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Research Article

Evaluation of the Antibacterial and Anti-biofilm Impact of Fenugreek (*Trigonella foenum-graecum* L.) Seed Extracts against some Antibiotic-Resistant Pathogenic Bacteria

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Abstract:

Background: The prevalence of multidrug-resistant bacteria has demonstrated a need to develop and use alternative antimicrobial agents. There has been a growing interest in medicinal plants and herbs and their extracts.

Objective: To identify the antibacterial and anti-biofilm activity of aqueous and alcoholic extracts of the Fenugreek (*Trigonella foenum-graecum* L.) seed against multidrug-resistant clinical bacterial isolates (Gram-positive *Staphylococcus aureus* and Gram-negative *Acinetobacter baumannii*).

Methods: Aqueous and alcoholic extracts were prepared from the seeds of the Fenugreek (*Trigonella foenum-graecum* L.) plant. A gas chromatography-mass spectrometer was used to identify the active compounds in the extracts. They were tested against multidrug-resistant bacterial isolates that produce biofilms (Gram-positive and Gram-negative), namely *Staphylococcus aureus* and *Acinetobacter baumannii*, which were isolated from Iraqi patients in Baghdad Medical City Hospitals (including Burns Specialized Hospital, Baghdad Teaching Hospital, and Al-Shahid Ghazi Al-Hariri Hospital for Surgical Specialties), Al Kadimyia Teaching Hospital, and Al-Kindy Teaching Hospital, from October 2023 to March 2024.

Result: The current study has proved that the aqueous and alcoholic extracts of Fenugreek seeds were effective as antibacterial and anti-biofilm against the studied bacterial isolates, in all the tested concentrations, with significant differences. The study also showed that the aqueous extract of Fenugreek was more effective as an anti-bacterial and anti-biofilm than the alcoholic extract. The aqueous extract demonstrated high inhibitory activity, ranging from 52.9% to 97.5%, while the inhibition of the alcoholic extract varied from 56.0% to 99.5%.

Conclusion: The present study supports the use of fenugreek seed extract to treat pathogenic bacteria that have developed a resistance to antibiotics.

Keywords: *Acinetobacter baumannii*; Antibacterial activity; Anti-biofilm impact; Multidrug-resistant bacteria; *Staphylococcus aureus*; *Trigonella foenum-graecum* L.

Introduction:

Antibiotic resistance, according to the World Health Organization (WHO), poses an imminent threat to worldwide public health. The emergence of multidrug-resistant bacteria worsens the situation, and humanity is destined the greater mortality and morbidity from microbial illnesses (1). The medicinal qualities of plants have long been identified. Conventional medicine is increasingly acknowledging the use of plant-derived antibacterial and other medications (2). New diseases cannot be managed by classical antibiotics (products of microorganisms or their synthetic equivalents) when they stop working (1). Approximately 80% of people globally use medicinal herbs as their major source of healthcare due to its

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Since the beginning of civilization, people have been interested in plant-based treatments. Since ancient times, medicinal plants have been used to make medicinal herbs, which are used to cure a wide range of ailments. This is due to the antimicrobial qualities of medicinal plants, which make them advantageous therapeutic sources (3). The growing interest in medicinal plants and their active ingredients is a result of microorganisms' developing resistance to commercially accessible treatments (4). Numerous herbal plant species have been shown in tests to be effective in eradicating bacteria, viruses, fungus, and parasites while reducing their cytopathogenic effects. This implies that they could be used to treat diseases all over the world (5).

An annual crop of the leguminous family, fenugreek is *Trigonella foenum-graecum* L. The ability of this

South Asian plant's seed to reduce blood glucose and cholesterol levels has been demonstrated to offer a number of health benefits, including the potential to help prevent and treat diabetes and coronary heart disease. "Foenum-graecum" means "Greek hay", suggesting that it was formerly a pasture crop. The Mediterranean region is thought to be the native home of fenugreek (6). Nonetheless, a large portion of the world now grows it as a spice. Sections of Europe, north Africa, west and south Asia, Argentina, Canada, the United States, and Australia have all been reported to plant it (7, 8). India is the world's largest producer of fenugreek (9). Numerous studies have linked the phenolic components of spices and herbs to their antibacterial, antioxidant, and therapeutic properties (10). Numerous pathogens, including bacteria and fungi, are susceptible to the antibacterial properties of fenugreek seed extract (11, 12). Fenugreek's phytoconstituents and derived products, including flavonoids, alkaloids, terpenoids, steroids, saponins, anthocyanin, tannin, and others, help to balance cholesterol, lower blood sugar, cure skin inflammation (wounds, rashes, boils), and treat arthritis, asthma, and sore throat. The presence of special compounds such alkaloids, flavonoids, phenols, tannins, and saponins gives the plant its therapeutic potential. (13).

