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Original Article

Molecular characterization of virulence genes and influence of *Xanthium strumarium* extract against two *Enterobacter* species isolated from some soil invertebrates

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Abstract

The development of bacterial antibiotic resistance poses a danger to healthcare systems worldwide. To reduce the spread of disease, researchers are looking for novel measures to control bacterial infections to reduce the spread of disease. The antibacterial properties of *Xanthium strumarium* methanolic and ethanolic extracts were evaluated against *Enterobacter cloacae* and *E. hormaeche* isolated from some invertebrates (*Porcellio laevis*, *Armadillidium* sp. (isopods), and *Archispirostreptus syriacus*). All *Enterobacter* strains tested positive for the presence of the virulence genes *csgA, csgD, AcrAB, fimH,* and *Hsp60.* Extracts of *X. strumarium* had significant anti-biofilm activity against *E. cloacae* and *E. hormaechei*. The disruption of established biofilm growth by the plant samples proved to be effective against *E. cloacae* and *E. hormaechei*. Both *E. cloacae* and *E. hormaechei* showed inhibition of biofilm formation and promotion of biofilm eradication in response to *X. strumarium* extract. Phenolic compounds such as ferulic acid, chlorogenic acid, trans-cinnamic acid, and flavonoids such as kaempferol were the most abundant components in the extract and might play crucial roles in the extract's antibacterial and antibiofilm action. Results suggest that ethanolic leaf extracts from *X. strumarium* show potential as a novel approach to prevent infections caused by *E. cloacae* and *E. hormaechei.*

Keywords: Antibiofilm; *Xanthium strumarium*; plant extracts; *Enterobacter* spp.; virulence genes

1. Introduction

Infections caused by *Enterobacter*, which is part of the family Enterobacteriaceae, are most often seen in healthcare institutes [1, 2]. Currently, there are 22 different *Enterobacter* species. Some species are known to cause human diseases, while others are not. Urinary tract infections (UTIs), lung infections, osteomyelitis, endocarditis, and soft tissue infections are some of the numerous nosocomial and, less often, community-acquired diseases caused by *Enterobacter* species [3,4]. Bacteria of the genus *Enterobacter* may be found in different environments, including the gastrointestinal tracts of mammals, as well as on human skin, in water, in certain foods, in soil, and in sewage [5, 6].

Nosocomial infections caused by *Enterobacter* species were first recognized in the 1970s. *Enterobacter* is a prevalent infection that may be isolated from surgical wounds, respiratory sputum, and blood in the intensive care unit (ICU) [6,7]. *Enterobacter* has developed a resistance to a wide variety of previously effective antibiotics. Carbapenem-resistant Enterobacteriaceae (CRE) was included in the critical priority category for an urgent need to develop new treatments when the World Health Organization released an updated list of antibiotic-resistant bacteria [7- 9].

The annual *X. strumarium* is a member of the Asteraceae family. *X. strumarium* which is available commercially between April and October in Taif, Saudi Arabia [10]. Leaves, fruit, and roots, in particular, are utilized as medicines in various societies [11]. Antitrypanosomal, hypoglycemic, diuretic anthelmintic, antifungal, antiulcerogenic, antileishmanial, and anti-inflammatory activity are some of the medical benefits attributed to this plant

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species [10, 12]. Moreover, it has neuroprotective effects on the brain and prevents human cancer cell growth in the laboratory [13, 14].

X. strumarium contains phenolic compounds including chlorogenic acids, ferulic acids, and thiazolidinediones; 1,3,5-tri-O-caffeoyl quinic acid, caffeic acid, and 1,5-di-O-caffeoyl quinic acid; monoterpene and sesquiterpene hydrocarbons; isoprenoids including β-sitosterol and stigmasterol; xanthanolide sesquiterpene lactones and triterpenoid saponins [14-16]. In addition, *X. strumarium* has large amounts of phenolic acids, alkaloids, and diterpenes, as well as considerable concentrations of glycosides, saponins, fixed oils, and phytosterols [17].

The objective of the current research was to evaluate the antibiofilm and antibacterial properties of ethanolic and methanolic leaf extracts of *X. strumarium* against *E. cloacae* and *E. hormaechei* isolated from some soil invertebrates.

2. Materials and Methods

2.1. *Enterobacter* strains

A total of 70 samples of soil invertebrates were collected between September 2021 and March 2022. Digestive tracts were obtained from *Porcellio laevis*, *Armadillidium* sp. (isopods), and *Archispirostreptus syriacus* (millipede). Gut contents were diluted and distributed on MacConkey agar medium, then incubated for 24 h at 37 °C to collect bacterial isolates, which were then characterized morphologically as *Enterobacter* and genetically as *E. cloacae* and *E. hormaechei* using 16S rDNA sequencing.

2.1.1. 16S rDNA gene sequencing

Genomic DNA was extracted from *Enterobacter* strains using a DNA extraction kit (Gena Bioscience, Germany) according to the manufacturer's instructions. The 16S rDNA primer of the 16S rDNA gene was used to amplify a DNA fragment of around 1465 base pairs Table 1, [18]. A DNA Analyzer 3146 Applied Bioscience was used for sequencing after the samples were purified using a QIAquick PCR purification kit (Germany), DNASTAR was used to edit and combine the sequencing texts, and the NCBI server was used to conduct the BLAST searches.

