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Phytochemical Investigation, Cytotoxic Effect, Antibacterial Effect and Isolation of Phenolic Acid of Iraqi Fenugreek Plant

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ABSTRACT

Objective: To examine the methanolic extracts of leaves of *Fenugreek* as antibacterial and cytotoxic effects, and phytochemical screening of crud extract with isolation of phenolic compound **Methods:** The antibacterial property of Fenugreek leaf extracts was evaluated towards two bacterial species utilizing the agar well diffusing technique. Additionally, the cytotoxic impact of Fenugreek leaf extract has been established by conducting using MTT test in 96-well plates. These MCF-7 cell line has been cultured using a medium of RPMI-1640 enriched by 10% Fetal bovine serum, along with 100 µg/mL streptomycin and 100 units/mL penicillin. These cells subsequently circulated by employing Trypsin-EDTA and afterward reseeded when they reached 80% confluence two times per week. The incubation of the cells was done at a temperature of 37 °C. The isolation of phenolic acid was achieved by the use of TLC, High-performance liquid chromatography (HPLC), as well as spectroscopic observations. **Results:** It has been established that Fenugreek leaves include flavonoids, triterpenoids, alkaloids, carbohydrates, and phenolic acid. Both Staphylococcus aureus and Escherichia exhibit susceptibility to the antibiotic capabilities of the methanol-based extract derived from Fenugreek leaves. Furthermore, the utilization of a plant extract derived from Fenugreek leaves has been seen to decelerate the growth of prostate cancer tumors, hence highlighting the significant anticancer attributes of this extract. **Conclusions:** It is observed from the research that extracts from Leaves of *Fenugreek* may be utilized to treat illnesses brought on by the examined organisms and indicate that whole plant extracts have cytotoxic effects against prostate cancer cells.

Keywords: *Fenugreek,* Methanolic extract, Anticancer, Antibacterial, and leaves

Introduction

Cancer has emerged as a highly formidable ailment during the twentieth century and has continued to exhibit an upward trend in prevalence throughout the 21st century. On a worldwide scale, it is projected that over 3,000 individuals will succumb to cancer daily, resulting in an approximate overall death rate of 650,000 [1]. According to the cited source, the annual rise in the incidence of cancer within the general population is 1.7%. Cancer comprises a pathological condition defined by the presence of genetically unstable cells and a disruption in the regulatory mechanisms that govern cellular development and differentiation [3]. There exists a wide array of cancer types, with breast as well as lung cancers being the most prevalent in the United States. The primary etiology of cancer is attributed to DNA damage, as deoxyribonucleic acid (DNA) is ubiquitously present within all cells of the human body and governs the regulation of cellular processes. The human body often possesses the capacity to mend damaged DNA, although it cannot heal malignant cells. Additionally, there are other strategies for mitigating the risk of developing cancer, such as adhering to a dietary regimen abundant in fruits, and vegetables including whole grains, sustaining a healthy body weight, minimizing excessive exposure to solar radiation, and consuming low-fat food items, among others. The standard therapeutic approaches for cancer encompass a range of interventions, such as surgical procedures, radiation therapy, biological therapy, hormone therapy, transplants of stem cells, as well as chemotherapy. The physician has the option to use a singular approach or a confluence of many methods, contingent upon the specific classification and site of the malignancy, the patient's age, and their overall state of well-being. Regrettably, the utilization of traditional therapies is accompanied by adverse consequences that render their overall efficacy questionable. To mitigate these adverse effects, the researchers are actively exploring alternative therapeutic approaches to address the issues mentioned above [4]. There seems to be a notable increase in the prioritization of research about alternative medicine and complementary therapies in the context of cancer care [5]. The current study is focused on investigating the natural compounds derived from plants, which have been proven to possess significant implications in the field of chemotherapy [6]. Numerous research has been undertaken on the utilization of plants across various ethnobotanical contexts. Approximately 3,000 plant species have been identified as potential sources of strong anticancer medicines, with qualities that can be utilized for the prevention or suppression of various types of malignancies [6]. In the US, been 4 categories of anticancer medicines derived from plants available in the market [7]. Numerous studies are now being done to identify new natural products and associated semi-synthetic equivalents as efficacious anticancer agents [8]. Trigonella foenum-graecum L. sometimes referred to as fenugreek, is an annual leguminous plant that belongs to the Fabaceae family. It is widely grown and harvested for the seeds that it produces, making it a valuable cash crop. The plant under consideration is indigenous to Afghanistan, Iraq, the Islamic Republic of Iran,

