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Research Paper

Ellagic acid, a polyphenolic compound, selectively induces ROS-mediated apoptosis in cancerous B-lymphocytes of CLL patients by directly targeting mitochondria

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ABSTRACT

To investigate the effects ofellagic acid (EA) on the cytotoxicity, B-lymphocytes isolated from CLL patients and healthy individuals. Flow cytometric assay was used to measure the percentage of apoptosis versus necrosis, intracellular active oxygen radicals (ROS), mitochondrial membrane potential (MMP) and the caspase-3 activity and then mitochondria were isolated from both groups B-lymphocytes and parameters of mitochondrial toxicity was investigated. Based on our results EA decreased the percentage of viable cells and induced apoptosis. EA increased ROS formation, mitochondria swelling, MMP decrease and cytochrome c release in mitochondria isolated from CLL BUT NOT healthy B-lymphocytes while pre-treatment with cyclosporine A and Butylated hydroxyl toluene (BHT) prevented these effects. Our results suggest that EA can act as an anti cancer candidate by directly and selectively targeting mitochondria could induce apoptosis through mitochondria pathway with increasing ROS production which finally ends in cytochrome c release, caspase 3 activation and apoptosis in cancerous B-lymphocytes isolated from CLL patients.

1. Introduction

Cancer is a leading cause of death in developed and developing countries [1,2]. Searching for new anticancer agents from plant and other sources is a promising approach which may lead to the discovery of many novel anti-cancer agents [3,4]. Chronic lymphocytic leukemia (CLL) is a neoplasm characterized by clonal proliferation and accumulation of immature lymphocytes in the blood, lymph nodes, spleen and bone marrow. CLL is the most common leukemia in Western countries, and it accounts for approximately one-third of all leukemias in the United States and other counties [5]. The median age for diagnosis of CLL is 65 years, and the disorder is higher in men than women [6]. However, despite the initial effectiveness of used anti-cancer drugs in patients with low-grade disease, resistant cells ultimately emerge, leaving no effective treatment options available. It is possible that drug-resistant CLL cells possess intrinsic defect(s) in their ability to undergo apoptosis. Apoptosis is a physiological cell suicide program that is essential for the regulation of development, the maintenance of homeostasis and the prevention of tumorigenesis [7]. Evading the apoptotic program is one of the hallmarks of cancer and represents an important mechanism in clinical resistance to therapies [8,9]. This is particularly true for chronic lymphocytic leukemia (CLL), a currently incurable condition that is clearly characterized by impaired apoptosis [10]. The development of therapeutic strategies that target apoptosis in CLL is therefore a very important issue [11]. CLL is typically sensitive to a variety of cytotoxic drugs, but the disease is considered incurable [12]. Treatment is generally recommended to control symptoms and reduce bulk of disease but without substantially improving survival [13]. New targets such as mitochondria has facilitated the development of new drugs with a view to improving clinical outcomes for this neoplasm [14]. It is now clear that mitochondria are central to cell death, cell differentiation, innate immune system, hypoxia sensing, metabolism of calcium and amino acids, iron sulfur center and heme biosynthesis [15]. Disruption to these processes contributes to several pathologies, such as cancer, making mitochondria an important therapeutic target [16]. In

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cancer cells, mitochondria have an essential role in the neoplasic phenotype, especially resistance to apoptosis, limitless proliferative potential and metabolic reprogramming [17]. It has been suggested that the most efficient mitochondrially targeted therapies would affect mitochondrial processes that participate in the establishment of several of this features [18]. Consistently, alteration in mitochondrial dynamics (i.e. fission and fusion) and damage to mitochondrial macromolecules, can induce cell death by apoptosis or autophagy [19]. Besides, mitochondria control the programmed cell death via several mechanisms that can be activated by disruption of mitochondrial bioenergetics, alteration of cellular redox balance and release or activation of pro-apoptotic factors [20,21]. So mitochondria are currently regarded as central organelles in mediating intrinsic death signals and might provide a novel target for new chemotherapeutics [18].

Ellagic acid is a naturally occurring phenolic compound that is contained in ellagitannins in grapes, nuts, strawberries, black currents, raspberries, green tea, pomegranates, and the stem and bark of Eucalyptus globulus and as well as royal jelly and honey [22]. EA exerts potent preventive and therapeutic effects against several types of cancers, including colon cancer, breast cancer, prostate cancer, skin cancer, esophageal cancer, and osteogenic sarcoma. The anti-carcinogenic properties of EA have drawn increasing attention globally [23].

In our study for first time we evaluated anti-cancer effect of EA on B-lymphocytes obtained from CLL patients. We isolated B-lymphocytes from CLL patients and healthy donors and then isolated mitochondria from both groups. Parameters such as cell viability, apoptosis versus necrosis %, caspase 3 activity, ROS formation and collapse of MMP in intact B-lymphocytes and succinate dehydrogenases activity, ROS formation, collapse of MMP, mitochondrial swelling and release of cytochrome C in isolated mitochondria was tested.

2. Materials and methods

2.1. Patients

Ten newly diagnosed, untreated CLL patients were enrolled into the study. Diagnosis of CLL was made on the basis of clinical examination, morphological and immunological criteria. Peripheral blood (PB) samples were obtained from the patients after informed consent. Age-matched controls were obtained from 10 healthy donors. This study was approved by the Shahid Beheshti University of Medical Sciences research ethics committee and all the patients and healthy donors signed an informed consent form.

2.2. B-lymphocyte isolation

Blood samples were obtained from the CLL patient and healthy donors during a routine diagnosis at Shohadaye Tajrish Hospital. B lymphocytes were isolated immediately using Ficoll gradient centrifugation. After 1 h of incubation at 37 °C in 5% CO_2, adhesive mononuclear cells were removed. Those non-adherent lymphocytes were thoroughly washed with the Hank's solution. T lymphocytes were removed using anti-CD3 dynabeads. The purification of B lymphocytes was assessed by flow cytometry with anti-CD19 antibodies with FACS. This cell preparation contained about 95% CD19 (B lymphocyte antigen) positive cells. It was added stromal cell-derived factor-1 to rescue B-lymphocytes from apoptosis [24].

2.3. Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann [25]. The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The dehydrogenases using NADH or NADPH as co-enzyme can convert the yellow form of the MTT salt to insoluble, purple formazan crystals [26]. Formazan solution is read spectrophotometrically after the crystals are dissolved in organic solvent (DMSO). B-lymphocytes (1×10^4 cells/well) were incubated in 96-well plates in the presence or absence of EA for 48 h in a final volume of 50 μl. At the end of the treatment, 20 μl of MTT (5 mg/ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple–blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the absorbance was measured at 570 nm on ELISA reader. Each concentration was tested in three different experiments run in three replicates for each sample.

2.4. Measurement of apoptosis versus necrosis

The healthy and CLL B-lymphocytes exposed to EA in duration 24 h and at times 6,12 and 24 h, apoptosis versus necrosis % were measured. In the early stages of apoptosis phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can, therefore, be used as a sensitive probe for the exposure of PS on the cell membrane and hence as a marker of apoptosis. Briefly, double-staining for FITC-Annexin V binding and for cellular DNA using propidium iodide (PI) was performed in duplicate. After washing twice times in PBS, the B-lymphocytes were resuspended in binding buffer. FITC-Annexin V was added to a final concentration of 10 μM and the cells were incubated in the dark for 10 min. The cells were then washed again in PBS, centrifuged at $300 \times g$ and resuspended in binding buffer. Prior to flow cytometric analysis 10 μM PI in binding buffer was added to each sample. The fluorescence signals of annexin V and PI were measured by flow cytometry on the FL1 and FL3 channels with gating for $CD19^+$ B-lymphocytes based on CD19 and side scattering, respectively. Only annexin V-positive (+) and PI-negative (−) cells were defined as apoptotic [27].

