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Research article

Menthol induces extracellular vesicle regulation of apoptosis via ATG3 and caspase-3 in acute leukemic cells

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ABSTRACT

Leukemia is one of the most deadly cancers in Thailand. Natural compounds have been developed for cancer treatment. Menthol, a peppermint compound, has shown pharmacological properties such as anti-cancer activity. However, the mechanism of menthol inducing extracellular vesicles in leukemic cells is not yet understood. In this study, we investigated the effects of menthol on leukemic extracellular vesicles and their role in apoptosis. NB4 and Molt-4 leukemic cells were cultured with menthol in various concentrations and times. Bioinformatic analysis was used to investigate target proteins of extracellular vesicle and apoptosis, followed by mRNA and protein expression by RT–PCR and western blotting, respectively. Our findings indicate that menthol inhibits leukemic cell proliferation and increases extracellular vesicles. Furthermore, menthol treated leukemic extracellular vesicles induce apoptosis and upregulate the expression of ATG3 and caspase-3 in both mRNA and protein levels. These results suggest that menthol has an antileukemic effect through ATG3 and caspase-3 in apoptosis of leukemic extracellular vesicles.

1. Introduction

Leukemia is a blood -forming cancer characterized by the impairment of hematopoietic stem cells, leading to uncontrolled abnormal blood cell production. In 2022, leukemia ranks among the 10 most common cancer types [1]. Leukemia can be classified into four main types, including acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphoid leukemia (CLL). Acute leukemia is rapid onset and poor progression. Moreover, the relapse of the disease often happens in some cases from chemotherapy induction [2]. At present, there are several cancer treatments such as chemotherapy, radiotherapy, immunotherapy, targeted therapy, and bone marrow transplantation. However, chemotherapy is the most commonly used treatment for leukemia and also leads to adverse effects, including vomiting, diarrhea, anemia, bleeding, and others. Therefore, alternative treatments have been developed to reduce side effects due to drug toxicity and the nonspecificity of chemotherapy [3–5]. Alternative treatments using natural products are commonly used for drug development in cancer therapy. Natural compounds have

Abbreviations: EV, Extracellular vesicle; Con-EV, Extracellular vesicle derived from untreated leukemic cell; Men-EV, Extracellular vesicle derived from menthol-treated leukemic cell; ATG3, Autophagy related 3.

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shown multiple biological activities, including antibacterial, antifungal, anti-inflammatory, and anticancer properties. Natural bioactive compounds can act as anticancer agents affecting various types of cancer cells [6]. Moreover, extending the understanding of signaling pathways and mechanisms involved in natural compound -induced cancer cell death is still important for developing therapeutic drugs [7].

Menthol is the most bioactive compound found in peppermint leaves. It has pharmacological and biological activities such as antibacterial, antiviral, antifungal, antioxidant, and anticancer activities. Moreover, menthol exerts several protective effects, including chemoprotective, radioprotective, and neuroprotective effects [8]. For example, menthol has been shown to induce bladder cancer (T24) cell death through mitochondrial membrane depolarization [9]. Menthol also exerts anticancer effects through inhibition of cell growth, cell cycle and cell migration of prostate cancer (DU145) cells [10]. Furthermore, menthol induces G2/M phase arrest in epidermoid carcinoma (A431) cells, leading to cancer cell death [11]. In previous study, menthol showed an effect on apoptosis induction via the caspase cascade and p53/MDM2 signaling pathway, and autophagy enhancement via the ATG3/mTOR signaling pathway [12]. However, the mechanism underlying the release of leukemic extracellular vesicles remains unknown.

Extracellular vesicles (EVs), small lipid bilayer packages, have been studied in cell–cell communication nowadays [13]. EVs consist of several signaling molecules, such as protein, RNA, and DNA, that are necessary for diverse biological activities. It has shown that leukemic EVs are essential to support the microenvironment and neighboring leukemic cells, resulting in cell survival, metastasis, and cell growth [14]. Almost chemotherapy drugs are associated with the induction of EV release, which increases risks for chemotherapy-resistant activity, such as modulating the immune response and sending survival signals [15]. On the other hand, several natural compounds impact signaling molecules in EVs, thereby slowing cancer progression and preventing cancer cell survival [14]. For example, EVs from cancer cells treated with curcumin, a natural compound from turmeric, reduces the severity of cancer cells, such as drug resistance and metastasis [16,17]. However, there is a lack of evidence on the effect of menthol on leukemic EV production and its mechanism related to apoptosis induction. Therefore, this study aims to investigate the effect of menthol on leukemic EV production and elucidate the mechanisms by which EVs derived from menthol-treated leukemic cells are involved in apoptosis.

2. Materials and methods

2.1. Leukemic cell culture

The NB4 cell line, human acute promyelocyte, and Molt-4 cell line, T-lymphocyte were received from Cell Line Service GmbH (Eppelheim, Germany). RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin as an antibiotic (Gibco Life Technologies, Waltham, MA, USA) was used to culture leukemic cells in a humidified incubator at 37 °C with 5 % CO_2. The medium was replaced every 2–3 days.

