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FENUGREEK (*TRIGONELLA FOENUM-GRAECUM L.*) EXTRACTS ARE INDUCING DOSE-DEPENDENT HORMETIC RESPONSE AND CYTOTOXIC EFFECTS IN CASE OF HUMAN BREAST CANCER CELL LINES

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ABSTRACT: The effects of aqueous and ethanolic extracts of fenugreek (*Trigonella foenum-graecum* L.) seeds have been studied on the T-47D and ZR-75-1 human breast cancer cell lines. The aqueous fenugreek extract was cytotoxic at higher concentrations, while at lower concentrations increased cell viability, suggesting the induction of a dose dependent hormetic response in case of both cell lines. The alcoholic fenugreek extract was markedly cytotoxic to both breast cancer cell types, and multiple cell division defects together with apoptosis were apparent in the affected cells. The viability of breast cancerous cells was examined time dependently with the MTT proliferation assay. Both types of the extracts showed significant differences in all treatments ($P<0.05$).

Keywords: fenugreek, *Trigonella foenum-graecum*, breast cancer, hormesis, cytotoxicity

INTRODUCTION:

Breast cancer is one of the most frequently diagnosed cancer all over the world, and therefore, finding novel remedies that would prevent such a disease or increase the efficacy of currently used chemo- and radiotherapies are of great importance (American Cancer Society, 2016). In this respect, there is an ongoing quest across the world for identification of natural bioactive compounds or even plant extracts with anti-cancer properties (Dietz *et al*., 2016).

Fenugreek (*Trigonella foenum-graecum*) seems to be a good source of bioactive compounds with not only anti-cancer but several other pharmacological effects (Yadav *et al*., 2014; Moradi and Moradi, 2013; Mehrafarin *et al*., 2010). The analgesic effect of fenugreek leaf extracts has been demonstrated on a rats using the spinal 5-HT system or purinoceptors (Parvizpur *et al*., 2006; Parvizpur *et al*., 2004). The hepatoprotective activity of fenugreek seed aqueous extract and seed specific polyphenol extract have been confirmed on rat models (Kumar and Bhandari, 2013; Kaviarasan *et al*., 2008). The hypolipidemic effects of fenugreek were studied on animal models, and has been found to lower triglycerides, total cholesterol and low density lipoprotein levels (Al-Habori and Raman, 1998; Al-Habori *et al*., 1998; Petit *et al*., 1995; Petit *et al*., 1993; Stark and Madar, 1993; Valette *et al*., 1984). The hypolipidemic effects may be due to saponins, a class of molecule present in fenugreek that is transformed in the gastrointestinal tract to sapogenins. Sapogenins increase biliary cholesterol secretion, lowering serum cholesterol levels (Sauvaire *et al*., 1991; Yoshikawa *et al*., 1997; Varshney and Sharma, 1996).

Hypoglycemic effects have been attributed among others to the amino acid 4-hydroxyisoleucine (4-HIL) from fenugreek seeds that increases glucose-induced insulin release *in vitro* in human and rat pancreatic islet cells (Broca *et al*., 2004; Sauvaire *et al*., 1998). In addition to stimulating insulin secretion, 4-HIL reduced insulin resistance in muscle and/or liver by activating insulin receptor substrate-associated phosphoinositide 3 (PI3) kinase activity (for review see Jetté *et al*., 2008). Furthermore 4-HIL could inhibit palmitate-induced, ROS-associated inflammation and restored insulin sensitivity by regulating insulin receptor substrate-1 function (Maurya *et al*., 2014). Another *in vitro* study indicated that fenugreek seed extract could phosphorylate a number of proteins, including the insulin receptor, insulin receptor substrate 1 and p85 subunit of PI3-K, in both 3T3-L1 adipocytes and human hepatoma HepG2 cells, leading to the activation of insulin-signaling pathway in adipocytes and liver cells (Vijayakumar *et al*., 2005). It was also shown in insulin resistant HepG2 cells that 4-HIL did improve insulin resistance by different

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mechanisms: (i) 4-HIL reduced TNF-α levels by affecting the protein expression of the TACE/TIMP3 (TNF-α converting enzyme/tissue inhibitor of metalloproteinase 3) system, and (ii) 4-HIL stimulated the expression of insulin receptor substrate-1 (IRS-1) and glucose transporter type 4 (GLUT4), (Gao *et al*., 2015). In a comparative study using HepG2 cells was demonstrated that an aqueous fenugreek seed extract, 4-HIL and metformin exert similar anti-hyperglycemic effects by enhanced insulin signaling, gene expression, and increasing glucose uptake (Naicker *et al*., 2016). The anti-inflammatory effects of 4-HIL in TNF-α-induced insulin resistance in C2C12 myotubes was also studied, and showed that 4-HIL reverses the insulin resistance by the activation of AMP-activated protein kinase (AMPK) and inhibition of suppressor of cytokine signaling-3(SOCS-3), (Gautam *et al*., 2016). Besides 4-HL, the diosgenin, a saponin from fenugreek was found to induce changes in lipid profile of plasma, liver, heart and brain in high-fat diet-streptozotocin (HFD-STZ)-induced diabetic rats (Naidu *et al.*, 2015). Raju and Bird (2006) demonstrated that oral administration fenugreek seeds could alleviate the hepatic steatosis by modulation of plasma and liver TNF-alpha levels in Zucker obese (fa/fa) rats. Moreover, fenugreek reduced insulin resistance by increasing the number of insulin receptors in humans (Raghuram *et al*., 1994), adiponectin levels, and PPARγ protein expression in rats (Mohammadi *et al*., 2016). Another experiment with hydroalcoholic fenugreek seed extract increased body weight and glucose uptake, reduced plasma glucose, glycosylated hemoglobin, liver glucose transport, pro-inflammatory cytokines, pancreatic enzymes, and restored depleted glycogen in muscle and liver in dose dependent manner, including prevention of lipid peroxidation and restoration of liver and pancreas GSH and SOD activities (Joshi *et al*., 2015).