2.1.2. Antimicrobial susceptibility test

By guidelines established by the Clinical and Laboratory Standards Institute (CLSI), USA, the antibiotic sensitivity of *Enterobacter* strains was investigated using the disc diffusion technique [19, 20]. This study was performed using 12 commercially available antibiotics: ampicillin (10 µg), sulfamethoxazole/trimethoprim (25 µg), amkacillin (30 µg), carbecillin (100 µg), oxacillin (5 µg), cefatrizine (10 µg), penicillin (10 µg), gentamicin (10µg), cefoxitin (30 µg), ciprofloxacin (5 µg), amoxicillin/clavulanic acid (30 µg) and erythromycin (15 µg).

2.1.3. Recognition of antibiotic resistance genes

PCR was conducted to determine the presence/absence of pathogenicity genes (*csgA*, *csgD*, *AcrAB, fimH*, *Hsp60,* and *irP*) in *Enterobacter* isolates [18, 19], amplification conditions, primer sequences, and amplicon sizes are listed in Table 1. PCR was performed using the GoTaq® Green Master Mix (Promega, USA). The expected sizes of the amplicons were determined through electrophoresis on a 1.5% agarose gel using 100 pb. DNA marker.

2.2. Extracts of *X. strumarium* leaves

2.2.1. Collection of *X. strumarium* leaves and extraction procedure

Leaves of *X. strumarium* were collected from a high-altitude region (Al-Hada) in the Taif Governorate of Saudi Arabia. The fresh leaves were air-dried and ground into a fine powder before being extracted using 95% ethanol and methanol at room temperature for three days. A pure filtrate was obtained by centrifuging both extracts at 7000 rpm for fifteen minutes and filtering them three times using Whatman filter paper (No.1). The extracts (pellets) were suspended in a 1% solution of dimethylsulfoxide (DMSO) after being filtered through a Buchner funnel using a rotary vacuum evaporator at 30 °C. The extracts were kept at 4 °C before the HPLC analysis.

2.2.2. Identification of the *X. strumarium* extracts components

Phenol and flavonoid components were found in the extracts [10] using Agilent 1260 infinity HPLC Series (Agilent, USA) connected with a quaternary pump with minor adjustments. The Kinetex® HLPC column (Phenomenex, USA) was used with the following specifications: 5 m EVO C18 100 mm 4.6 mm maintaining the temperature 30 °C throughout the procedure. This separation was achieved using a ternary linear elution gradient of (A) HPLC grade water 0.2% and H_3PO_4 (v/v), (B) methanol,

Table 1. Primer sequences and amplicon sizes of virulence genes of *Enterobacter* species.

Primers	Sequence	Size (bp)	Annealing temperature
16S rDNA	(F) TCC AGA TTA CAA CTT CAC CAG G (R) CAA TTC ATA TCT TGT AAC G	1465	56
csgA	(F) ATTGCAGCAATCGTAGTTTCTGG (R) ATWGAYCTGTCATCAGAGCCCTGG	230	55
csgD	(F) TGAAARYTGGCCGCATATCAATG (R) ACGCCTGAGGTTATCGTTTGCC	243	55
AcrAB	(F) ATCAGCGGCCGGATTGGTAAA (R) CGGGTTCGGGAAAATAGCGCG	312	58
fimH	(F) TGCAGAACGGATAAGCCGTGG (R) GCAGTCACCTGCCCTCCGGTA	192	55
Hsp60	(F) GGTAGAAGAAGGCGTGGTTGC (R) ATGCATTCGGTGGTGATCATCAG	350	58
irP	(F) TGAATCGCGGGTGTCTTATGC (R) TCCCTCAATAAAGCCCACGCT	238	58

and (C) acetonitrile. A total of 20 µL was subsequently injected. At 284 nm, an AVWD detector was employed to identify phenols and flavonoids.

2.2.3. Antibacterial activity of *X. strumarium* extracts

2.2.3.1. Disc diffusion test

The antibacterial efficacy of the *X. strumarium* leaf extracts was determined using the agar disc diffusion method in triplicate [20]. The *Enterobacter* was cultured at 37 °C in a liquid medium for 24h. Saline water and 0.5 turbidity standard *Enterobacter* suspension were plated on Mueller-Hinton agar plates. The *X. strumarium* leaf extract (10 µL/disc) was plated onto the agar surface on a sterile filter disc. After 2 h at 4 °C, the MHA plates were incubated at 37 °C for 24 h. The zone of suppression of cell growth surrounding the discs was used for assessing the antibacterial activities [21].

