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Allium jesdianum Extract Induces Oxidative Stress and Necroptosis in Human Colorectal Cancer (HT-29) Cell Line

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HIGHLIGHTS

- *Allium jesdianum* extract inhibits proliferation of HT-29 cells
- *Allium jesdianum* extract induces necroptosis in HT-29 cells
- *Allium jesdianum* extract induces oxidative stress in HT-29 cells

Abstract: This study aimed to evaluate the toxic impact of hydro-alcoholic *Allium jesdianum* extract (AJE) on the growth of HT-29 human colorectal cancer cell line. Phytochemical analysis using gas chromatography and mass spectroscopy (GCMS) was done to determine the bioactive components of AJE. HT-29 cells exposed to 0 (control), 25, 50, and 100 μg/mL of AJE for 48 hours. Cell survival, colony numbers, flow cytometry, oxidative stress, and gene expression were examined to evaluate the toxic impacts of the AJE. Twelve different phyto-constituents with peak areas were determined by the GCMS analysis. The major compounds were Allicin and α-Pinene. AJE considerably reduced the viability and colony numbers of the HT-29 cells. The AJE concentration-dependently increased necrosis, but not apoptosis in the HT-29 cells. AJE upregulated the expression of necroptosis-associated genes including RIPK1, RIPK3, and MLKL in a concentration-dependent manner. AJE also dose-dependently enhanced MDA contents and reactive oxygen species (ROS) level and diminished antioxidant enzyme level in the HT-29 cells. These data collectively indicated that AJE prevented the growth of the HT-29 cells by inducing oxidative stress, and activation necroptosis signaling pathways.

Keywords: necroptosis; oxidative stress; colorectal cancer; *Allium jesdianum.*

INTRODUCTION

Colorectal cancer is one of the most common cancer worldwide. Today, several methods used to treat cancer, but the response to treatment has very weak and often accompanied by undesirable side effects. Therefore, the production of drugs with high efficacy and less toxicity is necessary [1, 2]. Many compounds have studied to target cancer cells and the success of these compounds as an anti-cancer agent depend on their ability to activate pathways that cause cancer cells to die [3].

In recent years, focusing on cancer management has led to research on natural anticancer agents that are safe and acceptable among patients [4]. Phyto-bioactive compounds can induce ROS (reactive oxygen species) formation in malignant cells [5, 6]. Catalase (CAT) and Superoxide dismutase (SOD) are major enzymatic antioxidants involved in neutralizing ROS in the malignant cells [7, 8]. Among these natural compounds, the extract of *Allium jesdianum* (AJE) has been used to treat inflammatory diseases such as rheumatoid arthritis and relieve the pain of kidney stones in Eastern medicine. This plant belongs to the Alliaceae family and is native to Iran. Studies show that AJE has antioxidant and antipyretic effects [9]. Despite the beneficial effects of this plant, its effectiveness has barely investigated on cancer [10].

Cancer cells killing by cytotoxic therapies require the activation or reactivation of the cell death pathways such as apoptosis, autophagy, and necroptosis. Targeting cell death components are used as a valuable therapeutic strategy for a wide range of cancers. Necroptosis is a regulated mode of cell death with morphological features similar to both necrosis and apoptosis, which plays a main role in guarding against many diseases and cancers. Moreover, necroptosis can be activated as another pathway to cell death when cancer cells acquire resistance to apoptosis. Besides, the increase of oxidative stress activates critical regulators for triggering of necroptosis including RIPK1 (receptor-interacting protein kinase-1), RIPK3, and MLKL (mixed lineage kinase domain-like protein). Binding activated RIPK1 to RIP3 leads to phosphorylation of RIP3 and generates the necrosome complex, which then interacts with MLKL and causes phosphorylation and oligomerization of MLKL. MLKL oligomerization forms pores through disrupting membrane permeabilization subsequently, necroptosis is occurred [11].

The purpose of the present research was to investigate the impact of AJE against colon cancer cell (HT-29) growth and whether AJE can activate the necroptosis pathway, and inducing oxidative stress in these cells.

MATERIAL AND METHODS

Preparation of the extract

The AJ was collected from altitudes of Zagros in Shahrekord of Iran in June 2019. The herbarium voucher specimen (Number: A151640100AP) was identified in the Herbarium of Agricultural and Natural Research Centre in Ahvaz. After separating and wash of the aerial parts of the plant, they dried at room temperature. To prepare the extract by the Soxhlet device, the powdered plant was soaked in alcohol 80% for 48 hours. The prepared extract was placed in the -80 freezer until use.

