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Abstract

The effects of a novel nutrient formulation Epican Forte (EF) were evaluated on proliferation and induction of apoptosis using non-cytotoxic concentrations against HTLV-1 positive (HuT-102 & C91-PL) and negative (CEM & Jurkat) cells.

EF showed anti-proliferative effect as determined by MTT assay and TGF mRNA protein expression using RT-PCR. EF resulted in the down-regulation of TGF-α and an up-regulation in TGF-β2.

EF caused a significant increase in apoptotic cells in the preG$_1$ phase. These results were confirmed using Cell Death ELISA and Annexin V-FITC. Induction of apoptosis was caused by an up-regulation of p53, p21 and Bax protein levels and a down-regulation of Bcl-2α protein expression level.
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Keywords: Apoptosis; HTLV-1; Epican Forte; Antioxidants; Amino acids; Leukemia; Ascorbic acid (AA); Epigallocatechin-3-gallate (EGCG)

1. Introduction

Human T-cell lymphotropic virus type-1 (HTLV-1) is the causative agent of adult T-cell leukemia (ATL), a fatal disease with average survival time of less than 1 year. Only 5% of the infected patients develop the disease after a very long latency period of 30–40 years. The virus is usually transmitted sexually, transplacentally from mother to child, through breast-feeding, or through blood transfusion.

Rath's work published in 1991 [1] indicated that the most critical pathomechanism of cancer, which is unrestricted cellular proliferation and enzymatic destruction of the connective tissue can be controlled by nutrients essential for maintaining connective tissue integrity. Research in this field has led to the development of Epican Forte (EF). EF has been shown to be effective in inhibiting cell invasion, proliferation and angiogenesis of various solid cancers [2–6].

AA was shown to inhibit the retroviral enzyme, reverse transcriptase (RT), activity in chronically, acutely and latently infected cell lines in vitro [7–12]. AA inhibited cell division in prostate cancer through the production of H_2O_2 [13]. Vitamin C has a crucial role in the production and structure of collagen fibers and is involved in the strengthening of ECM, thus preventing the spread of cancer [14]. EGCG has been shown to possess chemo-preventive, anti-mutagenic, anti-inflammatory and anti-cancerous activities mainly due to its pro-apoptotic effects [15–17]. EGCG inhibited the proliferation of acute myeloblastic leukemia cells and the inhibitory concentrations tested on leukemic cells exhibited no growth inhibitory effects on normal lymphocytes [18]. Using EGCG as a nutrient synergy component indicated a high anti-cancer activity [19]. NAC raises the intracellular GSH levels, which is a defense against oxidative stress, necessary for mixed lymphocyte reactions like T-cell proliferation, T- and B-cell

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differentiation, cytotoxic T-cell activity and natural killer cell activity, and the regulation of signal transduction [20,21]. It also reduced the invasiveness and metastasis potential of melanoma cells and the tissue invasion of endothelial cells [22]. L-Lysine can help decrease the breakdown of collagen fibers in extracellular matrix (ECM), basement membranes, and the walls of blood vessels, thus leading to the reduction of the ability of cancer cells to undergo metastasis [23,24]. The trace metal selenium inhibited the production of MMP-2 and MMP-9 by fibrosarcoma cells [25]. Also, it interfered with the activity of MMP-9 and reduced the migration of endothelial cells through ECM [22,26]. Metabolites of selenium induced apoptosis in cancer cells [27]. Arginine is a precursor for the production on nitrogen oxide (NO) which acts as an inducer of apoptosis [28–30]. Cancer patients excrete more hydroxyproline, which is a product of L-proline, in their urine than healthy individuals. Moreover, hydroxyproline in cancer tissue is low. It is likely that proline synthesis cannot keep up with the extra demand the body exerts when it is going through stress or disease [31,32]. The role of both copper and manganese induction of apoptosis has resulted in cell cycle arrest in G_1 phase with apoptosis which was p53 dependant [33,34]. It is reported that supplemental calcium is associated with a decreased risk of colon cancer [35–39]. However, this has been a controversial issue [40,41].

The objective of this study is to evaluate the anti-cancer effectiveness of EF, by inhibition of cell proliferation and induction of apoptosis on several HTLV-1 positive and negative malignant leukemia cell lines.

2. Materials and methods

2.1. Cell lines and treatment

Four leukemia cell lines were used. C91-PL and HuT-102 are HTLV-1 positive cell lines whereas CEM and Jurkat are HTLV-1 negative. C91-PL and HuT-102 cells (a gift from A. Gessain, Institut Pasteur, Paris, France) are ATL-derived HTLV-1 infected $CD4^+$ T-cell lines that upon replication constitutively express the retrovirus HTLV-1. CEM and Jurkat are uninfected human T-cell line obtained from patients with T-cell leukemia. CEM cells are T-lymphoblastoid cell lines originally derived from peripheral blood of a Caucasian female infected with Acute Lymphoblastic Leukemia (ALL), Jurkat cell lines were primarily established from the peripheral blood of a patient with ALL [42]. The advantage of using Jurkat cells is that they have the same markers as of the normal lymphocytes. Cells were suspended in RPMI 1640 with 25 mM of the indicator Hepes. Ten percent heat-inactivated Fetal Bovine Serum (Gibco-BRL, Paisley, Scotland), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Paisley, Scotland) were added to the media. The cells were kept in a humidified incubator (37 °C) at 95% air and 5% CO_2.

2.2. Epican Forte

Epican Forte was obtained from Dr. Rath Research Institute and it has the following ingredients in a 1 mg/ml solution: 900 μM of ascorbic acid, 1.1 mM of L-lysine, 1.1 mM of L-proline, 500 μM of L-arginine, 250 μM of *N*-acetyl L-cysteine, 150 μM of epigallocatechin gallate, 85 μM of selenium, 7 μM of copper and 4 μM of manganese.

2.3. Isolation of freshly activated human mononuclear lymphocytes

Blood was collected from healthy donors and used for the isolation of human mononuclear blood lymphocytes. Isolation was done on a Ficoll–Isopaque gradient (1.077) and used immediately. Lymphocytes were cultured using RPMI 1640 complete growth medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} β-mercaptoethanol and phytohemaglutinin (5 μg/ml, total protein concentration) for 4 days. Cytotoxicity of EF was evaluated using the Trypan Blue exclusion method.

