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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Oxidative stress induced apoptosis mediated anticancer activity of *Rhus typhina* fruits extract in human colon cancer

Lan Wang[1] · Ming Lu Xu[2] · Ling Xin[1] · Chi Ma[3] · Guoying Yu[1] · Kandasamy Saravanakumar[4] · Myeong-Hyeon Wang[4]

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Abstract

The present work was tested the anti-proliferation activity of *Rhus typhina* fruits extract (RTFE) in HEK293 and HT-29 cells, and studied their underlying mechanisms through the analysis of oxidative stress and apoptosis-related genes expressions by flow cytometer and RT-qPCR. In addition, the bioactive constituents were identified from RTFE by chromatography and NMR analysis. Among the various type of the fractions from the RTFE, the ethyl acetate fraction (EAF) was showed the potent anti proliferation effect in HT-29 cells. Thus EAF was selected for the further extensive analysis. Cytotoxcity and RT-qPCR results revealed that the treatment of EAF significantly inhibit the growth of the HT-29 cells by the up regulating of Bax and down regulation of Bcl-2, Survivn, AIF and SOD-2 gene expressions in time and dose dependent manner. Followed by sub G1 accumulation and the G0/G1 phase arrest, ROS production, and mitochondrial transmembrane potential ($\Delta\Psi m$). Finally, the active anti-proliferations agents such as luteolin and luteolin-7-O-glucuronide were identified from EAF by NMR. This work suggests that EAF could be a functional food to treat the chronic diseases.

Keywords Rhus typhina · Apoptosis · Cell cycle · Reactive oxygen species

Introduction

Rhus typhina (Staghorn sumac) is a main forestation species in North China and its fruits extracts (RTFE) are commonly used to make the traditional Chinese beverage such as "sumac-ade" and "Rhus juice". The RTFE based Chinese beverages are considered as folk medicine because of its pharmacological functions such as anti-haemorrhoidal, antiseptic, diuretic, and anti-stomachic pain (Kossah et al. 2010). The *R. typhina* fruits (RTF) are rich in polyphenols and linoleic acids, vitamins, minerals and organic acids (Kossah et al. 2009). The delocalization of aromatic rings in plant phenolics containing phenolic hydroxyl groups possess unpaired electrons, which shows the better stability and less reactivity than other antioxidants (Pereira et al. 2009). Furthermore, the toxicity of polyphenols is lower than that of destroyed free radicals (Micota et al. 2016). Much evidences are suggests that polyphenols of fruits and vegetables are play a beneficial role in cancer chemoprevention (Shin et al. 2010). Therefore, the understanding of the mechanism of polyphenols mediated antioxidant or pro-oxidant properties is essential in chemoprevention of various chronic diseases.

Oxidative stress with an excess of reactive oxygen species (ROS) has been proved to be involved in a variety of physiological processes such as proliferation, apoptosis and necrosis (Masgras et al. 2012). The role of oxidative stress has been investigated in cancer drug development and disease progression for several decades (Sosa et al. 2013; Gupta et al. 2009). The adequate level of ROS could induce the mitochondrial damage, which is involved in the early stages of apoptosis (Ravindran et al. 2011). The p53 is a particular important protein, which is in the response to the

✉ Myeong-Hyeon Wang
mhwang@kangwon.ac.kr

1 Colloge of Life Science, Henan Normal University, Henan Province, Xinxiang 453-007, China

2 Henan Institute of Science and Technology, Henan Province, Xinxiang 453-003, China

3 Xinxiang medical university, Henan Province, Xinxiang 453-003, China

4 Department of Medical Biotechnology, College of Biomedical Sciences, Kangwon National University, Chuncheon, Gangwon 200-701, Republic of Korea

genotoxic stress pathways including the apoptosis, cell cycle arrest, senescence and other physiological processes (Vousden and Prives 2009). Apoptosis induced by p53 is a central mechanism of tumor suppression through the intrinsic mitochondria-mediated pathway (Schuler et al. 2000). The Bcl-2-family proteins are regulate the cancer cell proliferation through apoptosis pathway (Reed 2008). Survivin is one of the apoptosis family protein, which is binds to the mitotic spindle and inhibits the apoptosis by down regulation of p53 (Hoffman et al. 2002). The expression of the apoptosis-inducing factor (AIF) with nicotinamide adenine dinucleotide (NADH) oxidase are affect the apoptosis associated oxidative stress (Daugasa et al. 2000).

The cell cycle is also very sensitive and can be altered by oxidative stress signals (Bloom and Cross 2007). ROS are considered as second messengers, which can be induced by chemotherapeutic drugs resulting the cell cycle arrest followed by cell death (Pyo et al. 2013). However, the antioxidant defense system of cells includes superoxide dismutases, catalase, and glutathione could defense against of ROS mediated cancer cell death (Tor et al. 2014). The recent reports are indicated that ROS may be an important progenitors in colorectal cancer (Wang et al. 2011). The aim of the present study was to determine the anticancer activities of the ethyl acetate fraction (EAF) of RTF against human colon cancer cells (HT29), and their underlying mechanisms of the oxidative stressed-mediated the cell cycle arrest and apoptosis.

