

## Original Research Article

# Antiproliferative and proapoptotic activities of ferulic acid in breast and liver cancer cell lines

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Sent for review: 5 July 2019

Revised accepted: 22 November 2019

### Abstract

**Purpose:** To explore the potential anticancer activities of ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA) on two different human cancers cell lines, viz, breast (MCF-7) and hepatocellular (HepG2).

**Methods:** MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity, Annexin V staining, enzyme-linked immunosorbent assay (ELISA), as well as caspase-8 and -9 activation assays were used to evaluate the proapoptotic and antiproliferative potentials of ferulic acid (FA) on MCF-7 and HepG2 cell lines.

**Results:** Ferulic acid exerted cytotoxic effects on MCF-7 and HepG2 cell lines with half-maximal inhibitory concentration (IC<sub>50</sub>) of 75.4 and 81.38 µg/mL, respectively, at 48 h. Annexin V staining revealed evidence of apoptosis. Caspase-8 and -9 levels were elevated in both cell lines after incubation with ferulic acid.

**Conclusion:** The findings of this study suggest that ferulic acid has promising therapeutic potentials for the treatment of breast and liver cancers by inducing apoptosis via activation of caspase-8 and -9 pathways.

**Keywords:** Ferulic acid, Apoptosis, Antiproliferative activity, Breast cancer, Caspase, Liver cancer

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## INTRODUCTION

Ferulic acid is a natural phenolic phytochemical present in the cell walls of many plants [1]. The literature on ferulic acid (FA) reported various biological Activities [2,3]. Its anti-oxidative and anti-inflammatory actions are mediated through free radical scavenging activity, which inhibits lipid peroxidation and apoptosis under normal

and stressful physiological conditions [1,2,4,5]. In hepatic disorders, FA was reported to protect from oxidative stress by high glucose levels and decrease cell apoptosis [2].

The previous studies on FA's effect against breast, cervical, lung, pancreatic, colorectal, prostatic, thyroid, bone, and skin cancers demonstrated potential anticancer activity [2]. It

was reported that H<sub>2</sub>O<sub>2</sub> induced Bcl-2 dependent apoptosis was inhibited by FA in cancer cells without affecting the expression of Bcl-2, resulting in the upregulation of immune system recognition of the cells and apoptosis [2]. Also, it decreases cancer cell viability while inhibiting migration and invasion [6]. When combined with PARP inhibitors and  $\delta$ -tocotrienol, FA significantly inhibited cancer cell proliferation [3].

Additionally, FA arrested the cell cycle in the G0/G1 phase of ECV304 endothelial cells [7]. Along with the previous findings, FA was found to exert antitumor activity and inhibit metastasis in MDA-MB-231 (breast adenocarcinoma) cells and downregulates EGFR in HCT 15 (colorectal cancer) and MCF-7 cell lines [8-10]. In MIA PaCa-2 human pancreatic cancer, FA decreased cell viability and migration and prevented rapid progression of pancreatic cancer in combination with aspirin [6,11]. FA was also shown to modulate p21, p53, NF $\kappa$ B, Bax, and caspase-3 molecules in radiosensitizing cervical and lung cancers [12].

Overall, based on the mechanisms through which chemotherapeutic agents are active, it has been emphasized that the induction of apoptosis is an important mechanism to target tumor cells [13,14].

Because of the potential anticancer effects of FA, and the lack of reports on its proapoptotic activity through the induction of caspase pathway, this study aims to explore the possible anti-proliferative and proapoptotic activities of FA on human breast and hepatocellular cancer cells (MCF-7 and HepG2, respectively) through induction of the caspases pathway.

## EXPERIMENTAL

### Chemicals and reagents

Ferulic acid, dimethyl sulfoxide (DMSO), MTT, fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and all related compounds were bought from Sigma-Aldrich Co. (USA). Caspase-8 and Caspase-9 colorimetric assay kits (AB39700 and AB65608) were obtained from Abcam (USA). Annexin V-ELISA apoptosis detection kit (Cat no. 61-ANNHU-E01) was purchased from ALPCO (USA).

### Cell culture

MCF-7 and HepG2 cell lines were purchased from American Type Culture Collection (ATCC) and grown in 75-cm<sup>2</sup> TC flasks (Thermo Scientific) with culture media consisting of DMEM containing

standard antibiotics (streptomycin and penicillin, 100 U/mL and 100  $\mu$ g/mL, respectively), in addition to, FBS (10 % v/v). The cells were incubated in a 95 % humidified incubator at standard conditions (37 °C, and 5 % CO<sub>2</sub>), and the experiments were conducted when 80% confluency was achieved.

