RESEARCH ARTICLE

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Influence of Static Magnetic Field on HeLa and Huo2 Cells in the Presence of *Aloe vera* Extract

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Abstract

This research aimed to assess the impact of static magnetic field (SMF) on apoptosis rate and cell cycle progression in the presence of *Aloe vera* Crude Extract (ACE) in normal (Huo2) and cancer cells (HeLa). The specimens were split into one untreated group (control) and two experimental groups, including treatment with ACE (Alo) and compound treatment with SMF and ACE (Alo+SMF). MTT assay determined the IC50 value, and flow cytometry was employed to evaluate cell cycle distribution and apoptosis rates. Statistical analysis was carried out through a two-way ANOVA followed by Tukey's post hoc test. Our results showed that combination treatment with SMF (10 mT) and ACE (Alo+SMF) significantly inhibited the cell proliferation. This increased the cell number in G2/M stage and early apoptosis in cancer cells compared to ACE treated cells after 24 and 48h but reduced the number of Huo2 cells in G2/M phase and early apoptosis after 24h. The effect of AEC on HeLa cells was intensified with increasing the SMF exposure time, such that the early apoptosis rate in Alo+SMF group had an approximate 4-fold increase compared to Alo group. This research proposes that the combination treatment accelerates the apoptosis induction of HeLa cell. During the interphase, there were significant differences between the cancer and healthy cells concerning the cell cycle. Moreover, exposure time may play an important role in the impact SMF on both healthy and cancer cells in the presence of AEC.

Keywords: Magnetic field- plant extract- apoptosis- cell cycle- HeLa cells

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Introduction

Available evidence shows that a therapy with both SMFs and anticancer medications can affect cell proliferation. SMFs have been shown to enhance the anticancer effect of chemotherapeutic drugs, offering a novel approach to cancer treatment (Sengupta and Balla, 2018).

Findings have shown that SMFs may act synergistically with antineoplastic drugs (Liu et al., 2011; Sun et al., 2012; Kamalipooya et al., 2017). In addition to the studies on the possible synergistic effects of SMF and chemotherapeutic agents on cancer cells, herbal extracts are considered as potential anticancer sources owing to the rapid development of natural resources in terms of efficacy and safety (Pawar et al., 2018; Qamar et al., 2019). There exist myriad kinds of cytotoxic products derived from medicinal plants. *Aloe vera* is one of these plants which has long been known and used for pharmaceutical purposes. This plant contains more than 75 active compounds, including vitamins, sugars, enzymes, amino acids, and around 12 types of anthraquinoids. These substances are phenolic compounds associated with many functions of the plant (Niciforovic et al., 2007; Chen et al., 2014; Guo and Mei, 2016). Grimau (1997) investigated the antitumor effects of five types of purified anthraquinoid compounds from *Aloe vera* on susceptible and resistant human K562 leukemia. He observed that these compounds increased cell death and reduced cell proliferation. Aloe emodin, a type of anthraquinoid compound found in *Aloe vera*, inhibits gene expression in the cell cycle, reduces cell proliferation, and induces apoptosis in the cell lines of human liver cancer (Kuo et al., 2002). This natural anthraquinone is also able to significantly reduce cell proliferation and cause apoptosis in the treated human colon cancer cells through activating caspase 9 and 6 (Suboj et al., 2012).

Different studies are currently being conducted to reform anticancer compounds, produce new drugs (via natural products and herbal extracts), discover new methods, or promote alternative strategies such as radiation or magnetic field treatment. Given the difference between healthy and cancer cells and the impact of magnetic fields on cells, we decided to conduct research

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in this field. Our primary goal was to explore the impact of SMF in the presence of AEC and compare apoptosis rates and cell cycle progression in the HeLa cell line to typical fibroblast. These experiments offer unique insight into biomagnetism and open new doors to reducing the side effects of herbal anticancer drugs.

Materials and Methods

Chemicals

Trypsin-EDTA and penicillin-streptomycin were purchased from Bioidea, and cell culture reagents and fetal bovine serum (FBS) were obtained from Gibco (Great Britain). Dulbecco's Modified Eagle's Medium (DMEM) and MTT salt, Propidium iodide (PI), and Triton X-100 were provided by Sigma-Aldrich Chemicals. Ribonuclease A (RNase A) was bought from Fermentas-Life sciences (EU), and dimethylsulphoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Trypsin- EDTA, penicillin-streptomycin, fetal bovine serum (FBS), and RNase-A were deposited as stock solutions.

