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Chemopreventive Activities of *Trigonella foenum graecum* Against Hepatocellular Carcinoma Cell Line

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and most current therapies are of limited efficacy. *Trigonella foenum-graecum* is a traditional herbal plant with antitumor activity, although the mechanisms of its activity remain unclear. Herein, a crude ethanol extract was prepared from *T. foenum-graecum* seed and its anticancer mechanism was evaluated, using SNU-423 cell line. Growth-inhibitory effect and apoptosis induction of SNU-423 cells were evidenced by MTT assay, cell morphology alteration and expression of p53, proapoptotic protein, Bax and proliferating cell nuclear antigen (PCNA) after (100~500 µg/ml) *T. foenum-graecum* treatment for 48 h. Our results revealed that *T. foenum-graecum* treatment for 48 h showed a cytotoxic effect and apoptosis induction in a dose-dependent manner that was mediated by upregulation of p53, Bax, PCNA in SNU-423 cells. Our data introduced *T. foenum-graecum* as a promising nontoxic herbal with therapeutic potential to induce apoptosis in SNU-423 cells through p53, Bax, and PCNA upregulation in caspase-3 dependent manner.

Keywords: *Trigonella foenum-graecum*, Hepatocellular carcinoma, Cytotoxic, Herbal plant, Antitumor activity, SNU-423, Apoptosis, Growth inhibitory, Cancer

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Introduction

Cancer is an atypical cell growth that starts with a genetic mutation that modifies the function of the cell, breaks down cellular relationships and connections, and eventually results in the production of oncogenes. It is either acquired or

inherited. Metabolic reprogramming is required for cancer cells to initiate the creation and spread of tumors. The survival mechanisms of cancer cells primarily rely on altering their own flux across metabolic pathways due to the heightened

demands on bioenergetic and biosynthetic pathways, as well as the moderation of oxidative stress (Simone Perna *et al.*, 2022). The International Agency for Research on Cancer (IARC) lists a number of external influences that can lead to cancer, such as alcohol, medications, tobacco, sunshine, and so forth (Zahavi and Weiner, 2020). Early identification of cancer can improve the body's response to medical therapies; there are currently technological approaches available for this purpose.

Worldwide, cancer is the second most common cause of death. An estimated 9.6 million fatalities were attributed to cancer in 2018 alone. According to Endalkachew Nibret *et al.* (2021), low- and middle-income nations accounted for almost 70% of these fatalities. Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, accounting for 9.1% of all cancers and an estimated incidence of 746,000 new cases every year. According to 692,000 cases, it is regarded as the third cause of cancer-related mortality. The highest incidence rates of HCC (around 85% of cases) are present in East Asia and sub-Saharan Africa (Ferlay *et al.*, 2015). Geographic patterns in incidence and mortality are comparable for liver cancer since the disease has a relatively poor prognosis (overall ratio of mortality to incidence of 0.95).

Recently, increasing attention has been focused to identify the naturally occurring anticancer agents, particularly those present in dietary and medicinal plants due to their bioactive substances (Kwon *et al.*, 2007). Most of these bioactive substances exert their cancer chemotherapeutic activity by modulating signal transduction pathways that are involved in cell cycle progression, proliferation, and triggering apoptotic cell death. In addition, induction of tumor suppressor genes in tumor cells has become promising therapeutic indicator for tumor treatment response in employing a plant derived-bioactive substance to decrease breast cancer mortality.

In many developed countries, herbal medicines are achieving attractiveness as alternative and courtesy therapies (Omobuwajo *et al.*, 2011). There are certain plants that are used as food or medicine. Numerous biological and pharmacological activities are displayed by these plants. According to Ferrow and Avila (1999), the U.S. Food and Drug Administration classifies fenugreek (*Trigonella foenum-graecum* L.), a legume crop used as a spice in cooking, as "Generally Recognized as Safe" when consumed in small amounts. It is one of the oldest medicinal plants known because of its incredible therapeutic and medical qualities, and it has long been used as a traditional medicine in many different parts of the world (Mebazaa *et al.*, 2009; Naidu *et al.*, 2011).