and Pakistan, and has since been brought to several other nations [9]. The utilization of fenugreek seed extract as a flavoring component is employed in the production of alternative syrups that possess the taste of maple nor hydrolyzed plant-based proteins [10]. Furthermore, it finds application as a flavoring agent for tobacco, a fundamental constituent for perfumes, and an important source of steroid sapogenin chemicals within the medicinal sector [11]. Fenugreek plants are frequently ingested as a culinary vegetable and serve as a seasoning component in Indian cuisine, where the plant is usually referred to as 'methi' [10,12]. Fenugreek has historically been employed for alleviating symptoms associated with many respiratory conditions, such as colds, bronchial problems, influenza, asthma, catarrh, pleurisy, sinusitis, sore throat, pneumonia, hay fever, tuberculosis, laryngitis, as well as emphysema. Moreover, several investigations have indicated that the plant known as fenugreek has several physiologically active chemicals that have been extensively characterized for their pharmaceutical characteristics (13,14). The painkilling properties of fenugreek leaf extracts were shown in rats using the 5-HT paradigm [15]. Hepatoprotective properties of seed extracts were observed in mice exhibiting dyslipidemia along with oxidative stress resulting from alcohol as well as monosodium glutamate exposure [16,17]. Multiple studies have provided evidence supporting the regulatory effects of fenugreek on total cholesterol, triglyceride, at levels of low-density lipoprotein (LDL) [18,19]. This potential has been established in studies utilizing rodent models. Previous studies have reported that saponins, a primary component of fenugreek, undergo conversion into sapogenins inside the gastrointestinal system, resulting in a reduction in blood cholesterol levels [20,21]. The antidiabetic effectiveness of fenugreek has been shown through its ability to enhance insulin production and reduce diabetes-related insulin resistance in several tissues. Additionally, the study elucidated that fenugreek seeds stimulated the activity of insulin receptor substrate-associated phosphoinositide 3 kinase (PI3K) [22]. Multiple genes, including the insulin receptor, are phosphorylated by the PI3K p85 subunit to do this, as demonstrated in vitro. A fenugreek seed extract has also been shown to reduce lipid peroxidation by regulating the activities of antioxidant enzymes [23,24]. This was discovered after much research was conducted. The research also showed that antioxidant capability significantly reduced cisplatin-induced nephrotoxicity and hepatotoxicity, suggesting a promising strategy for mitigating the harmful effects of chemotherapy [25]. It has also been suggested that fenugreek seeds can protect against lipid peroxidation and increase the activity of antioxidant enzymes [26]. This idea is supported by a plethora of studies. The putative anti-cancer properties of fenugreek have been described, however, there has been little investigation of these properties in both laboratory (in vitro) and live organism (in vivo) investigations. These studies have explored the effects of fenugreek on many forms of cancer, especially breast cancer[27]. Fenugreek is composed of several chemical elements, among which steroidal sapogenins are included. The presence of a Diosgenin compound has been identified in the lipid-rich embryo of fenugreek. Fenugreek has been shown to contain two furastanol

glycosides, which are precursors of diosgenin with an opened F-ring. These glycosides were additionally discovered as hederagin glycosides. The stem contains alkaloids, including nicotinic acid, trigocoumarin, trimethyl coumarin, as well as trigonelline. The mucilage constitutes the most prominent component of the seeds [28]. The composition of the substance includes around 28% mucilage, a volatile oil, and two alkaloids, namely trigonelline and choline. Additionally, it contains flavonoids such as quercetin, rutin, vitexin, isovitexin, and phenolic acid [29,30]. The objective of this work is to conduct a phytochemical examination of plant leaves and assess the in vitro cytotoxic and antibacterial properties of an Iraqi fenugreek leaf extract when applied to the MCF-7 breast cancer cell line, which originates from an Iraqi patient suffering from cancer. This study is deemed distinctive due to its pioneering nature in focusing on an Iraqi community, as well as its investigation of the isolation of phenolic acid.

Figure 1: Fenugreek green leaves

Materials and Methods

Plant material

The collection of fenugreek leaves was placed in October 2022 in the Makishifa Salah Aldin location. The leaves underwent a thorough washing process, followed by drying in a shaded area, as well as were afterward pulverized into a fine powder using a mechanical grinder.

