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Effect of some natural plants' extract for P38 downregulation on breast cancer

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Abstract

Introduction: Breast cancer is the most common cancer diagnosis and the leading cause of death for women worldwide. A multitude of women favor alternative therapies to address their ailments, mitigate pharmaceutical adverse effects, or postpone illness progression**. Objective:** This study investigates the application of economical, environmentally sustainable ethanol-extracted Fenugreek and green tea in treating breast cancer. **Methods:** The impact of these plant extracts on MCF-7 cells was evaluated utilizing MTT, qRT-PCR, and ELISA methodologies. The findings indicated that Fenugreek extract inflicted cellular damage. Similar to green tea, Fenugreek elevated LDH levels in cells. Fenugreek extracts induced apoptosis in MCF-7 cells more than green tea extracts**. Results:** Fenugreek extracts promote dendritic cell maturation by modulating the JNK, p38 MAPK, and NF-κB pathways, resulting in elevated IL-1β, IL-6, IL-8, and TNF-α levels. One of the initial RAS/RAF/MEK/ERK-associated breast tumors is associated with Raf-1 expression. In MCF-7, P38 and Raf1 exhibited down-regulation, whereas TNFα, IL1β, IL6, and IL8 shown up-regulation. **Conclusion:** There are no significant effects from green tea extract. Ultimately, fenugreek extract decreased apoptosis and pro-inflammatory cytokines.

Keywords: Fenugreek. Green tea. P38 gene.

Introduction

Breast carcinoma constitutes one-third of malignancies in Egypt. Worldwide, about 1 million cases are diagnosed annually. Multidisciplinary breast cancer treatment includes surgery, radiation, chemotherapy, Breast cancer is the most diagnosed cancer and the leading cause of death among women. The treatment for breast cancer requires a multidisciplinary approach that includes surgery, radiation, chemotherapy, and hormone therapy. Despite a 40-year decline in mortality rates, around 40,000 Americans still die from breast cancer annually. Even with decades of advancements in new drugs and personalized therapies, patients continue to experience significant physical and psychological stress. Recurrence and metastasis further complicate the prognoses for breast cancer patients **[1-2]**.

Chemicals sourced from nature or produced industrially can aid in the prevention or treatment of cancer. Chemoprevention can be especially beneficial for advanced breast cancer patients who have limited treatment options. Innovative natural therapeutics have the potential to kill cancer cells while minimizing side effects, enhancing selectivity, and reducing overall toxicity and hormones **[1-2]**. Despite a 40-year decline, 40,000 Americans die from breast cancer annually. Despite decades of new drugs and personalized therapy, patients still feel physical and psychological stress **[3]**. Recurrence and metastasis aggravate breast cancer

prognoses. Modern breast cancer treatments have severe side effects, spurring drug development [4]. The cause of breast cancer is uncertain. Phytotherapeutics treat cancer and other disorders [5]. Different herbal medicines, such as phenolic acids, flavonoids, and sesquiterpenes, destroy cancer cells [6]. Overall treatment failure, especially in metastatic and locally advanced illness, contributes. Thus, innovative breast cancer treatments are urgently needed.

Chemicals from nature or industry prevent or treat cancer. Chemoprevention may help advanced breast cancer patients with few treatment alternatives. Innovative natural therapeutics kill cancer cells, reduce side effects, boost selectivity, and reduce toxicity [7]. Fenugreek is used in traditional treatments for diabetes, hypercholesterolemia, wounds, inflammation, and gastrointestinal issues. It may combat cancer, although how is uncertain [8]. Numerous studies have found that fenugreek contains dietary fiber, galactomannans, antioxidants, steroidal sapogenins, and amino acids including 4-hydroxyisoleucine. These chemicals have anti-diabetic, anti-leukemic, antipyretic, anti-nociceptive, anti-tumor, hypocholesterolemic, and hypoglycemic activities. Apoptosis, tumor suppressor genes, and TNF-decrease are induced by fenugreek leaves, seeds, and components [9].

Green tea (GT) is a popular medicinal drink worldwide. Recently plucked tea leaves contain 30% phenolics. Over 90% of GT's polyphenols are catechins. EGCG dominates green tea catechins with 50–80% GT catechins. In addition, epicatechin, epicatechin-3-gallate, and epigallocatechin are physiologically active [10]. These drugs may fight cancer. Thus, GT or its constituents show promise as cancer prevention therapy in animal and clinical models [11].

Activated p38 MAPKs regulate proinflammatory mediator synthesis, cell proliferation, differentiation, and survival in response to stress and cytokines. Found four isoforms: α, β, γ, and δ [12]. Raf-encoded MAP3K directly interacts with membrane-associated Ras GTPases. RAF1 phosphorylation of MEK1/2 activates ERK1/2. In cell division, death, differentiation, and migration, activated ERKs influence gene expression [13].

LDH indicates necrosis. LDH may indicate necrosis [14]. Chan et al. [15] called necrosis uncontrolled cell death. Studies indicated that TNF-α can produce necroptosis (programmed necrosis). The ability of necroptosis to bypass apoptotic resistance and elicit powerful anticancer immune responses has been extensively researched. This supports the drug's cancer treatment potential. Necroptosis produces IL-6 and IL-8 [16]. Cytokines boost neutrophil chemotaxis and degranulation in the tumor microenvironment (TME). Wound repair, innate, and adaptive immunity require interleukin-1. Like IL-1α and IL-1β, family members are associated with tumorigenicity and cancer therapy resistance [17].

The aim of this work was to investigate the effects of fenugreek seeds and green tea extracts against MCF-7 breast cancer cells through MTT assays. The RT-PCR assay was evaluated on gene expressions of p38, Raf1, TNF, and IL-8, whereas the ELISA assay measured IL-1β and IL-6 cytokines.

Materials and Methods

Ethical statement and Informed Consent

The experiment was approved by the Ethics Committee of Faculty of medicine, Menofia University (No. pHy59). Informed consent was obtained from all participants involved in the study, with all procedures explained in detail before participation.