2.2. Cell proliferation inhibition assay by MTT

NB4 and Molt-4 leukemic cells were seeded into 96-well plates, treated with menthol at various concentrations (0, 100, 200, 300 μg/mL) and incubated for 24 and 48 h at 37 °C with 5 % CO_2. PBMCs were isolated and seeded following a previous protocol (Ethics approval no. MU-CIRB 2022/116.0411) [6]. After that, 10 μl of MTT solution (5 mg/mL) was added and incubated for 4 h at 37 °C before adding 100 μl of 10 % SDS in 0.01 M HCl. The formazan crystal was solubilized, and its absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Then, the half maximal inhibitory concentration (IC50) was calculated and used in further experiments. Moreover, inhibition of PBMCs after Menthol treatment was also examined to analyze the possible effect of menthol on normal blood cells [12]. Furthermore, NB4 and Molt-4 leukemic cells were treated with 0.2 μg/ml daunorubicin and combination of daunorubicin and IC_{50} menthol. Afterward, cell inhibition of these conditions was determined using MTT.

2.3. Isolation of leukemic extracellular vesicles

Leukemic cells (3 x 10^6 cells) were treated with 250 μg/ml (−) Menthol (Sigma–Aldrich, Schnelldorf, Germany, Cat No. 63660) for 48 h as a Menthol-treated EV (Men-EV). The concentration of menthol was the half-maximal inhibitory concentration (IC50) according to MTT experiment. Leukemic cells were treated with 1 % DMSO as a control group (Con-EV). The conditioned media were harvested in total at least 30 ml. and stored at −20 °C until isolation. First, the conditioned media were centrifuged at 300×*g* for 5 min at 4 °C to remove cells. Second, the supernatant was centrifuged at 1500×*g* for 15 min at 4 °C to remove cell debris as much as possible. Third, the supernatant was centrifuged at 17,000×*g* for 30 min at 4 °C to obtain the pellet of EVs. Then, this pellet was washed with PBS and centrifuged at 17,000×*g* for 30 min at 4 °C. The EV pellet was resuspended in 50 μl of PBS and stored at −80 °C until further experiments. EV protein was determined by Bradford assay (Bio-Rad laboratory, USA).

2.4. Quantification of extracellular vesicles

Standard fluorescent polystyrene particles with sizes of 0.22 μm and 1.35 μm were used to create gates in the scatter plot. Briefly, isolated EVs were stained with FITC-conjugated Annexin V (BioLegend, USA) and then incubated for 15 min at room temperature. Finally, BD Trucount™ beads (BD Biosciences, San Diego, CA, USA) in 1x Annexin V binding buffer (BD Biosciences, San Diego, CA,

USA) were added to the EV sample, followed by flow cytometry analysis using a FACSCantoII flow cytometer (BD bioscience, Palo Alto, CA, USA). The EV population in P3 was restricted to no more than 10,000 cells. The absolute count of Annexin V^+ EVs was manually calculated using the following formula:

$$\text{Annexin V}^+\text{EV number} = (\text{Number of events in containing population(P4)} / \text{Number of events in absolute count bead region(P5)}) * (\text{Total number of Trucount bead per test} / \text{Test volume}) * \text{dilution factor}$$

2.5. Prediction of target proteins in extracellular vesicles (EVs) involved in apoptosis by bioinformatic tools

The target proteins were obtained from the computational tool STITCH database. The keyword for searching was "Menthol", and the species target was *Homo sapiens*. The minimum required interaction score was medium confidence (0.400). The maximum number of interactors to show was no more than 40 interactors. The published proteomic data were obtained from a previous study [18]. Two sources of data were combined by Draw Venn Diagram. The intercept proteins between menthol responsive proteins and proteins in leukemic EVs were obtained and compared by score from the STITCH database. Finally, the highest score proteins involved in cell death mechanisms were selected as candidate proteins.

Based on previous predictions, the network interaction between target proteins in EVs and apoptosis proteins was constructed by the STITCH database. Briefly, target proteins in EVs and representative proteins in apoptosis, including caspase3 (CASP3), caspase8 (CASP8), and caspase9 (CASP9), were input. The minimum required interaction score was medium confidence (0.400), and the maximum number of interactors to show in the 1st shell and 2nd shell was 15 and 0, respectively.

2.6. Apoptosis analysis with FITC-conjugated Annexin V/PI staining by flow cytometry

Leukemic cells were treated with 0.2 μg/ml daunorubicin, IC50 menthol and combination of daunorubicin and menthol for 48 h or 1 μg/ml Con-EVs or Men-EVs for 24 h. After that, leukemic cells were collected and centrifuged to wash the cells with PBS. Leukemic cells were stained with 2 μl FITC-conjugated Annexin V (Biolegend, USA) and 2 μl 50 μg/ml propidium iodide (Sigma–Aldrich, Schnelldorf, Germany) and then incubated for 15 min at room temperature in the dark. Finally, leukemic cells were analyzed by a FACSCantoII flow cytometer and FACSDiva software (BD bioscience, Palo Alto, CA, USA).

2.7. Determination of mRNA expression by quantitative real-time polymerase chain reaction (RT–qPCR)

Leukemic cells were treated with 1 μg/ml Con-EVs or Men-EVs for 24 h and then pellets of leukemic cells were harvested by centrifugation at 1500 rpm for 5 min. RNA was extracted using GENEzol™ reagent (New England Biolab, Inc., Ipswich, MA, USA) following the manufacturer's instructions. The concentration of RNA from extraction was measured by a Nanodrop2000 (Thermo Scientific, Waltham, MA, USA). One microgram of extracted RNA was converted to cDNA by using a RevertAid first strand cDNA synthesis kit (Thermo Scientific, Waltham, MA, USA). cDNA was mixed in Luna real-time PCR mastermix (New England Biolab, Inc., Ipswich, MA, USA) and designed primers. The primer sequences that were used in this study included;

ATG3 FP: 5′-CACGACTATGGTTGTTTGGCTATG-3′,
ATG3 RP: 5′-TTTAGTGGGAGTGGAAGGTGG-3′,
CASP3 FP: 5′-TTCAGAGGGGATCGTTGTAGAAGTC-3′,
CASP3 RP: 5′-CAAGCTTGTCGGCATACTGTTTCAG-3′,
GAPDH FP: 5′-GCACCGTCAAGGCTGAGAA-3′,
GAPDH RP: 5′-AGGTCCACCACTGACACGTTG-3′.