2.2.3.2. Evaluation of MICs (minimal inhibitory concentrations) and MBCs (minimal bactericidal concentrations)

Three sets of MICs and MBCs were performed on a 96-well microtiter plate (Nunc, Roskilde, Denmark) [20]. The *Enterobacter* suspension was made by diluting an overnight culture of a McFarland (0.5). Deferent doses ranging from 0.012 to 50 mg/ml of methanolic and ethanolic *X. strumarium* leaf extracts were produced in nutrient broth (5 ml). 100 µl of each extract dilution was added to 95 µl of nutrient broth and 5 µl of *Enterobacter* inoculum on microtiter plates. Control wells without *X. strumarium* extract were supplemented with 195 µl of nutrient broth and 5 µl of bacterial inoculum. The MICs and MBCs were calculated after the plates were incubated at 37 °C for 18-24 h [22]. Subculturing 20 µl of the clear wells from the MIC test on MHA allowed for the determination of MBC.

2.3. Biofilm formation

Using a crystal violet assay, the ability of Enterobacter strains to form biofilms on U-bottomed 96-well microtiter polystyrene plates was examined [22]. In brief, cells of *Enterobacter* were cultured in a Trypticase Soy broth medium at 37 °C for 24h. Then, Microtiter plates comprising wells containing sterile TSB were inoculated with 200 µl of a 1:100 dilution of the culture in TSB supplemented with 2% (w/v) glucose. Plates were incubated with cultures for 24 hours at 37 °C then washed twice with phosphate buffer saline and air dried. Cells were adhered to a glass slide and stained for 5 min with 1% crystal violet (Merck, France) in 100 µl. After filling, the wells were rinsed with 300 µl of sterile distilled water and air-dried. The optical density of the wells was measured at 570 nm to identify the presence of biofilm.

2.4. Biofilm inhibition

The potential of *X. strumarium* leaf methanolic and ethanolic extracts to inhibit Enterobacter isolates' formation of biofilms at MICs was evaluated. The crystal violet test was used to determine the production of biofilms [22]. The following formula was used for calculating the percentage of biofilm inhibition:

% inhibition = 100 – ((OD570 sample)/ (OD570 control) × 100).

2.5. Statistical analysis

For every treatment, three replicated with a minimum of four plants were utilized. The significance of the variation in mean values for each replicate was determined. All data were analyzed using one-way analysis of variance (ANOVA), and the significance of the differences between the treatments was assessed using at least significant difference (LSD) [23].

3. Results

3.1. Isolation and identification of *Enterobacter* isolates

3.1.1. Isolation of *Enterobacter* species

A total of 17 isolates were identified as *Enterobacter* species names and locations of the investigated invertebrates are presented in Table (2). Ten *Enterobacter* isolates were obtained from millipede guts, four of which (ETU-5, ETU-6, ETU-7, and KTU-12) were collected from Wady Ghazal, Taif, and six (ETU-8, ETU-9, ETU-10, ETU-13, ETU-14, and ETU-15) from Al-Shafa, Taif. Four *Enterobacter* isolates (ETU-1, ETU-2, ETU-3, and ETU-4) were isolated from the gut of an isopod, *Porcellio laevis*, collected from the Taif University Garden, Hawia, Taif. Moreover, two *Enterobacter* isolates (ETU-16 and ETU-17) were isolated from the gut of an isopod collected from Al-Shafa, Taif and only *Enterobacter* isolate ETU-11 was

Table 2. The source and locations of *Enterobacter cloacae* and *Enterobacter hormaechei* that was isolated from some invertebrates in Taif, Saudi Arabia.

Isolates	Species	source	Locations
ETU-1	*Enterobacter cloacae*	soft isopods	Hawia, Taif
ETU -2	*Enterobacter cloacae*	soft isopods	Hawia, Taif
ETU -3	*Enterobacter cloacae*	soft isopods	Hawia, Taif
ETU -4	*Enterobacter cloacae*	soft isopods	Hawia, Taif
ETU -5	*Enterobacter cloacae*	millipedes	Wady Ghazal, Taif
ETU -6	*Enterobacter cloacae*	fmillipedes	Wady Ghazal, Taif
ETU -7	*Enterobacter hormaechei*	millipedes	Wady Ghazal, Taif
ETU -8	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU -9	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU -10	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU -11	*Enterobacter hormaechei*	soft isopods	Wady Ghazal, Taif
ETU -12	*Enterobacter hormaechei*	millipedes	Wady Ghazal, Taif
ETU -13	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU -14	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU -15	*Enterobacter hormaechei*	millipedes	Shafa, Taif
ETU-16	*Enterobacter hormaechei*	soft isopods	Shafa, Taif
ETU-17	*Enterobacter hormaechei*	soft isopods	Shafa, Taif

isolated from the gut of an isopod collected from Wady Ghazal, Taif, Saudi Arabia.