Experimental work

Extraction method (cold method)

A total of 200 gms of ground plant material was placed in 1000 milliliters of methanol above room temperature while being periodically agitated. Following a period of three days, the components that were found to be soluble in methanol were separated using the

process of filtration. The filtrate was subjected to vacuum evaporation utilizing a rotary evaporator until complete dryness was achieved. A residue with a dark greenish hue was acquired. One-half of the leftover is sent to the lab for phytochemical testing and cytotoxic as well as antibacterial activity tests, while the other half is suspended in 100 ml water along successively separated using ethyl acetate (100 ml) up to the organic layer becomes clear lacking color. The final ethyl acetate fraction is then dried throughout anhydrous sodium sulfate, purified, and percolated to dryness to isolate the phenolic compound.

Preliminary phytochemical examination of crude extracts

Crude collects, fractions, as well as powder samples were analyzed by phytochemical methods for identification and screening of reactive chemical ingredients in the plants for medicinal purposes under investigation.

Alkaloid test: Then heated up 8 ml of 1% HCl, added 0.5-0.6 g of every herb extract as well as a fraction, followed by filtration of the resulting solution. The presence or absence of the alkaloids has been identified by treating 2 ml of the filtrate solution with each of Mayer's as well as Dragendorff's solutions and then observing the degree of turbidity or precipitation development.

Test for saponins: Boiling water was used to disperse 0.5 g of every botanical extract as well as a fraction in a tube meant for testing, and then the mixture was cooled as well as agitated quickly to create a froth.

Test for flavonoids: The lipid layer was separated by shaking 0.5 g of every single plant extraction and fractions in petroleum ether. After filtering, 20 ml of 80% ethanol had been added to the defatted residual. A test tube containing 3 ml of each filtrate along with 4 ml of 1% aluminum chloride diluted using methanol has been employed for observing the resulting color change. The existence of flavonoids was evidenced by the production of a yellow tint.

Test for sterols and terpenes: To remove the dye, 0.5 g of extraction, as well as fraction, was agitated with petroleum ether. Ten milliliters of chloroform were used to extract the remaining material, and the resulting chloroform layers were dried using anhydrous sodium sulfate. Five milliliters of the coat of chloroform, 0.25 milliliters of acetic anhydride, as well as a couple of drops of strong sulphuric acid, were combined. The existence of sterol/terpenes was shown to be associated with a variety of hues. Sterols were represented by the green hue, whereas terpenes and triterpenes by the pinkish-purple range.

Test for phenol: De-ionized water is used to create a neutralized ferric chloride (FeCl3) solution, followed by 0.5 g will dissolve therein with a few drops of the solution. For a dark, long-lasting precipitate, add a solution of sodium hydroxide to the mixture. If a color change occurs (to blue, red, green, or purple), phenols are present.

Thin Layer Chromatography Examination of Ethyl acetate extract

The phenolic acid species found within the ethyl acetate sample was identified using a technique called thin-layer chromatography. Gallic acid was found during preliminary TLC testing, the most effective solvent solutions for extraction and maximum spot number have been tested for confirmation. The TLC data are displayed in Figure 2. A fraction weighing only a few milligrams was immersed in around one milliliter of 100% methanol, then spotted onto an analytical TLC plate covered using silica gel GF254 before being processed in mobile phases consisting of chloroform, acetone, as well as formic acid (75:16.5:8.5).

HPLC Conditions for Analyzed Fractions Analysis of phenolic acid

Phenols were analyzed by fast liquid chromatography (FLC) column separation under ideal conditions. Nuclear C18-DB 3um particle size column (50 x 2.0 mm internal diameter), Trifluoroacetic acid (TFA acid) mixed with deionized water (solvent A) and methanol (solvent B), pH 2.5 (mobile phase), were used as linear-gradient mobile phase components. For the next 15 minutes, run a gradient spanning 0%B to 100%B. Rate of flow: 1.1 ml/min. UV light at 285 nm is used for detection.

Equipment

The recovered peaks have been examined by UV -Vis 10 A- SPD while being separated on a Shimadzu 10AV-LC fitted with a Shimadzu LC-10A binary delivery pump.

Maintenance of cell cultures

RPMI-1640 was enriched using 10% Fetal bovine serum, as well as 100 g/mL streptomycin and 100 units/mL penicillin for sustaining MCF-7 cells. Trypsin-EDTA reseeding was performed at 80% expansion twice weekly, and cells endured in a 37 °C incubator[31,32].

