# Terpenic fraction of *Pterodon pubescens* inhibits NF-kB and ERK 1/2 activation and deregulates gene expression in leukemia cells

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**ABSTRACT**

**Background**

Plant derived compounds have shown to be important sources of several anti-cancer agents and since the cell cycle deregulation and tumor growth are intimately linked, the discovery of new substances targeting events in this biochemical pathway has been of great value. The anti-leukemia effect of the ethanolic extract of *Pterodon pubescens* seeds (EEPp) was previously demonstrated. On the other hand, a terpenic subfraction (SF5) of EEPp showing farnesol, geranylgeraniol and vouacapan derivatives presents an anti-tumor action by inducing apoptosis of leukemia cells. This work studied the SF5 antiproliferative mechanisms in leukemia cells since they are still unclear.

**Methods**

The DNA synthesis of K562 cells was assessed by [$^{3}$H]-methyl-thymidine incorporation and cell cycle by flow cytometry. The expression of cyclins D1 and E2, p21 and c-myc was evaluated by semi-quantitative RT-PCR. ERK 1/2 and NF-κB activation was evaluated by western blotting.

**Results**

SF5 induced a higher inhibition level of DNA synthesis and cell growth than the Hexanic fraction of EEPp, the fraction that SF5 is originated, and also arrested the leukemia cell cycle in G1 phase. Down-regulation of cyclin E2 and up-regulation of cell cycle inhibitor p21 gene expression were also observed in K562 cells treated with SF5. Furthermore, SF5 reduced the mRNA level of c-myc transcription factor and inhibited the MAP kinase ERK 1/2 and NF-κB activation.

**Conclusions**

This work suggests that the SF5 anti-leukemic action is linked to inhibition of ERKs, NF-κB and c-myc signaling pathways, which results in reduced E2 Cyclin mRNA expression and cell cycle arrest in G1 phase.

**Keywords:** *Pterodon pubescens* seeds, Leukemia, Cell cycle, Cell signaling

## Background

Cancer has been the largest single cause of death in both men and women. Then, the search for new anti-tumor agents has increased, and since the cell cycle deregulation and tumor growth are intimately linked [1,2], the discovery of new substances targeting events in this biochemical pathway has been of great value. So, phytochemical study combined to pharmacological investigations permit to reveal new compounds from plants with potential anti-cancer properties. Eukaryotic cell cycle progression is tightly regulated by sequential activation and inactivation of cyclin-dependent kinases (CDKs), positively regulated by cyclins and negatively by cyclin-dependent kinase inhibitors [2,3]. Inappropriate expression, regulation and/or mutations of cyclin-dependent kinases, cyclins and its regulator proteins have been described in various cancers [1]. Cyclins D and E are fundamental G1-phase cyclins, while cyclins A and B allow cells to traverse the S and $G_2$M phase of cell cycle, respectively. Therefore, the cell cycle is controlled by intracellular signaling mechanisms. One of them is the Mitogen Activated Protein Kinase (MAPK) pathway, which is formed by a cascade of kinases, generally referred to as Raf/MEK/ERK. Activated extracellular signal-regulated kinases (ERKs) translocate into the nucleus and regulate the activity of transcription factors like c-myc and other ones [4,5], resulting in the expression of genes required for cell cycle progression like cyclin D1, cyclin E and p27 [6,7]. In addition, the Nuclear Factor kappa B (NF-κB) transcription factor stimulates the transcription of diverse genes modulating the cell cycle as cyclins D1 [8] and cyclin E [9], or influences the epigenetic regulation of gene transcription modulating the expression of DNA methyl transferase activity [10]. The hypermethylation of the promoter region of a gene usually represses its transcription.

Anti-tumor effect has been demonstrated for terpenes derivatives in medicinal plants [11]. *Pterodon pubescens* (Pp) Benth. (Leguminosae), popularly known as “Sucupira branca,” is a native tree widely distributed over the central region of Brazil, which the seeds are used in folk medicine to treat rheumatic and inflammatory diseases [12], both activities scientifically demonstrated [13,14]. Recently, anti-tumor effects have also been demonstrated for *Pterodon pubescens* [15].

Phytochemical studies of hexanic fraction of EEPp have shown linear terpenes, as farnesol and geranylgeraniol, and diterpenes with vouacapan skeletal [16-19]. Recent work reported that the SF5 hexanic subfraction of EEPp, rich in vouacapan and geranylgeraniol diterpene derivatives, and also farnesol derivatives, showed anti-tumor effect by inducing leukemia cells apoptosis [19], but its effects on the cell cycle are still obscure. This work investigated the effects of SF5 terpenic subfraction of this plant on leukemia cells proliferation and its mechanisms of action.

