ORIGINAL PAPER

Anticancer effects of *Artemisia campestris* extract on acute myeloid leukemia cells: an ex vivo study

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Abstract

Cure rates for acute myeloid leukemia (AML) remain suboptimal; thus, new treatment strategies are needed for this deadly disease. *Artemisia campestris* leaves hold significant value in traditional medicine. Despite extensive research conducted on this plant globally, the specific anti-AML properties of the leaves have received limited investigation. This study aims to explore the potential anti-leukemic activities of the ethyl acetate extract derived from *Artemisia campestris* (EAEAC), using mononuclear cells from bone marrow of thirteen AML patients. To this end, cytotoxic effects were evaluated using the MTT assay, and the mechanisms of cell death were investigated through various methods, including propidium iodide staining, annexin V/propidium iodide double staining, mitochondrial depolarization, and caspase-3/7 activation assays. Results demonstrated that EAEAC induced cell apoptosis by increasing DNA fragmentation, causing mitochondrial depolarization, and activating caspases 3/7. On the other hand, we assessed EAEAC's effect on two leukemia stem cell subpopulations, with results suggesting a potential decrease in their frequencies (three/five patients).

Introduction

Acute myeloid leukemia (AML) represents a malignant hematopoietic neoplasm characterized by the halting of myeloid differentiation, rapid proliferation, and the suppression of apoptosis in leukemic blasts originating from the hematopoietic stem/progenitor cells found in the bone marrow [1]. It is the most prevalent form of acute leukemia in adults and is associated with a low probability of survival [2]. For younger and fitter patients, the standard treatment for AML involves intensive cytotoxic therapy using a combination of anthracycline and cytarabine (AraC). Despite the efficacy of current therapies, resistance to treatment and relapse remain significant challenges. Moreover, research has indicated that leukemia stem cells (LSC) in AML support the proliferation of leukemia cells and offer defense against chemotherapy drugs [3–6]. Initial reports suggested

that AML LSC shared a common limited immunophenotype (CD34+CD38−) and in being rare populations [7, 8]. In a study of 200 AML patients at diagnosis, researchers analyzed CD34 and CD38 expression in bone marrow samples, with a focus on the CD34+CD38− population. They discovered that a high level (> 1%) of CD34+CD38− blasts was associated with advanced age, adverse cytogenetics, lower rates of complete response post-induction, and shorter disease-free survival. Multivariate analysis further identified a percentage of CD34+CD38− leukemic cells greater than 1% as an independent predictor of disease-free survival and overall survival [9].

Previous study has shown that there are both populations coexisting in most patients with LSC potential [10]. The more mature LSC population, characterized by Lin-CD34+CD38+CD123+/loCD110-CD45RA+, most closely mirrors normal granulocyte-macrophage progenitors (GMP), while the immature LSC population exhibits a previously uncharacterized progenitor functionally similar to lymphoid-primed multipotential progenitors (LMPP) and are characterized by Lin-CD34+CD38−CD90−CD45RA+ [10]. These distinguishing features of LSC phenotype have facilitated the characterization of minimal residual disease

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and helps target these populations in the context of research for new treatments against AML [11, 12].

Furthermore, as defects in apoptosis are important mechanisms of tumor development and drug resistance [13], the induction of this pathway was identified as a target for treatment of many cancer, especially with relapse [14]. The apoptotic cell death mechanism is divided into two major pathways: intrinsic and extrinsic. Extrinsic pathway is death receptor-mediated apoptosis which activates the FAS-associated death domain and forms the death-inducing signaling complex, which processes downstream caspases including caspase-8,-7,-6, and -3 [15]. Concerning the intrinsic pathway, it is mitochondria-mediated apoptosis which is mediated by cytochrome C release and activation of caspase-9, stimulating effector caspases, caspase-3.

Plants are a source of molecules that can induce apoptosis in the context of cancers, including AML, such as phenols, flavonoids, and terpenes [16]. Indeed, several plants have shown anticancer effects, notably in hematological cancers, particularly *Artemisia campestris* L (AC) [17, 18]. This polymorphic species with various subspecies and varieties, part of the Asteraceae family, enjoys global distribution which is found in North Africa, especially in the middle and southern regions of Tunisia [19–21]. *Artemisia campestris* (AC), especially leaves, are widely used in traditional medicine for their digestive, analgesic, and antihypertensive properties [19]. Numerous studies have highlighted the antivenin, anti-inflammatory, antirheumatic, antimicrobial, and digestive system benefits of AC species [22, 23].

Recently, the leaves of this plant have garnered significantly interest for their anticancer proprieties [19]. For instance, Akrout and colleagues demonstrated the cytotoxic activity of AC against a colon cancer cell line, HT-29 [24]. Another study showed the effect of AC on breast cancer cell line MCF-7 and ovarian cancer cell line OVCAR [25]. Recent research conducted by our team illuminated the anticancer potential of the ethyl acetate extract from *Artemisia campestris* (EAEAC) against multiple myeloma cells. Interestingly, EAEAC demonstrates significant potentialities against human multiple myeloma cells U266 with IC_{50} values of 62.12 ± 1.09 μg/mL and 43.39 ± 1.27 μg/mL after 24 and 48 h of incubation, respectively [26].

On the other hand, only a limited number of studies have explored the effects of the Artemisia genus on AML [27]. Furtheremore, no specific studies have been conducted on the effects of AC on AML. Building upon our previous findings related to multiple myeloma, our aim is to study the impact of EAEAC on a primary culture of bone marrow mononuclear cells (BM-MNC). To this end, cytotoxic effects were evaluated using the MTT assay, and the mechanisms of cell death were investigated through various methods, including Annexin V (AV)/propidium iodide (PI) double staining, propidium iodide staining, mitochondrial depolarization, and caspase-3/7 activation assays. LSC frequency was evaluated by flow cytometry using a panel of five antibodies (CD45, CD123, CD34, CD38, and CD90).

