University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

Effect of Ethanolic Extract of *Ferula assa-foetida* on Modulating SMAD4 Tumor Suppressor Gene Expression in Caco-2 Cell Line Derived from Human Colorectal Adenocarcinoma

Ali Abolhasanzadeh Parizi [1], **Milad Lagzian** [2]

[1] Dept. of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: ali.abolhasanzade95@gmail.com

[2*] *Corresponding author*, Dept. of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran. E-mail: m.lagzian@science.usb.ac.ir.

ARTICLE INFO

Article type:
Research Article

Article history:
Received: 1 February 2024
Revised: 10 March 2024
Accepted: 25 April 2024

Keywords:
Ferula assa-foetida,
SMAD4,
Colorectal cancer,
Gene expression,
TGF-β signaling

ABSTRACT

This study investigated the effect of ethanolic extract of *Ferula assa-foetida* on the expression of SMAD4, a critical tumor suppressor gene, in the Caco-2 colorectal cancer cell line. Loss of SMAD4 function has been associated with advanced stages of colorectal cancer and poor prognosis. The research employed qRT-PCR analysis to measure changes in SMAD4 expression following treatment with various concentrations (50, 100, and 200 µg/mL) of *F. assa-foetida* extract. Cell viability assays confirmed that the extract maintained cell survival rates above 90% across all concentrations, indicating minimal cytotoxicity. The results demonstrated a significant dose-dependent increase in SMAD4 expression, with the highest concentration (200 µg/mL) inducing a 4.8-fold increase compared to untreated controls ($p < 0.0001$). Statistical analysis revealed high precision and reliability of the findings ($R^2 = 0.996$). The extract induced progressive upregulation of SMAD4, with 1.7-fold, 2.3-fold, and 4.8-fold increases observed at 50, 100, and 200 µg/mL, respectively. RNA quality assessment and qRT-PCR optimization confirmed the reliability of gene expression measurements, with consistent GAPDH expression serving as an internal control. These findings suggest that *F. assa-foetida* extract can modulate SMAD4 expression without significant cytotoxicity in colorectal cancer cells, warranting further investigation of its effects on the TGF-β signaling pathway. This study provides initial molecular evidence examining the traditional medicinal applications of *F. assa-foetida* at the gene expression level, though additional research is needed to fully understand its mechanisms of action and potential therapeutic implications.

Introduction

Colorectal cancer (CRC) is a major global health concern, ranking as the third most common cancer and the second leading cause of cancer-related deaths worldwide. The complexity of CRC development involves a multifactorial interplay of genetic, environmental, and dietary factors. Among the genetic factors, tumor suppressor genes play a critical role in maintaining cellular integrity and preventing tumorigenesis (Fearon and Vogelstein, 1990). The SMAD4 gene, a key component of the transforming growth factor-beta (TGF-β) signaling pathway, is particularly noteworthy. This pathway is essential for

DOI: https://doi.org/ 10.22111/JEP.2024.50509.1076
© The author(s)
Publisher: University of Sistan and Baluchestan

How to Cite: Abolhasanzadeh Parizi, A., Lagzian, M. (2024). Effect of Ethanolic Extract of Ferula assa-foetida on Modulating SMAD4 Tumor Suppressor Gene Expression in Caco-2 Cell Line Derived from Human Colorectal Adenocarcinoma. *Journal of Epigenetics*, 5(1), 41-49. https://doi.org/ 10.22111/JEP.2024.50509.1076

regulating cell proliferation, differentiation, and apoptosis. Loss of SMAD4 function due to mutations or deletions has been associated with advanced stages of colorectal cancer and correlates with poor prognosis and increased metastasis (Papageorgis et al., 2011).

F. assa-foetida, commonly known as Asafoetida, has a long history of use in traditional medicine for its various therapeutic properties (Sirizi et al., 2023). Recent studies have begun to explore its potential anticancer effects, particularly in relation to colorectal cancer. The oleo-gum-resin extracted from *F. assa-foetida* contains a variety of bioactive compounds, including sulfur-containing compounds, phenolic acids, and flavonoids, which have been shown to exhibit significant cytotoxicity against various cancer cell lines (Elarabany et al., 2023, Sirizi et al., 2023, Moulazadeh et al., 2022). For instance, research has demonstrated that extracts from *F. assa-foetida* can inhibit cell viability and induce apoptosis in colorectal cancer cell lines such as HT-29 and Caco-2 (Elarabany et al., 2023). The mechanisms by which *F. assa-foetida* exerts its anticancer effects are diverse. Studies indicate that its active components can modulate key signaling pathways involved in cell survival and apoptosis (Mahendra and Bisht, 2012, Nouioura et al., 2024). For example, the essential oil of *this plant* has been reported to increase the expression of pro-apoptotic proteins such as PUMA, BIM, BIK, and BAK in cancer cells (Mahendra and Bisht, 2012). Furthermore, it has been shown to reduce the expression of anti-apoptotic proteins, thereby promoting programmed cell death. This dual action not only highlights the potential of *F. assa-foetida* as a therapeutic agent but also emphasizes its role in restoring the balance between pro-apoptotic and anti-apoptotic signals in cancer cells (Mustafa et al., 2024). In addition to its direct effects on cancer cell viability and apoptosis, *F. assa-foetida* may also influence the expression of critical tumor suppressor genes such as SMAD4. The modulation of SMAD4 expression by natural compounds could provide a novel approach to enhance TGF-β signaling in colorectal cancer cells, potentially reversing the malignant phenotype associated with SMAD4 loss (Zhao et al., 2018). Given that alterations in SMAD4 expression are frequently observed in colorectal tumors, investigating the impact of *F. assa-foetida* extract on this gene could reveal important insights into its anticancer mechanisms.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is an effective method for evaluating changes in gene expression levels in response to treatment with bioactive compounds (Bustin et al., 2009). This technique allows for precise quantification of mRNA levels, providing valuable data on how the extract influences SMAD4 expression in colorectal cancer models. By employing qRT-PCR in our study, we aim to elucidate the relationship between *F. assa-foetida* treatment and SMAD4 expression changes in Caco-2 cells.