## Results

### Effect of SF5 on leukemic cell growth

SF5 treatment reduced the K562 cell growth in a time and concentration dependent manner. As shown in Figure 1, significant reductions of 51.9 ± 12.7%, 66.2 ± 7.8% and 57.7 ± 19.6% in cell growth was observed after treating cells with 30 µg/mL SF5 for 24 h, 48 h and 72 h, respectively, compared to control culture. This inhibition increased to 83.6 ± 4.1%, 91.9 ± 1.5% and 85.4 ± 3.9%, respectively, with SF5 at 50 µg/mL. The significant inhibition indexes of SF5 at 24 h and 48 h were similar ($p>0.05$) to those of the traditional antineoplasic MTX (84.8 ± 7.0%, 95.2 ± 1.8% and 97.7 ± 24.5% at 24 h, 48 h and 72 h, respectively). The HEX subfractionation resulted in a subfraction (SF5) with greater inhibitory effect on cell growth.

### Effect of SF5 on DNA synthesis and cell cycle

Since the SF5 inhibited K562 leukemic cell growth, then its effect on DNA synthesis (Figure 2A) and cell cycle (Figure 2B) of these cells were analyzed. Both HEX and SF5 inhibited significantly the $^{3}$H-methyl-thymidine incorporation to DNA of K562 cells, compared to control culture, SF5 showing higher inhibitory indexes on cell proliferation than the original HEX fraction (Figure 2A). The cell cycle profile of K562 leukemic cells was modified by treatment with SF5 (30 µg/mL). Cells were accumulated in G1 phase with concomitant reduction in S and G2/M phases (Figure 2B).

Again, SF5 induced more intensive anti-leukemia effect than the original fraction (HEX). However, the mechanisms that underlie this effect remain unknown.

**Effect of SF5 on gene expression**

Cyclins play a key role in controlling cell cycle progression by positively regulating the CDKs activities at an appropriate time in the cell cycle. Since D1 and E2 cyclins regulate the transition of cell cycle from G1 into S phase and SF5 induced G1 arrest in K562 cells (Figure 2B), these cyclins expression was studied after treating these cells with SF5 (50 µg/mL). Treatment with this sub-fraction increased the mRNA levels of D1 cyclin at all times studied while it reduced the E2 cyclin at 6 h and increased it afterwards (Figure 3) in K562 cells. Cyclin-dependent kinase inhibitors (CKIs) control cell cycle progression by negatively regulating the CDKs activities. In this work, SF5 markedly increased p21 mRNA levels of K562 cells (Figure 3). The expression of c-myc gene, which codifies the important transcription factor c-myc, that stimulates cell survival and proliferation, was reduced by treatment with SF5 (Figure 3).

**Effect of SF5 on ERK 1/2 and NF-κB activation**

The SF5 effects on intracellular cell signaling for cell proliferation were evaluated by determining the level of phosphorylated-ERK 1/2 form and the NF-κB transcription factor translocation to the nucleus. Control culture showed a lower level of nuclear NF-κB expression at 1 h, which was progressively increased at 2 and 4 h, while K562 cells treated with SF5 at 50 µg/mL reduced nuclear NF-κB expression at 2 h and 4 h (Figure 4A). The level of total ERK 1/2 protein expression is higher than the phosphorylated form in all culture conditions (Figure 4B). Treatment with SF5 inhibited the ERK 1/2 phosphorylation (Figure 4B).

## Discussion

Previous studies with the EEPp reported an anti-leukemia effect by arresting K562 cell cycle in G1 phase [15]. Similar results were demonstrated for melanoma cells [18]. SF5, a terpenic sub-fraction of hexanic fraction from EEPp, contains epoxyfarnesol (32%), geranylgeraniol (4.6%) and 7β-acetoxy-vouacapan (1.9%) derivatives, methyl 6α-acetoxy 7β-hydroxivouacapan 17β-oate (6%) and 6α-7β-diacetoxivouacapan 14β-oate (20.6%) [19]. Previous studies reported cytotoxicity and apoptosis of leukemia cells by treatment with SF5 [19]. This work demonstrated that SF5 inhibits leukemia cell proliferation by reducing transcription factor and ERK 1/2 activation, and also altering gene expression of cell cycle regulators. Comparative analysis of the SF5 anti-proliferative effects with its original hexanic fraction of EEPp (HEX) shows higher effects on leukemic cells growth, DNA synthesis and cell cycle inhibition for SF5. The higher cell growth inhibition of SF5, compared to HEX, must be related to their different chemical composition. HEX contains other compounds that are not present in SF5, which may be contributing to the reduced inhibitory effect of HEX by neutralizing the inhibitory action of SF5 compounds or inducing activation effects. Inhibition of tumor cell proliferation has been demonstrated for traditional chemotherapeutic agents and potential anti-cancer drugs [22], as observed with MTX in this work. SF5 cytotoxic effects were also demonstrated on other tumor cell lines although they were lower than those on leukemia cells (data not shown). The finding of a terpenic sub-fraction with anti-tumor effect higher than the hexanic fraction of the original extract is of great value since it can represent a potential anti-tumor agent with fewer side effects. The anti-proliferative effect of SF5, here observed, was not due to ethanol since control cultures containing this one did not alter this response (data not shown).