Cytotoxicity Assays

The MTT test was used to assess fenugreek's cytotoxicity in 96-well plates [33,34]. The wells were planted with 1 104 cell lines. The cells were stimulated with different fenugreek dosages after 24 hours or immediately after a condensed monolayer. This treatment was three rounds repeated. In cell viability testing, these cells had been rinsed with PBS after 24 hours. A 2 mg/mL MTT solution diluted to 28 liters was then added to the growth medium. Shaking the samples at 37°C for 2.5 hours concluded. To dissolve any leftover crystals, 37 degrees Celsius incubation for 15 minutes after the MTT solution was not used. After rejecting MTT, this was done. Shaking the plates with 130 L of DMSO-containing water solution did this [35]. Three absorbance measurements were taken using a 492 nm-calibrated microplate reader. The formula used to calculate cytotoxicity, which evaluates cell growth inhibition, was:

Cytotoxicity = A-B/A *100

Where A and B are the optical density of control and the optical density of test

Inhibition rate = A- B/A*100
Here, A represents the reference density, B represents the average specimens density (refer to [38, 39]).

Reagents and materials.

Table 1: Reagents Used for the Analysis of cytotoxic Activity

No.	Items	Company	Country
1	Trypsin/EDTA	Capricorn	Germany
2	DMSO	Santacruz Biotechnology	USA
3	RPMI 1640	Capricorn	Germany
4	MTT stain	Bio-World	USA
5	Fetal bovine serum	Capricorn	Germany

Instruments

Table 2: Instruments Used for the Assay of Cytotoxic Activity

No.	Item	Company	Country
1	CO_2 incubator	Cypress Diagnostics	Belgium
2	Microtiter reader	Gennex Lab	USA
3	Laminar flow hood	K & K Scientific Supplier	Korea
4	Micropipette	Cypress Diagnostics	Belgium
5	Cell culture plates	Santa Cruz Biotechnology	USA

Statistical analysis:

Utilizing GraphPad Prism 6 [40], we some unpaired data regarding the t-test we collected. The average standard deviation from measurements done in triplicate was used to create the values[41].

Antibacterial Activity

The antibacterial properties of Fenugreek were evaluated using the gram-positive bacterial strain Staphylococcus aureus (S. aureus). The bacterial strains were incubated at a temperature of 37°C on M–H agar plates. Inoculations were obtained from the recently cultivated plates and transferred into tubes containing 50 ml of nutrient broth (NB). This was done to assess the impact of Fenugreek on the growth curve of the bacteria. The bacterial species were cultivated until the optical density (OD) at 600nm reached 0.1, which corresponds to a bacterial concentration of 108 colony-forming units per milliliter (CFU/ml). Subsequently, the bacteria were introduced into a 50 ml volume of new NB medium, which was supplemented with varying quantities of Fenugreek (62.5, 125, 250, 500 µg/ml). After 24 hrs. incubation at 37°C, with gentle agitation. Bacterial growth with the help of optical density (OD) is calculated along a spectrophotometer after 12 hours [42,43].

Statistical Analysis

The mathematical examination of the information was analyzed using GraphPad Prism 6 [44]. The outcomes were provided as mean ± standard deviation (SD) across three duplicates per operation [45].

Results and Discussion

The initial stage in the isolation of desired organic goods from the foundational elements involves the process of extraction. The specific method of extraction is contingent upon the nature of the material being separated. Two extraction methods were employed, utilizing varying temperatures, for the extraction of a specific component from a plant. The determination of the optimal method as well as a solvent to feed extraction was made by evaluating the percent of yield achieved from every approach, as well as conducting a thin-layer chromatography (TLC) examination on the natural extract to identify its elements. The careful consideration of solvent choice is of utmost importance in the process of solvent extraction. Factors such as selectivity, the ability to dissolve, expense, and safety must be taken into account when selecting solvents. This selection process should be guided by the principle of similarity as well as impermissibility, where solvents that have a polarity value that closely matches that of the substance are expected to yield superior results, while those with contrasting polarities are probably going to be less effective.

Phytochemical screening of crude extracts

Several qualitative phytochemical testing tests were conducted to analyze the chemical structure of the basic extract. The test outcomes are significant as they provide valuable insights into the types of secondary metabolites that are present in the plant, thereby aiding in the development of an appropriate procedure for isolating these compounds from each of the extracts. The initial analysis of crude leaf extracts indicated the existence of flavonoids, alkaloids, phenols, as well as terpenoids. The findings of these examinations are succinctly depicted in Table 3.

Table 3: Phytochemical composition of leaves, in qualitative terms.

