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*Basic Study*

**Ferula Assafoetida Induced Colon Cancer Cells Differentiation Through JNK/MAPK Signalling Pathway Activation**

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**Abstract**

BACKGROUND

Colon cancer is a major health problem with increasing mortality rates worldwide.

AIM

Is to evaluate the ability of *Ferula assafoetida* (*F. assafoetida*) to induce differentiation of colon cancer cells to function as normal cells.

METHODS

The cytotoxic effect of *F. assafoetida* was assessed against Caco cells using the MTT assay. Cell cycle analysis and apoptosis were assessed using Cytell™ cell system. The total antioxidant (TA), glutathione (GSH), malondialdehyde (MDA) concentrations, and alkaline phosphatase (ALP) activity were also assessed. The *JNK* and *MAPK* expressions were evaluated using qRt-PCR.

RESULTS

There was a significant increase in the cell number treated with *F. assafoetida* ( $53.85 \pm 0.03\%$ ), and those treated with NaBT ( $54.6 \pm 0.10\%$ ) in sub-G1 phase, compared to the

untreated cells ( $0.78 \pm 0.03\%$ ,  $p < 0.001$ ). Apoptosis was significantly increased in the Caco cells treated with *F. assafoetida* ( $49.1 \pm 0.14\%$ ) compared to those treated with NaBT ( $27.3 \pm 0.10\%$ ,  $p < 0.001$ ), and untreated cells ( $11.1 \pm 0.02\%$ ,  $p < 0.001$ ). DNA degradation was observed in Caco cells treated with *F. assafoetida* in a dose-dependent manner, where complete degradation occurred at the dose of  $IC_{50}$  ( $342.9 \mu\text{g/mL}$ ). *F. Asafoetida* induced a significant increase in the TA concentration and GSH, while a significant decrease in the MDA levels ( $p < 0.001$ , for all). Also, there was a significant increase in ALP activity in Caco cells ( $0.53 \pm 0.26 \text{ U/mL}$ ) compared to the control cells ( $0.05 \pm 0.02 \text{ U/mL}$ ,  $p = 0.045$ ). There was a significant upregulation of *JNK* and *MAPK* expression in Caco cells treated with *F. assafoetida* compared to the controls [ $2.59 \pm 0.01$  ( $p < 0.001$ ), and  $3.62 \pm 0.01$  ( $p < 0.001$ ); respectively].

## CONCLUSION

*F. Asafoetida* is a potentially successful therapeutic and differentiating agent for colon cancer.

## INTRODUCTION

Colorectal cancer (CRC) is a malignant tumor arises from the colonic or rectal cells, where it affects about 150,000 new individuals yearly[1]. It ranked third in cancer diagnosis and cancer mortality in the United States[2]. Despite the advances in the treatment strategies for CRC including chemotherapy, radiation therapy, surgery, and immunotherapy, the outcome of the patients is very poor. As most patients showed an increased incidence of metastasis, disease recurrence, drug resistance, and toxicity[3]. Therefore, extensive research to investigate for other treatment modalities for CRC patients is highly required, especially those developed from natural botanical compounds[4].

The colonic epithelium is continuously under rapid proliferation by the stem cells located in the base of the colonic crypts. The proliferated cells migrate to the upper part

of the crypts where they differentiate to the terminal phenotype[5]. The colon cancer cells are characterized by lacking the differentiated phenotype as the cells were rapidly produced before reaching full differentiation. Alternatively, it was proposed that cancer cells can undergo dedifferentiation into cells with “stem-like” properties known as cancer stem cells (CSCs)[6,7]. These CSCs have self-renewal properties which are responsible for the low tumor grade, increased drug resistance, and poor patients’ prognosis[8,9]. Therefore, it was found that increased levels of CSCs molecular markers including *OCT4*, *NANOG*, and *SOX2* associated with advanced tumor stage and inferior patients’ outcomes[10].

Indeed, the differentiation of colon cells depends on many factors including growth factors, vitamins, hormones, and the intestinal microbiota[11]. The intestinal microbiota secrete many metabolites such as sodium butyrate (NaBT) which is a short-chain fatty acid (SCFA) that is present abundantly in the colon lumen. It has an important role in colon cell proliferation and differentiation[12]. Additionally, it has an *in vitro* anticarcinogenic effect through suppressing the histone deacetylase (HDAC) activity and modulating the energy metabolism of the cells[13,14].