Materials and methods

Extraction of R. typhina fruits (RTF)

RTF were harvested from Xinxiang city in Henan Province, China. The dried RTF were pulverized into fine powder. RTF powder (658 g) was extracted three times using the methanol at 60 °C for 12 h. Then, the extracts were combined, filtered and concentrated using the vacuum rotary evaporator (CCA-1110; EYELA, Tokyo, Japan) under reduced pressure and lyophilized. The yield of methanolic extracts (315.9 g) were suspended in water and fractionated by the different polarity solvent such as hexane, dichloromethane, ethyl acetate, *n*-butanol and water. Finally, final yield RTF extracts (RTFEs) such as 37.6 g of the hexane fraction (HF), 2.18 g of dichloromethane fraction (DF), 173.8 g of ethyl acetate fraction (EAF), 32.7 g of *n*-butanol fraction (BF), and 46.2 g of water fractions (WF) were subjected to the further experimental study.

Cell viability

The cytotoxic effect of the various RTFEs were tested in the cell lines such as HT-29 and HEK293 by MTT assay as described elsewhere (Saravanakumar et al. 2015). In brief, the cell lines HT-29 (3×10^5 cells.mL^{-1}) and HEK293 (3×10^5 cells.mL^{-1}) were seeded in 96 well plates containing the Roswell Park Memorial Institute (RPMI)-1640 and Dulbecco's Modified Eagle's medium (DMEM) respectively, supplemented with 10% of fetal bovine serum (FBS; Gibco, USA) and 1% streptomycin–penicillin in 5% CO_2 incubator at 37 °C for 24 h. Then the different concentration of RTFEs were treated to cells (HT-29 and HEK293) for 48 h. Cell viability was evaluated by standard MTT assay (Mosmann 1983).

Morphological changes

The HT-29 cells (2×10^5 cells.mL^{-1}) were seeded in 6-well plates and cultured 5% CO_2 incubator at 37 °C for 24 h. After the incubation periods the cells were treated with the different concentrations of EAF (0, 12.5, 25, 50, and 75 μg.mL^{-1}) for 48 h. The effect of EAF in morphological of the HT-29 cells were observed by the inverted and phase-contrast microscope (Olympus, Japan) at ×400 magnification.

Determination of apoptosis by Annexin V-FITC/PI staining

The apoptosis proportion of HT-29 cells was determined according to manufactures instructions of an Annexin V-FITC apoptosis detection kit. The HT-29 cells (2×10^5) were seeded into 6-well plates and incubated in 5% CO_2 incubator at 37 °C for 24 h, then the cells were treated with various concentration of EAE (12.5, 25, and 50 μg.mL^{-1}) for 48 h. After the treatments the cells were collected and followed the Annexin V-FITC conjugate and propidium iodide (PI) staining in the dark condition for 10 min. Then, the apoptotic rates of the stained cells were analyzed using flow cytometry (Becton–Dickinson, USA) as described earlier (Saravanakumar et al. 2019).

Cell cycle analysis

The HT-29 cells (2×10^5 Cells) were seeded into 6-well plates and incubated in 5% CO_2 incubator at 37 °C for 24 h, then the cells were treated with various concentration of EAE (0, 12.5, 25, and 50 μg.mL^{-1}) for 24 and 48 h. After the incubation periods the cells were stained with PI stock solution (50 μg.ml^{-1} of PI and 100 μg.mL^{-1} RNase A in PBS) for 30 min in the dark, DNA content was measured using a FACS flow cytometer analysis system (BD FACS Calibur, BD, USA).

Measure the intracellular ROS generation

The effect of the EAF on intracellular ROS level in HT-29 cells was detected with the fluorescence probe 2′,7′-

dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Invitrogen), using a flow cytometer according to the method described elsewhere (Jiang and Li 2014 and Kummara et al. 2016). A total of the 10,000 cells were analyzed for each treatment by flow cytometer. The experiments was carried out with 3 times and the data was presented as mean ± standard error.

Detection of mitochondrial membrane potential

The HT-29 cells (2×10^5 cells.mL^{-1}) were seeded in 6-well plates contain RPMI-1640 with 10% of FBS and 1% streptomycin–penicillin then placed in 5% CO_2 incubator at 37 °C for 24 h. After reaching the 80–90% of the confluences the cells were treated with different concentration of EAF (0, 25, 50, 75 µg.mL^{-1}) for 48 h. After the treatment periods, the cells were washed with PBS and then incubated for 30 min with 25 nM $DiOC_6(3)$ in the dark. The mitochondrial membrane potential was analyzed using 10,000 individual cells by FACS system. The experiments was carried out with 3 times and the data was presented as mean ± standard error.

The apoptotic-relative gene mRNA level expression

The HT-29 cells (2×10^5 cells.mL^{-1}) were seeded in 6-well plates contain RPMI-1640 with 10% of FBS and 1% streptomycin–penicillin then placed in 5% CO_2 incubator at 37 °C for 24 h. Then the cells (HT29) were treated with different concentration of EAF (0, 25, 50, 75 µg.mL^{-1}) for 48 h. After the treatment, the cells were harvested using the scraper then the total RNA was extracted from the treated cells using Trizol reagent (Rio et al. 2010). RNA were reverse transcribed to cDNA for RT-PCR analysis of apoptotic-related gene expression, the primer sequence referenced from our previous study (Wang et al. 2011).

Identification of compound1 and compound 2 in the EAF

The active fraction of EAF (32.4 g) was subjected to the open silica gel column chromatography followed by active compounds were purified using the thin layer chromatography, and high-performance liquid chromatography (HPLC). Finally, the yield compound 1 and 2 were identified using the NMR.