Then, a Bio-Rad CFX96 touch™ real-time PCR system (Bio-Rad, Hercules, CA, USA) was used to quantify the threshold cycle (CT) of the sample. The mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method using GAPDH as an internal control.

2.8. Determination of protein expression by Western blot

Western blotting was performed to characterize EVs and determine the expression of target proteins. NB4 and Molt-4 leukemic cells were treated with 1 μg/ml Con-EVs or Men-EVs for 24 h. The cells were harvested and washed with PBS. Both leukemic cells and EVs were extracted by RIPA lysis buffer (Merck Millipore, Burlington, MA, USA) containing 1 % protease inhibitors (Merck Millipore, Burlington, MA, USA). The cell and EV lysates were centrifuged at 14,000×*g* for 10 min, and the supernatants were collected. The concentration of proteins was measured by a Dual-Range BCA Protein Assay Kit (Visual Protein, Taipei, Taiwan). Protein lysates (10–20 μg) were loaded and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Bio-Rad, Inc., Hercules, CA, USA). Then, proteins on SDS gels were transferred to nitrocellulose membranes (Bio-Rad, Inc., Hercules, CA, USA). After that, the nitrocellulose membrane was blocked with 5 % skim milk in TBST or EveryBlot Blocking Buffer (Bio-Rad, Inc., Hercules, CA, USA) at room temperature and then incubated with primary antibodies against CD9, CD63 (Abcam Inc., Cambridge, UK), ATG3, caspase-3 and β-actin (Cell signaling, Danvers, MA, USA). Next, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, including HRP-linked anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA, USA) and HRP-linked anti-rabbit antibody (Merck Millipore, Burlington, MA, USA). Finally, enhanced chemiluminescence (ECL) substrate (Merck Millipore, Burlington, MA, USA and Bio-Rad, Inc., Hercules, CA, USA) was added to the membrane, and the signal was detected by the ChemiDoc™ MP imaging system (Bio-Rad, Inc., Hercules, CA, USA). The band intensity of each target protein was quantified

using Image Lab™ software (Bio-Rad, Inc., Hercules, CA, USA) and normalized to β-Actin.

2.9. Statistical analysis

All experiments were performed in triplicate. All data are represented as the mean ± standard error of the mean (S.E.M). The graphs were generated with GraphPad Prism version 8.0 (GraphPad Inc., San Diego, CA, USA). Statistical analyses were performed in GraphPad Prism version 8.0. Student's t-test was used to compare two groups. A p-value <0.05 was considered statistically significant.

3. Results

1. Menthol inhibits NB4 and Molt-4 leukemic cell proliferation.

The inhibition of NB4 and Molt-4 leukemic cells was assessed by using MTT assay. Menthol significantly increased cell inhibition in both NB4 and Molt-4 leukemic cells at 24 and 48 h as shown in Fig. 1A and B. The IC_{50} concentrations of menthol were 296.7 and 250.9 μg/mL in NB4 cells at 24 and 48 h, respectively. For Molt-4 leukemic cells, IC_{50} concentrations were 283.8 and 257.6 μg/mL after 24 and 48 h of treatment, respectively. The lower concentration at 48 h was selected for further experiments. Moreover, less cytotoxicity was found in healthy PBMCs as shown in Fig. 1C. In addition, daunorubicin illustrated a significant increase in cell inhibition in both NB4 and Molt-4 leukemic cells compared to untreated NB4 and Molt-4 leukemic cells. The average cell inhibition of NB4 and Molt-4 leukemic cells treated with 0.2 μg/ml daunorubicin was 52.7 % and 57.1 %, respectively. The cell inhibitions of NB4 and Molt-4 leukemic cells treated with combination of menthol and daunorubicin were significantly increased compared to menthol alone or daunorubicin alone as shown in Fig. 1D. Furthermore, the percentage of apoptosis in NB4 and Molt-4 leukemic cells treated with combination of menthol and daunorubicin was significantly increased compared to menthol alone or daunorubicin alone, respectively as shown in Fig. 1E.

2. Characterization of menthol-treated leukemic extracellular vesicle.

Untreated-EV (Con-EV) and Menthol-EV (Men-EV) were characterized by Western blot with CD9 and CD63 markers which are common transmembrane markers for EV [19]. CD9 and CD63 of Con-EV and Men-EV were shown in Fig. 2A. CD9 of Men-EV was qualitatively decreased compared to CD9 of Con-EV. According to Fig. 2B, Scatter plot of Con-EV and Men-EV of NB4 and Molt-4