Plants extracts

In December 2022, Cairo University's Faculty of Science approved 10 mg of finely powdered Trigonella foenum-graecum (Fenugreek) seeds and Camellia sinensis (Green Tea) leaves were carefully dissolved and sterilized using ethanol to prepare plant extracts. One kilogram of GT leaves and fenugreek seeds were macerated in 70% ethanol for 48 hours. Transfer 500 µg/µL supernatant to a sterile tube and refrigerate at 4 °C until use. The final concentration was 100 µg/µL, and 1 mL of DMSO was used to dissolve each sterilized extract. After incubation at 4°C, the sample was used.

Analysis of phytocompounds and molecular assays

HPLC identification of green tea leaf phenolic components and flavonoids: The phenolic and flavonoid content of fenugreek seeds and GT leaves extract was measured using HPLC. Herrera-Carrera et al. [18] use an Agilent 1100 HPLC with an automated injector, 1100 quaternary pump, in-line degasser, autosampler, dual-wavelength UV/vis detector, and acquisition system.

Agilent's ODS-C18 reversed-phase Zorbax octadecylsilane column was used at room temperature. Phenols and flavonoids were separated at 1 mL/min using a gradient of two solvent systems, (A) acetic acid-water (2:98 v/v) and (B) acetic acid-acetonitrile-water (2:30:68 v/v). The mobile phase composition was 90% A and 10% B at time zero and 0% A and 100% B at 30 minutes. Using co-eluted pure standards' chromatography retention times, substances were identified. Phenols and flavonoids were measured at

325 and 280 nm.

Propagation of Cell line

Cells MCF-7 for breast cancer came from VACSERA in Giza, Egypt. The cells were grown in a 75 mL cell culture flask with 5% CO_2 and a humid environment. The medium used was RPMI 1640, which had 4 mM sodium pyruvate, 4 mM L-glutamine, and 5% heat-inactivated bovine serum albumin (BSA). Cells that had been cultured were shot with Zeiss A-Plan 10x objective lenses on upside-down microscopes.

MTT assay with CC50 cytotoxic dose

Extracts were tested for lethal effects and CC50 by seeding 10 x 10^3 MCF-7 cells in 96-well plates and incubating for a duration. Cells were grown overnight at 37 ºC in 5% CO_2 with different extract concentrations (0.3-5 mg/mL). A 0.5 mg/mL concentration results. To each well, add 10 µL of MTT labeling reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) from Sigma-Aldrich MTT cell growth test kit (Germany Placed plate in 5% CO_2 chamber at 37 ºC and humidified for 4 hours.

Each well received 100 εl of solubilization solution and was incubated overnight at 37 ºC in a 5% humidified CO_2 atmosphere. Cell viability and cytotoxicity were measured by formazan dye absorbance at 570 nm.

Cytotoxicity Detection and necrotic activity

The Abcam LDH test kit (cat. no. ab65393, USA) checked how cytotoxic and necrotic the treated cells were in a culture setting. In 96-well plates, 10 × 10^3 MCF-7 cells were put into each well and 600 εg/mL of fenugreek seeds and GT were added. After being kept at 37 °C for 24 hours, the media were spun at 600 x g for 10 minutes at 4 °C. The cell-lysate supernatant was moved to a different 96-well plate **[19]**.

As directed by the maker, 40 εl of treated cells were mixed with 60 µL of LDH reaction mixture (20 εl LDH substrate and 40 εl LDH buffer). This was left to sit at room temperature for one hour. A 450 nm microplate reader was used to measure LDH **[19]**. Non-treated (NT) cells are used as the negative control and Triton 100-X is used as the positive control in the equation for relative LDH production. Here, X = sample absorbance at 450 nm. The results were shown as fold change.

Evaluation of cell growth and shape

To help the cells divide, 10 x 10^4 copies of each type were put into each well of a 6-well plate. The cells were washed twice with PBS, trypsinized with the right amount of enzyme, and then kept at 37°C for three minutes. Lastly, enough full RPMI medium was given to the cells that had been trypsinized. Hemocytometers were used to count living cells and inverted telescopes to look at the shape of cells **[20]**. **cDNA synthesis**

TRIzol (Invitrogen, PureLink, Thermo Fisher Scientific, USA) kits were used to separate and clean up the total RNA from cells. It was standardized to 100 ng/µL and then mixed back with water that didn't contain RNase. You can make complementary DNA (cDNA) from 1 µg of pure total RNA and M-MLV reverse transcriptase (Promega, USA). Following the maker's directions, oligo (dT) primers were used to reverse transcribe total RNA at 45 °C for an hour and then it was heated to 95 °C for 5 minutes. Until you need it, keep cDNA at -20 °C **[21]**.

qRT-PCR

Gene activity was measured using qRT-PCR. Relative gene expression of P38, Raf1, TNF-α, and IL-8 was evaluated using the Quantitative SYBR Green PCR Kit (Qiagen, USA) and appropriate primers (Table 1). B-actin expression levels normalized qRT-PCR data. To perform PCR, mix 0.2 µM primers, 2 µL of produced cDNA, 0.25 µL of RNase inhibitor (25 U/µL), 10 µL of SYBR green, and nuclease-free water in 25 µL. PCR was done 35 times (94°C for 30 seconds, 60°C for 15 seconds, and 72°C for 30 seconds) after 5 minutes at 94°C **[21]**.

Table 1. Oligonucleotide primer sequences used for mRNA quantification of the studied genes.

Description	Primer sequences 5'- 3'
P38 sense	CTACGGCTCGGTGTGTGCTGC
P38 antisense	CTGAACGTGGTCATCGGTAAGC
TNF- α sense	AGGCAGTCAGATCATCTT
TNF- α antisense	AGCTGCCCCTCAGCTTGA
Raf-1 sense	TTTCCTGGATCATGTTCCCCT
Raf-1 antisense	ACTTTGGTGCTACAGTGCTCA
IL-8 sense	ACTGAGAGTGATTGAGAGTG
IL-8 antisense	AACCCTCTGCACCCAGTTTTC
beta-actin sense	ACCGTGAAAAGATGACCCAG
beta-actin antisense	CCATACCCAAGAAGGAAGGC

Source: Own authorship.