3.1.2. Molecular genotyping of *Enterobacter* isolates according to 16S-rDNA gene

The 16S rDNA gene was amplified and sequenced from each *Enterobacter* isolate, fragments were aligned to the 16S rDNA sequences of similar *Enterobacter* isolates available in the NCBI database. The *Enterobacter* isolate sequences were deposited at NCBI GenBank with accession numbers (OQ164616–OQ164632). The BLAST analysis revealed that the fragments of 16S rDNA were most closely related to *Enterobacter* sequences in the NCBI database. The similarity between *Enterobacter* isolates obtained in the current study and related strains from the NCBI database ranged from 98 to 100 %, with zero E value. For example, the *Enterobacter* isolates (ETU-1, ETU-2, ETU-3, ETU-4, ETU-5, and ETU-6) with accession numbers OQ164616, OQ164617, OQ164618, OQ164619, OQ164620, and OQ164621 were identified as *E. cloacae* with high similarity to *E. cloacae* OP413041 and *Enterobacter cloacae* MW281774. On the other hand, *Enterobacter* isolates from (ETU-7 to ETU-17) with accession number OQ164622 to OQ164632 were identified as *E. hormaechei* with high similarity to *E. hormaechei* OQ421693, *E. hormaechei* OP048976 and *E. hormaechei* ON384641, respectively (Table 3, Figure 1).

3.1.3. Antimicrobial susceptibility

A total of 12 antibiotics were examined for their effectiveness against different *Enterobacter* strains. The overall susceptibility, intermediate susceptibility, and resistance values were determined (Table 4). All *Enterobacter* strains showed a high percentage of resistance against carbenicillin (100%), cefoxitin (100 %), amoxicillin (100 %), erythromycin (100 %), and penicillin (100 %). Ciprofloxacin (70.4%) and ampicillin (82.4 %) were moderately susceptible. Intermediate resistance was found against ampicillin (29.4 %) and oxacillin (35.3 %). Moreover, all the *Enterobacter* isolates were sensitive to sulfamethoxazole / Trimethoprim and gentamicin.

3.1.4. Detection of virulence genes in *Enterobacter* species

The presence of virulence genes in *Enterobacter* species is shown in Table (5) and Figure 2. The virulence genes *csgA, csgD, AcrAB, fimH,* and *Hsp60* were recorded in all *Enterobacter* strains (Table 5). However, all virulence genes *csgA, csgD, AcrAB, fimH, Hsp60,* and *irP* were recorded only in 52.9% of all *Enterobacter* strains. Moreover, the *irP* gene was recorded in nearly 66.7% of *Enterobacter cloacae* strains (ETU-1, ETU-2, ETU-4, and ETU-5) and it was recorded in about 54.5% of *Enterobacter hormaechei* strains (ETU-7, ETU-8, ETU-10, ETU-14,

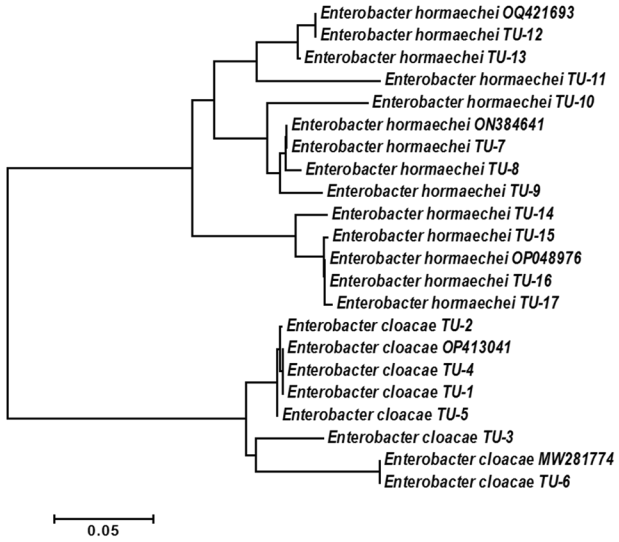

Fig. 1. Neighbour-Joining phylogenetic tree based on 16S rDNA gene sequence of *Enterobacter* species isolated from some invertebrates in Taif region, Saudi Arabia.

Table 3. The NCBI BLAST search results for *Enterobacter* isolated from invertebrate animals in Taif, Saudi Arabia.

Isolates	Species	Query coverage %	E value	Ident %	Accession number
ETU-1	*Enterobacter cloacae*	100.00	0.00	99.00	OQ164616
ETU -2	*Enterobacter cloacae*	100.00	0.00	100.00	OQ164617
ETU -3	*Enterobacter cloacae*	99.00	0.00	99.00	OQ164618
ETU -4	*Enterobacter cloacae*	100.00	0.00	100.00	OQ164619
ETU -5	*Enterobacter cloacae*	99.00	0.00	99.00	OQ164620
ETU -6	*Enterobacter cloacae*	100.00	0.00	100.00	OQ164621
ETU -7	*Enterobacter hormaechei*	99.00	0.00	100.00	OQ164622
ETU -8	*Enterobacter hormaechei*	100.00	0.00	99.00	OQ164623
ETU -9	*Enterobacter hormaechei*	100.00	0.00	99.00	OQ164624
ETU -10	*Enterobacter hormaechei*	99.00	0.00	100.00	OQ164625
ETU -11	*Enterobacter hormaechei*	98.00	0.00	99.00	OQ164626
ETU -12	*Enterobacter hormaechei*	100.00	0.00	99.00	OQ164627
ETU -13	*Enterobacter hormaechei*	100.00	0.00	99.00	OQ164628
ETU -14	*Enterobacter hormaechei*	99.00	0.00	100.00	OQ164629
ETU -15	*Enterobacter hormaechei*	100.00	0.00	100.00	OQ164630
ETU-16	*Enterobacter hormaechei*	100.00	0.00	100.00	OQ164631
ETU-17	*Enterobacter hormaechei*	99.00	0.00	100.00	OQ164632

Table 4. Antibiotic resistance profile of *Enterobacter* isolates.