Culture of cells

Normal skin fibroblast cells (Hu02) and human cervical cancer HeLa cell line were prepared in the Iranian Biological and genetics Reserves Center (IBRC) and explanted in DMEM with 10% FBS and 1% penicillin/streptomycin (100 Units/ml penicillin, 100 µg/ml streptomycin). Afterwards, they were incubated in humidified 5% CO_2 atmosphere at 37°C (Sabo et al., 2002; Kamalipooya et al., 2017) for 48 h prior to changing the media. After 48 h, they were replanted once the cells reached ~ 70 - 80% confluence on the surface of the flasks. In each phase, cells were washed twice by phosphate buffered saline (PBS); thus, non-adherent cells were thrown away from adherent cells. Both cell types were allowed to grow until reaching a density of cells about 5×10^4 cells/cm^2 per flask. The medium was substituted with a fresh medium 1 h before the treatments.

Magnetic generator and incubating system

A locally intended generator exerted the magnetic field (MF). The generator was composed of two wire coils with 3.0 mm diameter, 3 Ω resistance, 2 H self-inductance, and 200°C heat resistance.

These two coils guided the magnetic field over two iron blades with a cross section of 10 cm^2 and 1 meter height. A 220 V AC power supply with a variable transformer and a single-phase full-wave rectifier was applied to provide the electrical power. A switching power supply is able to use a maximum direct current of 16 A with a potential difference of up to 50 V to generate various MF intensities.

Using a 13610.93 PHYWE (Gottingen, Germany) teslameter, the field between iron blades was calculated, and pulsation was examined via an oscilloscope (8040, Leader Electronics Co., Yokohama, Japan). To cool off the system, a gas chiller with optimum temperature control was applied. The chiller was comprised of a condenser, an engine, an evaporator, and refrigerant gas which covered the exterior surface of the coil. The system was further equipped with a rectangular cube ($23\times20\times50$ cm^3) incubator. Humidity, CO_2 pressure, and the temperature of the air surrounding the flasks were controlled by three various sensors (Kamalipooya et al., 2017).

Preparation of Aloe vera extract

Aloe vera plants were obtained from department of Plant Biology, Tarbiat modares University- Tehran. The fresh leaf gel was isolated, ground by a blender, and extracted with normal saline solution (0.9%). The extract was boiled for 20 minutes, following by filtration on filter paper and Buchner funnel under reduced pressure. The filtrate was dried at 70°C. Aliquots of the extract powder (50µg) were dissolved in 1 mL of complete cell culture medium and used for determination of cell toxicity.

Herbal extract and SMF treatments

To assess the impact of SMF and extract of *Aloe vera*, normal fibroblast cells (Hu02) and Human cervical cancer (HeLa) were applied. Each type was divided into three groups, namely control, treatment with IC_{50} concentration of AEC (50µg/ml), and treatment with AEC in the presence of SMF. The experiments were carried out over two various treatment times (24 and 48 h). According to Kamalipooya (2017), 10mT intensity of was selected for investigations.

Colorimetric assay and IC_{50} determination

The MTT assay determined the cell viability as stated in the previous study (Satari et al., 2018). This colorimetric technique measures a tetrazolium component (MTT) reduction by the mitochondria of metabolically active cells into an insoluble formazan product. Briefly, by trypsinization, cells were picked, calculated, and planted in 96-well flat-bottomed plates. Experiments were repeated five times. After 24 h, the cells attached to the flasks were ready for experiment. The culture medium was removed and replaced by 100 µl of AEC (50µg/ml) and was ready for experiment. The MTT stock was prepared through dissolving 5 mg MTT per ml of PBS and filtered through a 0.22 µm filter prior to dilution with DMEM at 10% v/v concentration. Next, the plates were incubated at 37°C for 4 h. 100µl of DMSO was added to each well and mixed completely to dissolve the dark blue crystals. After a few minutes, the plates were read on a BIO-TECH MQX200 Elisa reader using a test wavelength of 540 nm.