Materials and methods

Chemicals

Chemicals as dimethyl sulfoxide (DMSO), 3,3′-dihexyloxacarbocyanine iodide DiOC6-(3), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Ficoll were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytarabine (AraC) was purchased from Pfizer (France). FxCycle PI/RNase-staining solution was purchased from ThermoFisher Scientific. The AV/PI Cell Apoptosis Kit was purchased from Invitrogen. CellEvent caspase-3/7 green flow cytometry assay kit was purchased from Eugene. Cell culture reagents, including RPMI 1640, fetal bovine serum (FBS), 1% penicillin-streptomycin, ʟ-glutamine, and Phosphate buffered-saline (PBS) were ordered from PAN-Biotech.

Sample collection

Leaves of AC were collected in July 2020 from the Foussana area in west-central Tunisia (Kasserine Governorate) and were identified by using the Tunisia flora as previously described [26]. Briefly, the leaves were air dried, crushed, and stored plastic bags at − 18 °C [26].

Extracts preparation

The EAEAC was prepared following a previously documented procedure [26]. To obtain the ethyl acetate extract, 25 g of finely ground leaf material was macerated in 200 mL of hexane for 24 h. After that, the obtained supernatant was, filtered, collected, and then dried at 40°. The dry residue was carefully collected, their and stored at 4 °C. The dry residue from ethyl acetate was dissolved in methanol to obtain a stcok solution of 25 mg/mL.

Clinical characteristics of patients

Thirteen bone marrow aspirates (designated as AML#1-AML#13) were obtained from untreated or relapsed AML patients, with mean age 47.6 ± 15, after informed consent from Aziza Othmena Hospital. The diagnosis of AML was performed according to World Health Organization classification. Demographic, clinical, and laboratory features of patients are shown in Table 1. This study was approved by Pasteur Institute Ethic Committee (2021/09/E).

Table 1 Biological and clinical characteristics of the 13 AML patients included in the study

Patient	Sex	Age (year)	Stage	Karyotype	Blasts (%)	Cytogenetic risk	Complete remission post-induction
AML#1	F	36	Relapse	Complex	79	High	No
AML#2	F	39	Relapse	Trisomy 8	39	Intermediate	No
AML#3	F	35	Relapse	Monosomy	60	High	No
AML#4	F	57	Diagnostic	Normal	76	Intermediate	Yes
AML#5	F	25	Diagnostic	Normal	25	Intermediate	Yes
AML#6	F	65	Diagnostic	T (8,21)	68	Low	N/A
AML#7	M	39	Diagnostic	Complex	100	High	No
AML#8	M	38	Diagnostic	Inversion 3	69	High	No
AML#9	M	29	Diagnostic	Normal	77	Intermediate	No
AML#10	F	72	Diagnostic	Deletion 5q	72	High	No
AML#11	M	65	Diagnostic	N/A	65	N/A	No
AML#12	M	65	Diagnostic	Deletion 6q	65	Intermediate	N/A
AML#13	F	54	Diagnostic	T (16,16)	49	Low	Yes

N/A Not available

Mononuclear cell isolation and cell culture

Bone marrow samples were freshly aspirated from patients. Approximately two to four milliliters of BM samples were diluted in PBS, followed by Ficoll gradient separation. The layer containing mononuclear cells was carefully collected and washed twice in PBS. BM-MNC were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin at 37 °C and 5% CO_2.

MTT assay

The MTT assay was employed as previously provided to evaluate cell viability after exposure to EAEAC. Concisely, BM-MNC of seven patients (AML#1, AML#2, AML#3, AML#4, AML#11, AML#12, and AML#13) were initially seeded in 96-well culture plates (2×10^5 cells/mL) and incubated for 24 h. Subsequently, the cells were treated or not with concentrations ranging from 0.5 to 500 µg/mL, for 48 h. The corresponding dilution of methanol served as the vehicle control. Triplicate cultures were set up for each condition. Cell viability was assessed by measuring absorbance at 490 nm using a microplate reader (EXL800, BioTek, Winooski, VT, USA).

Annexin V/PI-apoptosis detection assay

To evaluate apoptosis, Annexin V/PI double staining was employed according Limam's work [26]. BM-MNC of three patients (AML#5, LAM#6, and AML#7) were seeded in 24-well plates in triplicate, preincubated for 24 h, and subsequently treated with EAEAC (50 and 100 µg/mL) or

AraC (100 µM). Flow cytometry analysis of 10^4 events per sample was conducted and analyzed utilizing the BD FACSDiva 7 software. The evaluation encompassed four distinct cell subpopulations: viable cells (AV−/PI−), early apoptotic cells (AV+/PI−), late apoptotic and/or secondary necrotic cells (AV+/PI+), and necrotic/damaged cells (AV−/PI+).

Cell cycle analysis

To analyze cell cycle distribution, the BM-MNC of three patients (AML#4, AML#5, and AML#6) was stained with FxCycle PI/RNase-staining solution and examined using flow cytometry. After a 24 h culture period, BM-MNC (2×10^5 cells/mL) were incubated with 50 or 100 µg/mL of EAEAC, or 100 µM of AraC, for an additional 24 h. Subsequently, the cells were fixed in 70% ethanol at − 20 °C for 1 h and stained with 500 µL of FxCycle PI/RNase-staining solution for 30 min. A minimum of 10^4 events were gated for each experiment using a BD FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data analysis was performed using BD FACSDiva 7 software (BD Biosciences, Franklin Lakes, NJ, USA).

Measurement of transmembrane mitochondrial potential (Δψ_m)

The assessment of mitochondrial depolarization was conducted by measuring the decrease in green fluorescence using the BD FACSCanto II flow cytometer (BD Biosciences). The lipophilic fluorescent dye 3,3′-dihexyloxacarbocyanine iodide DiOC6-(3) was employed to examine mitochondrial membrane potential (ΔΨm) alterations. In summary, the BM-MNC of three patients (AML#6, AML#7,

and AML#11) were seeded and incubated for 24 h. Subsequently, cells were treated with two different concentrations of EAEAC (50 or 100 µg/mL) or AraC (100 µM). After 24 h of treatment, cells were incubated with 40 nM DiOC6-(3) for 15 min at 37 °C. Data analysis was performed as described previously.

Caspase-3/7 activity assay

The caspase 3/7 activation was evaluated by measuring cleavage of the Ac-DEVD substrate using the CellEvent caspase-3/7 green flow cytometry assay kit. In short, the BM-MNC of three patients (AML#6, AML#7, and AML#11) were treated with 50 or 100 µg/mL of EAEAC or with 100 µM AraC as positive control for 24 h. Afterwards, cells were processed according to the manufacturer instructions, and samples were analyzed as mentioned before [28].