The current study intended to assess the antibacterial and anti-biofilm effects of aqueous and alcoholic Fenugreek seed extracts against antibiotic-resistant bacteria, given the plant's medical significance and the paucity of research on its efficacy against antibiotic-resistant pathogenic bacteria. Additionally, gas chromatography-mass spectrometer (GC-MS) analysis was performed to determine the bioactive ingredients in fenugreek.

Materials and Methods

Identification of Bacterial Isolates: Samples of wound, burns, and sputum specimens were collected from male and female Iraqi patients admitted to Baghdad Medical City Hospitals (including Burns Specialized Hospital, Baghdad Teaching Hospital, and Al-Shahid Ghazi Al-Hariri Hospital for Surgical Specialties), Al Kadimyia Teaching Hospital, and Al-Kindy Teaching Hospital. From the period from October 2023 to March 2024. Five bacterial isolates were collected from Iraqi patients, diagnosed by preliminary diagnostic laboratory tests using Gram stain, and different biochemical tests (Motility test, Hemolysis, Catalase production, Coagulase, Oxidase test, Urease production, Nitrate Reduction, Indole production, MR (Methyl Red), VP (Voges Proskauer) according to Bergey's manual (14), in addition to the VITEK2 system. Three of them were Gram-positive (*Staphylococcus aureus*), and two were Gram-negative (*Acinetobacter baumannii*). The ability of these isolates to produce biofilms was tested according to Pokhrel *et al.et al.*. (15), and they were vigorous biofilm producers.

Antibiotic susceptibility test: Susceptibility to various classes of antibiotics was tested by the disk diffusion method (Kirby-Bauer method) according to Clinical Laboratory Standard Institute recommendations (CLSI, 2023). The following antibiotics were used against*A. baumannii* Piperacillin, Ticarcillin-clavulanate, Ceftazidime, Imipenem, Gentamicin, Amikacin, Tetracycline, Ciprofloxacin, Levofloxacin, and Trimethoprime-sulfamethoxazole. While these antibiotics were used against*S. aureus* Azithromycin, Nitrofuration, Cefoxitin, Doxycycline, Levofloxacin, Rifampin, Clindamycin, Vancomycin, Chloramphenicol, and Trimethoprime-sulfamethoxazole.

Preparation of Extract: For the current study, the Fenugreek (*Trigonella foenum-graecum*) seeds were purchased from the local markets in Baghdad. A botanist (specialist in plant classification) in the Department of Biology / College of Science for Women identified the samples. Taxonomical Classification According to (17), *Trigonella foenum-graecum* L. was classified as follows: Kingdom: Plantae, Division: Magnoliophyta, Class: Magnoliopsida, Order: Fabales, Family: Fabaceae Genus: Trigonella, Species: *Trigonella foenum-graecum* Linn.

Preparation of Alcoholic Fenugreek Seed Extract (FSE): Sultana *et al.et al.*. (2009) performed an alcoholic extraction of fenugreek seeds (16). With a few modifications, in separate experiments, 100 g of fenugreek seeds were extracted using the solvent aqueous alcohol methanol: Water, ethanol (1:1:3) (80% v/v) (500 mL) for 8 hours under Soxhlet on a water bath. To concentrate the extract, a rotary evaporator was utilized.

Preparation of Hot Aqueous Fenugreek Seed Extract: To obtain the plant's hot watery extract, the following procedure was used (18): Weigh 100 grams of plant powder and place it in a 1-liter glass beaker. Add 500 milliliters of boiling distilled water. It was placed in a shaking incubator at 45 degrees Celsius for 30 minutes. The mixture was filtered through gauze and centrifuged at 4000 rpm for 20 minutes. The liquid was collected and concentrated using an evaporator rotary device under vacuum pressure and at 45°C, after which the sample was placed in an oven at 45°C for (22-24) hours to obtain the dry powder, which was stored in the refrigerator until use.