As shown in Figure 1, the MTT assay specified the IC_{50} value based different AEC concentrations (1, 10, 50, 100, and 500µg/ml). IC_{50} was defined as the AEC resulting in 50% cell viability loss in comparison with untreated cells in both 24 h and 48 h treated samples (Damiani et al., 2019). According to this procedure, the calculated IC_{50} value of the AEC was 50 µg/ml for HeLa cell line after 24 and 48 hours. The IC_{50} value of HeLa (50 µg/ml) was used for all the treatments.

Staining, data collection, and cell cycle determination

Trypsin-EDTA was applied to the harvest cultured cells, which were then resuspended in DMEM at a concentration of 1.0×10^6 cells/flask. After that, the cells were suspended in PBS and centrifuged for 5 minutes at 200g at 4°C. The supernatant was decanted, and the cells

were gradually resuspended in 0.5 ml PBS.

The cells were stabilized through adding up to 4.5 ml of cold ethanol (70%). Fixed cells were left at 4°C for 24 hours for further analysis, at which point, as mentioned above, they were once again centrifuged, washed one time with cold PBS, and centrifuged again. Centrifuged cells were resuspended in 0.5 ml PBS encompassing 10 µl of 10 mg/ml RNase-A and 1 µl triton- X100, and incubated at 37°C for 10 minutes. The incubated cells were stained via adding up to 10 µl of 1 mg/ml propidium iodide (Kamalipooya et al., 2017). An LSR II flow cytometer was applied to measure the flow cytometry (Becton Dickinson). Once the linear amplification was performed, PI fluorescence was gathered with a 575/25 nm band pass filter, orange-red fluorescence (FL2). The flow cytometry data were deposited based on the flow cytometry standard (FCS) format (Kamalipooya et al., 2017).

Apoptosis/necrosis detection

To detect the cell count in different stages of its death, at least 5×10^5 cells/ml were harvested and resuspended in annexin V binding buffer according to the manufacturer's instructions (annexin V apoptosis detection kit FITC, eBio science, USA; 5 µl PI and 5µl annexin V- FITC/100µl binding buffer). 200µl binding buffer was added after incubation at room temperature for 15 min, and the samples were analyzed for PI and FITC signals by LSR II flow cytometer (Tavasoli et al., 2009). As observed in Figure 2, based on fluorescence signals, the cells were categorized into viable (FITC negative, PI negative), early apoptotic (FITC positive, PI negative), late apoptotic (FITC positive, PI positive), and necrotic (FITC negative, PI positive). The relative numbers of cells in each section were statistically analyzed (Wang et al., 2013), and the apoptosis rate was then assessed through flow cytometry.

Statistical analysis

Statistical analysis was carried out via a two-way ANOVA followed by Tukey's post hoc test. The experiments were independently carried out for three times, and data were presented as the mean values ± standard deviation (M ± SD). The differences were considered significant at $P < 0.05$.

Results

MTT assay

To determine the cytotoxicity effects of herbal extract, Huo2 and HeLa cells were incubated with different concentrations (1, 10, 50, 100 and 500µg/ml) of AEC. The viability (%) was measured 24 and 48 hours after treatment. The inhibitory concentration 50% (IC_{50}) of the AEC was calculated based on the survival curves via MTT assay. The assay revealed that various concentrations of the extract had different impacts on the viability percentage of both cell kinds. The effect of the AEC on cancer and healthy cells was not the same. The mortality rate rose in cancer cells by the increase in AEC concentration. As illustrated in Figure 1, cell viability (%) in HeLa cells significantly decreased ($P < 0.05$) by treatment with AEC as compared with control group in a dose-dependent way. At 50 µg/ml concentration, the lowest and highest cell death rates were observed in normal and cancer cells, respectively; therefore, based on MTT assay, at this concentration, the viability percentage was 59.44% and 57.84% in HeLa cell 24 and 48 h after treatment, respectively. In all treatments, IC_{50} value (50µg/ml) was considered for both cells and both times.