Extract	Flavonoids	Alkaloids	Phenols	Saponin	Terpenoids
Leaves	+	+	+	+	+

Thin layer chromatography examination of ethyl acetate

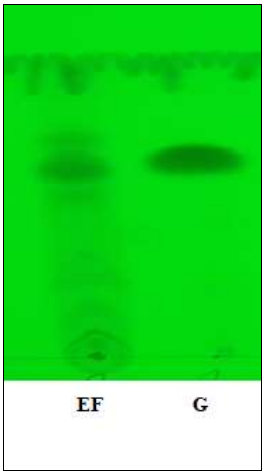


Figure: Gallic acid (G) & ethyl acetate (EF) fractions are separated using thin layer chromatography using Silica Gel GF254. Created with the Solvent System (Chloroform: Acetone: Formic Acid (75: 16.5: 8.5)), UV Light 254 nm Detection.

HPLC of Ethyl Acetate Fraction

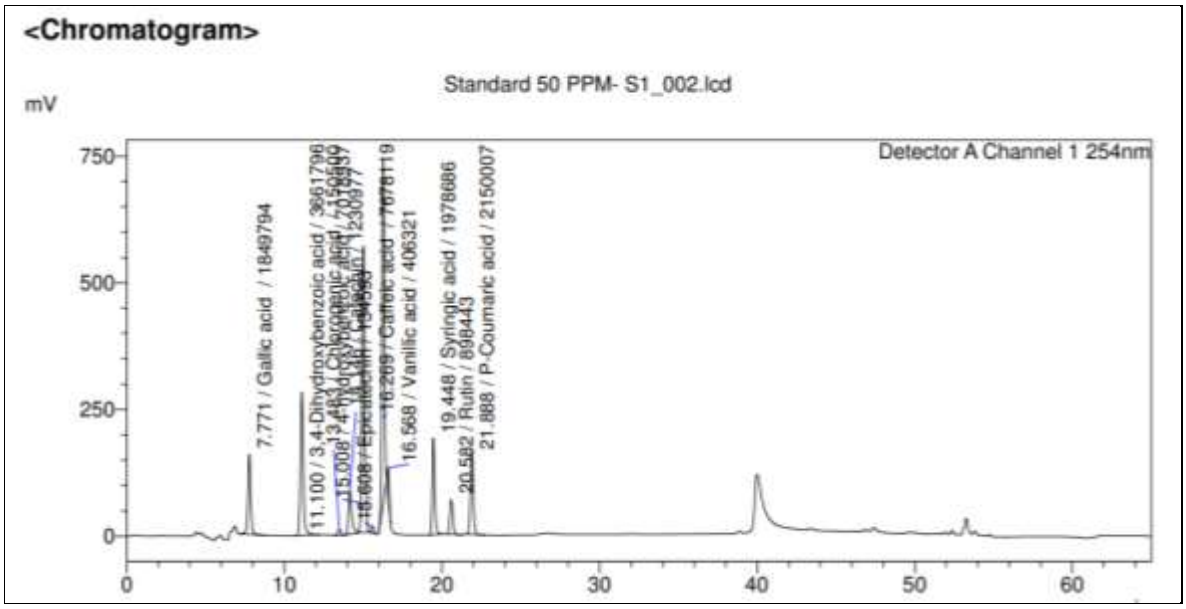


Figure 3: HPLC Chromatogram of Ethyl Acetate

Table 4: Retention Times in Minutes of Standard Materials Standard materials

Detector A

ID#	Name	Ret. Time	Area	Height	NTP	Asymmetry	Resolution	Mark
1	Gallic acid	7.771	1849794	157708	11219	1.015	--	

3,4,5-trihydroxybenzoic acid

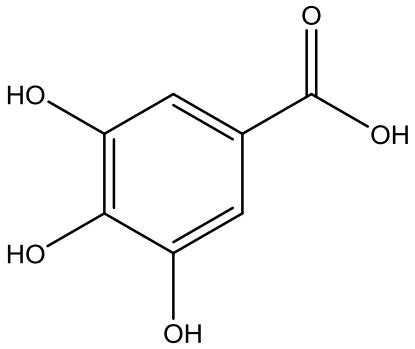
Chemical Formula: $C_7H_6O_5$

Exact Mass: 170.02

Molecular Weight: 170.12

m/z: 170.02 (100.0%), 171.02 (7.6%), 172.03 (1.0%)