2.4. Cell proliferation and LDH cytotoxic test

The cells were distributed in 96 well plate microtitre plates at a 1×10^5 density. Cells were incubated for 24 h prior to treatment in RPMI complete growth medium. Treatment with the test compound was then carried out daily for 2 or 4 days depending on the experimental conditions. Cytotoxicity and cell proliferation were determined using the Cell Titer96™ non-radioactive cell proliferation assay and the CytoTox 96 non-radioactive Cytotoxicity assay following the manufacturer's directions (Promega Corp. Madison, WI). The proliferation assay is an MTT-based method which measures the ability of the mitochondria of metabolically active cells to convert tetrazolium salt into a formazan product and its absorbance is recorded at 570 nm. The CytoTox 96 Assay quantitatively measures the enzyme lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released from the cells upon lysis. Released LDH in culture supernatants is measured with a coupled enzymatic assay which results in the conversion of a tetrazolium salt into a red formazan product, the absorbance of which is measured at 490 nm.

2.5. Flow cytometry analysis of DNA content

Cells suspended in complete growth medium were distributed in 25 cm^2 tissue culture flasks and incubated for 24 h. The cells were then treated daily with EF at varying concentrations for 2 or 4 days. Cells were then spun down, washed twice with phosphate buffered saline (PBS) (Gibco-BRL, Paisley, Scotland), permeablized with 70% ethanol, treated with 1% RNase and finally stained with propidium iodide (Molecular Probes, Engene, Oregon) (100 μg/ml

final concentration). The distribution of cell cycle phases with different DNA content was determined using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The increase in the amount of cells in the pre G_1 (<2*n*) phase indicate an increase in apoptosis. Diploid G_0/G_1 cells are indicated by (2*n*), S-phase is (>2*n* but <4*n*), and G_2/M-phase is (4*n*) and the DNA content was determined using Cell Quest software.

2.6. Annexin V-FITC

The Annexin-V-Fluos Staining Kit (Roche Mannheim, Germany) was used to verify whether EF exerted an apoptotic or necrotic effect. This was done by growing the cells in the presence and absence of different non-cytotoxic concentrations of the test compound for 4 days. Cells were harvested and washed twice with cold PBS. Then, cells were gently suspended in a solution containing 100 μl of 1× binding buffer, 50 μl of Annexin V-FITC, 50 μl of propidium iodide (50 μg/ml) and incubated in the dark at room temperature for 15 min. Cells were subjected to flow cytometry analyses using a FACScalibur (Becton Dickinson) machine. This technique allows the detection of the binding of Annexin V-FITC to the cell surface, which is an early indication of apoptosis. Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with a high affinity for phospholipids. To differentiate between apoptotic and necrotic cells, PI was applied simultaneously which is used for dye exclusion tests and thus allows the distinction between necrotic cells (Annexin-V positive, PI positive stained cell cluster) and apoptotic cells (Annexin-V positively, PI negative stained cell cluster) [43]. CellQuest™ software (Becton Dickinson) analysis was used in order to determine which fraction of the cells was apoptotic or necrotic. Cell cycle distribution and DNA ploidy status were determined using a FL2-area/FL2-width dot plot using Modfit LT 2.0™ software (Verity Software Inc) after the exclusion of cell doublets and aggregates.

2.7. Cell death by ELISA

An ELISA kit was used to assess cell death (Roche Molecular Biochemicals, Mannheim, Germany). The ELISA kit quantitatively detects cytosolic histone-associated DNA fragments. Briefly, cells were seeded in 96-well culture plates at a density of 1×10^4 cells per well. After treatment, cells were lysed and whole nuclei were spun down by centrifugation. An aliquot obtained from the supernatant was used as an antigen source in a sandwich ELISA with primary anti-histone monoclonal antibody coated to the streptavidin-coated well. This was followed by the addition of a second anti-DNA monoclonal antibody coupled to peroxidase. By measuring the amount of peroxidase on the antibodies, the number of histones was determined. Peroxidase activity was determined photometrically at 405 nm with 2,2′-azino-di (3-ethylbenzthiazolin-sulfonate) (ABTS) used as a substrate.

2.8. Protein extraction and Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 4% protease inhibitors and 1% phosphatase inhibitors. Cells were kept in boiling water for 5 min and then centrifuged at 14,000 rpm for 10 min after cooling down. The supernatants were collected and kept on ice. Protein concentrations were determined using the DC BioRad protein assay kit (BioRad Laboratories, Hercules, CA) with bovine serum albumin used as a standard. A total of 30 μg of cellular protein was loaded onto 10% SDS-polyacrylamide gels. The separated protein bands were then transferred electrically to PVDF membranes (NEN Life Sciences Products, Boston, MA). The following primary antibodies, obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), were used for immunoblotting: Bcl-2α (N-19), p53 (FL-393), p21 (F-5) and Bax. Subsequently, membranes were probed with horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA). The actin antibody (Biogenesis, England) was used to ensure equal protein loading. The detection of the protein bands was done using an enhanced chemiluminescence system according to the manufacturer's instructions and results were obtained on film developed using a Xomat (Amersham, Pharmacia, Biotech).

2.9. Analysis of TGF mRNA by RT-PCR

Total RNA was isolated from cells using the SV Total RNA Isolation Kit (Promega, Madison, WI) following the manufacturer's directions. Precipitated nucleic acids were washed with 75% ethanol, dried and resuspended in water. To transcribe total RNA into cDNA, 10 U of avian myeloblastosis reverse transcriptase was used (Promega). Oligo(dT) 17 primer (50 pM), 0.2 mM each of deoxynucleotide triphosphate (dATP, dGTP, dCTP, and dTTP; Promega), reaction buffer (50 mM Tris–HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol) were added in a total volume of 50 μl for 60 min at 42 °C. Polymerase Chain Reaction (PCR) was used to amplify cDNA using specific oligonucleotide primers (TGFα; TGFβ1 and TGFβ2) designed to detect the target cDNAs (Table 1) [44].