Flavonoids have high pharmacological action and excessive anxiety in these constituents has been stimulated by the prospective health benefits arising from the antioxidant activity of these polyphenolic compounds (Reihani and Azhar, 2012). The seed extract contains another bioactive compound, which is called diosgenin that induces apoptosis in HT-29 human colon cancer cells (Raju *et al.*, 2004); besides it plays a role in osteoclastogenesis, invasion, and proliferation inhibition through the downregulation of Akt, I kappa B kinase activation, and NF kappa B-regulated gene expression in tumor cells (Shishodia and Aggarwal, 2006). Furthermore, some constituent of alkaloids, "trigonelline," has a potential role in cancer treatment (Bhalke *et al.*, 2009). Another active agent identified in Fenugreek is Protodioscin, which induces apoptosis in the leukemic cell line HL-60 (Hibasami *et al.*, 2003).

The prototypic molecular change associated with cancer is mutation of tumor suppressor p53, which is inactivated in 50% of human cancers (Yu, 2006). Key players in the regulation of cell cycle arrest and apoptosis, the p53 protein is governed by an intricate web of posttranslational modifications that affect its stability, activity, and interactions with other molecules (Vousden and

Lu, 2002). A p53 mutation develops late in the hepatocarcinogenesis of HCC (Ng *et al.*, 1995). Recently, many studies from our laboratory and others focused on p53 role in the pathogenesis and development, diagnosis and treatment, and therapeutic effects and prognosis of HCC (Breuhahn *et al.*, 2006; Farinati *et al.*, 2006).

The 36 kDa molecule known as proliferating cell nuclear antigen, or PCNA, is highly conserved across species and functions as a crucial regulator of the cell cycle. PCNA is an evolutionally well-conserved protein found in all eukaryotic species from yeast to humans, as well as in archaea. Its functions are related to vital cellular processes such as DNA replication, chromatin remodeling, DNA repair, sister chromatid cohesion and cell cycle control (Stoimenov and Helleday, 2009). PCNA role and interactions are modulated by posttranslational regulation, whose exact mechanisms are controversial and not completely understood. Many reports showed posttranslational regulation of PCNA modifications, including phosphorylation (Loor *et al.*, 1997), acetylation (Wang *et al.*, 2006) and methyl esterification (Hoelz *et al.*, 2006). In addition, PCNA is important to determine its role in proliferative activity in different tumors including HCC (Peng *et al.*, 2005).

The present study was aimed at evaluating the therapeutic effect of Fenugreek crude extract (FCE) against immortalized HCC cell line, SNU-423 (Seoul National University-423). In addition, we focused on the role of FCE on p53, Bax, and PCNA protein expression levels as one of the key proteins involved in apoptosis induction in SNU-423 cell line.

Materials and Methods

Seeds of *Trigonella foenum-graecum* were purchased and collected from the local market. The seeds were dried and crushed into coarse powder, which was used for extraction with alcohol (95% v/v) using Soxhlet apparatus. The extracts were evaporated to dryness under controlled temperature (35-40°C). The extracts

were stored in air tight containers under refrigeration. These dried extracts were dissolved in respective solvents and used for further analysis.

Cell Culture:

Since 1982, Korean cancer patients have provided SNU (Seoul National University) with cell lines. Park became the founder of the cell line SNU-423 (Seoul National University-423). A male Korean patient with primary hepatocellular carcinoma provided the sample. A detection of HBV DNA was made in the multinucleated cultured cells. The cell lines are generally used to examine the expression of HBV and IGF. The ERK, TGF- β , NF- κ B and Akt/mTOR pathways are active in this cell line. SNU-423 has a doubling time of 72 h and maintained in Dulbecco's modified essential media (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 100 Units/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ atmosphere.