Statistical analysis

Results were expressed as the mean ± standard error (SEM, $n = 3$). Statistically significant differences were determined using a one-way ANOVA and Duncan's post hoc test by SPSS 11.5 software.

Results

Cytotoxicity and anti-proliferation effect of EAF

Firstly, all type of extracts were screened against HT-29 cells based on inhibitory effects by the MTT assay. Among the different extracts, the ethyl acetate fraction (EAF) was showed the strongest inhibitory effect in HT-29 cells (Fig. 1a). Thus, it was used for the further bioactivity assays and isolation of compounds. The cytotoxicity of EAF (12.5, 25, 50, and 75 µg.mL^{-1}) was tested in HEK293 and HT29 cells and its effect was compared with commercial standard of 5-FUat 40 µg.mL^{-1} as positive control. The different concentration of EAF (12.5, 25, 50, and 75 µg.mL^{-1}) treatments was significantly inhibits the proliferation of HT-29 cells by 2.4, 9.8, 38.9, 48.1%, and 8.7, 16.8, 41.6, 52.7% with 24 and 48 h respectively (Fig. 1b). While the EAF was not exhibited cytotoxicity in HEK293 cells (Fig. 2). The 5-FU was inhibited 54.6% and 62.5% of HT-29 cells proliferation for 24 and 48 h of treatments (Fig. 1b).

EAF induced the morphological changes

After treatment with EAF for 48 h, the significant morphological change of HT-29 cells was observed under a light microscopy (Fig. 1c–f). The HT-29 cells without the EAF treatment displayed normal, healthy shape with a distinct cytoskeleton. After incubation with 12.5, 25, and 50 µg.mL^{-1} of EAF for 48 h, the cellular morphology of HT-29 cells showed the distort shape, the membranes were damaged, the chromatin became more condensed, more apoptosomes and necrosis occurred with the increasing of EAF concentration. On the other hand the treatment of the EAF in the HEK293 cells did not showed any significant changes compared with untreated control (Fig. 2b–e).

EAF induced the apoptosis by Annexin V-FITC/ PI staining

The apoptosis induced by EAF was determined by Annexin V-FITC and PI staining analysis. Figure 3a–e indicated that the ratio of early-stage apoptotic cells increased from 2.59%, 4.56 to 6.95% after treatment with EAF (12.5, 25, and 50 µg.mL^{-1}) for 48 h, while the ratio of late-stage apoptotic/necrotic cells increased from 7.72%, 13.62 to 31.28% (Fig. 3e).

Cell cycle arrest

To estimate that the treatment of the EAF whether affect cell cycle of HT-29. We measured the cell cycle arrest using PI staining by flow cytometer after the treatment of EAF for 24