PCR reactions were conducted in 100 μl of 75 mM Tris–HCl, pH 9, 20 mM $(NH_4)_2SO_4$ 0.01% Tween 20, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of DNA polymerase, 50 pM of each primer and 2 ml of the cDNA mixture. PCR was performed according to the following program: denaturation for 45 s at 94 °C, annealing at 50 °C for 45 s, and elongation at 72 °C for 45 s, followed by a final 5 min at 72 °C. For each oligonucleotide pair and for every RNA sample, a preliminary analysis was conducted to define the appropriate range of cycles consistent with an exponential increase in the amount of DNA product. Ten microliters of the PCR products were separated by running them on 3% agarose gels stained with ethidium bromide; the intensity of the luminescent bands was evaluated using the Molecular Analyst/PC image anal-

Table 1
Synthetic oligonucleotides and experimental conditions used for RT-PCR analysis

Gene	Position	Size	Sequence	Number of cycles	T^0 hybridization
Ribosomal phosphoprotein	344–363 850–830	486	5′GTTCACCAAGGAGGACCTCA3′ 3′AGACACCTCTGCCTAATGTG5′	28	50
TGF-β1	731–752 1415–1392	661	5′GAAGTCACCCGCGTGCTAATGG3′ 3′GGATGTAAACCTCGGACCTGTGTG5′	32	50
TGF-β2	1–22 214–192	192	5′TTCGCAGGTATCGATGGCACCT3′ 3′CGTCGTATTAACGACGGAAGCGG5′	37–40	50
TGF-α	3538–3557 3930–3911	373	5′ATGTTGTTCCCTGCAAGTCC3′ 3′ACTATGGAGAGGGGTCGCTT5′	30	50

ysis software (BioRad Laboratories, Hercules, CA). Results were expressed as relative densitometric units, normalized to the values of the phosphoribosomal protein mRNA used as an internal control. Control PCR was performed by using RNA samples which were not exposed to reverse transcription, to detect the presence of any amplified fragment(s) corresponding to genomic DNA.

2.10. Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher's least significant differences at $p \leq 0.05$ (Fisher PLSD). An effect was considered significant when the value (±) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

3. Results

3.1. Effects of EF on cytotoxicity and proliferation

The cytotoxicity of EF was evaluated at 48 and 96 h of exposure using four leukemic cell lines. Cells were grown in the presence and absence of different concentrations of the test compound and the infectious dose at which 50% of the cells died was determined (IC_{50}). The corresponding reduction of proliferation was recorded at the IC_{50}.

The results showed a dose-dependant decrease in viable cell counts in all tested cell lines. In the case of the positive cell lines, HuT-102 showed an IC_{50} of 586 and 500 μg/ml at 48 and 96 h, respectively with significant corresponding significant decrease in proliferation of 38% and 9% at IC_{50} as compared to the control ($p < 0.05$). While in the case of C91-PL cell line, the IC_{50} at 48 h of exposure was 772 and 365 μg/ml at 96 h. A significant corresponding inhibition of proliferation was 18% after 48 h and 35% after 96 h ($p < 0.05$). Concerning the negative HTLV-1 cell lines, IC_{50} for CEM's was 450 μg/ml at 48 h and 380 μg/ml at 96 h of treatment with a corresponding significant inhibition of proliferation of 53% at 48 h and 30% at 96 h as compared to the control ($p < 0.05$). In the case of Jurkat, the IC_{50} was 615 μg/ml at 48 h and 400 μg/ml at 96 h. Corresponding cell proliferation was significant with 55% decline at 48 h and 72.4% at 96 h compared to the control ($p < 0.05$) (Fig. 1a and b). No significant cytotoxic effects were noted when EF was tested on activated fresh human mononuclear lymphocytes ($p > 0.05$) (Fig. 1c).

3.2. Effects of EF on the transforming growth factor (TGF) expression

In order to further verify the effects of EF on cell proliferation, its effects on the expression of TGF mRNA using RT-PCR was investigated. TGF is a growth factor that regulates essential functions such as proliferation, differentiation and apoptosis.

Cells were treated with different non-cytotoxic concentrations of the compound (*C*, 200, 250, 300, 350 and 500 μg/ml).

RT-PCR was used to amplify RNA isolated from the cells. Then the RNA was separated by agarose gels electrophoresis. The effects of EF on the expression of proteins involved in cell cycle arrest, such as TGF-α, TGF-β1 and TGF-β2 were evaluated by RT-PCR.

As shown in Fig. 2a and b, the in the presence of increasing concentrations of EF a gradual down-regulation of TGF-α (an anti-apoptotic protein) and up-regulation of TGF-β2 (pro-apoptotic protein) was observed. However there was no change in TGF-β1 expression (Fig. 2a and b).

3.3. Effects of EF on cell cycle progression and apoptosis

The growth inhibitory effects of EF on cell cycle progression and/or induction of apoptosis were assessed using flow cytometry and ELISA-using flow cytometry and ELISA-based apoptosis assay.

3.3.1. DNA flow cytometry analyses

The DNA content of different cells treated with EF was determined using FACScan Flow Cytometer which measures the intensity of the signal emitted by the DNA stained with propidium iodide. DNA content is indicative of the different phases of the cell cycle. An increase in cells in the pre G_1 phase corresponds to an increase in apoptosis.