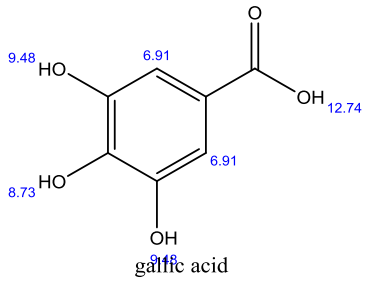
Elemental Analysis: C, 49.42; H, 3.56; O, 47.02



gallic acid

Figure 4: Chemical structure of isolate gallic acid shown by Mass spectroscopy

ChemNMR ^{1}H Estimation



Estimation quality is indicated by color: good, medium, rough

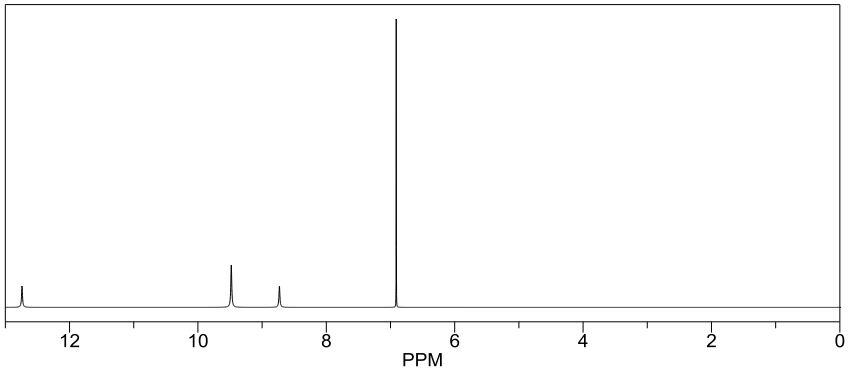
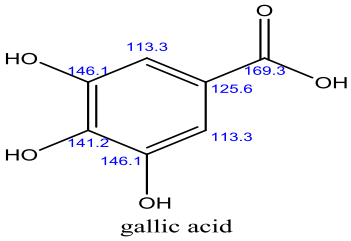


Figure 5: ^{1}H NMR of Isolated Compound gallic acid from Ethyl acetate Fraction

ChemNMR ^{13}C Estimation



Estimation quality is indicated by color: good, medium, rough

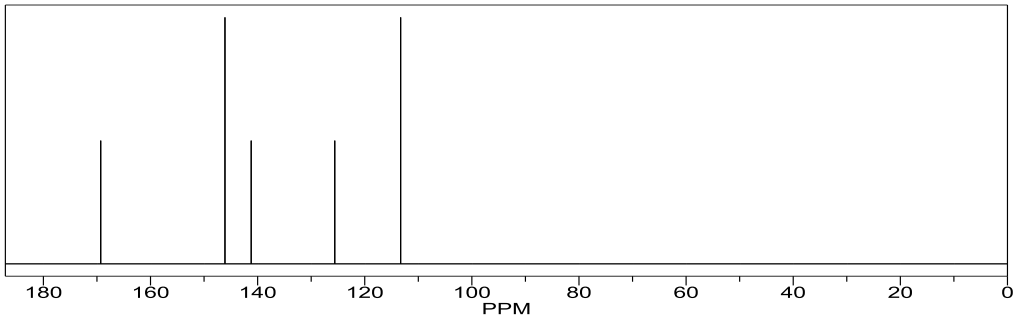


Figure 6: ^{13}C NMR of Isolated Compound gallic acid from Ethyl acetate Fraction

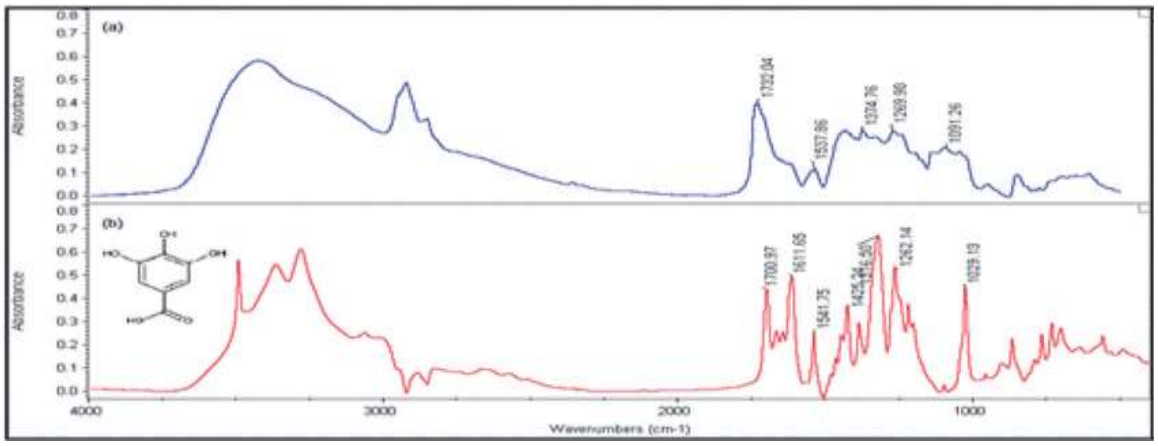


Figure 7: UV spectra of Isolated Compound gallic acid with standard.