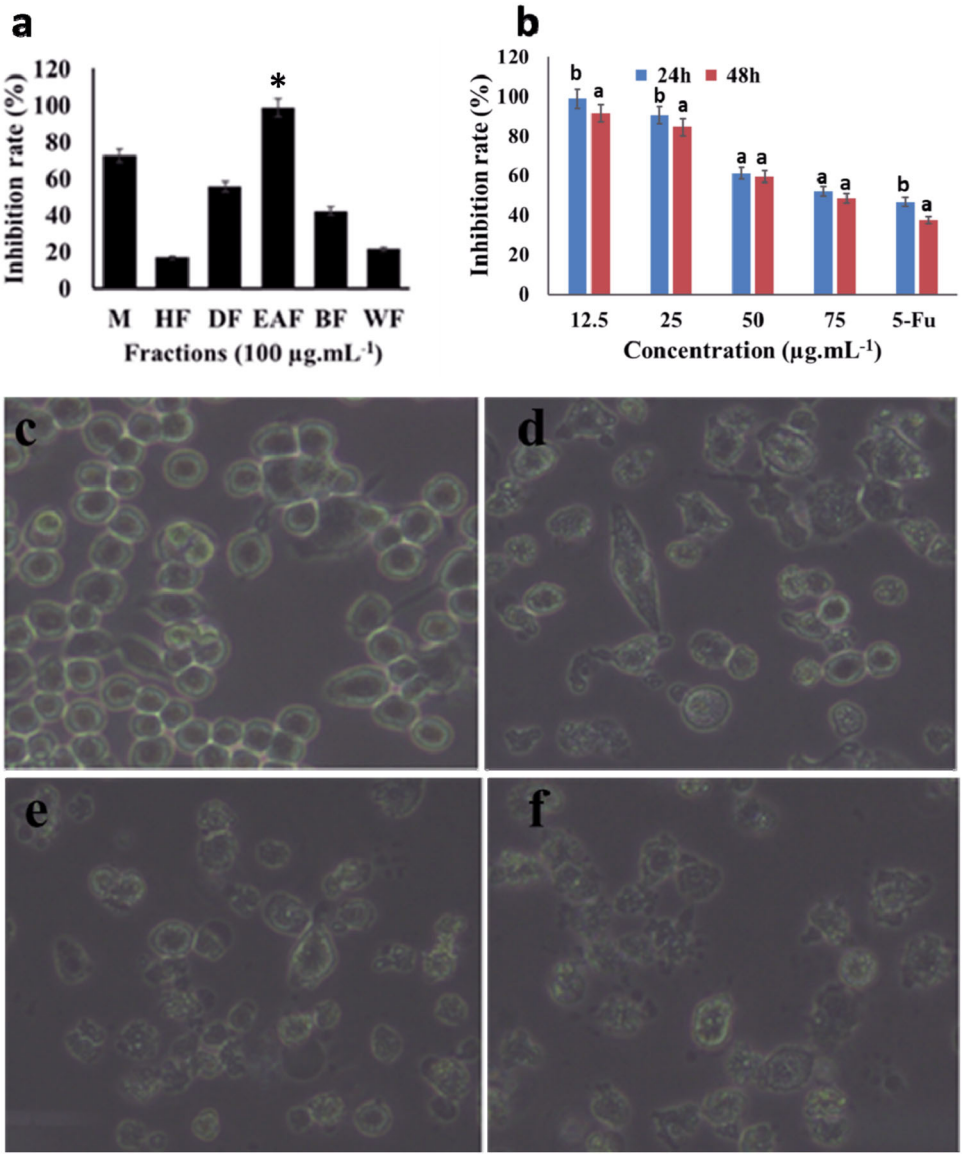

Fig. 1 Cytotoxic effect of various solvent fractions of RTFE on HT-29 (**a**), inhibitory effect of EAF in HT29 cells after the 24 and 48 h of treatments, where 5-FU at 40 µg/mL (**b**). Light microscopic observation of cellular changes in HT-29 cells untreated (**c**), treated with different concentration EAF 12.5 µg.mL^{-1} (**d**), 25 µg.mL^{-1} (**e**), and 50 µg.mL^{-1} (**f**). In Fig. 1a *$p < 0.05$ was significantly differ with other extracts RTFE; Data are represented as mean ± standard error (SEM, $n = 3$). The values not sharing the same alphabets significantly varied at $p < 0.05$

and 48 h by cellular DNA was analysis. Results showed that the percentage of cells in the sub-G_1 region was increased at a time and dose-dependent manner. Treatment with 12.5, 25, 50, and 75 µg.mL^{-1} of EAF was significantly increased sub-G_1 populations from 4.29% and 23.21 to 69.33% and 68.26%, respectively (Table 1). The percentage of cells in G_0/G_1 decreased from 70.78% and 60.24 to 29.26% and 31.21%, respectively. This indicated that EAF induces sub-G1 accumulation and G0/G1 arrest of HT-29 cells.

EAF induced decreasing of mitochondrial membrane potential (ΔΨm)

The loss of ΔΨm is associated with apoptotic cell death. To determine whether EAF induced the mitochondrial membrane potential of HT-29 cells, the pre-incubation of HT-29 cells with EAF was examined using the fluorescent probe $DiOC_6$ (3). Results showed that the loss of mitochondrial membrane potential in HT-29 cells was significantly increased from 0.74 to 26.72% (Fig. 4a–e). This indicates that the EAF-induced apoptosis occurs through destroying mitochondrial homeostasis.

ROS generation

Some studies have demonstrated that cell proliferation is related the induction of oxidative stress and generation of ROS in cells (Nita and Grzybowski 2016). Therefore, we examined whether the bioactivity of EAF was associated with ROS generation. As shown in Fig. 5a–g, treatment HT-29 cells with 0, 12.5, 25, and 50 µg.mL^{-1} of EAF for 48 h induced ROS level increasing in a concentration dependent