Apoptosis/necrosis measurements

To measure the changes in apoptosis/necrosis, a flow cytometric analysis was carried out on harvested single cells, labeled with annexin V/FITC. Tables 1 and 2 show the result of this test for both cell types, indicating the percentage of cells in both living and death phases. To better explain the behavior of cells under SMF exposure, the percentage of necrotic and apoptotic cells was calculated. The result revealed insignificant differences between Huo2 and HeLa cells control groups regarding survival rate; however, the mean viability percent of Hu02

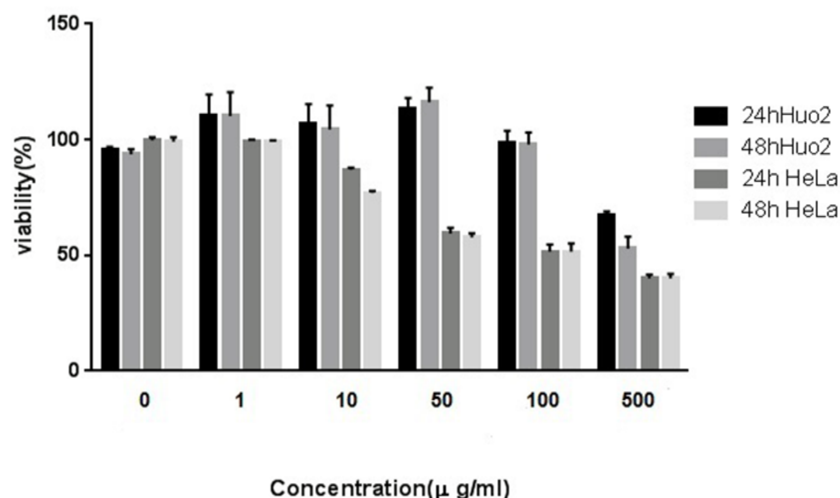

Figure 1. Effect of Different Concentration of Aloe vera Extract on Percentage Viability of Hela Cell Lines and Huo2 Cells after 24h and 48h. The viability percentage of two types cells at different concentrations (0, 1, 10, 50, 100 and 500 µg/ml) were evaluated in vitro utilizing MTT assay. Viability of HeLa cells dropped significantly compared with Huo2-treated cells at concentrations higher than 10 µg/ml.