2.5. Determination of caspase-3 activity

Caspase-3 activity was determined in cell lysate of lymphocytes from different treatments using “Sigma's caspase-3 assay kit (CASP-3-C)”. In brief, this colorimetric assay is based on the hydrolysis of substrate peptide, Ac-DEVD-pNA, through caspase-3. The released moiety (p-nitroaniline) has a high absorbance at 405 nm. The concentration of the p-nitroaniline (μM) released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined p-nitroaniline solutions [28].

2.6. Determination ROS in intact B-lymphocytes

CLL and healthy B-lymphocytes ($1x10^6$ cells) were treated with both vehicle (solvent of EA) and EA for 6, 12 and 24 h. After treatment, B-lymphocytes were washed with PBS. H_2DCFDA (10 μM) was used to measure intracellular reactive oxygen species as previously described [29,30]. This agent diffuses into cells and is sequestered intracellularly by de-esterification. Subsequent reaction with peroxides generates fluorescent 5-chloromethyl-2′, 7′-dichlorofluorescein (DCF). Cells were read on the FL1 channel of a Cyflow Space-Partec. Mean of fluorescence intensities were

compared between treated and untreated CLL and healthy B-lymphocytes.

2.7. Determination of MMP in intact B-lymphocytes

MMP was determined in B-lymphocytes with both vehicle and EA at 6,12 and 24 h by flow cytometry using a lipophilic cationic $\Delta\Psi_m$ dependent fluorescent dye rhodamine 123, which was analyzed in fluorescence detection channel 1 (FL-1). B-lymphocytes with intact mitochondria excite an intense green fluorescence due to the formation of the dye aggregates, whereas the monomer dye fluoresce green in cells with a disrupted mitochondrial membrane [31]. All aliquots were incubated at room temperature for 20 min in the dark with 2 μL of the dye diluted in PBS at a concentration of 2.5 μg/mL and were analyzed immediately with flow cytometry. Data, from at least 10,000 events per sample, were recorded and processed using Cyflow Space-Partec.

2.8. B-lymphocytes lysis and isolation of mitochondria

B-lymphocytes were lysed and isolated mitochondria from CLL and healthy B-lymphocytes. Mitochondria were isolated from the B-lymphocytes by mechanical lysis and differential centrifugation. Briefly, B-lymphocytes were washed with cold PBS at 4 °C and centrifuged at 450 × *g*. The pellet was resuspended in cold isolation buffer (75 mmol/L sucrose, 20 mmol/L HEPES, 225 mmol/L mannitol, 0.5 mmol/L EDTA, pH 7.2), and the cells were disrupted by homogenization. Nonlysed B-lymphocytes and nuclei were spun down by centrifugation at 750 × *g* for 20 min. The supernatant was further spun at 10,000 × *g* for 10 min twice. The pellet, designated as the mitochondrial fraction, was suspended in assay buffer (140 mmol/L KCL, 10 mmol/L NaCl, 2 mmol/L MgCl2, 0.5 mmol/L KH2PO4, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH). The isolation of mitochondria was determined by measurement of succinate dehydrogenase. The assay buffer was supplemented with 1 mg/mL rotenone and 10 mmol/L succinate immediately before use [24].

2.9. Succinate dehydrogenases activity assay using MTT test

The activity of mitochondrial complex II (succinate dehydrogenases) was assayed by measuring the reduction of MTT (3-[4,5–dimethylthiazol–2–yl]-2,5-diphenyltetrazoliumbromide). Briefly, 100 μL of mitochondrial suspensions (1 mg protein/mL) was incubated with different concentrations of EA (0, 5, 10, 20, 50 and 100 μl) at 37 °C for 1 h; then, 25 μl of 0.4% of MTT was added to the medium and incubated at 37 °C for 30 min. The product of formazan crystals was dissolved in 100 μl DMSO and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow hermo, Austria) [32].

2.10. Determination of mitochondrial swelling on isolated mitochondria

Purified mitochondria were isolated as described above. Mitochondria suspensions (at 100 μg protein per well) were incubated in 96-well plates at 25 °C in swelling buffer (140 mmol/ L KCl, 10 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.5 mmol/L KH_2PO_4, 20 mmol/L HEPES, 0.5 mmol/L EGTA; adjusted to pH 7.2 with KOH) supplemented with 1 mg/mL rotenone and 10 mmol/L succinate. Various concentration of EA were added after 10 min of preincubation. The MPT pore inhibitor such as cyclosporine A and inducer MPT pore such as Ca^{+2} were added upon initiation of the preincubation period. Mitochondrial swelling was measured spectrophotometrically in duration 1 h. This method equates mitochondrial membrane permeability transition with high-amplitude swelling of the mitochondria. Mitochondrial swelling results in a decrease in absorbance monitored at 540 nm [24].

2.11. Determination of ROS formation on isolated mitochondria

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA. Briefly, purified mitochondria were isolated as described above and were placed in respiration buffer (0.32 mM sucrose, 10 mM Tris, 20 mM Mops, 50 μM EGTA, 0.5 mM $MgCl_2$, 0.1 mM KH_2PO_4 and 5 mM sodium succinate). Following this step, DCFH-DA was added (final concentration, 10 μM) and then added various concentration ellagic acid, in 1.5-mL eppendorf tubes (at 1000 μg protein per ml) at 37 °C for an hour. Then, the fluorescence intensity of DCF was measured using Shimadzu RF-5000U fluorescence spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 527 nm .

Most of the researchers like us use DCFH-DA to measure intracellular generation of H_2O_2 and other oxidants or monitor redox signaling changes in cells in response to intra- or extracellular activation with oxidative stimulus. However, based on some previously published works the intracellular redox chemistry of DCFH is complex and there are several limitations associated with the DCF assay for intracellular H_2O_2 measurement. One of these limitations which is relevant specifically for our assays include cytochrome c, a heme protein that is released from mitochondria to the cytosol during apoptosis, is capable of oxidizing DCFH directly or indirectly via a peroxidase-type mechanism, forming DCF . The increase in DCF fluorescence that occurs during apoptosis of cells loaded with DCFH-DA has frequently been associated with enhanced oxidant production. It is necessary to keep these limitations in mind for proper interpretation of data obtained with the DCFH-DA probe. Even if differences in DCF fluorescence are documented under control and experimental conditions, one should consider the various limitations for proper interpretation of the results [30,33].

2.12. Determination of MMP on isolated mitochondria

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. The mitochondrial fractions (1000 μg protein/ml) were incubated with 10 μM of rhodamine 123 in MMP assay buffer (220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 50 μM EGTA, 5 mM sodium succinate, 10 mM HEPES, and 2 μM Rotenone) and then added various concentration EA in 1.5-mL eppendorf tubes (at 1000 μg protein per ml) at 37 °C for an hour. The fluorescence was monitored using Shimadzu RF-5000U fluorescence spectrophotometer at the excitation and emission wavelength of 490 nm and 535 nm, respectively [32].

2.13. Determination of cytochrome c release from isolated mitochondria

Mitochondria obtained CLL and healthy B-lymphocytes were incubated in 1.5-mL eppendorf tubes at 37 °C for various periods. Inhibitor of MPT pore, cyclosporine A at the final concentration 5 μmol/l and anti-oxidant BHT also at the final concentration 5 μmol/l were added 15 min before the addition of ellagic acid. Following the incubation, tubes were centrifuged at 10,000 × *g*. The supernatant contained the cytochrome c released from the mitochondria (cytosolic fraction), and the pellet consisted of the mitochondrial fraction. The concentration of cytochrome c was determined through using the Quantikine Human Cytochrome c Immunoassay kit provided by R and D Systems, Inc. (Minneapolis, Minn). Briefly, a monoclonal antibody specific for human

cytochrome c was pre-coated onto the microplate. 100 μL of conjugate (containing monoclonal antibody specific for cytochrome c conjugated to horseradish peroxidase) and 50 μL of control and test group were added to each well of the microplate. One microgram of protein from each supernatant fraction was added to the sample wells. All of the standards, controls and test were added to two wells of the microplate. After 2 h of incubation, the substrate solution (100 μL) was added to each well and incubated for 30 min. After 100 μL of the stop solution was added to each well; the optical density of each well was determined through the aforementioned microplate spectrophotometer set to 450 nm [34].