Gas chromatography and mass spectroscopy (GCMS) analysis

GCMS analysis of a hydro-alcoholic extract of the AJ was done on GC 7890A equipped with MS 5975C detector and HP-5ms capillary column (30 × 0.25 m, and 0.25 µm). The capillary columns were heated from 60ºC to 180ºC (5ºC per min); from 180ºC to 280ºC for 30 min, and maintained at 280ºC for 6 min. The GCMS results were interpreted using the NIST (National Institute Standard and Technology) database. The spectra of the unknown components were determined by comparing with the known components registered in the NIST library [12].

Experimental design

Human NIH-3T3 (non-tumorogenic) and HT-29 (colorectal cancer) cell lines was bought from Genetic National Center of Iran. The cells were cultured in DMEM/F12 medium with streptomycin /penicillin (1%), and 10% FBS. They kept in a cell culture incubator. HT-29 cells treated with 0 (control), 25, 50, and 100 μg/mL of AJE for 48 hours. The exposure time of AJE were chosen on the basis of MTT test. AJE was diluted in the

DMEM/F12 medium. To determine the role of necroptosis, AJE co-treated with 3 mM Nec-1 (Necrostatine-1, a necroptosis inhibitor), and the survival rates of the HT-29 cells were evaluated by MTT test.

Cell viability

The HT-29 cells harvested in a 96-well plate (10,000 cells/ well). After 48 hours, the culture media replaced with different concentrations of AJE for 12, 24, 48, and 72 hours. Then, the cells exposed to the MTT solution (0.5 mg/mL) for 4 hours at 37°C. When the supernatants removed, Dimethyl sulfoxide (100 µL) added to the wells. A microplate reader (BioRad, CA) used to read the absorbance at 570 nm [13]. The MTT test was repeated 6 times for each group.

Colony assay

The HT-29 cells seeded on 6-well plates (300 cells/well) and exposed to different concentrations of AJE diluted in complete media. After 14 days, the cells incubated in the crystal violet solution (0.1 %) for 10 minutes. Then, the wells washed and air-dried to counting the colonies. A cell colony was defined as a group formation of at least 50 cells and counted using Image J software (Bethesda, Maryland, USA) [14]. The colony assay was repeated 6 times for each group.

Annexin V-FITC/propidium iodide assay

The HT-29 cells harvested in T-25 flasks (1×10^6 cells) and treated with different concentrations of AJE. After 48 hours, an Annexin V-FITC/ propidium iodide (PI) kit (Thermofisher, USA) was applies to quantify the necrosis, and apoptosis by a flow cytometer (Becton, CA). The $FITC^-/PI^-$ cells were viable, $FITC^+/PI^-$ cells were in early apoptosis and $FITC^+/PI^+$ cells were in the necrosis stage. WinMDI 2.9 software was used to analyzing the data [15]. The Annexin / PI assessments were done in triplicate.

Real-time polymerase chain reaction

The RNA was extracted from the HT-29 cells (1×10^7 cells) using RNeasy kit (Qiagen, USA). A cDNA synthesis kit (Fermntase, CA) was applied to generate cDNA from the extracted RNAs. The cDNAs (2 µL) were amplified in the PCR reaction solution (25 µL) containing SYBR Green (12 µL), and 0.3 µL of each primer (Table 1). The following program for 45 cycles considered to PCR amplification: 95ºC for 10 seconds, 95ºC for 15 seconds, 51-57 ºC for 20 seconds (annealing), and 60ºC for 20 seconds. GAPDH gene as a housekeeping gene was used to normalize the relative gene expression. Data were analyzed by the $2^{-\Delta\Delta CT}$ method [16]. The Real-time assays were performed in triplicate.

Table 1. Primer sequences

Genes		Primer sequencing	AT
GAPDH	Forward	5′-GCAAGAGCACAAGAGGAAGA-3′	55
	Reverse	5′-ACTGTGAGGAGGGGAGATTC-3′	57
RIPK1	Forward	5′-GCACAGCAAAGACCTTACG -3′	51
	Reverse	5′-TTGTTCCAAAGCCATGTGAG -3′	53
RIPK3	Forward	5′-CAAGGAGGGACAGAAATGGA-3′	55
	Reverse	5′-TTGTGGAACCTTGCTCCTCTT-3′	57
MLKL	Forward	5'-AGAGCTCCAGTGGCCATAAA-3'	55
	Reverse	5'-TACGCAGGATGTTGGGAGAT-3'	55

AT: Annealing temperature

Determining MDA content, ROS level, and antioxidant enzyme activity

After treatment, the lysis of the gathered specimens have been performed, and the protein amounts of HT-29 cells measured via a BCA assay kit (Thermofisher, USA). When the centrifuging of cell lysates was done, the contents of MDA and activities of the CAT and SOD were evaluated on the basis of the related guidelines (ZellBio, GmbH, Germany). With regard to the Company's guidelines, the reactive oxygen species

(ROS) amount measured by a dichloro-dihydrofluorescein diacetate (DCFH-DA) determination kit (Sigma, USA). Measurement of the ROS level was done via a spectro-fluorometer (LS50B, USA; Em: 570 nm, Ex: 490 nm). The measurement of the oxidative stress biomarkers was repeated 6 times for each group.