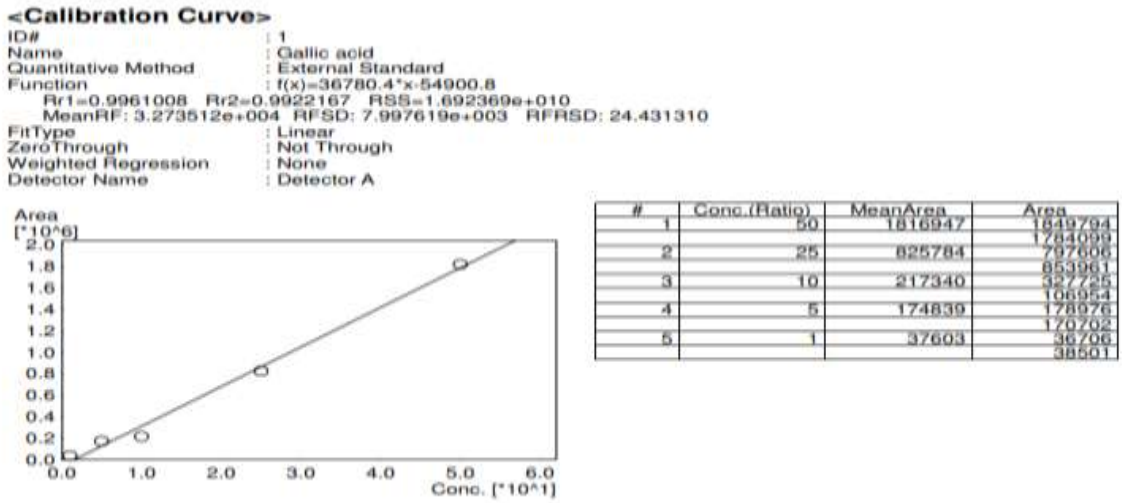


Figure 8: Calibration curve of Isolated Compound gallic acid from Ethyl acetate Fraction.

Effect of cytotoxicity *Fenugreek* Against MCF-7 breast cancer cell line

Fenugreek's cytotoxicity impact on cancer cells has been studied. Fenugreek's potential to prevent breast cancer growth MCF-7 cell line has been studied to determine its antiproliferative effect. Fenugreek, has already studied the cytotoxicity impact on the MCF-7 cell line, as depicted in Figure (9).

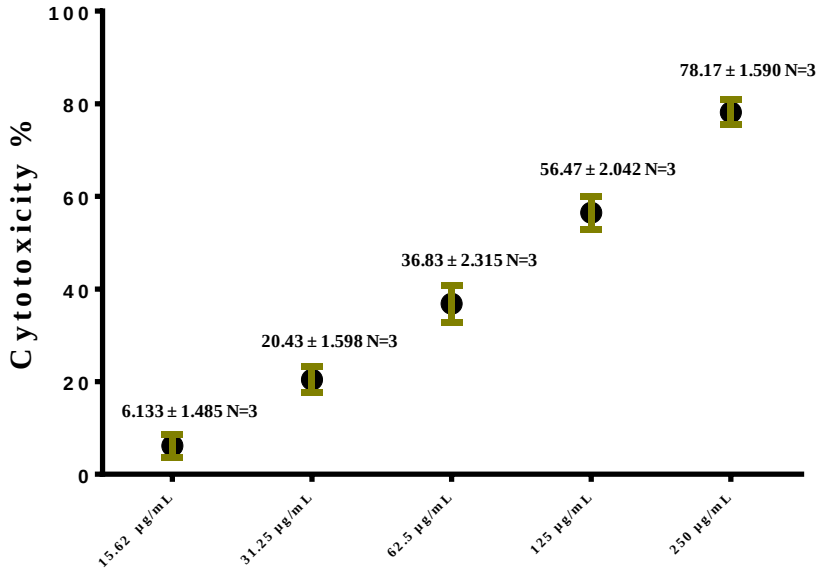


Figure 9: Cytotoxicity of *Fenugreek* extract in MCF-7 cells.