2.14. Statistical analysis

Results are presented as mean ± SD. Assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test when appropriate. Statistical significance was set at $p < 0.05$. And the parameters of mitochondrial dysfunction were analyzed by two way ANOVA and Bonferonie posttest. In all graph were expressed as mean ± SEM and $p < 0.05$ was considered statistically significant. The flow cytometric data was analyzed by Flow Max.

3. Results

3.1. Cellular assay

3.1.1. Viability assay

For determination of anti-cancer effect of EA we used MTT assay. First we measured the probable cytotoxicity of EA on healthy B-lymphocytes. Our results on healthy B-lymphocytes showed that EA wascytotoxic at the highest concentration (200 mM), we therefore did not use this concentration when we decided to measure cytotoxicity of EA on CLL B-lymphocytes. Our results with MTT assayshowed that even at the lowest concentration (5 μM) EA has toxic effecttoward healthy B-lymphocytes. As shown in graph B, EA at concentrations of 20, 50 and 100 μM, significantly ($p < 0.001$) reduced cell viability down to 59, 38 and 17 % respectively (Fig. 1, graph B), while no toxicitywas recordedat these concentrations on healthy B-lymphocytes (Fig. 1, graph A).

3.1.2. Measurement apoptosis and necrosis

The apoptosis was further quantified by the externalization of PS, assessed by annexin V-PI double staining at 6,12 and 24 h following the exposure with ellagic acid. The percentage of annexin V^+/PI^- cells (CD19-gated) increased to 6.73 , 11.35 and 28.16 in CLL B-lymphocytes at 6,12 and 24 h after exposure with 25 μM ellagic acid, respectively, were significantly higher than those of untreated cancerous control B-lymphocytes (all $p < 0.01$) (Fig. 2E–H). While for healthy B-lymphocytes, EA (25 μM) treatment did not induce early apoptosis (Fig. 2A–D). Based on these data, EA appeared to have selective significant apoptotic activity toward CLL B-lymphocytes. Besides, we tested the pre treating effect of BHT and Cs.A on EA induced apoptosis in CLL B-lymphocytes within 24 h, Our findings showed that both compounds significantly decreased apoptosis% in EA treated cancerous B-lymphocytes (Fig. 2I and J). These results again confirms the role of a ROS mediated mitochondrial pathway in EA induced apoptosis.

3.1.3. Caspase 3 assay

Caspase-3 is activated in the cell under apoptosis signaling through both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. As shown in Fig. 3, EA significantly increased the activity of apoptosis final mediator, caspae-3 in CLL B-lymphocytes. Increased caspase-3 activity was not shown in healthy B-lymphocytes. To figure out the upstream mechanism involved in EA induced caspase-3 activation we examined the pretreating effect of Z-IETD a caspase 8 inhibitor and cyclosporine A (Cs.A), an MPT pore sealing agent and Butylated hydroxytoluene (BHT), a ROS scavenger on EA treated CLL B-lymphocytes. Our results showed that only Cs.A and BHT but not Z-IETD prevented EA induced caspase 3 activation ($p < 0.001$) suggesting that EA activates a ROS-mediated mitochondrial intrinsic pathway in cancerous B-lymphocytes which could end in apoptosis.

3.1.4. Determination ROS in intact B-lymphocytes

In our study, EA treatment caused a significant increase in intracellular ROS levels in CLL B-lymphocytes (Fig. 4B) BUT NOT in healthy B-lymphocytes (Fig. 4A). In CLL B-lymphocytes, EA induced increase in intracellular ROS, which was not induced in healthy B-lymphocytes.

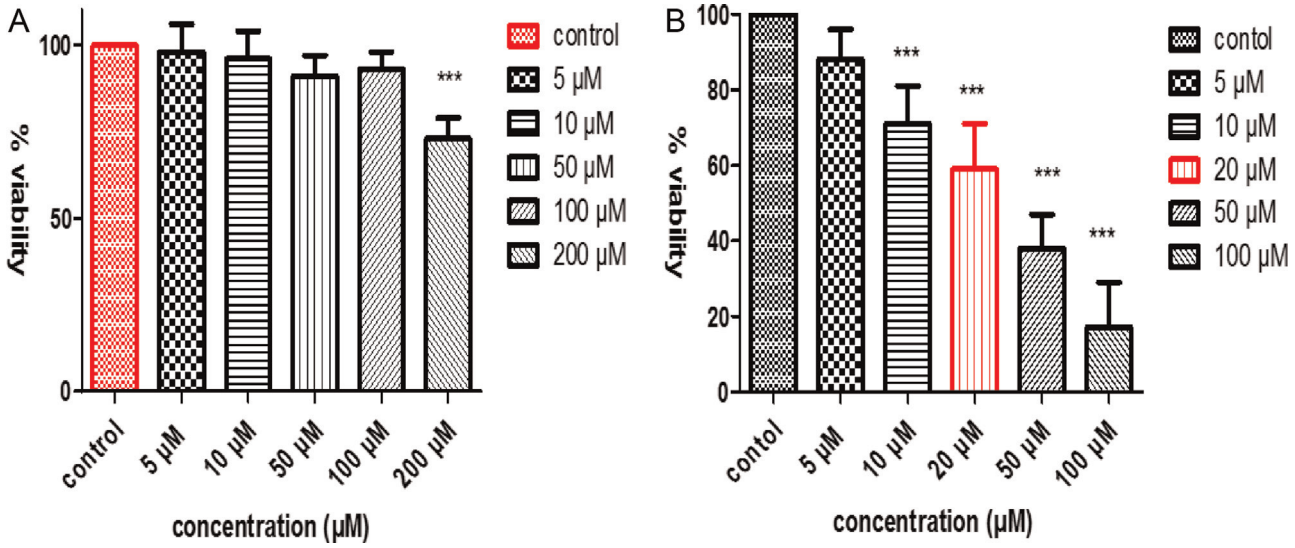

Fig. 1. Cell viability, B-lymphocytes from CLL and healthy donors, at 1×10^4 cells/well, were seeded on 96-well plates. EA at 20, 50 and 100 μM concentrations was incubated for 48 h. The absorbance representing the viability of B-lymphocytes was determined by the ELISA reader at 570 nm. Data presented as mean ± SD. The significant level was $p < 0.001$, $n = 5$.