Cyclin D is the first cyclin expressed by cells in response to favorable growth conditions [1]. In conjunction with CDK4/6, cyclin D mediates the initial phosphorylation of Retinoblastoma protein (pRb). The kinase activity of cyclin E/CDK2 complex then acts to maintain pRb in the hyper-phosphorylated state [2]. Any factor affecting these kinases activities could abrogate the normal inactivation of pRb and cause accumulation of cells in $G_1$ phase. The mechanisms of *in vitro* anti-cancer activity of SF5 seem to be similar to those of EEPp [15], by inhibiting cyclin E2 gene

expression, resulting in cell cycle arrest in G1 phase. SF5 also increased cyclin D1 gene expression as did EEPp [15]. Other cells also show an increase in D1 cyclin expression associated with inhibition of cell proliferation [23].

Investigation of SF5 effects on gene expression of p21 showed increased mRNA expression, which may be collaborating to the cell cycle inhibition. Several reports describe up-regulation of p21 [24] by anti-cancer drugs. According to the literature, p21 plays a direct role in mediating p53-induced G1 arrest [25]. On the other hand, as K562 cells have their *TP53* tumor suppressor gene mutated [26], the effects of increased p21 gene expression on the cell cycle arrest in this work must be related to a p53-independent mechanism, as occurs during cellular senescence [27] and differentiation [26, 28]. The increased mRNA level of both cyclin D1 and p21, by treatment with SF5, can result in higher levels of these proteins and formation of p21-CDK4-cyclin D1 and/or p21-CDK6-Cyclin D1 complexes, decreasing CDK4 and CDK6-associated kinase activities in K562 cells [29]. Sequestration of cell cycle inhibitors (p21 and p27) is well established for D-cyclins [3]. Furthermore, the SF5 inhibited cyclin E mRNA expression that can lead to reduced formation of CDK2-cyclin E protein complex and consequently reduced CDK2 kinase activity. Cyclin E2 down-regulation has been reported as an important effect of anti-cancer drugs [30].

Gene expression of cyclin and cell cycle inhibitors depends on different transcription factors and MAP kinases activities [31-33]. Since the ERKs signaling pathway stimulates c-myc expression [5], the cyclin E2 gene transcription is activated by c-myc [34], and that SF5 inhibited both ERK 1/2 activation and c-myc and cyclin E2 gene expression, the cell cycle arrest in this work may be related to inhibition of these MAP kinase protein and c-myc function.

The up-regulated expression of cyclin D1 and p21 by SF5, despite of reduced activation of ERK 1/2, suggests that these proteins expression can be submitted to other regulatory mechanisms. Indeed, p21 and p27 gene expression can be regulated by the AP-1 transcription factor [35] or promoter gene methylation [36]. Otherwise, D1 cyclin gene expression is under regulation of other transcription factors as AP1 and STATs [37].

In addition, the NF-κB transcription factor, activated in certain cancers [38], is linked to the expression of different genes associated with cell proliferation as c-myc [39] and E2 [40]. NF-κB has been an important target for anti-cancer treatment and this study demonstrated that SF5 inhibited NF-κB translocation to the nucleus, thus probably inhibiting specific gene expression, as here demonstrated for cyclin E2 and c-myc.

## Conclusions

Concluding, SF5 showed minor chemical complexity and higher *in vitro* anti-leukemia action than HEX, which can represent reduction of side effects for a potential anti-tumoral agent. SF5 anti-leukemic action is linked to inhibition of intracellular signaling, as reduced activation level of ERK 1/2 and NF-κB transcription factor and altered expression of cell cycle regulatory proteins, as demonstrated by increased p21 and down-regulated c-myc and cyclin E2 gene expression.

## Methods

### Materials and Reagents

RPMI 1640 medium, streptomycin sulfate, propidium iodide, triton X-100, RNAse, sodium dodecyl sulfate, PPO, POPOP, Phytohemaglutinin A (PHA), proteases inhibitor and serum bovine albumin were purchased from Sigma Chemical Co., USA. Penicillin was purchased from Fontoura-Wyerth, Brazil. Fetal calf serum (FCS) was purchased from Cultilab, Brazil. Also, $^{3}$H-metil-thymidine was purchased from Amersham Life Science, USA. For PCR, dNTP mix, Trizol, first-strand buffer, ribonuclease inhibitor, DTT, SuperScriptTM II RNAse H Reverse Transcriptase, random primers, $MgCl_2$ and Taq polymerase platinum were purchased from Invitrogen, USA. Primers for cyclins D1 and E2, c-myc, p21 and beta actin were purchased from Imprint, Brazil. Antibodies anti-human:

p65, Histone, ERK 1/2 and p-ERK 1/2 were purchased from Santa Cruz Biotechnology, USA. ECL kit and film for western blotting were purchased from Amersham Biosciences, USA.