Considering above description, this study aims to explore the effect of *Ferula assa-foetida* extract on the expression of the SMAD4 tumor suppressor gene in Caco-2 cell lines as a model for colorectal cancer. Understanding how this natural extract modulates tumor suppressor gene expression may pave the way for developing novel therapeutic strategies that leverage traditional medicinal knowledge alongside modern molecular biology techniques. As research continues to uncover the anticancer properties of natural compounds like *F. assa-foetida*, there is potential for these agents to contribute significantly to future colorectal cancer therapies.

Methods

Plant Material and Extract Preparation

The plant material used in this study consisted of mixed stem, root, and leaves of *F. assa-foetida*, which were collected from a natural habitat in Taftan area (Sistan and Baluchestan providence, Iran) during mid spring of 2023. The collected plant parts were thoroughly washed with distilled water to remove any soil and impurities, then air-dried in a shaded area to prevent degradation of bioactive compounds. Once dried, the plant materials were ground into a fine powder using a mechanical grinder. The powdered material was then subjected to extraction using 80% ethanol as the extraction solvent. A known weight of the powdered plant material was mixed with the solvent in a ratio of 1:10 (w/v) and allowed to macerate for 48 hours at room temperature with occasional stirring. Following maceration, the mixture was filtered through Whatman No. 1 filter

paper to separate the solid residues from the liquid extract. The resulting filtrate was concentrated using a rotary evaporator at reduced pressure and temperature to yield a viscous extract. This concentrated extract was then stored at -20°C until further use in biological assays.

Cell Line and Culture Conditions

The Caco-2 cell line (HTB-37), derived from human colorectal adenocarcinoma, was obtained from American Type Culture Collection (ATCC) and used as a model for colorectal cancer in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% non-essential amino acids. The culture was maintained in a humidified incubator at 37°C with 5% CO2. Upon reaching approximately 80% confluence, the cells were passaged using trypsin-EDTA solution to detach them from the culture flask. For experimental treatments, Caco-2 cells were seeded at a density of 10^5 cells/mL in 6-well plates and allowed to adhere for 24 hours before exposure to the *F. assa-foetida* extract. The medium was replaced every 12 hours to ensure optimal growth conditions prior to treatment.

Treatment Protocol

Following the establishment of Caco-2 cell cultures, the treatment protocol was initiated to evaluate the effects of the extract on SMAD4 expression. The cells were divided into several groups, including a control group and three experimental groups treated with varying 50, 100, and 200 µg/mL concentrations of the extract. Prior to treatment, the culture medium was replaced with fresh DMEM with low-serum conditions (1% FBS) to minimize any potential interference from serum components. The prepared extract was then added to the respective wells, and the cells were incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. The control group received only ethanol at the same final concentration as used for the extract dilution to account for solvent effects.

Cell Viability Assay

To assess the cytotoxic effects of *F. assa-foetida* extract on Caco-2 cells and to ensure that the observed changes in SMAD4 expression were not attributable to cell death, an MTT assay was conducted (Kumar et al., 2018). Following the treatment with varying concentrations of the extract (50, 100, and 200 µg/mL) for 24 hours, the culture medium was removed, and the cells were incubated with 0.5 mg/mL of MTT solution in fresh DMEM without serum for 3 hours at 37°C in a 5% CO_2 incubator. During this incubation, metabolically active cells reduced MTT to insoluble purple formazan crystals. After the incubation period, the MTT solution was carefully removed, and the crystals were dissolved by adding 150 µL of dimethyl sulfoxide (DMSO) per well, followed by gentle shaking to ensure complete dissolution. The absorbance of the resulting solution was measured at 570 nm using a microplate reader, with 630 nm as a reference wavelength to correct for background absorbance. The percentage of cell viability was calculated using the formula:

$$(1)\ Cell\,Viability\ (\%) = \left(\frac{\text{Absorbance of Control Cells}}{\text{Absorbance of Treated Cells}}\right) \times 100$$