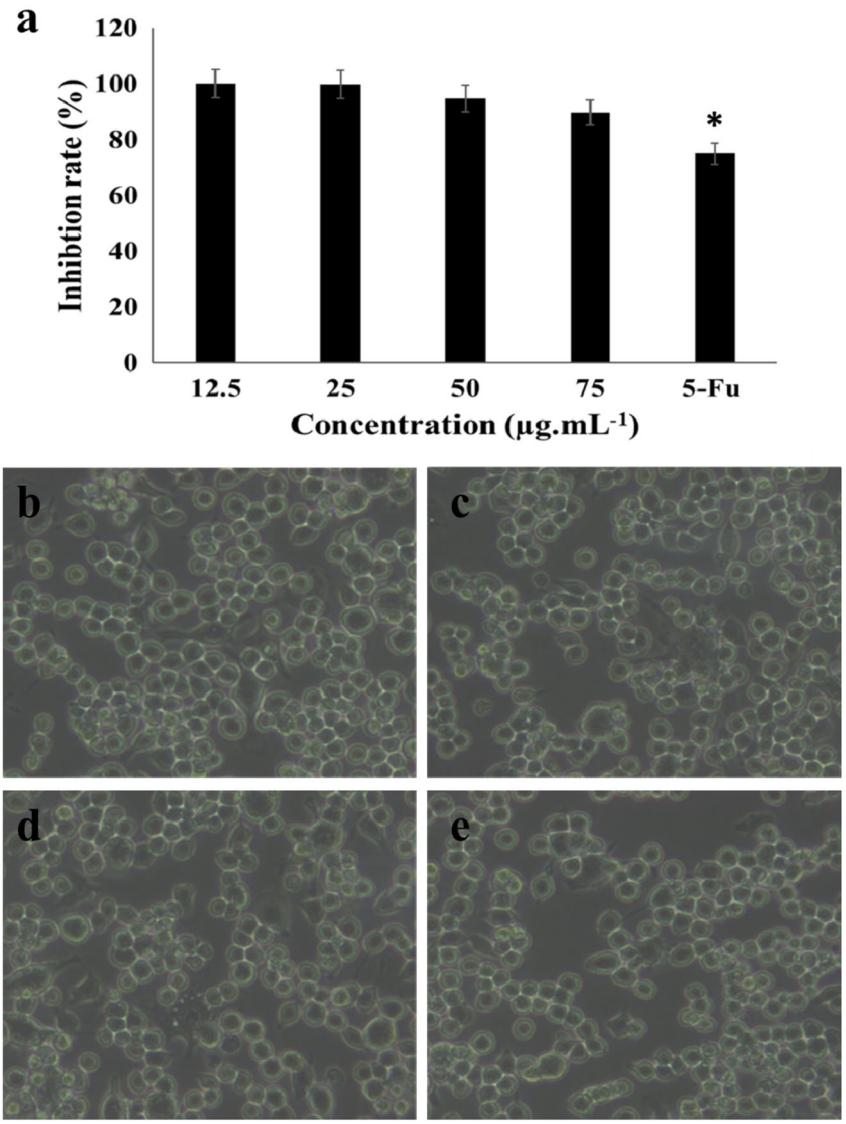

Fig. 2 Inhibitory effect of EAF in HEK293 cells after 48 h of treatment, where 5-FU at 40 μg. mL^{-1} (**a**). Light microscopic observation of cellular changes in HEK293 cells, untreated (**b**), treated with different concentration EAF 12.5 μg.mL^{-1} (**c**), 25 μg.mL^{-1}(**d**), and 50 μg. mL^{-1} (**e**). Data are represented as mean ± standard error (SEM, $n = 3$), in Fig. 2a *$p < 0.05$ was significantly differ with extracts RTFE (12.5 μg.mL^{-1})

manner. Comparatively, the antioxidants (10 mM NAC) reversed ROS generation, these results indicate that EAF induced the apoptosis by increasing ROS level.

Apoptotic-related gene expression in HT-29 cells

To explore the molecular mechanism of EAF triggered anticancer activity, we examined the apoptotic and anti-oxidant related gene expression by qRT-PCR. As shown in Fig. 6a, b, the p53 and Bax expression level was up-regulated while the Bcl-2 level was down-regulated in HT-29 cells after treatment with 25, 50, and 75 μg.mL^{-1} of the EAF. An increased Bax/Bcl-2 ratio contributes to apoptosis in PKO cells (Agarwal et al. 2004). The expression level of Survivin was decreased with the increasing concentration of EAF. AIF expression was significantly down-regulated after treatment with 25, 50, and 75 μg.mL^{-1} of the EAF. One of the antioxidant enzymes such as SOD_2 expression level was dramatically decreased with the EAF treatments at concentration depended manner (Fig. 6b).

Identification of compound 1 and compound 2 in the EAF

The EAF was loaded on a silica gel column and the column was eluted with dichloromethane-methanol (gradient from 100% dichloromethane to 100% methanol). The eluate was combined based on thin layer chromatography (TLC) results, yielding 22 fractions (RTFE1-22). Active fractions RTFE-4 (2.56 g), RTFE-15 (3.21 mg) were recrystallized with 100% ethyl acetate and purified by HPLC to give compounds **1** (2.12 mg), **2** (2.94 mg), respectively. Their structures were shown in Fig. 7. Compound **1** was identified as Luteolin, yellow powder; FAB-MS m/z: $[M + H]^+$, 287; ^1H NMR

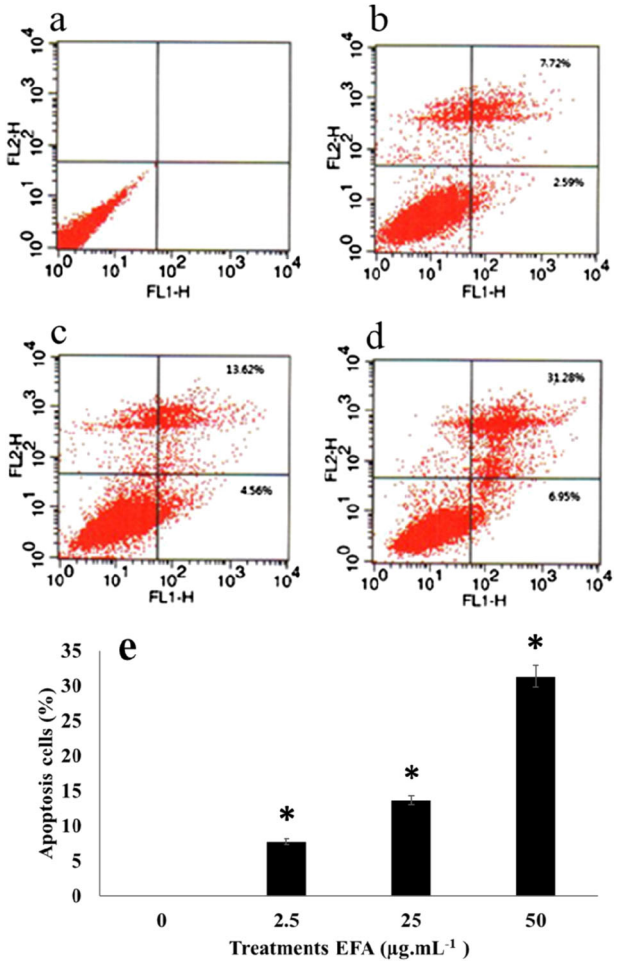

Fig. 3 Flow cytometry analysis of apoptosis cells in EAF treated or untreated HT29 cells. Untreated (**a**), treated with EAF 2.5 µg.mL^{-1} (**b**), 25 µg.mL^{-1} (**c**), and 50 µg.mL^{-1} (**d**). The Flow cytometry analysis was carried out after the 48 h treatments by using annexin V-FITC/PI staining. The lower right indicates the percentage of early apoptotic cells; the upper right indicates the percentage of late apoptotic and necrosis cells. Data are represented as mean ± standard error (SEM, $n = 3$), in Fig. 3e *$p < 0.05$ was significantly differ with untreated control (0)