Proinflammatory cytokines measurement by ELISA

After giving 600 µg/ml of each extract to MCF-7 cells for 72 hours, human ELISA kits (ab181421 and ab100575 Abcam, USA) were used to measure the amounts of IL-1α and IL-6. The extracts were put in 96-well plates with cell cultures overnight and were subjected to them for 0 to 72 hours. At each point in time, 1X cell lysis buffer (Invitrogen, Thermo Fisher Scientific, USA) was used to break down the cells. The ELISA plate reader mixed 100 µL of broken-down cells with 100 µL of reference solution and 50 µL of 1X

biotinylated antibody. It did this for two hours at room temperature. After 100 µL of 1X streptavidin-HRP solution was added, the sample wells were left to sit in the dark for 30 minutes. The sample wells were mixed with 100 µL of TMB chromogen substrate solution and left to sit at room temperature for 15 minutes without any light. To stop the reaction, 100 µL of a stop solution was put into each well. 450 nm was used to measure cytokines **[22]**.

Data analysis

Excel made the histograms and plots. The ΔΔCt method was used to assess mRNA expression levels in qRT-PCR using the equations: (1) ΔCt = gene - β-actin, (2) ΔΔCt = sample - control, and (3) fold change quantification = $2^{(-\Delta\Delta Ct)}$. A two-tailed t-test was used for statistical analysis, with a significance level of P-value ≤ 0.05. **[23]**.

Results

Fenugreek seeds and green tea influence on MCF-7 cell

The MTT test checked how alive the MCF-7 cells were after being treated with the extract. Four times, ethanolic extract concentrations of 0.3, 0.6, 1.25, 2.5, and 5 mg/mL were tried. The t-test hypothesis is used in Table 2 to look at the mean value of four separate events with a 0.05 significance level. Green tea and ethanolic fenugreek seeds both reduced the survival of MCF-7 cells in a dose-dependent way (Figure 1). Cancer cells are not as likely to be hurt by the ethanolic fenugreek seed product. If you mix fenugreek seeds and green tea in ethanol, the CC50 numbers for killing MCF-7 cells were 1.25 mg/mL and 2.0 mg/ml, respectively. The average outcomes of giving both extracts' CC50 amounts were compared to those of NT cells and cells treated with ethanol in three separate tests. Fenugreek seeds increased the growth of MCF-7 cells by five times, but the ethanolic fluid from GT leaves did not. Compared to the negative control (NT MCF-7 cells) and the positive control (etanol-treated cells), ethanol-treated fenugreek seeds changed the shape of the cells and lowered the number of them. Ethanolic GT had no effect on the form or number of cells (Table 3).

Table 2. Statistical of MCF-7 cells toxicity.

Treatment	Concentration mg/mL	Mean	Standard deviation (S.D.)	p-value
fenugreek seeds	0.0	0.55	0.07	
	0.3	0.33	0.10	0.00917222
	0.6	0.15	0.07	0.000203
	1.25	0.07	0.01	0.00001
	2.5	0.04	0.01	0.0000
	5.0	0.03	0.02	0.0000
GT	0.0	0.58	0.05	
	0.3	0.51	0.05	0.1210
	0.6	0.49	0.05	0.0448
	1.5	0.44	0.06	0.0141
	2.5	0.45	0.08	0.0401
	5.0	0.31	0.05	0.0003

Source: Own authorship.

Table 3. Statistical parameters of cell viability.

Statistical measurements	Control		Plants extract	
	NT	Ethanol 70%	fenugreek seeds	green tea
Mean ± SD	235000± 21213	215000± 7071	45000± 7071	225000± 21213
p-values		0.333	0.007**	0.684

Source: Own authorship.

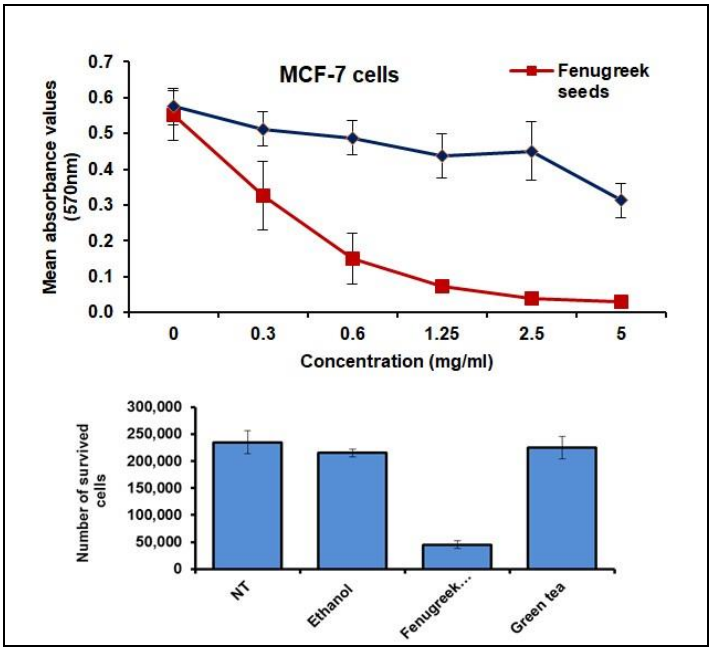


Figure 1. GT extract and C fenugreek seed kill MCF-7. CC50, n=3, MTT experiments showed MCF-7 cells treated with ethanolic fenugreek seed or GT extracts. Post-fenugreek seed or GT extract treatment, cell survival. Error bars show the standard deviation of two independent experiments. Student's two-tailed t-test determined value significance. (**) indicates p≤0.01 and (*) indicates p≤0.05. Inverted microscope images of cell shape and population after 24 hours of ethanolic fenugreek seeds or GT extract treatment vs positive control and NT cells. Source: Own authorship.

Cellular damage in MCF-7 cells

When cells are damaged from the inside or the outside, LDH is released from the cytoplasm into the extracellular world. It works well as a tissue and cell damage and toxicity indicator because it stays stable in cell culture settings. Table 4 and Figure 2 show that treating cells with ethanolic fenugreek seed raises the production of LDH in a way that depends on the amount, compared to GT extract. It grew a lot faster than NT cells, which were used as a negative control.

Table 4. Relative LDH production in MCF-7 cells.

Statistical measurements	Control			Plants extract	
	NT	Ethanol 70%	Triton 100X	fenugreek seeds	green tea
Mean ± SD	0.06± 0.01	0.07± 0.02	0.33± 0.15	0.23± 0.10	0.09± 0.00
Relative produced LDH	1.08	1.33	5.79	3.30	1.11
p-values		0.6014	0.0134	0.0184	0.2230

Source: Own authorship.