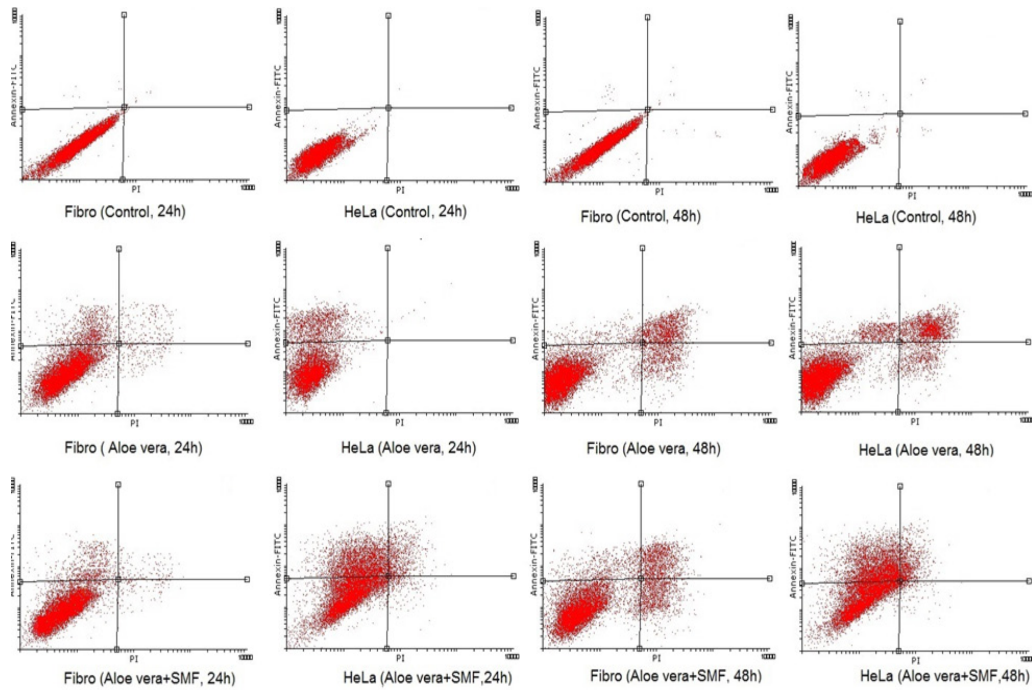

Figure 2. Flow Cytometric Analysis of Early and Late Apoptosis in HeLa Cells Line and Fibroblast Cells (Huo2) Treated with Aloe vera Extract (IC_{50}, 50 μg/ml), Aloe vera Extract (IC_{50}) in the Presence of Static Magnetic Field (SMF,10mT) and Control after 24 and 48 hours. Annexin V and PI quadrant gating: (left bottom) viable, (left top) early apoptotic, (right top) late apoptotic and (right bottom) necrotic cells.

Table 1. The Distribution Percentage of Apoptosis and Necrosis Rate (% Mean ± SD) of Fibroblast Cells at 24 and 48 Hours by Using Flow Cytometry

Fibroblast groups	Live cells 24-hours	Live cells 48-hours	Necrotic cells 24-hours	Necrotic cells 48-hours	Early apoptotic cells 24-hours	Early apoptotic cells 48-hours	Late apoptotic cells 24-hours	Late apoptotic cells 48-hours
Control	99.25±0.54 a	95.36±0.63 a	0.08±0.02 a	0.91±0.05 a	0.18±0.09 a	1.38±0.52 a	0.06±0.02 a	2.25±0.22 a
Alo	80.57±1.49b*	78.22±1.06 b*	4.17±0.12 b*	7.19±0.10 b*	13.22±0.10 b*	3.14±0.12 b	3.48±0.02 a	10.5±0.50 b*
Alo +SMF	85.1±1.51 c*	70.33±1.12 c*	1.69±0.56 c*	10.71±0.68 c*	10.54±0.48 b*	6.56±0.54 c*	2.35±0.94 a	11.67±0.10 c

Letters a, b and c refer to the groups' Significant Difference from each other according to Tukey test in two time 24 and 48h ($P < 0.05$). Groups with the same letter had not significant difference each other. * means Significant Difference value is $p<0.0001$

cells in (Alo+SMF) group was 85.1% while that of HeLa cells was 60.69% after 24 h (Tables 1 and 2).

In SMF+ Alo group, the fraction of early and late apoptotic Huo2 cells was dramatically reduced as compared with Huo2 cells in Alo group after 24 h. In the SMF+Alo group, the cell fraction in the early and late apoptosis of HeLa significantly increased in comparison with HeLa control and Alo groups after 24 h ($P<0.0001$). The percentage of cancer cells in the initial stage of apoptosis showed a major increase (mean difference = + 26.42) in Alo+SMF group compared to the Alo group after 48 h($p<0.0001$). This indicates that treatment with SMF+Alo resulted in a 4-fold increase in primary apoptosis in cancer cells at 48 h. The findings suggest that exposure time may play an important role concerning the effect of AEC on normal and cancer cells (Figure 3).

Cell cycle analysis

In each cell cycle phase, the cell number was obtained through flow cytometry to evaluate the possible impacts

Table 2. The Distribution Percentage of Apoptosis and Necrosis Rate (%Mean± SD) of HeLa Cells at 24 and 48 hours by Using Flow Cytometry

HeLa Groups	Live cells 24-hours	Live cells 48-hours	Necrotic cells 24-hours	Necrotic cells 48-hours	Early apoptotic cells 24-hours	Early apoptotic cells 48-hours	Late apoptotic cells 24-hours	Late apoptotic cells 48-hours
Control	99.58±0.41a	94.08±0.22 a	0.09±0.05 a	3.91±0.29 a	0.11±0.08	1.20±0.35 a	0.09±0.05 a	0.54±0.12a
Alo	76.15±1.52 b*	60. 59±1.13 b*	0±0.07 a	12.12±0.64 b*	20.17±2.05 b*	8.45±0.53 b*	0.12±0.04 a	19.41±0.12 b*
Alo +SMF	60.69±1.25 c*	54.98±2.63 c	2.12±0.06 b*	1.15±0.38 c*	28.97± 0.11 c*	34.87±0.70 c*	6.72±0.64 b*	7.67±0.09 c*