Isolates	Antibiotic Profile
ETU-1	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -2	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -3	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -4	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -5	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -6	*Amp, Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -7	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -8	*Car, Amk, Caz, Pen, Cip, Fox, Eth, Amc*
ETU -9	*Amp, Car, Caz, Oxa, Pen, Cip, Fox, Eth, Amc*
ETU -10	*Amp, Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
ETU -11	*Amp, Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
ETU -12	*Amp, Car, Amk, Caz, Oxa, Pen, Fox, Eth, Amc*
ETU -13	*Car, Amk, Pen, Cip, Fox, Eth, Amc*
ETU -14	*Car, Caz, Oxa, Pen, Fox, Eth, Amc*
ETU -15	*Car, Amk,Caz, Pen, Cip, Fox, Eth, Amc*
ETU-16	*Amp, Car, Oxa, Pen, Fox, Eth, Amc*
ETU-17	*Car, Amk,Caz, Pen, Cip, Fox, Eth, Amc*

Sxt = sulfamethoxazole / Trimethoprim (25µg), Amp = Ampicillin (10µg), Car = Carbecillin (100 µg), Amk = Amkacillin (30µg), Caz = Cefatrizine (10µg), Oxa = Oxacillin (5 µg), Pen = Pencillin (10 µg), Cip = Ciprofloxacin (5 µg), Gen = Gentamicin (10µg), Fox = Cefoxitin (30 µg), Eth = Erythromycin (15 µg), and Amc = Amoxicillin (30 µg).

Table 5. Virulence genes *csgA, csgD, AcrAB, fimH, Hsp60* and *irP* recorded in *Enterobacter* isolates.

Isolates	Virulence genes
ETU-1	*csgA, csgD, fimH, Hsp60, irP*
ETU -2	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -3	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU -4	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -5	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -6	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU -7	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -8	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -9	*csgA, csgD, fimH, Hsp60*
ETU -10	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -11	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU -12	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU -13	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU -14	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU -15	*csgA, csgD, AcrAB, fimH, Hsp60, irP*
ETU-16	*csgA, csgD, AcrAB, fimH, Hsp60*
ETU-17	*csgA, csgD, AcrAB, fimH, Hsp60, irP*

ETU-15, and ETU-17).

3.2. The potential of *X. strumarium* extract against *Enterobacter* isolates

3.2.1. Chemical composition of *X. strumarium* leaves extracts

The chemical composition of methanolic and ethanolic leaves extracts from *X. strumarium* are summarized in Figure 3. A total of 21 components were detected which were divided into five flavonoids and 16 phenolic components. Also, a total of 13 compounds were detected in ethanolic extract, while 17 compounds were identified in methanolic extract. HPLC results showed differences between extracts about compound numbers and quantities. The major components of the ethanolic extract were Benzoic acid, Resvertol, Myricetin, and Kampherol, while those of the methanolic extract were Caffeic acid, Ellagic, Resvertol, Quercitin, Rosemarinic, Myricetin and Kampherol (Figure 3).

3.2.2. Antibacterial activity of *X. strumarium* extracts against *Enterobacter* species (Disc diffusion)

Methanolic and ethanolic extracts of *X. strumarium* leaves were tested for their antibacterial activity against *Enterobacter* isolates using the disc diffusion method (Table 6). *X. strumarium* ethanolic extract showed strong inhibitory activity on 30% of the strains. *X. strumarium* methanolic extract demonstrated a strong inhibitory action on 16.67% of the isolates. The ethanolic extract was active against 96.66% of the isolates as compared to the methanolic extract which was active against 86.66% of the isolates, supporting the effectiveness of the ethanolic extract compared to the methanolic extract.

3.3. Biofilm formation and inhibition

3.3.1. Biofilm formation on polystyrene surface

The bacterial isolates' capability to form biofilms on polystyrene surfaces is presented in Table 7. The bacterial isolates capability was allocated as follows: 29.4 % were highly positive biofilm producers with OD_{570} estimates