Leukemia stem cells' frequency analysis

The influence of EAEAC and AraC on LSC frequency was performed with fluorochrome conjugated antibodies. Using a panel of monoclonal antibodies, namely CD45, CD123, CD34, CD38, and CD90, these monoclonal antibodies were composed of markers conjugated to distinct fluorochromes: CD45 (V500, clone J.33), CD123 (PE, clone 9F5), CD34 (PercP, clone 581), CD38 (APCH7, clone LS198-4-3), and CD90 (BV421, clone 5E10). All monoclonal antibodies were from Becton Dickinson (BD Biosciences, San Jose, CA). BM-MNC of five patients (AML#5, AML#6, AML#8, AML#9, and AML#10) were seeded in 24-well plates at a density of 2×10^5 cells/mL, preincubated for 24 h, and subsequently treated with EAEAC (50 and 100 µg/mL) or AraC (100 µM) during 48 h. BM-MNC were incubated. After staining, samples suspended in 500 µl of cellWash. The sample acquisition was performed using the BD FACSLyric flow cytometer (Becton Dickinson, San Jose, CA, USA). The CellQuest™ program was used for data acquisition and for

analysis. The instrument fluidic was accurately washed prior to sample acquisition to minimize the risk of carry-over and/or false-positive events. The flow-cytometric run was continued to achieve maximum cellular acquisition.

Statistical analysis

All the ex vivo experiments were performed at last with three samples in triplicate. Representative results are shown as means ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA, USA). Two group comparisons were performed with a two-tailed Student's t-test whereas multiple group comparisons were performed with a one-way ANOVA test with multiple comparison option. The differences between two experimental groups were statistically significant when the obtained p value was < 0.05.
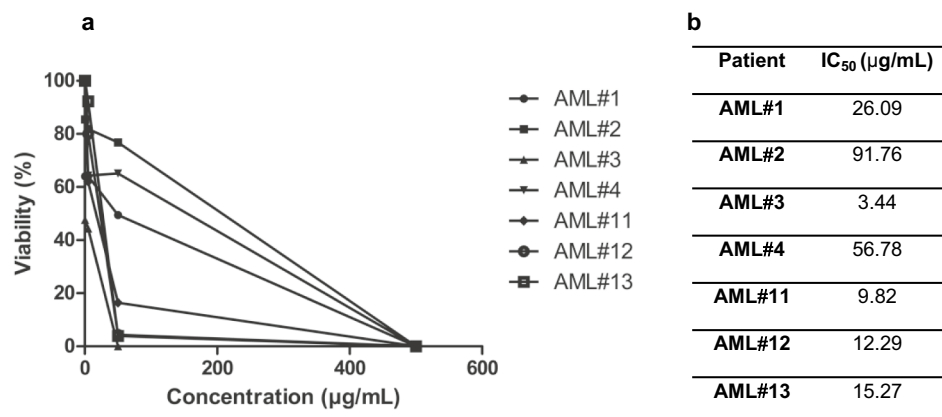
Results

EAEAC exhibited cytotoxic activity on BM-MNC

The BM-MNC of seven patients (AML#1, AML#2, AML#3, AML#4, AML#11, AML#12, and AML#13) were incubated for 48 h with increasing concentrations of EAEAC to assess anti-AML potential. Data showed a high variability in response to EAEAC with IC_{50} value ranged from 3.44 µg/mL for AML#3 to 91.76 µg/mL for AML#2 (Fig. 1a). Interestingly, 57% of the patients (AML#3, AML#11, AML#12, and AML#13) presented an IC_{50} less than 20 µg/mL (Fig. 1b).

EAEAC-induced apoptosis in BM-MNC

Programmed cell death is identifiable by several specific markers, such as the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This characteristic marker is detectable by the

Fig. 1 Evaluation of EAEAC-induced cytotoxic effects on AML cells. **a** AML cells were seeded in 96-well plates at the density of 2×10^5 cells/well for 24 h. Then, cells were cultured with 0, 5–500 µg/mL EAEAC for 48 h, and their viability assayed using a colorimetric MTT assay. **b** The IC_{50} values of EAEAC for seven AML cells with different characteristics were calculated using GraphPad software

Patient	IC$_{50}$ (µg/mL)
AML#1	26.09
AML#2	91.76
AML#3	3.44
AML#4	56.78
AML#11	9.82
AML#12	12.29
AML#13	15.27

double-staining AV/PI where the AV+/PI− population is considered apoptotic cells. The cytometry dot plot of patient AML#6, presented in Fig. 2a, showed a clear shift from the state of living cells (in the lower left quadrant AV−/PI−) to apoptotic cells (right one AV+/PI−) under the effect of AraC, but also of EAEAC. Remarkably, after 24 h of treatment with EAEAC, the apoptotic proportion increased from 8.2% (control) to 16.4% (50 μg/mL) and 17.6% (100 μg/mL). Interestingly, the doubling of the AV+/PI− population was also observed in patients AML#5 (1.8-fold) and AML#7 (2.35-fold) after treatment with 50 μg/mL of EAEAC.

To confirm these findings, a statistical study was conducted using the average of the three patients (Fig. 2c). This study demonstrated that our results are significant ($p < 0.05$) and that EAEAC-induced BM-MNC cell death through the apoptosis pathway. Additionally, the treatment with EAEAC appeared to be dose independent since no notable difference was observed between the concentrations 50 μg/mL and 100 μg/mL. Similarly, EAEAC did not seem to induce necrosis as is the case with AraC treatment (Fig. 2c). For late apoptosis (AV+/PI+), we obtained large fluctuations between the three patients, preventing us from drawing conclusions. To validate these results, we observed another characteristic of apoptosis, DNA fragmentation. For this purpose, DNA staining with PI, followed by flow cytometry analysis, was performed. The results, still with the same patient AML#6, are illustrated in Fig. 2b. The data demonstrated an interesting increase in the sub-G1 proportion, recognized as the population representing fragmented DNA or aneuploid nuclei [18]. Specifically, this designated population showed an increase from 27 to 32.1% and 43.4% after exposure to 50 μg/mL and 100 μg/mL of EAEAC, respectively. Similarly, a comparable result was observed with two other patients, AML#4 and AML#5, confirming the trend. The statistical analysis using these three patients showed that our results were significant (Fig. 2d) ($p < 0.05$), confirming that EAEAC-induced DNA fragmentation in BM-MNC, and consequently, apoptosis.