Gas-Chromatography–Mass Spectrometry (GC–MS) Analysis of Fenugreek Seed Extracts: The active chemicals in the FSE were discovered using a Japanese gas chromatography-mass spectrometer (Shimadzu QP-2010 Plus). This was performed by comparing the absorbance of the unknown chemicals to that of the stored and known components in the National Institute of Standards and Technology

(NIST) database. The different components were identified by comparing the mass spectral data with the Wiley 229 mass spectrometry libraries and the National Institute of Standards and Technology (NIST12 or NIST62).

Antibacterial Activity of Fenugreek Seed Extracts by Determination of Minimum Inhibitory Concentration (MIC): The microtiter plate technique was performed to analyze the MIC and MBC of Alcoholic and aqueous Fenugreek Seed Extract against Gram-negative and Gram-positive bacterial isolates, using a 96-well microtiter plate aided by the resazurin dye in Mueller-Hinton broth (MHB), as described by Strompfová *et al.* (19).

Anti-biofilm Activity of Alcoholic and Aqueous Fenugreek Seed Extract: Anti-biofilm activity of FSE was done according to Haney *et al.*. (20), with some modifications as follows: Using 180 microliters of Mueller-Hinton broth supplemented with 1% glucose, microtiter plates were created by adding 20 microliters of suspended bacteria and 10 microliters of alcoholic and aqueous fenugreek seed extract at the Sub-MIC concentration. Microplates are incubated for a full day at 37°C. The clinging cells are cleansed. The liquid media is disposed of, and the wells are dried at 37°C for a maximum of one hour. It is then colored for 15 minutes using 200µl of 1% crystal violet. The microplate wells stained with crystal violet are then washed by distilled water to get rid of the stain. Following air drying of the wells, the biofilm dye lining the microplate walls is re-solubilized using 200 µl of 95% ethanol. The optical density (OD) at 580 nm is measured over a period of 5 - 10 minutes using a spectrophotometer, more precisely an ELISA reader. The following formula was used to determine the biofilm inhibition percentage:

$$Biofilm\ reduction\ (\%) = \left(\frac{OD_{control}}{OD_{sample}}\right) \times 100\ \%$$

Statistics Analysis: The IBM SPSS 26.0 program was used for continuous data, and the mean, standard error of the mean, and probability were determined using the independent t-test and ANOVA table. Pearson's chi-square was used to determine associations between variables (21).

Results:

Identification of Bacterial Isolates: The identification of five bacterial isolates showed that it had *Staphylococcus aureus* and *Acinetobacter baumannii* (Table 1), confirmed by using the VITEK 2 system. A microtiter plate assay was also used to detect the ability of the bacterial isolates to form a biofilm, through a rapid screening method that was sensitive enough as a quantitative method for biofilm screening. The results were based on the OD 580 value of the five chosen clinical bacterial isolates that appeared to be strong biofilm producers.

Table (1): Microscopic and biochemical characteristics of bacterial isolates

Characteristics	*Staphylococcus aureus*	*Acinetobacter baumannii*
Gram stain	Positive	Negative
Shape	Cocci	Coccobacillus
Capsule	Non-Capsulated	Capsulated
Spore	Non-Sporing	Non-Sporing
Motility	Negative (Non-motile)	Negative (Non-motile)
Hemolysis	Positive Beta	Negative
Catalase	Positive	Positive
Coagulase	Positive	Negative
Oxidase	Negative	Negative
Urease	Positive	Negative
Nitrate Reduction	Positive	Negative
Indole	Negative	Negative
MR (Methyl Red)	Negative	Negative
VP (Voges Proskauer)	Positive	Negative
Citrate	Positive	Positive

Antibiotic susceptibility test: The Kirby-Bauer method (Disc Diffusion Method) had been employed to evaluate the susceptibility of all Five isolates of bacteria. All *A. baumannii* isolates showed high resistance to all the aminoglycoside antibiotics used. In contrast, all Staphylococcus aureus isolates were resistant to Azithromycin, Doxycycline, Chloramphenicol, Clindamycin, Levofloxacin, Trimethoprime-sulfamethoxazole, Levofloxacin, Gentamycin.

GC-MS Analysis of Alcoholic and Aqueous Fenugreek Seed Extract: The chemical compositions of fenugreek seed alcoholic and aqueous extracts (FSE) were determined by GC-MS (Figures 1 and 2). Tables 2 and 3 list their compositions and the percentage of the composition area.