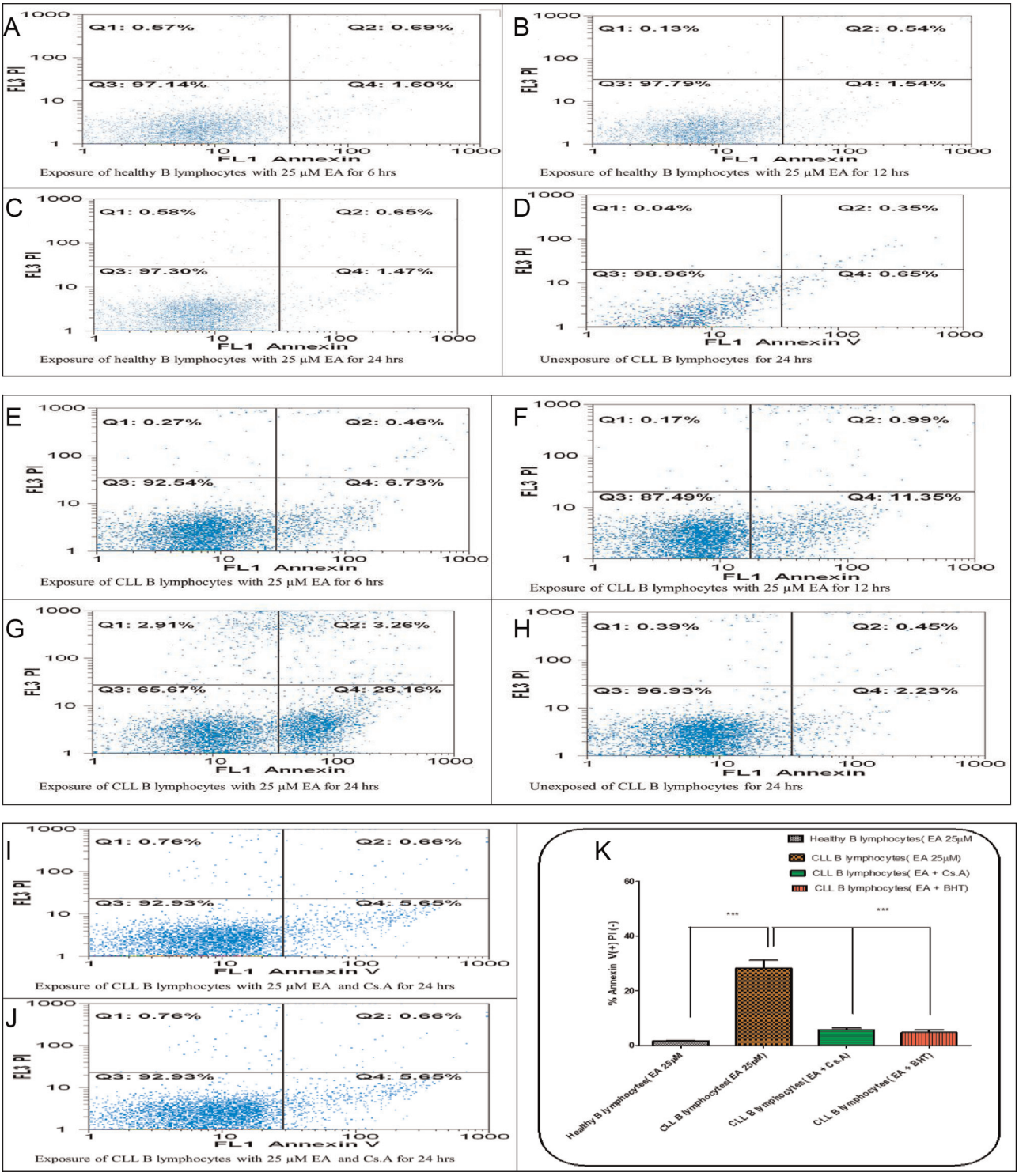

Fig. 2. EA induced apoptosis in CLL B-lymphocytes. Freshly isolated and purified CLL B-lymphocytes were treated with either EA-free medium containing 0.05% (v/v) DMSO (unexposed control) or 25 μM EA for 6, 12 and 24 h. Early apoptosis was measured by the annexin V assay using flow cytometry at 6, 12 and 24 h after incubation in both groups. As shown in this figure, pretreatment with BHT and Cs.A significantly inhibited apoptosis in the CLL mitochondria (graph J, graph I). The summarized apoptotic data was demonstrated at graph K. Results are expressed as means ± SD, $n=5$, $^{*}p<0.01$ vs. respective control.

3.1.5. *Determination MMP in intact B-lymphocytes*

To search for the indication of mechanisms involved in apoptosis, we examined the effects of EA on $\Delta\Psi_m$. The treatment with EA (25 μM for 24 h) had higher percentage of low $\Delta\Psi_m$ in CLL B-lymphocytes in comparison to the healthy B-lymphocytes (Fig. 5).The change of $\Delta\Psi_m$ in CLL B-lymphocytes has been shown with arrow.

3.2. *Mitochondria assay*

3.2.1. *Succinate dehydrogenase activity*

Evaluations of EA for potential activity on mitochondria obtained from both CLL and healthy B-lymphocytes were carried out by studying the inhibitory effects of the compound on succinate dehydrogenase activity using the MTT assay. EA (10, 50 μM) strongly inhibited succinate dehydrogenase activity in only CLL

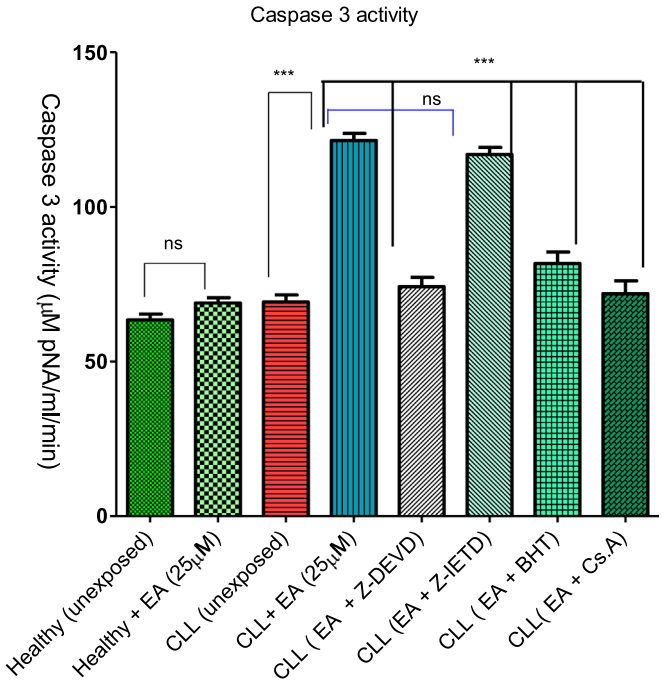

Fig. 3. CLL and healthy B-lymphocytes (10^6 cells/mL) were incubated in RPMI 1640 medium in conventional condition (37 °C and 5% CO_2-air) following the addition of EA to both groups. Caspase-3 activity was determined by Sigma-Aldrich kit. The kit determines produced pNA that is released from the interaction of caspase-3 and AC-DEVD-pNA (peptide substrate). As shown in figure, EA significantly increased the activity of caspae-3 in CLL B-lymphocytes BUT NOT in healthy B-lymphocytes. However only Cs.A (5 μM) and BHT (5 μM) but not Z-IETD (10 μM) prevented EA induced caspase 3 activation. Values are expressed as mean ± SD of three separate experiments ($n=5$).***:Significant difference in comparison with healthy control ($p<0.001$).

BUT NOT healthy B-lymphocyte mitochondria(Fig. 6, graph B). In healthy B-lymphocytes the inhibitory effect was shown only at the highestconcentrations 100 and 200 μM (Fig. 6, graph A).

3.2.2. ROS formation assay

ROS plays a role during cell apoptosis. We examined whether the level of ROS in CLL and healthy mitochondria were affected by EA with DCFH-DA staining. As shown by Fig. 7 graph B, treatment with EA at 5, 10 and 20 μM for 1 h,significantly induced ROS generation ($p<0.05$) in CLL mitochondria. These results suggested that EA induced ROS generation might underlie its effect on promoting CLL cell apoptosis. However as shown at Fig. 7 graph A, treatment with EA at above concentrations did not induce ROS generation in healthy mitochondria.