**Plant material collection and extraction**

*Pterodon pubescens* seeds were collected by Luciana Pontes Coelho in Goiás, Brazil and was taxonomically identified by Haroldo Cavalcante de Lima at the Departamento de Botânica Sistemática, Jardim Botânico do Estado do Rio de Janeiro, Rio de Janeiro, RJ, Brazil, where a voucher of the collected specimen has been deposited (RB 350279). The *Pterodon pubescens* seeds were powdered in liquid nitrogen and submitted to 100% ethanol extraction (15 g/100 mL) at room temperature for 15 days as described by Silva and collaborators [12]. The EEPp was obtained (50% yield, w/w) after ethanol evaporation. Afterwards, the EEPp was fractionated by liquid-liquid partition chromatography using hexane. The obtained hexanic fraction (HEX, 55% yielding) was submitted to a column chromatography (42 x 2.5 cm) on silica gel (Art. 7733 – Kieselgel 60, 35-70 MESH ASTM, Merck), eluted successively with hexane (500 mL), hexane/dichloromethane (1:1) (500 mL), hexane/ethyl acetate (1:1) (500 mL), ethyl acetate (500 mL), ethanol (500 mL) and 2% acetic acid in ethanol (500 mL), as previously described by Pereira and collaborators [19]. After gas chromatography analysis of eluted samples, they were joined in eight sub-fractions according to their chromatographic profile similarity. The HEX GC profile shows two regions of important peaks, the first one in the retention time range of 2.0 and 5.0 seconds and the second one between 6.0 and 10.0 seconds. The GC analysis of the most cytotoxic subfraction to leukemia cells, subfraction 5 (SF5, 32.9% of HEX fraction), indicates that it corresponds to the second region of HEX fraction, containing the furane diterpenes methyl-6α-acetoxy-7β-hydroxyvouacapan-17β-oate and 6α, 7β-diacetoxy vouacapan 14β-oate, 7β-acetoxy vouacapan derivative, besides epoxyfarnesol and geranylgeraniol derivatives (Pereira et al, Oncology Reports , 2011, 25:215-21). HEX and SF5 were previously diluted at different concentrations with RPMI 1640 supplemented with 10% FCS.

**Cell proliferation assays**

The human chronic myelogenous leukemia cell line K562 (CCL-243) was purchased from the American Type Culture Collection (ATCC) and was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. The effects of the plant samples on cell growth were evaluated by treatment of cells (2.5 x $10^5$ cells/mL) with HEX or SF5 at different concentrations for 72 h at 37$^o$C and 5% $CO_2$. Viable cells were counted in a 12 h interval using the trypan blue dye exclusion method. To assess DNA synthesis, 25 μL/well of 10 μCi/mL [$^3$H]-methyl-thymidine ($^3$H-Tdr; Amersham Biosciences, Brazil) were added for the last 24 h of culture. Then, the cells were harvested on filter paper and processed for $^3$H-Tdr radioactivity determination by liquid scintillation.

**Cell cycle analysis**

To determine the distribution of DNA content in K562 cells, these ones were treated with DNA staining solution as previously described [19]. Cells (2.5 x $10^5$/mL) were incubated either in the absence (control) or in the presence of 30 μg/mL HEX or SF5 concentrations for 36 h at 37$^o$C and 5% $CO_2$. After centrifugation (400 x g) for 5 min, 1 x $10^6$ viable cells were suspended in DNA staining solution (0.3% Triton X-100 and 50 μg/mL propidium iodide [PI] in 43 mM citrate buffer) and maintained for 15 minutes at room temperature in the dark. Later, the samples were treated with 50 μg/mL ribonuclease A (Sigma Chemical Co., St. Louis, MO, USA) in 43 mM citrate buffer pH 8.2 for 15 minutes at room temperature. PI fluorescence was measured (100,000 events per sample) using a FACSCalibur flow cytometer (Beckton Dickson, USA). The excitation wavelength for PI was 488 nm and the emission detected at 585 ± 15 nm. Analysis was made with the WinMDI 2.8 software.

**Imunoblotting**

Cells (2.5 x $10^5$ cells/mL) were incubated with 50 μg/mL SF5 for different times. The whole cell lysate was prepared for evaluation of phosphorylated (p-ERK 1/2) and total ERK 1/2 protein expression. The cells were washed twice in PBS, suspended in lysis buffer (50 mM HEPES pH 6.4,

1 mM $MgCl_2$, 10 mM EDTA, and 1% Triton X-100), left 30 minutes on ice and centrifuged at 4°C for 10 minutes. The supernatant was stored in a freezer. Nuclear extract was prepared as previously described [20] for evaluation of NF-κB activation. The protein concentration was measured by Lowry assay [21] using bovine serum albumin as standard. Proteins were denatured for 5 min in sample buffer (glycerol, β-mercaptoethanol, 10% SDS, 10 N NaOH and bromophenol blue) and 30 μg of protein per sample were resolved in a 12% or 15% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Bio-Rad). Blots of nuclear extract (for NF-κB and histone) or whole cell extract (for p-ERK 1/2 and total ERK 1/2) were blocked for 2 h at room temperature with 2% BSA and 0.1% Tween in PBS and then incubated overnight with the specific primary antibodies anti-p65 or anti-pERK 1/2 in PBS containing 0.5% BSA and 0.1% Tween. The membranes were washed three times, soaked for 1 h with secondary antibody (horseradish peroxidase-linked anti-mouse IgG), and washed again with PBS-Tween 0.1%. Finally, blots were developed using the enhanced chemiluminescence method (ECL; Amersham). Blots were reincubated with anti-histone or anti-ERK 1/2 as loading control of nuclear or whole cell extracts, respectively.