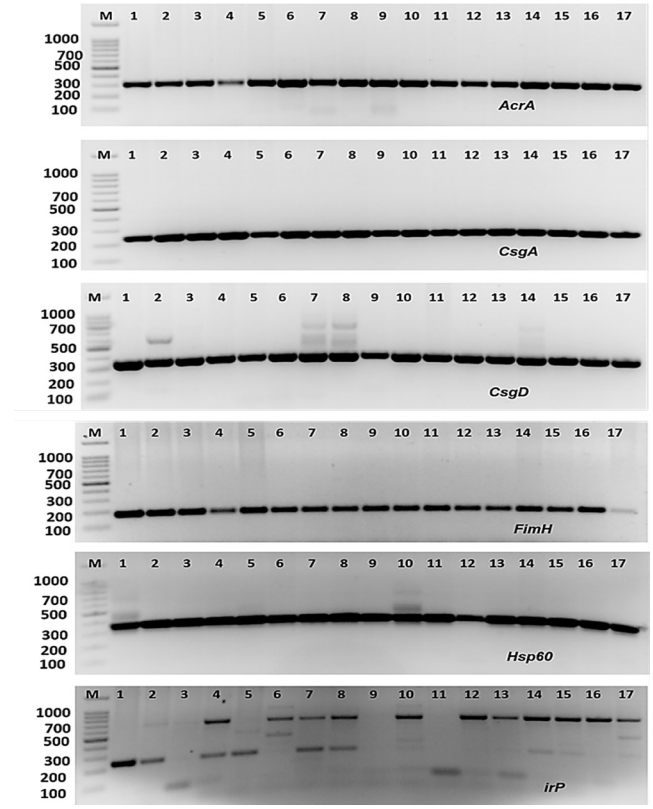

Fig. 2. Amplification of virulence genes of *Enterobacter* species isolates by single PCR. Amplification of *AcrA* gene (312 bp), amplification of *CsgA* gene (230 bp), amplification of *CsgD* gene (343 bp), amplification of *FimH* gene (192 bp), and amplification of *Hsp60* gene (350). M: 100-bp DNA ladder.

between 0.815 and 1.231, and 70.6 % were low-grade positive with OD_{570} estimates between 0.423 and 0.773.

3.3.2. Biofilm inhibition

Biofilm development by *Enterobacter* isolates was inhibited by ethanolic and methanolic extracts of *X. strumarium* (Table 7). The isolates selection in the current study was based on their tendency to create biofilms. Both extracts showed significant biofilm inhibition effectiveness, with 11 strains categorized as low-grade or highly positive biofilms.

3.3.3. Antibiofilm activity

The biofilm inhibition levels observed in the current study ranged between 84.3% and 97.8%, suggesting that the ethanolic extract of *X. strumarium* leaves had broad biofilm inhibition action across all 17 tested isolates. Around 80% of the highly positive isolates were negative for biofilm. Moreover, following treatment, 10 of the low-grade positive isolates (75%) became biofilm-negative.

Methanolic extract showed biofilm-inhibiting properties, with most isolates showing activity between 74.2 and 97.1 %. Additionally, the methanolic extract had similar outcomes as the ethanolic extract produced for the four highly positive biofilm isolates. Four of the low-grade positive isolates tested negative for biofilm. Despite a drastic reduction in biofilm after treatment with the two extracts, isolate No.10 maintained its original biofilm phenotype (Table 7). The capacity of isolate No. 1 to develop a biofilm was not affected by the methanolic extract.

4. Discussion

It is well established that the discriminating power of the full 16S rDNA gene sequence is rather good [18]. The phylogenetic tree of this family based on the 16S rDNA gene is very inconsistent, varying greatly across different techniques and different sets of bacteria [18, 24]. The presence of this gene in all bacteria guarantees that they may be correctly classified into their respective genera and species [10]. Therefore, sequencing is a practical method suitable for many microorganisms, particularly those isolated from nature or other animals. Similarities were found between invertebrate-isolated *E. cloacae* and *E. hormaechei* 16S rDNA gene sequences and GenBank sequences, suggesting that sequencing may be more sensitive than culture-dependent morphological and microscopic identi-

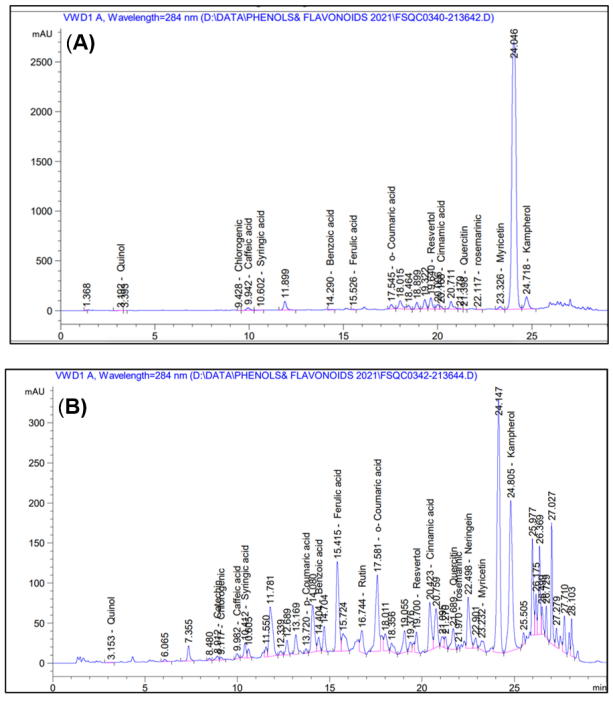

Fig. 3. HPLC analysis for phenol and flavonoid compounds of *X. strumarium* leaves extracts, A) ethanolic extract and B) methanolic extract.