3.2.3. MMP assay in isolated mitochondria

To search for the identification of mechanisms involved in apoptosis, we examined the effects of EA on membrane permeability of mitochondria ($\Delta\Psi_m$) in isolated mitochondria from both groups. Treatment with different concentrations of EA (5, 10 and 20 μM for 1 h) induced significant decrease in $\Delta\Psi_m$ only in mitochondria obtained from CLL patient lymphocytes in comparison to the their untreated control (Fig. 8, graph B). Treatment with EA (5, 10 and 20 μM for 1 h) did not induce $\Delta\Psi_m$ collapse in mitochondria obtained from normal lymphocytes in comparison to their untreated control (Fig. 8, graph A). Furthermore, 5 μM cyclosporine A (an MPT blocker) strongly inhibited the decline of $\Delta\Psi_m$ induced by 10 μM of EA in CLL B-lymphocyte mitochondria ($p<0.05$). An MPT inducer, $CaCl_2$ (50 μM) was also used as a positive control in $\Delta\Psi_m$ assay (Fig. 8).

3.2.4. Mitochondrial swelling in isolated mitochondria

Induction of mitochondrial swelling in isolated lymphocyte mitochondria was monitored by following 540 nm absorbance (A540) decrease. EA addition (5, 10 and 20 μM) resulted in an extensive mitochondrial swelling in CLL mitochondria obtained from B lymphocytes of CLL patients (Fig. 9, graph B). EA addition to healthy mitochondria (5, 10 and 20 μM) not resulted mitochondrial swelling (Fig. 9, graph A). Furthermore, 5 μM cyclosporine A (an MPT blocker) strongly inhibited the decline of swelling in isolated B-lymphocytes mitochondria exposed to 10 μM of EA ($p<0.05$). An MPT inducer, $CaCl_2$ (50 μM) was also used as a positive control in MMP assay (Fig. 9).

3.2.5. Cytochrome c release

Our results showed that the EA significantly caused mitochondrial swelling and collapse of the mitochondrial membrane potential. These events could result in mitochondrial permeability transition and release of cytochrome c from mitochondria into the cytosolic fraction. As shown in Fig. 10, EA (10 μM) induced significant ($p<0.05$) release of cytochrome c in CLL mitochondria isolated from CLL patients but not healthy mitochondria. Significantly, the pretreatment of EA-treated mitochondria with the MPT inhibitor of cyclosporine A (Cs.A) and butylated hydroxyl toluene (BHT), an antioxidant, inhibited cytochrome c release as compared with EA-treated group (10 μM) ($p<0.05$), indicating the role of oxidative stress and MPT pore opening in cytochrome c release.

4. Discussion

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in adults in Western countries [35]. Despite advances in the treatment of chronic leukemias, CLL continues to be an incurable disease with median survival varying from 18 months to 12 years depending on severity [36]. Fludarabine has now been shown to be the most effective agent in the treatment of the disease, more effective than the previous standard therapy with chlorambucil. However, the toxicity of fludarabine has led to controversy over whether the drug should be used as first-line treatment or only after chlorambucil proved unsuccessful [37]. CLL is commonly considered as an instance for a malignancy of failed apoptosis. Cell division must occur, presumably in 'proliferation centers' in tissue microenvironments, accounting for the inexorable rise in white blood cell counts in some patients and evidenced by the shortening of telomeres. However, lack of apoptosis is considered a major component of the dysregulation of normal B-cell homeostasis in all subsets of this malignancy [38]. Evasion of apoptosis is a hallmark of CLL, Therefore there are callings for new strategies to induce apoptosis in this malignancy [10]. Natural polyphenols, the most abundant antioxidants in human diet, have many potential benefits in human health [39]. The relationship between natural polyphenols, apoptosis and cancer was identified by studies on the ability of these compounds to act as cancer chemo-preventive and/or chemotherapeutic agents [40]. Our result showed that EA as a natural polyphenol is selectively toxic on B-lymphocytes obtained from CLL patients (Fig. 1). The management of cancer through non selective drug therapies often results in deleterious effects on the normal healthy human cells which can result in other adverse reactions on different parts of the body. Our results also showed that EA has no toxicity on healthy B-lymphocytes at concentrations used (Fig. 1).

Defected apoptosis signaling represents a major causative factor in the development and progression of CLL. The ability of CLL B-lymphocytes to evade engagement of apoptosis can play a significant role in their resistance to conventional therapeutic

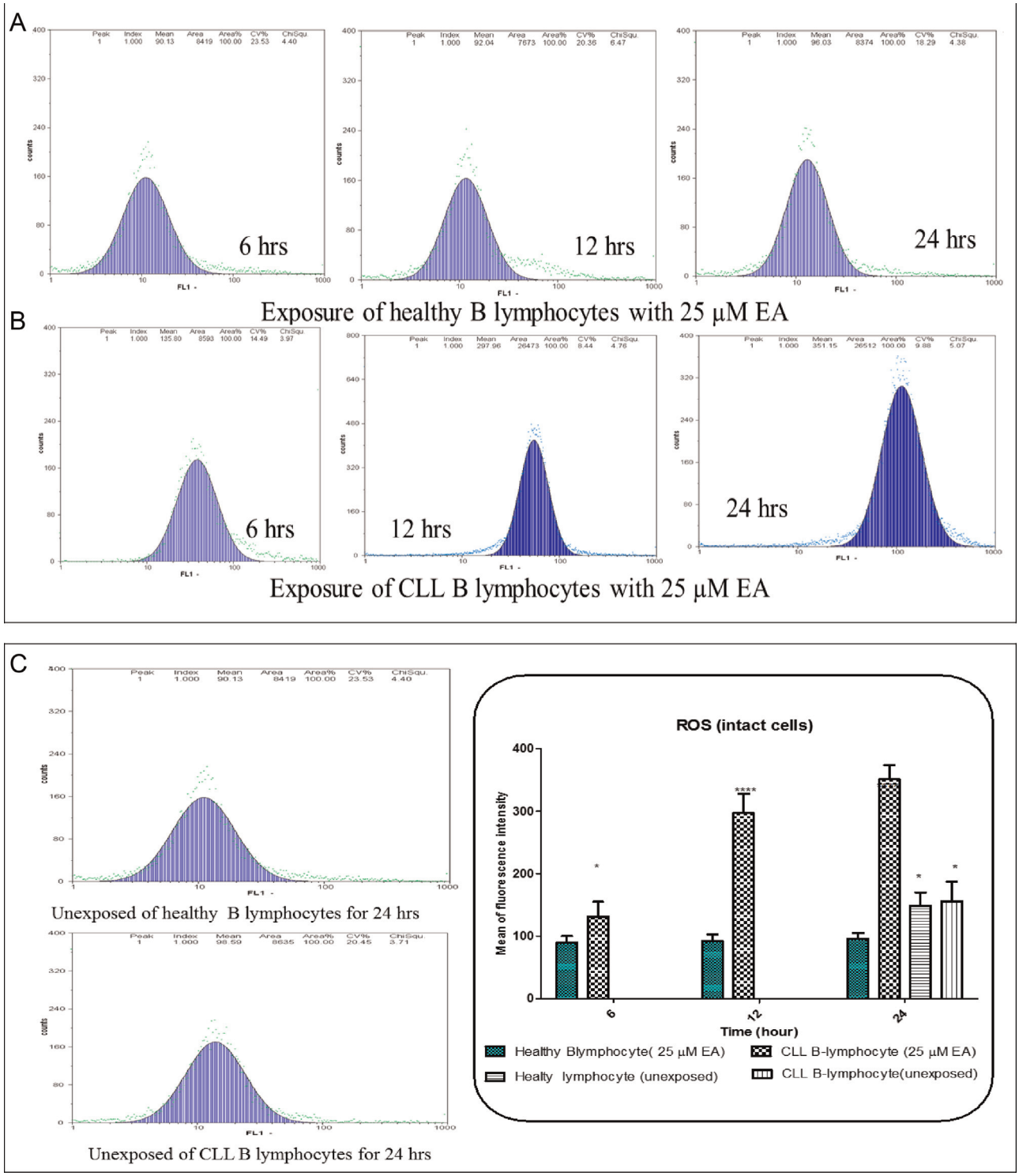

Fig. 4. Effects of EA on CLL and healthy B lymphocytes. EA-induced ROS generation in CLL but not in healthy B-lymphocytes (A and B). Changes mean of fluorescence intensity in ROS generation in CLL and healthy B-lymphocytes treated with EA 25 μM for 0–24 h summarized in graph D. Values (mean ± S.E.) are from three independent experiments (D). $^{*}p < 0.05$, $^{**}p < 0.01$ for difference in ROS generation between healthy and CLL in time interval (D).