EAEAC induced a mitochondrial depolarization

Mitochondrial depolarization ($\Delta\Psi m$), recognized as a pivotal step in apoptosis and essential for the activation of caspases, was evaluated by flow cytometry after DiOC6-(3) staining. As shown in Fig. 3a, the treatment of BM-MNC of AML#11 with EAEAC or AraC for 24 h highly decreased the fluorescence intensity, correlating with an increase in the fraction of cells exhibiting $\Delta\Psi m$ loss from 11.4% for untreated cells to 47.8% for 50 μg/mL EAEAC, 57% for 100 μg/mL EAEAC, and 69.9% for positive control (AraC).

The statistical analysis conducted on three different BM-MNC (AML#6, AML#7, and AML#11) revealed the significance of the increase in mitochondrial depolarization

fractions following treatment with EAEAC. Specifically, both EAEAC and AraC led to an increase in the proportion of $\Delta\Psi m$ (from 23% in control to 49.08% for 50 μg/mL EAEAC, 56.3% for 100 μg/mL EAEAC, and 58.97% for 100 μM AraC) ($p < 0.05$) (Fig. 3c).

EAEAC activated caspase-3/7

Caspases 3 and 7 play crucial roles in apoptosis by initiating the cleavage of various cellular proteins, ultimately leading to cell death. AML#6, AML#7, and AML#11 cells were treated with two concentrations of EAEAC (50 or 100 μg/mL) or AraC (100 μM) for 24 h. The untreated group served as vehicle control while AraC-treated group presented the positive control. Figure 3b illustrates the effect of EAEAC in AML#7, resulting in an amplification of this enzymatic activity to 26% and 40.5% with 50 μg/mL and 100 μg/mL of EAEAC, respectively, compared to the control, which is about 10.7%. Moreover, these results are very similar to those found with the positive control (43.3%), confirming the high potential of the EAEAC. The statistical analysis using the average of the three patients clearly demonstrated the significance of our results. Specifically, on average, EAEAC remarkably caused a 1.6-fold (25.8%, 50 μg/mL) and 2.7-fold (41.8%, 100 μg/mL) enhancement in Caspases 3/7-activated cell proportion compared to control (15.5%) ($p < 0.05$) (Fig. 3d).

EAEAC showed potential decrease in subpopulation frequencies

Gordon's research has identified two distinct subpopulations of LSC. These subpopulations are characterized by specific markers, which allow for their differentiation. The LMPP-like population (CD38−) is less differentiated compared to the GMP-like population (CD38+), reflecting their more stem-like, multipotent nature versus a more committed progenitor state [10].