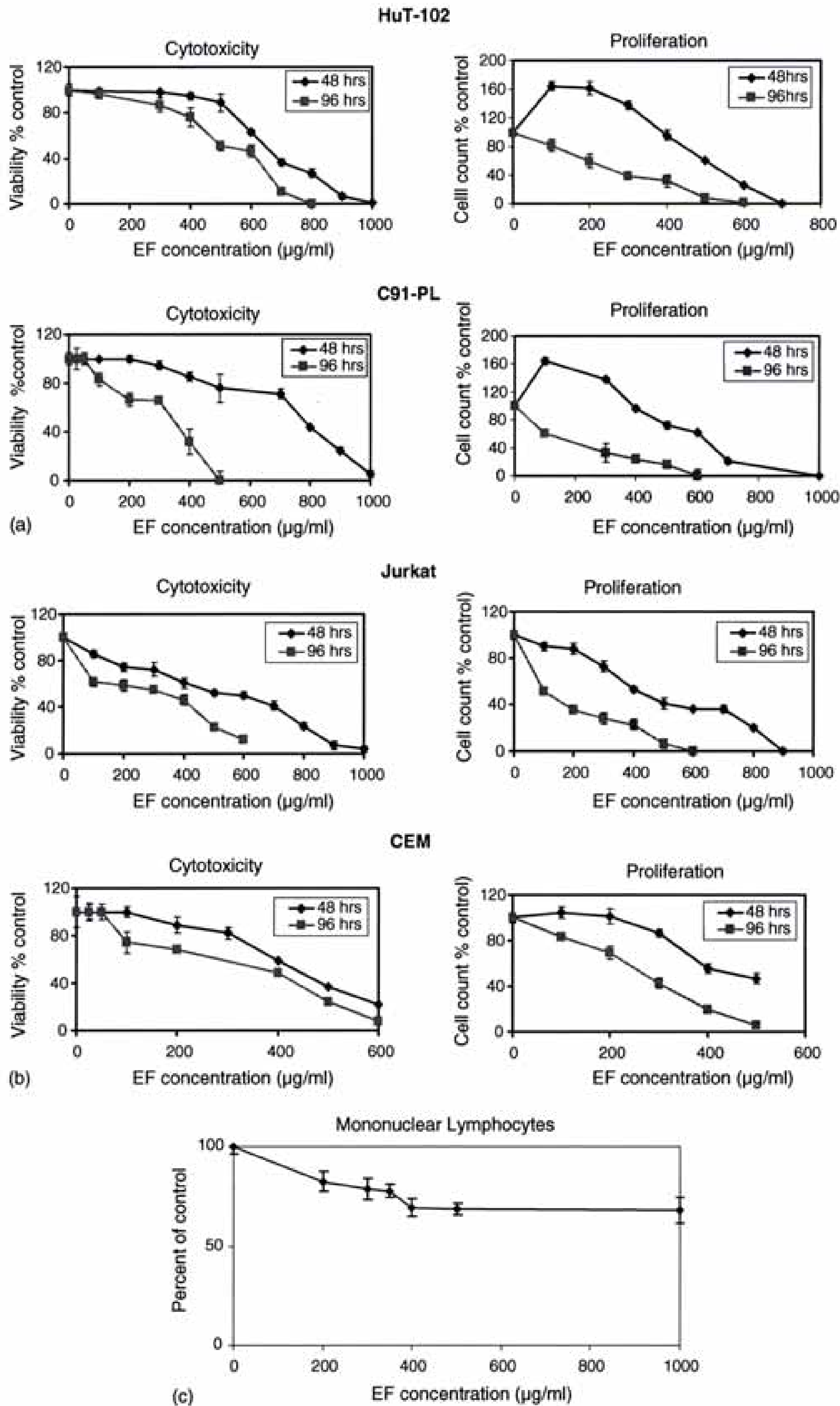

Fig. 1. (a–c) EF inhibits the proliferation of (a) HTLV-1 positive (HuT-102 and C91-PL), (b) HTLV-1 negative (CEM and Jurkat) and on (c) freshly activated human lymphocytes in a dose-dependent manner at non-cytotoxic concentrations Each value is the mean ± S.D. of three separate experiments done in triplicate.

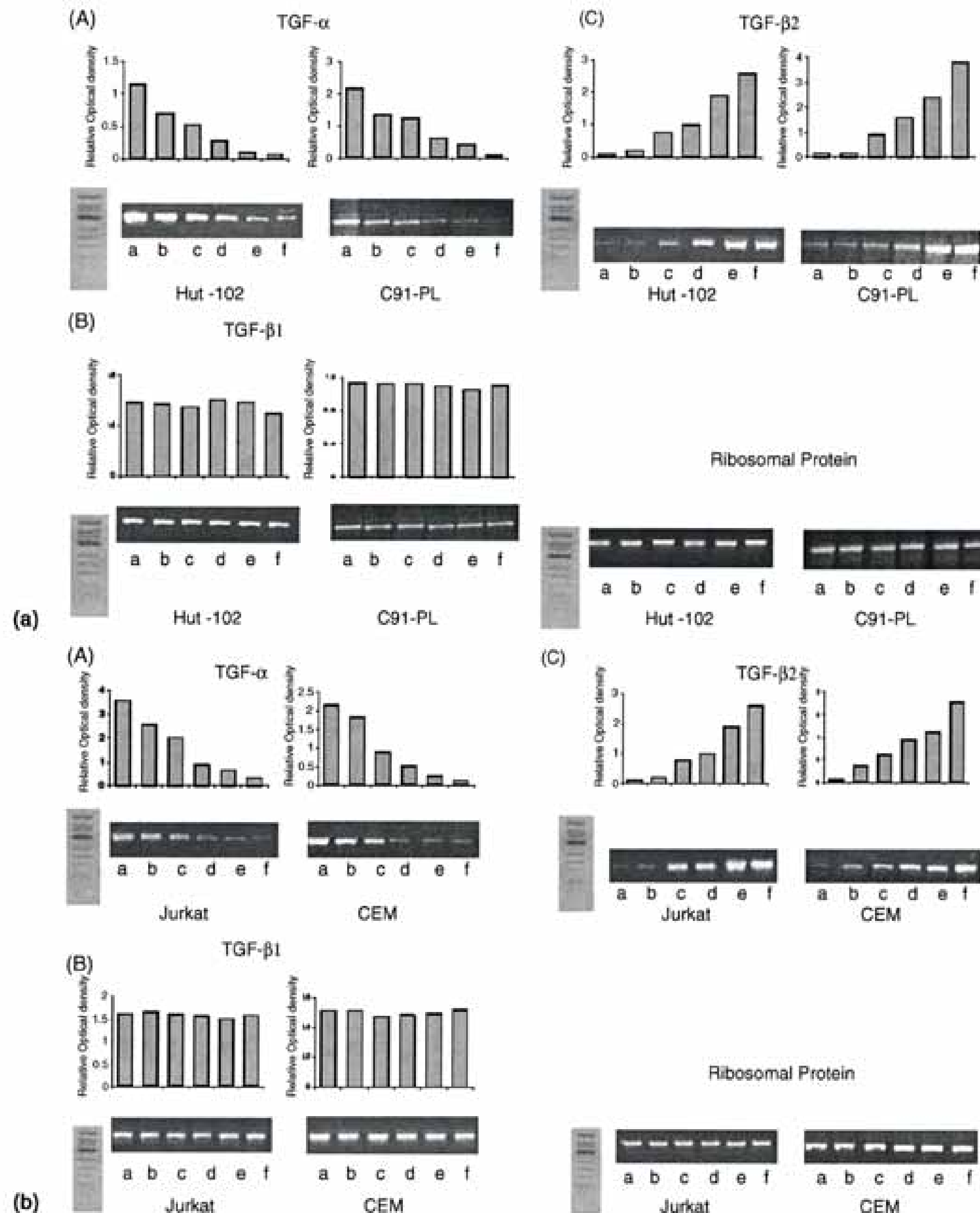

Fig. 2. (a–b) The effects of EF on the mRNA expression, using both HTLV-1 positive and negative cells, at 96 h of exposure. The expression of TGF α, β 1 and β 2 expression was determined using RT-PCR. The results are expressed as relative densitometry units normalized to the values of the phosphoribosomal protein mRNA used as an internal control. EF dose-dependently reduced the level of TGF-α mRNA expression and caused the up-regulation of the levels of TGFβ2 mRNA expression. However, TGFβ1 mRNA expression levels remained unchanged in HTLV-1 negative and positive leukemia cells. EF (μg/ml) control (a), 200 (b), 250 (c), 300 (d), 350 (e) and 500 (f).