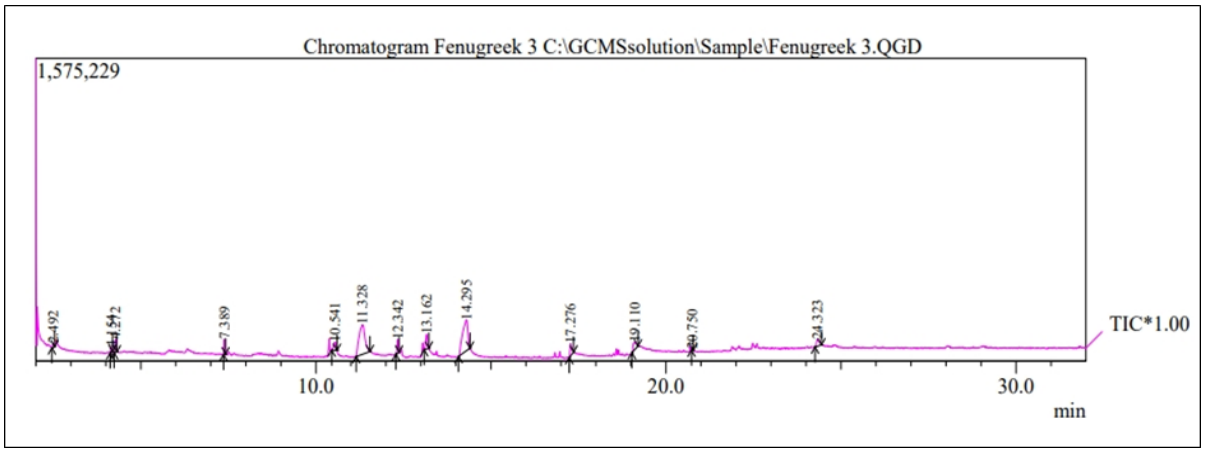

Figure 1: Chromatogram obtained from the GC/MS with the alcoholic Fenugreek extract

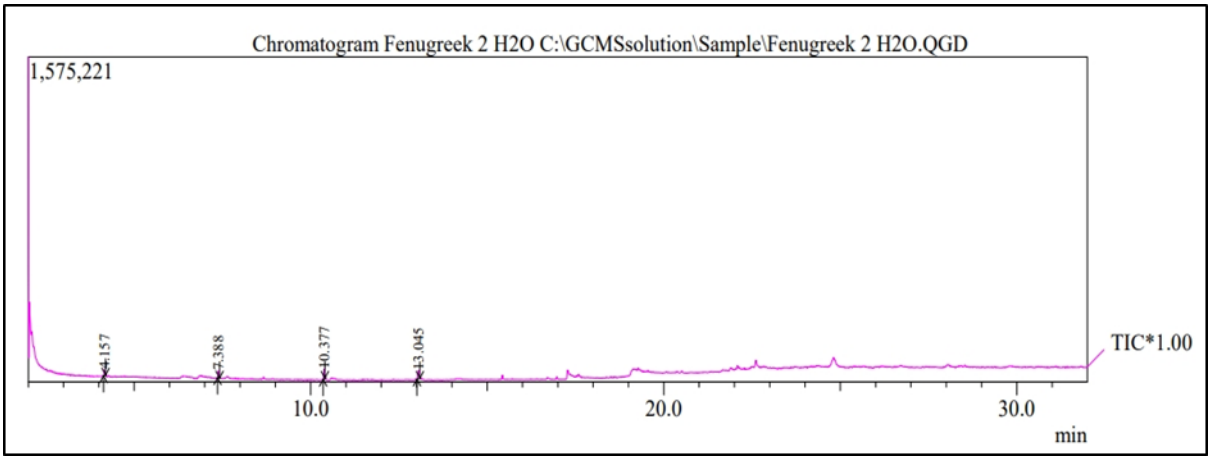

Figure 2: Chromatogram obtained from the GC/MS with the aqueous Fenugreek extract

Table 2: GC-MS analysis results of alcoholic Fenugreek seeds extract

Active group	Area%
Alkaloids	47.6
Terpenoids	9.4
Coumarin	39.0
Flavonoid	4.0
Total	100.0