RNA Extraction

Total RNA was extracted from the Caco-2 cells using TRIzol reagent, following the manufacturer's protocol. After the treatment period with *F. assa-foetida* extract, the cells were washed twice with phosphate-buffered saline (PBS) to remove any residual medium and extract. Subsequently, 1 mL of TRIzol reagent was added directly to each well to lyse the cells. The cell lysates were mixed thoroughly and incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complexes. Following this, 200 µL of chloroform was added to each sample, and the mixtures were shaken vigorously for 15 seconds before being allowed to sit for another 2-3 minutes. The samples were then centrifuged at 12,000 × g for 15 minutes at 4°C to separate the phases. The aqueous phase containing the RNA was carefully transferred to a new tube, and an equal volume of isopropanol was added to precipitate the RNA. After incubation at -20°C for at least 1 hour, the samples were centrifuged again at 12,000 × g for 10 minutes at 4°C. The resulting RNA pellet was washed with 75% ethanol, air-dried, and then dissolved in RNase-free water. The quality and quantity of the extracted RNA were assessed using a spectrophotometer, ensuring that the samples were suitable for subsequent quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed to evaluate the expression levels of the SMAD4 tumor suppressor gene in response to treatment with *F. assa-foetida* extract. First, complementary DNA (cDNA) was synthesized from the extracted RNA using Rocketscript™ reverse transcriptase kit (Bioneer, Korea), employing an oligo-dT random hexamer mix as the primer. The reaction mixture consisted of 1 µg of total RNA, 1 µL of oligo-dT random hexamer mix, and the appropriate volume of reverse transcription reagents, which were incubated at 42°C for 60 minutes followed by heat inactivation at 70°C for 5 minutes. The synthesized cDNA was directly used for qRT-PCR analysis. For the amplification of SMAD4 and GAPDH genes, specific primers were used according to table 1.

Table 1. Primer sequences used for qRT-PCR.

Gene	Size bp	Strand	Sequence (5' → 3')	Length	Tm°C	Location
SMAD4	146	F	CTCATGTGATCTATGCCCGTC	21	60.0	272-292
		R	AGGTGATACAACTCGTTCGTAGT	23	60.8	417-395
GAPDH	116	F	TGTGGGCATCAATGGATTTGG	21	60.9	231-251
		R	ACACCATGTATTCCGGGTCAAT	22	61.4	346-325

The qRT-PCR reactions were conducted in a final volume of 20 µL, comprising 10 µL of SYBR Green PCR Master Mix, 2 µL of cDNA template, 0.5 µL of each primer (10 µM), and 7 µL of nuclease-free water. The thermal cycling conditions included an initial denaturation step at 95°C for 5 minutes to activate the enzyme, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 57°C for 15 seconds, and extension at 72°C for 15 seconds. A melting curve analysis was performed at the end of the amplification to verify the specificity of the PCR products. The relative expression levels of SMAD4 were normalized to GAPDH using the Pfaffl method (Pfaffl, 2001), allowing for quantification of changes in gene expression in response to treatment with *F. assa-foetida* extract by considering the efficiency for each gene amplification.

Statistical Analysis

Statistical analysis was performed to evaluate the significance of the results obtained from the qRT-PCR and cell viability assays. The data were analyzed using GraphPad Prism 8 (GraphPad Software), and results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Differences between the treatment groups and the control group were assessed using one-way analysis of variance (ANOVA), followed by post hoc Tukey's test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. As already described the expression levels of SMAD4 were normalized to GAPDH, and relative expression was calculated using the Pfaffl method. Cell viability percentages obtained from the MTT assay were also analyzed to ensure that observed changes in gene expression were not confounded by cytotoxic effects. All statistical tests were conducted with appropriate assumptions checked, ensuring the validity of the results presented in this study.

Results

Effect of F. assa-foetida Extract on Cell Viability

The effect of *F. assa-foetida* extract on the viability of Caco-2 cells was assessed using the MTT assay to ensure that the observed changes in SMAD4 expression were not due to cytotoxicity (Fig. 1). The results indicated that treatment with the extract did not significantly affect cell viability across all concentrations tested. Specifically, the percentage of viable cells was measured as follows: for the control group, an average viability of 100% was recorded, while treatment with 50 µg/mL, 100 µg/mL, and 200 µg/mL concentrations of the extract resulted in average viabilities of 95%, 91%, and 90.3%, in three independent experiments respectively. Further analysis revealed that the viability percentages for the treated groups were not significantly lower than the control group and remained above 90% for all concentrations, indicating no meaningful cytotoxic effects. These findings suggest that the extract is well-tolerated by Caco-2 cells and does not induce significant cell death at the concentrations tested, thereby

supporting the validity of subsequent analyses of SMAD4 expression changes.