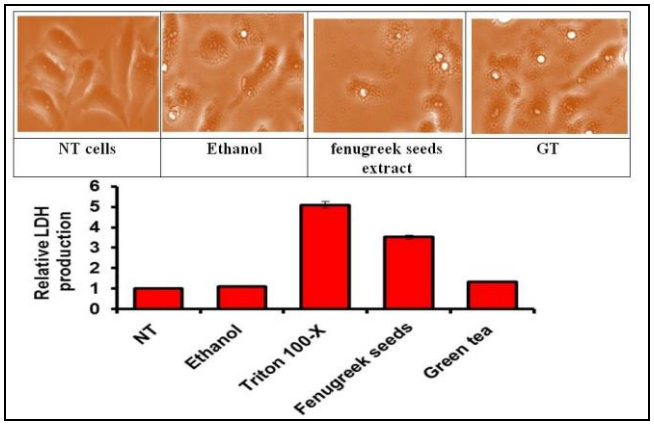


Figure 2. Damaged cells produce LDH. Comparative LDH production from MCF-7 cells treated with fenugreek seeds or GT extract vs Triton 100-X-treated and NT cells. The standard deviation of three replicates was displayed by error bars. Student's two-tailed t-test yields $p<0.01$ for differentiated values. Source: Own authorship.

Modulatory Role of fenugreek seeds and GT

These genes were checked using qRT-PCR to see how much P38, Raf1, TNFα, and IL-8 were expressed in MCF-7 cells. This was done to see how fenugreek seeds and GT extracts affected signal transmission (Tables 5 and 6). Compared to cells that were treated with ethanol or not, fenugreek seeds changed gene expression in a way that depended on amount and time. P38 levels dropped a lot when fenugreek seeds were added (Figure 3). Also, Raf1 went down (Figure 3). To be fair, GT slightly increased P38 and Raf1. The fenugreek seeds had a big effect on the levels of TNF-α and IL-8 ($p=0.00088$ and 0.01744), but they only had a small effect on GT (Figure 4).

Table 5. Quantification of relative gene expression of P38 and Raf-1

genes	Treatment	Fold change	p-values
P38	NT	1.000 ± 0.000	-
	Ethanol	1.079 ± 0.040	0.06637
	fenugreek seeds	5.101 ± 0.067	0.00245**
	GT	1.203 ± 0.149	0.10589
Raf 1	NT	1.000 ± 0.000	-
	Ethanol	1.088 ± 0.034	0.69382
	fenugreek seeds	5.300 ± 0.195	0.00066**
	GT	1.305 ± 0.154	0.42160

Source: Own authorship.

Table 6. Relative cytokines expression of TNF-α and IL-8.

Cytokines	Treatment	Fold change	P-values
TNF-α	NT	1.000 ± 0.000	-
	Ethanol	1.306 ± 0.177	0.07778
	fenugreek seeds	5.600 ± 0.200	0.00076**
	GT	1.209 ± 0.500	0.58328
IL-8	NT	0.900 ± 0.097	-
	Ethanol	1.448 ± 0.040	0.28989
	fenugreek seeds	5.700 ± 0.202	0.01755**
	GT	0.960 ± 0.392	0.75440

Source: Own authorship.

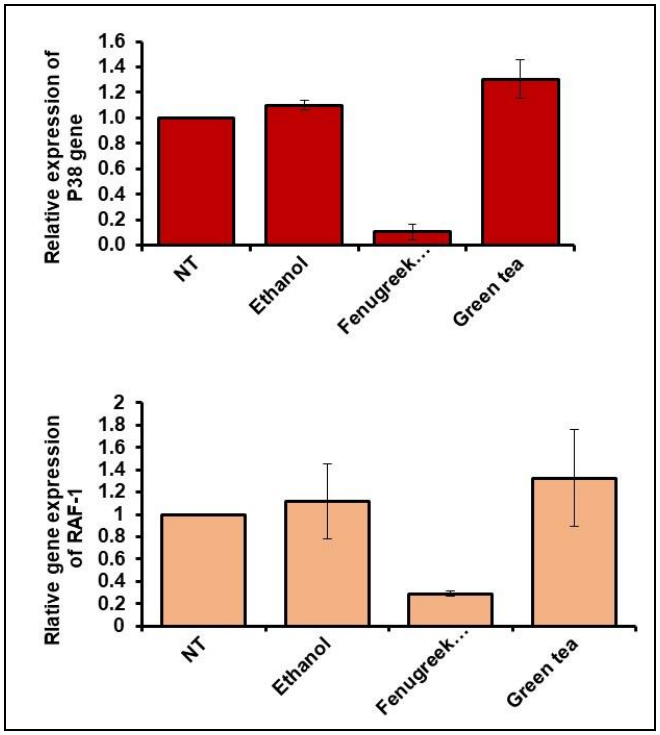
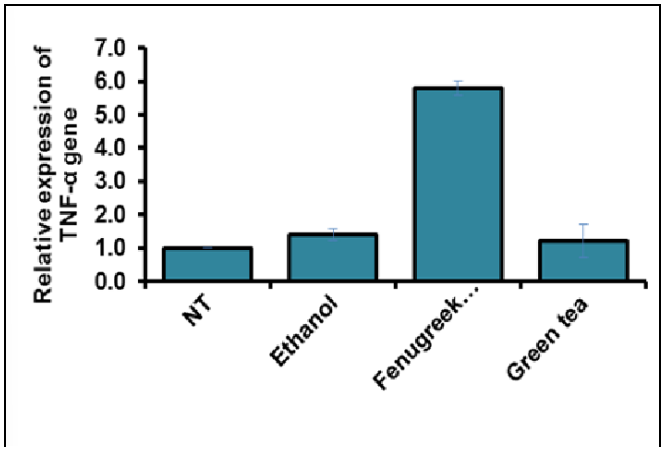


Figure 3 Plant extract-treated MCF-7 cells express P38 and Raf 1. MCF-7 vs. NT steady-state P38 gene mRNA fold change. MCF-7 vs. NT steady-state Raf1 gene mRNA fold change. B-actin mRNA levels were the internal control, while etanol-treated cells were the positive control. The standard deviation of two independent experiments was illustrated by error bars. We used a two-tailed t-test, with $p<0.01$. Source: Own authorship.