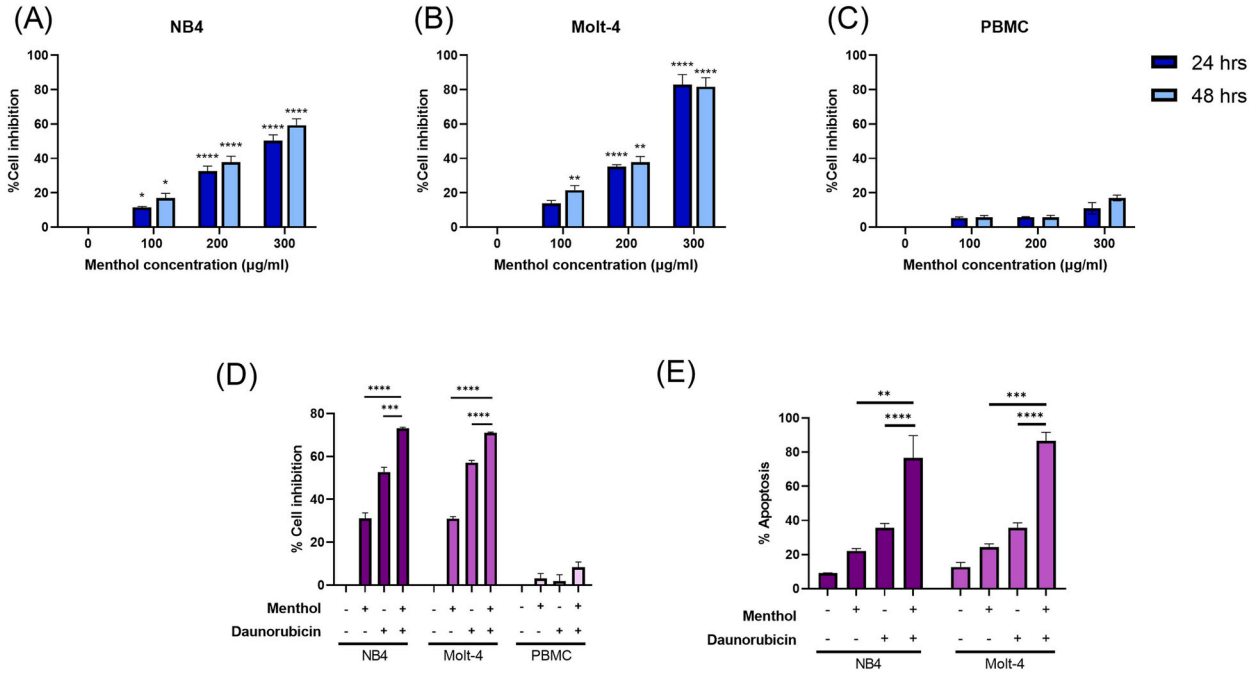

Fig. 1. Menthol inhibited NB4 and Molt-4 proliferation. (A) NB4 and (B) Molt-4 cells were examined for cell inhibition that showed a significant increase in both 24 and 48 h using MTT assay. While (C) PBMC was not inhibited by menthol. (D) The cell inhibition of NB4 and Molt-4 after treatment with menthol, daunorubicin and combination of daunorubicin and menthol was significantly increased while less effect on PBMC. (E) The percentage of apoptosis after treatment with combination of daunorubicin and menthol was significantly increased compared to menthol alone or daunorubicin alone, respectively. Error bar was defined as S.E.M, n = 3. Statistical analysis was performed by Student's t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ were considered statistically significant difference compared to control.

illustrated the population of EV size 0.22–1.35 μm. In addition, the population of Annexin V positive EVs (Annexin V^+ EVs) were detected in both Con-EV and Men-EV derived from NB4 and Molt-4. Annexin V^+ EVs were calculated by using the above formula. The mean total numbers of Annexin V^+ EVs from control-NB4 and Molt-4 were 13,472 ± 876 particles/μl and 18,846 ± 2160 particles/μl, respectively while, from Menthol-treated NB4 and Molt-4 were 35,644 ± 7245 particles/μl and 35,108 ± 2748 particles/μl, respectively. The result showed that menthol significantly increased the total number of Annexin V^+ EVs release compared to EVs release of untreated leukemic cells or control (Fig. 2C). Both Con-EV and Men-EV were defined as large Annexin V^+ EVs whose size ranges between 0.22–1.35 μm.

3. Candidate proteins in leukemic extracellular vesicle involved in apoptosis pathway

According to menthol responsive target proteins from STITCH, the database generated 36 proteins that have an interaction score of more than 0.400 as shown in supplementary data 5. In the case of proteomic profile, there are 2014 proteins that Molt-4 leukemic EV illustrated in supplementary data 6. Proteins from menthol responsive target proteins and proteins in leukemic EV were combined as shown in Venn diagram in Fig. 3B. The target proteins in EV of menthol from combination contained Topoisomerase II alpha (TOP2A), Topoisomerase II beta (TOP2B), Topoisomerase I alpha (TOP1A), caspase-3 (CASP3), and Autophagy related 3 (ATG3). A network of proteins in EV and representative apoptosis proteins including caspase-8 (CASP8) and caspase-9 (CASP9) were constructedas shown in Fig. 3B. Therefore, our study chose ATG3 and CASP3 as representative proteins in this apoptosis network.