The antioxidant properties of fenugreek seeds were suggested to contribute to its health promoting effects (for review see Srinivasan, 2014). In an ethanol toxicity rat study, an aqueous extract of fenugreek seeds prevented the rise in lipid peroxidation and enhanced antioxidant potential (Thirunavukkarasu *et al*., 2003). In a CCl4 toxicity rat study, the administration of fenugreek seeds and seed extract were effective in protecting the liver and kidneys through their antioxidant properties by alteration of the antioxidant enzyme activities (Mbarki *et al*., 2017). These results are supported by *in vitro* evidence in diabetic human erythrocytes, so that polyphenol acids from fenugreek seeds showed a concentration-dependent inhibition of lipid peroxidation (Kaviarasan *et al*., 2004). Moreover, rat based experimental results are suggesting that fenugreek co-administration has a powerful antioxidant effect, and might serve as a novel and promising preventive strategy against cisplatin-induced nephron- and hepatotoxicities in some cancer therapy (Hegazy *et al*., 2015). Another study concluded that fenugreek seed extract reduced cisplatin induced reproductive toxicity in rats by the suppression of testicular oxidative stress, apoptosis and inflammations (Hamza *et al*., 2016). Interestingly, a 15-day fenugreek seed treatment applied to diabetic rats besides the hypoglycemic effect, had improved the altered levels of H_2O_2, MDA, 4HNE, and the activities of SOD, GPx, and CAT enzymes by increasing the expression of these genes in liver and brain (Sharma *et al*., 2015). In a comparative study, the antioxidant potential of fenugreek leaf and seed extracts were evaluated, including total phenolics, free radical scavenging assay, superoxide anion radical scavenging activity, reducing power, lipid peroxidation, ferric thiocyanate assay, hydroxyl radical scavenging activity and DNA damage protective activities (Singh *et al*., 2014). Results showed that leaf extract had the lowest free radical and superoxide anion radical and hydroxyl radical scavenging activities with the highest reducing power. The leaf extract also showed the maximum DNA damage protection activity and higher concentration of phytochemicals. However, the seed extract showed the maximum inhibition of lipid peroxidation. Inclusion of fenugreek seeds in the diet of colon carcinogenesis induced rats significantly decreased lipid peroxidation with simultaneous enhancement of circulating antioxidants like ascorbic acid, vitamin E, reduced glutathione, glutathione peroxidase, glutathione S-transferase, superoxide dismutase and catalase (Devasena and Menon, 2002).

The above mentioned experiments are strongly pinpointing to the anti-cancer effects of fenugreek (for review see Jesus *et al*., 2016; Sung *et al*., 2012; Aggarwal *et al*., 2008). In case of colon cancer, the fenugreek seeds anti-cancer effects were attributed to its fiber, flavonoids or saponins that modulated beta-glucuronidase and mucinase activities (Devasena and Menon, 2003). Much of attention was given to diosgenin from fenugreek seed since it could suppress the colonic aberrant crypt foci formation, and induced apoptosis in human colon cancer cells (Raju *et al*., 2004). The pure and fenugreek isolated diosgenin prevented telomerase activity by down regulating the human *telomerase reverse transcriptase gene (hTERT)* gene expression which is critical for telomerase activity and the survival of A549 lung cancer cells (Rahmati-Yamchi *et al*., 2014). In case of some human breast cancer cell lines, was shown that diosgenin inhibited pAkt expression and Akt kinase activity without affecting PI3 kinase levels, resulting in the inhibition of its downstream targets, NF-kappaB, Bcl-2, survivin and XIAP (Srinivasan *et al*., 2009). The Raf/MEK/ERK pathway, another functional downstream target of Akt, was also inhibited by diosgenin in estrogen receptor featuring (ER+) MCF-7 cells by causing G1 cell cycle arrest, and down regulating cyclin D1, cdk-2 and cdk-4 expression leading to the inhibition of cell proliferation and induction of apoptosis (Srinivasan *et al*., 2009). Moreover, a chloroform-based fenugreek seed extract could effectively reduce the viability of MCF-7 breast cancer cells through induction of apoptosis associated with increased expression of Caspase 3, 8, 9, p53, Fas, FADD, Bax and Bak in a time-and dose-dependent manner (Khoja *et al*., 2011). Cells were exposed to 50

µg/mL chloroform-based fenugreek seed extract for 24 hours, and such a treatment induced apoptosis in 23.2% of cells, while a 48-hour exposure caused 73.8% of cells to enter apoptosis. In another study, a methanol based fenugreek seed extract was assessed on MCF-7 breast cancer cells, and was found to that at least 90% of methanol extract induced apoptosis in breast cell is mediated by Fas receptor-independently of either FADD, Caspase 8 or 3, as well as p53 interdependently (Alshatwi *et al*., 2013). Furthermore, in case of protodioscin, another bioactive compound found in fenugreek seeds, was shown to strongly inhibit the growth of HL-60 leukemic cells, but had little effect on gastric cancer cell line KATO III *in vitro* (Hibasami *et al*., 2003). Apoptosis in the HL-60 cells might have been related to the concentration- and time-dependent fragmentation of DNA by protodiosgenin. In case of an aqueous fenugreek extract has been found a very selective cytotoxicity against cancer cell lines such as T-cell lymphoma (TCP), B-cell lymphomas, Thyroid Papillary carcinoma (FRO) and breast cancer (MCF7), while no significant cell cytotoxicity was detected amongst normal cells, including human lymphocytes and meningioma (Alsemari *et al*., 2014). All the above mentioned data are clearly indicating that fenugreek presents selective cytotoxicity in case of different type of cancer cells, and such effects could be triggered by different bioactive compounds through several cellular mechanisms whose complexity needs further analysis. In order to shed light on the fenugreek bioactive compound content and the generated anti-cancer effects, we carried out the UHPLC-ESI-MS characterization of two (aqueous and alcoholic) fenugreek seed extracts (Vígh *et al*., accepted for publication). In the current paper we are reporting the aqueous and alcoholic extracts effect on human T-47D and ZR-75-1 breast cancer cell lines by correlating the generated effects with the bioactive compound content of the analyzed extracts.

MATERIALS AND METHODS:

Preparation of plant extracts

The fenugreek seeds were obtained from TRIGONELLA MED. LTD., Mosonmagyaróvár (Hungary), and two types of extracts were prepared as follows. For the aqueous extract 5g fenugreek dried seeds were prepared for 5 min in 100 ml boiling water. After cooling at room temperature, the extract was centrifuged (10 min, 4000 rpm) and filtered firstly through a Whatman filter paper (Sigma Aldrich), and stored in refrigerator until administration. The obtained samples were stored at 4 °C. For the hydroalcoholic extract, 5 g dried fenugreeks seeds were extracted two times with 500 ml ethanol –water (1:1) by stirring for 4hr at 40°C. The obtained fenugreek solutions were centrifuged at 4000 rpm for 10 min at room temperature, and the ethanol was removed from the samples with a rotation vacuum evaporator. The obtained samples were stored at 4 °C. The aqueous and alcoholic extract samples before being diluted in the cell culturing media were filtered through a 0,45 µm filter.

Cell culture

Human breast cancer cell lines T-47D and ZR-75-1 were obtained from ATCC (American Type Culture Collection, USA). These ductal carcinoma cell lines were grown in RPMI-1640 (Lonza) culture medium supplemented with 1% Antibiotic Antimycotic Solution (Invitrogen-Gibco), 10% heat-inactivated FBS (Fetal Bovine Serum; Sigma) and 0,1% 1 mM Na-pyruvate (Biochromag). All cell culture experiments were carried out at 37°C in 5% CO_2 incubator.