Statistical Analysis

Data were analyzed in SPSS (version 22.0, USA) using one-way analysis of variance, followed by posthoc pairwise comparison. The *p*-values < 0.05 were statistically significant. Each assay was done at least three times.

RESULTS

The phytochemical analysis

The phyto-components of the hydro-alcoholic extract of AJ identified by GCMS analysis. The main active compounds in the AJE are provided in Table 2, and the GCMS chromatogram is illustrated in Fig. 1. The heights of the peaks show the relative concentrations of the phyto-components of the AJE. The major compounds with anticancer property were included Allicin, α-Pinene, and γ-tocopherol.

Table 2. GCMS analysis of the AJE.

S.No.	Name of compound	Area (%)
1	Dimethyl-sulfide	5.6
2	Allyl alcohol	7.5
3	Allicin	5.4
4	Cyclopentasiloxane-decamethyl	10.1
5	β-Pinene	8.3
6	α-Pinene	26.4
7	2-pentylfuran	6.7
8	2-phenyl-5-methylindole	13.8
0	Methyl ethyl cyclopentene	3.4
10	γ-Tocopherol	5.7
11	Pentasiloxane, dodecamethyl	1.6
12	Bicyclo[4.3.0]nonane, 3-methylene	2.8

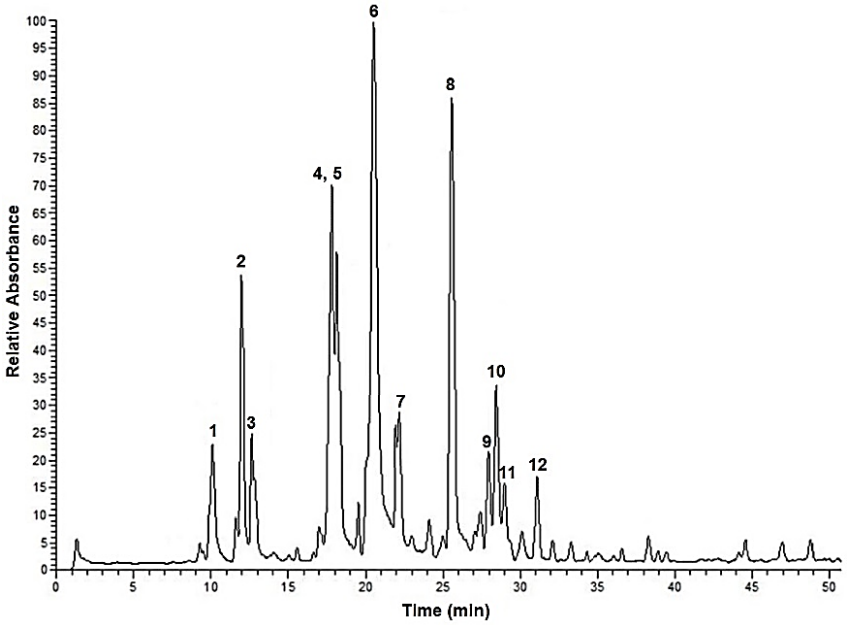

Figure 1. GCMS chromatogram of AJE. The numbers represent the separated main bioactive constitutes of AJE.

Cell Viability

No obvious reduction in the survival rate of HT-29 cells occurred within 12 or 24 hours of exposure to different concentrations of the AJE. A considerable diminish in viable rate from 100% in control to 85.3%, 67.1%, and 49.8% in 25, 50, and 100 µg/mL AJE-exposed cells, respectively, observed within 48 hours of exposure. No considerable changes in the viability percentage of HT-29 cells occurred within 72 hours of treatment with different concentrations of AJE compared to the 48 hours of incubation (Table 3). As shown in Fig. 2, treatment with Nec-1 plus AJE induced a considerable increase in the viability of the HT-29 cells compared with the only AJE-exposed cells ($P < 0.05$). AJE had no noticeable effects on the viability of the NIH-3T3 normal cells (Table 4).