Cell Cytotoxicity (MTT Assay) of SNU-423 Cell Line:

SNU-423 cells were cultured as described above till mid-log phase. Cells were harvested and resuspended in growth media to make a stock cell suspension containing 20,000 cells/ml. 100 μ l of this stock cell suspension was added to the wells of a 96-well plate. The cells were given a full day to attach and proliferate. A stock solution containing 100 mg/ml was prepared by weighing the crude extract and diluting it with DMSO. To create a secondary working solution, this stock solution was further diluted using culture media. The working solution was added to the wells such that final concentrations of range 0~2000 μ g/ml of *Trigonella foenum-graecum* were obtained. Each experiment was performed in triplicate in parallel for each concentration. Control was performed in which only culture media and DMSO were added. The cells were then incubated at 37°C in a 5% CO₂, 95% air atmosphere. After 72 h of incubation, the culture medium was removed and the cells were washed twice with phosphate buffered saline (PBS). Then 20 μ l of 5 mg/ml MTT [3-(4,5-

dimethylthiazol-2-yl) - 2, 5- diphenyltetrazolium bromide] was added to each well. For four hours, the cells were incubated at 37°C. After discarding the supernatant, 100 µl of DMSO was added to each well. The mixture was shaken for five minutes on a microvibrator, and the absorbance at 570 nm (A), which is a measure of cell viability, was recorded. Inhibition ratio (I%) was calculated using the following equation:

$$\text{Formula I\%} = (\text{A control} - \text{A treated}) / \text{A control} \times 100.$$

Morphological Analysis:

Morphological observation of SNU-423 cells treated with *Trigonella foenum-graecum* was done to determine the changes induced by treatment. All the cells were exposed to increasing concentrations (100~500 µg/ml range) of *T. foenum-graecum* for 48 h and cell images were taken using an inverted phase contrast microscope at 200x magnification.

Flow Cytometric Analysis:

Cells were seeded at a density of $3-5 \times 10^5/10\text{-cm}^2$ plate and incubated for 24 h before treatment. Media were changed to media containing 100~500 µg/ml range of *Trigonella foenum-graecum* for 48 h. Cells were harvested by trypsinization, washed with PBS, and fixed with ice-cold 70% ethanol while vortexing. Finally, the cells were washed and resuspended in PBS containing 5 µg/ml RNase-A and 50 µg/ml of propidium iodide for cell cycle analysis. Cell cycle analysis was performed, using FAC Scan Flow Cytometer according to the manufacturer's protocol.

Western Blotting Analysis:

Western blotting analysis was performed on plated cells in 6-well plates. Cells were lysed with a 1% NP-40 containing buffer supplemented with a 1x cocktail of protease and phosphatase inhibitors (phosphatase inhibitor cocktail I and II, Sigma) at 4°C for 30 min. Lysates were centrifuged at 10,000 g at 4°C for 15 min and supernatants collected. The protein concentration of the supernatant was determined using the BCA assay

(Pierce, Rockford, IL). Samples were mixed in a ratio of 1:2 in Laemmli buffer and denatured by heating at 98°C for 5 min. Forty µg of protein was separated on 10% Tris-SDS-PAGE gels at 100 V for 1 h. For western blotting, the separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes at 380 mA for 1 h. Western blot analysis was carried out using specific primary antibodies for p53 and Bax and PCNA and anti-poly(ADP-ribose) polymerase antibodies. The expression of β-actin was used as a normalization control for protein loading. The membranes were blocked with TBS plus 5% nonfat milk (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) followed by incubation overnight with primary antibodies diluted in blocking solution for antibodies (1~1000). After that, the proper horseradish peroxidase-conjugated secondary antibodies were added and incubated for an additional hour. For detection, an ECL kit was used according to the manufacturer's instructions. The corresponding relative density of p53 and PCNA bands was calculated by Quantity One software.