<sup>17</sup> C-JUN N-terminal kinases (*JNKs*) are members of the mitogen-activated protein kinase (*MAPK*) signalling pathway. It is also called stress- activated protein kinase pathway, as it has a notable role in stress and inflammatory cascades in the body[15]. The *JNK/MAPK* pathway has been implicated in many cellular processes including cellular proliferation, migration, apoptosis, immunity regulation, wound healing, and metabolism[16-20]. Additionally, it was proved that the *JNK/MAPK* pathway has a pivotal role in tumorigenesis and embryonic cell development[21,22]. Moreover, the activated *MAPK* pathway was reported to associate with cancer cell resistance to the chemotherapeutic drugs as well as targeted therapies[23].

<sup>19</sup> *Ferula Assafoetida* (*F. assafoetida*), is an oleo-gum-resin that is extracted from the stems of *Ferula* plants, which are members of the *Umbelliferae* family[24]. It is used as a flavouring additives, anticonvulsant and anti-inflammatory medicinal herpes in many countries including <sup>1</sup> Central Asia, particularly West Afghanistan, Iraq, Turkey, Eastern

Iran, Europe, and North Africa[25]. *F. assafoetida* has many biological functions, especially on gastrointestinal health such as promoting salivary glands secretion, bile acid production, and increased activity of the pancreatic and intestinal digestive enzymes[24,26]. It was also well recognized for its antioxidant[27], anti-diabetic[28], hypotensive, antispasmodic[29], antimicrobial[30], and neuroprotective effect[31].

Moreover, many studies reported the potent chemo-preventive effect of *F. assafoetida* against the development of different cancers including colon, breast, and cutaneous cancer using experimental animal models[32-34]. Based upon the previous series highlighted the anti-inflammatory, antioxidant, and anticarcinogenic effect of *F. assafoetida* [27-34]. We thought to assess its role in colon cancer cell differentiation. To the best of our knowledge, there were no reports in literature addressed the differentiating function of *F. assafoetida* on colon cancer. Therefore, we aimed to assess the role of *F. assafoetida* as a differentiating agent for colon cancer cells to normal functioning cells. This will be performed through investigating the efficacy of *F. assafoetida* in inhibiting the proliferation of colon cancer cells, induction of cell cycle arrest and apoptosis. Additionally, determination of alkaline phosphatase (ALP) as a colonocyte differentiation marker, and the effect of *F. assafoetida* on PI3K/AKT pathway. Moreover, assessing the role of *F. assafoetida* on cellular oxidative stress. The study will be performed in the human colon carcinoma cells (Caco) cell line, in comparison to Na. butyrate. This will provide a novel differentiating agent that could introduce another strategy for the treatment of colon cancer patients.

## **MATERIALS AND METHODS**

### **Preparation of plant extract**

The *F. assafoetida* was purchased from the Egyptian Company for Medical Herbs, Egypt. The resin was cut with a knife and crushed into small pieces by using a blender. Then, the resin was soaked in the extraction material either absolute ethanol or water. The *F. assafoetida* was extracted using the cold maceration method, where the resin was macerated in the solvent for three days on a shaker at room temperature. Then, the

resin was filtered using 101 Double Rings filter paper. The diluted liquid extract was concentrated with a rotary evaporator to obtain a viscous liquid. This liquid was finally dried in the dryer for one day to remove the remaining traces of solvent.

#### Ethanol extract preparation

For the preparation of ethanolic extract, more than 100 gm of dried oleo-resin-gum was pulverized. Then 100 gm of the obtained powder was soaked in 1000 mL of 70% ethanol for 48 h at room temperature. The solvent was filtered with Whatman filter paper (grade 40) and soaked in 70% ethanol again. This process was conducted four times. The solvent was evaporated using rotary evaporator and the collected extract was freeze dried and finally stored in the refrigerator at 4 °C until needed.

