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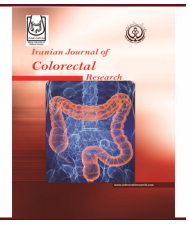

Anti-proliferative and Apoptotic Potential of Ferula Asafoetida's Essential Oil Via Inhibiting NF-kB and TNF-alpha Receptors Pathway

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Abstract

Background: The current investigation was conducted with the aim to elucidate and determine the anti-cancer efficacy *in vitro* on colon carcinoma human cell lines and to determine its probable mode of action.
Methods: Anti-proliferative and apoptotic effect of various doses of essential oil from *Ferula asafoetida* was investigated on colon cancer cell lines i.e. CW620 and CT26. WT. The percentage cytotoxicity was determined by MTT assay. In addition, expression studies were carried out to determine the mode of action by determining the levels of NF-kB, TNF-alpha, TGF-beta and Caspases.
Results: The potent anti-proliferative activity has been observed in both the cell lines. The percentage cytotoxicity is directly proportional to the increasing concentration of the essential oil. The same was also confirmed in the expression studies which shows significant upregulation of the antiapoptotic characteristics and down regulation of pro-carcinogenic factors.
Conclusion: The essential oil shows potent anti-cancer activity and shows the role in gene regulation to attenuate the colon cancer.

Keywords: Colon cancer, *Ferula asafoetida*, Essential oil, Anti-cancer properties, Caspases, Inflammation

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Introduction

Colorectal cancer (CRC) is one of the most common type of cancer in oncologic pathology. CRC is among the second most leading cause of cancer death worldwide and the third most common cancer, accounting for approximately 1.2 million new cases and 608,000 deaths per year (1, 2). Several endogenous factors, as well as lifestyle and dietary factors, related to CRC risk have been identified (3). It is assumed that the entire process of hepatocarcinogenesis involves the collaborative action of several cellular mechanisms such as a change in the tumour microenvironment, neuroinflammation, oxidative stress, and hypoxia along with alternative molecular mechanisms, as well as the transcription and activation of cytokines, chemokines, and growth factors, DNA damage, and DNA methylation. Pro-inflammatory (such as Interleukin-6, or IL-6, Tumour Necrosis

Factor, or TNF-α) and anti-inflammatory cytokines (Transforming Growth Factors α and β or TGF-α and β), different transcription factors (NF-κB, STAT-3), and their signalling pathways are involved in HCC development (4, 5).

The expression of IL-6 and TNF-α during chronic hepatic injury activates downstream targets of the STAT3 transcription factor, which drives neoplastic transformation in the liver microenvironment. Further, TNF-α promotes hepatic tumour growth and HCC recurrence. Jing et al. (6) discovered that TNF- overexpression promotes HCC by activating hepatic progenitor cells (HPCs), and that TNF- knockdown inhibited HPC activation and proliferation, lowering tumour incidence. This confirmed that TNF- α plays a significant role in liver injury and prognosis.

Activated NF- κB is a frequent and early event in HCC, irrespective of aetiology and it is linked with the attainment of a transformed phenotype during hepatocarcinogenesis. As a result, NF- κB is proposed to be a key mediator of hepatic injury, fibrosis, and HCC.

TGF- is expressed at a low level in normal liver cells. The combined actions of different cytokines secreted as a chronic inflammatory response following hepatic injury persistently upregulate TGF- in the liver, allowing regeneration of hepatocytes, hepatocyte proliferation, hepatocyte dysplasia, and finally the development of HCC (7). TGF- α is also up-regulated in HCC and it plays a critical role in HCC progression by inducing tumour cell migration and invasion (8). The inflammatory response caused by viral (microbial attack) or non-viral etiologies (sterile attacks) made proinflammatory cytokines through inflammasome-dependent or independent pathways. The inflammasome component serves as a platform for caspase activation. Proinflammatory cytokines, through activation of transcription factors or by some unknown mechanisms create the hepatic atmosphere appropriate for cellular transformation.

There has been increased demand for identification of new novel and potent chemotherapeutic or anti-cancer agents with high efficiency and negligible toxicity. This need is due to ever increase in the incidence rate of the CRC and major limitations of the existing therapeutic alternatives. Currently, more than 40% of the drugs available in the market are derived from natural sources (9).