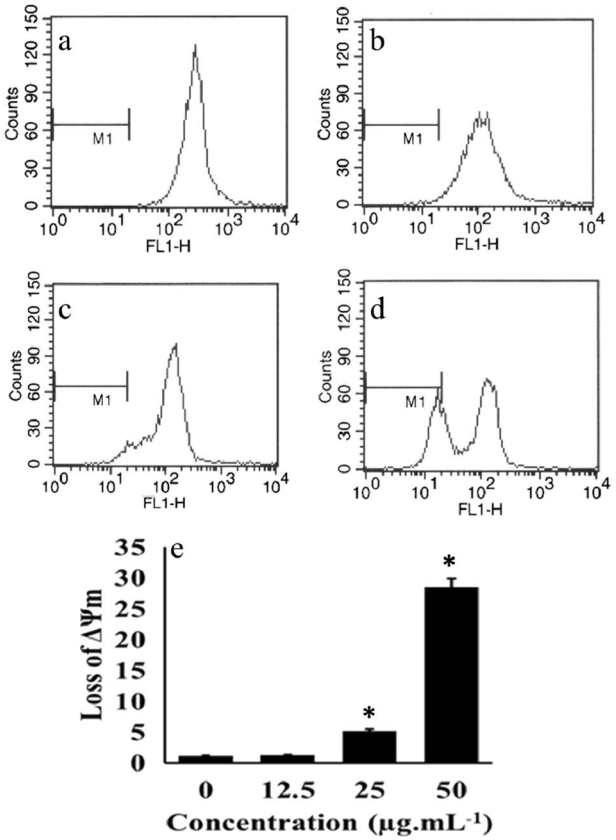

Fig. 4 Effect of EAF on mitochondrial membrane potential (ΔΨm) in HT 29 cells after 48 h of treatments. Untreated cells (**a**), cells treated with 12.5 µg.mL^{-1} (**b**), 25 µg.mL^{-1} (**c**), and 50 µg.mL^{-1} (**d**). Determination of ΔΨm loss in HT 29 due to the treatment of EAF (**e**). Data are represented as mean ± standard error (SEM, $n = 3$), in Fig. 4e *$p < 0.05$ was significantly differ with control (0)

Table 1 Analysis of cell cycle progression after treatment of different concentration of EAF (12.5, 25, 50, and 75 µg.mL^{-1}) for 24 and 48 h by flow cytometer

Time	Concentration of EAF (µg/mL)	SubG$_1$	G$_0$/G$_1$	S	G$_2$/M
24 h	0	1.35 ± 0.25^a	70.28 ± 1.07^e	18.21 ± 0.87^d	10.16 ± 0.67^c
	12.5	4.29 ± 0.38^b	69.03 ± 0.94^d	10.12 ± 0.27^c	16.56 ± 0.64^d
	25	23.53 ± 0.84^c	66.78 ± 1.37^c	3.74 ± 0.28^b	4.15 ± 0.19^b
	50	60.99 ± 1.26^d	37.68 ± 0.94^b	0.99 ± 0.18^a	0.55 ± 0.24^a
	75	69.33 ± 0.95^e	29.26 ± 0.15^a	1.10 ± 0.27^a	0.45 ± 0.17^a
48 h	0	12.75 ± 0.87^a	73.81 ± 2.34^e	4.26 ± 0.15^c	9.36 ± 1.04^d
	12.5	23.21 ± 0.28^b	60.24 ± 2.08^d	2.98 ± 0.38^b	13.63 ± 0.48^e
	25	31.80 ± 1.06^c	56.44 ± 2.05^c	9.25 ± 0.67^d	3.96 ± 0.35^c
	50	46.32 ± 1.35^d	52.24 ± 1.29^b	0.63 ± 0.35^a	0.93 ± 0.29^b
	75	68.26 ± 3.28^e	31.21 ± 1.03^a	0.32 ± 0.15^a	0.27 ± 0.24^a

Data are the mean ± SE (SEM, $n = 3$). The values not sharing the same alphabets were significantly varied at $p < 0.05$