Results

Effects of Trigonella foenum-graecum on the Inhibition of SNU-423 Cell Proliferation:

Exponentially growing SNU-423 cell line was cultured continuously in the absence or presence of different concentrations of *Trigonella foenum-graecum*. The effects of tested *T. foenum-graecum* on cell growth were assessed by the MTT assay after 48 h of incubation with *T. foenum-graecum*. The concentration of 50% inhibition of SNU-423 cell viability was calculated as IC₅₀, using a Semilogarithmic plotting of the % of cell viability versus concentration of the tested extract. The (IC₅₀ = 1000 µg/ml) dose of *T. foenum-graecum* showed a significant decrease in cell survival of SNU-423 cells compared to control group at 48 h (Fig.1). As shown in Figure 1, treatment by concentrations range of 0~2000 µg/ml for 48 h of *T. foenum-graecum* showed a significant dose-dependent cytotoxic effects on SNU-423 cell line with complete elimination of all cells at a dose

over 1000 $\mu\text{g}/\text{ml}$ (Fig. 1). Our results showed that *T. foenum-graecum* significantly decreases SNU-

423 cell viability in dose-dependent manner.

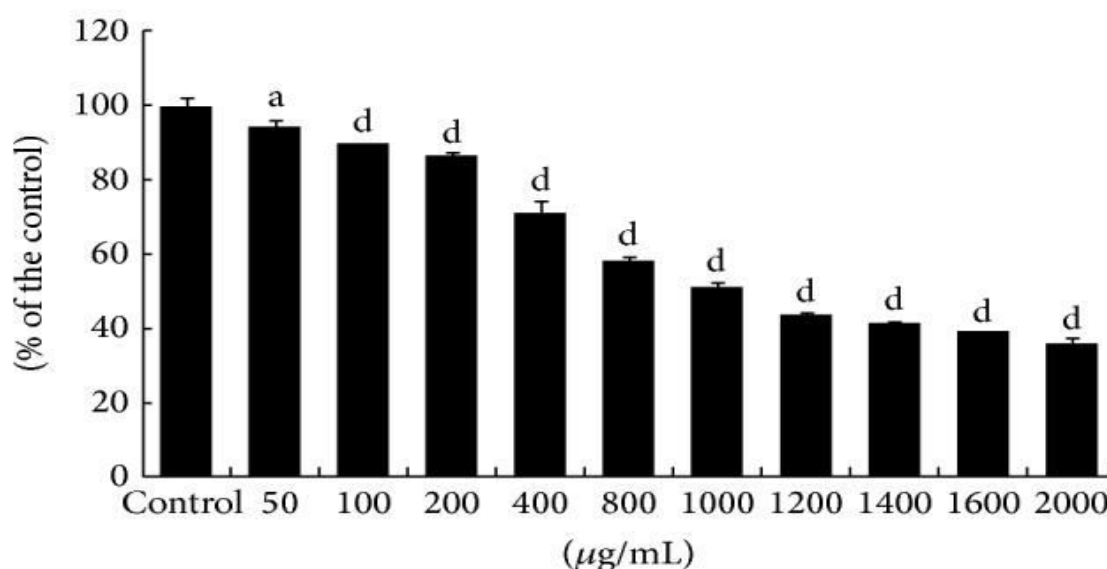


Fig. 1: Effect of *Trigonella foenum-graecum* on cell viability of SNU-423 cells. MTT assay was performed to detect the living cells.

Significant decrease of cell viability was observed at 100 $\mu\text{g}/\text{ml}$ and above concentrations of *T. foenum-graecum* compared to control untreated group ($P < 0.01$, unpaired t-test).