Based on this work, we focused on evaluating the effect of EAEAC, as well as AraC for the first time, on these two subpopulations using a panel of specific antibodies. Table 2 reports the results obtained with five patients (AML#5, AML#6, AML#8, AML#9, and AML#10), showing notable heterogeneity between these two distinct LSC subpopulations. Interestingly, we observed a potential reduction in LMMP-like (from 5.22 to 1.72%) and GMPP-like (from 19.09 to 6.86%) subpopulation frequencies in one patient (AML#5) after 24 h of treatment with a dose of 50 μg/mL. In addition, with a dose of 100 μg/mL of EAEAC and 100 μM of AraC, a similar trend was observed in the LMMP-like subpopulation, though the effect was less pronounced; no effect was observed on the GMP-like population.
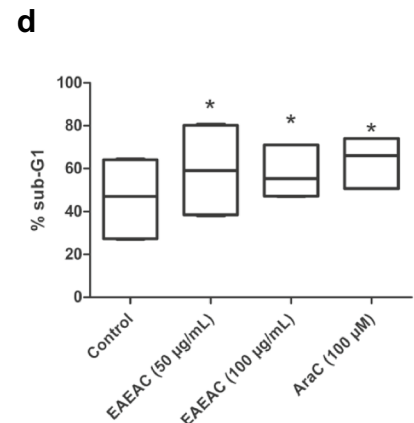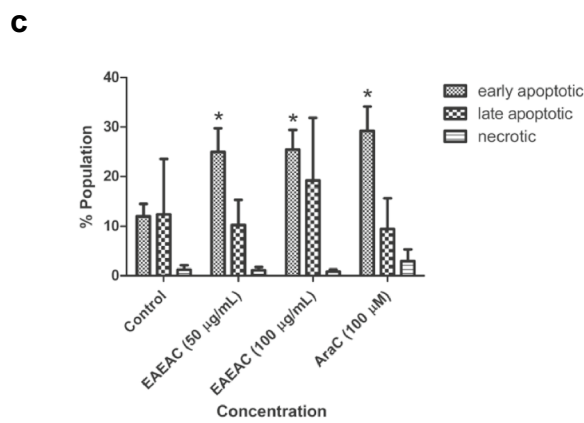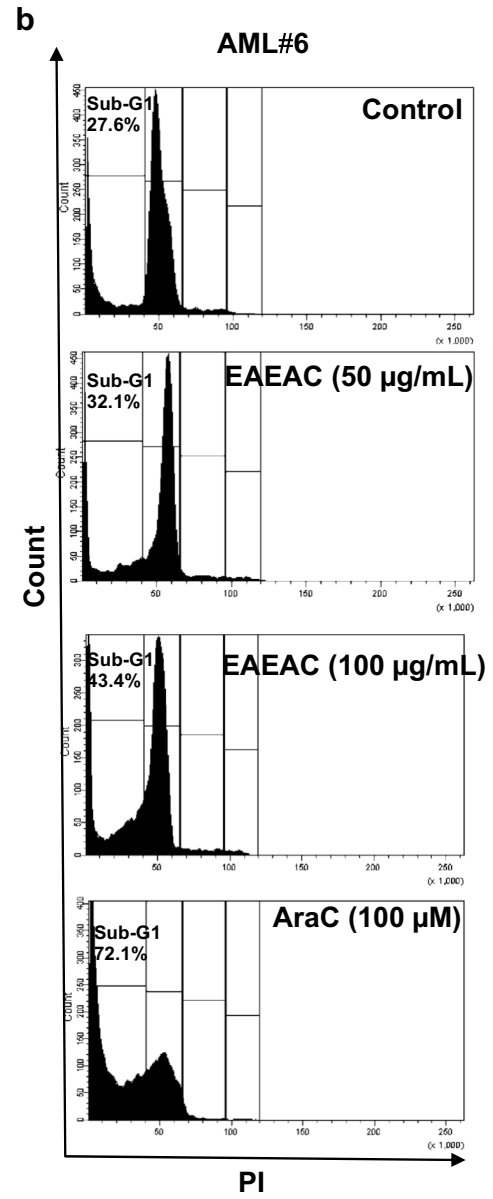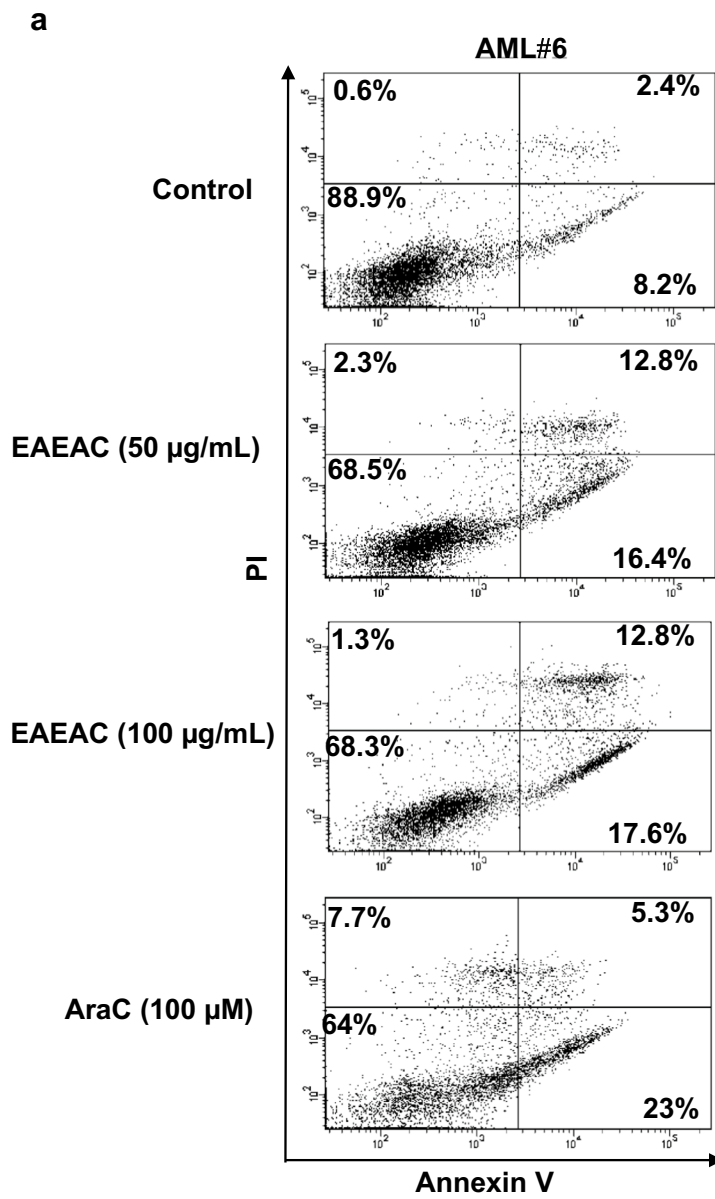
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◄**Fig. 2** Flow cytometry analysis of apoptosis and cell distribution (sub-G1) of BM-MNC. **a** Representative histograms of cell apoptosis induction in AML#6 cells treated for 24 h with EAEAC (50 and 100 µg/mL) or AraC (100 µM). **b** Representative histograms of cell cycle distribution depicting apoptosis in AML#6 cells treated for 24 h with EAEAC (50 and 100 µg/mL) or AraC (100 µM). **c** Analysis of cell apoptosis induction of three BM-MNC (AML#5, LAM#6, and AML#7). The percent of early apoptotic, late apoptotic, and necrotic cells is measured as the percentage of the number of cells relative to the number of total cells. Quantification of apoptotic and necrotic cells were analyzed by BD FACSDiva 7 software. (mean ± standard error) following 24 h treatment with EAEAC or AraC. **d** Analysis of cell population at sub-G1 phase of three BM-MNC (AML#4, AML#5, and AML#6). The percent of sub-G1 is measured as the percentage of the number of cells in the sub-G1 population relative to the number of total cells. Quantification of sub-G1 phase was analyzed by BD FACSDiva 7 software. (mean ± standard error) following 24 h treatment with EAEAC or AraC. *$p < 0.05$ shows significant differences compared to the control group

Furthermore, in AML#6, there was only a decrease in GMP-like subpopulation from 28.03% for control cells to 21.12%, 23.55%, and 26.96% for 50 µg/mL EAEAC, 100 µg/mL of EAEAC, and 100 µM of AraC, respectively (Fig. 4). Regarding the other patients, EAEAC did not have a significant effect on the two cancer stem cell subpopulations, except for patient AML#8, where a dose of 50 µg/mL showed a slight reduction in the LMMP-like subpopulation. On the other hand, despite the conventional chemotherapy AraC not having a notable effect on AML#8, a slight decrease in the LMMP-like population (from 0.68 to 0.59%) was observed in AML#9. In addition, in patient AML#10, there was a significant decrease in the same population, dropping from 1.95 to 0.81%, along with a reduction in the GMP-like population, which decreased from 0.71 to 0.53%. We conducted a statistical analysis on these results and found that the difference is not significant; therefore, we could not draw definitive conclusions. However, these preliminary findings remain very interesting.