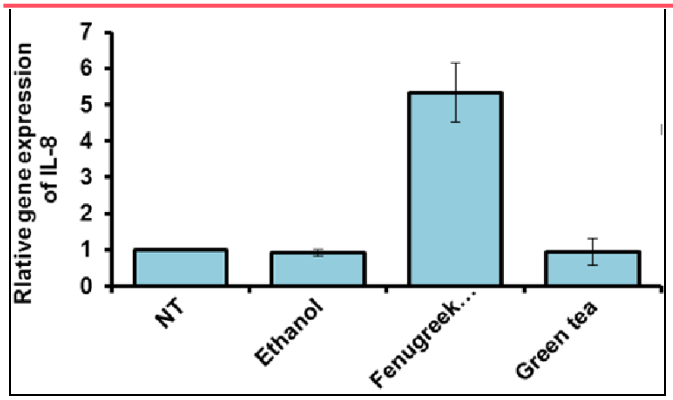


Figure 4. Anti-inflammatory indicators TNF-α and IL-8 levels in MCF-7 cells treated with plant extracts were compared to NT cells and ethanol-treated cells. Source: Own authorship.

Fenugreek seeds and GT stimulatory effects

ELISA was used to look into the link between treating cells with extract and the production of proinflammatory markers by those cells. Adding fenugreek seeds to NT cells and ethanol-treated cells increased the production of IL-β and IL-6 over time, hitting 600 and 330 pm/mL, respectively (Figure 5). The cells that were treated with GT made very little IL-β and IL-6 (107 and 130 pm/mL, respectively). Fenugreek seeds change the release of immune markers in cells that have been treated.

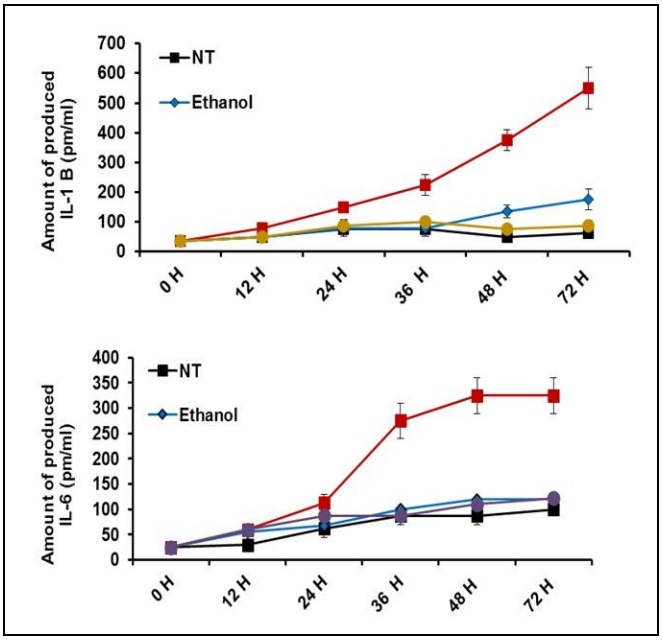


Figure 5. Proinflammatory indicators IL-β and IL-6 levels in MCF-7 cells treated with plant extracts. The study compared IL-α levels in MCF-7 cells treated with 1.25 and 2.0 mg/mL fenugreek seed extract to ethanol-treated and non-treated (NT) cells at specific time intervals. (b) Chemokine IL-6 (pm/mL) in MCF-7 cells treated with 2.0 mg/ml GT extract at defined time intervals compared to ethanol-treated and NT cells. Error bars showed two replicates. S.D. Source: Own authorship.

Tables 7 and 8 show the results of the HPLC analysis regarding the total phenolic and flavonoid phytocompounds in the ethanolic extract of GT leaves and extract of fenugreek seeds, respectfully.

Table 7. HPLC analysis report of total phenolic and flavonoid phytocompounds in ethanolic extract of GT leaves.

Phenolic Compounds	Formula	RT (min)	Concentration (µg/mL)	Flavonoid Compounds	Formula	RT (min)	Concentration (µg/mL)
Gallic Acid	$C_6H_2OH_3CO_2H$	4.4	0.56	**Myricetin**	C15H10O8	3.0	2.33
Pyrogallol	C6H3OH3	5.0	2.66	**Rutin**	C27H30O16	4.2	11.36
Cinnamic Acid	C9H8O2	6.5	4.22	**Naringin**	C27H32O14	5.1	4.14
Chlorogenic Acid	C16H18O9	7.89	9.27	**Quercetin**	C15H10O7	6.8	5.88
Catechol	C6H4OH2	9.2	10.14	**Kaempferol**	C15H10O6	8.0	5.07
Isoferulic Acid	C10H10O4	11.0	0.78	**Luteolin**	C15H10O6	9.0	10.43
Caffeic Acid	C9H8O4	11.5	11.89	**Catechin**	C15H14O6	12.0	7.69
Ferulic Acid	C10H10O4	12.2	1.09	**Epicatechin**	C15H14O6	18.0	0.98
Protocatechuic Acid	C7H6O4	14.2	0.09				

Source: Own authorship.

Table 8. HPLC analysis report of total phenolic and flavonoid phytocompounds in ethanolic extract of fenugreek seeds.

Phenolic Compounds	Formula	RT (min)	Concentration (µg/mL)	Flavonoid Compounds	Formula	RT (min)	Concentration (µg/mL)
Gallic Acid	$C_6H_2OH_3CO_2H$	5.02	7.56	**Myricetin**	C15H10O8	3.0	5.33
Pyrogallol	C6H30O16	5.0	2.66	**Vanillin**	C8H8O3	5,0	7.65
Rutin	C27H8O3	6.5	11.22	**Apigenin**	C15H10O5	5.1	14.12
Chlorogenic Acid	C16H18O9	7.22	8.79	**Quercetin**	C15H10O7	6.8	15.30
Catechol	C6H4OH2	9.2	10.5	**Kaempferol 1**	C15H10O6	8.0	6.17
Caffeic Acid	C9H8O4	11.5	15.13	**Luteolin**	C15H10O6	9.0	6.46
P-Coumric Acid	C9H8O3	12.1	4.22	**Catechin**	C15H14O6	12.0	9.52
o-Coumric Acid	C9H8O3	12.5	1.33	**Genistein**	C15H10O5	18.0	3.98

Source: Own authorship.