regimens [10]. We proved that EA selectively induced apoptosis in CLL but not healthy normal B-lymphocytes (Figs. 2 and 3). According to accepted models, apoptotic cell death can be resulted from activation of two different but interrelated molecular cascades. An extrinsic pathway transduces the extracellular stimulus, protein death ligands, through plasmatic membrane [41]. Besides, an intrinsic pathway controls and monitors the intracellular environment, the mitochondria, which evaluate the molecular signals leading to death or survival. Pro apoptotic signals such as oxidative stress, damage in DNA, and alterations in mitochondrial membrane trigger apoptosis activation [42].

Findings obtained in this study proved that EA selectively rises intracellular ROS formation in CLL B-lymphocytes (Fig. 4). The intrinsic pathway of cell death is especially susceptible to ROS. Previously, it has been shown that exogenous addition of H_2O_2 has an inhibitory effect on Na^+/H^+ exchanger activity resulting in

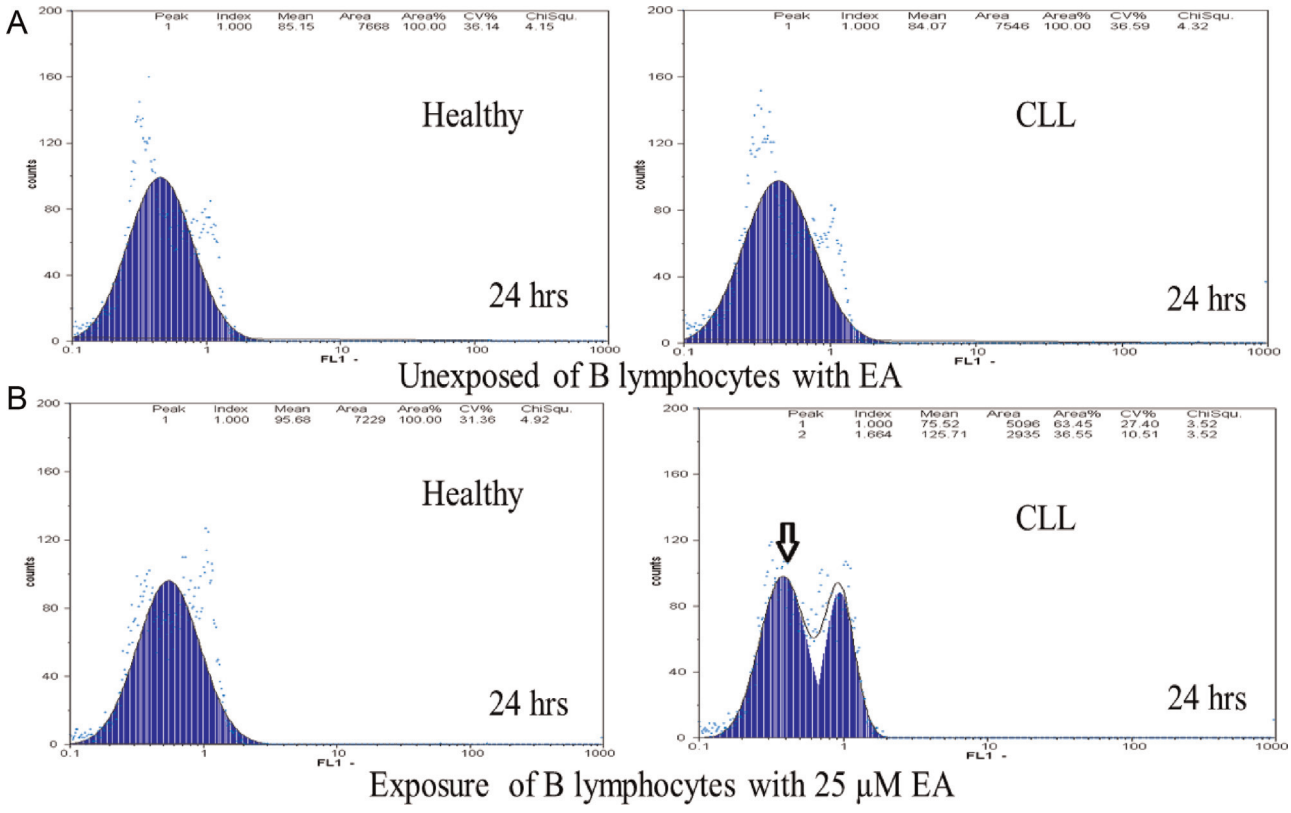

Fig. 5. The effect of EA on $\Delta\Psi_m$ of healthy and CLL B-lymphocytes (A and B). Freshly isolated purified B-lymphocytes were incubated with 25 μM EA for 24 h. $\Delta\Psi_m$ was measured following rohodamine 123 staining with flow cytometry. The presented data revealed that exposure to EA caused a significant decrease in $\Delta\Psi_m$ in CLL B-lymphocytes compared with those suspended in the EA-free medium with 0.05% (v/v) DMSO (untreated CLL control).

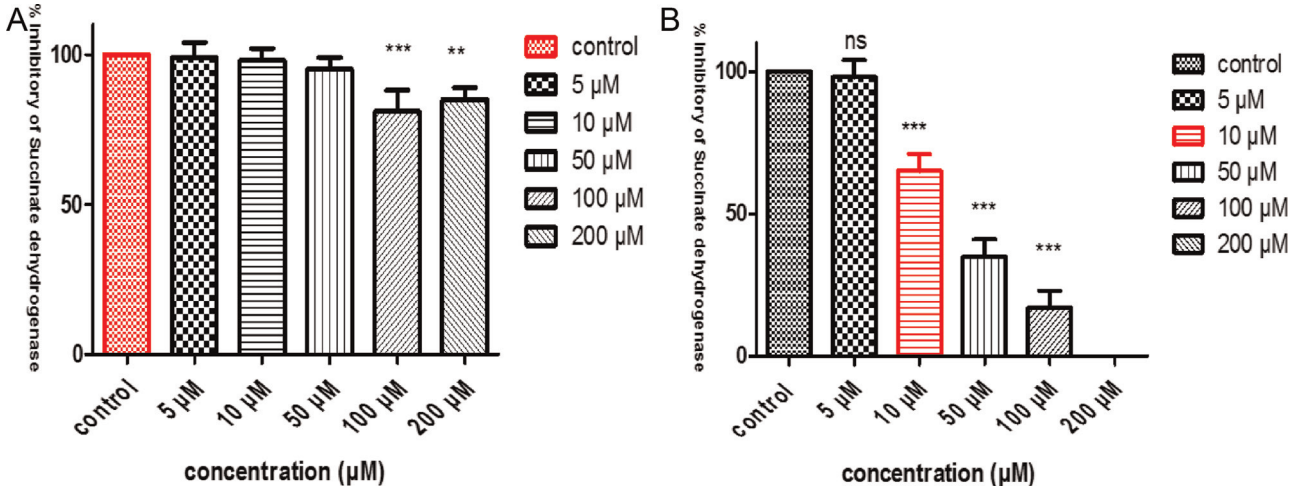

Fig. 6. Succinate dehydrogenase activity. The effect of EA on succinate dehydrogenase activity in both healthy and CLL mitochondria obtained from human lymphocytes were evaluated by MTT assay following 1 h of treatment. Values are mean ± SD of three determinations (graph A and B).