MTT (cytotoxicity) assay

Cells were used in this studies when 80% confluence was reached in T75 flasks (Greiner Bio-One GmbH). Cells were washed twice with sterile PBS, and harvested with Trypsin in the incubator. For counting the cells we used Trypan blue dye (Sigma) exclusion method and counted with Bürker chamber. After it the cells were seeded into 96-well plates at a density of 10^4 cells/well and left to attach to the plates. After 24 hours cells were incubated for two and three days with various concentrations (10-5-2.5-1.25-0.62-0.31-0.16-0.08-0.04 v/v%) of extracts. After the exposure time removed the extracts and the cells were incubated with ten microliter of MTT (5 mg/ml) (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) at 37°C for three hours. After dissolving the formazan crystals in MTT solution, plates were read in a microplate reader (BioTek EL808) at 570 nm. This experiment was performed in sextuplicates and repeated three times.

Immunostaining and microscopy

The treatments with both fenugreek extracts were carried out in six-well plates and at the examined concentration range. At the bottom of each well, a coverslip was laid and cells were let to grow on the surface of coverslip. After 2- and 3-day treatments the cells were fixed with formaldehyde and stained as described by Mathe *et al*. (2004) and Lemos *et al*. (2000). The microtubules were detected with YL1/2 rat monoclonal anti-α-tubulin antibody (Sera Lab, Inc.), and POLO like kinase-1 (Plk1) was stained with mouse monoclonal Anti-hPlk1 antibody (P5998 Sigma-Aldrich), while the DNA was counterstained with the DAPI dye (Molecular Probes). Digital images of optical sections were collected with an Olympus Cell R inverted fluorescent microscope system equipped with a Hamamatsu CCD camera controlled by the Olympus XcellenceR imaging software, using a Plapon 60x objective.

Statistical analysis

Statistical analysis were performed by the SPSS 16.0 software. Statistical differences among treated and untreated cells were determined by one-way ANOVA (Analysis of Variance). To compare several groups was applied the Tukey post-hoc test, and mean differences with $p< 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION:

We set to analyze the effects of our fenugreek extracts on human cancerous breast cells by looking at

T-47D and ZR-75-1 cell lines. The T-47D is considered a luminal A subtype of breast cancer cell line that is endocrine (estrogen receptor expression) and often chemotherapy responsive (Holliday and Speirs, 2011). The ZR-75-1 is luminal B subtype of breast cancer cell line that is usually endocrine responsive and shows some kind of variability with respect to chemotherapy. The two analyzed cell lines are not genetically identical. There have been genomic differences detected, ZR-75-1 cells are bearing the normal p53 allele, while T-47D cells are featuring a mutant allele of p53 gene (Huovinen *et al*., 2011). The ZR-75-1 cells were shown to contain the normal BRCA1/2 alleles (Gilardini Montani *et al*., 2013), while in case of T-47D cell line only indirect evidences are supporting the presence of normal BRCA1/2 alleles. It was also demonstrated that the hMAT gene expression shows a 10-fold increase in T-47D cells as compared to the normal expression levels detected for the ZR-75-1 cells (Bandyopadhyay *et al*., 1996). These experimental data are indicating that the T-47D and ZR-75-1 cell lines could be considered different subtypes of breast cancer, so different we can presume some specificity regarding cancer prognosis and treatment responses.

Aqueous fenugreek seed extract generates hormetic response in case of treated T-47D cultured cells

The aqueous fenugreek extract possible effects on the viability of T-47D breast cancer cells was tested in 2 to 3 days long treatments with the culture media containing several dilutions of the original extract, and for every dilution MTT assays were performed (for results see Fig. 1 and 2). Surprisingly, for the 2-day treatment experiment, we could observe that in case of 10 – 0.63% concentration range of aqueous fenugreek extract that the treated cancerous cell viability was approximately 6 times lower than in case of the control untreated cells. We estimate that the hypothetical LD50 value specific to this extract would fall in between the 0.31- 0.16% concentration range. Throughout the 0.16 – 0.04% extract-specific concentration interval, the viability of the treated cells was increasing progressively, so that at 0.08% and 0.04% concentrations the treated cell viability exceeded approximately 1.3 times those of the untreated control cells. Such a concentration dependent viability-toxicity profile was proposed to be specific to hormetic responses (Mitchell, 2007; Calabrese and Baldwin, 2000; Calabrese, 1999; Calabrese and Baldwin, 1999).

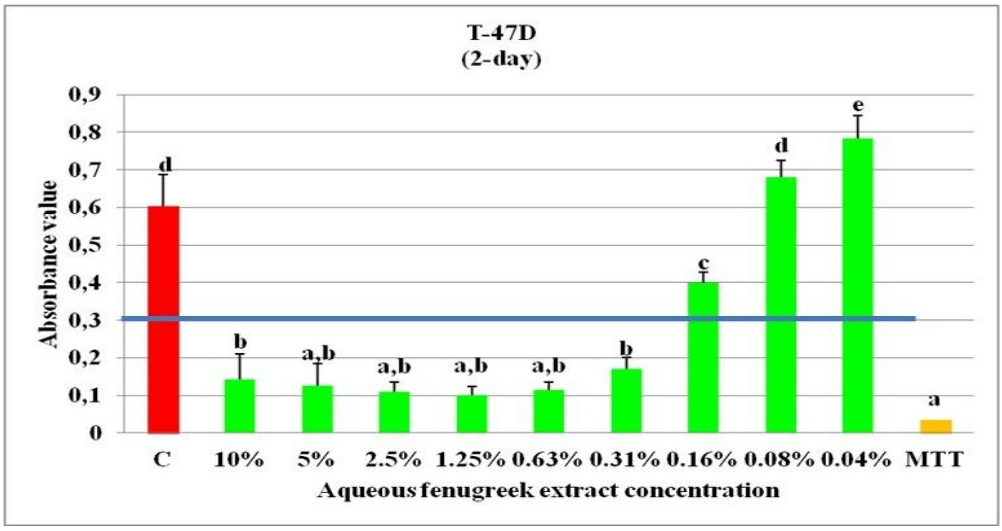

Fig. 1 Cell viability after 2-day treatment using aqueous fenugreek extract. The T-47D cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the hypothetical LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at p<0.05.

Looking at the 3-day treatments, in the 10 – 0.31% concentration range of aqueous fenugreek extract, the viability of treated cancerous cells was 8 times lower, yet constant, throughout the assessed concentration range as compared to control untreated cells (see Fig.2). The 0.16% concentration seems to be close to the hypothetical LD50 value in this experiment. Across the 0.16 - 0.04% concentration range, the viability of treated cells was steadily increasing. At 0.08% extract concentrations, the viability of treated cells equaled those specific to the control cells, while at 0.04% extract concentration the viability of cells surpassed showed a 1.2 fold increase as compared to the values of untreated control cells. It seems therefore likely that the 3-day treatment recapitulates the 2-day experiment, and once again the dose dependent hormetic effect was evident.