Table 3. The viability percentage of HT-29 cells after different concentration (µg/mL) and exposure times of AJE (pilot study).

Groups	12 hours	24 hours	48 hours	72 hours
Control	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
AJE25	99.4 ± 3.2	92.4 ± 4.1	85.3 ±6.7	86.8 ± 4.9
AJE50	98.8 ± 2.9	84.6 ± 5.2	67.1 ±5.4*#	47.7 ± 5.3*#
AJE100	98.3 ± 3.7	82.5± 5.7	49.8 ± 4.8*#	44.5 ± 4.5*#
AJE200	96.3 ± 3.4	80.8± 6.5	25.7 ± 3.6**##	19.4 ± 4.1***##

Values are presented as mean ± SD (n=6). The concentration of 200 µg/mL AJE was considered as lethal dose and didn't use in this study. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$, ## $p < 0.01$; * and # respectively show comparison to 12 and 24 hours.

Table 4. The effects of different concentrations AJE on the NIH-3T3 cells

Groups	Control	AJE25	AJE50	AJE100
Viability (%)	99.01 ± 0.07	99.6 ± 0.09	101 ± 0.53	100.1 ± 0.48
Colony numbers	1.28 ± 0.14	1.18 ± 0.13	1.11 ± 0.07	1.23 ± 0.13
Early apoptosis (%)	1.26 ± 0.39	1.19 ± 0.23	1.31 ± 0.22	1.25 ± 0.17
Late apoptosis (%)	1.41 ± 0.14	1.37 ± 0.24	1.39 ± 0.12	1.44 ± 0.13
Necrosis (%)	0.53 ± 0.08	0.49 ± 0.05	0.41 ± 0.11	0.45 ± 0.07
CAT (U/ mg protein)	160.2 ± 11.4	166.2 ± 10.7	176.4± 13.1	177.5 ± 14.3
SOD (U/mg protein)	147.2 ± 9.3	145.6 ± 8.4	155.3 ± 10.5	157.7 ± 11.2
MDA (nmol/mg protein)	0.045 ± 0.00	0.041 ± 0.00	0.036 ± 0.00	0.035 ± 0.0
DCF formation (% of control)	100 ± 0.00	100.1 ± 3.2	92.8 ± 6.3	91.6 ± 0.17

Values are presented as mean ± SD.

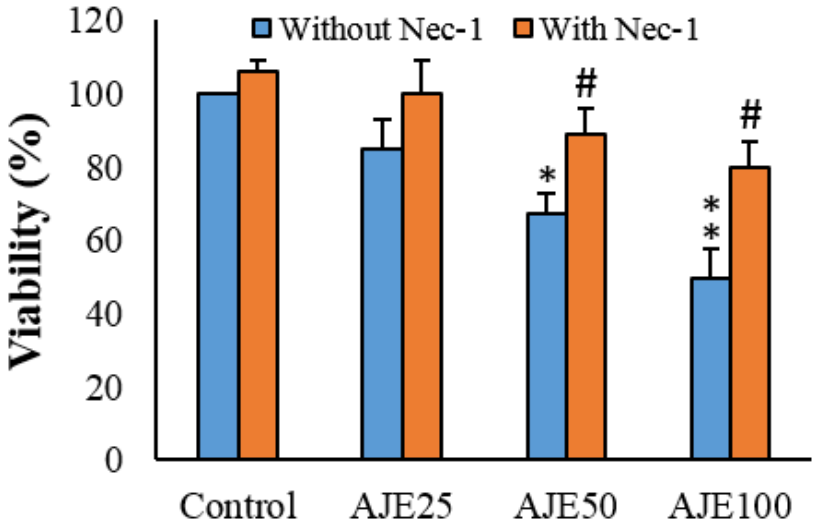

Figure 2. Viability percentage in control and experimental groups (mean ± SD). *P < 0.05, **P < 0.01, #P < 0.05; * and # show comparison to the control and without Nec-1.

Colony assay

The colony formation of HT-29 cells diminished slightly in the AJE25 group. The colony numbers reduced considerably in treatment with 50 or 100 µg/mL AJE compared to the control cells (P < 0.01, and P < 0.001, respectively) [Fig. 3]. AJE had no noticeable effects on the colony formation of the NIH-3T3 cells (Table 4).