**RT-PCR for mRNA expression analysis**

Total RNA of samples was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen). After treatment with DNAse I and the RNA integrity confirmed by a denaturing agarose gel electrophoresis, reverse transcription reaction was performed. The cDNA was brought into 25 μL mixture of PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 3.2 μg of each primer, and 2.0 U of Taq DNA polymerase platinum (Invitrogen). Primers, annealing temperature and cycles used: β-actin forward primer: 5´-TCCTGTGGCATCCACGAAACT-3´, reverse primer: 5´-GAAGCATTTGCGGTGGACGAT-3´ (59°C, 30 cycles, 314 pb); Cyclin D1 forward primer: 5´-TTGCTGCCCTTCTCCATGAT-3´, reverse primer: 5´-TCCCAACTGAAACCCAATGC-3´ (55°C, 30 cycles, 334 pb); Cyclin E2 forward primer: 5´-GATTTGTCCTTGGAGAACGG-3´, reverse primer: 5´-TTGGGTGTTGGTTCTTTGGTT-3´

(57°C, 30 cycles, 499 pb), $p21^{CIP/KIP}$ forward primer: 5´-TTGCTGCCCTTCTCCATGAT-3´, reverse primer: 5´-TCCCAACTGAAACCCAATGC-3´ (55°C, 30 cycles, 334 pb), c-myc forward primer: 5´-GTCCTCGGATTCTCTGCTC-3´, reverse primer: 5´-GACTCTGACACTGTCCAACT-3´, (60°C, 30 cycles, 342 pb). All genes examined were normalized to a housekeeping gene encoding β-actin. PCR was performed in a Perkin Elmer GeneAmp PCR System 9600. Each PCR cycle consisted of denaturation at 94°C for 30 seconds, annealing at specific temperature for 30 seconds, and extension at 72°C for 1 minute. Semi-quantitative analysis of the products was available by 2% agarose gel electrophoresis and products band intensities determined by using Lab Image software (Germany).

**Statistical analysis**

Significant differences between pairs of groups were accessed using Student's *t* test, with a significance level set at $p<0.05$.

## Authors' Contributions

Pereira MF and Martino T carried out the experiments. Sabino KCC designed and coordinated the study. Barja-Fidalgo C (Cell biology), Albano RM (Molecular biology), Paes MC (Cell biology), Coelho MGP (Phytochemistry) and Dalmau SR (Flow cytometry) contributed to experiments with their important specific expertise. All authors approved the final manuscript.

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**LEGENDS**

**Figure 1.** Effects of hexanic fraction (HEX) and SF5 hexanic sub-fraction on the K562 leukemia cell growth. A. 30 µg/mL, B. 50 µg/mL. Cells (1 x $10^5$/mL) were cultured for 24, 48 and 72 h either in the absence (control) or presence of HEX, SF5 or methotrexate (MTX, 50 µg/mL). Viable cells were counted by Trypan blue dye exclusion. Results represent the mean ± SD of three independent experiments. $^a$ $p<0.05$, $^b$ $p<0.01$ and $^c$ $p<0.001$, versus control by *t* Student's test.

**Figure 2.** Effects of hexanic fraction (HEX) and SF5 hexanic subfraction on K562 cell proliferation. A. $^3$H-methyl-thymidine incorporation in DNA. B. Cell cycle analysis. Cells (1 x $10^5$/mL) were cultured for 72 h in the absence (control) or presence of different HEX or SF5 concentrations. $^3$H-methyl-thymidine 0.25 µCi/well was added 24 h before the ending of culture. For cell cycle analysis cells were treated with HEX or SF5, both at 30 µg/mL, for 36 h. Hypodiploidnuclei and debris were discarded from analysis. Results express means ± SD of three independent experiments. All inhibition indexes were significantly different from control cultures. $^a$ $p<0.05$ and $^c$ $p<0.001$ versus HEX fraction by *t* Student's test.

**Figure 3.** Effects of SF5 on the cyclins, p21 and c-myc expression in K562 cells. Cells (2.5 x $10^5$/mL) were cultured in the absence (control) or presence of SF5 (50 µg/mL) for 36 h. RNA was

extracted and the RT-PCR products were shown by agarose gel electrophoresis as described in "Methods." The β-actin mRNA was used as loading control.

**Figure 4.** Effects of SF5 on the NF-κB and ERK 1/2 activation. A. NF-κB protein expression. B. Phosphorylated ERK expression. Nuclear extract (NF-κB) and whole cell lysate (pERK) were prepared as described in "Material and Methods," after culture of K562 cells ($2.5 \times 10^5$/mL) with SF5 (50 μg/mL) for the indicated times. Proteins (50 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, probed with anti-p65 or anti-p-ERK and developed by chemiluminescence. Histone was used as a loading control for nuclear extracts and total ERK 1/2 for whole cell lysates.