#### Water extract preparation

One hundred grams (100 g) of *F. assafoetida* was soaked in 600 mL distilled water for 72 h at room temperature. Then, the extract was filtered and dried at 4°C using a rotary evaporator.

#### Determination of Total Flavonoid Content in *F. assafoetida*

Total flavonoid content of the *F. assafoetida* ethanolic extract was detected by the classical Aluminium chloride (AlCl<sub>3</sub>) colorimetric method[35]. In brief, 20 µL of 1% AlCl<sub>3</sub> dissolved in ethanol was mixed with the same volume of the *F. assafoetida*. After incubation for 10 minutes, the absorption was detected at 490 nm against a blank sample formed of the same mixture without AlCl<sub>3</sub>. A standard calibration curve was plotted using different concentrations of quercetin (0-250 µg/mL), where the total flavonoids were measured as mg quercetin equivalent (QE)/g dry weight (DW).

#### Determination of Total Phenolic Compounds in *F. assafoetida*

Total phenolics amount of the *F. assafoetida* ethanolic extract was assessed using Folin-Ciocalteu method[36]. Briefly, 100 µL sample of *F. assafoetida* ethanolic extract was mixed with 800 µL Na<sub>2</sub>CO<sub>3</sub> (700 mmol/L) and 200 µL of 10% Folin-Ciocalteu. After incubation for two hours at 25°C, the absorbance was measured at 765 nm. The phenolic content was expressed as gallic acid equivalent (GAE)/g DW according to the standard calibration curve of gallic acid.

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### Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC/MS analysis of *F. assafoetida* extract was performed using Agilent Technologies GC-MS 7890A spectrophotometer (Agilent, USA), equipped with autoinjector (Agilent 7693A Automatic liquid sampler). The mass selective detector (5975 C) was operated in full scan mode. The ionization source was supplied with a voltage at 70 eV. The GC was fused with silica capillary column; Hewlett-Packard 5MS (30 m × 0.25 mm; 0.25 µm film thickness). The oven temperature was held at 80°C for two min and increased to 300°C isothermally over 20 min. Helium was used as carrier gas with flow rate of 1.5 mL/min. Injector temperature was adjusted at 280°C. The injected volume of *F. assafoetida* extract was prepared at One µL and was analysed by GC twice. The separated sample components were identified by comparing their mass spectra with those recorded standards data base in Agilent's own Retention Time Locked (RTL) libraries. The analysis was performed at the National Institute of Oceanography and Fisheries, Alexandria University Egypt.

### Maintenance of The Caco Cell Line

The Caco-2 cells were obtained from the American Type Culture Collection ATCC, Manassas, VA, USA). The cells were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing in RPMI-1640 medium (RPMI-1640, Sigma-Aldrich, USA), supplemented with antibiotic-free 10% foetal bovine serum (FBS, Sigma, USA), 100 U/mL penicillin, and 2- mg/mL streptomycin. The Caco-2 cells were subcultivated after trypsinization (Trypsin-EDTA, Cambrex Bioscience Verviers, Belgium) once or twice per week and re-suspended in a complete medium in a 1: 5 split ratio to maintain it in the exponential growth phase. They were maintained as a monolayer in T75 cell culture flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37 °C in a humidified 5% CO<sub>2</sub> incubator Revco, GS laboratory equipment, RCO 3000 TVBB, USA).

Cytotoxicity Assay

The cell Viability and cytotoxicity of all extracts were done <sup>13</sup> utilizing the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide thiazolyl (MTT) assay [37]. Caco cells were seeded (5x10<sup>5</sup>/ well) in 96-well flat-bottomed microlitre plates <sup>10</sup> in RPMI-1640 media supplemented with FBS and incubated overnight at 37 °C in a humidified 5% CO<sub>2</sub> incubator. The cells were placed in a fresh medium containing serial dilutions of NaBT (0.65-10 mg/mL), ethanolic and water extracts of *F. assafoetida* (100-1600 <sup>18</sup> µg/mL). The cells were incubated for 24 hours at 37 °C and 5% CO<sub>2</sub>. A <sup>6</sup> 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and Shaked for 5 minutes, then the mixture was incubated in the dark for 4 hours. The medium was then removed after centrifugation and 100 µl of isopropanol was added for the assessment of cell viability. The absorbance was measured by the ELISA reader (TECAN Sunrise™, Germany) at 570 <sup>7</sup> nm. Cell viability was measured by dividing the absorbance of the treated cells by that of the untreated cell. All experiments were done in triplicate. The 50% inhibitory concentration (IC<sub>50</sub>) was calculated through the sigmoidal dose response curve-fitting equation <sup>25</sup> using GraphPad Prism 8.4.2 (GraphPad Software, La Jolla, CA, USA), where the least IC<sub>50</sub> was chosen.