Morphological Changes:

The morphological changes observed in SNU-423 treated with or without *T. foenum-graecum* treatment for 48 h are shown in Figure 2. Morphological alterations of SNU-423 cell line after (100~500 $\mu\text{g}/\text{ml}$) *T. foenum-graecum* treatment for 48 h were observed under phase contrast inverted microscope. The cells indicated the most prominent effects after *T. foenum-graecum* treatment starting at 24 h~48 h. Changes in morphology were found in concentration-dependent manner. Cells exposed to concentration range of 100~500 $\mu\text{g}/\text{ml}$ of *T. foenum-graecum* for 48 h altered the normal morphology of the cells and cell adhesion capacity in comparison to control (Fig. 2). The effect of *T. foenum-graecum* on treated cells started at 100 $\mu\text{g}/\text{ml}$ in which the cells lost their typical morphology and appeared smaller in size, shrunken and rounded.

Furthermore, treatment of the cells with the same concentrations for 72 h killed most of the cells (data not shown).

Western Blot Analysis:

To analyze the intracellular mechanism for the observed increase in apoptosis in *T. foenum-graecum* treated SNU-423 cells, the protein expression levels of the p53, Bax as a well-known tumor suppressor protein and proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle, were examined. The p53 tumor suppressor protein is a key regulator of cell cycle arrest and of apoptosis. The p53 is typically activated and accumulates in response to DNA damage, using its DNA-binding ability to regulate various genes involved in cell cycle regulation and the induction of apoptosis. To understand the mechanism behind the decreased cell viability after *T. foenum-graecum* treatment, we examined the p53 protein expression for different time intervals. Western blot data showed that p53 protein expression was upregulated after *T. foenum-graecum* treatment (500 $\mu\text{g}/\text{ml}$) for 48 h compared to untreated control cells (Fig. 3a) and

this expression was sustained till 72 h of treatment, suggesting that *T. foenum-graecum* treatment triggered p53 protein expression and