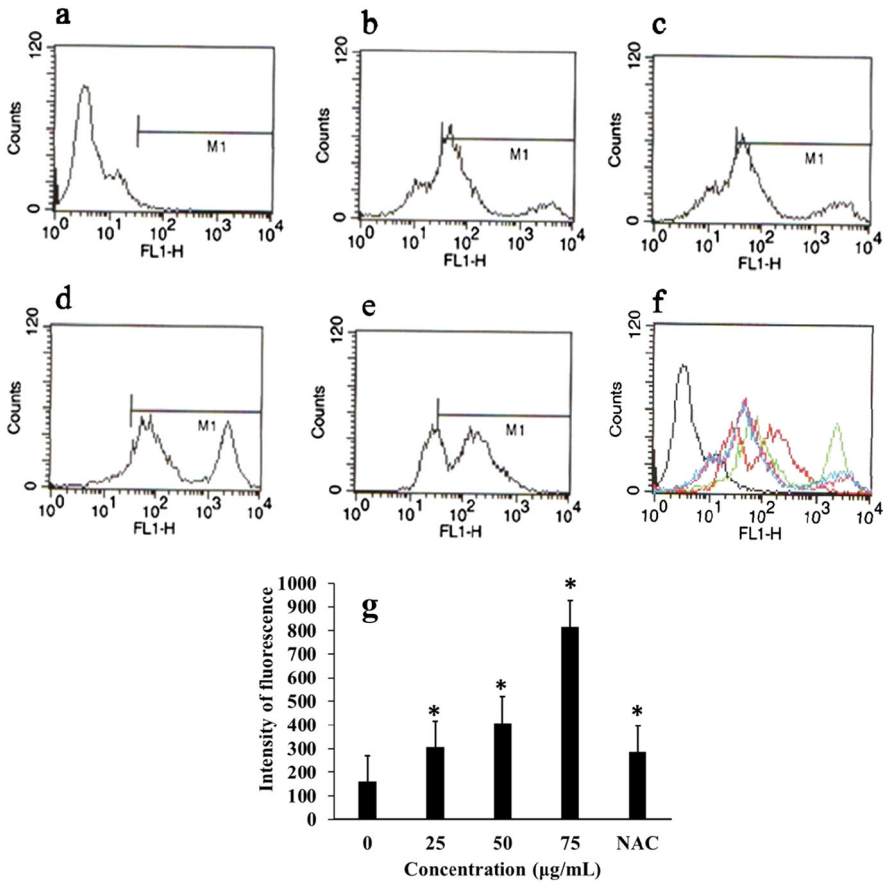

Fig. 5 Effect of EAF on generation of intracellular ROS in HT29 cells untreated (**a**) or treated with 25 μg.mL^{-1} of EAF (**b**), 50 μg.mL^{-1} of EAF (**c**), 75 μg.mL^{-1} of EAF (**d**), and NAC 10 mM (**e**). Comparison of ROS generation in HT29 cells treated with different concentration of EAF by flow cytometer (**f**), Competitive analysis of ROS production in in HT29 cells treated with different concentration of EAF in terms of intensity of fluorescence (**g**)

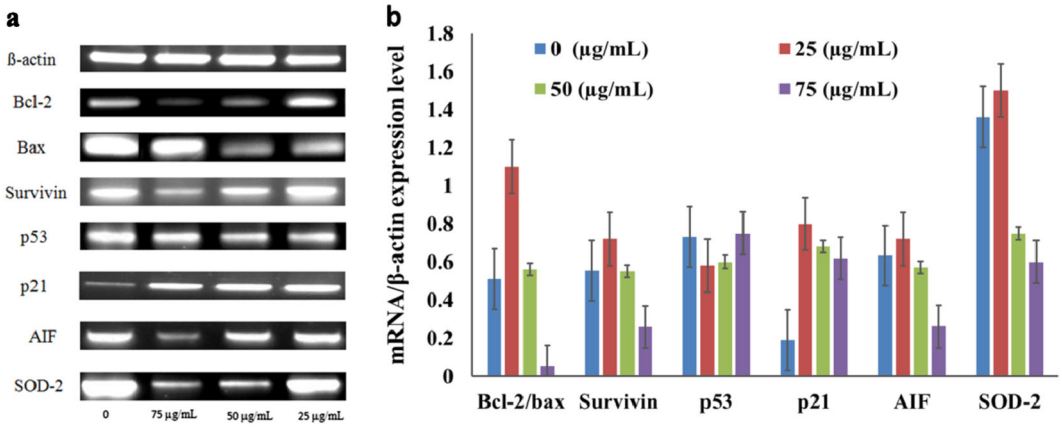

Fig. 6 Analysis of the apoptosis-related gene expression using the agarose gel electrophoresis (**a**), quantification of apoptosis-related gene expression in terms of mRNA/β-actin expression using RT-qPCR (**b**)

(DMSO-d_6, 400 MHz): δ 12.94 (1H, OH-5), 7.47 (1H, d, J = 8.1 Hz, H-6′), 7.42 (1H, s, H-2′), 6.90 (1H, d, J = 8.1, H-5′), 6.79 (1H, s, H-3), 6.46 (1H, br s, H-8), 6.14 (1H, br s, H-6). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 181.63 (C-4), 164.31 (C-7), 163.65 (C-2), 161.36 (C-9), 157.28 (C-5), 150.28 (C-4′), 146.08 (C-3′), 121.63 (C-1′), 119.35 (C-6′), 116.17 (C-5′), 113.67 (C-2′), 103.68 (C-10), 103.15 (C-3), 99.86 (C-6), 94.07 (d, C-8) (Zheng et al. 2008) (Fig. 7a).