4. Men-EV promotes NB4 and Molt-4 leukemic cell apoptosis.

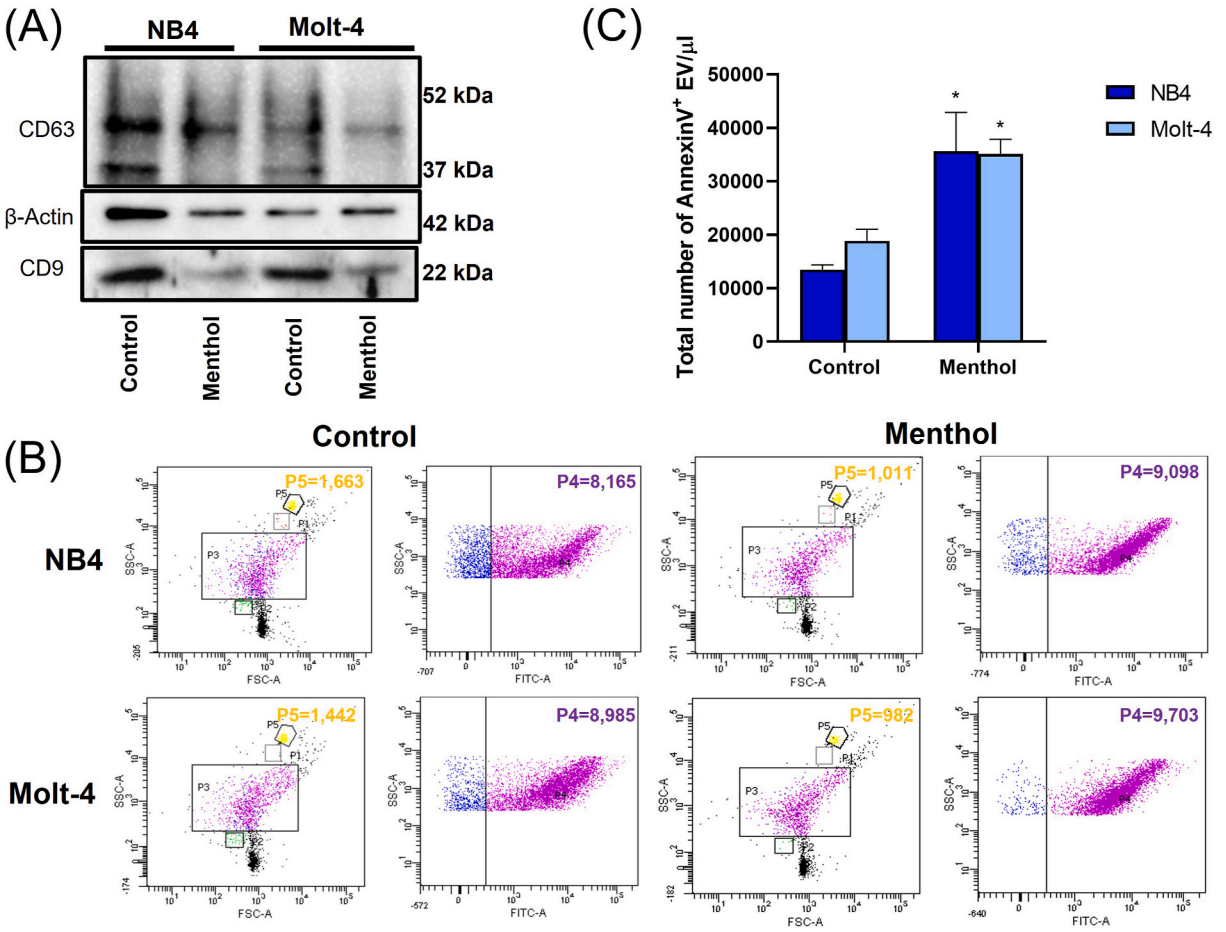

Fig. 2. Characterization of Con-EV and Men-EV derived from leukemic cells, and total number of Annexin V+ leukemic extracellular vesicle was increased in Menthol-treated leukemic cells compared to control. (A) Western blot of CD63, β-actin, and CD9 of leukemic extracellular vesicle. (B) Scatter plot of Annexin V^+ EV population size 0.22 μm–1.35 μm. P3 is EV population size 0.22 μm–1.35 μm; P4 is Annexin V^+ EV population from P3; P5 is Trucount beads population. (C) Total number of Annexin V^+ EV released from leukemic cells after treated with Menthol. Error bar was defined as S.E.M, N = 3. Statistical analysis was performed by Student's t-test. *$p < 0.05$ was considered statistically significant compared to control.

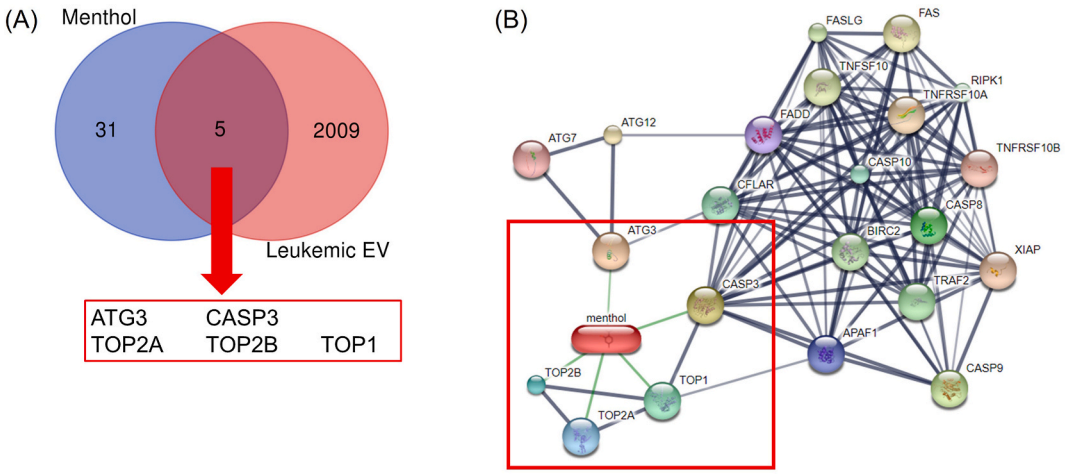

Fig. 3. ATG3 and caspase-3 are candidate proteins predicted from bioinformatic tools. (A) A construction of Venn Diagram between Menthol responsive proteins and proteins in leukemic EV originated from public data. (B) A network of interaction between proteins in leukemic EV and representative proteins in apoptosis signaling pathway. Red box is targeting proteins in EV. Topoisomerase II alpha; TOP2A, Topoisomerase II beta; TOP2B, Topoisomerase I alpha; TOP1A, caspase-3; CASP3, and Autophagy related 3; ATG3, Caspase-8; CASP8, Caspase-9; CASP9.