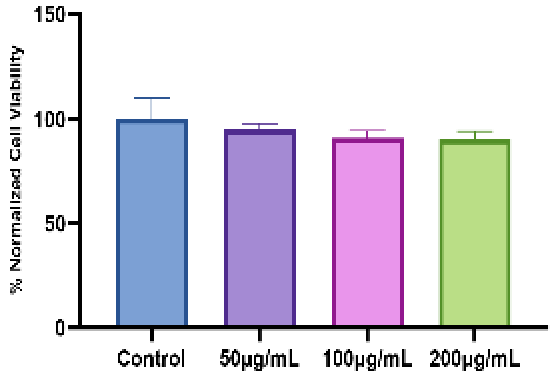


Fig. 1. Effect of *F. assa-foetida* Extract on Caco-2 Cell Viability. The viability of Caco-2 cells was assessed using the MTT assay following treatment with varying concentrations of the extract (50 µg/mL, 100 µg/mL, and 200 µg/mL) for 24 hours. The results are expressed as the percentage of viable cells compared to the control group (untreated cells), which was set at 100%. No significant cytotoxicity was observed at any of the tested concentrations.

RNA Quality and qRT-PCR Optimization

The quality and concentration of the extracted RNA were assessed using a nano-drop spectrophotometer (Fig. 2a), and the results are summarized in Table 2. The quality of the extracted RNA was assessed to ensure its suitability for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. The RNA samples exhibited high purity, as indicated by the R260/280 ratios, which were consistently around 2.05 to 2.10 across all samples. These values suggest that the RNA is free from significant protein contamination, which is essential for reliable downstream applications. Additionally, the R260/230 ratios were also within acceptable limits, indicating minimal contamination from phenolic compounds or other impurities.

To ensure the specificity of the primers used for qRT-PCR, gel electrophoresis was performed on PCR products amplified from cDNA templates using SMAD4 and GAPDH primers. The gel image, shown in Fig. 2b, confirms that the primers generated distinct bands corresponding to the expected product sizes without any non-specific amplification or primer-dimer formations demonstrating that both primer pairs functioned effectively without contamination or unexpected by-products. These optimizations confirmed that the extracted RNA was suitable for subsequent qRT-PCR analysis to evaluate SMAD4 expression levels in response to *F. assa-foetida* treatment.

Table. 2. Concentration and quality assessment of RNA extracted from caco-2 cells treated with *F. assa-foetida* extract. The table presents the RNA concentrations (ng/µl) alongside the r260/280 and r260/230 absorbance ratios for control and treated samples (50 µg/ml, 100 µg/ml, and 200 µg/ml), indicating the purity and suitability of RNA for subsequent qRT-PCR analysis.

Sample	Conc. (ng/µL)	$R_{260/280nm}$	$R_{260/230nm}$
Control	1917.3	2.05	2.19
50µg/mL	1810.5	2.06	2.16
100µg/mL	2527	2.10	1.99
200µg/mL	1342.2	2.08	2.05

The standard curve analysis for both SMAD4 and GAPDH genes was conducted to evaluate the efficiency and reliability of the qRT-PCR assays. Part a of Fig. 3 presents the melting curve analysis generated from tenfold serial dilutions of both genes. The red curve represents the SMAD4 gene, while the green curve corresponds to the GAPDH gene. The absence of any mis-amplified segments is indicated by the distinct single peaks observed for both genes, confirming that the assays are specific and that non-specific amplification is unlikely. This specificity is crucial for accurate quantification of gene expression levels. In parts b and c of Fig. 3, the standard curves for GAPDH and SMAD4 are displayed, respectively. The efficiency of the GAPDH gene was calculated to be 1.04, while the efficiency for SMAD4 was determined to be 1.00. Both values indicate that the PCR reactions are highly efficient, as efficiencies close to 1.0 are desirable for accurate quantification. Additionally, the R^2 values for both genes exceeded 0.99, demonstrating a strong linear relationship between the log of the template concentration and the corresponding CT values. These results confirm that both SMAD4 and GAPDH assays are reliable and suitable for quantifying gene expression changes in response to treatment with *F. assa-foetida* extract.