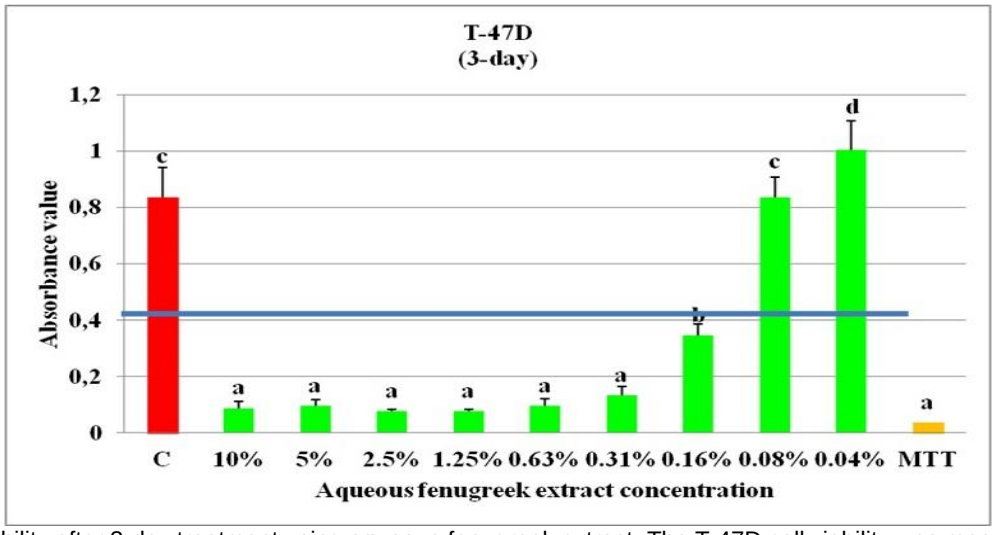

Fig. 2 Cell viability after 3-day treatment using aqueous fenugreek extract. The T-47D cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

Alcoholic fenugreek seed extract induces marked cytotoxicity on T-47D cultured cells

We have assessed the alcoholic fenugreek extract effect with respect to the viability of T-47D breast cancer cells in 2 and 3 days long experiments by treating them within culture media containing a dilutions of the original extract (for results see Fig. 3 and 4). In 2-day treatment experiments, we could observe that throughout the analyzed concentration range the cancerous cell viability was significantly reduced. Among the observed values some minimal fluctuations were detected. Therefore, the assessed concentration range was associated with a 6-12 fold decrease of viability in case of treated cells as compared to the control cells. No obvious dose dependent biphasic effect could be detected suggesting that the assessed extract shows a more pronounced cytotoxic effect without any indication of hormesis.

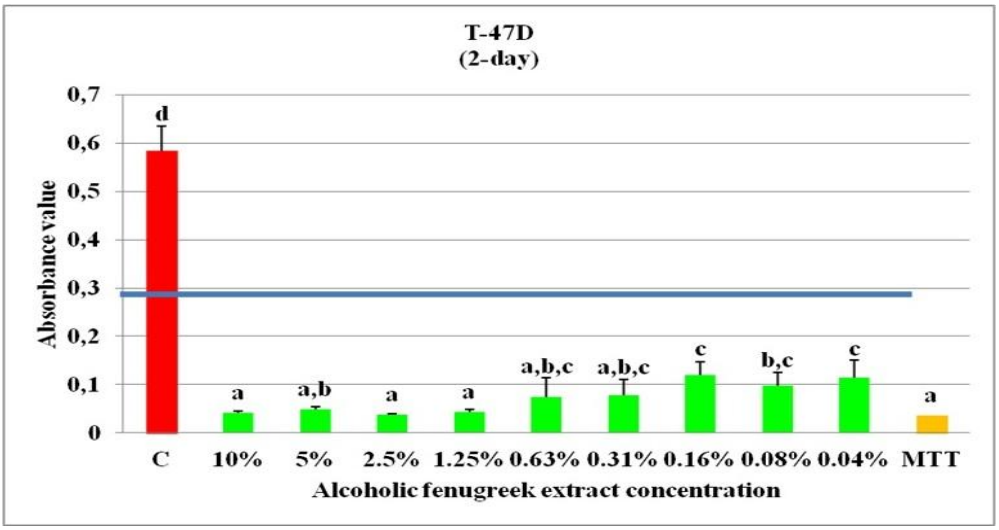

Fig. 3 Cell viability after 2-day treatment using alcoholic fenugreek extract. The T-47D cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

At the 3-day treatment experiments, we could observe that the viability was greatly reduced for treated cancerous cells at all concentration assessed (see Fig. 4.). The observed significantly low viability values showed some but minor degree of variation. Accordingly, the viability of treated cells was approximately 8-16 times lower as compared to control cells. It is also evident that the 3-day long applied alcoholic fenugreek extract concentrations are featuring a greater level of toxicity than those observed in the 2-day treatment experiments. Once again no obvious signs of hormesis could be detected suggesting that the alcoholic fenugreek extract does feature a more pronounced cytotoxic effect.

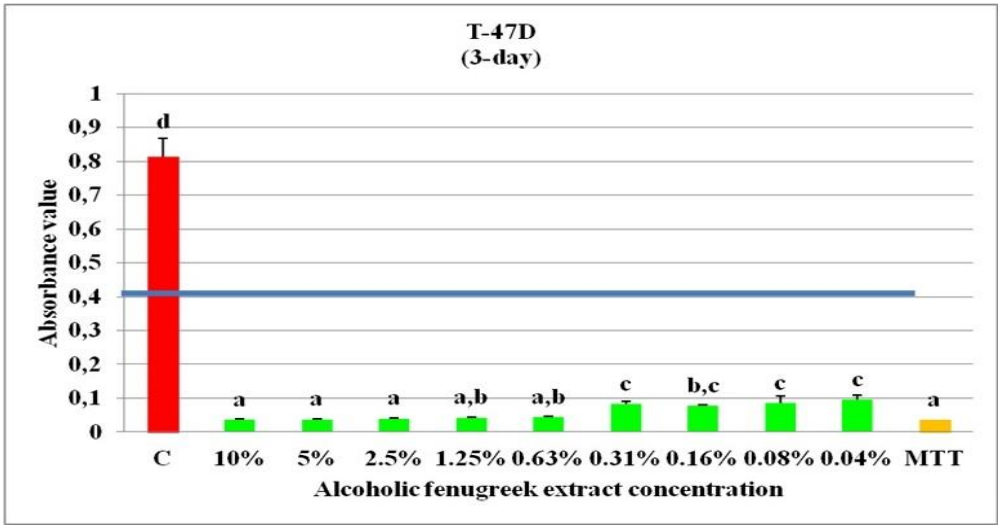

Fig. 4 Cell viability after 3-day treatment using alcoholic fenugreek extract. The T-47D cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

