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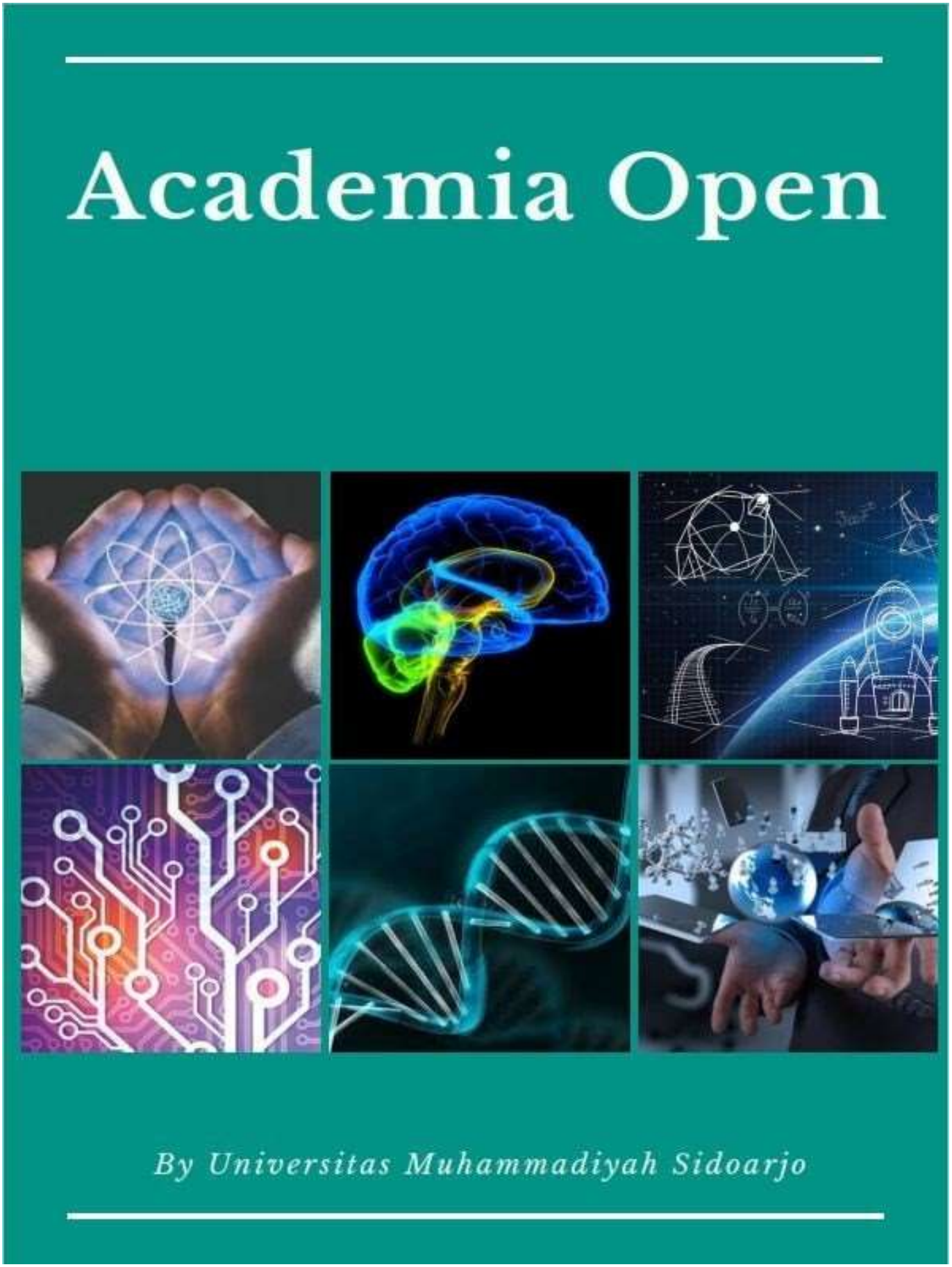

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The Antimicrobial Activity of Extracted Shiga Toxin from E. coli O157:H7 on Different Types of Bacteria and Fungi

