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MOLECULAR STUDY AND ASSESSMENT OF THE MEDICINAL POTENTIAL OF THE *LEPIDIUM SATIVUM* PLANT EXTRACTS AGAINST ANTIBIOTIC RESISTANCE *E. COLI*

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SUMMARY: This study includes isolation of Escherichia coli *from different sources of human infection (urine, wounds, and burn), and 40 isolates of E. coli were obtained from 150 samples. According to the cultural characteristics, morphological features, and biochemical examination in addition to the API system, the* E. coli *isolates were identified. Antibiotic sensitivity for these isolates was tested, which included 14 antimicrobials. According to the resistance of isolates to antimicrobials, the latter were classified into 10 groups. The isolates varied in their resistance to tested antimicrobials. Isolates E7, E8, E9, E10, and E11 were resistant to all antimicrobials while E1, E2, and E20 were resistant to only one antimicrobial. The results also revealed that imipenem and amikacin were the most powerful antibiotics against all isolates. All isolates were screened for their ability to produce extended spectrum* β-lactamase *enzyme (ESBL). A total of 24 isolates (60%) were found to be ESBL producers. Agarose gel electrophoresis was used and the plasmid contents showed variation in their size; more than one DNA band appeared in the case of some isolates. The reported results indicated the dissemination of plasmids among* E. coli *isolates that may carry resistance genes against wide spectrum antibiotics used clinically. Antibacterial activity of water, methanol, and ethanol extracts of the whole plant of* Lepidium sativum *was carried out using agar plate. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of extract inhibiting the visible growth of each organism on the agar plate. The results showed that the MIC of watery extract was 4% for all chosen isolates, the MIC of ethanolic extract was 5% for isolates E7, E8, and E10 and 6% for E9, and the MIC of methanolic extract was 5% for all isolates under study. The phytochemical screening revealed the presence of alkaloids, flavonoids, tannis, phenols, and saponins in both aqueous and methanolic extract, but saponins was absent in the ethanolic extract.*

Key words: Escherichia coli, Antibiotic resistance, β-lactamase, Lepidium sativum, Antibacterial activity.

INTRODUCTION

Escherichia coli are naturally found in the intestinal tracts of all warm-blooded animals and humans. Most forms of the bacteria are not pathogenic and serve useful function in the intestine. Pathogenic strains of *E. coli* are transferred to seafood through sewage pollution of the coastal environment or by contamination after harvest. *E. coli* food infection causes abdominal cramping, water or bloody diarrhea, fever, nausea, and vomiting (1,2). From

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the late 1990s, multidrug resistant *E. coli* that produce extended spectrum β-lactamases (ESBLs) such as the CTX-M enzymes have emerged within the community setting as an important cause of urinary tract infection. Recent reports have also described ESBL-producing *E. coli* as a cause of blood stream infections (3). Such development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substance, chemotherapeutic agents, and agrochemicals that combine antimicrobial efficacy with low toxicity and minor environmental impact.

Natural products offer an untold diversity of chemical structure. These natural compounds often serve as lead molecules whose activities can be enhanced by manipulation through combination with chemicals and by synthetic chemistry (4,5). An important source of natural product is plants that are rich in a wide variety of secondary metabolites such as tannis, terpenoids, alkaloids, and flavonoids. These metabolites have been found in vitro to have antimicrobial properties (6,7).

Many plants have been evaluated not only for their inherent antimicrobial activity but also for their action as a resistance-modifying agent (8,9). The enhancement of antibiotic resistance by natural or synthetic nonconventional antibiotics has lead to the classification of these compounds as a modifier of antibiotic activity. The known success of these traditional therapies has guided the search for new chemotherapeutic alternatives to fight a variety of infections caused by drug resistant bacteria (10).

Lepidium sativum, known as pepper cress or Elrashad, belongs to the family Brassicaceae (cruciferae). The seeds and leaves of the plant contain volatile oils. *L. sativum* plant and its seeds are considered one of the popular medicinal herbs used in the community of Saudi Arabia, Sudan, and some other Arabic countries as a good mediator for bone fracture healing in the human skeleton. The plant is known to contain imidazole, lepidine, semilepidinoside A and B, β-carotenes, ascorbic acid, linoleic acid, oleic acid, palmitic acid, stearic acid, sinapic acid, and sinapin. A number of recent studies pointed out the traditional uses of *L. sativum* seeds extract in controlling many clinical problems. They were used as antiasthmatic antiscorbutic, aperient, diuretic, galactogogue, poultice, and stimulant. It has been shown to possess antiasthmatic and bronchodilatory potential (11).

The objective of the present study on *L. sativum* was to estimate the possible antibacterial activity of extracts at different concentrations against pathogenic *E. coli*.