Discussion

Second most prevalent disease in women is breast cancer. It affects several cell types, making it difficult to prevent globally. Breast cancer research and prevention have advanced in the past decade.

Cancer drugs are being made safer by scientists. Botanical remedies matter. Healthy phytochemicals include polyphenols, flavonoids, and terpenoids. These compounds have been extensively studied to improve health and lifestyle **[24]**. Plant-based diets may boost biological activity by improving chemical absorption **[24]**. Egyptians treated ailments with herbs for centuries. Egyptians understood how to employ herbs for medicine. Some companies remain open. This study

examined breast cancer treatment using fenugreek seeds and green tea **[25]**. The same photochemical components were found in varied levels in fenugreek seeds and green tea by species in quantitative HPLC tests. This study examined whether fenugreek seeds and green tea could treat breast cancer. Fenugreek and green tea have varied levels of the same phytochemicals by species, according to HPLC.

Health and healing have been achieved using fenugreek. Traditional usage and medical benefits of the plant have been verified by many investigations. Scientific evidence is lacking to explain this plant's function **[26]**.

Recent research has examined bioactive compounds in food and medical plants' chemo-preventive activities. This herb fights cancer. New study examines T's tumor-fighting potential. Cell death escape, angiogenesis, proliferative signaling, tumor-promoting inflammation, invasion, metastasis, and genomic instability are affected by Foenum-graecum extracts and isolated compounds **[27]**. Seed water destroyed T-, B-, thyroid papillary carcinoma, and breast cancer cells **[27]**. The phytochemical, sample makeup, extraction process, solvent polarity and make-up, particle size, solid-solvent ratio, and physical circumstances determine how bioactive compounds are extracted from plant materials. Solvent matters when extraction temperature and time are the same **[27]**. This investigation found the optimal removal method using 70% ethanol and water.

Plant phenolics, glycosides, terpenoids, alkaloids, polyacetylenes, and sterols are extracted with ethanol. Mixtures of water and ethanol make amino acids, organic acids, and carbohydrates easier to extract. Thus, more chemicals than phenolics are discarded. Total phenolic and flavonoid content in fenugreek seed and green tea leaf extracts were measured.

The 70% ethanol solutions had the greatest flavonoids and phenolics. Samples with 70% ethanol were tested by HPLC (Table 2). The samples' compounds were identified by comparing their spectra and retention time data to recognized substances and published research. Standard acids included arbutin, cinnamic, methylcynnamic, gallic, ferulic, coumaric, and caffeic acids. Eight flavonoids were abundant in green tea extract. The concentrations of rutin, luteolin, and catechin were 11.36, 10.43, and 7.69 µg/mL, respectively. The least kaempferol, quercetin, myricetin, and naringin was detected.

GT contains less ferulic, isoferulic, gallic, and protocatechuic acids than other plants. It also possesses the greatest catechol, chlorogenic, and caffeic acid. Pyrogallol was determined to be 2.66 µg/mL, while cinnamic acid was 4.22 µg/mL. HPLC measured total phenolics in Iranian green tea water extract. The extraction method altered phenolic content **[28, 29]**. Thus, green tea has more catechins than gallic acid. Tea (Camellia sinensis), the world's most popular drink, contains polyphenols, antioxidants, and alkaloids. Multiple HPLC experiments have detected catechins (epicatechin gallate) and alkaloids (caffeine) in tea leaves. HPLC detected flavonoids, gallic acid, caffeine, and four catechins (C, GCG, EC, and EGC) in 50% ethanol extracts **[30]**.

Gallic, catechol, chlorogenic, and caffeineic acids predominated in fenugreek seeds. P-coumaric acid, pyrogallol, and o-coumaric acid were rare. The plants contained quercetin, apigenin, catechin, vanillin, luteolin, and kaempferol. Lots of Genistein and Myricetin but low amounts. The findings support Lohvina et al. **[30]**, who demonstrated that 70% ethanol may extract phenolic components from fenugreek seeds.

The HPLC-ESI-MS test demonstrates polyphenolic profiles contain flavone C-glycosides, apigenin or luteolin aglycones, and glycones. Flavone apigenin 6-C-betachinovopyranosyl-8-C-luteolin 8-C-β-glucopyranoside, Quercetin, vitexin, kaempferol, luteolin, and apigenin were found in fenugreek seeds by HPLC **[30]**. Benayad et al. **[31]** found 32 flavonoid glycosides (apigenin, luteolin, and kaempferol as aglycones) and phenolic acids (hydroxycinnamic and caffeic acid) in raw fenugreek seeds using HPLC-DAD-ESI. HPLC testing found flavones to be the major phenolic in fenugreek seed juices. The removal and cleaning process may affect it.

Our investigation found flavonoid glycosides in 70% ethanol fenugreek seed extracts. Many studies have identified flavonoids in fearnek seeds. Fenugreek seed products may fight free radicals due to flavonoids, according to Ahmed et al. **[32]**. Lutein didn't remove DPPH free radicals as well as quercetin, myricetin, and kaempferol, according to Hirano et al. Flavonoids' aglycones have more antioxidants than glycosides. Lutein aglycone prevented membrane bilayer hydroperoxide accumulation better than its 3-, 4′-, and 7-O-glucosides **[32]**.