At non-cytotoxic doses of EF, cell cycle distribution changes became apparent in all ATL cells treated with the test compound. The apoptotic effects of EF were indicated by the significant increase in the preG$_1$ (apoptosis) phase and the decrease in the S phase of the cell cycle in all tested ATL cells. Flow cytometry results showed an increase in cell apoptosis at 48 and 96 h. At 48 h, the HTLV-1 positive cell line HuT-102 showed a striking 70-fold increase in the cell cycle distribution in the preG$_1$ at ET concentration of 350 μg/ml. In the other HTLV-1 positive cell line, C91-PL, a 7.6-fold increase in cell cycle distribution in the preG$_1$ phase was noted. Regarding other, HTLV-1 negative cell lines CEM and Jurkat, the flow cytometry data showed a 48.4- and 60.5-fold increase in cells in the preG$_1$ phase respectively at 350 μg/ml of EF (Data not shown). A similar increase in cell cycle distribution in the preG$_1$ was also seen at 96 h with the around 20-fold increase in the two negative cell lines and one of the positive cell lines; C91-PL, while a much higher increase in the case of HuT-102 of around 53% (Fig. 3a and b).

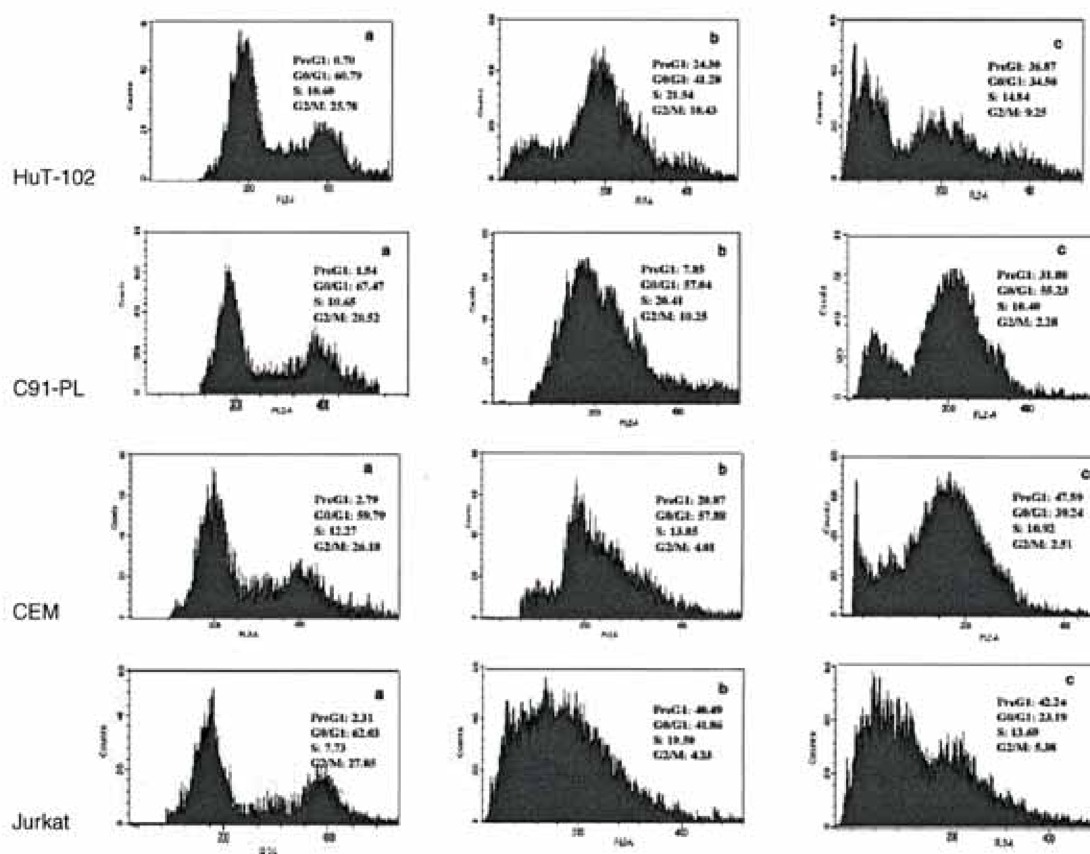

Fig. 3. (a) Flow cytometry data of HTLV-1 positive leukemia cells treated with EF shows an induction in cell cycle progression. Cells were grown under standard conditions at 50% confluence, were treated with different concentrations of EF for 96 h. Cells were then harvested for flow cytometric analysis of DNA content by PI staining. Distribution of cell cycle phases with different DNA contents was determined using a FACScan flow cytometer. The percentage of cells in the G_1, S and G_2/M phases of the cell cycle were determined using Cell Quest and are indicated at the top right of each figure. The histograms shown here represent the typical result from at least two independent experiments.

3.3.2. Cell death by ELISA

A hallmark of apoptosis is the fragmentation of DNA forming the DNA ladder. To further verify the results of flow cytometry we used Cell Death ELISA assay which uses optical density to quantitatively measure cytosolic histone associated DNA fragments, the indicators of cell death.

The ELISA results (Fig. 4) showed that EF induced apoptosis in all the cell lines tested. The results in all cell lines were significant at the 95% confidence level ($p < 0.05$). The HTLV-1 positive cell lines, C91-PL and HuT-102 showed a similar increase in cell death ranging from 2.7- to 3.1-fold was shown in HTLV-1 positive cell lines at 48 and 96 h of treatment with EF.

In the case of the HTLV-1 negative cell lines, a 5.4-fold increase in cell death was observed in the case of CEM and a 2.8-fold increase in the case of Jurkat at 500 μg/ml of EF at 48 h contact as compared to the control. Similar reductions in cell death ranging from 4.7- to 4.8-fold increase were observed in those cell lines at 96 h of EF treatment (350 μg/ml) as compared to the control (Fig. 4).