Table 3: GC-MS analysis results of aqueous Fenugreek seeds Extract

Active groups	Area%
Terpenoids	66.1
Resins	33.9
Total	100.0

According to the results of GC-MS analysis, the fenugreek seeds alcoholic extract (FSE) contained 47.6% Alkaloids. Six varied retention times were discovered, which were (4.154), (11.328), (12.342), (19.110), (20.750) and (24.323) minutes, respectively. The three varied retention times of 39.0% Coumarin were (2.492), (13.162), and (14.295) minutes, respectively. The three varied retention times of 9.4% Terpenoids were (4.272), (7.389), and (10.541) minutes, respectively. The retention time of 4.0% Flavonoid was (17.276). The result of the aqueous extract appeared to contain 66.1% Terpenoids at three varied retention times of (4.157), (7.388) and (10.377) minutes, respectively. The retention time of 33.9% resins was (13.045).

Antibacterial Activity of Fenugreek Seed Extracts by Determination of Minimum Inhibitory Concentration: The aim was to detect the minimum inhibitory concentrations (MICs) of alcoholic and aqueous FSE against isolates of the control groups (P+, N-). All of the test wells were blue at first, but after two to four hours of incubation, a few of them developed a pink colour, which could indicate the growth of bacteria (Figure 3: A and B). The presence of alkaloids and polyphenols, which acted as antibacterial agents through many pathways, as

previously described, could be responsible for the greater antibacterial activity of the extracts, Table 4.

Table 4: MICs of alcoholic and aqueous Fenugreek seed extract against *S. aureus* and *A. baumannii* determined via microtiter plate method

Isolates	alcoholic FSE MIC (mg\ml)	alcoholic FSE Sub-MIC (mg\ml)	Aqueous FSE MIC (mg\ml)	Aqueous FSE Sub-MIC mg\ml
S1	500.0	250.0	83.0	42.0
S2	250.0	125.0	41.5	21.0
S3	62.5	31.3	20.8	10.4
A1	250.0	125.0	83.0	41.5
A2	125.0	62.5	41.5	20.8

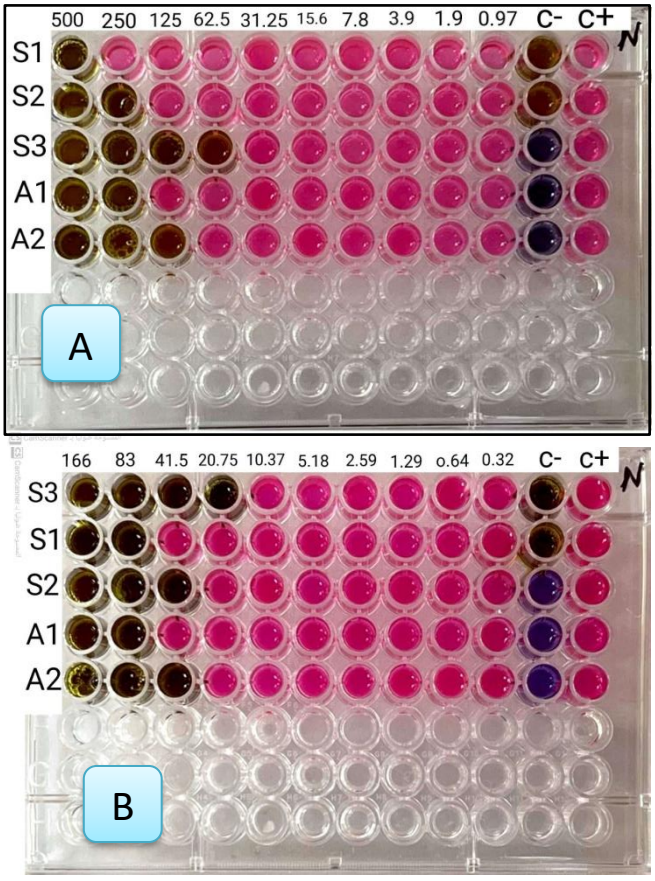

Figure 3: Microtiter plate 96-well for determination of the MICs of alcoholic FSE (A) and aqueous FSE (B) against *S. aureus* and *A. baumannii*

Anti-biofilm Activity of Fenugreek Seed Extracts:
For determining an agent's efficiency against biofilm production, an ELISA microplate reader was used. The effect of the alcoholic and aqueous Fenugreek seed extracts on the production or inhibition of a biofilm of the studied isolates that produced a strong biofilm was investigated using the sub-minimal inhibition concentrations of alcoholic and aqueous Fenugreek seed extracts (in the aqueous extract it was from 10.37 to 41.5 mg\ml and in the alcohol extract it was from 31.25 to 250 mg\ml), Table 5.