High polyphenol (quercetin, myricetin, and kaempferol) products are antioxidant-active, according to HPLC. Functional groups in nucleotides and glycosylation give quercetin, myricetin, and kaempferol antioxidant properties. MCF-7 human breast cancer cells treated with DMSO were tested for fenugreek seed and green tea leaf extract toxicity using the MTT assay. MCF-7 cells were treated overnight to determine the CC50, or extract content, that kills 50% of them. In previous studies, fenugreek seeds lowered cell viability depending on amount and time **[31]**. This is the first

study to indicate fenugreek sprout products offer these benefits. Sebastian and Thampan **[33]** discovered that a 50 ɛg/mL ethanolic fenugreek seed extract destroyed 70% of MCF-7 cells in 72 hours. Ahmed et al. found MCF-7 FSMEs IC50 values **[31]**. Seed products destroyed breast, pancreatic, and prostate cancer cells. Fenugreek seeds showed 1.25 mg/mL IC50 values, while green tea had 1.0 mg/mL. So fenugreek is really damaging to cells. These extracts contained mostly phenolic and flavonoid seed compounds. Phytochemicals may have induced cytotoxicity. Many research has examined the chemistry, bioactivities, and effects of fenugreek seeds, stems, and other portions on MCF-7 cell survival and proliferation. MCF-7 breast cancer cells were destroyed by whole plant methanol extracts (IC50 = 65 µg/mL) **[8]**. Similar cells were destroyed by chloroform extract (IC50 = 41.6 µg/mL) **[34]**. The MTT test showed that green tea extract had a small effect on HT-29 (human colorectal cancer) and 3T3 (mouse normal fibroblast) cell lines after 48 hours. The amount and duration of green tea aqueous extract may affect metabolic pathways and anti-proliferative actions. However, the extract did not affect 3T3 cells, proving its safety for normal cells **[29]**.

Seo et al. examined green tea's glyceroglycolipid, non-phenolic, and phenolic components. Green tea's toxicity was tested in human HepG2 and normal AML12 hepatocytes. Neither HepG2 nor AML12 cells were appreciably destroyed by any of the three 10µg/mL green tea samples. At 100µg/mL PF (catechins and caffeine), AML12 and HepG2 cell viability decreased, but treatment with 100µg/mL NPF increased cell viability to over 60% **[35]**.

We examined how ethanolic fenugreek seed and GT extracts affected MCF-7 cell shape and density to determine their growth potential. MCF-7 cells treated with alcohol-soaked fenugreek seeds had fewer cells and changed shape than NT MCF-7 cells and positive control cells. Ethanolic GT did not alter cell shape or number. Crude fenugreek extracts inhibited drug-sensitive and drug-resistant cancer cells. LDH assays detect cell-mediated damage and substances. LDH is released during necrosis and apoptosis when cell membranes collapse. Hiebl et al. **[36]** state LDH in cell supernatants can indicate cell viability and membrane degradation. We discovered that LDH increased and cell survival decreased. The MTT assay examined cell viability, and LDH release into the medium measured cytotoxicity, supporting a prior result **[37]** on cytotoxicity but disagreeing on antiproliferation. We wanted to see our dose impact viable cell numbers and morphology.

High LDH levels indicate cell injury. LDH is an essential enzyme in living cells. Three times the concentration of fenugreek seed extracts increased LDH release. GT extracts increased cytotoxicity by one-fold. MCF-7 cells generated LDH, suggesting fenugreek compounds can kill cancer cells. It also has necrosis. The fenugreek extracts altered caspase-3 and caspase-6 levels, LDH activity, and nucleosomal DNA breaking.

Understanding and developing new cancer treatments requires understanding the p38 MAPK pathway in the tumor microenvironment and breast cancer growth. Signaling pathways are crucial to the genetic and molecular alterations that cause cancer. Blocking the p38/MAPK signaling pathway and producing too much DR5 killed cells. MAPK signaling pathways greatly affect apoptosis when chemotherapy medications are used **[38]**.

The study found that fenugreek seeds predominantly reduce P38 and Raf1. After 24 hours of treatment with 1.25 mg/mL of fenugreek seeds in MCF-7 cells, TNFα and IL-8 gene levels increased by 6.3 and 5.5 times, respectively, compared to untreated cells. We found that fenugreek seeds enhance P38 and TNFα, depending on the amount and duration. Green tea (GT) at 2.0 mg/mL for 24 hours increases P38 and Raf1 gene expression in MCF-7 cells.

Different researchers can utilize fenugreek extract to treat cancer cells, according to our findings. By modifying gene activity, fenugreek and its primary component can decrease tumors by causing apoptosis, activating tumor suppressor genes, and inhibiting tumor necrosis factor. Adding fenugreek to MCF-7 cells dramatically reduced P38 and Raf1. Same as other T. report fragments. When TGFb is present, Tfg prevents EGFR and Akt phosphorylation. In addition, p38 MAPK activity decreases. When TNFα is present, diosgenin (D) reduces NF-kB, IKK, and IkBa phosphorylation and degradation. Methanol extract of fenugreek reduced p38MAPK expression in DMBA-TPA-induced skin cancer animals. Akt's downstream target Raf/MEK/ERK was blocked by diosgenin in ER+ MCF-7 cells. It stopped the G1 cell cycle and decreased cyclin D1, cdk-2, and cdk-4. This inhibited cell development and killed them **[39]**.

When EGF was present, capsaicin inhibited human fibrosarcoma cells from migrating and spreading. It blocked EGFR-dependent signaling pathways like FAK/Akt, PKC/Raf/ERK, p38 MAPK, and AP-1 and decreased MMP-9. Melanomas account for 8% of BRAF-positive malignancies. The three RAF isoforms, MEK1/2 downstream, and ERK1/2 create a continuous signaling module that controls bodily processes. EK and RAF1 may inhibit mutant KRAS signaling via MAPK **[40]**. EGFR-blocking medications such cetuximab, leucovorin, and oxaliplatin treat colon cancer. The RAF gene changes most in colon cancer. In 10% of

metastatic colon cancer patients **[41]**. A clinical trial found that BRAF inhibitor monotherapy only helps half of cancer patients. These results confirm that fenugreek extract decreases P38 and Raf1 gene levels and alters their communication in MCF-7 breast cancer cells.

Capsaici (CPS) may increase P38 and JNK phosphorylation, killing cells. CPS treatment activated caspase-3, -8, and -9 via P38 and JNK MAPK pathways, causing apoptosis. Liu et al. **[42]** found that an ethanolic extract of Marrubium Vulgare activated p38 MAPK, cell death genes, and cell cycle regulators while inhibiting cell death genes. Magnoflorine aids autophagy by activating p38 and inhibiting AKT/mTOR. This makes breast cancer cells more susceptible to doxorubicin-induced death **[43]**.