MATERIALS AND METHODS

Specimens collection

A total of 150 samples were collected from different sources of human infection (urine, wound, and burn) from Hawler Teaching, Rizgary Teaching, Raparin, and Emergency hospitals in Erbil city. Then the collected samples were identified by performing an API 20E test and also by studying the cultural, morphological, and some biochemical features as IMViC, gelatenase, catalase oxidase, urease, and SIM (12).

Antibiotics Resistance test

To test the sensitivity of *E. coli* isolates to a variety of antimicrobials under study, Muller Hinton agar was used as a growth medium after sterilization and cooling at 45°C. The final concentration of each antimicrobial as explained in Table 1 was added to the medium and poured into sterile petri dishes. After solidification, the plates were inoculated by the streaking method with *E. coli* and then incubated at 37°C for 24 hours. The results were recorded next day (13).

Detection of ESBLs in *E. coli*

ESBLs are plasmid-mediated enzymes produced by a number of gram-negative bacteria (14). A double-disk diffusion test was performed with amoxillin-clavulanic acid surrounded by aztreonam and third-generation cephalosporin disks cefotaxime and ceftazidime (15).

Extraction of plasmid DNA

The plasmid was extracted using GF-1 plasmid DNA extraction kit (Vivantis Company, Ukrania).

Study of plasmid characteristics purified from *E. coli* isolates by agarose gel electrophoresis technique.

The method described by Sambrook and Russell (16) was followed in this study.

Selection of medicinal plant

L. sativum (stems and leaves) were obtained from the local market in Erbil city. The plant was then washed, air dried, and crushed. It was finally stored in polyethylene sack in refrigerator at 4°C. The alcoholic (ethanolic and methanolic) and water extracts were prepared according to Parekh *et al.* (17) with slight modification. Ten grams of plant powder under study were soaked in 100 ml solvent (ethanol and methanol) and water for 3 days with occasional shaking. After this the contents were filtered through eight-layered muslin cloth and then through Whatman No.1 filter paper and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and put in a sterile bottle and stored in the refrigerator.

Table 1: Antimicrobials used in this study.

Antibiotics	Symbol	Stock solution (ig/ml)	Final concentration (ig/ml)	Solvent
Ampicillin	AMP	10	50	Distilled water
Amoxillin-clavulanic acid	AMOC	10	20	Distilled water
Pipercillin	PIP	10	30	Distilled water
Pipercillin/Tazoboctam	PIPT	10	20	Distilled water
Cefazolin	CFZ	10	30	Distilled water
Cefoxitin	CFX	10	50	Distilled water
Cefotaxime	CFT	10	30	Distilled water
Ceftazidime	CAZ	10	30	Saturated $NaHCO_3$ solution
Cefepime	CFP	10	20	Distilled water
Imipenem	IPM	10	10	Distilled water
Amikacin	AK	10	30	Distilled water
Gentamicin	GN	10	30	Distilled water
Ciprofloxacin	CIP	10	30	Distilled water
Tetracyclin	TE	10	20	Distilled water

Agar dilution method

The agar dilution method approved by the NCCLS was followed with the following modification: a series dilution of each extract ranging from 1% (v/v) to 10% (v/v) was prepared in the sterilized nutrient agar (the concentrations were added to the sterilized nutrient agar at 45°C). The plates were left for solidification, and then were inoculated with the bacterial isolates that showed the highest resistance to the antimicrobials during the streaking method. The suspension of the isolates was prepared by inoculating the sterilized nutrient broth with 3-5 colonies of each bacterial isolates; the inoculums were incubated at 37°C for 90 minutes (18). Inoculated plates were incubated at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of extract inhibiting the visible growth of each organism on the agar plates. The presence of one or two colonies was disregarded (19).

Qualitative analysis of phytochemicals

The method followed was described by Buchanan and Gibbson (20). Ten milliliters of the plant extract was mixed with HCl, and then tested with picric acid. The appearance of yellow precipitate referred to alkaloids.

Flavonoids

Ten milliliters of 50% ethanol was added to 10 ml 50% KOH. This solution was then mixed with an equal volume of plant extract. The appearance of yellow color referred to a positive result (21).

Tannis

Ten milliliters from the plant extract was divided into two equal parts. Then drops of CH_3COOPb were added to the first part. The appearance of a white pellet meant a positive result. To the second part were added drops of 1% $FeCl_3$. The formation of bluish green color meant a positive result (20).

Phenols

Three milliliters of plant extract was added to 2 ml of potassium hexacyanoferrate and 2 ml of $FeCl_3$. The formation of bluish green color meant a positive result (22).

Saponin

Five milliliters of plant extract was extremely shacked for 30 seconds, and then left in a vertical case for 15 minutes. The appearance of foam meant the presence of saponin (20).