Aqueous fenugreek seed extract induces hormetic response in case of ZR-75-1 cultured cells

We have analyzed the aqueous fenugreek seed extract effects regarding the viability of ZR-75-1 breast cancer cells by carrying out 2 and 3 days long treatments within culture media containing different dilutions of the original extract (see Fig. 5 and 6). The 2-day treatment showed that in the 10 – 0.31% concentration range of aqueous fenugreek extract the treated cancerous cell viability was approximately 3.4 times lower than in control untreated cells. At the 0.16% concentration the viability of treated cells seems to be close to what one would expect for the LD50 value in present experiment. Throughout the 0.16 – 0.04% extract-specific concentration range, the viability of the treated cells was increasing, so that at 0.08% equaled the viability of control untreated cells, while at 0.04% concentration, the viability of treated cell exceeded 1.2 times the untreated control cells. The viability profile of ZR-75-1 cells associated with the 2-day treatments of aqueous fenugreek seed extract, once again recapitulates the hormetic dose response that has already been described for the T-47D cells.

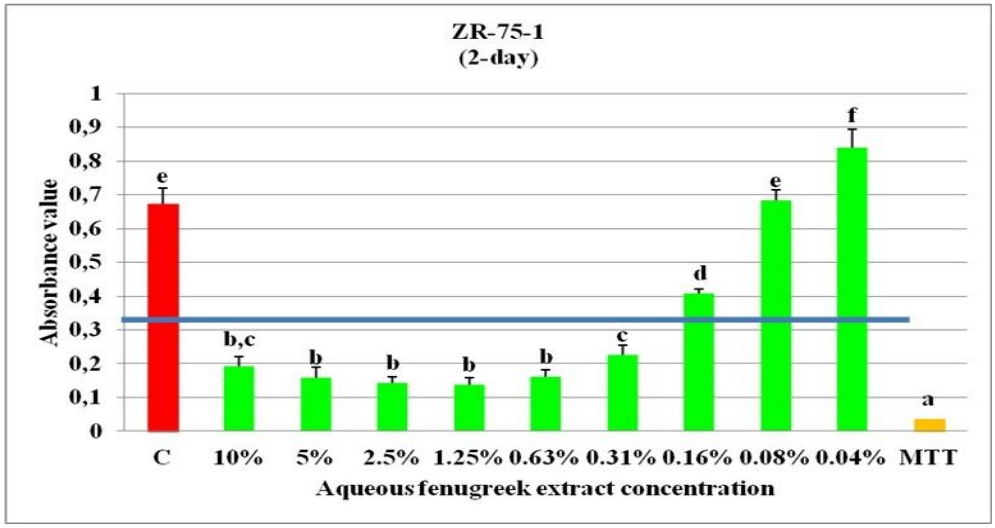

Fig. 5 Cell viability after 2-day treatment using aqueous fenugreek extract. The ZR-75-1 cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

Looking at the 3-day long treatments, at the 10 – 0.63% concentration interval, the viability of treated cancerous cells was markedly reduced, and constant, showing at about 8 times lower values as compared to control untreated cells (see Fig. 6). The LD50 value is expected to fit in between 0.31 - 0.16% concentration interval in this experiment. In the 0.31 - 0.04% concentration range, the viability of treated cells was increasing progressively. At 0.08% extract concentration, the viability of treated cells was similar to the untreated control cells. However, at 0.04% extract concentration, the viability of treated cells

surpassed 1.2x the values observed for untreated control cells. Therefore, the 3-day treatment seems to reiterate the 2-day experiment, and once again a hormetic dose response was observed.

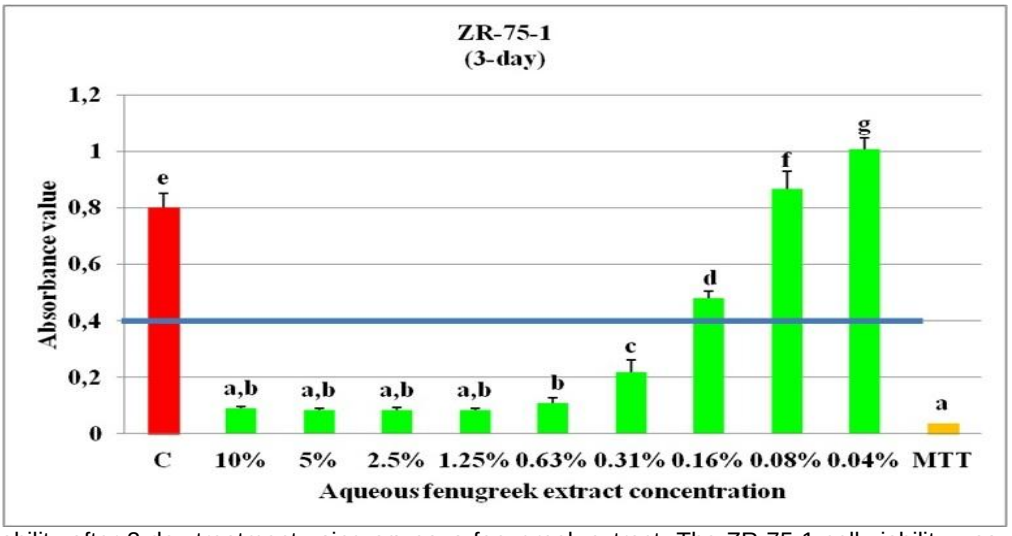

Fig. 6 Cell viability after 3-day treatment using aqueous fenugreek extract. The ZR-75-1 cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

Alcoholic fenugreek seed extract is associated with a strong cytotoxic effect in case of ZR-75-1 cultured cells

We also analyzed the alcoholic fenugreek extract ability to influence the viability of ZR-75-1 breast cancer cells in 2 to 3 days treatments, while the culture media contained different concentrations of the original extract (see Fig. 7 and 8). The 2-day treatment experiment showed that in the 10 – 0.63% concentration interval, the treated cancerous cell viability was at least 11 times lower than of untreated control cells. Next, in the 0.63-0.08% concentration interval, the viability of treated cells increased slightly but remained still reduced, being 4.8 times lower than the control. However, neither any of the seen viability values were close to what would be the hypothetical value of LD50. The observed data are strongly indicating that the alcoholic fenugreek seed extract is featuring a pronounced toxicity in 2-day experiments.