Diosgenin, the primary steroidal sapogenin in fenugreek seeds, kills cancer cells by blocking communication pathways that promote inflammation and life. Early research by Shishodia and Aggarwal **[44]** demonstrated that diosgenin reduced NF-κB activation and osteoclast formation in RAW 264.7 macrophage cells with TNF-α. The effects of diosgenin on NF-kB and STAT3 communication. Diosgenin inhibits TNFα-activated NF-kB and STAT3 signaling pathways in tumor cells. As a cancer treatment, diosgenin slows cell proliferation, expansion, and angiogenesis and kills cells.

Diosgenin inhibits receptor-activated NFKB ligand-induced osteoclastogenesis, TNF-induced invasion, and tumor cell proliferation in osteosarcoma. Diosgenin inhibited Akt activity to prevent TNF from activating NFkB, IkBα kinase, IkBα phosphorylation, degradation, p65 phosphorylation, and p65 nuclear translocation **[44]**. Researchers observed that diosgenin reduces NF-kB-regulated gene expression, increases apoptosis, and prevents cell proliferation, invasion, and bone resorption. Fenugreek diosgenin increased the mortality ratio (Bax/Bcl-2) by 1.6 times and activated caspase-3 and -9 by 1.6 and 1.2 times in S and G2/M HEp-2 cells. PARP is cut by diosgenin.

Mitochondrial toxins activate Bax, p53, p21, p38, JNK, and ERK. This releases mitochondrial cytochrome c and activates caspases. ROS can initiate intracellular signaling pathways by oxidizing phosphatases like PTEN or PTP at cysteine residues in their active sites or directly oxidizing kinases like Src. The PI3K/Akt cascade, MAPKs pathways (ERK1/2, p38, and JNK), and Src/PKD1-mediated NF-kB activation are active. Propolis polyphenols activate Bax, p53, p21, p38, JNK, and ERK. Cell death occurs when cytochrome c is released and the caspase cascade begins **[45]**.

Wound healing and innate and adaptive protection require IL-1 family inflammatory cytokines. Family members of the IL-1α and IL-1β genes are connected to tumor formation and cancer drug resistance. Transactivating transcription factors like NF-κB and AP-1 are crucial for IL-1β signaling pathways to function. To treat breast cancer induced by IL-1 and NF-κB, effective NF-κB inhibitors are necessary **[46]**. Resveratrol blocks the RAF/MAPK pathway, stopping interleukin-6-induced stomach cancer. Cucurbitacin IIb inhibits BRAF, Raf1, MEK1/2, and ERK2 in liver cancer (549) cell lines **[47]**. SK-MEL-28 melanoma cells with 22β-hydroxytingenone exhibit reduced BRAF, NRAS, and KRAS **[48]**.

Most research on IL1α, IL-6, and IL-8 focuses on their role in tumor development. IL-1β, IL-6, and IL-8 levels were significantly higher in cells treated with fenugreek seed extract for 24 hours compared to untreated cells. The release of IL-1β, IL-6, and IL-8 was not significantly altered by green tea leaf extract. Interestingly, fenugreek hydro-alcoholic extract reduced inflammatory markers like TNF-α and IL-1β in a rat model of diabetes **[49]**. Giving rats Freund's adjuvant plus fenugreek extract reduced their levels of TNF-α, IL-1α, IL-1β, and IL-6. This may be because IL-1 converts CD4+ T cells to Th17 cells. Research indicates that IL², IL6, and IL8 significantly enhance inflammation and tumor invasion in animal models of breast, colon, lung, head, neck, and melanoma. Fenugreek flavonoids such luteolin, hypericin, rutin, quercetin, and others benefit cytokines. Laboratory and animal experiments indicate that luteolin effectively inhibits inflammation and MAPK and NF-κB function compared to other phenolic substances. Macrophages produced increased cytokines and phagocytosis with Echinacea extracts. Higher levels of TNF-α, IL-1, IL-6, and IFN-β were seen **[50]**.

Dehydroandrographolide regulates the release of TNF-α, IL-6, IL-1β, and IL-10 in human blood based on its quantity. As shown in mice, it may turn down inflammatory genes. Studies on animals with H22 tumors indicate that APS injections increase IL-2, IL-6, and TNF-α production **[51]**. Fenugreek and other plants contain diosgenin, a steroidal saponin. Saponin inhibits TNF-activated genes. This affects cell proliferation, survival, and invasion (cyclin D1, COX-2, c-myc, IAP1, Bcl-2, Bcl-xL, Bfl-1/A1, TRAF1, and cFLIP). Diosgenin increased TNF and cell-killing chemotherapeutic medicines. Diosgenin inhibits NF-κB-regulated gene expression, decreases cell growth, invasion, and osteoclastogenesis, and accelerates cytokine and chemotherapy-induced mortality **[52]**.

Curcumin reduces inflammation by inhibiting TNF-α-induced NF-κB activity and nuclear translocation **[53]**. It inhibits inflammatory hormones such as IL-1β, IL-2, IL-5, IL-6, IL-8, IL-12, and IL-18 **[54-56]**. While

Fenugreek extract treatment increased LDH and pro-inflammatory markers (TNFα, IL1β, IL6, and IL8), another study indicated that chemotherapy-induced transient pro-inflammatory indicators damaged the immune system. Immunotherapy and adjuvant treatment aim to alter the Tumor Microenvironment (TME) to kill cancer cells. This helps normal tissues respond to treatment with fewer negative effects.

Conclusion

Fenugreek extracts can help dendritic cells mature by controlling the JNK, p38 MAPK, and NF-κB pathways and increasing the release of IL-1β, IL-6, IL-8, and TNF-α. Raf-1 was found in some of the first breast cancers linked to RAS/RAF/MEK/ERK. Things like P38 and Raf1 were turned down in MCF-7, but TNFα, IL1², IL6, and IL8 were turned up. The green tea extract wasn't important.

CRediT

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Ethical Approval

The experiment was approved by the Ethics Committee of Faculty of medicine, Menofia University (No. pHy59).

Informed Consent

Informed consent was obtained from all participants involved in the study, with all procedures explained in detail before participation

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Data Sharing Statement

The datasets created and analyzed during this study are available upon reasonable request from the responsible author.

Conflict of Interest

The authors declare no competing interests.

Similarity Check

It was applied by Ithenticate@.

Application of Artificial Intelligence (AI)

Not applicable.

Peer Review Process

It was performed.

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