic potential than the activation loop D835Y mutation. Ann Hematol. 2018; 97(5): 773-80. doi: 10.1007/s00277-018-3245-5.

[31] Holliday J, Cleaver MP. Medicinal value of the caterpillar fungi species of the genus Cordyceps (Fr.) Link (Ascomycetes). A Review. Int J Med Mushrooms. 2008; 10(3): 219-34. doi: 10.1615/IntJMedMushr.v10.i3.30.

[32] Tima S, Tapingkae T, To-Anun C, Noireung P, Intaparn P, Chaiyana W, et al. Antileukaemic cell proliferation and cytotoxic activity of edible golden Cordyceps (Cordyceps militaris) extracts. Evid Based Complement Alternat Med. 2022; 2022: 5347718. doi: 10.1155/2022/5347718.

[33] Do T, Nguyen HC. Extraction of cordycepic acid from the fruiting body of Cordyceps militaris (L.). BioTechnologia [Internet]. 2019; 100(3): 219-26. Available from: doi.org/10.5114/bta.2019.87581.

[34] Oh JY, Choi WS, Lee CH, Park HJ. The ethyl acetate extract of Cordyceps militaris inhibits IgE-mediated allergic responses in mast cells and passive cutaneous anaphylaxis reaction in mice. J Ethnopharmacol. 2011; 135(2): 422-9. doi: 10.1016/j.jep.2011.03.030.

[35] Eiamthaworn K, Kaewkod T, Bovonsombut S, Tragoolpua Y. Efficacy of Cordyceps militaris Extracts against Some Skin Pathogenic Bacteria and Antioxidant Activity. J Fungi (Basel). 2022; 8(4): 327. doi: 10.3390/jof8040327.

[36] Wang M, Meng XY, Yang RL, Qin T, Wang XY, Zhang KY, et al. Cordyceps militaris polysaccharides can enhance the immunity and antioxidation activity in immunosuppressed mice. Carbohydr Polym. 2012; 89(2): 461-6. doi: 10.1016/j.carbpol.2012.03.029.

[37] El-Hagrassi A, Daba G, Elkateeb W, Ahmed E, El-Dein AN, Fayad W, et al. In vitro bioactive potential and chemical analysis of the n-hexane extract of the medicinal mushroom, Cordyceps militaris. Malays J Microbiol [Internet]. 2020; 16(1): 40-8. Available from: http://mjm.usm.my/uploads/issues/1578/Formatted%20MJM-19-0346_checked_COLOUR.pdf.

[38] Tian T, Song L, Zheng Q, Hu X, Yu R. Induction of apoptosis by Cordyceps militaris fraction in human chronic myeloid leukemia K562 cells involved with mitochondrial dysfunction. Pharmacogn Mag. 2014; 10(39): 325-31. doi: 10.4103/0973-1296.137374.

[39] Liao Y, Ling J, Zhang G, Liu F, Tao S, Han Z, et al. Cordycepin induces cell cycle arrest and apoptosis by inducing DNA damage and up-regulation of p53 in Leukemia cells. Cell Cycle. 2015; 14(5): 761-71. doi: 10.1080/15384101.2014.1000097.