Inhibition of siderophore production in E. coli O157:H7 strain

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Abstract

E. coli O157:H7 strain is a frequent source of foodborne infections and has been linked to a number of human illnesses, siderophores is important virulence factor for this bacterium used to take up the necessary iron from the environment. The current study aimed to inhibit the production of siderophore in the E. coli O157:H7strain using plants, natural materials and organic acids which may be used as anti-virulence drugs against these bacteria.

The results showed the ability of the E .coli O157:H7 strain to produce siderophore, which was found to be a hydroxamate and catecholate type, Our PCR results shows that bacteria have two the genes entC and iucA genes which encoded enterobactin and aerobactin siderophores. In siderophore inhibition assays, sub-minimum inhibitory concentrations were used. Results showed that Rhus, Citric acid and Sinjar honey were inhibit siderophore production and also it was found that rhus affected the iucA gene, as the gene disappeared completely, but did not affect the entC gene and citric acid caused disappearance of the two genes, also it was found that Sinjar honey caused iucA gene disappearance, but did not affect the entC gene.

Our results indicates the possibility of using Rhus, Citric acid and Sinjar honey as anti-virulence agents against this bacteria and future additional studies are required to find out the active compounds that led to the inhibition of serophore production .

Keywords: *E .coli O157:H7, Siderophore, Rhus, Citric acid, Honey.*

INTRODUCTION

Shiga-toxigenic Escherichia coli (STEC), often referred to as verotoxigenic (VTEC) or enterohemorragic (EHEC) E. coli is a frequent source of foodborne infections. The most noteworthy STEC serotype, E. coli O157:H7 strain has been associated with a number of human sicknesses, including mild diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS), (Puligundla and Lim, 2022).

E. coli O157:H7 strains are capable to produce either the Shiga toxin types 1 (Stx1), or 2 (Stx2), or both (Geresu and Regassa, 2021). Early antibiotic treatment can prevent the development of the hemolytic uremic syndrome from a Shiga toxin-producing E. coli O157:H7 infection. Research has revealed that E. coli O157:H7 strain has significantly much more antibiotic resistance than before. This may be influenced by the extreme and inappropriate use of antibiotics in both humans and animals food. According to

studies, E. coli O157:H7 has developed varying degrees of resistance to several commonly used antibacterial medications (Haile at al., 2022).

Iron is a crucial metal cofactor in enzymes involved in the biofilm development, ribosome assembly, oxidative stress, synthesis of DNA and repair, and citric acid cycles (Wu et al., 2022). In respond to an inadequate supply of iron, the organisms create siderophores to take up the necessary iron from the environment. siderophores are organic chelators molecules with very small molecular weight that are particularly selective for the Fe (III). Because of their strong affinity for iron, siderophores may remove iron from molecules like ferritin, transferrin, and lactoferrin. Due to their impacts on pathogen pathogenicity, the majority of siderophores have become more significant as a result of this characteristic. The term "hypervirulent" refers to pathogens that create a large number of siderophores, whereas "lower virulent" refers to pathogens that are unable to make siderophores during infection (Cavas and Kirkiz, 2022).

The siderophore may acquire new or enhanced features as a result of differentiation in siderophore biosynthesis, making the bacteria more virulent. Enterobactin, salmochelin, yersiniabactin, and aerobactin are the four forms of siderophores that different E. coli strains create (Cavas and Kirkiz, 2022). Siderophores are particularly useful for avoiding drug resistance associated with membrane by utilizing their iron moving ability to get medications into cells via combine the siderophores and antibiotic using the Trojan horse strategy. A siderophore is connected to the antibiotic that is incapable to pass through the membrane barrier of bacteria. When the cognate receptor recognizes the complex of siderophore-Fe (III), it transports the medication along with complex over the outer membrane. The pathogen may be destroyed by a variety of methods when the combination of drug -siderophore enters the cytoplasmic material, including drug release, antibacterial agent activity as a whole, and preventing iron absorption (Ribeiro and Simões, 2019).

The goal of the current study is to inhibit the production of siderophore in the E. coli O157:H7strain using plant, natural materials and organic acids which may be used as anti-virulence drugs against this bacterium.

Materials and Methods:

Bacterial strain:

E. coli O157:H7 strain which used in current study was obtained from Biology department / college of science / University of the Mosul, Iraq.