Table 5: OD of fenugreek seed extracts against bacterial isolates

Bacterial isolates	Alcoholic FSE inhibition % mean ± SE	Aqueous FSE inhibition % mean ± SE
Staphylococcus aureus		
S1	90.8 ± 3.47 ^{A}	85.5 ± 2.95 ^{B}
S2	95.4 ± 2.07 ^{A}	72.7 ± 2.88 ^{B}
S3	87.0 ± 3.59 ^{A}	52.9 ± 6.52 ^{B}
Acinetobacter baumannii		
A1	99.5 ± 0.36 ^{A}	97.5 ± 1.35 ^{A}
A2	56.0 ± 8.52 ^{A}	80.6 ± 3.68 ^{B}

***Duncan test: Similar letters indicate no significant differences (P > 0.05) between the inhibition ratios of extracts used to inhibit the growth of the same bacterial isolate.**

Discussion:
Genetically flexible bacteria can adjust to a variety of environmental stressors, such as the presence of antibiotic drugs. As a result, bacteria that live close to organisms that produce antibiotics through developing defenses that enable them to endure their presence. Growing antibiotic resistance is a global health concern. This boosted efforts to find therapeutic medications other than antibiotics (22).
Five *S. aureus and A. baumannii* isolates that were known to generate biofilms and exhibit multidrug resistance were chosen for the investigation. These were the perfect amounts to stop *S. aureus and A. baumannii* from growing. In the current study, fenugreek seed extracts caused a reaction in both Gram-positive and Gram-negative bacterial strains in agreement with the findings of Behzadi *et al.* (23) and Alenazy (24). Gram-positive and Gram-negative bacterial strains react differently, which can be explained by significant structural differences. For instance, the bacterial walls of the former have many holes and thin peptidoglycan layers, among other virulence characteristics.
On the other hand, both aqueous and alcoholic extracts exhibited a higher activity on most of the bacterial strains. This disagrees with the findings of Okoh *et al.* (25) and Singh *et al.* (26), who reported that extraction from both ethanol and aqueous extracts did not exhibit any effect on the bacterial species, because the fenugreek seed extracts contained alkaloids and coumarin, terpenoids, and flavonoids as primary chemicals, which have different biological activities, such as, antioxidant, anti-carcinogenic, antimicrobial, antibiofilm, anti-fungal and anti-inflammatory characteristics (27, 28). Accordingly, the antibacterial

activity of the extracts is due to the presence of active secondary metabolites in both the aqueous and alcoholic extracts.

The most harmful virulence factor in pathogenic bacteria isolated from clinical sources is biofilm, so it was worthwhile to investigate how fenugreek seed extracts, both alcoholic and aqueous, inhibited the production of biofilms. The findings demonstrated that both the alcohol and aqueous extracts inhibited the formation of biofilms by bacteria, with highly significant differences. It was evident that the bacterial isolates were slightly more sensitive to the alcoholic extract than to the aqueous extract. The results demonstrate a considerable drop in optical density after treatment with the experimental compounds.

By targeting one or more steps of biofilm development, the active secondary metabolites found in both alcoholic and aqueous extracts eliminate the bacteria's capacity to produce pathogenicity through biofilm formation. This enhanced effectiveness; which may be attributed to the greater solubility of bioactive compounds—such as alkaloids, flavonoids, and saponins—in alcohol compared to water. These compounds are known to disrupt bacterial cell walls or interfere with metabolic processes, leading to bacterial inhibition. Overall, while alcoholic fenugreek seed extracts demonstrated superior antibacterial activity, the results also highlight the importance of considering strain-specific responses when evaluating the potential of plant-based antimicrobial agents. Further studies are recommended to isolate and identify the specific active compounds responsible for the antibacterial effects.

Limitations:

The current study was limited to examining the effect of fenugreek extracts on a limited number of pathogenic bacteria and did not include in vivo experiments. The efficacy of the extract was not compared with conventional antibiotics, which limits the comprehensive evaluation of its therapeutic value.