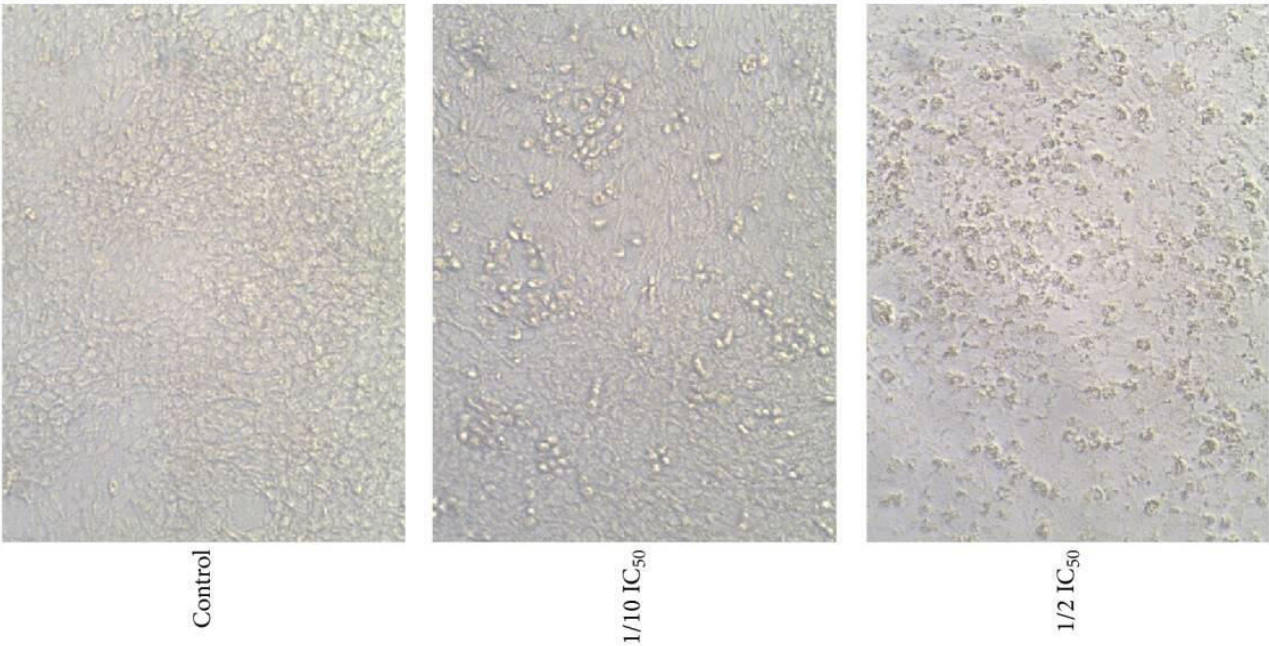


Fig. 2: *Trigonella foenum-graecum* induced changes in SNU-423 cell line morphology. The results revealed that the control cells showed a typical polygonal and intact appearance, whereas the 100~500 µg/ml range of *Trigonella foenum-graecum* treated cells displayed morphological changes with preapoptotic characteristics.

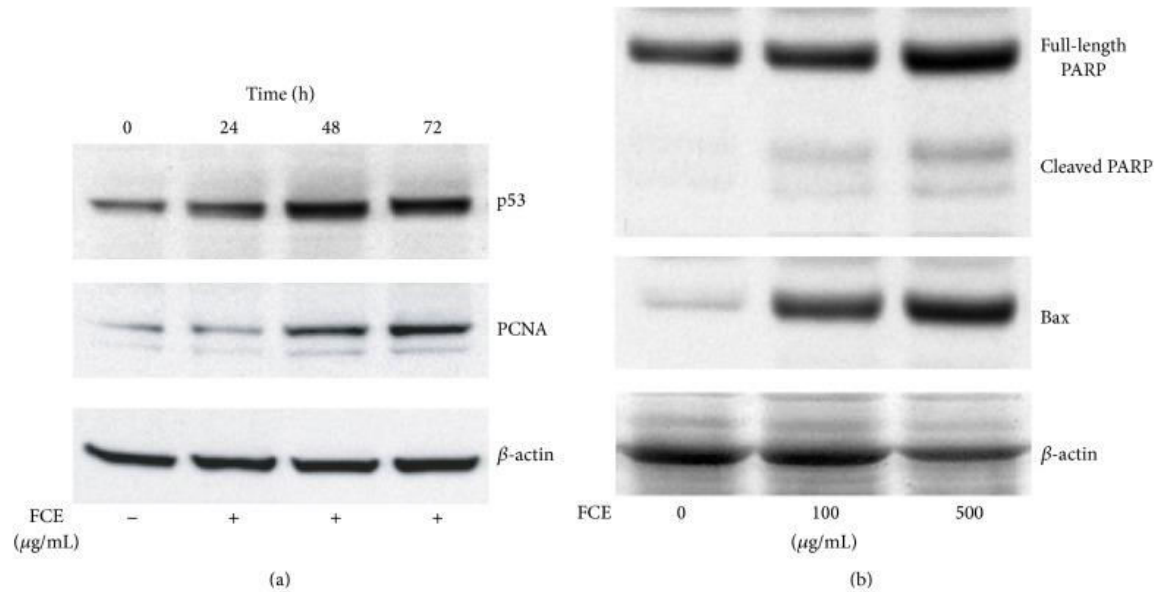


Fig. 3(a): *T. foenum-graecum* upregulated p53 and PCNA expression levels in SNU-423 cells line, using western blot analysis. Cells were treated with or without (500 µg/ml) FCE for different time intervals. Our data showed a significant increase in p53 and PCNA protein expression, respectively. Blots were normalized for total protein loading. Representative data from three independent experiments are shown. (3b): Apoptosis indicated by poly(ADP-ribose) polymerase (PARP) cleavage. Western blot analysis for poly(ADP-ribose) polymerase (upper