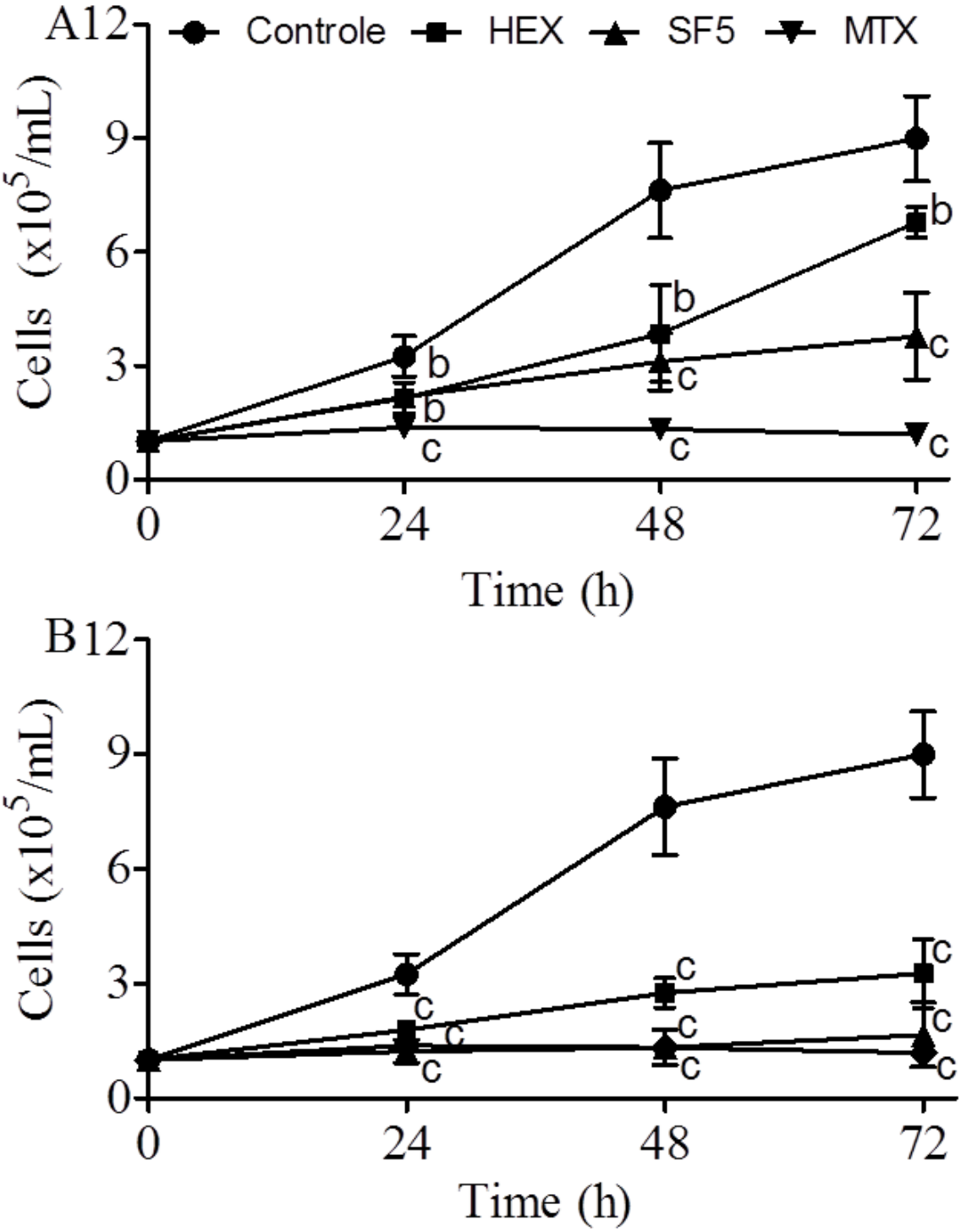
A12
Controle
HEX
SF5
MTX
Cells (x10^5/mL)
0
3
6
9
0
24
48
72
Time (h)
b
b
b
c
c
c
B12
Cells (x10^5/mL)
0
3
6
9
0
24
48
72
Time (h)
c
c
c
c
c
c
c
c