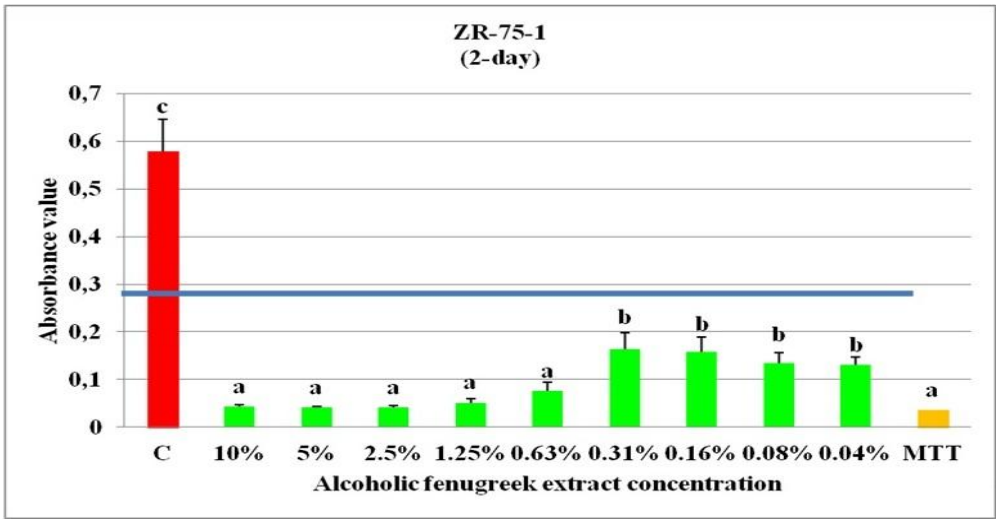

Fig. 7 Cell viability after 2-day treatment using alcoholic fenugreek extract. The ZR-75-1 cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

At the 3-day treatment experiments, we could observe throughout the analyzed concentration interval that the viability was even more pronouncedly reduced in case of treated cancerous cells. The observed viability values showed some minor variation throughout the monitored concentration interval (see Fig.8). The viability of treated cells was approximately 20 times lower as compared to control cells in the concentration interval 10-0.63%. Next, in the concentration interval 0.31-0.04%, the viability of treated cells was about 9 times lower than in case of control untreated cells. All the observed viability values were very far from the

expected LD50 value. It is obvious that the 3-day treatment with alcoholic fenugreek extract showed the most pronounced toxic effect in case of the ZR-75-1 cancerous cell line.

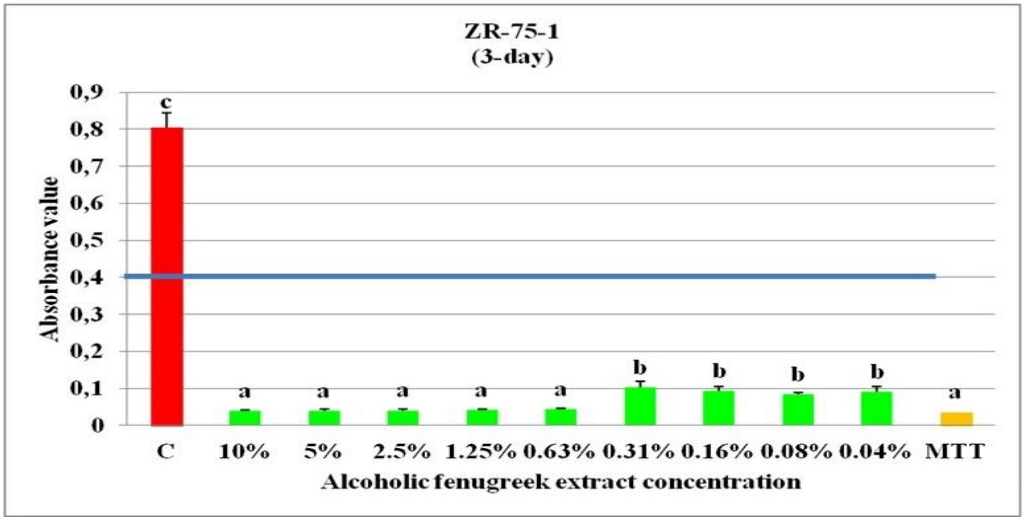

Fig. 8 Cell viability after 3-day treatment using alcoholic fenugreek extract. The ZR-75-1 cell viability was measured by MTT assay. The applied concentrations are indicated in %. **C**-stands for untreated or positive control cells. Blue line indicates the LD50. **MTT**- represents the negative control. One-Way ANOVA Tukey test was used for statistical analysis. Different letters on columns indicate statistical significance at $p<0.05$.

Immunostaining reveals multiple cell division defects for fenugreek seed extracts treated breast cancer cells

Both fenugreek seed extracts described in current paper are affecting the viability of treated human breast cancer cell lines. In order to find out how these extracts are influencing the treated cells we set to analyze by immunostaining their phenotype, hoping that the identified defects would reveal details regarding the affected cellular phenomena. Such an approach would correspond to a phenotypic study, but contrary to a classical genetic analysis where mutant alleles generated phenotypes are described, in our experiments the extracts are responsible for the impeded biological phenomenon. Both fenugreek seed extracts are affecting the viability of the analyzed cancerous cell lines in a dose dependent fashion, and by immunostaining we were able to detect multiple cellular defects. We could identify cell division defects and apoptosis like characteristics. Therefore, we were able to detect normal looking interphase and dividing cells, together with multi-nucleated presumably aneuploid cells, other cells with small or multi-polar spindles, and cells showing nuclear blobbing or fragmented chromosomes, the latter being specific to apoptosis (Fig. 9-10.). We also looked at the structure of mitotic spindles, and by monitoring the Polo like kinase-1 (Plk1) subcellular localization we taught to gain more information about mitotic entry, spindle assembly, chromosome alignment, sister chromatid segregation, metaphase-anaphase transition and cytokinesis. It has been demonstrated that Plk1 regulates almost every aspect of mitotic events, and its overexpression is a marker for poor prognosis in many cancers (Weng *et al*., 2016). In the case of fenugreek extracts treated cells we were able to found that Plk–1 in some instances is associated with centrosomes, while in others shows some accumulation along the central spindle microtubules of the spindle apparatus (Fig. 10.).