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Abstract

General Background: Shiga toxin–producing Escherichia coli O157:H7 is a major foodborne pathogen associated with severe human disease and increasing antimicrobial resistance. **Specific Background:** Bacterial toxins have gained attention as alternative antimicrobial agents, yet their activity depends strongly on effective purification and characterization. Knowledge Gap: Limited evidence is available regarding the antimicrobial spectrum of purified Shiga toxin against diverse bacterial and fungal pathogens isolated from food sources. Aims: This study aimed to isolate E. coli O157:H7 from dairy and meat products in Baghdad and to evaluate the antimicrobial activity of purified Shiga toxin. Results: Thirty E. coli O157:H7 isolates were obtained from 300 samples. Shiga toxin was successfully purified using ammonium sulfate precipitation, dialysis, and ion-exchange chromatography, and showed inhibitory activity against several pathogenic bacteria and Candida albicans, with the lowest MIC observed for the fungus. Novelty: The study demonstrates selective antimicrobial efficacy of purified Shiga toxin against both bacterial and fungal pathogens. Implications: These findings highlight the potential of bacterial toxins as candidate agents for controlling foodborne and multidrug-resistant microorganisms.

Keywords : Shiga Toxin, Escherichia coli O157:H7, Antimicrobial Activity, Ion-Exchange Chromatography, Foodborne Pathogens

Highlight :

- Purified toxin showed strongest growth inhibition against Candida albicans at the lowest tested concentration.
- Meat-derived samples exhibited higher contamination rates compared with dairy products.
- Selective susceptibility was observed, with Klebsiella pneumoniae demonstrating marked sensitivity.

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Introduction

Enterobacteriaceae represent a large and diverse group of bacteria, most of which naturally reside in the intestines of humans and animals [1]. This family includes over 30 genera and 120 species; however, more than 95% of clinically relevant strains belong to only 10 genera and fewer than 25 species. Species classification is based on phenotypic traits such as carbohydrate fermentation patterns and amino acid bond degradation Intestinal bacteria thrive on simple media and are mostly facultative anaerobes. They grow rapidly, forming colonies on solid media and causing turbidity in liquid media within 12-18 hours of incubation. All intestinal bacteria ferment glucose [3]. Among them, Escherichia coli is categorized into pathogenic and non-pathogenic groups. While non-pathogenic E. coli coexists harmlessly in the intestines of warm-blooded animals, they can occasionally cause opportunistic infections. Pathogenic E. coli strains, however, invade the epithelial lining of the large intestine, multiply within host cells, and spread to adjacent cells, leading to tissue damage. This process manifests as diarrhea accompanied by blood, mucus, and abdominal pain [4,5]. Key pathogenic mechanisms include the formation of attachment and effacing (A/E) lesions, which are encoded by chromosomal genes located at the Locus of Enterocyte Effacement (LEE). This destruction reduces intestinal mucosal absorption, disrupts electrolyte balance, and results in diarrhea [6]. E. coli O157:H7, a zoonotic pathogen, is a leading cause of foodborne outbreaks. Ruminants, particularly cattle, are primary carriers of this serotype. Humans are typically infected through contaminated food (e.g., meat, milk, fruits, leafy vegetables), water, or contact with infected individuals or animals. E. coli O157:H7 is frequently implicated in foodborne illnesses, breaking down the intestinal mucosa and producing Shiga toxin (Stx), which causes hemorrhagic colitis and hemolytic-uremic syndrome [7]. The A/E phenotype is regulated by pathogenicity islands (PAIs), specifically at the LEE locus. Both A/E and Stx expression are influenced by complex regulatory mechanisms, including environmental signals and key proteins critical for host colonization, particularly in cattle [8]. These bacteria carry the hlyA gene, located on a plasmid, which encodes enter hemolysin A. They also possess the *eaea* gene on their chromosome, encoding the adhesive protein Intimin, crucial for molecular diagnostics of E. coli O157:H7 [9]. Discovered in 1945 as a cause of infantile diarrhea in England, E. coli O157:H7 causes infections ranging from mild diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (HUS) [10]. The bacteria produce two toxins—Shiga toxin (Stx) and vero toxin (VT)—encoded by stx1 and stx2 genes, respectively. Furthermore,The virulence of E. coli O157:H7 is largely attributed to its production of two potent cytotoxins: Shiga toxin (Stx) and vero toxin (VT). These toxins are encoded by bacteriophages—viral elements integrated into the bacterial genome—which play a crucial role in the horizontal gene transfer of virulence factors. Upon release, Stx and VT target endothelial cells, particularly in the kidneys and intestines, leading to inflammation, vascular damage, and systemic complications characteristic of HUS. The public health significance of E. coli O157:H7 continues to prompt research into its pathogenic mechanisms, routes of transmission, and strategies for prevention and treatment.