### Cell treatment

The Caco cells (5x10<sup>5</sup> cells/well) were divided into three groups; the first group which treated with the ethanolic extract of *F. assafoetida* (343.6 µg/mL), the second which treated with the NaBT (3.3 mg/mL), and the untreated cells were considered as a control group. All group of cells were <sup>27</sup> incubated for 48 hours in humidified 5% CO<sub>2</sub> incubator at 37 °C for further analysis.

### Cell Cycle Analysis

The cell cycle analysis was determined using Cytell™ cell cycle kit (GE Healthcare Japan, Tokyo, Japan), according to the manufacture instructions. The cell groups were <sup>22</sup> incubated for 24 hours in a 5% CO<sub>2</sub> incubator at 37°C. The DNA content and the cell cycle phases were detected using Cytell™ cell imaging system (GE Healthcare Japan).

### **Apoptosis Assay**

Apoptosis assay was determined using the Invitrogen™ Alexa Fluor™ 488 annexin V/Dead Cell Apoptosis Kit (Invitrogen™, ThermoFisher Scientific, USA) according to the manufacturer's instruction. Briefly, all groups of cells (cells treated with *F. assafoetida*, NaBT, and untreated cells) were washed with cold PBS and centrifuged. Then cells were resuspended in 100 µL annexin-binding buffer ( $1 \times 10^6$  cells/mL), five µL Alexa Fluor 488 annexin V, and one µL (100 µg/mL) propidium iodide (PI) working solution. The cell groups were incubated for 15 minutes at room temperature, then 400 µL 1X annexin-binding buffer was added and the cells were kept on ice for detection of apoptotic cells using Cytell TM cell imaging system (GE Healthcare Japan).

### **DNA fragmentation Assessment**

Assessment of DNA fragmentation was performed based on the detection of DNA ladder appearance at late stage of apoptosis as described by (Elmore *et al.*, 2007). DNA was extracted from untreated Caco cells (as a control) and those treated with *F. assafoetida*. The DNA was prepared at a concentration of 300-600 ng/µL. Then it was run in pre-stained agarose gel electrophoresis 1.5%. The DNA was visualized and photographed under ultraviolet illumination[38].

### **Antioxidant Activity**

The total antioxidant (TA), glutathione reductase (GSH), and Malondialdehyde (MDA) concentrations in the treated and untreated Caco cells were assessed using Biodiagnostic kits (Cat.no. TA 25 13, GR25 11, and MD 25 29; respectively, Cairo, Egypt). According to the manufacture instructions. The TA, GSH, and MDA concentrations were measured calorimetrically at 505 nm, 405 nm, and 534 nm; respectively, using a spectrophotometer (UV-2505, LaboMed, inc. USA).

### **Effect of Ferula Assafoetida on ALP activity**

The ALP activity was determined in the Caco cell groups according to the instructions of the <sup>26</sup> assay kit (Cat. No. E-BC-K091-M, Elabscience, USA). The samples were performed in triplicate and the absorbance was measured at 500-530nm using microplate reader (TECAN Sunrise<sup>TM</sup>, Germany).