3.3.3. Annexin V-FITC

In order to investigate whether EF had an apoptotic or necrotic effect on cells, the Annexin-V flow-cytometric approach was used. The use of EF has resulted in an intense increase of Annexin-V staining typical of apoptosis in all the cells tested (Fig. 5a and b). A much higher increase in the proportion of apoptotic to necrotic cells after treatment with EF (%) was noted.

3.4. Effects of EF on the expression of apoptosis related proteins

The effects of EF on expression of certain important regulatory proteins involved in apoptosis were examined by Western blot using β-actin as a control to ensure equal loading. The expression of four proteins was tested: Bcl-2α, an anti-apoptotic protein, Bax, a pro-apoptotic protein, p53, a tumor suppressor gene, and p21, a cyclin dependent kinase inhibitor. Cells were grown in the presence of non-cytotoxic concentrations of the test compound (*C*, 200, 250, 300 and 350 μg/ml). The results showed that EF up-regulated the expression of p53, p21 and Bax protein levels and a down-

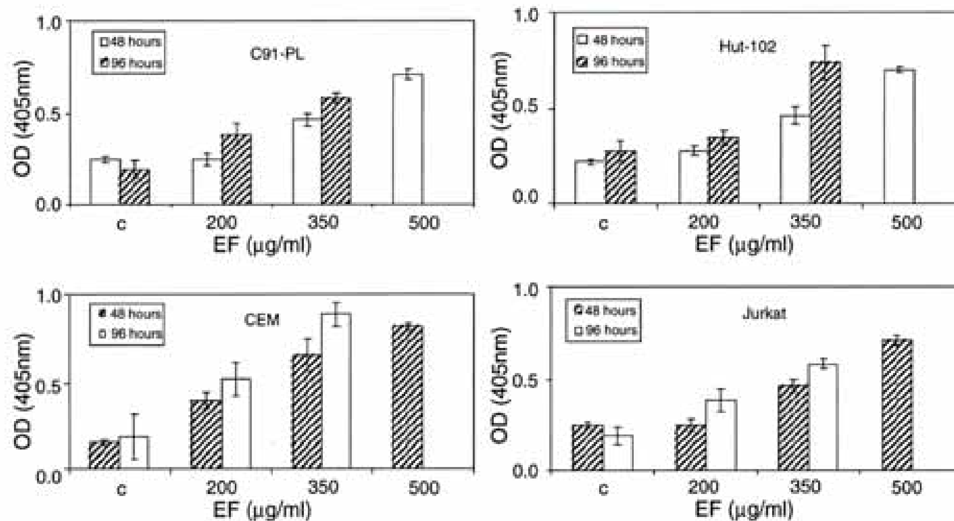

Fig. 4. EF induces apoptosis in ATL cells. Apoptosis was assessed using an ELISA kit, which quantitatively detects cytosolic histone-associated DNA fragments, as described in Section 2. Each value is the mean ± S.D. of three separate experiments done in triplicate.

regulated Bcl-2α protein in a dose-dependent manner for all the cells tested (Fig. 6a and b). Based on the quantification of data by scanning densitometry, EF showed a significant increase in the Bax/Bcl-2 ratio ($p < 0.05$) (Fig. 6c).

4. Discussion

There is no effective treatment for ATL. Hence, there is an urgent need for evaluating new compounds for the treatment of this cancer. Currently used experimental therapies have been associated with severe side effects. In this context, naturally derived compounds are likely to have better effectiveness with minimal cytotoxic effects. Our previous work with a novel compound, EF has shown promising results against a variety of cancers. The data presented in this study shows that EF exerts anti-proliferative effects in all ATL tested cells by inducing apoptosis. This was associated with the effect on TGF-α, TGF-β1 and TGF-β2 proteins involved in cell cycle arrest. As such, EF induced a decrease in the anti-apoptotic protein TGF-α levels, an increase in the pro-apoptotic protein TGF-β2 levels, and no change in TGF-β1 levels. Thus the results confirm inhibitory effect of EF on ATL cells proliferation.

The effect of EF on down-regulation of TGF-α level may have significant clinical implications. TGF-α is an important mediator of growth stimulation [45]. This effect is mediated through the NF-κB pathway [46]. Another significant finding is the up-regulation of the pro-apoptotic protein TGF-β2 by EF. This protein exerts its functions by regulating the expression of the apoptosis associated proteins Bcl-xL and bcl-2α in vivo [47]. However, the TGF-β1 expression in all the cell lines was not affected. The TGF-β1 belongs to a family of proteins that can induce apoptosis in a variety of cell types and its over expression has been shown to produce anti-proliferative effects in T and B-cells [48,49]. Based on those results, the anti-proliferative effect of EF involves the down-regulation of TGF-α and the up-regulation of TGF-β2 levels.

Induction of apoptosis by EF was evaluated using flow cytometry. In all the cells tested there was a marked increase in cell cycle distribution in the pre G_1 phase indicating a pro-apoptotic activity of EF. Those findings were further confirmed using cell death ELISA detection assay and by testing the levels of various proteins involved in apoptosis using Western blot analyses.

In all tested cell lines EF induced the expression of p53, p21 and Bax protein levels and down-regulated Bcl-2α protein expression in a dose-dependent manner. Bcl-2 is an important family of anti-apoptotic proteins. The up-regulation of this protein causes the immortality of cells and their transformation to malignancy by preventing the release of cytochrome *C* from the mitochondria thus blocking a major apoptotic pathway [50]. Bax is a pro-apoptotic protein that acts in opposition to Bax causing the release of cytochrome *C* from the mitochondria. Hence, Bax and Bcl-2 are antagonists and a balance between them is required for normal cells to function. p53, known as the guardian of the genome, is a tumor suppressor gene which triggers apoptosis when damage occurs in the cell. A posttranscriptional mechanism that stabilizes this protein is thought to be responsible for its up-regulation [51–53]. p21 is a cyclin-dependant kinase inhibitor that causes preG_1 phase arrest. p53 mediates cellular response to oxidative stress and genotoxic damage. After this protein is activated, it accumulates in the nucleus triggering the expression of p21 besides other proteins resulting in inhibition of cell proliferation by G_1 or G_2 cell cycle arrest and apoptosis. This important regulatory function makes any mutation or inactivation of p53 a possible pathway for cancer induction. EF showed that it exerted its strong apoptotic effect through the up-regulation of pro-apoptotic proteins.