### Determination of cell viability

The colorimetric assay of MTT was conducted assessing the cytotoxic activity of FA on the tested cell lines. Briefly, 96-well plates were used to culture cell lines at a density of 5  $\times$  10<sup>6</sup> cells/mL and were incubated for 24 hours at 37 °C, 95 % humidified air and 5 % CO<sub>2</sub>. Various concentrations of FA (0, 1, 10, 50, 100, 200, and 500  $\mu$ g/mL) in triplicates were added to the culture medium, and it was incubated for three-time intervals (24, 48 and 72 h). Then, the tested cells got washed once using phosphate-buffered saline (PBS).

Subsequently, 100  $\mu$ L MTT in DMEM was added, they were incubated for 3 h. The formed formazan was solubilized in DMSO (100  $\mu$ L) followed by 15 min incubation. Thereafter, absorbance was measured at 570 nm and computed as proliferation rate (PR) using Eq 1.

$$PR (\%) = (At/Ac)100 \dots\dots\dots (1)$$

where At and Ac are the absorbance of treated cells and control cells, respectively.

The cytotoxicity of FA was calculated from PR in terms of its inhibition rate (IR) as in Eq 2.

$$IR = 100 - PR \dots\dots\dots (2)$$

The IC<sub>50</sub> value is reported for each cell line.

### Annexin V-ELISA assay

Human Annexin V levels were determined using annexin V ELISA assay kit in the treated cell lines at 48 h at a 100  $\mu$ g/mL concentration of FA compared to control in duplicates. Briefly, to determine Annexin V levels, the cells lysate was obtained by incubating the cells with 50  $\mu$ L cell lysis buffer on ice for 15 min and centrifuging it at 10,000 g for 10 min at 4 °C. Serial dilutions of Human Annexin V standard at 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 ng/mL were made and transferred to two 8-well flat-bottom microwell strips in duplicates. Sample diluent (100  $\mu$ L) was added to the two blank wells. The cytosolic extracts (50  $\mu$ L) were transferred to a microwell strip in duplicates, and 50  $\mu$ L of sample diluent in addition to 50  $\mu$ L of biotin-conjugate were added

and incubated for 2 h at 24 °C on a microplate shaker (400 rpm).

The microwell strip, streptavidin-HRP (100 µL), was added then incubated at 25 °C for one hour after being washed. After washing the strips, tetramethylbenzidine (TMB) substrate solution (100 µl) was added and then incubated at 25 °C for 10 min. The enzyme reaction was terminated once the highest standard turned to a dark blue color by adding Phosphoric acid (100 µL of 1 M). The absorbance of the wells was obtained at 450 nm after blanking utilizing the blank wells, and the concentration of human Annexin V was determined by creating a standard curve from the standards and interpolating the sample absorbance values and multiplying by the dilution factor (x2).

### Caspases-8 and -9 activation assay

Caspases 8 and 9 assay kits were used to assess the potential of FA to induce caspases-8's in addition to -9's activity in the tested cell lines. Two concentrations of FA (100 and 200 µg/mL) were tested. In summary, to assess caspase-8 activity, the cells were incubated in ice with cell lysis buffer (50 µL) for 15 min followed by 10 min centrifugation (10,000 ×g at 4 °C). On the other hand, for caspase-9 activation assay, the cytosolic extracts were obtained from the lysed cells and transferred to a 96-well microplate at a concentration of 100 - 200 µg total protein in 50 µL Cell Lysis Buffer per well, 2X reaction buffer (with 10mM Dithiothreitol, DTT) and either substrate IETD-pNA for caspase-8 or LEHD-pNA for caspase-9 were added. For additional background controls, free from cells lysate, was used. The samples were read at 405 nm, and the fold increase was calculated by contrasting the absorbance of both the treated and untreated control samples.

### Statistical analysis

Analysis of results was done using GraphPad Prism 6.0.1 software and the data presented as mean ± SEM. Student's t-test was used to evaluate statistical differences between means.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Inhibitory effect of FA

Plotting the inhibition rate as a function of the logarithm of FA concentration showed a sigmoidal curve pattern. The inhibition rate approaches a plateau as the Log of FA concentration increases, demonstrating the

higher rates of inhibition on HepG2 followed by MCF-7 at 48 h. The  $IC_{50}$  values of FA for MCF-7 cells were 143.8, 75.4, and 85.6 µg/mL at 24, 48, and 72 h, respectively, while the  $IC_{50}$  values for HepG2 cells were 150.7, 81.38, and 210.4 µg/mL at 24, 48, and 72 h, respectively. For Annexin V and caspase-8 and -9 assays, 48 h incubation time was selected, and a dose of 100 µg/mL was used for Annexin V test, and two doses, 100 and 200 µg/mL, were used for caspase assay to demonstrate the dose-response relationship.