panel) and Bax protein expression (lower panel). Cells were treated with or without 100–500 µg/m; range of *T. foenum-graecum*, for 48 h. Blots were normalized for total protein loading. induced apoptosis in SNU-423 cells. Our results give clear evidence that the proliferation activity and apoptosis induction are altered by *T. foenum-graecum* treatment in SNU-423 cell line. Furthermore, western blot analysis showed that the occurrence of apoptosis was confirmed by cleavage of poly(ADP-ribose) polymerase most notably at 48 h after *T. foenum-graecum* treatment (100, 500 µg/ml), which is consistent with induced significant growth inhibition (Fig. 3b; upper panel). Apoptosis indicated by poly(ADP-ribose) polymerase (PARP) cleavage, Poly(ADP-ribose) polymerase (upper panel) and Bax protein expression (lower panel) were detected by Western blot analysis. Cells were treated with or without 100–500 µg/ml range of *T. foenum-graecum* for 48 h. Blots were normalized for total protein loading.

Discussion

Despite the combined efforts of governments and scientists worldwide, there is constant increase in the incidence of hepatocellular carcinoma during the last two decades (El-Serag, 2004). Successful HCC treatment requires an adequate therapeutic index reflecting the treatment's specific effects on target cells and its lack of clinically significant effects on the host. Fenugreek is commonly used as a spice in food preparations due to the strong flavor and aroma and is used in traditional medicines as leads for therapeutic drug development in modern medicine. Pharmacological features of Fenugreek seed are known such as hypoglycaemic, hypercholesterolaemic, gastroprotective, and hepatoprotective activity (Thirunavukkarasu *et al.*, 2003; Kaviarasan and Anuradha, 2007).

In the present study, the effects of tested *T. foenum-graecum* on cell growth were assessed by the MTT assay after 48 h of incubation with *T. foenum-graecum*. The concentration of 50% inhibition of SNU-423 cell viability was calculated as IC₅₀, using a Semilogarithmic plotting of the % of cell viability versus concentration of the tested

extract. The (IC₅₀ = 1000 µg/ml) dose of *T. foenum-graecum* showed a significant decrease in cell survival of SNU-423 cells compared to control group at 48 h. Our results agreed with the previous findings where they found difference in the sensitivity of the cell lines (Vijayan *et al.*, 2004). The reduction in percentage of cell viability after 48 h of treatment showed that *T. foenum-graecum* significantly decreases SNU-423 cell viability in dose-dependent manner. Significant decrease of cell viability was observed at 100 µg/ml and above concentrations of *T. foenum-graecum* compared to control untreated group. Our results are also in agreement with the previous findings in which the extract was found more cytotoxic to cancerous cells than normal cells (Vijayarathna and Sasidharan, 2012) due to the sensitivity of cancerous cells towards the flavanoids (Das *et al.*, 2010). The morphological changes in the SNU-423 cells were observed more prominent in treated cells showing extensive blebbing and vacuolation, suggesting autophagic mechanism of cell death (McGill and Fisher, 1997).

Alterations of p53 protein have been observed during hepatocarcinogenesis (Hussein, 2004) and the overexpression of p53 correlates with a high level of proliferation of cell nuclear antigen (PCNA), HCC dedifferentiation, and advanced HCC stages (Hu *et al.*, 2007). The present study examined the intracellular mechanism for the observed increase in apoptosis in *T. foenum-graecum* treated SNU-423 cells, the protein expression levels of the p53, Bax as a well-known tumor suppressor protein and proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle. The p53 tumor suppressor protein is a key regulator of cell cycle arrest and of apoptosis. During DNA damage, the p53 is normally activated and accumulated to exert its DNA-binding activity for the regulation of different genes that are involved in cell cycle regulation and apoptosis induction. To understand the mechanism behind the decreased cell viability after *T. foenum-graecum* treatment, we examined