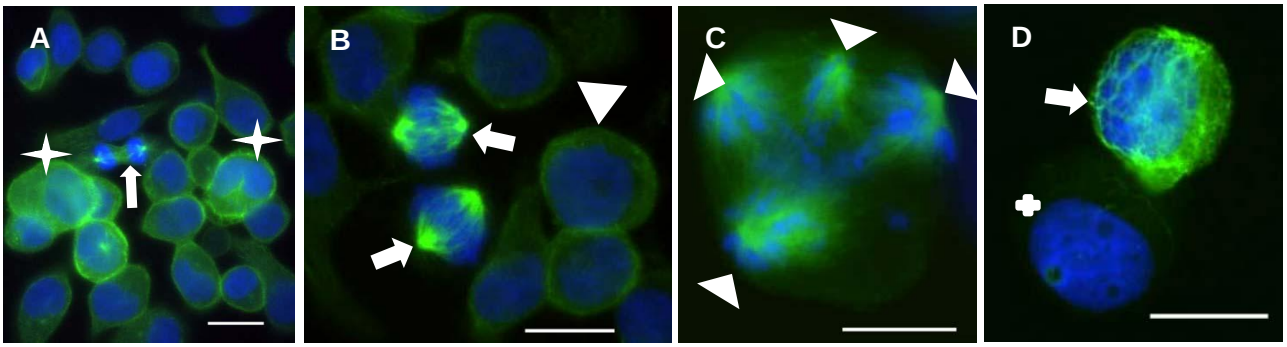

Fig. 9 Immunostaining of fenugreek extract treated cancer cells.
Note. A. Untreated cells in interphase together with giant nuclei cells (star) and normal anaphase (arrow). **B.** Untreated cells at interphase (arrowhead) and mitotic metaphase (arrow). **C.** Extract treated cell with abnormal multipolar spindle showing accumulation of chromosomes at spindle poles (arrowhead). **D.** Extract treated cell showing nuclear blobbing and abnormal microtubule network (arrow) as compared to a normal interphase cell (plus).Green indicates microtubules and blue shows the chromosome specific DNA. The scale bar represents 20µm.

Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii
Vol. 26 issue 4, 2016, pp.435- 448

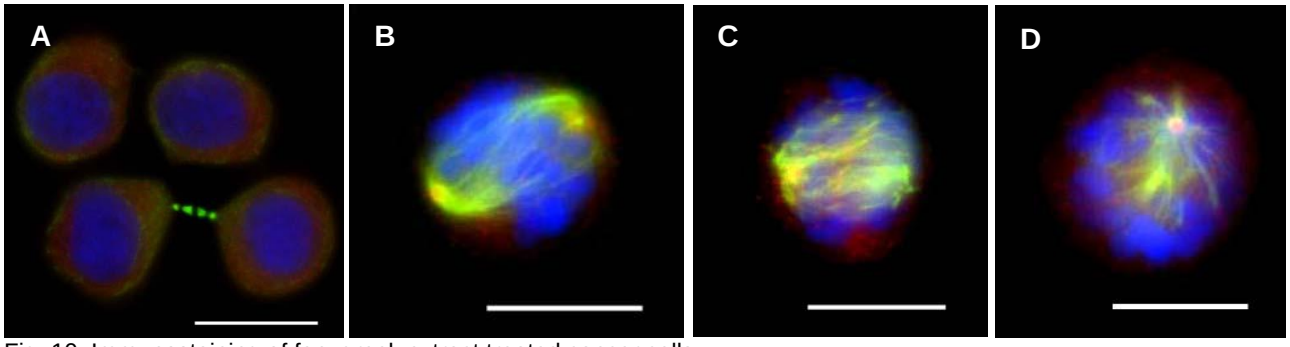

Fig. 10. Immunostaining of fenugreek extract treated cancer cells.
Note. A. Normal cells in interphase (star) and cytokinesis (arrow). **B.** cell at mitotic metaphase-anaphase transition with centrosomes at spindle poles and PLK associated with centrosomes but not kinetochores (arrow). **C.** Extract treated cell with abnormal bipolar spindle showing normal centrosome at one pole with Plk-1 accumulation (arrow), while the opposite pole is much broader with no centrosome (arrowhead) and some PLK accumulation along the spindle microtubules (plus). **D.** Extract treated cell with abnormal monopolar spindle showing PLK accumulation at the spindle pole associated centrosome (arrow). Green indicates microtubules, red stands for Plk, and blue shows the chromosome specific DNA. The scale bar represents 20μm.

Despite the fact that the performed immunostaining revealed certain cellular defects presumably generated by the fenugreek extract based treatments, we did not carry out a quantitative analysis since after 2- and 3-day treatments usually the cell number was greatly reduced. However, when we looked at the highly viable cells coming from the very low concentration aqueous fenugreek treatments, we were able to detect relatively more normal looking cells, though defects were also apparent. To gain a much clearer picture it will be necessary to carefully assess the phenotypes all treated cells by FACS analyses.

CONCLUSIONS:

Our studies are indicating specific cell viability effects for the aqueous and alcoholic fenugreek seed extracts as assessed on the T-47D, ZR-75-1 human breast cancer cell lines. In case of aqueous fenugreek seed extract, a concentration dependent hormetic response was observed, so that at high concentrations the extract was cytotoxic, while at lower concentrations it increased the treated cell viability, and ultimately at the lowest concentrations the viability of the treated cancerous cells exceeded at about 1.2 times those of control cells (see Table 1). Moreover, the duration of aqueous fenugreek extract treatment has also influenced the cell viability since the longer, 3-day treatments were producing more severe effects as compared to shorter 2-day treatments with the corresponding extract. It is also interesting to notice that the T-47D and ZR-75-1 cells were featuring similar concentration- and time-dependent viability profiles. Some minor but significant differences observed after the 2-day treatment are suggesting that the T-47D cells were more responsive to the applied aqueous fenugreek seed extract than the ZR-75-1cells.

Table 1.
Concentration dependent variation of T-47D and ZR-75-1 cells' viability.

Aqueous fenugreek extract treated cells	2-day treatment		3-day treatment	
	conc. range (%)	viability	conc. range (%)	viability
T-47D	10-0.31	6x↓	10-0.31	8x↓
	0.31-0.16	LD50	0.16	LD50
	0.08	1.15x↑	0.08	1x
	0.04	1.3x↑	0.04	1.2x↑
ZR-75-1	10-0.31	3.4x↓	10-0.63	8x↓
	0.16	LD50	0.31-0.16	LD50
	0.08	1x	0.08	1x
	0.04	1.2x↑	0.04	1.2x↑

Note:↑ indicates increased , while ↓ symbolizes decreased cell viability values.

It remains an open question how to explain the hormesis specific low dose stimulation and high dose inhibition seen in case of the aqueous fenugreek seed extract. Similar hormetic dose responses were described in numerous studies for chemicals and radiation, while for the explanation of the phenomenon was evoked the cellular adaptive stress response (for review see Calabrese *et al*., 2012). Hormesis requires a different set of interpretations for the dose-specific responses. In general terms, at high concentrations within the toxicological range, cellular damage will occur, while as the concentration falls below the threshold, the low concentration specific stimulation more likely will represent the manifestation of an adaptive stress response facilitated by vitagenes. Genes encoding heat shock proteins, heme-oxygenase, thioredoxin and glutathione system and sirtuin proteins were proposed to act like vitagenes. Such vitagenes will activate antioxidant enzymes, chaperons, phase 2 enzymes, cytoprotective proteins and pathways like Keap1/Nrf2/ARE. The emerging picture of hormetic low dose stimulatory effect looks rather complicated, and requires a system biology type of approach, which will be the subject of our future research. Nevertheless

it seems logic that at lower concentrations, the aqueous fenugreek extract composition will change due to the dilution of the original stock solution, and these concentration differences could elicit different biological responses with either deterministic or stochastic nature. An aqueous fenugreek extract had already been assessed and was found a very selective cytotoxicity against certain cancer cell lines such as T-cell lymphoma (TCP), B-cell lymphomas, Thyroid Papillary carcinoma (FRO) and breast cancer (MCF7), while no significant cell cytotoxicity was detected amongst normal cells, including human lymphocytes and meningioma (Alsemari *et al*., 2014).