Compound **2** was identified as Luteolin 7-O-glucuronide, straw yellow powder; FAB-MS m/z: $[M + H]^+$, 463; ^{1}H NMR

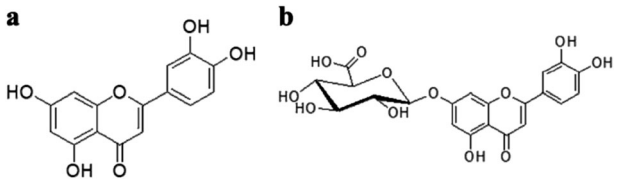

Fig. 7 The structures of compounds 1-Luteolin (**a**) and compound 2-Luteolin 7-O-glucuronide (**b**)

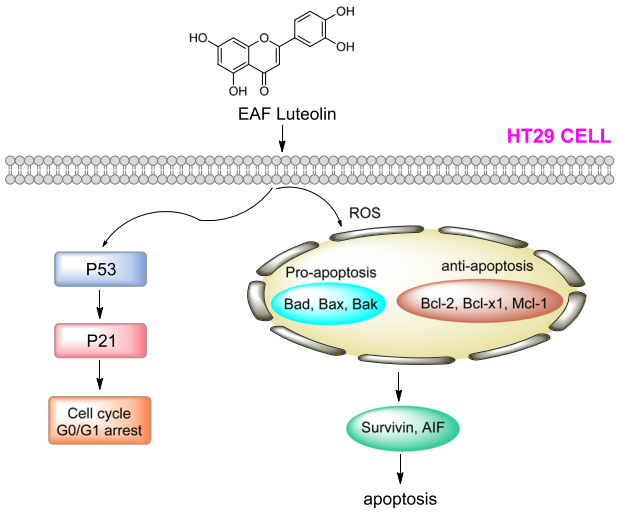

Fig. 8 Possible molecular mechanisms of EAF induced cell death in HT-29 cells

(DMSO-d_6, 400 MHz): δ H 12.89 (1H, s, 5-OH) 7.49 (1H, d, $J = 8.1$ Hz, H-6′), 7.44 (1H, s, H-2′), 6.92 (1H, d, $J = 8.1$, H-5′), 6.80 (1H, s, H-3), 6.76 (1H, br s, H-8), 6.46 (1H, br s, H-6), 5.28 (1H, d, $J = 6.6$ Hz, H-1″), 4.01 (1H, D, $J = 9.6$ Hz, H-5″) 3.80-3.10 (m, sugar-H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 182.23 (C-4), 170.12 (C-6″) 164.81 (C-2), 163.25 (C-7), 161.48 (C-5), 157.26 (C-9), 150.28 (C-4′), 146.08 (C-3′), 121.63 (C-6′), 119.45 (C-1′), 116.27 (C-5′), 113.87 (C-2′), 105.68 (C-10), 103.45 (C-3), 100.18 (C-1″), 99.86 (C-6), 95.07 (d, C-8), 75.96 (C-5″), 75.38 (C-3″), 73.16 (C-2″), 71.85 (C-4″), and 172.32 (C-6″) (Ma et al. 2018) (Fig. 7b).

Discussion

The EAF causes the predominant apoptosis in HT29 cells evidenced by sub-G1 accumulation and cells morphological changes, ROS generation and regulating apoptosis related gene in HT29 cells. EAF could destroy the functions of mitochondria, which lead to the loss of $\Delta\Psi$m. The increasing level of ROS subsequently activated the intrinsic apoptosis pathway. Base on these results, we summarized the possible anti-apoptotic mechanisms of EAF in Fig. 8.

The EAF can up-regulate the gene p53 and p21 expression and lead to the G_0/G_1 phase arrest. Mitochondrial outer membrane permeabilization (MOMP) was a crucial event of intrinsic pathway which controlled by Bcl-2 family members, and it requires the activation of Bax or Bak (Youle and Strasser 2008). Our results suggested that EAF made the mitochondrial outer membrane permeabilization and activates the Bax. The increased level of Bax/Bcl-2 ratio is implied that EFA may be induce the cell death in HT29 cells via the classic intrinsic apoptotic pathway. Li and co-workers has been reported that survivin is repressed in the G1 phase (Li and Altieri 1999), similarly our results also exhibited that the treatment of EFA was significantly inhibited the expression level of survivin which result the G_0/G_1arrest in HT-29 cells. Thus, EAF induce the survivin repression in the G1 phase. The AIF expression was significantly down-regulated, therefore AIF contribute to p53-mediated apoptosis.

An increase of ROS is a typical phenomenon in the process of intrinsic apoptosis pathway (Ying et al. 2008). Antioxidant enzymes such as SOD2 and phospholipid hydroperoxide glutathione peroxidase (PHGPx) generally regulate the ROS produced in mitochondria, which can potently protect the mitochondria from oxidative damage. Several studies have shown that SOD2 deficiency is associated with apoptotic cell death. Similarly, our results was found the SOD2 expression level was dramatically decreased with the EAF treatments depending upon the concentration increase.

Luteolin (3′,4′,5,7-tetrahydroxyflavone), is a subclass of flavonoids, possesses cancer chemo preventive and chemotherapeutic potential, and it can modulate the ROS levels, and inhibit the topoisomerases I and II, reduce the NF-κB and AP-1 activity, stabilization of p53 (Lopez-lazaro 2009). Evidence demonstrates that Akt, STAT3, HSP90 and Bcl-2 family proteins are the main targets of luteolin (Tuorkey 2016). The flavonoid luteolin, also found can prevent toxic response (Iakovleva et al. 2015), little report about the bioactivity of luteolin 7-O-β-glucuronide except its allelopathic activity (Clifford et al. 2005). In conclusion, our results demonstrated that the antiproliferative effects of EAF was occurred through the ROS generation and regulation of the apoptosis related gene expression. Also this work emphasized that the further in vitro molecular elucidation followed by animal model experiments are required for successful application of RTFE derived compound in future chemotherapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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