Finally, the aim of the study this study emphasizes the importance of purification methods and explores the promising potential of bacterial toxins in the fight against multidrug-resistant strains.

Materials and Methods

Sampling and study area

A total of 300 samples of dairy and meat products were collected from different areas of Baghdad, Iraq, from 21 February to 25 April 2023. Of these, 57 samples were collected from Abu Ghraib, 48 from Bab Al-Muazzam, 36 from Hay Al-Adl, 72 from Al-Shulla, 45 from Al-Hurriyah, and 42 from Al-Baya'. Samples were collected in sterile plastic containers and transported immediately to the laboratories of the College of Health and Medical Technologies in Baghdad for culture, isolation, and diagnosis using conventional methods. After collecting the samples, a swab was taken from each sample in the sterile plastic container and culture in 5 mL of Brain Heart infusion Broth. The mixture was then mixed and incubated at 37 °C for 24 hours to activate the samples.

Bacterial Isolation

A suitable number of samples were taken using a bacterial inoculation loop from the dilutions prepared from the liquid culture medium of Brain Heart Infusion Broth to be inoculated onto the Sorbitol MacConkey Agar medium prepared in advance according to the manufacturer's instructions. The plates were incubated at 37 °C for 24 hours to detect the presence of E. coli O157:H7 by the SMAC culture medium. The colonies were colorless or slightly brown and transparent, characterized by their inability to ferment sorbitol [11]. Then, it was inoculated onto the CHROMagar E. coli O157:H7 medium by streaking and incubated for 24 hours at 37 °C. Growth was observed, and the bacteria that fermented lactose were distinguished; they appear as dark pink to reddish colonies [12]. Diagnosis was performed using the VITEK 2 diagnostic system [13]. Antibiotic susceptibility testing of bacteria was performed using the VITEK 2 system.

Shiga-like Toxin Purification from E. coli O157:H7:

Preparation of Crude Toxin:

Bacteria were cultured in Tryptic Soy Broth at 37°C for 24 hours with shaking. Cells were collected via centrifugation, resuspended in Tris-HCl buffer (pH 8.6), and disrupted using sonication. Cell debris was removed by centrifugation, and ammonium sulfate was added to 45% saturation at 4°C to precipitate proteins. The precipitate was dissolved, dialyzed, and centrifuged to obtain the crude toxin [14].

Ammonium Sulfate Precipitation and Dialysis:

Proteins were precipitated stepwise by adding ammonium sulfate at different saturation levels. The precipitate was dissolved in phosphate buffer, and enzymatic activity was measured to identify the optimal saturation level. The dialyzed sample was centrifuged to remove insoluble material [15].

Purification Using Ion-Exchange Chromatography:

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DEAE-Cellulose resin was prepared and packed into a column. Proteins were separated by washing the column with a buffer and eluting with stepwise sodium chloride concentrations (0.1-1M). Absorbance of eluates was measured at 280 nm to monitor protein separation [16].

Protein Concentration Determination (Bradford Method):

Coomassie Brilliant Blue G-250 was used as a colorimetric reagent. Absorbance at 595 nm was measured, and a standard curve with bovine serum albumin was prepared for protein quantification [17].

Determination of Minimum Inhibitory Concentration (MIC):

The broth microdilution method was used with Mueller-Hinton Broth as a diluent. Serial dilutions of the test sample were inoculated with bacterial suspension and incubated at 37°C for 24 hours. Bacterial growth was assessed by measuring optical density at 450 nm, and turbidity was used to indicate growth inhibition [18].

Results

Physicochemical samples parameters and bacteriological

The diagnostic result showed the growth of 30 *E. coli* O157:H7 isolates due to their resistance to this antibiotic, while the remaining isolates gave a negative result. *E. coli* grew on CHROMagar as intense blue colonies, unlike the O157 strain, which grew as purple colonies, as shown in Figure 3 (a,b) below. Some bacterial species were identified visually based on their cultural characteristics. Some isolates appeared on Sorbitol MacConkey Agar (SMAC) medium (Figure 2), with no colonies of E. coliO157:H7 on the culture media containing the isolates identified using cultural characteristics. The characteristics of *E. coli* O157:H7 were small and pale yellow in color due to their inability to ferment and consume sorbitol, and their number was 30 isolates. For other *E. coli* isolates, the colonies were pink, and the results showed that the number of *E. coli* isolates diagnosed in this medium was 5 isolates. The results also showed the presence of *Klebsiella bacteria* (4isolates), which were diagnosed in this medium.