The current study checks the cytotoxic effect of Fenugreek on cancer cells. The anticancer efficacy of Fenugreek was assessed by an investigation of its capacity to impede the multiplication of cancerous cells. The findings of this investigation demonstrated a substantial and statistically significant cytotoxic effect on mankind's tumor cell lines, as seen in Figure 3.1. The findings indicate that Fenugreek possesses inhibition capacity over the proliferation of cell lines, although this effect seems contingent upon the concentration that contains the substance. Herbal remedies have long been employed in conventional medical practices for several ages, serving as natural treatments that possess well-documented therapeutic properties in various domains. These include the prevention of cardiovascular ailments, as well as the provision of anti-inflammatory, antibacterial, and anticancer effects. Moreover, the development of immunity to cancer treatment has compelled researchers to shift their focus toward natural compounds derived from plants as well as marine sources. The current scientific research extensively examines the anticancer abilities of various compounds derived from plants. However, there is a growing acknowledgment that the beneficial impacts of plants are somewhat a result of the intricate interaction among the combination of compounds found in the entire plant. This interaction may appear complementary, synergistic, antagonistic, or a combination thereof, rather than solely relying on individual constituent agents by default.

Antibacterial activity

In recent times, there has been a significant focus on the study of extracts and physiologically active chemicals derived from widely recognized plant species. Medicinal plant usage has important importance in addressing the fundamental healthcare requirements in underdeveloped nations. These plants have the potential to serve as a

novel reservoir of antibiotics, fungicides, and antiviral compounds that exhibit substantial efficacy against pathogenic microbes. The antibacterial property of the methanol extract of Fenugreek generated by maceration demonstrates significance in inhibiting the growth of Gram-positive microorganisms. Methanolic extracts anti-bacterial natural derived from Fenugreek have been observed against S. aureus. The traditional application of this botanical specimen in the management of infectious ailments exhibits considerable potential, particularly in combating bacterial pathogens. The ongoing process involves the purifying of the bioactive constituent(s) from the extracts, intending to enhance our comprehension of potential antibacterial and antifungal properties.

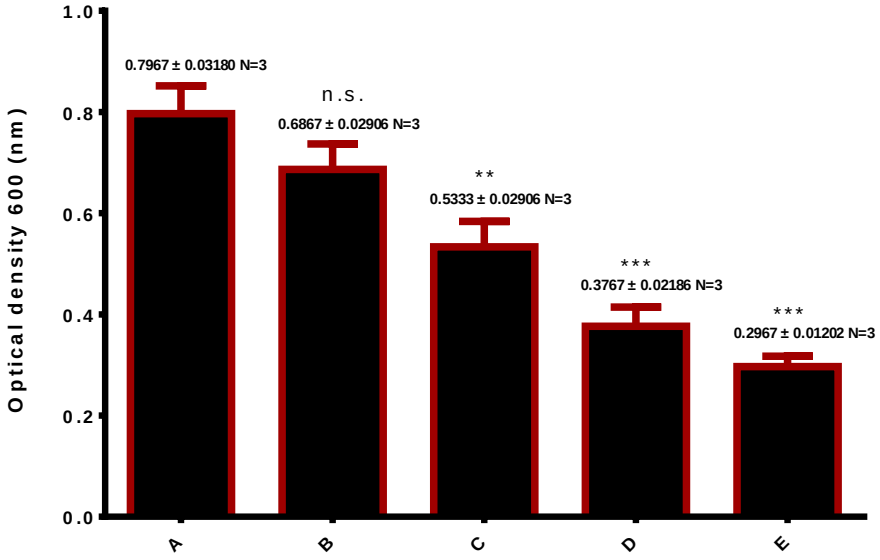


Figure 10: Antibacterial activity of Fenugreek against *S. aureus*. The obtained outcomes are provided as the mean ± SD. A Control untreated bacteria. B, Bacteria treated with F at concentration 62. 5 µg/ml. C, Bacteria treated with F at a concentration of 125 µg/ml. D, Bacteria treated with F at a concentration of 250 µg/ml. E, Bacteria treated with F at a concentration of 500 µg/ml.

Conclusions

The achieved conclusions from the present research are:

- The phytochemical analysis of Fenugreek growing in Iraq reveals the existence of several extracted phytochemicals with specific chemical characteristics obtained from the plant's leaves.
- HPLC, and Liquid chromatography-tandem mass spectrometry (LC-MS-MS), are robust methods utilized for the determination of substances inside a combination, relying on their molecular weight, retention duration, and MS/MS fragmentation patterns, which are essential for further investigations.

- The majority of the findings from this study align with the outcomes of previous research conducted by foreign scholars on the same plant species.
- The initial investigation has provided empirical data indicating that the methanolic extract derived from a cold technique of Iraqi Fenugreek leaves demonstrates noteworthy cytotoxic activity concerning cells of breast cancer, as well as antibacterial properties.

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