Leukemic cell lines, NB4 and Molt-4, were treated with 1 μg/ml Con-EV and Men-EV for 24 h. After that, an apoptosis of NB4 and Molt-4 which were exposed to Con-EV and Men-EV was investigated by flow cytometry. The scatter plot of apoptosis was shown in Fig. 4A. In NB4, the percentage of total apoptosis of Con-EV and Men-EV were 8.00 ± 2.45, and 21.33 ± 4.04, respectively. While in Molt-4, the percentage of total apoptosis of Con-EV and Men-EV were 7.10 ± 1.16, and 21.80 ± 4.12, respectively. The percentage of apoptosis were increased in Men-EV compared to Con-EV in both NB4 and Molt-4 leukemic cells (Fig. 4B). Therefore, EV from Men-EV derived from leukemic cells (NB4 and Molt-4) increased the percentage of apoptosis in NB4 and Molt-4 leukemic cells.

5. Men-EV modulates ATG3 and Casp3 mRNA expression levels in NB4 and Molt-4 leukemic cells.

ATG3 and Casp3 from previous result of bioinformatic were selected to investigate ATG3 and Casp3 mRNA expression after exposed with Con-EV and Men-EV. Leukemic cells were treated with 1 μg/ml Con-EV and Men-EV derived from leukemic extracellular vesicle for 24 h. Then, cells were collected, and were extracted RNA for RT-qPCR. The results (Fig. 5) have shown that both ATG3 and Casp3 mRNA expression of NB4 and Molt-4 were significantly upregulated by Men-EV compared to Con-EV. The average relative ATG3 mRNA expressions after inducing with Men-EV were 3.27 ± 0.50 and 1.51 ± 0.03 in NB4 and Molt-4, respectively while average relative Casp3 mRNA expression, were 1.44 ± 0.49 and 1.60 ± 0.15 in NB4 and Molt-4 leukemic cells, respectively. These results indicated that Men-EV might modulate ATG3 and Casp3 genes.

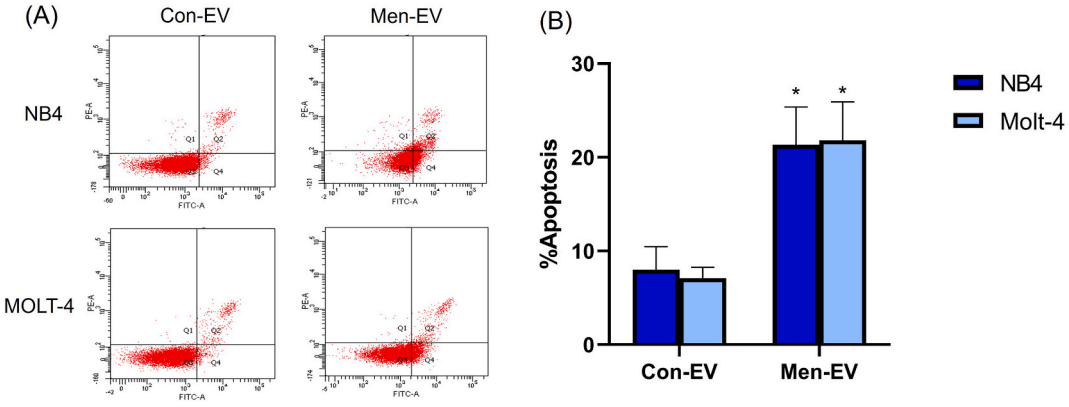

Fig. 4. Apoptosis of NB4 and Molt-4 was exposed by Men-EV. NB4 and Molt-4 leukemic cells were treated with 1 μg/ml Con-EV and Men-EV for 24 h and apoptosis was measured by Annexin V^+/PI staining and detected by flow cytometer. (A) Scatter plot of population of cell undergoing apoptosis by flow cytometry analysis Q1: necrosis; Q2: late apoptosis; Q3: live cells; Q4: early apoptosis. (B) Percents of total apoptosis of leukemic cells after exposed with Con-EV and Men-EV. Error bar was defined as S.E.M, n = 3. Statistical analysis was performed by Student's t-test. Statistical analysis was performed by Student's t-test. *$p < 0.05$ was statistically significant compared to Con-EV.

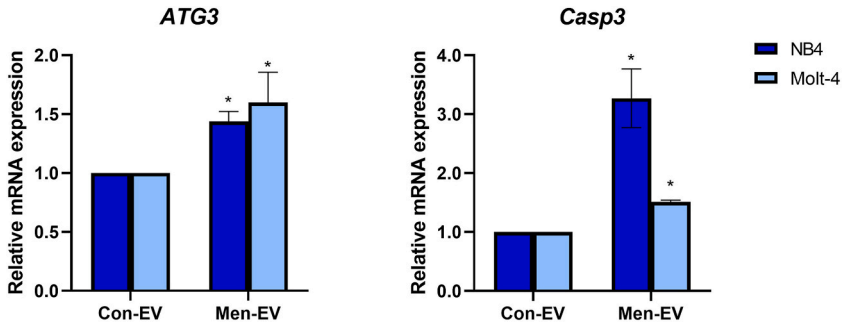

Fig. 5. ATG3 and Casp3 mRNA of NB4 and Molt-4 were upregulated after exposed with Men-EV. NB4 and Molt-4 leukemic cells were treated with 1 μg/ml Con-EV and Men-EV for 24 h and ATG3 and caspase-3 mRNA were determined by RT-qPCR and normalized by GAPDH. Relative mRNA expression of Men-EV of ATG3 and Casp-3 were compared to Con-EV. Error bar was defined as S.E.M, n = 3. Statistical analysis was performed by Student's *t*-test. *$p < 0.05$ was statistically significantly compared to Con-EV.