The fact that chemical composition of fenugreek seed extracts could be correlated with the induced biological effect is further supplemented by our experimental results. When the T-47D and ZR-75-1 cells were treated with alcoholic fenugreek seed extracts they were featuring throughout whole concentration range and treatment duration markedly reduced viability, and no hormetic response was evident. It seems likely that the alcoholic extract showed a more pronounced cytotoxic effect than the aqueous fenugreek seed extract. Indirect immunostaining based evidences are indicating that the alcoholic fenugreek seed extract could induce apoptosis, though multiple mitotic spindle defects were also observable even at very low extract concentrations (Fig.9-10). When the bioactive compound profile of the aqueous and alcoholic fenugreek extracts were compared several differences were evident (Vígh *et al.*, 2017). Bioactive compounds like 4-HIL, asparagine, adenine, adenosine, adenosine 3',5'-cyclic monophosphate, cytidine and guanine were identified only in the aqueous fenugreek extract. Other compounds like aspartic acid, scopoletin, resveratrol, naringenin, chrysoeriol, tricin, luteolin-8-C-(2"-O-(E)-p-coumaroylglycoside), tricin-7-O-glucoside, genistein, vitexin, medicarpin, scoparin, 5'-S-Methyl-5'-thioadenosine, flavin mononucleotide, xanthine, soyasaponin I, protoneogitogenin-S5, ursolic acid and B3 vitamin were found only in the alcoholic fenugreek extract. In case of alcoholic fenugreek extract specific naringenin, vitexin and isovitexin it has been shown to modulate the SIRT6 *in vitro* H3K9 deacetylation activity, though the exact physiological significance of this phenomenon remains obscure (Singh *et al*., 2014).The SIRT6 is a master regulator of glucose homeostasis by controlling the expression of multiple glycolytic genes (Dominy *et al*., 2012; Liu *et al*., 2012;). What is even more interesting is the fact that diosgenin seemed to be present only in the alcoholic extract at very low levels, while protodioscin and protodiosgenin were absent from both extracts. This it means that neither diosgenin nor protodioscin and protodiosgenin are responsible for the toxicity of our fenugreek seed extracts as it would have been expected based on experimental results on MCF-7 human breast cancer line (Srinivasan *et al*.,2009) and HL-60 human leukemic cells (Hibasami *et al*., 2003).

A comparative study had also shown that the aqueous extract had higher antioxidant capacity and lower total flavonoid and total phenolic content, while the alcoholic extract had lower antioxidant capacity and higher total flavonoid and total phenolic contents (Vígh *et al*., 2017, accepted for publication). It remains an open question if the different chemical compositions, and the aqueous extract higher antioxidant capacity or the alcoholic extract total flavonoid content could contribute to the differences seen in relation to the inhibitory effects on the viability of T-47D and ZR-75-1 human breast cancer cell lines. However, the fact that 4-HIL is present only in aqueous extract and knowing some of its beneficial effects it seems worthwhile assessing the putative correlations with respect to the increased viability seen at very low concentrations. It has been shown that 4-HIL could inhibit the secretion of TNFα in 3T3-L1 adipocytes (Yu *et al*., 2013), and reduction of TNFα could contribute to the survival of some cancerous cells (Aggarwal *et al*., 2012).

Future studies of our aqueous and alcoholic fenugreek extracts will focus on the assessment of their estrogen receptor modulator effect, taking into account the fact that estrogenic activity of fenugreek has already been reported (Sreeja *et al*., 2010), while the T-47D and ZR-75-1 human breast cancer cell lines are classified as estrogen receptor (ER) positive, and the estrogen receptor was shown to promote the growth of breast tumor cells. (Ford *et al*., 2011). Other plant extract based studies carried out on T-47D and ZR-75-1 cells showed to reduce significantly the mRNA and protein levels of estrogen receptor α and such an effect could be correlated with activation of proteasomes, and inhibition of estrogen-stimulated proliferation (Wang *et al.*, 2013).

Despite the fact that many more papers are presenting evidences for the anti-cancer properties of fenugreek seeds (Goyal *et al*., 2016; Chatterjee *et al*., 2012; Shabbeer *et al*., 2009; Sebastian and Thampan, 2007; Amin *et al*., 2005), it remains largely controversial its beneficial effects since very few clinical studies have been reported for fenugreek in the treatment of cancers (Swaroop *et al*., 2015; Alsemari *et al*., 2014), and some genotoxicity has been also detected (Ouzir *et al*., 2016; Flammang *et al*., 2004). Alsemari *et al*. (2014) are reporting for the first time the clinical profile of a human case of primary CNS T cell lymphoma that responded to fenugreek treatment and resulted in tumor regression, while Swaroop *et al*. (2015) are describing the beneficial effects of fenugreek seed extract treatment in case of polycystic ovary syndrome. Other clinical studies are indicating the beneficial effects of fenugreek based treatments of type 2 diabetes patients (Verma *et al*., 2016), and a fenugreek based dietary supplement could improve testosterone levels, healthy sperm profile, mental alertness, cardiovascular health and overall performance in human male subjects (Maheshwari *et al*., 2017). It is also interesting that Assad and Khan (2016) demonstrated that fenugreek seeds did not reveal any sedative effect but significant antianxiety effects were detected. However, consumption of fenugreek in humans, rodents, rabbits and chickens induced teratogenic effects from congenital malformations to death, while in rats, mice and rabbits

males testicular toxicity and infertility associated oxidative stress and DNA damage were detected (Ouzir *et al*., 2016).

All together our data are suggesting that the aqueous and alcoholic fenugreek seed extracts hold promise for consideration in the complementary therapy for breast cancer patients. However, further studies are needed to carefully assess their potential effects before any step is taken regarding their use as complementary remedies. In this respect it would be important to carefully evaluate all possible anti-cancer, anti-diabetic, anti-hypercholesterolemic, anti-inflammatory properties of our fenugreek extracts since results obtained using other fenugreek sources may not be totally applicable worldwide. Evidences are suggesting that the soil and climatic conditions, the applied extraction method could influence the chemical composition of fenugreek seeds and consequently their health-promoting effects (Alsemari *et al*., 2014; Vígh *et al.*, 2017, accepted for publication).

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