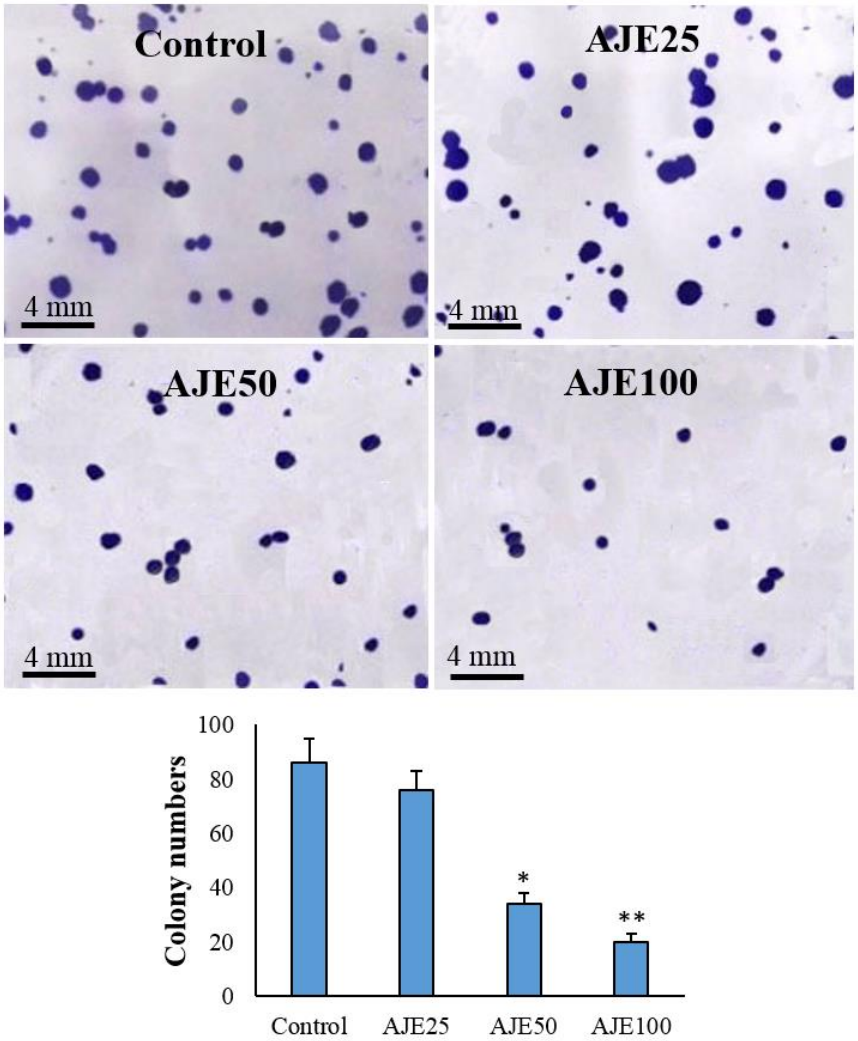

Figure 3. Colony number in different groups (scale bars: 4 mm). Each assay was done 6 times. Values are presented as mean ± SD. *P < 0.01, **P < 0.001; * indicates comparison to the control.

Annexin V-FITC/Propidium assay

The inducing apoptosis or necrosis by the AJE in the HT-29 cells was examined by Annexin V/PI staining.

AJE at different concentrations could not change the percentage of apoptosis. AJE dose-dependently could significantly enhance the necrosis percentage of the HT-29 cells (Fig. 4). AJE had no noticeable effects on the apoptosis or necrosis percentages of the NIH-3T3 cells (Table 4).