Letters a, b and c refer to the groups' Significant Difference from each other according to Tukey test in two time 24 and 48h ($P < 0.05$). Groups with the same letter had not significant difference each other. * means Significant Difference value is $p<0.0001$

Table 3. The Distribution Percentage of Cell Cycle Phases of Huo2 cells at 24 and 48 Hours

Cell cycle phases	G0/G1		S		G2/M	
Groups	24-hours	48-hours	24-hours	48-hours	24-hours	48-hours
Control	73.11±0.86a	70.97±0.08 a	10.16±0.16 a	10.07±0.21a	14.22±0.02 a	17.44±0.28 a
Aloe	55.05±0.81b*	57.23±0.74 b*	16.16±0.06 b*	12.91±0.12 b*	27.35±0.94 b*	29.35±0.32 b*
SMF+Alo	68.77±0.13 c *	62.06±0.06 c*	10.51±0.16 a	14.00±0.05 c	20.11±0.29c*	23.69±0.11 c*

Letters a, b and c refer to the groups' Significant Difference from each other according to Tukey test in two time 24 and 48h ($P < 0.05$). Groups with the same letter had not significant difference each other. * means Significant Difference value is $p<0.0001$

Table 4. The Distribution Percentage of Cell Cycle Phases of HeLa Cells at 24 and 48 hours

Cell cycle phases	G0/G1		S		G2/M	
Groups	24-hours	48-hours	24-hours	48-hours	24-hours	48-hours
Control	70.61±0.39 a	72.47±0.17 a	10.03±0.19 a	8.46±0.35 a	18.50±0.05 a	18.83±0.48 a
Aloe	52.79±0.64 b*	50.32±0.28 b*	14.12±0.55 b*	14.94±0.56 b*	31.19±0.75 b*	33.07±1.65 b*
SMF+Alo	54.13±0.4c	46.12±0.11 c*	10.56± 0.14a	13.90±0.25b*	34.92±0.07c*	39.24±0.56c*

Letters a, b and c refer to the groups' Significant Difference from each other according to Tukey test in two time 24 and 48h ($P < 0.05$). Groups with the same letter had not significant difference each other. * means Significant Difference value is $p<0.0001$.

of SMF in presence of AEC on cell cycle distribution (Figure 4).

The combination of AEC (50µg/ml) and SMF treatment significantly reduced the population of Hu02 cells in G2/M phase (approximately 7.24% and 5.66%) compared to AEC-treated cells, respectively, after 24 and 48 h (Table 3). However, this combination resulted in a significant increased G2/M arrest (approximately 2.94% and 5.07%) in HeLa cells compared to AEC-treated group, respectively, after 24 and 48 h (Table 4).

Discussion

According to the present findings, the combination of AEC and SMF treatment increased the necrosis and the early and late apoptosis and reduced living cells in HeLa cells compared to AEC-treated group after 24 h. However, this combination led to a significant reduce in the population of necrotic cells and early and late apoptosis in SMF+Alo group in fibroblast cells (Huo2) compared with AEC-treated group after 24 h. In one study, within 48 hours of treatment with these compounds, the number of G0/G1 phase cells decreased and the cell population of S