cytosolic acidification, which creates a conducive environment for apoptosis [43]. The inner mitochondrial protein, ANT, is also a target of ROS modulation by virtue of its redox-sensitive cysteines, providing an additional mechanism by which drug-induced ROS production may activate mitochondrial apoptosis [44] Besides the mitochondrion is a main source of reactive oxygen species (ROS) [45]. Our findings also showed that CLL but not healthy B-lymphocytes treated with EA experience collapse of mitochondria membrane potential (MMP) (Fig. 5). This confirmed that probably EA selectively targets mitochondria only in CLL B-lymphocytes. Our results on isolated mitochondria also confirmed that EA selectively inhibited succinate dehydrogenase only in CLL but not healthy mitochondria (Fig. 6). ROS formation significantly also increased only in CLL but not healthy mitochondria (Fig. 7). In fact, many anti-tumor agents have been reported to act as pro-oxidant agents for instance inhibitors of mitochondrial respiratory chain, chemicals resemble ubiquinone and inhibitors of mitochondrial antioxidant systems which all can eventually disturb the mitochondrial respiratory chain and lead to further increase in mitochondrial oxidative stress. Therefore mitochondria may appear as an important target for these compounds [46].

MMP is a universal feature of cell death and is often considered as the “point of no return” in the cascade of events leading to apoptosis [47]. Shortly after the discovery that MMP is frequently

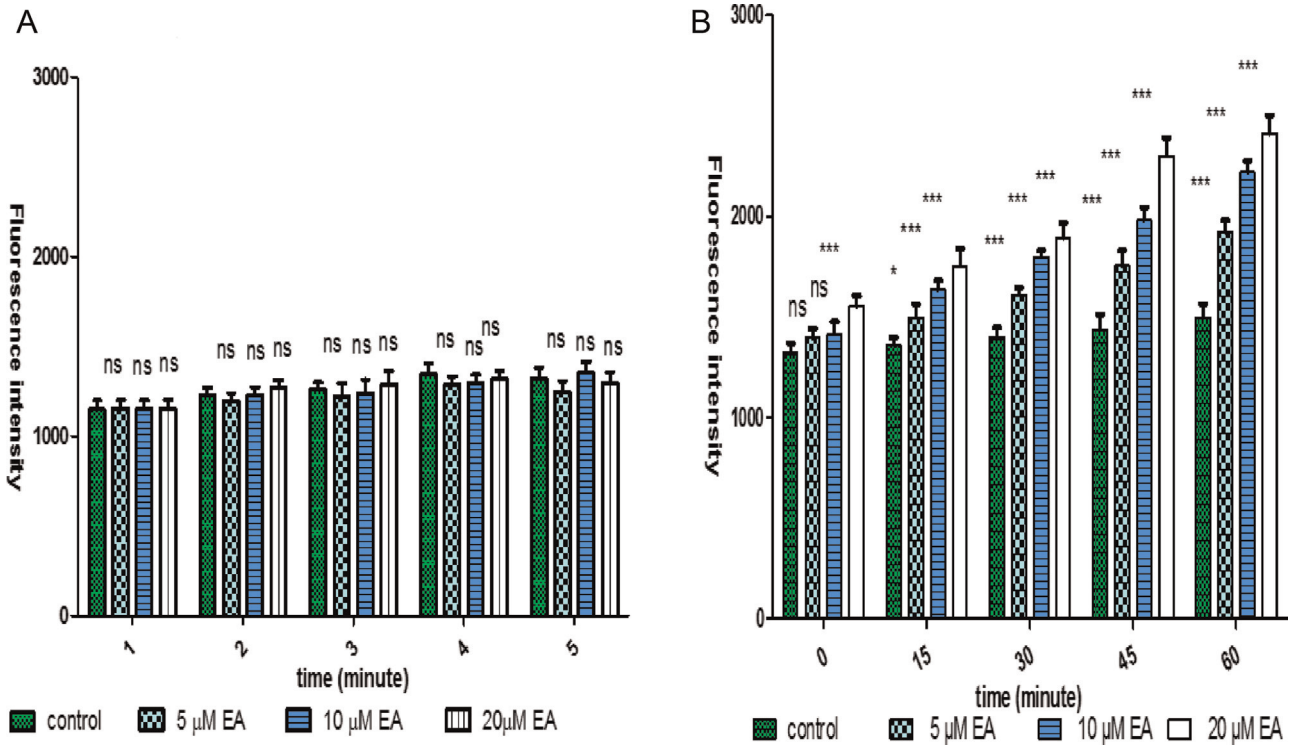

Fig. 7. The effect of EA on ROS formation in both healthy and CLL mitochondria. Freshly isolated purified mitochondria were obtained from both healthy and CLL human donors incubated with EA for 1 h. ROS was measured by DCFH staining with spectrofluorimetric method. The fluorescence intensity of DCF was enhanced by EA in comparison to control of each group during 1 h of incubation. $n=5$. *The minimum significant level was $p<0.05$.

impaired in cancer, mitochondria have become an attractive target to induce apoptosis and to overcome resistance to chemotherapy [48]. Currently, more than 20 mitochondrion-targeted compounds have been reported to induce apoptosis selectively in malignant cell lines [49]. We showed that EA could selectively induces collapse of MMP, mitochondrial swelling and cytochrome c release in CLL mitochondria and as well as in intact CLL B-lymphocytes with almost no effect on healthy B-lymphocytes and their mitochondria (Figs. 5 and 8–10), suggesting directly targeting of mitochondrial as a possible mechanism for the anticancer effect of phenolic compound.

One of our determinative findings was that EA induced cytochrome C release prevented by BHT (a ROS scavenger) and Cs.A (an inhibitor of MPT pore). Despite the previously mentioned fact that cytochrome c , by itself can act as a pollutant by oxidizing DCFH directly or indirectly via a peroxidase-type mechanism, forming DCF and demonstrating the false ROS formation during apoptosis of cells loaded with DCFH-DA, this result confirms that