### Assessment of MAPK and JNK Genes Expression

Total RNA was purified from the treated and untreated Caco <sup>1</sup> cells using Direct-zol RNA Miniprep Plus (Cat# R2072, ZYMO RESEARCH CORP. USA), in accordance with the manufacturer's instructions. The amount and the purity of the extracted RNA was detected using Beckman dual spectrophotometer (USA). Complementary DNA (cDNA) followed by <sup>29</sup> Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by utilizing the <sup>1</sup> SuperScript IV One-Step RT-PCR system (Cat# 12594100, Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. The relative expression levels of MAPK and JNK using was determined relative to the GAPDH housekeeping gene. The primer sequences of the assessed genes were; MAPK “forward: TCTCCCGCACAAAATAAGG, reverse: TCGTCCAACCTCCATGTCAAA”, JNK: “forward: GCTGCCCTGTACCCACATCT, reverse: GCTGCACCTGTGCTAAAGGA”, and GAPDH: “forward: GAAAACGCTGACTCAGAACAC, reverse: TTTGCACTGGTACGTGTTGAT”. The qPCR reactions were run in triplicate using the Step One Applied Biosystem machine, (Foster city, USA). Data were analysed using the  $2^{-\Delta\Delta Ct}$  comparative approach according to the Schmittgen and Livak, 2001[39].

### Statistical Analysis

Data were analysed <sup>6</sup> using IBM© SPSS© Statistics version 22 (IBM© Corp., Armonk, NY, USA). The results were expressed as mean and standard deviation (SD) according to the Kolmogorov-Smirnov and Shapiro- Wilk normality tests. Comparison between variables was done <sup>11</sup> using the Student's *t*-test and the one-way analysis of variance (ANOVA) with post hoc Tukey's test. Cytotoxicity curves were done using <sup>4</sup> GraphPad

Prism 8.4.2. (San Diego, CA, USA). All tests were performed as two-tailed, and the significant level was adjusted at p-value  $\leq 0.05$ . All experiment were done at triplicate.

## **RESULTS**

Cytotoxicity Assay of *F. assafoetida* on Caco cells

The cytotoxicity effect of *F. assafoetida* on Caco cells was determined according to the concentration-dependent decrease in the growth rate of the cells. Doses that inhibited cell growth to 50% (IC<sub>50</sub>) were 343.6  $\mu\text{g}/\text{mL}$  for ethanol extract of *F. assafoetida* and 3.3 mg/mL for NaBT (**Figure 1A**).

Determination of The Total Phenolic and Flavonoid Compounds of The *F. assafoetida* Ethanolic Extract

The total phenolic and flavonoid content present in the ethanolic extract of *F. assafoetida* extract were 3.101% and 0.797%, respectively.

### **Phytochemical Analysis of The Ethanolic Extract of *F. assafoetida* Using GC-MS**

GC-MS analysis of the ethanolic extract of *F. assafoetida* revealed the presence of 26 phytochemical compounds. The identification of the phytochemical compounds was based on the peak area, retention time, and the molecular formula (**Table 1, Figure 1B**). The major compounds were coumarins (31.17%), Torreyol ( $\delta$ -Cadinol) (7.63%), L-ascorbic acid 6-stearate (7.46%), 2-Mercapto-3,4-dimethyl-2,3-dihydrothiophene (7.29%), Cadinene (4.22%), Aristolene (3.54%), 1-heptatriacotanol (3.18%), Vanillin (3.07%), beta-guaiene (3.03%), Dotriacontane (2.69%), Octadecanoic acid,4-hydroxy-methylester (2.45%), Dasycarpidan-1-methanol acetate (2.32%), 2-Mercapto-3,4-dimethyl-2,3-dihydrothiophene (2.29%), 3,4 Dimethyl-thiophene (2.28%), 2,4,4-Trimethyl-3-(3-methylbuta-1,3-dienyl) cyclohexanone (2.27%), Tetracosane 2,6,10,15,19,23-hexamethyl (2.13%), phenyl aziridine carboxylate (1.68%), isolongifolene (1.61%), Rhodopin (1.54%), Styrene (1.48%), N,N,N-Trimethyl-1,4-phenylenediamine (1.46%), 1-chloro Octadecane (1.23%), 3-Oxo-20-methyl-11-alpha-hydroxyconanine-1,4-diene (1%), 10,12-

Tricosadiynoic acid methyl ester (1%), and bicyclo[4,4,0]dec-1-ene, 2-isopropyle-5-methyl-9-methylene (0.92%).