Discussion

For over four decades, the standard treatment for AML has remained largely unchanged, relying on intensive chemotherapy with AraC and an anthracycline as the cornerstone drugs. While the majority of patients achieve remission, up to 70% of adults and 30% of children do not survive beyond five years after their initial clinical response due to disease relapse. This highlights the urgent and unmet need for novel drugs to enable a sustainable recovery in patients with AML [29].

Previous studies have shown the anticancer potential of EAEAC on multiple myeloma refractory cells [26]. Building on these findings, we conducted an ex vivo study on BM-MNC freshly collected from patients with AML. The MTT assay evaluating the cytotoxicity of EAEAC on AML cells revealed a significant dose-dependent cytotoxic effect, with IC$_{50}$ values ranging from 3.44 to 91.76 µg/mL, indicating variability in sensitivity among patient cells. At the highest concentration (500 µg/mL), EAEAC demonstrated maximum cytotoxicity, significantly reducing cell viability. These variations can be attributed to individual differences in genetic makeup, disease stage, and cellular characteristics. Further investigation was conducted to identify the type of cell death, employing a panel of tests. We used AraC as a positive control based on an effective concentration of 100 µM [30]. This concentration was chosen based on the literature as well as optimizations conducted in the laboratory. Due to challenging ex vivo conditions, we were unable to perform all tests for every patient. Nonetheless, we ensured that we had at least three patients for each type of experiment to enable statistical analysis.

Apoptosis plays a crucial role in cancer therapy, as it is a mechanism through which cancer cells can be eliminated. To evaluate whether EAEAC acts on leukemic cells through this pathway, the activity of the AV-PI assay was assessed. The results indicated that, after 24 h of treatment with EAEAC, the apoptotic proportion remarkably increased in BM-MNC of three AML patients. Moreover, DNA fragmentation, another hallmark of apoptosis, was analyzed by examining changes in the sub-G1 fraction of the cell cycle. Increased DNA damage was observed with 50 and 100 µg/mL concentrations of EAEAC, confirming the results obtained with the AV-PI assay. These findings highlight that EAEAC induces BM-MNC cell death through apoptosis. This aligns with our previous study, which reported that EAEAC induces both apoptotic and necrotic cell death in hematologic cancers [26]. Mancuso's research on *Artemisia annua* highlights that the isolated compound artemisinin is highly effective in treating hematologic cancers, particularly leukemia, multiple myeloma, and lymphoma. Artemisinin's mechanisms of action include inducing oxidative stress response, inhibiting proliferation, and triggering various types of cell death, such as apoptosis, among others [31].

DNA damage is a critical factor that can trigger the mitochondrial pathway of apoptosis, also known as the intrinsic pathway. When cells experience significant DNA damage, it can activate a series of signaling events that result in the permeabilization of the mitochondrial outer membrane, leading to the release of cytochrome c into the cytosol. Once in the cytosol, the apoptosome forms, which then activates procaspase-9. This activation subsequently leads to the activation of caspase-3 and other downstream caspases, resulting in irreversible changes and cell death. To evaluate the effect of EAEAC on the mitochondrial membrane, the DIOC-3-(6) assay was performed. Our results showed a significant reduction in fluorescence in EAEAC-treated cells, indicating a substantial decrease in mitochondrial

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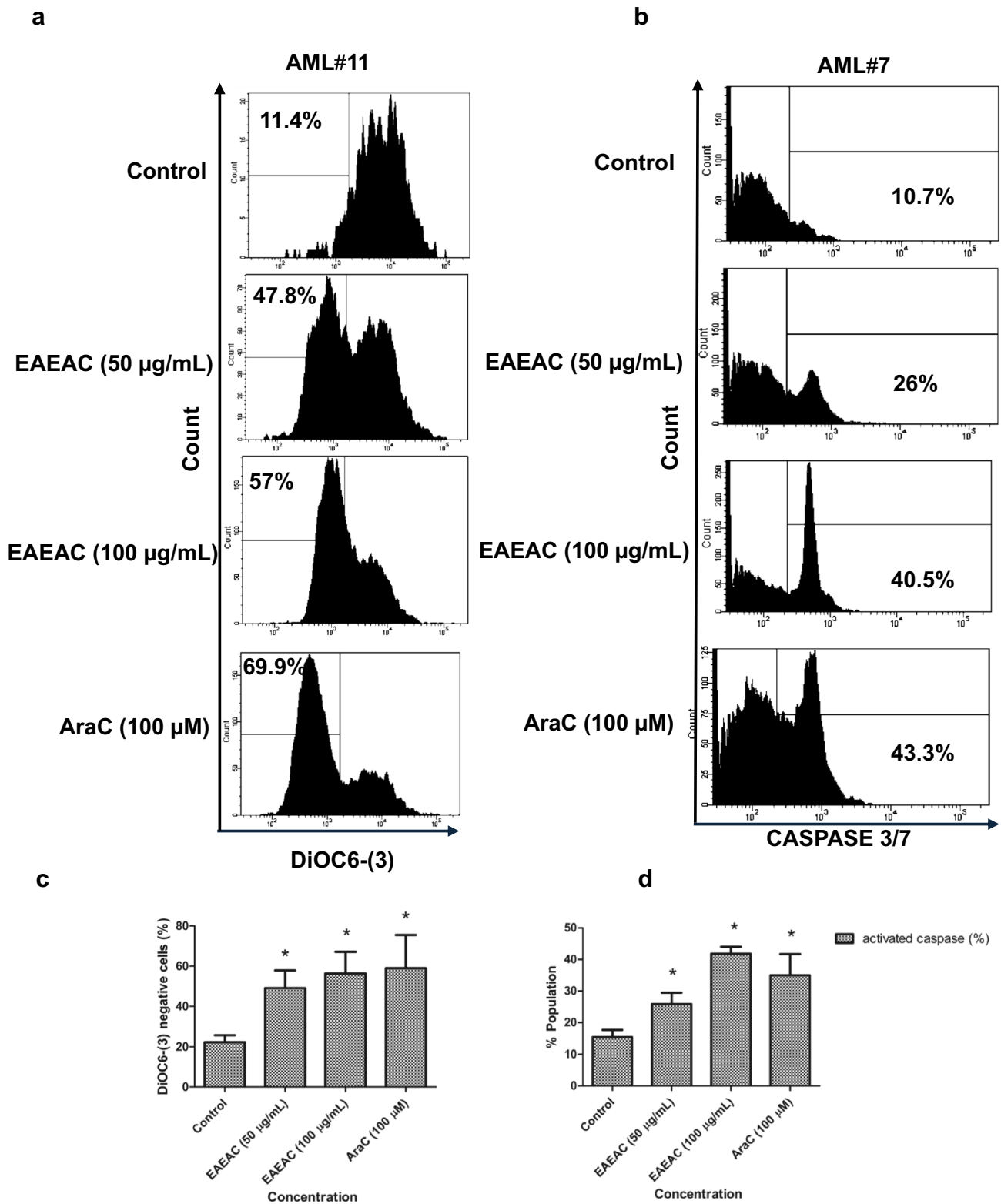
membrane potential. These results were further confirmed by cell caspase activation of BM-MNC on treatment with EAEAC. The activation of executioner caspases 3/7 provides mechanistic insight into the involvement of apoptotic