Induction of CRCs can take place due to any of the following mechanisms, i.e., chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI) (10). In addition to these, diet also has been experimentally and epidemiologically indicated to increase the colon cancer risk. There are increasing epidemiological evidences to suggest a close association between the insulin resistance and colonic adenomas and cancers. It has been reported that the consumption of diet rich in calories resulted in the development of insulin resistance with increased levels of insulin, triglycerides, and non-esterified fatty acids. These circulating factors stimulates the proliferation of colonic cells, leading to generation of reactive oxygen species and their intermediates. Thus, long-term exposures can result in the induction of colon cancer. There is a continued thrust in the area of identification of potential agents that could inhibit colon cancer in various animal models. The probable mechanism by which induction can occur includes a localized loss of barrier function. The loss can result due to a leaky gut, leading to local inflammatory response characterized by activation of COX-2 and elevation of reactive oxygen species and their intermediates (10).

Numerous plants and plant-based products have significantly shown promising anticancer potentials. Various attempts have been made to purify and characterize the potential constituents isolated from natural products as a future anti-cancer agents. Auyurveda provides holistic approach for the treatment of various diseases. It may provide an effective alternative to single purified active compounds in the treatment of cancer. *Charaka* and *Sushruta* samhitas, the two well-known Ayurvedic classics, describes cancer as a inflammatory or non-inflammatory swelling and mention them as either *Granthi* (minor neoplasm) or *Arbuda* (major neoplasm) (9). In our earlier studies on liver cancer cell lines, we have reported potent anticancer potential for dithiolane rich fraction of *F. asafoetida* (11).

Ferula asafoetida belongs to the family *Umbelliferae*. It is widely used as a traditional medicine in different parts of the world, in the treatment of several diseases such as asthma, bronchitis, stomach ache, ulcer, intestinal parasites and epilepsy (12-14). Asafoetida is used mainly for the stomach related ailments and digestion in Ayurveda. Higwastaka, a popular polyherbal formulation of Asafoetida is used as digestive. Asafoetida along with other dietary spices facilitates digestion by promoting the activities of digestive enzymes in pancreas and small intestine, stimulating the bile acid production (15). In our previous study, we had first reported theanti-proliferative as well as pro-apoptotic potentials of dithiolane-rich essential oil of *F. asafoetida* on human liver cancer cell lines (11).

An important hallmark of cancer cells is the evasion of apoptosis (15). However, most of the existing chemotherapies work by inducing apoptosis as well as by producing direct toxicity. Since many plant extracts exert their effects through apoptosis (15), evaluating the ability of plant extracts to induce apoptosis is crucial. Therefore, in this study, we focused on the apoptotic mechanism evoked by the essential oil (EO) to combat diseases, which might help understand the toxicity-inducing mechanisms that mediate the action of the EO.

Materials and Methods

Plant Material and Its Preparation of Essential Oil (EO)

Fresh resins of *Ferula asafoetida*, used in the present study, were gifted by National Foods, Vadodara and the sample were authenticated by Dr. Vasant A. Patel, Department of Botany, Smt. S.M.P. Science College, Hemachandracharya North Gujarat University, Gujarat, India. Specimen sample of *Ferula asafoetida* was submitted and stored at Institute of Science, Nirma University, Ahmedabad, Gujarat, India with the voucher no. ISNU/FA/CN-120421/01.

As reported earlier (11), the EO was extracted by microwave-assisted hydro-distillation of 100 gms resin n in 50 mL of water. The whole system is kept in microwave-assisted hydro-distillation with set power and temperature at 750 W at 100 °C for 30 min. The EO was collected through a separating funnel and then stored in the refrigerator. Extraction efficiency was found to be 8.3%±0.23% (11). The Study with which submitted and approved by the Institutional Ethics Committee. The code is as under IEC/NU/23/IS/7.

Cell Culture

Human colon cancer cell lines viz., SW620 (Human colorectal adenocarcinoma) and CT26. WT (Mouse colon carcinoma) were obtained from NCCS, Pune, India and (ATCC, USA). The cell lines were maintained under growth conditions with 37 °C, 95% Humidity, 5 % CO_2 provided the medium of Leibovitz's L-15+10 % FBS and RPMI-1640 (HiMedia, India)+10 % FBS (HiMedia, India), respectively. The cell lines were trypsinized and cells were suspended into fresh flasks and supplementing with fresh culture medium.

The isolated EO was diluted in ethanol to obtain a stock solution corresponding to 0.50% (volume/volume). The above stock solutions were then used for preparation of serial dilutions corresponding to 10-fold high concentrations than the desired final concentrations of each test item ranging from 0.016 % to 1 %. As a positive control, Doxorubicin hydrochloride was dissolved in DMSO to obtain the primary stock solution of 20 mM. The stock solution was used for preparation of serial dilutions corresponding to 10-fold high concentrations than the desired final concentrations ranging from 10 µM to 1000 µM in cells.