6. Men-EV regulates ATG3 and caspase-3 protein expression in NB4 and Molt-4 leukemic cells.

To analyze the protein expression of ATG3 and caspase-3 protein expression when exposed with Con-EV and Men-EV, leukemic cells, NB4 and Molt-4, were treated with 1 μg/ml Con-EV and Men-EV for 24 h and their proteins were harvested, ATG3 and caspase-3 protein expression were determined by Western blot analysis. The band protein intensities of NB4 and Molt-4 after exposed with Con-EV and Men-EV were shown in Fig. 6A. Following Fig. 6B, the results of Western blot have shown that both ATG3 and caspase-3 protein expressions were significantly increased in NB4 and Molt-4 leukemic cells after exposed with Men-EV compared to Con-EV. The average relative ATG3 protein expressions after inducing with Men-EV were 1.37 ± 0.11 and 1.25 ± 0.07 in NB4 and Molt-4, respectively while for caspase-3, were 1.17 ± 0.05 and 1.17 ± 0.06 in NB4 and Molt-4 leukemic cells, respectively. These findings demonstrated that Men-EV induced both ATG3 and caspase-3 proteins in NB4 and Molt-4 leukemic cells.

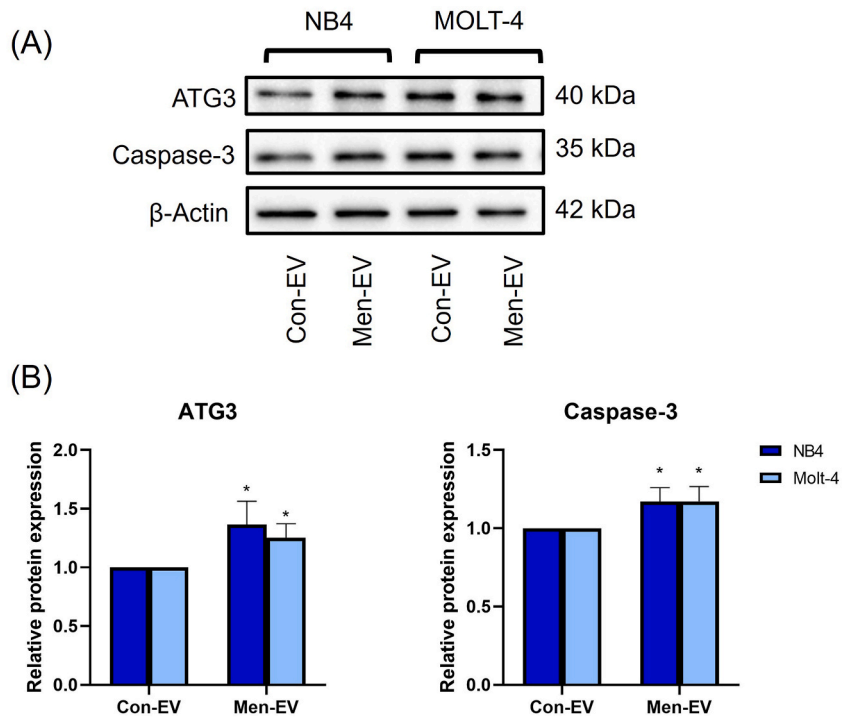

Fig. 6. ATG3 and caspase-3 proteins of NB4 and Molt-4 were increased by Men-EV. NB4 and Molt-4 leukemic cells were treated with 1 μg/ml Con-EV and Men-EV for 24 h and ATG3 and caspase-3 proteins were determined by Western blot and normalized by β-actin. (A) ATG3 and caspase-3 band intensities of Western blot. (B) Relative protein expression of ATG3 and caspase-3 compared to Con-EV. Error bar was defined as S.E.M, n = 3. Statistical analysis was performed by Student's t-test. *$p < 0.05$ was statistically significantly compared to Con-EV.

4. Discussion

Acute leukemia is a severe problem affecting many people worldwide. There are numerous treatment approaches against leukemia, such as chemotherapy, targeted therapy, or radiation, but disease readmission is still an issue. Natural compounds might reduce this problem. Our study emphasized the mechanism of extracellular vesicles (EVs) activity, which is a signal of proliferation, disease remission and drug resistance in cancer. More research is needed to investigate the effect of menthol on the release of leukemic EVs and to understand the regulatory mechanism of EVs derived from menthol-treated leukemic cells on ATG3 and caspase-3 in leukemic cells.

Natural products have become popular as alternative treatments for leukemia. Bioactive compounds from natural sources, such as bioactive peptides from ginger [20] and sesamin from sesame seeds [21], showed antileukemic properties with minimal effects on PBMCs. Menthol is the most abundant compound found in peppermint leaves and exerts various pharmacological effects, such as anticancer, anti-inflammatory, and antimicrobial effects [10,22,23]. Our previous work clearly demonstrated that menthol could induce both apoptosis and autophagy in NB4 and Molt-4 cells through the caspase cascade, p53/MDM2 and the ATG3/mTOR pathway [12]. In recent research, menthol also showed the inhibitory effect on other leukemic cells, such as WEHI-3 leukemia cell [24] and Du 145 prostate cancer cell line [25]. In this study, menthol inhibited NB4 and Molt-4 leukemic cell proliferation in a dose-dependent manner, while it had less effect on normal PBMCs. However, the regulatory mechanism of EVs derived from menthol-treated leukemic cells (Men-EVs) remains to be elucidated.