Screening for Siderophore production:

The E. coli O157:H7 strain was cultured in a Tris-Minimal Succinate medium (TMS) which was made according to (Sebulsky et al., 2004), for 24 hours at 37 ℃ with 100 rpm shaking. German Hermle cooling centrifuge was used to remove the cells by spinning at 11,000 rpm for 10 minutes at four ºC. Then the aliquot was sterilized by filtering it through a 0.45 μm Millipor filter to get rid of the remnants of bacterial cells (Al-Mawla, 2005). The sterile filter is taken and the following tests were performed on it:

FeCl3 test:

1ml of 2% FeCl3.6H2O suspension was added to 1 ml of sterile filter culture. If siderophore is present, an orange or reddish-brown color will appear (Jalal and Helm, 1990).

Determined the Type of Siderophore :

A FeCl3 test, tetrazolium test and Arnow test was performed according to (Aravinth, 2012). Briefly, in Fecl3 tests, A UV-Vis spectrophotometer was used to scan the mixture from 300 nm to 600 nm after adding 2ml of FeCl3 solution. The tetrazolium test, 2drops of 2N sodium hydroxide was added to a small amount of tetrazolium and 1ml of sterile filter culture. The red color indicates hydroxamate type. In Arnow test, 1ml of sterile filter culture was mixed with 1ml of each of 1mM HCl, nitrite-molybdate reagent, and 2N NaOH. A maximum absorbance at 510 nm confirmed catcolate type.

Detection of siderphores encoding genes using PCR technique:

The bacterial DNA was extracted using the DNA extraction kit supplied by Geneaid Gompany, according to the proven method .The Nanodrop device (Germany/Implen) was used to measure the concentration. We employed 2 particular pairs of primers (Australia\ Macrogen) to check for the presence of the entC and IucA genes in E. coli O157:H7 strain (Table 1). The PCR conditions were established in accordance with the conclusions of the research conducted by (Searle et al., 2015; Tivendale et al., 2004) (Table 2) and the results were seen on a 1.5% agarose gel.

Table1. Primers used in current study.

Gene	Size (bp)	Primer Sequences (**5′-3′**)		Reference
entC	438	F	- GACTCAGGCGATGAAAGAGG-	(Searle *et al*., 2015)
		R	-TGCAATCCAAAAACGTTCAA-	
IucA	1,482	F	-ATGAGAATCATTATTGACATAATT-	(Tivendale *et al*., 2004)
		R	-CTCACGGGTGAAAATATTTT -	

Table 2. The PCR program used for the detection of genes

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final extension
entC	95 C°, 1 cycle, 2m	95 C°, 35 cycle, 30sec	55 C°, 35 cycle, 1m	72 C°, 35 cycle, 1m	72 C°, 1 cycle, 5m
IucA	94 C°, 1 cycle, 3m	94 C°, 26 cycle, 1m	55 C°, 26 cycle, 1m	72 C°, 26 cycle, 1m	72 C°, 1cycle, 10m

Inhibition of Siderophore production:

Inhibitory materials:

The inhibitory materials (Rhus, peppermint), (propolis, and honey) were obtained from the local markets in Nineveh Governorate, while citric acid and ascorbic acid supplied from (England /BDH)

Plants and propolis alcoholic extracts preparation:

Alcoholic extracts of plants were created, according to (Al-saidy et al., 2013), by combining 20gm of previously prepared plants with 200ml of 70% ethanol in an ice bath. 40°C was used for both the extraction and drying procedures. The powder was kept in sterilized tubes at -20°C. Alcoholic extract of

propolis was prepared according to (Hegazi and Abd El Hady, 2002).

Preparation of stock solutions:

The stock solutions of honey was prepared according to (Al-Noman, 1998), Propolis stock solution was made at aconcentration of 500mg/ml according to (Hegazi and Abd El Hadi. 2002). stock solutions of organic acid were prepared in aconcentration of 500mg/ml according to (Tabak et al., 2003).The stock solutions of Rhus and peppermint extract was made at aconcentration of 400mg/ml and the minimum inhibitory concentration and Sub-minimum inhibitory concentration were Determined according to a procedure describe by (Al-Noamy, 2020).

Phenotypic and molecular detection of the inhibitory material effect:

Inhibitory effect plant and propolis alcoholic extracts, honey and organic acids on siderophore production after treating the isolate with a Sub-MIC concentrations was determined by repeating the previous steps of phenotypic and molecular methods for siderophore production and encoding genes .

Results:

The Fecl3 test was used to investigate the presence of siderophore in bacteria, as the results showed the capability of the E. coli O157:H7 strain to produce siderophore, which was inferred by the appearance of a reddish-orange color (Figure 1) by the addition of aqueous ferric chloride to the cell-free filtrate, as a result of the reaction Iron and siderophore present in the cell-free filtrate.