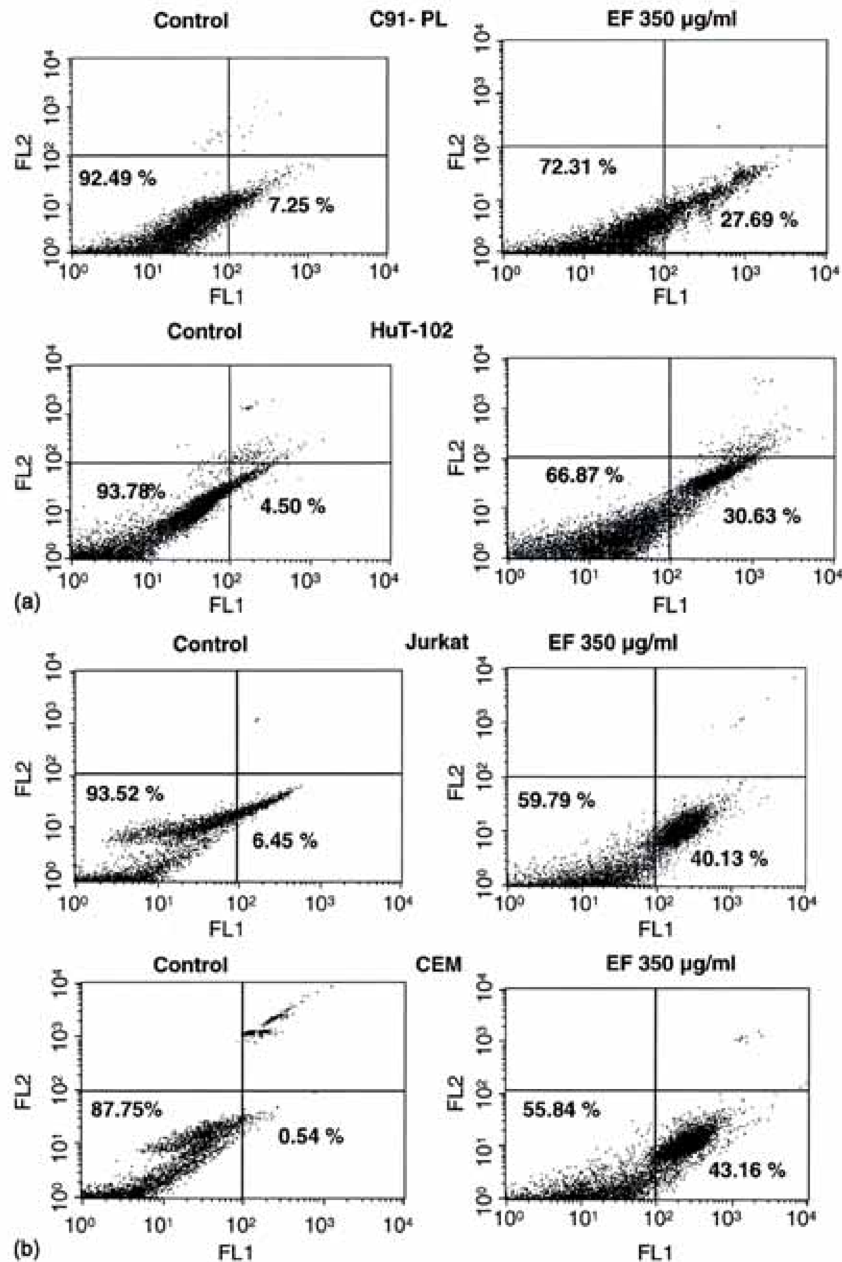

Fig. 5. (a) The effects of EF on enhancing apoptosis in both HTLV-1 positive and negative cells after staining with both Annexin V and PI and analyzed by flow cytometry. Cells in the left bottom quadrant (Annexin V-negative, PI negative) are viable. Cells in the bottom right quadrant (Annexin V-positive, PI-negative) are in the early stages of apoptosis. Finally, cells in the top right quadrant (Annexin V-positive, PI-positive) are in later stages of apoptosis and necrosis.

EF has low cytotoxicity with high anti-proliferative potency and its strong pro-apoptotic effects. These effects may be attributed to a synergy among the different components and to triggering of different cellular mechanisms involved in cancer and metastasis, as indicated in other studies [2–6]. As presented before, EF consists of the following components: AA, NAC, EGCG, L-lysine dihydrochloride, L-proline, L-arginine, magnesium, calcium, selenium, copper and manganese. The role of individual nutrients such as selenium, copper and calcium on induction of apoptosis and cell cycle arrest has been investigated in different cell lines. However, there is little known on the role of L-arginine, L-lysine and L-proline in cell cycle arrest and apoptosis. Mechanistic aspects of nutrient synergy on malignant cell proliferation and apoptosis have not been investigated, besides the work by Roomi et al. [2–6].

Among EF ingredients, EGCG, a major polyphenolic constituent of green tea, has been shown to block angiogenesis, carcinogenesis, metastasis and proliferation of tumor cells. EGCG induces cell cycle arrest and growth inhibition in NBT-II bladder tumor cells. Its action is mediated by the down-regulation of cyclin D1-cdk4/6-Rb, protein machinery