the p53 protein expression for different time intervals. Western blot data showed that p53 protein expression was upregulated after *T. foenum-graecum* treatment (500 µg/ml) for 48 h compared to untreated control cells (Fig. 3a) and this expression was sustained till 72 h of treatment, suggesting that *T. foenum-graecum* treatment triggered p53 protein expression and induced apoptosis in SNU-423 cells. The distinct chemopreventive mechanism is p53-dependent and involves the binding of Gadd45a and two BER-mediated repair proteins, PCNA and apurinic/apyrimidinic endonuclease (APE1/Ref-1) (Jung *et al.*, 2013). Furthermore, it was reported that PCNA gene is induced by p53, while PCNA protein interacts with p53-controlled proteins Gadd45, MyD118, CR6, and p21, in the process of deciding cell fate (Azam *et al.*, 2001). The binding partner of Gadd45a can decide the activity that mediates, in other words, the binding of PCNA and p21WAF1/CIP1 or Cdc2 to Gadd45a can mediate DNA repair or cell cycle arrest, respectively. In addition, the possibility that p53 may be involved in the induction of human PCNA was reported earlier; the human PCNA promoter has a p53 binding site and is transactivated by wild type p53 which in turn leads to DNA repair (Chang *et al.*, 1999). In contrast, if PCNA is slightly upregulated in cell, apoptosis also occurs (Shivakumar *et al.*, 1995). The dual role of PCNA in HepG2 cells could be explained by the presence of acidic and basic PCNA isoforms, according to previous reports (Paunesku *et al.*, 2001; Naryzhny and Lee, 2003).

Our results give clear evidence that the proliferation activity and apoptosis induction are altered by *T. foenum-graecum* treatment in SNU-423 cell line. Furthermore, western blot analysis showed that the occurrence of apoptosis was confirmed by cleavage of poly(ADP-ribose) polymerase most notably at 48 h after *T. foenum-graecum* treatment (100, 500 µg/ml), which is consistent with induced significant growth inhibition (Fig. 3b; upper panel). Apoptosis indicated by poly(ADP-ribose) polymerase (PARP) cleavage. Cells were treated with or without 100–500 µg/ml range of *T. foenum-graecum* for 48 h.

Blots were normalized for total protein loading. As PCNA is known to be a key regulator of cell cycle progression, we investigated the protein expression levels of PCNA with or without FCE treatment (500 µg/ml) for different time intervals. The PCNA serves as a cofactor for DNA polymerase delta in S-phase and is involved in DNA repair during DNA synthesis (Taylor *et al.*, 1997). The temporal pattern of PCNA protein expression was upregulated and that is consistent with p53 expression pattern after FCE treatment (500 µg/ml) for 48 and 72 h compared to untreated control cells. PCNA was found valuable in studying the proliferative activity in different tumors including HCC (Loor *et al.*, 1997; Goswami, 2012). Our results give clear evidence that the proliferation activity and apoptosis induction are altered by *T. foenum-graecum* treatment in SNU-423 cell line. Furthermore, western blot analysis showed that the occurrence of apoptosis was confirmed by cleavage of poly(ADP-ribose) polymerase most notably at 48 h after *T. foenum-graecum* treatment (100, 500 µg/ml), which is consistent with induced significant growth inhibition (Fig. 3b; upper panel).

Conclusion

The mechanism of *T. foenum-graecum* induced cell death was via inhibiting SNU-423 cell viability and inducing apoptosis. *T. foenum-graecum* enhances apoptosis induction in SNU-423 cell in p53, Bax and PCNA-dependent pathway and phase arrest that was confirmed by cell cycle analysis. Despite the finding of p53 involvement in *T. foenum-graecum* cell viability inhibition and apoptosis induction, the exact downstream target of p53 is unclear, which requires further investigation. However, we suggest that induction of p53, Bax, and PCNA-dependent pathway which mediated apoptosis with caspase-3 activation is, at least in part, a possible explanation for the anti-liver-carcinogenic effect of *T. foenum-graecum* which in turn might be a potential strategy for Hepatocellular carcinoma treatment.

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