Table 6. Antibacterial activity of *X. strumarium* leaves extracts against *Entrobacter* isolates using disc diffusion.

X. strumarium	**isolates**				
	(+ + + +) *n* (%)	**(+ + +) *n* (%)**	**(+ +) *n* (%)**	**(+) *n* (%)**	**(-) *n* (%)**
Ethanolic extract	9 (30.00%)	11 (36.66%)	7 (23.33%)	2 (6.66%)	1 (3.33%)
Methanolic extract	5 (16.67%)	8 (26.67%)	11 (36.66%)	2 (6.66%)	4 (13.33%)

(+ + + +) strong inhibitory action, (+ + +) complete inhibitory action, (+ +) partial inhibitory action, (+) slight inhibitory action, and (-) no inhibitory action, n: number of isolates.

Table 7. Antibiofilm potentialities of ethanolic and methanolic *Xanthium strumarium* leaf extracts against *Enterobacter* isolates.

Isolates	Biofilm formation OD570±SD	Ethanolic extract OD570±SD	Inhibition (%)	Methanolic extract OD570±SD	Inhibition (%)
ETU-1	0.423±0.062	0.066±0.016*	84.3	0.109±0.033*	74.2
ETU -2	1.221±0.120	0.137±0.024*	88.7	0.145±0.203*	88.1
ETU -3	0.616±0.211	0.049±0.016*	92.1	0.055±0.011*	91.1
ETU -4	0.524±0.113	0.046±0.012**	91.2	0.062±0.165**	88.2
ETU -5	0.766±0.205	0.050±0.058*	93.4	0.070±0.031*	90.8
ETU -6	0.442±0.106	0.020±0.066*	95.5	0.025±0.012*	94.3
ETU -7	0.624±0.213	0.042±0.079**	93.2	0.036±0.089**	94.2
ETU -8	0.773±0.118	0.038±0.016*	95.1	0.041±0.090*	94.6
ETU -9	0.473±0.105	0.070±0.055***	85.2	0.072±0.046***	84.7
ETU -10	0.598±0.114	0.043±0.130**	92.8	0.050±0.067**	91.6
ETU -11	0.892±0.212	0.042±0.079**	95.2	0.054±0.053**	94.1
ETU -12	0.589±0.224	0.034±0.014**	94.2	0.049±0.202**	91.6
ETU -13	0.641±0.106	0.014±0.102*	97.8	0.020±0.067**	96.8
ETU -14	0.542±0.107	0.032±0.021*	94.1	0.081±0.108*	85.1
ETU -15	1.231±0.114	0.129±0.011*	89.5	0.171±0.086*	86.1
ETU-16	0.815±0.115	0.019±0.103*	97.6	0.024±0.111***	95.1
ETU-17	0833±0.211	0.029±0.021*	96.5	0.024±0.011*	97.1

*Isolates changed from low-grade positive to biofilm negative. ** Isolates changed from highly positive to low-grade positive. *** Isolates changed from highly positive to biofilm negative.

fication [18].

Identification at the species or strain level improves the ecological and/or clinical importance of the microbiota findings within most ecosystems, environments, and habitats as compared to identification at the genus level [25]. Host-associated microbial communities, for instance, often include both commensal and pathogenic species of the same genus, making species-level identification of *Enterobacter* isolates a necessity in many studies [24]. Furthermore, some bacterial taxa comprise species that are restricted to one or many locations and that live only in limited niches within a given ecosystem [26].

The Enterobacteriaceae family is a global health concern. It is part of the family Enterobacteriaceae and may be found in different environments. *E. cloacae* and *E. hormaechei* are the most isolated species of this bacterium, and they may be found in humans as well as animals [26, 27]. Nosocomial infections caused by species in this genus are becoming more common, especially in immunocompromised patients, those caring for newborns, and the elderly. They are also becoming common in emergency rooms, wards treating skin and soft tissue diseases, and urology wards [28, 29]. A new health concern has emerged due to the unexpected and exponential growth of antibiotic resistance across numerous bacterial species [10]. This highlights the critical need for the development of novel therapeutic medicines, particularly natural products, for the treatment of infections caused by *Enterobacter* species.

Given the current interest in the hunt for antibacterial and antibiofilm medications, plant chemicals have emerged as viable possibilities. *X. strumarium* stands out due to its therapeutic potential [10]. Both ethanolic and methanolic extracts of *X. strumarium* were tested for their antibacterial effects against *E. cloacae* and *E. hormaechei* strains obtained from invertebrates found in various areas of the Taif governorate. Inhibition of growth tests were used to examine the isolates. The antibacterial activity of *X. strumarium* leaf extracts was reported experimentally [10, 30]. Results from this research suggest that the phenolic and flavonoid chemicals present in *X. strumarium* leaf extracts are responsible for the plant's potent antimicrobial activity [10, 30]. It is evident that flavonoids such as quercetin [31], kaempferol, and catechin [21], exhibit great growth inhibition activity against *Enterobacter* isolates. Phenolic substances, whether they are single molecules or heavily polymerized, invariably have an aromatic ring with one or more hydroxyl substituents [32]. Their structure-activity connections reveal that their structural makeup is the primary factor in determining their radical scavenging and metal-chelating activities [33]. Gallic acid, which exhibited significant antioxidant and anti-bacterial efficacy, is an example of a phenolic acid with a high degree of hydroxylation, which boosts its antioxidant activity. Furthermore, the strong *Enterobacter* species growth inhibition activity in the *X. strumarium* leaf extracts seems to be related to the synergistic action of flavonoids and other phenolic compounds. Flavonoid free radical scavenging ability is proportional to the extent of their hydroxylation [34].