Conclusions:

The present study yielded important findings about the use of fenugreek seed extract to treat pathogenic bacteria that have developed a resistance to antibiotics. Natural substances found in these plant extracts can also be utilized.

Authors' declaration:

We hereby confirm that all the Figures and Tables in the manuscript are ours. The project was approved by the local ethical committee the College of Science for Women, University of Baghdad. According to document number (7017/22, dated 15,11, 2023).

Conflict of interest: None

Funding: None

Authors' Contributions:

Study conception & design: (Zahraa A. Zamil & Huda S. A. Al-Hayanni). Literature search: (Zahraa A. Zamil & Huda S. A. Al-Hayanni). Data acquisition: (Zahraa A. Zamil & Huda S. A. Al-Hayanni). Data analysis & interpretation: (Zahraa A. Zamil & Huda S. A. Al-Hayanni). Manuscript preparation: (Zahraa A. Zamil & Huda S. A. Al-Hayanni). Manuscript editing & review: (Zahraa A. Zamil & Huda S. A. Al-Hayanni).

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تقييم التأثير المضاد للبكتيريا والمضاد للأغشية الحيوية لمستخلصات بذور الحلبة (*Trigonellafoenum-graecum* L.) ضد بعض البكتيريا الممرِضة المقاومة للمضادات الحيوية

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الخلاصة:
الخلفية: أظهرت الزيادة في انتشار البكتيريا المقاومة للأدوية المتعددة الحاجة إلى تطوير واستخدام عوامل بديلة مضادة للميكروبات. وقد ازداد الاهتمام مؤخرًا بالنباتات الطبية والأعشاب ومستخلصاتها.
الهدف: تقييم الفعالية المضادة للبكتيريا والأغشية الحيوية للمستخلصات المائية والكحولية لبذور الحلبة (*Trigonella foenum-graecum* L.) ضد عزلات بكتيرية سريرية مقاومة للعديد من الأدوية (المكورات العنقودية الذهبية*Staphylococcus aureus* الموجبة لصبغة غرام، والراكدة البومانية *Acinetobacter baumannii*السالبة لصبغة غرام).
المنهجية: تم اختيار نبات الحلبة (*Trigonella foenum-graecum* L.) لهذه الدراسة، حيث تم تحضير مستخلصين مائي وكحولي من بذوره. واستخدم جهاز كروماتوغرافيا الغاز المقترن بمطياف الكتلة (GC-MS) لتحديد المركبات الفعالة في هذه المستخلصات. تم اختبار فعالية المستخلصات ضد العزلات البكتيرية المقاومة للأدوية المتعددة والمكوّنة للأغشية الحيوية (موجبة وسالبة لصبغة غرام)، وهي المكورات العنقودية الذهبية *Staphylococcus aureus*والراكدة البومانية *Acinetobacter baumannii*، والتي تم عزلها من مرضى عراقيين في مستشفيات بغداد.
النتائج: أظهرت الدراسة الحالية أن المستخلصين المائي والكحولي لبذور الحلبة لهما فعالية مضادة للبكتيريا ومضادة للأغشية الحيوية ضد العزلات البكتيرية المدروسة بجميع التراكيز المستخدمة، مع فروق معنوية ملحوظة. كما بينت الدراسة أن المستخلص المائي كان أكثر فعالية كمضاد للبكتيريا ومضاد للأغشية الحيوية مقارنة بالمستخلص الكحولي. وأظهر المستخلص المائي نشاطًا مثبطًا عاليًا تراوح بين 52.9% و97.5%، بينما تراوح تثبيط المستخلص الكحولي من 56.0% إلى 99.5%.
الاستنتاج: توفر هذه الدراسة نتائج واعدة تدعم استخدام مستخلصات الحلبة، في معالجة البكتيريا الممرضة المقاومة للمضادات الحيوية، حيث تحتوي هذه المستخلصات على مركبات طبيعية يمكن استخدامها، كما تم إثباته بواسطة تحليل جهاز كروماتوغرافيا الغاز - مطياف الكتلة (GC-MS).
الكلمات المفتاحية: الراكدة البومانية، فعالية مضادة للبكتيريا، مقاومة متعددة للأدوية، المكورات العنقودية الذهبية، الحلبة (*Trigonella foenum*-graecum L.)