pathways triggered by EAEAC. Furthermore, the decrease in mitochondrial membrane potential ($\Delta\Psi$m) signifies mitochondrial dysfunction induced by EAEAC, which is a hallmark of apoptosis. Our findings show notable similarities

◄**Fig. 3** Flow cytometry analysis of mitochondrial depolarization and caspase-3/7 activation of BM-MNC. **a** Representative histograms of ΔΨm loss in AML#11 cells treated for 24 h with EAEAC (50 and 100 µg/mL) or AraC (100 µM). **b** Representative histograms of caspase-3/7 activation in AML#7 cells treated for 24 h with EAEAC (50 and 100 µg/mL) or AraC (100 µM). **c** Analysis of the fraction of cells having a ΔΨm loss of three BM-MNC (AML#6, AML#7, and AML#11). Quantification of mitochondrial depolarization was analyzed by BD FACSDiva 7 software. (Mean ± standard error) following 24 h treatment with EAEAC or AraC. **d** Analysis of caspase-3/7 activation of three BM-MNC (AML#6, AML#7, and AML#11). Quantification of caspase activation was analyzed by BD FACSDiva 7 software. (Mean ± standard error) following 24 h treatment with EAEAC or AraC. *$p < 0.05$ shows significant differences compared to the control group

with studies on the methanolic extract of *Artemisia vulgaris*, which demonstrated its ability to inhibit chronic myeloid leukemia cell proliferation by inducing apoptosis through the release of cleaved poly (ADP-Ribose) polymerase (PARP) and caspase-3. Further investigations into the sub-fractions of *Artemisia vulgaris* identified ethyl acetate and chloroform as the most effective solvents for isolating anticancer bioactive compounds [32].

Interestingly, our results demonstrate the significant anticancer properties of EAEAC, an ethyl acetate extract derived from the plant *Artemisia campestris*. This species is known to contain a variety of bioactive compounds, including flavonoids, terpenoids, and phenolic acids, which have been documented to possess anticancer, anti-inflammatory, and antioxidant properties [20]. These compounds can interfere with multiple cellular pathways, including those regulating cell proliferation, survival, and apoptosis [17]. Precisely, EAEAC induces substantial cytotoxicity in AML cells through the induction of the mitochondrial pathway of apoptosis. The phytochemical composition of EAEAC, explored in our previous work, likely plays a crucial role in these observed effects. Specifically, the LC-MS analysis demonstrated that EAEAC contained a high rate of specific terpene lupeol (34.03 ± 4.83) and flavonoids (773.68 mg/g DE), including compounds like cirsiliol and luteolin [26]. These molecules are known to modulate the activity of key proteins involved in apoptotic pathways. Specifically, Metoui's study reported that the cirsiliol compound isolated from AC-dried leaves exhibited cytotoxic activity against ovarian cell lines OVCAR-3 and IGROV-1, as well as the human colon cell line HCT-116, at 15 µM, with inhibition percentage respective values of 53.7, 48.8, and 40.9% [25, 33].

Our study highlights, once again, the potent anticancer activity of EAEAC derived from *Artemisia campestris*. The extract's ability to induce apoptosis through mitochondrial disruption and caspase activation underscores its therapeutic potential. Future research should focus on isolating and characterizing the specific bioactive components of EAEAC responsible for its anti-AML potential.

Table 2 Quantification of LMMP-like and GMP-like subpopulation in five AML cells

Patient		LMMP-like (%) Lin-CD34+CD38−CD90−CD45RA+	GMP-like (%) Lin-CD34+CD38+CD123+CD45RA+
AML#5	Control	5.22	19.09
	EAEAC (50 µg/mL)	**1.72**	**6.86**
	EAEAC (100 µg/mL)	**2.15**	23.77
	AraC (100 µM)	**2.43**	29.31
AML#6	Control	5.06	28.03
	EAEAC (50 µg/mL)	7.99	**21.12**
	EAEAC (100 µg/mL)	6.86	**23.55**
	AraC (100 µM)	5.14	**26.96**
AML#8	Control	0.89	2.47
	EAEAC (50 µg/mL)	**0.65**	5.37
	EAEAC (100 µg/mL)	1.57	16.15
	AraC (100 µM)	1.55	6.50
AML#9	Control	0.68	3.74
	EAEAC (50 µg/mL)	2.19	7.20
	EAEAC (100 µg/mL)	4.19	8.25
	AraC (100 µM)	**0.59**	4.21
AML#10	Control	1.95	0.71
	EAEAC (50 µg/mL)	2.5	1.26
	EAEAC (100 µg/mL)	2.43	1.61
	AraC (100 µM)	**0.81**	**0.53**