Despite HPLC data showing the presence of ferulic acid, chlorogenic acid, and trans-cinnamic acid in the extracts of *X. strumarium*, the ethanolic extract of *X. strumarium* leaves was more efficient against *Enterobacter* isolates than the methanolic extract in the current investigation. In contrast to the ethanolic extract, which showed substantial levels of ferulic acid, the methanolic one showed high levels of chlorogenic acid. HPLC analysis revealed that the methanolic extract of *X. strumarium* contained more total phenols and higher flavonoid than the ethanolic extracts. Furthermore, the methanolic extract was more potent than the ethanolic extract with regards to the presence of quercetin, ellagic acid, caffeic acid, kam-

pherol, and catechin. Myricetin, resveratrol, benzoic acid, and kampherol were particularly abundant in the ethanolic extract. Researchers have shown that the phenolic chemicals included in plant extracts have a strong relationship with their antioxidant properties [35]. Inhibition rates and anti-biofilm activities shown by ethanolic and methanolic extracts of *X. strumarium* are distinct factors that can potentially be traced directly to the extracts' respective compositions. The development of *Enterobacter* species and other harmful bacteria was also substantially impeded by p-coumaric acid. p-coumaric acid binds to the genomic DNA of bacteria, preventing the DNA from replicating and disrupting biological processes [30]. Strong antibacterial action was shown by quinol against *Enterobacter* via disrupting the bacterial cell membrane and cell wall, increasing permeability, and altering gene expression [36]. However, the bactericidal properties of chlorogenic acid are weak [10].

The capacity of several *E. cloacae* and *E. hormaechei* isolates to form biofilms on polystyrene surfaces was tested, and the results showed that 23.33 % of the isolates were high-grade biofilm producers, while 50 % were low-grade positive producers. These results confirm that *Enterobacter* is the most common bacteria in biofilm-associated diseases and reveal the great ability of *Enterobacter* strains to generate biofilms [37]. Biofilm is an important virulence factor that causes about 80% of microbial infections and 65% of nosocomial infections [38]. Nasal colonization of the respiratory system, soft tissue infections, endocarditis, and urinary tract infections are only some of the disorders associated with the formation of biofilms [20]. Long-term bacterial persistence in the genitourinary tract is a major concern in urology, and biofilms are a major contributor to this problem [39, 40]. The alarming prevalence of biofilm-driven diseases and rising antibiotic resistance have prompted us to investigate the composition and complexity of *E. cloacae* biofilms [37, 41]. Since the production of biofilms by *Enterobacter* strains has been rarely investigated, we provide a detailed analysis that sheds light on the current knowledge of this topic. We used a crystal violet technique to measure bacterial adhesion to different surfaces as a preliminary test for biofilm development by *Enterobacter* strains. Different types of bacteria were colonizing various types of medical equipment, including enteral feeding tubes and Foley latex catheters. Multi-layer biofilm on silicone catheters and greater biofilm production by *Enterobacter sakazakii* on stainless steel and enteral have all been documented as examples of surface-dependent differential biofilm formation [22, 42]. The investigation revealed that myricetin hinders the development of biofilms by some strains of *Enterobacter*. Interestingly, the ethanolic extract exhibited more potent biofilm inhibitory capabilities than the methanolic extract. This finding implies that the components responsible for growth suppression and biofilm inhibition are similar [37, 41]. As a result of their specificity for Bap proteins builds Amyloid, flavonoids were able to inhibit the bacterial biofilm matrix effectively [22]. Myricetin prevents *E. coli* and other bacteria from forming biofilms by blocking a protein called curli [39].

Conclusion

In conclusion, leaf extracts of the medicinal plant *X. strumarium* were utilized against the pathogens *E. cloacae* and *E. hormaechei*. The extracts exhibited considerable biofilm inhibitory activity and potent antibacterial properties, attributed mainly to the phenolic and flavonoid components. *X. strumarium* leaf extracts could be used for treating or preventing *Enterobacter* infections. These extracts showed antibiofilm production for the pathogens *Enterobacter cloacae* and *E. hormaechei* isolates which showed positive biofilm synthesis. Therefore, the antibacterial and antibiofilm properties of ethanolic extract were more effective than methanolic extract, supporting the use of *X. strumarium* for treating infections caused by *Enterobacter* species.

Data Availability

The data presented in this study are available in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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