Figure 1: results of Fecl3 test

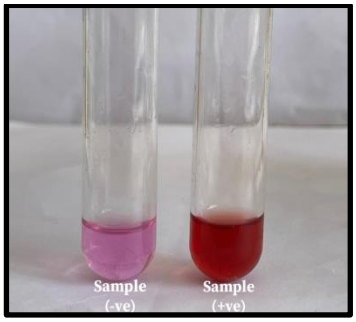


Figure 2: results tetrazolium salt

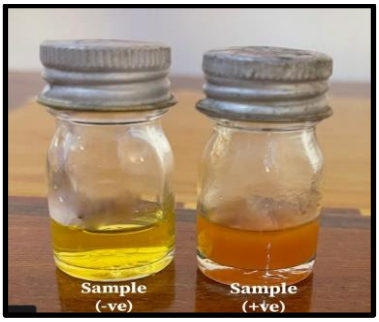
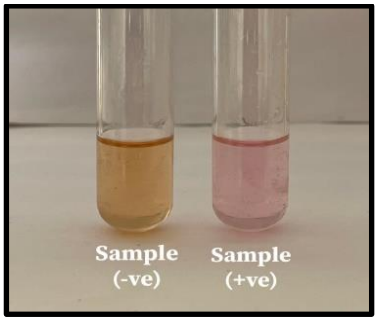


Figure 3: results of Arnow test



Hydroxamate-type siderophores were indicated by a 2% FeCl3 and a wide peak range between 400 and 450 nm whereas a peak between 450 -495 nm indicate the catacholate type of siderophore. The tetrazolium test detected the hydroxamate-type siderophores as a rich red color appears immediately (Figure 2). The arnow test detected the present of catacholate siderophore which was confirmed by an absorbance maximum of 510 nm in a UV-vis spectrophotometer and formation of pink color (Figure 3).

Siderophore encoding genes results by PCR technique showed at (Figure 4). The entC gene is in charge of enterobactin siderophore production, while iucA gene is in charge of aerobactin siderophore production. These tow genes were amplified by PCR. The Nanodrop device was used to measure the concentration of the DNA of the strain, which was (59 ng/microliter) and purity (1.835), and the gene entC was amplified to 438 base pairs, and the iucA gene was amplified to 1.482 base pair and compared with the DNA ladder. The results showed that the E .coli O157:H7 contains both genes.

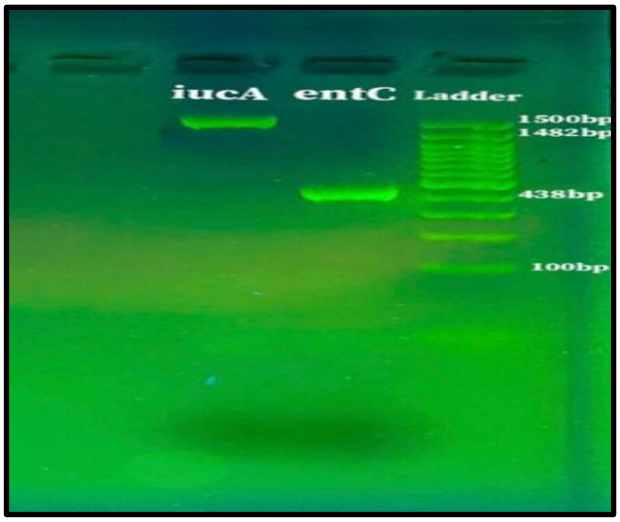


Figure (4): results of entC and iucA genes detection in E. coli O157:H7 strain by PCR and electrophoreses technique.

The minimum and sub-minimum inhibitory concentration of inhibitory substances are shown in the (Table 3).

Table (3): The MIC and Sub MIC of inhibitory substances.

inhibitory substances		Rhus	Peppermint	Propolis	Sidr honey	Sinjar honey	Citric acid	Sorbic acid
MIC and Sub-MIC	MIC	100mg/ml	250mg/ml	850µg/ml	25mg/ml	25mg/ml	62.5mg/ml	125mg/ml
	Sub-MIC	50mg/ml	125mg/ml	425µg/ml	12.5mg/ml	12.5mg/ml	31.25mg/ml	62.5mg/ml

In siderophore inhibition assays, sub-minimum inhibitory concentrations were used. The results of this study are listed in (Tables 4) where there was a difference in the efficacy of inhibitory substances in inhibiting the siderophore production. The siderophore were not inhibited by Peppermint, Propolis, Sidr honey and Sorbic acid.