EV is a small package of biological molecules, such as miRNA, mRNA, and proteins. There are several roles of EVs derived from cancer in contributing to cancer cell survival, promoting proliferation, and inhibiting the cell death signaling pathway in other cells [13]. Laurenzana et al. investigated the effect of leukemia derived-EVs (LEVs) on healthy umbilical cord blood HSPCs about dysregulating normal hematopoiesis [26]. Moreover, LEVs isolated from patient blood samples have been investigated as biomarkers for diagnosing and monitoring leukemia, providing valuable insights into disease status and treatment response [27,28] A previous study demonstrated that Raji-EV containing miR-106a inhibited caspase-3 cleavage in apoptosis and LC3II in autophagy in lymphoma cells [29]. In apoptosis, compounds like menthol activated leukemic cells to release more EVs than inactivated cells [30]. In contrast, some compounds inhibited EV secretion [31,32]. These phenomena might be dependent on the nature of the cells and the kind of stimuli.

Our candidate proteins for analysis in this study were ATG3 and caspase-3 which directly interacted with menthol in leukemic EV. ATG3 is an important protein in autophagy signaling that eliminates cancer cells. ATG3, conjugated with ATG4 and ATG7, catalyzes the conjugation between ATG8 and phosphatidylethanolamine (PE) to form autophagosomes [33]. ATG3 is usually used to indicate autophagy cell death status in many cancers such as HER-2 breast cancer cell [34] and oral cancer cell [35]. Caspase-3 is an essential executioner caspase that activates apoptosis. Procaspase-3 is activated by upstream caspases such as caspase-8 from the extrinsic pathway and caspase-9 from the intrinsic pathway. In addition, caspase-3 also plays a role in regulating EV secretion by budding from cells and is loaded into EVs to regulate caspase levels [36]. Together with evidence that caspase-3 was upregulated by menthol in many cancer cells such as non-small cell lung carcinoma [37] and human colon adenocarcinoma Caco2 cells [38]. The interaction between ATG3 and caspase-3 is mediated by CASP8 and FADD-like apoptosis regulator (CFLAR), an antiapoptotic protein to inhibit the action of caspase-8, leading to the downregulation of caspase-3 [39]. ATG3 and caspase-3 are usually used to indicate apoptosis and autophagy cell death from several natural product treatments [40,41].

In this study, we investigated the expression of ATG3 and caspase-3 mRNA and protein expression in leukemic cells exposed by Men-EV. Our findings reveal Men-EV, can induce apoptotic cell death in leukemic cells. In addition, Men-EVs can increase ATG3 and caspase-3 mRNA and protein levels in leukemic cells. Certain stimuli activate a unique release of EVs with consistent contents, such as hypoxia, chemotherapy, and some kinds of compounds such as curcumin [17,42,43]. From a previous report, apoptosis induction of leukemic cells was induced by mesenchymal stem cell (MSC)-derived EVs [44]. There was a report on K562 leukemic cells treated with curcumin released more miR-21-enriched exosomes, which inhibited angiogenesis in HUVECs by targeting RhoB. The invasive activity of HUVECs was reduced by exosomes from K562 cells treated with curcumin [17]. Similarly, EVs derived from colorectal cancer cells with Jianpi Jiedu Recipe treatment, a traditional Chinese medicine, also reduced metastasis in colorectal cancer cells by decreasing ITGBL1 levels in EVs. This EV derived from colorectal cancer cells with Jianpi Jiedu Recipe treatment will further reduce metastasis via TNFAIP3-NF-κB signaling [45]. The effect of EV release from cancer cells transfers biological molecules to distant cells in the microenvironment, thereby increasing the survival and proliferation of cancer cells. From evidence, natural products affect the characterization of EVs, which reduce progressiveness of cancer cells, such as metastasis and invasion of cancer. Our study contributes to the understanding of how EVs derived from menthol-treated leukemic cells can influence the expression of ATG3 and caspase-3, leading to the induction of apoptosis in acute leukemia.

The primary limitation is that the conclusions are based on the findings within two cell lines. How these findings extrapolate to the heterogeneity of AML is unknown and warrants mentioning. Several types of EVs could be investigated in the role of apoptosis and other cell death pathways. In addition, EVs from leukemia patients would be further developed as potential biomarkers.

5. Conclusion

In the present study, menthol inhibited leukemic cell proliferation and regulated apoptosis through leukemic extracellular vesicles. We successfully isolated Men-EVs to investigate their role in apoptosis. Our results suggest that menthol inhibits NB4 and Molt-4 leukemic cell proliferation. Additionally, menthol induces leukemic extracellular vesicle production, which can upregulate ATG3 and caspase-3 mRNA and protein expression. From these results, menthol could be developed as a candidate antileukemic drug in the future. In perspective, new information of EVs could be developed for the diagnosis and treatment of leukemia.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Kantorn Charoensedtasin: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mashima Naksawat:** Writing – original draft, Investigation, Formal analysis, Data curation. **Chosita Norkaew:** Investigation, Formal analysis, Data curation. **Wasinee Kheansaard:** Supervision, Methodology. **Sittiruk Roytrakul:** Supervision, Data curation. **Dalina Tanyong:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33081.

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