Cytotoxicity Assay

The Cytotoxicity assay was performed by following Verma et.al, 2019 (11). The cells were trypsinized and counted with a haemocytometer before being plated at a density of $5x10^3$ cells/well in a 96-well plate. To allow for exponential growth, the cells were incubated overnight under the desired growth conditions. Following incubation for 48 hours, the cells were treated with increasing concentrations of EO ranging from 0.0016 per cent to 0.1 per cent and doxorubicin concentrations ranging from 10 µM to 1000 µM in cells. Following incubation, the plates were removed and 20 µl of 5 mg/ml MTT 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to all wells, followed by another 3 h incubation at 37 °C. To dissolve formazan crystals, the supernatant was aspirated and 150 µl of DMSO were added to each well., The absorbance of each well was measured at 540 nm (16).

The percentage cytotoxicity corresponding to each treatment was calculated using the following formula:

% Cytotoxicity = (1-X/R) •100,

where X=absorbance of treated cells; R=absorbance of untreated cells.

For expression studies, the both cell lines were divided into eight sets each i.e., untreated (UT); Doxorubicin treated [D1-DOX 0.1 mg/DL; D2-DOX 0.2 mg/DL; D3-DOX 0.5 mg/Dl] and EO treated cells [S1-1 µL/ML; S2-10 µL/ML; S3-25 µL/ML; S4-50 µL/ML]

Gene Expression Studies

Total RNA was isolated using TRI reagent (Sigma-Aldrich), as per the manufacturer's instructions. The quantification RNA was determined using optical density at 260 and 280 nm (UV-2450, Spectrophotometer, Shimadzu (Japan)). Gene expression study was carried out for *NF-kB, TGF-b, TNF-a, Caspase 3, BAX* and *BCL2* and was assessed by RT-PCR. Reverse transcription (RT) was carried out using a First strand cDNA synthesis Kit (Thermo Scientific, USA). β-actin was used as the normalization control. (Table 1)

Polymerase chain reaction (PCR) was carried out as per the protocol of Chaudhary et al. (16). Relative quantities of different mRNA expressions were analyzed by Total Lab 1.0 software (Magnitec Ltd., Israel), normalized with β-actin expression.

Statistical Analysis

Graphpad Prism V.6 was used to perform statistical analysis. One way anova followed by turkey's multiple comparison test were used to determine significance level at 95% significant interval between untreated group (UT) and Dox treated and Samples. All the values are expressed as mean±SD of three independent experiments. Levels of significance is indicated as a- $P<0.05$; b- $P<0.01$; c- $P<0.001$ and d- $P<0.0001$.

Results

Effect of Ferula asafoetida's Essential Oil on Colon Cell Lines

The essential oil rich in Dithiolane was evaluated for its cytotoxic potentials by performing the MTT assay. The oil exhibited profound cytotoxic potentials on both the cell lines as we increase the dose. IC_{50} value of the oil was 10 µg/mL which was derived from the MTT assay through growth curve (Figure 1).

Table 1: Details of the Primer Sequence for the Gene expression study.

Name	5'-3' sequence	Accession No
NF-kB	F- GCTGCCAAAGAAGGACACGACA	NM_008689
	R- GGCAGGCTATTGCTCATCACAG	
TNF-a	F- GGTGCCTATGTCTCAGCCTCTT	NM_013693
	R- GCCATAGAACTGATGAGAGGGAG	
TGF-b	F- TGATACGCCTGAGTGGCTGTCT	NM_011577
	R- CACAAGAGCAGTGAGCGCTGAA	
Caspase 1	F- GGCACATTTCCAGGACTGACTG	NM_009807
	R- GCAAGACGTGTACGAGTGGTTG	
BAX	F- AGGATGCGTCCACCAAGAAGCT	NM_007527
	R- TCCGTGTCCACGTCAGCAATCA	
BCL-2	F- GGAGATACGGATTGCACAGGAG	NM_009741
	R- CTCCATACCAGACGGAAGATAAAG	
b-Actin	F- CATTGCTGACAGGATGCAGAAGG	NM_007393
	R- TGCTGGAAGGTGGACAGTGAGG	

NF-kB -Nuclear factor kappa of activated B cells; TNF-a - Tumour Necrosis Factor alpha; TGF-b - Transforming growth factor-β; BAX - BCL2 associated X; BCL-2 - B-cell lymphoma 2