Figure 1

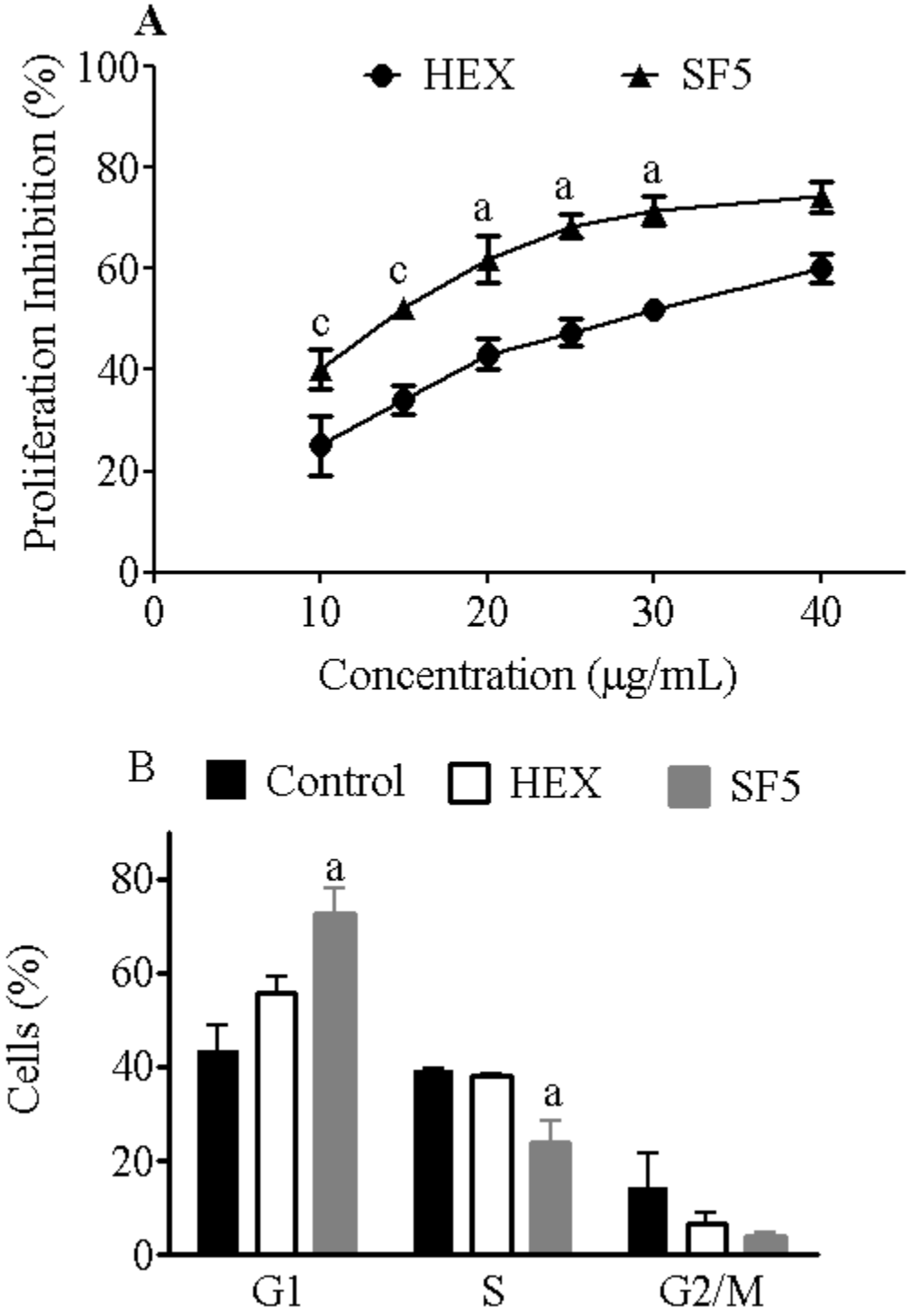
A
HEX
SF5
Proliferation Inhibition (%)
100
80
60
40
20
0
0
10
20
30
40
Concentration (μg/mL)
a
a
a
c
c
B
Control
HEX
SF5
Cells (%)
80
60
40
20
0
a
a
G1
S
G2/M