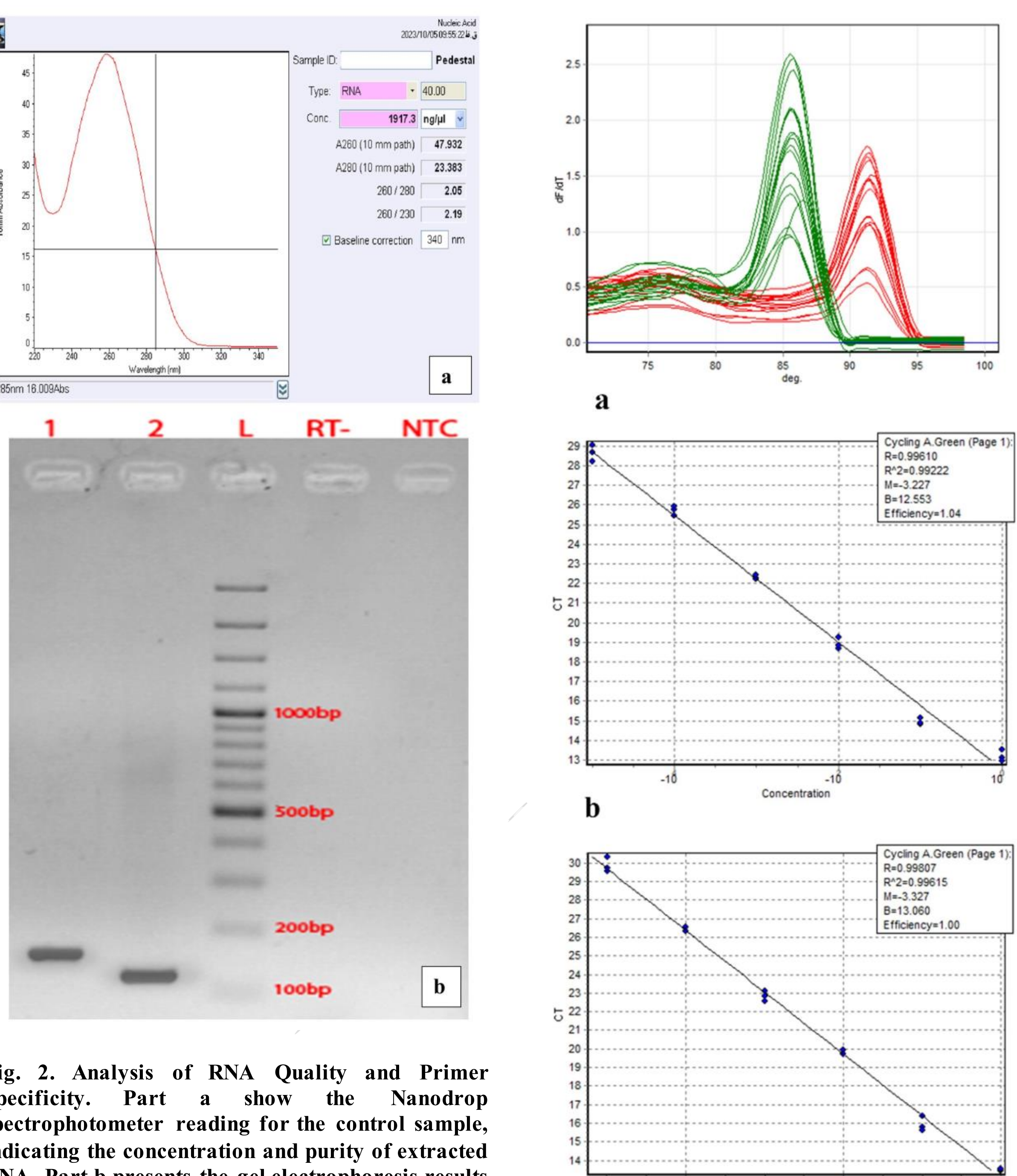


Fig. 2. Analysis of RNA Quality and Primer Specificity. Part a show the Nanodrop spectrophotometer reading for the control sample, indicating the concentration and purity of extracted RNA. Part b presents the gel electrophoresis results of PCR products amplified using SMAD4 and GAPDH primers, demonstrating specific amplification with distinct bands corresponding to the expected product sizes (116 and 146 bp). The lanes are arranged from left to right as follows: SMAD4, GAPDH, DNA size marker, RT minus control, and no template control (NTC) sample. No non-specific bands or primer-dimer formations were observed, confirming the specificity of the primers used for qRT-PCR analysis.

Fig. 3. Standard Curve and Melting Curve Analysis for SMAD4 and GAPDH Genes. Part a show the melting curve analysis of tenfold serial dilutions for both genes, with the red curve representing SMAD4 and the green curve representing GAPDH, indicating no mis-amplified segments. Parts b and c display the standard curves for GAPDH and SMAD4, respectively, with efficiencies of 1.04 and 1.00, and R^2 values exceeding 0.99 for both genes, confirming their reliability for qRT-PCR analysis.

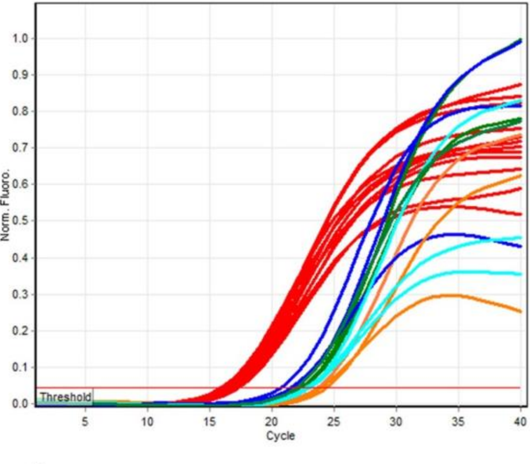
Expression Levels of SMAD4 in Caco-2 Cells

The analysis of the expression levels of the SMAD4 tumor suppressor gene in Caco-2 cells, following treatment with various concentrations of *F. assa-foetida* extract, was conducted using a one-way ANOVA test. The amplification plot presented in Fig. 4a illustrates the qRT-PCR results for each sample, demonstrating distinct amplification curves for the control and treated samples, which validates the successful quantification of SMAD4 expression.