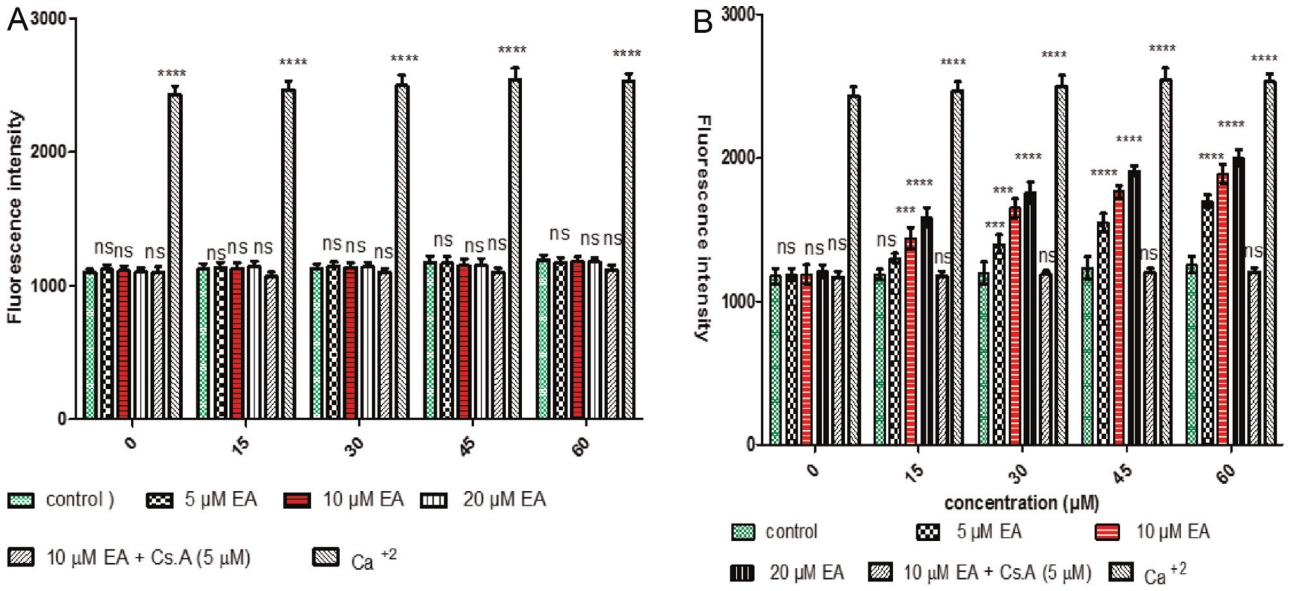

Fig. 8. The effect of EA on $\Delta\Psi_m$ decreasing. Freshly isolated purified mitochondria from healthy and CLL B-lymphocytes were treated as indicated above. $\Delta\Psi_m$ was measured by rhodamine 123 staining with spectrofluorescence method. The presented data revealed that EA-induced a decrease in $\Delta\Psi_m$ in CLL mitochondria obtained from CLL patients (graph B) BUT NOT in healthy mitochondria obtained from healthy donors (graph A). Data presented as fluorescence intensity. $n=5$. *The minimum significant level was $p<0.05$.

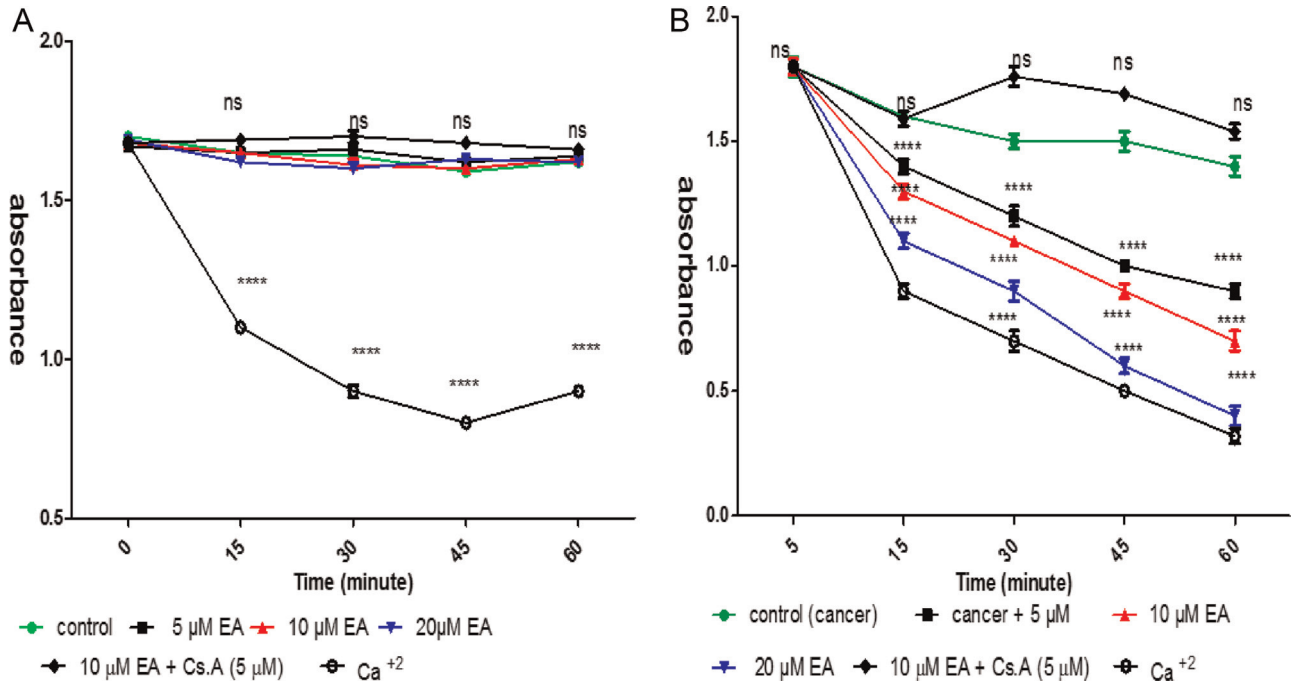

Fig. 9. Induction of mitochondrial swelling by EA. Addition of EA (5, 10 and 20 μM) induces mitochondrial swelling in CLL BUT NOT in healthy B lymphocyte mitochondria in a concentration depending manner. Mitochondria were suspended in swelling buffer and incubated for 1 h. The mitochondrial swelling was measured by following absorbance (λmax=540 nm) decrease.

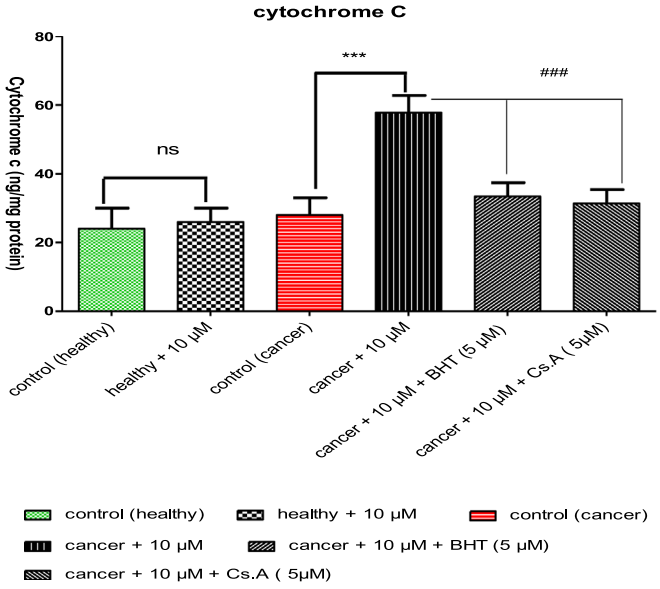

Fig. 10. EA (10 μM) on the cytochrome c release in the CLL and healthy mitochondria isolated from both group. As shown in this figure, pretreatment of with BHT and Cs.A significantly inhibited cytochrome c release in the CLL mitochondria BUT NOT in healthy mitochondria. The amount of expelled cytochrome c from mitochondrial fraction into the suspension buffer was determined using human Cytochrome c ELISA kit as described in above. Values presented as mean ± SD (n=5). *# Minimal significance level $p < 0.05$.

cytochrome c release measured in our study apparently did not have any interaction with reactive oxygen species (ROS) determined by DCFH-DA.

ROS may promote MPT (mitochondrial permeability transition) pore by causing oxidation of thiol groups on the adenine nucleotide translocator [50]. Reactive oxygen species (ROS) play a key role in promoting mitochondrial cytochrome c release and induction of apoptosis. ROS induce dissociation of cytochrome c from cardiolipin on the inner mitochondrial membrane, and cytochrome c may then be released via mitochondrial permeability transition (MPT)-dependent or MPT-independent mechanisms. Stimuli triggering apoptosis through mitochondria pathway, resulting in the release of cytochrome c from the mitochondrial intermembrane space (MPT pores) into cytoplasm and activation of downstream effectors including caspases [51].

Finally our results suggest that EA can act as an anti-cancer candidate by directly and selectively targeting mitochondria could induce cell death through ROS mediated mitochondrial pathway which finally ends in cytochrome c release, caspase 3 activation and apoptosis in cancerous B-lymphocytes isolated from CLL patients.

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