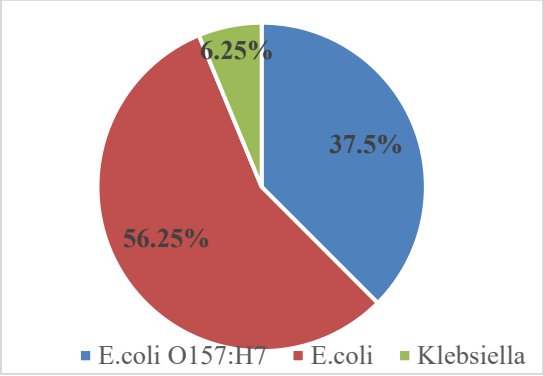

Figure 1. The percentages of bacteria isolated from milk and meat products

The incidence of *E. coli O157:H7* was higher in meat products than in milk products collected, as follows: 22 (73.33%) and 8 (26.67%) respectively, as shown in table 4

Table 1. *E.coli O157:H7* isolation ratio

Sample type	Number	(%)
Milk products	8	26.67
Meat products	22	73.33
The total	30	100

The highest percentages of bacterial contamination with *E. coli* were found in samples collected from Al-Adel district, with 25% for meat and 0% for milk. The highest percentage of contamination was in Al-Shu'ala district, with 14.3% for milk and 2.8% for milk in Abu Ghraib district. It is worth noting that the samples were collected randomly from markets in different areas of Baghdad. The reason for this may be due to the lack of hygiene in the slaughterhouses in these different areas during the slaughter process and the contamination of the carcass with waste and contaminated entrails, as bacteria from contaminated meat can be transferred to meat cutting equipment, thereby contaminating the meat,

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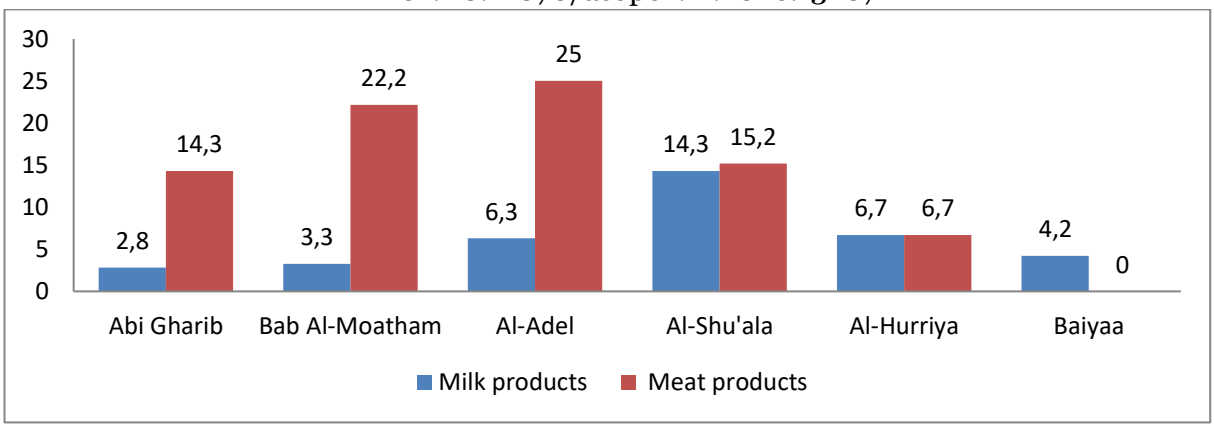

Figure 2. The percentage of product contamination in the collection areas of Baghdad city.

Precipitation of Shiga like toxin using ammonium sulfate

An experiment was conducted to precipitate the protein responsible for toxicity using ammonium sulfate, with optimal results achieved using different concentrations (0-45%) of ammonium sulfate during dialysis as a preliminary extraction of the positive raw protein. The best results were obtained when the protein was deposited at a concentration of 45%, with the effectiveness of the protein measured at a raw concentration of 12,750 PER protein after the completion of dialysis, as shown in Table 4-10. Dialysis was used to increase the protein concentration in preparation for the purification process using a purification column with gel. The solubility of proteins varies according to the ionic strength of the solution, and therefore according to the salt concentration. At low concentrations of ions (<0.5 m), the solubility of proteins increases with increasing salt concentration, as the presence of salt stabilizes various charged groups on the protein molecule, enhancing the solubility of the protein. Conversely, at high ionic strengths, the salt concentration increases, usually leading to a maximum point of protein solubility. Further increases in salt concentration limit the water available to dissolve the protein, causing the protein to precipitate due to insufficient water molecules to interact with the protein molecules.