#### **Effect of *F. assafoetida* on The Cell Cycle**

There was a significant increase in the cell number treated with *F. assafoetida* ( $53.85 \pm 0.03\%$ ), and those treated with NaBT ( $54.6 \pm 0.10\%$ ) in the sub-G1 phase, compared to the untreated control cells ( $0.78 \pm 0.03\%$ ,  $p < 0.001$ ). While there was a significant difference in the number of cells in the other cycle phases for those treated with *F. assafoetida* (G0/G1 phase ( $29.27 \pm 0.03\%$ ), S phase:  $12.76 \pm 0.05\%$ , G2/M:  $2.9 \pm 0.04\%$ , and  $> 4n$ :  $1.17 \pm .03\%$ ,  $p < 0.001$ ), and those treated with NaBT (G0/G1 phase  $25.6 \pm 0.01\%$ , S phase:  $10.65 \pm 0.01\%$ , G2/M:  $8.7 \pm 0.01\%$ , and  $> 4n$ :  $0.17 \pm 0.20\%$ ,  $p < 0.001$ ), compared to the control cells (G0/G1 phase ( $67.41 \pm 0.02\%$ ), S phase:  $30.73 \pm 0.11\%$ , G2/M:  $0.79 \pm .01\%$ , and  $> 4n$ :  $0.24 \pm 0.01\%$ , **Table 2, Figure 2A-C**).

#### **Effect of *F. assafoetida* on Apoptosis**

Apoptosis was significantly increased in the Caco cells treated with *F. assafoetida* ( $49.1 \pm 0.14\%$ ) in comparison to those treated with NaBT ( $27.3 \pm 0.10\%$ ,  $p < 0.001$ ), and the untreated cells ( $11.1 \pm 0.02\%$ ,  $p < 0.001$ ,  $F = 113152.6$ ,  $df = 2$ ). While the number of live cells in each group represented  $50.9 \pm 0.12\%$  in the Caco cell treated with *F. assafoetida*,  $72.7 \pm 0.10\%$  in those treated with NaBT, compared to  $88.9 \pm 0.10\%$  in control cells ( $p < 0.001$ ,  $F = 95649.6$ ,  $df = 2$ , **Figure 2 D-H**).

#### **DNA fragmentation Assessment**

The results of DNA fragmentation analysis showed complete DNA degradation in Caco cells treated with the ethanolic extract of *F. assafoetida* with the dose of  $IC_{50}$  ( $342.9 \mu\text{g/mL}$ ). While at the dose of half and forth of  $IC_{50}$ , there were slight DNA degradation when compared with untreated cells (**Figure 3A-C**).

#### **Role of *F. assafoetida* on Cellular Oxidative Stress**

There was a significant increase in the total antioxidant concentration in Caco cells treated with *F. assafoetida* ( $0.625 \pm 0.009$  nmol/mL) in comparison to those treated with NaBT ( $0.387 \pm 0.015$  nmol/mL), and untreated controls ( $0.138 \pm 0.001$  nmol/mL,  $p < 0.001$ ,  $F = 1833.6$ ,  $df = 2$ , **Figure 4A**). Additionally, there was a significant increase in the GSH concentration in Caco cells after treatment with Ferula Assafoetida ( $0.036 \pm 0.005$  nmol/mL) or Na butyrate ( $0.038 \pm 0.004$  nmol/mL), in comparison to the untreated cells ( $0.019 \pm 0.001$  nmol/mL,  $P < 0.001$ ,  $F = 40.3$ ,  $df = 2$ , **Figure 4B**). On the other hand, there was a significant decrease in the MDA concentration in Caco cells treated with *F. assafoetida* ( $0.370 \pm 0.004$  nmol/mL) in comparison to the control cells ( $0.886 \pm 0.005$  nmol/mL), however, it is higher than those treated with NaBT ( $0.242 \pm 0.031$  nmol/mL,  $p < 0.001$ ,  $F = 1044$ ,  $df = 2$ , **Figure 4C**).

#### **Effect of *F. assafoetida* on ALP activity**

There was a significant increase in ALP activity in Caco cells treated with *F. assafoetida* ( $0.53 \pm 0.26$  king unit/mL) in comparison to those untreated control cells ( $0.05 \pm 0.02$  king unit/mL,  $p = 0.045$ , **Figure 4D**).