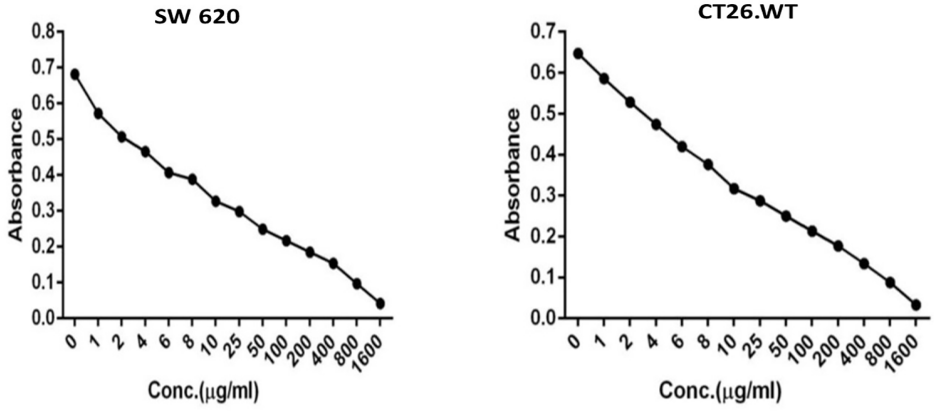

Figure 1: MTT assay for cytotoxicity for essential oil on cancer cell lines.

The isolated essential oil was found to be equally effective against both the cell lines.

The MTT assay was used to assess the cytotoxic potential of dithiolane-rich EO. The oil demonstrated significant cytotoxic potential on both cell lines in a dose-dependent manner. The IC_{50} value of the oil was determined using the growth curve derived from the MTT assay (Figure 1) and was found to be 7.21±0.29 µg/ml for HepG2 and 8.0±0.36 g/ml for SK-Hep1, respectively. In a dose-dependent manner, the isolated EO was found to be equally effective against both cell lines.

Mode of Action Determination of Ferula Asafoetida

Nuclear factor- (NF-κB) is a transcriptional master regulator of inflammatory response and cell death (6). Several studies substantiated the role of NF-κB in the development of hepatocellular injury, liver fibrosis, and HCC. TGF-b, a polypeptide that promotes cellular proliferation and transformation, has been thought to bear a close relationship with hepatocarcinogenesis. NF-kB and TGF-b plays a crucial role in cancer progression and considered to be one of the potent pro-carcinogenic factors. Following treatment with essential oil, both these pro carcinogenic factors were down regulated as shown in (Figure 2).

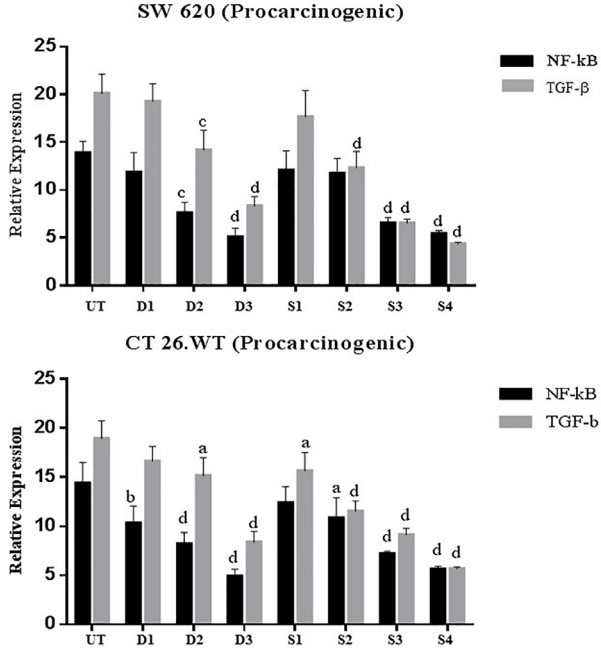

Figure 2: NF-kB and TGF-b expression in cell lines following treatment with assafoetida's essential oil; a-P<0.05; b- P<0.01; c- P<0.001 and d- P<0.0001; Grouping are as follows: untreated (UT); Doxorubicin treated [D1-DOX 0.1 mg/DL; D2-DOX 0.2 mg/DL; D3-DOX 0.5 mg/Dl] and EO treated cells [S1-1 µL/ML; S2-10 µL/ML; S3-25 µL/ML; S4-50 µL/ML]