Table 2.stages of extraction and purification of protein (toxin) from *E.coli O157:H7*

Purification step	Volume (ML)	OD.AT 595 nm.	Proteinconc.(mg / mL)
Crude extract	100	0.09	2250
Dialysis	60	0.51	12750
9	3	1.369	1026.75
10	3	1.435	1076.25
11	3	1.425	1068.75
12	3	1.395	1046.25
13	3	1.368	1026
14	3	1.382	1036.5

Purification of Shiga-like Toxin toxin from E.coli O157:H7 using ion exchange chromatograph

An experiment was conducted to purify the toxin produced by bacteria using the ion exchange purification technique, where the toxin was purified via a DEAE-cellulose column. After several washings, the result was the purification of the toxin at a concentration of 1,036.5 mg/mL, as measured using a spectrophotometer at a wavelength of 595 nm, as shown in Table 4-10. The optical density during purification peaked at a wavelength of 595 nm, ranging between 7 and 16, specifically peaking at 10. For the peaks at a wavelength of 280 nm, the range was between washing 8 and 16, with the highest peaks also at 10, indicating similar concentration heights of the peaks in these purifications, as illustrated in Figure 4-18, which shows the peaks throughout the purification of the toxin produced by E. coli O157:H7.

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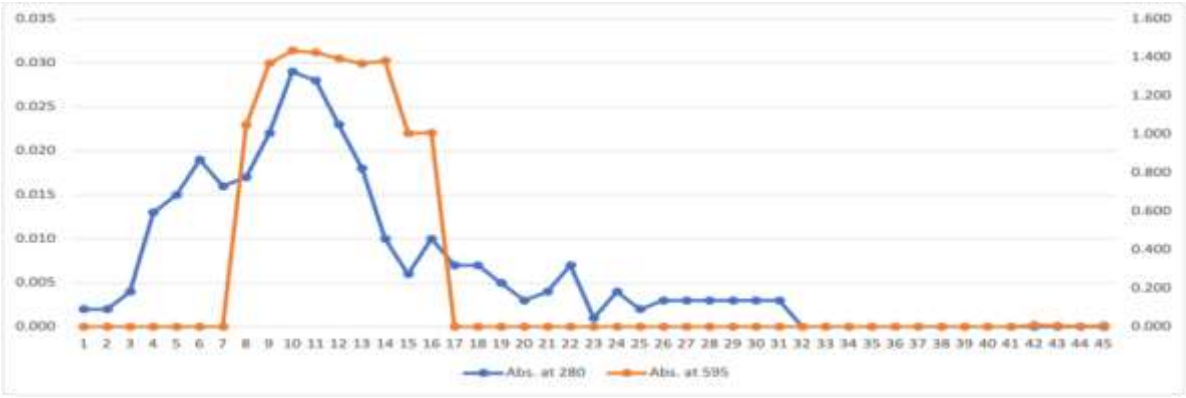
Figure 3. purification of semshiga-like Toxin from E.coli O157:H7 using ion exchange chromatography



The minimum inhibitory concentration of

Shiga-like Toxin purified from E.coli O157:H7

After the purification process of the poison, the effectiveness of the poison was tested against several types of bacteria by testing the minimum inhibitory concentration of the poison (MIC), where it was tested against several types of pathological bacteria negative and positive for cram dye, in addition to testing against a type of pathological fungus, Candida albicans, and the test result was as shown in Table 5.

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O.D of tested isolate	Conce of poison	Isolates
0.392	125	*Candida albicans*
0.266	500	Salmonella typhi
0.196	500	*Acinetobacter baumannii*
0.374	250	*Escherichia coli O157*
0.165	1000	*Proteus mirabilis*
0.157	250	*Klebsiella pneumoniae*
0.323	500	*Staphylococcus aureus*
0.321	500	*Epidermis*
0.161	1000	*Staphylococcus haemolyticus*
0.191	500	*Streptococcus mutans*
0.360	500	*Enterococcus faecalis*

Discussion

The researchers used special media designed to diagnose E. coli O157:H7, which does not ferment sorbitol, for the bacteria isolated from beef Results indicated by [19,20].