Figure 2

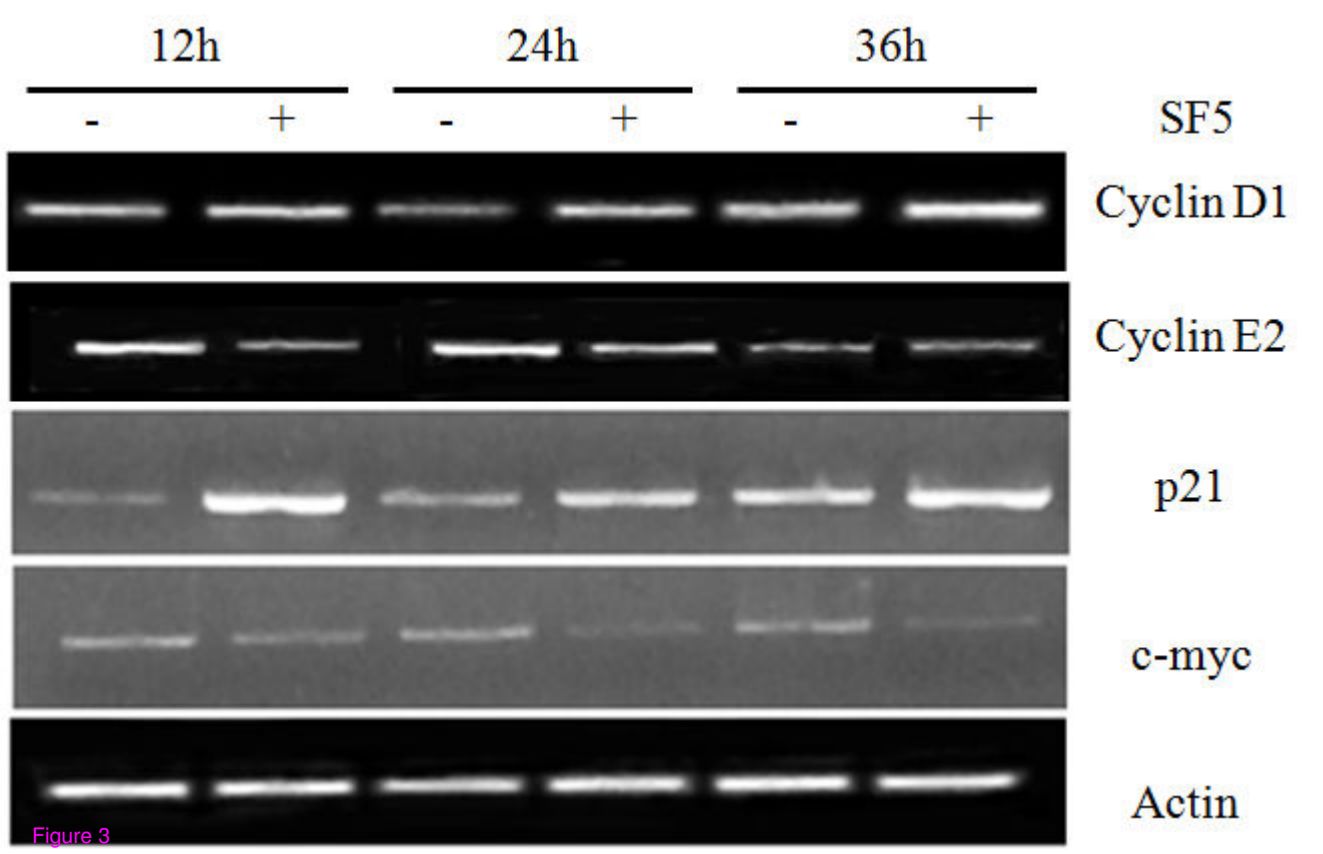


Figure 3

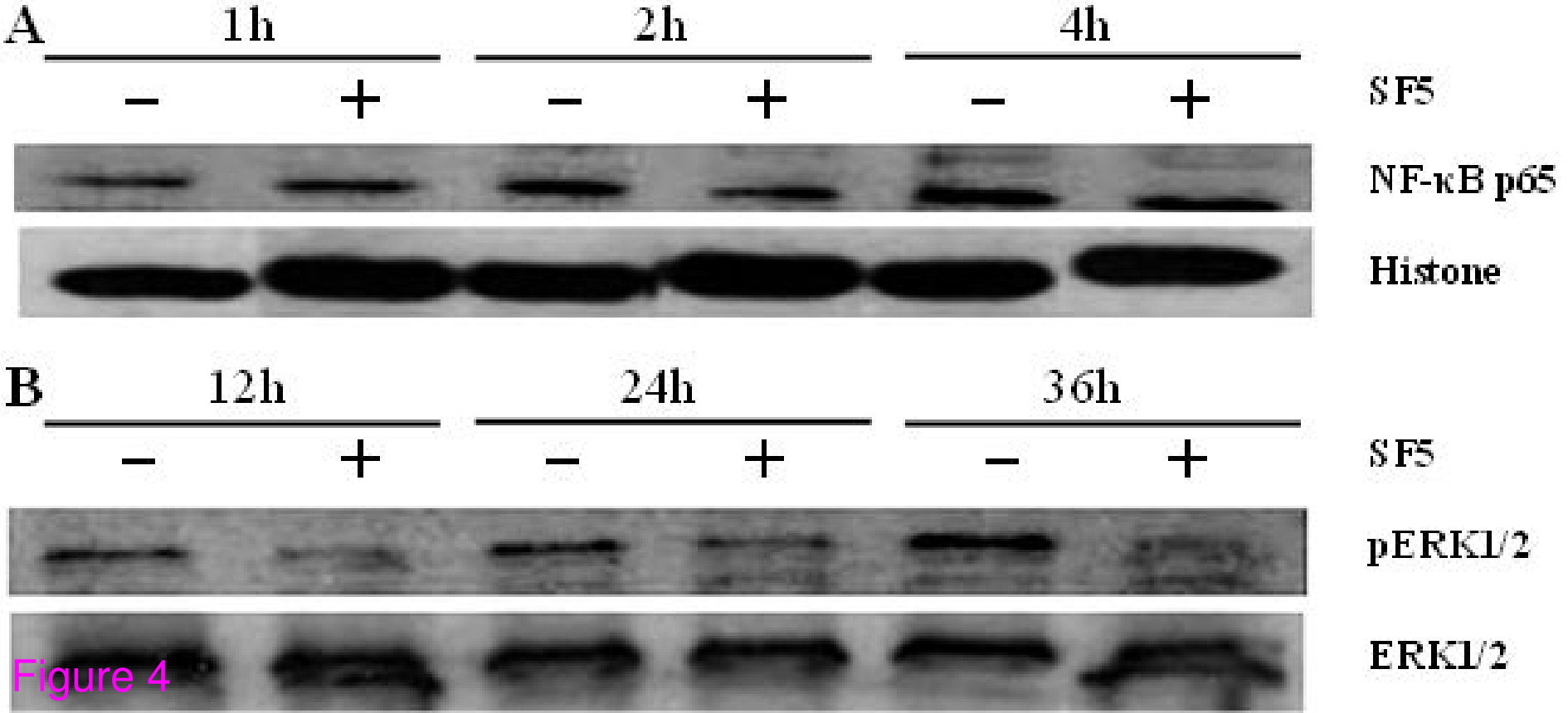


Figure 4