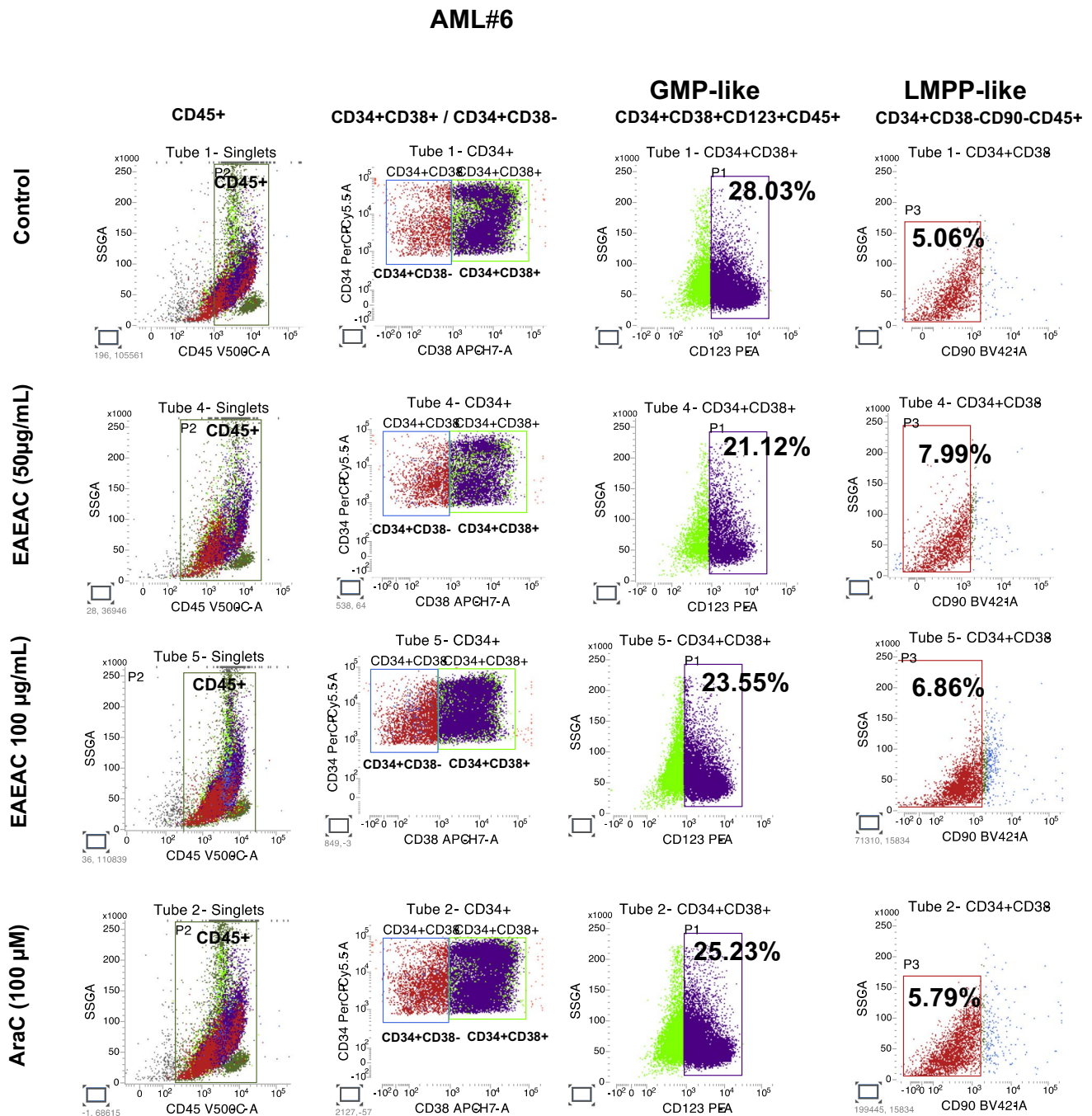Bold value indicates the decrease in the frequency of leukemic stem cells

Fig. 4 Flow cytometry analysis of LMPP-like and GMP-like subpopulations of AML#6

AML is notorious for its high rate of recurrence, even after initial successful treatment, largely due to the persistence of LSC [34–37]. Previous studies have indicated a correlation between the frequency of LSC at diagnosis and the rate of complete response after induction, as well as refractory disease in AML. Recently, Goardon's research has immunophenotypically identified two LSC subpopulations, namely GMP like and LMPP like [10]. Accordingly, we used a panel of four specific antibodies for each LSC subpopulation to evaluate, for the first time, the impact of EAEAC alongside conventional clinical treatment with AraC. Unfortunately, the preliminary results do not allow for definitive conclusions, which are expected given the limited number of patients used.

Nevertheless, we would like to highlight some interesting points in these results, particularly for AML#5, where

there was a threefold reduction in both the LMPP-like and GMP-like subpopulations following treatment with 50 µg/mL EAEAC. Furthermore, clinical data (Table 1) indicated a complete remission after induction therapy for this patient, demonstrating their sensitivity to the treatment. In addition, we observed that EAEAC and AraC were able to reduce the GMP-like population in AML#6, a patient with a low cytogenetic risk.

On the other hand, only AraC demonstrated a decrease in the frequency of both LSC subpopulations in AML#10, a patient with a high cytogenetic risk. Unfortunately, the patient did not achieve complete remission post-induction.

To summarize, our findings underscore the challenges in managing AML effectively, especially in cases where conventional treatments demonstrate limited efficacy. Both EAEAC and AraC had limited impact on reducing LSC. These results emphasize the importance of exploring targeted therapies that directly address the LSC population. For instance, compounds like parthenolide, which target AML progenitor and stem cell populations, represent promising avenues for future research and treatment strategies in AML [38].

Conclusion

Overall, these results collectively indicate that EAEAC exhibits multifaceted anti-leukemic properties, including cytotoxicity against BM-MNC, induction of apoptosis, disruption of mitochondrial function, and potentially reducing the frequency of LSC in an ex vivo model for some patients. This points toward the promising role of EAEAC as a potential therapeutic agent for AML. However, further investigations are necessary to elucidate the specific molecular mechanisms underpinning its action and to explore its clinical translational potential for more targeted and effective AML therapy.

Author contributions Mohamed Abdelkarim: conceptualization, methodology, investigation, formal analysis, and writing—original draft preparation, Rachid Kharrat, Fatma Ben Lakhal, Hiba Souia, Ines Limam, and Hend Ben Naji: performed experiments, methodology, and investigation. All authors have read and approved the final manuscript.

Data availability The datasets generated or analyzed during this study are not publicly available but can be requested from the corresponding author.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All experiments using human cells were exempted by the Institutional Review Board of Pasteur Institute (IRB Number: 2021/09/E).

Consent to participate Written informed consent was obtained from the patients.

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