### Annexin V levels in cells

To measure the levels of Annexin V and assess apoptosis induced by FA, Annexin V ELISA assay was performed at 48 h with 100 µg/mL of FA. The standard curve for Annexin V ELISA was generated and used to interpolate the Annexin V concentrations for each sample. Results revealed that Annexin V concentration significantly increased in the treated cells compared to the control (Table 1).

**Table 1:** Annexin V concentrations (ng/mL) in MCF-7 and HepG2 cell lines at 48 h

Annexin V	Control	100 µg/mL FA
MCF-7	0.321	0.896*
HepG2	0.438	1.350*

\*Annexin V concentration increased significantly in treated cell compared to control cells ( $p < 0.05$ )

### Caspase activity

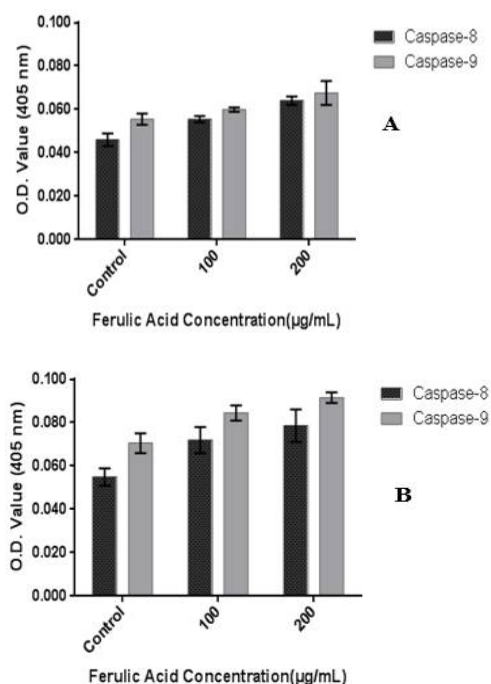
In order to investigate the possible role of caspase pathways in FA-induced apoptosis, the activities of caspases-8 and caspase-9 were determined at 48 h in response to treatment with FA at concentrations of 100 and 200 µg/ml. The results demonstrated that the activity of caspase 8 was significantly elevated in a dose-response manner in the FA treated MCF-7 cells ( $p < 0.05$ ), with a slight elevation in caspase-9 activity at a concentration of 200 µg/mL, ( $p < 0.073$ ), as shown in Figure 1. For the HepG2 cell line, the activity of both caspases -8 and -9 was elevated in a dose-response manner. FA at a concentration of 200 µg/mL induced both caspases significantly ( $p < 0.05$ ). Fold change of caspase activity was higher for caspase-8 than caspase-9 in MCF-7 cells. On the other hand, the activity was comparable for both caspases in HepG2 cells, as shown in Figure 2.

## DISCUSSION

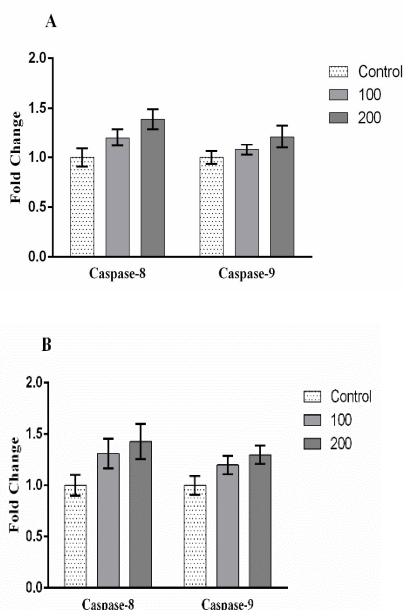
Even though chemotherapy has now progressed towards more targeted therapy against cancer, it still struggles with unpreventable complications

and side effects which is why research on new drugs is of great importance in the scientific field. For centuries, natural products of plant origin have been considered as a treasured source of potential anticancer drugs.

lines: (A) MCF-7 showing increasing fold change with FA concentration, with greater changes in caspase-8 compared to -9; (B) HepG2 showing increasing fold change with FA concentration, with significant difference from control at 200 µg/mL FA.