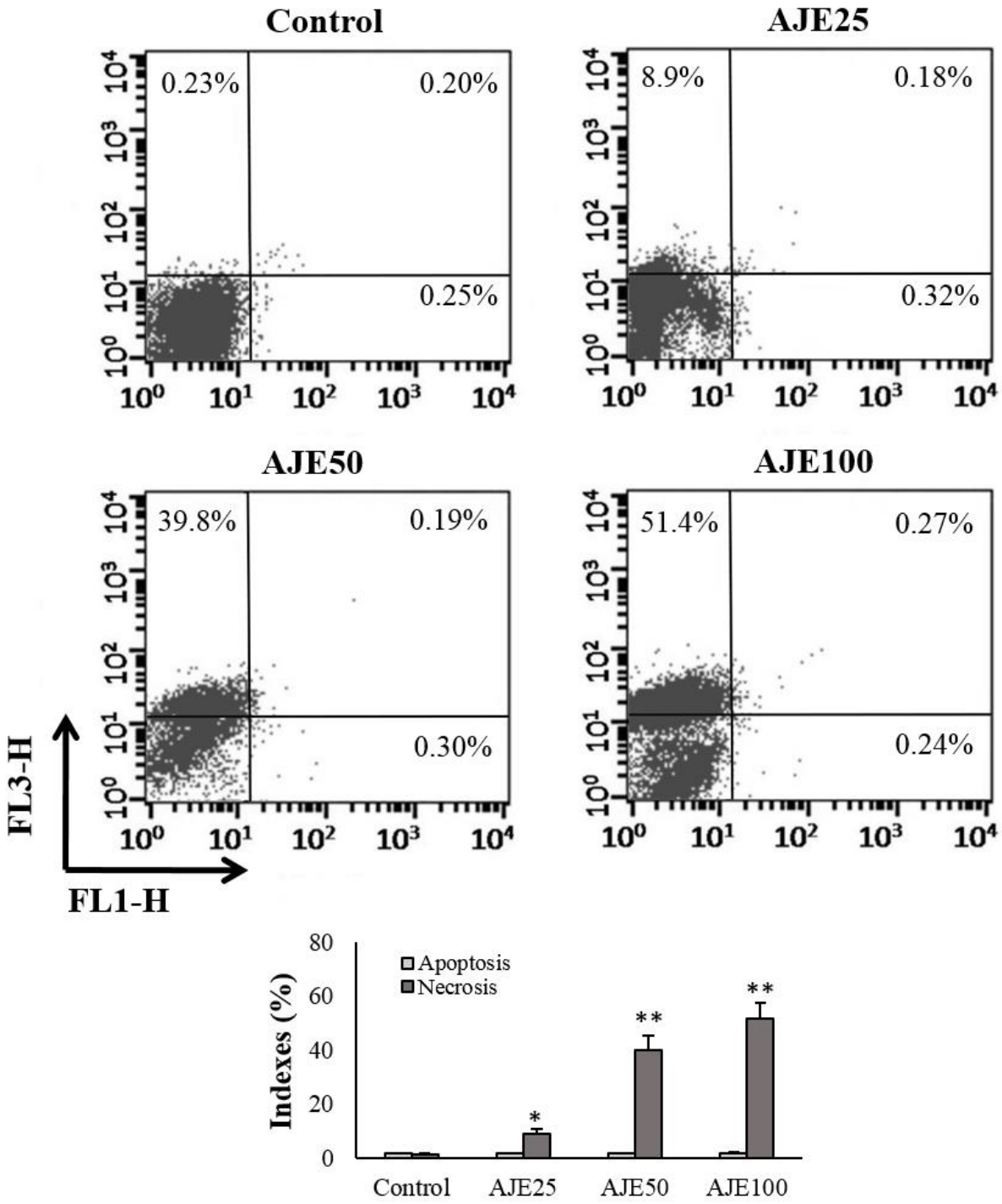

Figure 4. The results of Annexin/PI staining in different groups (mean ± SD). Lower left quadrant: live cells; Upper left quadrant: necrotic cells; Upper right quadrant: late apoptosis; Lower right quadrant: early apoptosis. **P* < 0.01, ***P* < 0.001; * indicates comparison to the control.

Real-time polymerase chain reaction

AJE-treated cells showed high mRNA expression of MLKL, RIPK1 and RIPK3 in a concentration-dependent manner. As sown in Fig. 5, the mRNA expression of MLKL, RIPK1 and RIPK3 was not obviously changed in the AJE25 group in comparison to the control. In the AJE50 and AJE100 groups, the mRNA expression of MLKL, RIPK1 and RIPK3 were considerably enhanced.