#### **Impact of *F. assafoetida* on JNK/MAPK Signalling pathway**

There was a significant upregulation of *JNK* expression in Caco cells treated with *F. assafoetida* compared to the untreated control cells, with a foldchange of  $2.59 \pm 0.01$  ( $p < 0.001$ ). Also, the *MAPK* expression was significantly upregulated in Caco cells treated with *F. assafoetida* relative to the controls, with a foldchange of  $3.62 \pm 0.01$  ( $p < 0.001$ , **Figure 5**)

### **DISCUSSION**

The CRC is a major health problem with increasing mortality rates worldwide. This is due to the presence of the dedifferentiated colon cancer cells which are responsible for the emergence of disease recurrence and resistance to the conventional chemotherapeutic drugs[40,41]. Therefore, new strategies aiming at modulating the

differentiation capabilities of cancer cells can be a promising line of successful treatment for colon cancer patients[42]. The current study showed that *F. assafoetida* has a potential anticancer activity through reprogramming and induction of colon cancer cell differentiation into functioning normal cells. This was supported with the significant increase in the ALP activity in Caco cells treated with *F. assafoetida* in comparison to those untreated cells. The ALP has been reported to be a well-established indicator for colon cell differentiation[43]. Moreover, Caco cells treated with *F. assafoetida* showed a significant upregulation of *JNK* and *MAPK* expression compared to the control cells. The *JNK/MAPK* signalling pathway is an important regulatory trigger for the differentiation of colon cells through the crosstalk with the *Wnt/β-catenin* pathway[44,45]. The *WNT/β-catenin* pathway has a pivotal function in maintaining stemness and differentiation of intestinal cancer cells (ISCs)[46]. Activation of *JNK/C-JUN* pathway results in increased expression of *WNT/β-catenin* downstream genes, such as *Ccdn1*, *Axin2*, and *Lgr5*, that leads to proliferation and differentiation of ISCs[45]. Additionally, *MAPK* signalling triggers *p38* activation, which is known as a crucial regulator of embryonic stem cell differentiation[47,48]. The activation of *p38MAPKs* pathway results in phosphorylation and inactivation of glycogen synthase kinase 3β (*GSK3β*), that leads to accumulation of the β-catenin[15,49].

Chen and his colleagues found that ferulic acid (FA; a component of *F. assafoetida*) inhibited colon cancer proliferation both *in vitro* and in BALB/c mice CRC model through the activation of *JNK* and *ERK* gene expression, which lead to stimulation of *BCL-2* and *BAX* required for the apoptosis pathway[50]. Similarly, Ren *et al*, demonstrated that the activation of *MAPK-JNK/c-Jun* pathway results in apoptosis stimulation in colon cancer cells[51]. Therefore, the *JNK/MAPK* signalling pathway could be a potential useful target for colon cancer therapy. However, these data should be validated in protein expression levels.

Sodium butyrate was recognized as an efficient epigenetic modulator and differentiating agent for colon cancer cells[14]. Therefore, we used it to assess the efficacy of *F. assafoetida* in colon cancer cell differentiation. The present data showed that

the number of Caco cells treated with *F. assafoetida* arrested at the sub-G1 phase that represents the cells undergoing apoptosis were significantly increased, compared to those treated with Na butyrate, and untreated control cells. Also, there was a significant decrease in the cell number treated with *F. assafoetida* in the S. (DNA synthesis) phase, compared to the control cells. Incomparable with these findings, Janicke *et al* concluded that ferulic acid exerts antiproliferative effects on Caco-2 cells through upregulating *RABGAP1* and *CEP2* gene expression, which were implicated in centrosome assembly, as well as the gene involved in S-phase checkpoint protein *SMC1 L1*[52]. While Bagheri *et al* found that *F. assafoetida* produces cell cycle arrest at G0/G1 phase that was responsible for its neuroprotective effect[53].