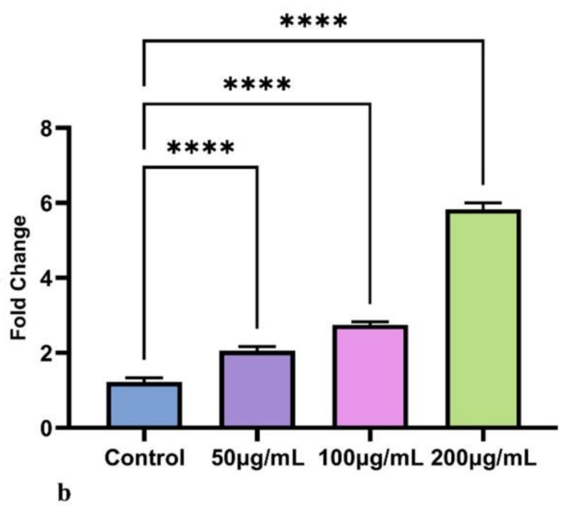
The one-way ANOVA revealed a statistically significant difference among the means of the treatment groups $p < 0.0001$, with an exceptionally high R-squared value of 0.996, indicating that 99.6% of the variance in SMAD4 expression can be explained by the treatment conditions. Dunnett's multiple comparisons test was performed to evaluate specific differences between the control group and each treatment concentration. The analysis revealed significant increases in SMAD4 expression across all treatment groups compared to the control. At 50 µg/mL, a mean difference of -0.835 (95% CI: -1.02 to -0.653) was observed. The 100 µg/mL concentration exhibited a larger increase with a mean difference of -1.53 (95% CI: -1.71 to -1.34), while the highest concentration of 200 µg/mL demonstrated the most substantial increase with a mean difference of -4.61 (95% CI: -4.79 to -4.43). All comparisons were highly significant ($p < 0.0001$).

The descriptive statistics further support these findings, with mean expression levels progressively increasing from 1.22 ± 0.112 in the control group to 2.06 ± 0.117, 2.75 ± 0.0817, and 5.83 ± 0.169 in the 50, 100, and 200 µg/mL treatment groups, respectively. The relatively small standard deviations across all groups indicate high precision in the measurements. As illustrated in Fig. 4b, the dose-dependent increase in SMAD4 expression was observed with statistical significance between all relevant groups.

These comprehensive statistical findings strongly support that *F. assa-foetida* extract has a potent, dose-dependent modulatory effect on SMAD4 expression in Caco-2 cells, potentially enhancing its tumor suppressor functions. The pronounced increase in SMAD4 expression observed at higher concentrations of the extract may contribute to improved regulation of the TGF-β signaling pathway, which is crucial for cellular processes such as growth inhibition and apoptosis. The robust statistical significance and high R-squared value provide strong evidence for the reliability of these findings and their potential biological importance.



a



b

Fig.4. Analysis of SMAD4 expression in Caco-2 cells treated with *F. assa-foetida* extract. Part a display the amplification plots for SMAD4 and GAPDH genes across various concentrations of the extract. The red curve represents the GAPDH gene, while the orange curve indicates the control SMAD4 expression. The cyan curve corresponds to the 50 µg/mL concentration of the extract, the green curve represents the 100 µg/mL concentration, and the blue curve illustrates the 200 µg/mL concentration. Part b presents the results of the ANOVA test, highlighting significant differences in SMAD4 expression levels among the control and treated groups. Statistical significance between relevant groups is indicated, demonstrating that treatment with the extract significantly enhances SMAD4 expression in a dose-dependent manner.

Discussion

The present study demonstrates that the ethanolic extract of *Ferula assa-foetida* significantly modulates the expression of the SMAD4 tumor suppressor gene in Caco-2 colorectal cancer cells. The findings reveal a dose-dependent increase in SMAD4 expression following treatment with various concentrations of the extract, suggesting a potential therapeutic mechanism through which *F. assa-foetida* may exert its anti-cancer effects.

The observation that cell viability remained above 90% across all treatment concentrations is particularly noteworthy, as it indicates that the increased SMAD4 expression is not a stress response to cytotoxicity but rather a specific molecular effect of the extract's bioactive compounds. This maintenance of cell viability while achieving significant changes in gene expression suggests that *F. assa-foetida* extract may act through epigenetic modulation rather than direct cytotoxic mechanisms, which could be advantageous from a therapeutic perspective. This is in contrast to conventional chemotherapeutic agents, such as 5-fluorouracil, which often induce significant cytotoxicity and side effects (Longley et al., 2003).

The robust upregulation of SMAD4 expression, particularly at the highest concentration of 200 µg/mL (approximately 4.8-fold increase compared to control), represents a significant finding in the context of colorectal cancer therapy. SMAD4, as a crucial mediator of the TGF-β signaling pathway, plays a vital role in regulating cell proliferation, differentiation, and apoptosis. The loss or downregulation of SMAD4 is frequently observed in colorectal cancer and is associated with poor prognosis and increased metastatic potential (Miyaki and Kuroki, 2003, Woodford-Richens et al., 2001). Therefore, the ability of *F. assa-foetida* extract to enhance SMAD4 expression could potentially help restore normal TGF-β signaling in colorectal cancer cells, similar to the effects observed with other natural compounds like curcumin (Shakibaei et al., 2014).