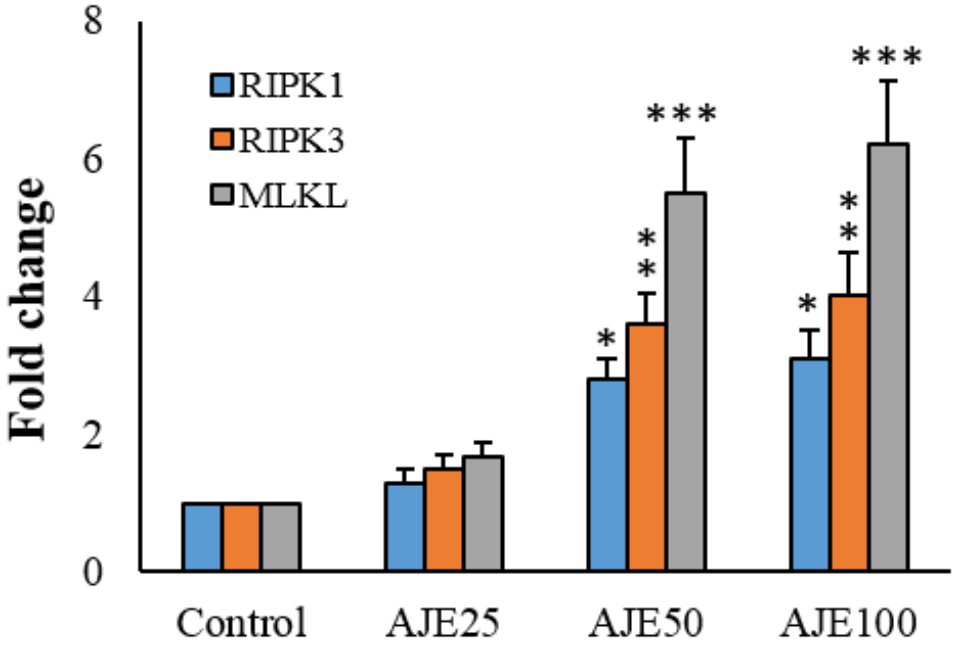

Figure 5. Gene expression in various groups (mean ± SD). Expression normalized to the average of a housekeeping gene (GAPDH). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; * indicates comparison to the control.

ROS level, MDA contents, and activities of antioxidant enzyme

ROS level, MDA content, and antioxidant enzymes activity of AJE-exposed cells were concentration-dependently changed in comparison to the control group. The oxidative stress biomarkers were not significantly changes in the AJE25 group. In the AJE50 and AJE100 groups, CAT and SOD activity considerably decreased compared to the control group. The ROS level, and MDA content obviously elevated in the AJE50 and AJE100 groups compared with the control (Fig. 6). AJE had no obvious effects on the ROS level, MDA content, and antioxidant enzymes activity of the NIH-3T3 cells (Table 4).

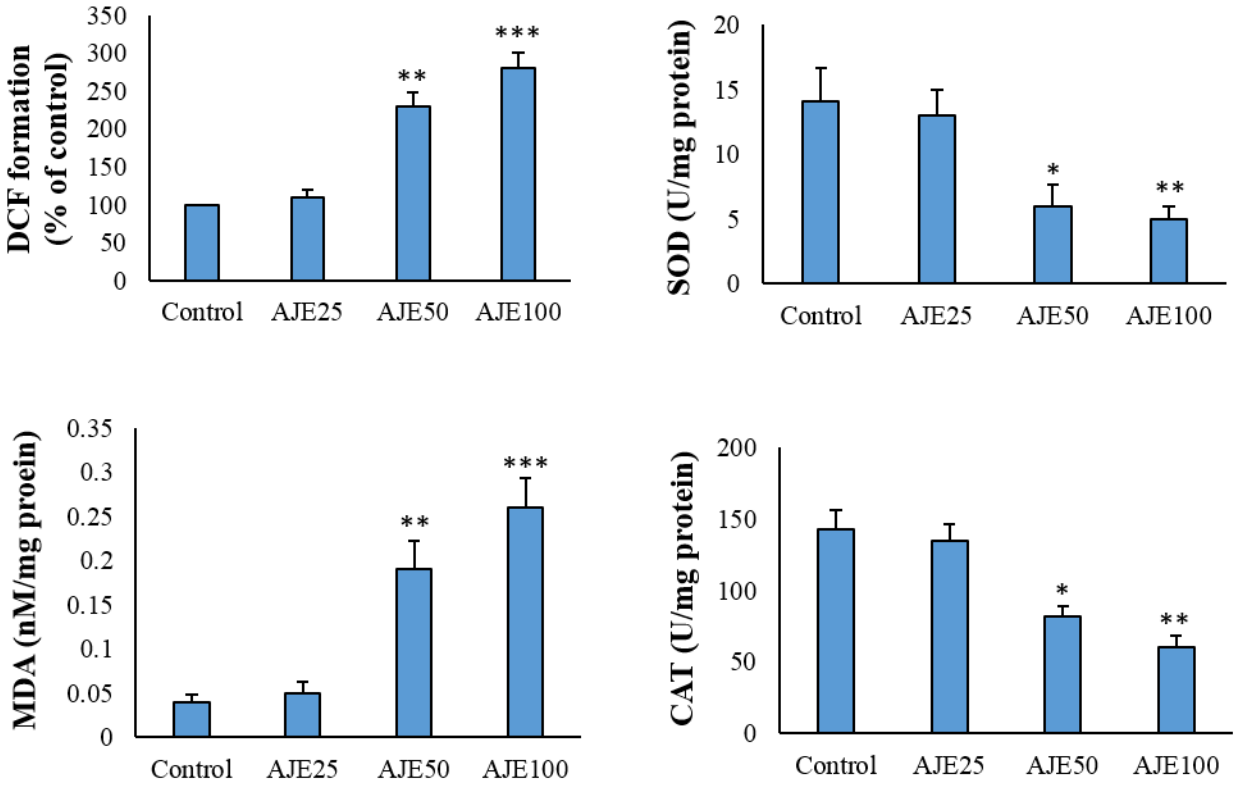

Figure 6. ROS levels, MDA contents, and antioxidant levels in different groups (mean ± SD). * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$; * indicates comparison to the control.