The current results showed that the efficacy of *F. assafoetida* to induce apoptosis in Caco cells was significantly higher than that of NaBT, compared to the untreated control cells. Additionally, there was a complete DNA degradation (which is a histological sign of apoptosis) in the Caco2 cells treated with the dose of IC<sub>50</sub> of *F. assafoetida*. These data are consistent with that of Elarabany *et al*[54], who informed that *F. assafoetida* exhibited an antitumor effect against colon cancer cells through decreasing cell viability and induction of apoptosis. Zhang *et al*, also reported that the antiproliferative effect of ferulic acid on breast cancer cells was accomplished through the induction of apoptosis[55]. Similarly, Efati *et al*, found that *F. assafoetida* L. extract zinc nanoparticles, exerts antiapoptotic and antioxidant functions on CRC and breast cancer cell lines through upregulating Bax and downregulating BCL-2 gene expression[56].

Regarding the impact of *F. assafoetida* on Cellular Oxidative Stress, the present data showed that *F. assafoetida* induced a significant increase in the total antioxidant concentration in Caco cells in comparison to those treated with NaBT, and untreated control cells. Also, it exhibited a significant increase in the GSH concentration in Caco cells similar to that treated with Na butyrate, in comparison to the untreated control cells. While it induced a significant decrease in MDA concentration in Caco cells compared to the untreated control cells. In consistence with these results, Mallikarjuna

*et al*[33], proved that *F. assafoetida* has a chemo-preventive activity against N-methyl-N-nitrosourea (MNU)-induced mammary tumor in rats through increasing GSH, catalase, and superoxide dismutase (SOD) antioxidant levels, that consequently, avert the process of carcinogenesis. Though *F. assafoetida* showed results superior to a well-known differentiating agent NaBT, however the data obtained were performed *in vitro* on a single cell line (Caco cells). Accordingly, these data should be validated in in-vivo model with different cancer cell types to properly assess the differentiating capabilities of *F. assafoetida*.

Moreover, the phytochemical analysis of the ethanolic extract of *F. assafoetida* revealed a large amount of phenolic and flavonoid content which proved to have antioxidant, anti-inflammatory, and antitumor effects[54,57,58]. This was illustrated by the presence of high amount of some beneficial components including *e.g.* coumarin (31.17%), L-ascorbic acid 6-stearate (7.46%), Cadinene (4.22%), as well as many sulfur-containing compounds. All these components have been reported to exhibit potent antioxidant, antimicrobial, neuroprotective, and anticancer functions[53,54,59,60].

The cytotoxicity assay of *F. assafoetida* showed that the concentration which inhibited the growth of Caco cells to 50% (IC<sub>50</sub>) was 343.6 µg/mL. These data are relevant to other series commonly used doses in rats ranged from low concentration of 10-100 mg/Kg for the treatment of colon cancer, to a high concentration of 200-400 mg/kg as an adjuvant for dementia therapy[32,61,62]. Indeed, *F. assafoetida* is widely used in many countries as flavouring additives because it has a good bioavailability, however, its use in infants should be cautioned as it can cause lethal methemoglobinemia[63]. On the other hand, Goudah *et al* reported that *F. assafoetida* is a safe gum extract at a dose of 250 mg/kg for a short-term administration up to 28 days. Also, a single oral dose up to 5000 mg/kg did not cause any toxicological effect in rats[64]. Therefore, *F. assafoetida* could be utilized as a natural remedy without obvious side effects in adults, however, further clinical studies are highly needed.

## **CONCLUSION**

In conclusion, the present study provided evidence that *F. assafoetida* could be considered a potential differentiating agent for colon cancer cells through increasing the ALP activity, as well as upregulation of *JNK* and *MAPK* gene expression. Additionally, it has important implications for cancer therapy through arresting cells in the sub-G1 phase, inducing cytotoxicity, DNA fragmentation, and apoptosis in the cancer cells compared to those treated with NaBT, and untreated control cells. Moreover, *F. assafoetida* has a protective impact against the cellular oxidative stress indicated by the significant increase in the TA concentration and GSH level, while a significant decrease in the MDA levels in the Caco cells compared to cells treated with NaBT, and the control cells. Therefore, more research should be directed to *F. assafoetida* as a useful, efficient, inexpensive, and novel therapeutic medicinal herbs for colon cancer patients.

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