Table (4): Phenotypic investigation of the inhibition of siderophore production

Inhibitory substances	Rhus	Peppermint	Propolis	Sidr honey	Sinjar honey	Citric acid	Sorbic acid
Siderophore production	+	-	-	-	+	+	-

(+)Siderophore production inhibition (–) no inhibition

In molecular investigation of the inhibition of siderophore production: The primary phenotypic inhibition results were relied upon to select the inhibitor substances in the molecular test. The effect of Citric acid, Sinjar honey and alcoholic extract of Rhus on siderophore genes was investigated. The (Figure 5) show that Citric acid removes both entC and iucA genes while Rhus and sinjar honey remove only iucA gene.as shown in

(Table 5). In figure 5, the number from 1 to 3 represents the effects of Rhus, Citric acid and Sinjar honey on entC gene while the number from 4 to 6 represents the effects of Rhus, Citric acid and Sinjar honey on iucA gene.

Figure (5): results of entC and iucA genes detection in E. coli O157:H7 after treated with sub-MIC concentration of inhibitory substances (1 - 3 represent iucA gene and 4 - 6 represent entC gene).

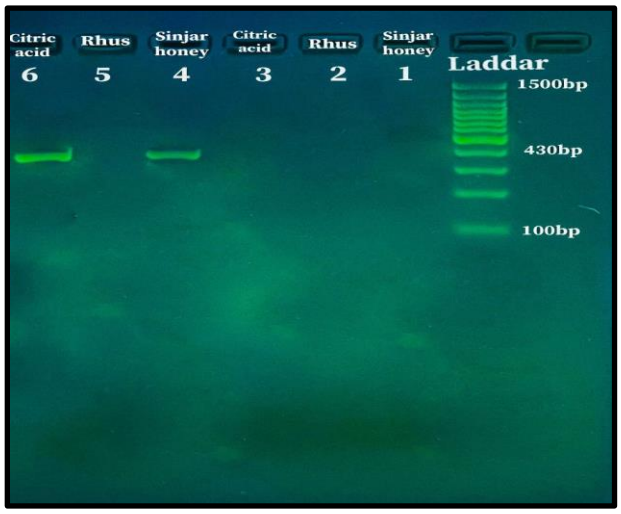


Table (5): The effects of rhus, Citric acid and Sinjar honey on siderophore genes

Inhibitor substances	The target gene	Inhibition result
Rhus	*entC*	-
	iucA	+
Citric acid	*entC*	+
	iucA	+
Sinjar honey	*entC*	-
	iucA	+

(+) No detected gene, (-) detected gene

Disscussion:

The E. coli O157:H7 strain was found to produce siderophore .which was conferred first by their ability to grow in TSM that contain succinate which engorge the strain to produce siderophore and was confirmed by FeCl3 test. The presence of a siderophore is shown by the yellow to orange coloration change in the culture supernatant upon the addition of FeCl3 (Figure 1). And our results are consistent with the study of the researcher Kumar et al., 2021 who found that most strains of E. coli have the capacity to produce siderophore.

In this study, the type of the siderophore produced by the E. coli O157:H7 strain was found to be a hydroxamate and catecholate type. Earlier reports suggest that some of E. coli O157:H7 strains produce hydroxamate and catecholate siderophores (Kumar et al., 2021). A wide peak between 400 and 450 nm in the bacterial siderophore culture filtrate that included 2% FeCl3 indicated the presence of a hydroxamate siderophore and a high peak at 495nm indicate the presence of a catecholate siderophore. This result has agreement with the result of Chowdappa (Chowdappa et al., 2020). Who said that ahigh peak at 420 and 450nm suggested a hydroxamate siderophores. The tetrazolium test results with deep red color showed a siderophore of the hydroxamate type. This result has agreement with the finding of (Nithyapriya et al., 2021) who detected the present of a hydroxamate siderophore in B. subtilis through red color appearance as shown in (Figure 2).

The Arnow technique relies on an acidic reaction between catechol and the nitrite-molybdate reagent that results in yellow color. When exposed to alkaline circumstances, the color changes to a pink\ red color (Ferreira et al., 2019). In this study we found that E.coli 0157:H7 have the ability to produce catecholate siderophore which was indicating by appearances of pink color as show in

(Figure 3). This result is in agreement with the result of Kumar et al., 2021 who found that most of E. coli bacteria have the ability to produce catecholate type.