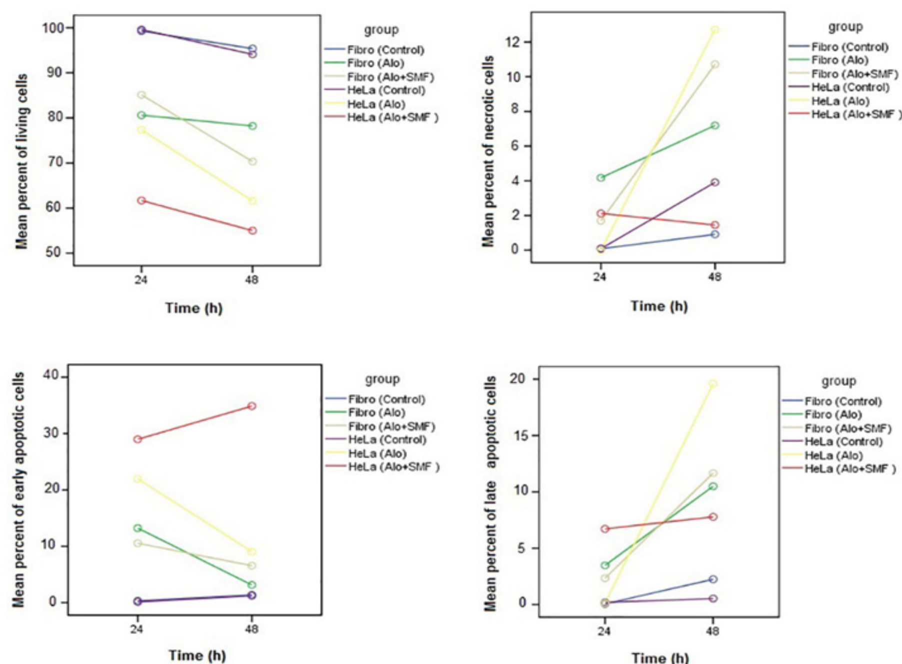

Figure 3. Time-Dependent Living Cell and Apoptotic and Necrotic Cell Death in Huo2 and HeLa Cells. The figures show HeLa cells line and fibroblast cells (Huo2) treated with Alo (Aloe vera extract (IC50, 50 µg/ml)), Alo+SMF (Aloe vera extract (IC_{50}) in the presence of static magnetic field (SMF,10mT)) and control HeLa cells line and Fibro (fibroblast cell) after 24 and 48 hours. Statistical analysis was performed using Univariate Analysis of Variance. Tukey's two ways ANOVA in apoptosis/necrosis analysis.

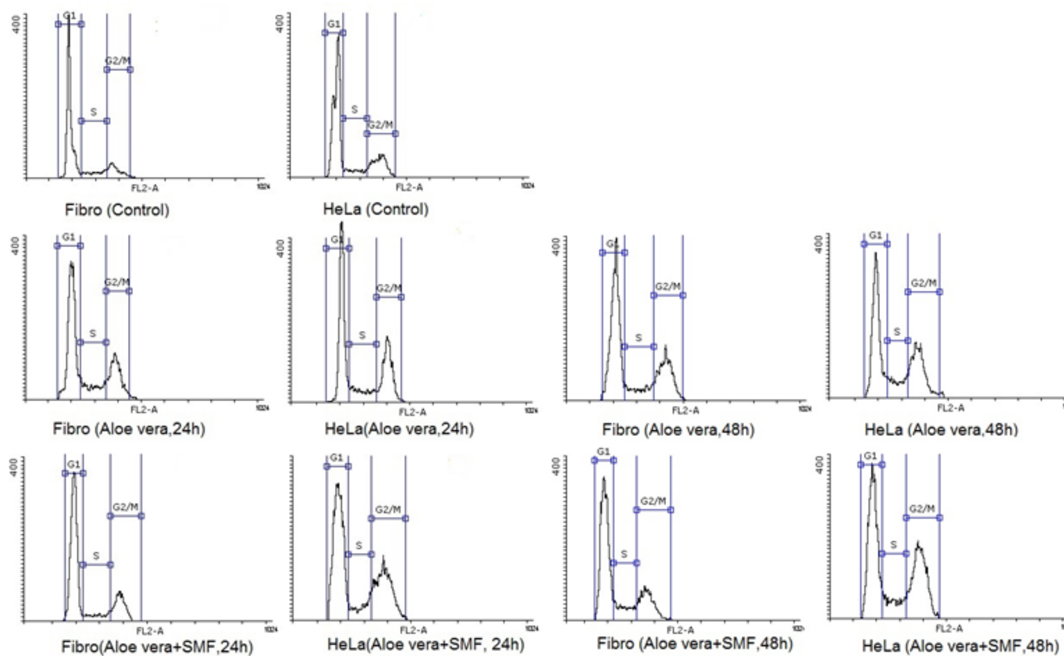

Figure 4. Analysis Cell Cycle Gating. The figure shows cell cycle stages of HeLa cells line and fibroblast cells(Huo2) in control and treated group with Aloe vera extract (Aloe vera,50µg/ml), Aloe vera extract in the presence of static magnetic field (Aloe vera+SMF)10mT after 24 h and 48h.