**Figure 1:** Effect of FA on the activation of caspases-8 and caspase -9. (A) incubation of MCF-7 cells with FA resulted in enhanced caspase -8 and -9 activity at 48 h after treatment, with statistically significant increases of caspase-8, \* $p < 0.05$ ; (B) incubation of HepG2 cells with high concentration of FA (200 µg/mL) resulted in significant enhancement of caspase -8 and -9 activity 48 h,  $p < 0.05$ . Background signal was subtracted



**Figure 2:** Fold-change in caspases 8 and -9 activity with increasing concentrations of FA in the two cell

Ferulic acid is a phenolic phytochemical natural compound present in the cell walls of many plants such as in rice, fruits, vegetables, and coffee [1]. It is a derivative of curcumin and has been identified as a component of medicinal gums and herbs including *Asafoetida* that has been studied on 4T1 breast cancer cells [15,16]. The literature on ferulic acid (FA) has reported various biological effects [3,4]. It was reported that FA antioxidative and anti-inflammatory actions are mediated through free radical scavenging, which prevents lipid peroxidation and apoptosis under normal physiological and stressful conditions through modulating Nrf2, p38, MMP, and mTOR, and suppression of IL-1 $\beta$ , TNF- $\alpha$  [1,2,4,5].

Furthermore, It has been shown that, in human pancreatic cancer (MIA PaCa-2), FA decreases cell viability, migration, and prevents the rapid progression of pancreatic cancer in combination with aspirin [6,11]. FA was also shown to modulate p21, p53, NF $\kappa$ B, Bax, and caspase-3 molecules in radiosensitizing cervical and lung cancers [12]. FA has also been shown to inhibit Melanin synthesis in B16 melanoma cells [17]. In addition, Zinc oxide (ZnO) nanoparticle-ferulic acid conjugate has been proven to induce apoptosis in Huh-7 hepatocellular carcinoma cells [18]. Overall, based on the mechanisms through which chemotherapeutic agents are active, it has been emphasized that the induction of apoptosis is an important mechanism to target tumor cells. This is backed up by evidence that suggests that neoplastic transformation involves alteration of the cell's apoptotic pathway [14,19].

The results of this study demonstrate the cytotoxic effect of FA on two cancer cell lines, namely MCF-7 and Hep G2 in a concentration and incubation time-dependent manner. These findings are in accordance with previously reported cytotoxic effect of FA on other different cell lines [3,6,9,11,19-21]. In addition, results from Annexin V analysis suggests that the observed cytotoxic effect could be attributed to the activation of an apoptotic pathway. Although the proapoptotic activity of FA has already been reported in different cancer cell lines, little is known about its effect on caspase-8 and-9 as a signal transduction pathway in the apoptotic process.

In general, apoptosis can occur through mainly two different activation pathways, intrinsic and

extrinsic pathways [22]. The intrinsic pathway includes changes in the potential of the mitochondrial transmembrane, leads to the release of cytochrome c, consequently activating caspase-9 [22]. While the extrinsic pathway begins through ligation of the death receptors, followed by oligomerization and caspase-8 activation. Both caspases-8 and -9 consequently activate caspase-3 and /or caspase-7, which is the main executor of apoptosis [23,24].

In the present study, caspases 8 and 9 were both induced by FA, but its effect on caspase-8 was more significant than caspase-9 on MCF-7 and Hep G2 cell lines indicating that both initiators of apoptosis are involved, but mainly through caspase-8's extrinsic pathway. The observed activation of caspase-9 could be due to the cross-communication of both the pathways, intrinsic and extrinsic, through the cleavage of the proapoptotic Bid, which is a Bcl-2 family member, that is mediated by caspase- 8. This process is considered as bridging element between the two apoptotic pathways [20,23]; thus, the increased activity of caspase-9 found in this study was a consequence of caspase-8 activation.

Further investigations are still required to elucidate the various mechanisms underlying the antiproliferative and proapoptotic effects induced by FA and to explore the apoptotic mediators involved in the above findings. Nevertheless, this study provides evidence that FA is an effective inducer of apoptosis in human cells MCF-7 and Hep-G2 through the induction of caspases-8 and -9 cascades.

## CONCLUSION

The findings of this study demonstrate that ferulic acid exhibits cytotoxic activity and induces apoptosis against the cancer cell lines, MCF-7 and Hep G2, via activation of caspase-8 and -9 pathways. Thus, FA is a potential therapeutic agent for the management of breast and hepatic cancers.

## DECLARATIONS

### Acknowledgement

This research was funded courtesy of an UOS-Boehringer Ingelheim award.

### Conflict of interest

The authors declare that no conflict of interest is associated with this work.

## Authors' contribution

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Eman Abu-Gharbieh, the corresponding author, was responsible for the research idea and experimental work design. Experimental work was done in Sharjah Institute for Medical Research by Mohammed ElKhazendar, Juhaina Chalak, and Arya Vinod. Wael M Abdel-Rahman and Eman Abu-Gharbieh supplied study materials. Statistical analysis was done by Mohammed ElKhazendar and Waseem El-Huneidi. The manuscript was prepared by Waseem El-Huneidi, Wael M Abdel-Rahman, and Mohammed ElKhazendar, and revised by Eman Abu-Gharbieh.

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