Previous research has demonstrated a connection between the bacteria's ability to produce siderophores and their pathogenicity. (Cavas and Kirkiz, 2022). E. coli strains can produce 4 types of siderophores whose are enterobactin, salmochelin, yersiniabactin and aerobactin.

Our PCR results shows the present of entC and iucA genes which encoded enterobactin and aerobactin siderophores respectively and agreement with phenotypic detection of siderophore. The catechol siderophore formed by E. coli, enterobactin, has the strongest affinity of any siderophore known to exist for the Fe (III) ion (Govindan et al., 2019). A study by Tivendale et al., 2004 and HANAN et al., 2020 shows the present of aerobactin in most of E .coli O157:H7 strain.

Plants are a potential area of alternative medicine for preventing E. coli O157:H7 pathogenesis because they contain active chemicals, are readily available in nature, are inexpensive, and have fewer adverse effects than antibiotics. As a result, a few plants with antimicrobial properties were chosen to investigate their capacity to obstruct siderphore production. The MIC and subsequent Sub-MIC values varied across the plant extracts, natural materials and organic acids under study (Table 3). This may be explained by variations in the active chemical composition (Nozohour et al., 2018). The result revealed the ability of alcoholic extracted of Rhus plant to inhibit the siderophore production. This inhibition is may be due to chemical composition such as hydrolysable tannins, flavonoids, gallic acid, and p-hydroxybenzoic acids, as well as phenolic acid methyl esters. Rhus extracts' have antibacterial properties and antioxidant activity. It affected both Gram-positive and Gram-negative foodborne and bacteria that are pathogen. Many investigations have shown that flavonoids and tannins can inhibit energy of metabolism, the function of cytoplasmic membrane, and the synthesis of nucleic acid. Moreover, flavonoids have been discovered to lessen membrane permeability, pathogenicity, porin on cell membranes, adhesion, and biofilm formation, all of which are essential for bacterial development (Shamsudin et al., 2022).

In the present study, by conducting a PCR to observe the effect of the extract on the detection of genes, it was found that Rhus affected the aerobactin gene, as the gene disappeared completely, but did not affect the enterobactin gene. A study by Alfonso et al., 2022 showed that gallic acid is a strong inhibitor of DNA gyrase. Also, they demonstrate the effective suppression of E. coli DNA topoisomerase IV by gallic acid and certain derivative of gallate.

The Federal Drug Administration (FDA) has identified and certified organic acids as safe drugs. Due to their low cost and ease of modification, organic acids have several benefits as antibacterial molecules (El Baaboua et al., 2018). In this study, we found that citric acid could inhibit siderophores production while sorbic acid does not affect siderophore production. A study by Gómez-García et al., 2019 showed that organic acids can prevent the growth of different type of bacteria, because of the lipophilic character of their undissociated state, they can enter cell membranes and alter the protons and associated anion concentrations in the cytoplasm material. Also, our PCR result after

treating the isolate with sub-MIC concentration of citric acid shows that the genes of aerobactin and enterobactin are disappeared. A study by Gómez-García et al., 2019 showed that the bases of purine and crucial enzymes are affected by organic acid, and also the viability of the bacterial is reduced.

Natural honey and propolis are inhibitor of gram-positive and gram-negative bacteria, regardless of the diversity of its production sources (AL-Sa'ady and Al-Mawla, 2019). Our results showed that the Sidr and Sinjar honey can inhibit bacterial growth and by growing the bacteria in sub-MIC concentration, it showed that sinjar honey can only inhibit siderophore production in E.coli O157:H7 strain. Our study agree with the study of Combarros-Fuertes et al 2019 who show that Manuka Honey can inhibit siderophore production in both E. coli and Staphylococcus aureus and this result is due to physiological alterations including membrane potential and integrity ofmembrane and can cause a notable metabolic instability in S. aureus as its main physiological impact, and it was able to limit efflux pump function in the E. coli strain. In the present study, by conducting a PCR to observe the effect of the sub-MIC concentration of sinjar honey on the detection of genes, it was found that it affected the aerobactin gene, as the gene disappeared completely, but did not affect the enterobactin gene. Lee et al., (2011) showed by genetic screening of E. coli that minimal quantities of honey in growth media dramatically inhibit genes that control of motility, QS signals, and virulence factors including siderophore.

We conclude from our results above the possibility of using Rhus, Citric acid and Sinjar honey as anti-virulence agents because of the importance of iron in the virulence and growth of bacteria and thus using them as alternative drugs in the case of antibiotic resistance and future additional studies are required to find out the active compounds that led to the inhibition of siderophore production in the inhibitory materials that were studied, as well as testing other inhibitors.

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