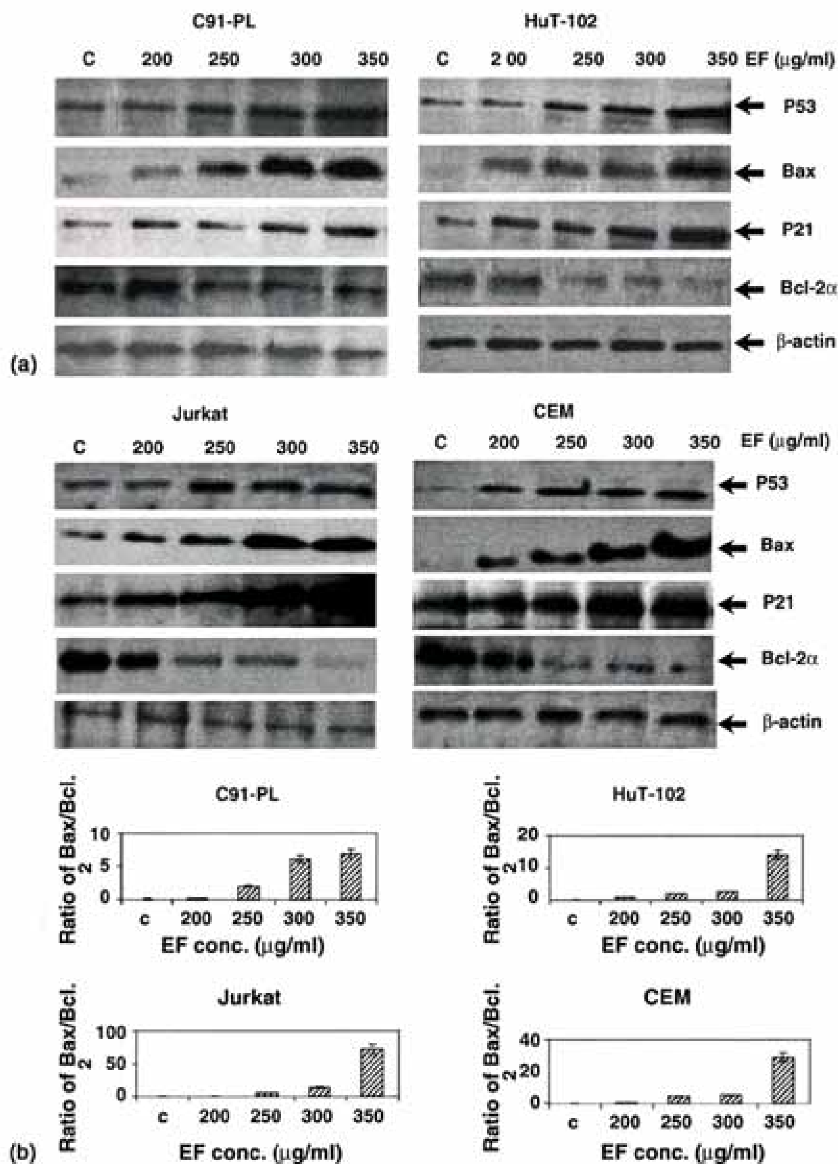

Fig. 6. Western blot analysis of P53, P21, Bax and Bcl-2α protein expression in HTLV-1 positive (a) and negative cells (b) treatment with EF for 4 days. EF was most effective in increasing the Bax/Bcl-2 ratio using densitometry analysis (c). EF up-regulates the levels of p53, p21, Bax proteins and down-regulates the level of Bcl-2α protein expression in all the cells tested in a dose-dependent manner. β-Actin has been used as a control to ensure equal loading. The immunoblots presented represent the typical results obtained in at least two independent experiments.

that is responsible for shifting cells from the G1 into the S-phase [54] EGCG also inhibited growth in CaSki human cervical cancer cell line, causing apoptosis, and it regulated gene expression in vitro [55]. Treatment of human prostate carcinoma cells with EGCG resulted in cell cycle arrest and apoptosis via modulation in the cki-cyclin-cdk machinery. This effect was mediated by an up-regulation in the protein expression of WAF1/p21 [56]. In addition, EGCG inhibited proliferation of human liver cancer cell line, Hep G2, by inducing apoptosis and blocking cell cycle progression in the G1 phase [57].

Vitamin C too has shown many interesting effects on cancer cells. Treatment of human bladder tumor cells, T24, with Vitamin C arrested those cells in G1 while cells in S-phase were arrested in G2/M [58]. Another study suggested that Vitamin C caused down-regulation of the viral oncoprotein E6 paralleled by a decrease of the activator protein (AP-1) members c-jun and c-fos. The down-regulation in E6 led to the up-regulation of pro-apoptotic p53 and Bax proteins but the down-regulation of Bcl-2 [59]. Vitamin C has also been found to play a protective role in the development of cervical intraepithelial neoplasia (CIN).

One of the studies on premalignant lesions of the rat mammary gland using methylselenocysteine, indicated that this nutrient reduces the expression of bcl-2 and modulates molecular pathways responsible for inhibiting cell proliferation and enhancing apoptosis by blocking clonal expansion of those lesions [60]. In another study on the effects of sodium

selenite using Murine B-lymphoma A20 cells, this compound affected the intracellular signaling involved in apoptosis. This effect was associated with the inhibition of Protein Kinase C (PKC), Nuclear Factor Kappa B (NF-κB), and inhibitor of apoptosis protein [61]. Research also showed that chronic exposure to selenite caused the up-regulation of cyclin dependent kinase inhibitor p21 and correlated with cell growth inhibition [62]. The effects of another form of selenium, the methylseleninic acid (MSA), on cell growth inhibition revealed that the treatment of H520 and H522 human lung cancer cell lines with that compound, resulted in cell cycle arrest at G1 and apoptosis [63]. The molecular and cellular effects of MSA showed that it can slow down the progression of cell cycle at different transition points without affecting the proportions of cells in different growth phases [64].

Among other EF components, a form of arginine, NG-monomethyl-L-arginine blocked the induction of apoptosis caused by endogeneous production of nitric oxide (NO) on human pancreatic carcinoma cell lines [65].

In studying the role of minerals on cell proliferation and differentiation, magnesium content directly correlated with proliferation in normal cells stimulating DNA and protein synthesis. Magnesium deprivation affected cell cycle control by up-regulating the cyclin inhibitor p27Kip thus influencing cyclin E-dependent kinase [66]. Calcium was shown to initiate the cell cycle arrest and apoptosis in human chronic myelogenous leukemia K562 cells stressing its vital role in these important pathways [67].

The study of the effects of copper on the PI3K/Akt signaling pathway in metal resistance in human breast cancer epithelial cells, MCF-7 cells, indicated that the pretreatment of these cells with copper resulted in higher cellular levels of p53 accompanied by accumulation of p21 protein, followed by cell cycle arrest in G1 phase and apoptosis [33].

In conclusion, EF has shown significant anti-proliferative and pro-apoptotic effects in tested HTLV-1 positive and negative Adult T-cell Leukemia cell lines. It inhibited growth of those cell lines by inducing apoptosis through modulation of TGF expression and up-regulation of the levels of p53, p21, Bax and the down-regulation of Bcl-2α. Taking into account the effectiveness of EF in addressing critical common mechanisms involved in cancer and its minimal cell toxicity compared to the pharmaceutical drugs further in vivo investigation of this compound is highly recommended and urged.

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