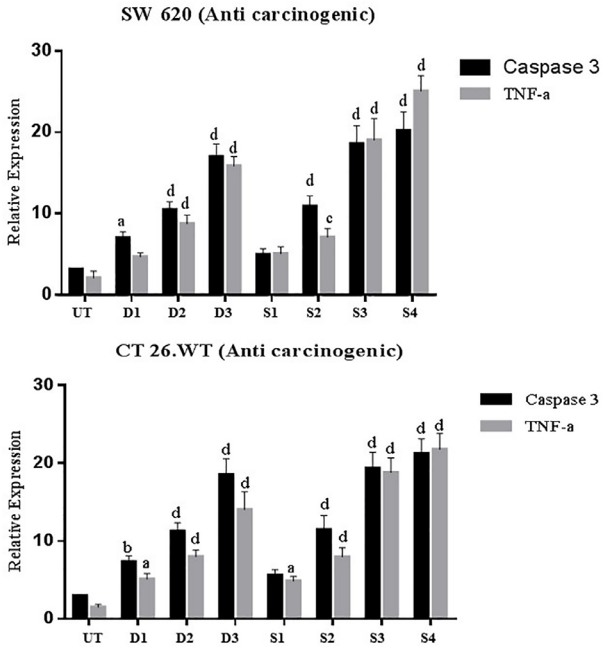

Figure 3: Caspase 3 and TNF-α expression in the cell lines following treatment with asafoetida essential oil; a-P<0.05; b-P<0.01; c- P<0.001 and d- P<0.0001; Grouping are as follows: untreated (UT); Doxorubicin treated [D1-DOX 0.1 mg/DL; D2-DOX 0.2 mg/DL; D3-DOX 0.5 mg/Dl] and EO treated cells [S1-1 µL/ML; S2-10 µL/ML; S3-25 µL/ML; S4-50 µL/ML]

Apoptosis inhibition is critical in the development of cancer. The caspase-3 signaling cascade is the primary pathway by which apoptosis is induced. Simultaneously, the anti-cancerous markers or the pro-apoptotic markers viz., i.e caspase 3 and TNF-alpha showed significant elevation following after treatment with essential oil (Figure 3).

Discussion

Currently, to inhibit unrestricted cell proliferation, maintaining homeostasis is the indispensable factor which needs to be exploited more through stabilizing the cell proliferation. By arresting the cell cycle in cancer cells and inducing apoptosis, we can achieve this target. Many research studies are already focusing on plant extracts which has the impact on metastasis on different kinds of cancer cells.

Anticancer potential for *T. cordifolia* has been shown in in-vitro experimentation (17). *A. Paniculuta* extracts have been shown to have anti-oncogenic properties. Oral administration of *C. asiatica* extracts has decreased the progression of solid and ascites tumors and even the life-span has also been improved in tumor bearing mice (18). In in-vitro experimentation, Turmeric has also been shown to inhibit tumor cell invasion and metastasis too (19). The mice bearing Dalton's lymphoma ascites (DLA), their life span got increased and there has been in reduction of tumor size just because of administration of *P. amarus* extract. Studying these outcomes, shows that there are some plants which have the anti-cancer properties with relatively less side-effects and studying them or their phytochemicals can generate a potent drug.

Nuclear factor-κB (NF-κB), which is a transcription factor, involves in the induction of several genes for cytokines and enzymes that play important functional roles in various cell types. Constitutively activated NF-kB, are the regulators to be seen in various cancer cells, cell lines, xenograft animal models or clinical sites. Constitutively activated NF-kB was observed in 66% of CRC cell lines and 40% of human CRCs. There are many reports that states constitutively activated NF-kB in almost 60-80% of CRCs.

Constitutively activated NF-kB promotes the proliferation of cancer cells and prevents the cancer cells from getting killed. After knocking down IKKg with siRNA (KD cells) to inhibit the constitutive activation of NF-kB, maximum apoptosis was induced in KD cells than in control cells by stimulation with TNF-a or 5-FU (20). Activation of activin receptor-like kinase (ALK) 1 and endoglin which causes angiogenesis which stimulates tumorigenesis by TGF- β1Both adaptive and innate immune responses got restrains by TGF-β1 and promotes the differentiation of immune-suppressive Tregs. And that's how tumorigenesis sustain by such effect on immune system which dampen anti-tumor immunity.

It is interesting that NF-κB signaling pathway is one of the key regulators of apoptosis, by blocking the anti-apoptotic proteins. Hence the apoptosis can be initiated via NF-κB inhibition, thereby inhibiting cell proliferation of cancer cells (21-23). In a similar study by Liu et at. (24) reported Riccardin D induced apoptosis in colon cell via NF-κB inhibition.