DISCUSSION

This study demonstrated cytotoxic impacts of AJE on the HT-29 cells. In line with our results, the methanolic extract of AJ exhibited anti-proliferative activity against cervical and leukemia cancer cells [10]. Among the phytocomponents identified by the GCMS, Allicin (a sulfur compounds), α-Pinene (a plenty terpenoids in nature), and γ-tocopherol have been reported to have anti-cancer impacts. The anti-cancer impacts of α-pinene against human hepatoma cell, human breast cancer cell, human prostate cancer cells and A549 (a lung cancer cell line) cells have been reported [17-20]. Antitumor activity of Allicin in gastric carcinoma, breast cancer, cervical cancer cells, and glioblastoma mainly by inhibiting cell proliferation has been shown [21-23]. It has been demonstrated that γ-tocopherol inhibits prostate malignancies in mice [24, 25].

Flow cytometry results have exhibited very low apoptosis, but high necrosis in the AJE-treated HT-29 cells. Thus, we examined the expression of the main necroptosis-regulators genes in the HT-29 cells. As mentioned in the results, AJE caused a high expression level of RIPK1, RIPK3, and MLKL genes in the HT-29 cells. The level of the RIPK1 and RIPK3 proteins is very low or silenced in colon cancer tissues [26]. The expression of RIPK3 protein diminished in breast cancer individuals that were associated with cancer progression [27]. Decreasing the MLKL expression has been reported in malignant cells [28-31]. Oxaliplatin and anthracyclines could activate necroptosis in malignant cell lines by inducing the expression of RIP3 and MLKL proteins [32].

The increased expression of necroptotic genes induced by AJE was accompanied by diminishing survival and proliferation of the HT-29 cells. These data indicate that AJE can effectively suppress the growth of the HT-29 cells by inducing necroptosis. To confirm the role of necroptosis in AJE-induced cell death, we evaluated the impacts of RIPK1 inhibitor Nec-1 on the HT-29 cells. The increasing viability of the HT-29 cells by Nec-1 confirms that necroptosis plays an important role in the anticancer impacts of AJE.

In the current research, the upregulation of RIPK1, RIPK3, and MLKL was accompanied by increasing ROS formation in the HT-29 cells. ROS participates in the modulating necroptosis in several cell types and enhances necrosome formation. It has been reported that mitochondrial ROS activated RIP1 autophosphorylation that enhanced RIPK1 recruitment of the RIPK3 aggregation [33, 34]. Bufalin inhibited the growth of breast cancer by increasing the expression of RIPK1/RIPK3 proteins and ROS production [35]. High expression of RIPK3 and MLKL proteins was accompanied by enhancing ROS formation in the Cobalt chloride-exposed HT-29 cells [36]. Due to the higher metabolic rate of cancer cells, they are more sensitive to ROS formation [37]. As evidenced in results, AJE concentration-dependently elevated MDA contents and ROS levels and reduced CAT, and SOD activity in the HT-29 cells. In line with these results, Kowalski and coauthors found that Vanadium complexes suppressed the growth of HT-29 cells by enhancing ROS production [38]. Phang and coauthors (2017) have demonstrated that Flavokawain-C prevents the growth of the HT-29 cells by increasing ROS formation and reducing SOD activity [39]. Bagheri and coauthors (2018) showed *Brucea javanica* fruit extract enhanced ROS generation in the HT-29 cells [40].

As shown in the results, the increased ROS formation in the AJE-exposed cells was accompanied by diminishing survival and proliferation of the HT-29 cells. Thus, AJE by inducing oxidative stress may kill the HT-29 cells.

CONCLUSION

In conclusion, the current study has evidenced that AJE concentration-dependently prevents the growth of colon cancer HT-29 cells. The toxic impact of AJE may be due to its anticancer components. The high cytotoxicity impacts of AJE on HT-29 cells depend on the activation of necroptosis signaling. In addition, AJE induced oxidative stress by enhancing ROS production and reducing antioxidant activity in the HT-29 cells. Further research will be necessary to increase insight into the cell death signal pathways in the AJE-exposed cancer cells.

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Conflicts of Interest: The authors declare no conflict of interest.

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