Results indicated by [21] show that only 10% of E. coli isolates were pathogenic for urinary tract infections, and the

bacteria exhibited variability in hemolysin production, indicating low virulence. The medium contains peptone, pyruvate, and sorbitol, which allows E. coli and its strains to grow rapidly. The medium is highly selective for E. coli because it contains tergitol, which inhibits the growth of Gram-positive and some Gram-negative bacteria without affecting the growth of E. coli and its strains. Selectivity is further enhanced by the addition of two antibiotics, cefsulodin and vancomycin, which target Pseudomonas spp. and Gram-positive bacteria such as Enterococci. The differentiation between the growing bacterial colonies occurs due to the chromogenic mixture, consisting of the basic substances 6-chloro-3-indoxyl-β-D-galactopyranoside and 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide. The first compound is cleaved by an enzyme produced by intestinal bacteria called β-D-galactosidase, giving the O157 strain a purple color. The second compound is cleaved by the enzyme β-D-glucuronidase produced by E. coli, causing its colonies to appear light blue. Many of the E. coli strains possess both enzymes, resulting in their colonies appearing dark blue. A recent study by[22] also agreed that E. coli O157 showed purple colonies on Chromagar agar, white colonies with sharp edges on blood agar, and 78% of them exhibited pink colonies on MacConkey agar.

Other bacterial species isolated

This is similar to the results of the researcher (14), who showed that the colonies appear pink when cultured. These bacterial isolates were also cultured on Cefixime Tolerate Sorbitol MacConkey Agar (CT-SMAC) medium, and the diagnostic result was the growth of isolates of the E. coli O157:H7 strain due to its resistance to this antibiotic, while the rest of the isolates gave a negative result. Many studies have reported similar results to what we found when tested with a medium containing the antibiotic Cefixime, as mentioned by (12), as shown in Table (2) below the results showed that 30 isolates of E. coli O157:H7 were obtained, representing 10% of the total samples, these results suggest that E. coli O157:H7 is a significant foodborne pathogen in Baghdad governorate. as shown in table 3 below:

These findings align with those of (8), who emphasized that environmental factors such as storage temperature, transportation conditions, humidity levels in storage areas, and slaughtering and handling practices play a crucial role in increasing E. coli contamination in food products. This supports the observed higher contamination rates in meat compared to dairy products in the present study. Furthermore, the current study confirmed that meat products, particularly ground beef, serve as a primary source of E. coli infection, which is consistent with the findings of (13). Their study indicated that E. coli O157:H7 can easily transfer from contaminated meat to cutting tools and preparation surfaces, thereby facilitating its spread.Regarding diagnostic methods, the present study demonstrated that the Vitek 2 system was effective in detecting *E.coli O157:H7*, which concurs with the results of (13). Their research highlighted that Vitek 2 is a highly accurate and rapid tool for identifying this bacterial strain.. As shown in Table 5

as mentioned by. [23] . As for milk, the poor storage conditions, with varying storage conditions of heat, humidity, and other conditions, can lead to contamination of milk with *E. coli*. Research has concurred with these findings, indicating that contamination is the cause of meat being present in stores for variable lengths of time and in an unfavorable manner, which is consistent with [24].

Other researchers have used this accurate system to diagnose *E. coli O157:H7*. This detection was considered a rapid and relatively inexpensive detection, with a high accuracy rate. It has been used by many researchers to detect colon bacteria, where it gave results similar to those reported in many studies, as mentioned in the study by [25] , which stated that 99% of the results were negative for the Sac analysis, as well as for PYrA,

while they were 99% positive for GlyA

The results of the local researcher (1) and the local results of the researcher [26] showed that the percentage of multidrug-resistant bacterial isolates was 100%. The results of the researcher [27] also showed similar results due to the fact that they were diseased isolates. The current research results were close to the results of the researcher Martinez et al., 2021 , which were 21% resistant to antibiotics and 63% sensitive to antibiotics .The main cause of this serious problem is due to the large increase in bacterial resistance to broad-spectrum antibiotics due to the excessive use of these antibiotics. Also, if the isolates are diseased or food, the diseased ones are more resistant than the food ones due to the reasons mentioned earlier (10)

Extraction and purification of E. coli O157: H7 from Shiga-like Toxin bacteria

Precipitation of Shiga like toxin using ammonium sulfate

This phenomenon of protein precipitation due to excess salt is known as salting out (3).