and G2/M phases increased in leukemia cells (Grimaudo et al., 1997). The results of a study showed that Aloe emodin reduced cell proliferation through blocking the cell cycle in the G2/M phase and inhibiting cyclin B1 in colon cancer cells (Suboj et al., 2012). Hussain et al., (2015) demonstrated that 60% ACE-treated HeLa cells showed increased amounts of cells in sub G0/G1 phase and reduced cells in S and G2 phases of the cell cycle in comparison with the untreated control. Our findings showed that AEC (50 µg/mL) caused a significant reduction in the number of cells in the G0/G1 phase while increasing the cells in S and G2 phase of the cell cycle in both cancer and normal cells in comparison to the untreated control groups following 24 and 48 h. In eukaryotic organisms, a vital cell cycle checkpoint is DNA the damage checkpoint of G2/M phase. To ensure that cells do not begin mitosis until after replication, damaged or incompletely-replicated DNA is sufficiently repaired (Löbrich and Jeggo, 2007). It seems that ACE caused certain problems in the transcriptional and repaired damage DNA, such that cellular rates increased over the two stages of the cell cycle. Nevertheless, in the presence of SMF, AEC had a different impact on normal cells, in a way that the amount of cells in the S and G2/M phase significantly decreased, and the cellular rate increased in the G0/G1 phase in compare to Alo group after 24 h. It appears that the field was able to reduce the AEC impacts on healthy cells (Huo2) down to a satisfactory level after 24 h, further confirmed by the rise in the rate of living cells in our statistical analysis of flow cytometry . After 48 hours, SMF exposure dramatically increased the AEC impact on HeLa cells. Accordingly, the number of cancer cells significantly decreased in the G0/G1 phase and remarkably increased in the G2-phase of the cell cycle. It is likely that SMF, as Okano (2012) stated, intensified the impact of the extract. The present results revealed that SMF exposure dramatically increased AEC effect and reduced the proliferation of HeLa cells in a time-dependent manner.

The SMF impact can be biophysical as its exposure could increase the concentration and life time of free radicals and the activity of reactive oxygen species (ROS) and other paramagnetic ions, possibly inducing apoptosis, oxidative stress, and genetic mutation (Ghodbane et al., 2013; Hajipour Verdom et al., 2018).

The S-phase checkpoint functions similar to a surveillance camera. Any issue with DNA replication triggers a “checkpoint”, a cascade of signaling events during the S-phase which puts the cell cycle phase on hold up until the issue is resolved (Li et al., 2019). The S-phase in both SMF+Alo groups (normal and cancer cells) decreased compared to Alo groups, approximately reaching the control levels as there was no significant difference between treated SMF+Alo groups after 24 hours. However, this influence was changed after 48 hours of exposure, where SMF significantly intensified the impact of AEC on normal cells (Huo2). Unlike cancer cells, the number of healthy cells significantly increased in the G0\G1 phase and decreased in the cell cycle G2-phase.

A previous investigation showed that SMF alone did not significantly affect the cell cycle arrest; however, it was able to intensify the effect of anticancer agents (Sarvestani et al., 2010).

SMF (600 µT to 9.4 T) influences the cell fate. The probable effects of SMF are dependent on the cell type, time, and field intensity (Marycz et al., 2018). Compared to normal cells, cancer cells undergo more genetic changes, some of which can distinguish its function and structure from normal cells, particularly affecting the uncontrolled growth of cells, DNA repair, and membrane

structure (Ertel et al., 2006; Chen et al., 2016; Rübben and Araujo, 2017; Jiang et al., 2019). Our data confirmed that the impact of SMF inhibition was dependent not only on time and dose, but also on the cell-type in the presence of AEC.

In conclusion, the results of this research revealed that SMF increased the anticancer impact of *Aloe vera* extract on HeLa cells and this effect was time-dependent. It seems that SMF reduced the impacts of *Aloe vera* extract on Huo2 cells. The effect of SMF on cell fate and molecular mechanisms and paths requires further thorough examinations.

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Conflicts of interest

None to declare.

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