The dose-dependent nature of SMAD4 upregulation, supported by strong statistical evidence (R-squared value of 0.996), suggests a specific and controlled molecular mechanism. This finding aligns with previous studies on *F. assa-foetida*'s bioactive compounds, particularly its sulfur-containing compounds and phenolic constituents, which have been shown to modulate various signaling pathways in cancer cells (Sattar and Iranshahi, 2017). The progressive increase in SMAD4 expression across treatment concentrations (1.7-fold at 50 µg/mL, 2.3-fold at 100 µg/mL, and 4.8-fold at 200 µg/mL) indicates a predictable and manageable dose-response relationship, which is crucial for potential therapeutic applications. This is comparable to the dose-dependent effects of other natural compounds, such as resveratrol, in modulating cancer-related pathways (Bishayee, 2009).

The high precision of measurements, indicated by the small standard deviations across all treatment groups, adds reliability to these findings. The careful validation of RNA quality and qRT-PCR optimization, including primer specificity confirmation and efficiency calculations, further strengthens the robustness of these results. The use of GAPDH as a reference gene, with consistent expression patterns across treatments, provides reliable normalization for the observed changes in SMAD4 expression.

These findings may have significant implications for understanding the traditional use of *F. assa-foetida* in medicine and its potential application in modern cancer therapy. The ability to upregulate SMAD4 without significant cytotoxicity suggests that the extract could potentially be developed as an adjuvant therapy in colorectal cancer treatment, particularly in cases where SMAD4 downregulation contributes to cancer progression and treatment resistance. Other natural compounds, such as epigallocatechin-3-gallate (EGCG) from green tea, have also shown potential as adjuvant therapies in cancer treatment (Maruyama et al., 2014, Shimizu et al., 2005).

However, several aspects warrant further investigation. Future studies should focus on identifying the specific bioactive compounds responsible for SMAD4 upregulation and elucidating the detailed molecular mechanisms involved. Additionally, investigation of potential synergistic effects with conventional chemotherapeutic agents could provide valuable insights for combination therapy approaches (Patel and Majumdar, 2009). The long-term stability of SMAD4 upregulation and its effects on downstream signaling pathways also need to be evaluated. Moreover, while the current study demonstrates significant effects in vitro, in vivo studies will be crucial to validate these findings in

a more complex biological context. The bioavailability of the active compounds, optimal dosing regimens, and potential side effects need to be carefully assessed before clinical applications can be considered. This is a common challenge in the development of natural compound-based therapies, as seen with curcumin (Anand et al., 2007).

These results contribute to the growing body of evidence supporting the anti-cancer properties of natural compounds and highlight the potential of traditional medicinal plants as sources of novel therapeutic agents. The ability of *F. assa-foetida* extract to modulate SMAD4 expression represents a promising direction for developing targeted approaches in colorectal cancer treatment, particularly in cases where SMAD4 dysfunction plays a crucial role in disease progression.

Conclusion

This study presents compelling evidence that ethanolic extract of *F. assa-foetida* significantly enhances SMAD4 tumor suppressor gene expression in Caco-2 colorectal cancer cells through a dose-dependent mechanism. The observation of substantial SMAD4 upregulation without meaningful cytotoxicity suggests that *F. assa-foetida* may act through epigenetic modulation rather than direct cell death pathways. The robust statistical validation and careful methodological controls strengthen the reliability of these findings. While these results are promising, further research is needed to identify the specific bioactive compounds responsible for SMAD4 upregulation, elucidate detailed molecular mechanisms, and evaluate potential synergistic effects with conventional chemotherapeutics. Additionally, in vivo studies will be crucial to validate these findings in more complex biological systems and assess bioavailability and optimal dosing regimens. This research not only contributes to our understanding of *F. assa-foetida*'s anticancer properties but also opens new avenues for developing targeted natural compound-based therapies for colorectal cancer, particularly in cases where SMAD4 dysfunction plays a central role in disease progression.

Acknowledgment

The authors gratefully appreciate the University of Sistan and Baluchestan for financial support of this research.

Conflict of Interest

The authors declare no conflict of interest

References

ANAND, P., KUNNUMAKKARA, A. B., NEWMAN, R. A. & AGGARWAL, B. B. 2007. Bioavailability of Curcumin: Problems and Promises. *Molecular Pharmaceutics,* 4, 807-818.

BISHAYEE, A. 2009. Cancer Prevention and Treatment with Resveratrol: From Rodent Studies to Clinical Trials. *Cancer Prevention Research,* 2, 409-418.

BUSTIN, S. A., BENES, V., GARSON, J. A., HELLEMANS, J., HUGGETT, J., KUBISTA, M., MUELLER, R., NOLAN, T., PFAFFL, M. W., SHIPLEY, G. L., VANDESOMPELE, J. & WITTWER, C. T. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem,* 55, 611-22.

ELARABANY, N., HAMAD, A. & ALZAMEL, N. M. 2023. Antitumor and Phytochemical Properties of Ferula assa-foetida L. Oleo-Gum-Resin against HT-29 Colorectal Cancer Cells In Vitro and in a Xenograft Mouse Model. *Molecules,* 28.