Dialysis is a process that typically involves the removal of salts from solutions, and it is often utilized to remove salts from proteins after salt deposition. Special tubes designed for this purpose, called dialysis tubes, resemble precursors and are equipped with pores whose diameters are measured by refraction [29]. Much research has consistently shown the efficacy of using the precipitation and dialysis processes to concentrate and purify Shiga-like toxin proteins from E. coli O157:H7 [30], indicating that the optimal concentration of ammonium sulfate for purification is around 60%. Additionally, [31] reported using the ammonium sulfate precipitation technique at various concentrations, achieving high efficacy and a concentration of the toxin extracted from E. coli O157:H7 that reached 3.2 mg per liter, highlighting the method's effectiveness in yielding desired results.

Ion exchange involves the exchange of ions of similar charge between a solution and a solid phase that does not dissolve in it and carries a fixed charge. The general principles dictate that the material is immobile with respect to the opposing charge of the exchanged ions; this material is termed an ion exchanger. The ion exchanger must possess a porous molecular structure that facilitates the movement of ions and solvent, and it maintains a constant charge [32]. These results align with previous research, notably the study conducted by Zhao et al. (2018), which utilized ion exchange technology to purify Shiga-like toxins from E. coli O157:H7, yielding excellent results regarding the concentration of protein toxins produced by the bacteria. Additionally, the findings corresponded with the results of [33], who employed ammonium sulfate extraction followed by ion exchange purification, reporting a purity ratio of nearly 85% for the Shiga-like toxin produced by E. coli O157:H7. This purity is considered excellent compared to other purification methods.(27) regarded ion exchange purification technology as an effective process for purifying toxin proteins derived from colonic bacteria. Their study indicated good concentration during extraction and purification, yielding high purity and clarity in SDS-PAGE analysis, where samples exhibited a clear concentration of approximately 3 mg per liter after purification.

The results indicated that the lowest effective concentration against bacteria was observed for Candida albicans at a concentration of 125 mg/mL. The optimal concentration that effectively killed bacteria and halted the growth of Klebsiella pneumoniae and Escherichia coli O157:H7 was 250 mg/mL. A concentration of 500 mg/mL was required to kill Salmonella typhi, Acinetobacter baumannii, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans, and Enterococcus faecalis, where the effectiveness at this concentration was comparable across these pathogens. The lowest concentration that resulted in the death of Proteus mirabilis and Staphylococcus haemolyticus was 1000 mg/mL.

Numerous studies have demonstrated the potential of these toxins, noting that the proteins interact destructively with the cell wall, which is evident from the observed lethal concentrations against bacteria and fungi. The variation in minimum lethal concentrations is attributed to differences in the cell wall structure of Gram-negative and Gram-positive bacteria, given the additional peptidoglycan layer found in Gram-positive bacteria compared to their Gram-negative counterparts. The purified Shiga-like toxin exhibits antibacterial and some antifungal properties, which was utilized in this experiment, allowing for interaction with the cell wall of various pathogens.

Research by (28) confirmed that the toxic composition of the Shiga-like toxin bears significant similarity to the rhizin protein secreted by certain plants, known for its high toxicity against various bacterial and fungal species. They found that the amino acid sequences of the rhizin protein closely resembled those found within the Shiga-like toxin. This study aims to explore the microbial effects of the toxin protein on pathogenic microorganisms affecting humans, marking a unique endeavor in utilizing this purified protein.

Additionally, several studies have utilized rhizin proteins similar to Shiga-like toxins against a variety of pathogenic bacteria and fungi, including Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Salmonella typhimurium, and Escherichia coli, with results demonstrating considerable efficacy [34]. Moreover, effectiveness was noted against Candida albicans and Aspergillus niger, although these proteins were less effective against soil-dwelling bacteria [35].

[36] also indicated that extracts containing these similar substances exhibited significant antibacterial and antifungal activity, inhibiting growth and reducing pathogenicity across various species

Conclusion

Limitation: In this study, a limited number

of samples were collected from specific areas in Baghdad, which may not comprehensively represent the overall situation of food contamination with E.coli O157:H7 in the city. This clearly requires a more robust sampling strategy. Further investigations focusing on large-scale sampling and advanced molecular analyses are crucial for developing effective strategies for bioremediation and treatment of E.coli O157:H7 contamination in Baghdad.

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