Many herbs or medicinal plants showed that that their essential oil can be used as an anti-cancer agent by using many signaling pathways. Anticancer properties against mouth, breast, lung, prostate, liver cancer, colon cancer, and brain cancer and even in leukemia has been reported in different EOs from different plants (25-30). Essential oil of *Illicium verum* showed the greatest cytotoxicity towards HCT 116 (31) . Lebanese sage essential oil possesses antitumor properties, however, the bioactive components and antitumor mechanisms are not known. When combining the three sage bioactive compounds, Linalyl acetate (Ly), Terpeniol (Te) and Camphor (Ca), they cause synergistic inhibition of the growth of two isogenic human colon cancer cell lines HCT-116 (p53+/+ and p53-/-) and which shows no effect on growth of FHs74Int normal human intestinal cell line (32).

The inhibitor concentration 50 (IC_{50}) value of thyme essential oil was found to be 0.347 mg/mL which treated DLD-1 cells exhibiting decreasing cell index values in increasing dose (33). High inhibitory effect on HT-29 cell proliferation was observed by Caper essential oil and aqueous infusion in time and

dose dependent manner and even, they induced the inhibition on nuclear factor jB (NF-jB) activity in a dose-dependent manner. On the other side, they did not show any effect on apoptosis in HT-29 cells. The results from *Zanthoxylum rhoifolium* Lam essential oil confirmed the cytotoxicity of essential oil against tumoral cells, but it is ineffective on non-tumoral cells (34).

In this study, we evaluated the anticancer properties of *F. asafoetida's* essential oil against SW 620 and CT26.WT (colorectal) cancer cell lines. Performing *in vitro* toxicological methods to study the cytotoxic and negative impacts on cell viability, MTT is extensively used. In order to increase the credibility of the results and to avoid misestimating the toxicity of the test extract, other tests need to be performed to assess cell viability in *in vitro* studies. In this study, the FAO has shown to have anticancerous properties where it reduces the viability and proliferation of colon cancer cell lines in a dose-dependent manner.

An extract is considered to be a promising one for further research, when its IC_{50} of crude extract against cancer cells doesn't exceed 30 μg/ml. This has been reported by the National Cancer Institute (NCI) (35). In the current study, it was found that the IC_{50} of the FAO extract falls within this defined value of NCI, therefore making the FAO a promising extract for cancer chemotherapy.

In the present study, the SW 620 and CT26.WT cells treated with FAO demonstrated the *in vitro* anticancer activity through the apoptotic pathway. Since caspase 3 promotes colon cancer resistance to radiotherapy and chemotherapy as well as colon cancer cells invasion and metastasis, thus to approach a promising agent for colon cancer treatment one should combine chemotherapy or radiotherapy with caspase 3 targeted agents (36). Tumor necrosis factor alpha (TNF-α) which was earlier identified as a factor that leads to rapid necrosis of transplantable tumors in mice, now it is considered a proinflammatory cytokine involved in the innate immune response (37-39). TNF-α act through activation of NF-κB and ERK1/2 signalling to up-regulate PD-L1 (Programmed cell death protein ligands) expression in colon cancer HCT116 cells. However, the activation of the caspases, including caspases and proapoptotic marker TNF-alpha, is implicated in both the intrinsic and extrinsic pathways of apoptosis and the genes which are procarcinogen i.e. NF-kB and TGF-B shows downfall in dose dependent manner. This shows that the pathway that FAO has taken to reduce cancer cells would be by regulating apoptotic pathway and probable mode of action. In recent studies, p53-inducible proapoptotic genes trigger apoptosis through both extrinsic and intrinsic apoptotic pathways (40-43). On the major shortcomings of the present study is the limited cell types explored for the efficacy studies.

Conclusion

According to our study, the essential oil shows potent anti-cancer activity and reveals the role in gene regulation to attenuate colon cancer. The efficacy needs to be explored in the animal models as well as to determine its mode of action.

Acknowledgment

The financial support and isolation and characterization of Essential oil from Ferula asafoetida was carried out by National Foods and experimentation was carried out at Institute of Science, Nirma University.

Author's Contribution

SV, Experimentation and characterization of oil; Manuscript preparation; SJ, Characterization of oil; VK, Analysis of results; SS, Analysis of results, Generation of Idea, interpretation of the results

Conflict of interest: None declared.

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