FEARON, E. R. & VOGELSTEIN, B. 1990. A genetic model for colorectal tumorigenesis. *Cell,* 61, 759-67.

KUMAR, P., NAGARAJAN, A. & UCHIL, P. D. 2018. Analysis of Cell Viability by the MTT Assay. *Cold Spring Harb Protoc,* 2018.

LONGLEY, D. B., HARKIN, D. P. & JOHNSTON, P. G. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer,* 3, 330-8.

MAHENDRA, P. & BISHT, S. 2012. Ferula asafoetida: Traditional uses and pharmacological activity. *Pharmacogn Rev,* 6, 141-6.

MARUYAMA, T., MURATA, S., NAKAYAMA, K., SANO, N., OGAWA, K., NOWATARI, T., TAMURA, T., NOZAKI, R., FUKUNAGA, K. & OHKOHCHI, N. 2014. (-)-Epigallocatechin-3-gallate suppresses liver metastasis of human colorectal cancer. *Oncol Rep,* 31, 625-33.

MIYAKI, M. & KUROKI, T. 2003. Role of Smad4 (DPC4) inactivation in human cancer. *Biochem Biophys Res Commun,* 306, 799-804.

MOULAZADEH, A., RANJBAR, R., DAKHILI ARDESTANI, A., RANJBAR, K., FARJADFAR, A., KOUHPAYEH, S. A., ABDOLLAHI, A. & NAJAFIPOUR, S. 2022. Cytotoxic effects of Trachyspermum ammi and Ferula assafoetida on MCF-7 and MDA-MB-468 breast cancer cell lines. *Beni-Suef University Journal of Basic and Applied Sciences,* 11, 147.

MUSTAFA, M., AHMAD, R., TANTRY, I. Q., AHMAD, W., SIDDIQUI, S., ALAM, M., ABBAS, K., MOINUDDIN, HASSAN, M. I., HABIB, S. & ISLAM, S. 2024. Apoptosis: A Comprehensive Overview of Signaling Pathways, Morphological Changes, and Physiological Significance and Therapeutic Implications. *Cells,* 13.

NOUIOURA, G., EL FADILI, M., EL BARNOSSI, A., LOUKILI, E. H., LAAROUSSI, H., BOUHRIM, M., GIESY, J. P., ABOUL-SOUD, M. A. M., AL-SHEIKH, Y. A., LYOUSSI, B. & DERWICH, E. H. 2024. Comprehensive analysis of different solvent extracts of Ferula communis L. fruit reveals phenolic compounds and their biological properties via in vitro and in silico assays. *Scientific Reports,* 14**,** 8325.

PAPAGEORGIS, P., CHENG, K., OZTURK, S., GONG, Y., LAMBERT, A. W., ABDOLMALEKY, H. M., ZHOU, J. R. & THIAGALINGAM, S. 2011. Smad4 inactivation promotes malignancy and drug resistance of colon cancer. *Cancer Res,* 71**,** 998-1008.

PATEL, B. B. & MAJUMDAR, A. P. 2009. Synergistic role of curcumin with current therapeutics in colorectal cancer: minireview. *Nutr Cancer,* 61**,** 842-6.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res,* 29**,** e45.

SATTAR, Z. & IRANSHAHI, M. 2017. Phytochemistry and pharmacology of Ferula persica Boiss.: A review. *Iranian Journal of Basic Medical Sciences,* 20**,** 1-8.

SHAKIBAEI, M., BUHRMANN, C., KRAEHE, P., SHAYAN, P., LUEDERS, C. & GOEL, A. 2014. Curcumin chemosensitizes 5-fluorouracil resistant MMR-deficient human colon cancer cells in high density cultures. *PLoS One,* 9**,** e85397.

SHIMIZU, M., DEGUCHI, A., LIM, J. T. E., MORIWAKI, H., KOPELOVICH, L. & WEINSTEIN, I. B. 2005. (−)-Epigallocatechin Gallate and Polyphenon E Inhibit Growth and Activation of the Epidermal Growth Factor Receptor and Human Epidermal Growth Factor Receptor-2 Signaling Pathways in Human Colon Cancer Cells. *Clinical Cancer Research,* 11**,** 2735-2746.

SIRIZI, M. A. G., ALIZADEH GHALENOEI, J., ALLAHTAVAKOLI, M., FOROUZANFAR, H. & BAGHERI, S. M. 2023. Anticancer potential of Ferula assa-foetida and its constituents, a powerful plant for cancer therapy. *World J Biol Chem,* 14**,** 28-39.

WOODFORD-RICHENS, K. L., ROWAN, A. J., GORMAN, P., HALFORD, S., BICKNELL, D. C., WASAN, H. S., ROYLANCE, R. R., BODMER, W. F. & TOMLINSON, I. P. 2001. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. *Proc Natl Acad Sci U S A,* 98**,** 9719-23.

ZHAO, M., MISHRA, L. & DENG, C. X. 2018. The role of TGF-β/SMAD4 signaling in cancer. *Int J Biol Sci,* 14**,** 111-123.