RESULTS AND DISCUSSION

Collection and identification of *E. coli*

A total of 150 samples were collected from different sources of human infections. After isolation the characteristics of isolates were studied through screening the cultural, morphological, and some biochemical properties. According to the above study, 40 isolates were obtained (23-26). Furthermore, API20E was performed to support the above results. The synonym number obtained for tested samples was (7144572) which indicated that all isolates were *E. coli* as in Figure 1.

Antibiotic resistance profile of *E. coli* isolates

The susceptibilities of 40 isolates were tested against 14 widely used antimicrobials (AMP, AMOC, PIP,

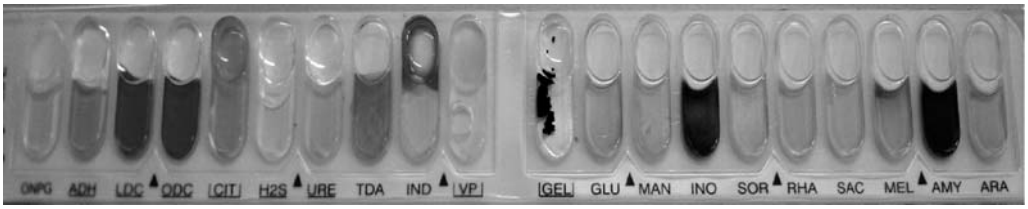

Figure 1: API 20E strip revealed E. coli.

PIPT, CFZ, CFX, CFT, CAZ, CFP, IPM, AK, GN, CIP, and TE). The bacterial isolates were divided into 10 groups according to their resistance to the antimicrobials under study. Table 2 shows the antibiogram groups of E. coli isolates and percent of resistance to antimicrobials. It is clear from Table 2 that (10) group was the most resistant group to antimicrobials under study compared with other groups. The resistance of other groups ranged between 64.28% and 7.1%.

Table 3 shows the resistance percentage of *E. coli* isolates to antimicrobials. The highest resistance was 100% for TE, while the lowest resistance was 0% for IMP and AMK.

This variation in antibiotic resistance on *E. coli* is attributed to the genes locating on the conjugant plasmid. The resistance genes may be transferred to the recipient *E. coli* by conjugation, transformation, or transduction processes or the resistance genes may be located on the bacterial chromosome and jumped to the resistant plasmid by the transposition process (many composite transposones contain genes for antibiotic resistance. This is found in both gram-positive and gram-negative bacteria) (27). Using inaccurate concentration of antibiotics or drug or unnecessary of medicine appointment leads to the resistance of sensitive bacteria, the resistance to multiresistance, in addition to weakening the immunty system in some human due to poor nutrition or heredity factors make bacteria to be more resistant (28).

Frequency of ESBL production by *E. coli*

Forty isolates of *E. coli* were screened for ESBL production by double-disk diffusion method (Figure 2). Of this 24 (60%) isolates were found to be ESBL positive, whereas 16 (40%) were ESBL negative.

Mahesh *et al.* (29) in India reported that the production of ESBL was noted in *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas spp.* at the rate 66.78%, 60.27%, and 58.86%, respectively. Tawfiq (30) in Baghdad found that the prevalence of ESBL production among isolates of gram-negative bacteria was 78.6%

Table 2: Antibiogram groups of E. coli isolates and percent of resistance to antimicrobials.

Antibiogram group	No. of isolates	% of resistance	AMP	AMOC	PIP	PIPT	CFZ	CFX	CFT	CAZ	CFP	IMP	AK	GN	CIP	TE
1	3	7.1	S	S	S	S	S	S	S	S	S	S	S	S	S	R
2	2	14.2	S	S	R	S	S	S	S	S	S	S	S	S	S	R
3	6	21.42	S	R	R	S	S	S	S	S	S	S	S	S	S	R
4	5	28.57	S	R	R	S	S	S	S	S	S	S	S	R	S	R
5	4	35.71	S	R	S	S	S	S	R	S	R	S	S	S	S	R
6	2	42.85	S	S	S	S	R	S	R	R	R	S	S	R	S	R
7	6	50	R	R	S	S	R	R	R	S	R	S	S	S	S	R
8	2	57.14	R	R	R	S	S	S	R	R	R	S	S	R	S	R
9	4	64.28	R	R	S	S	R	R	R	R	R	S	S	S	R	R
10	6	71.42	R	R	R	S	R	R	R	R	R	S	S	S	R	R

Table 3: The resistance of E. coli isolates to antimicrobials.

Antimicrobials	No. of resistance isolates	Resistance %
AMP*	26	65
AMOC	22	55
PIP	21	52.5
PIPT	5	12.5
CFZ	20	50
CFX	10	25
CFT	25	62.5
CAZ	19	47.5
CFP	23	57.5
IMP	0.00	0.00
AMK	0.00	0.00
GN	12	30
CIP	9	22.5
TE	40	100

*Abbreviations are given in Table 1.

and was observed in *E. coli*, *K. pneumoniae,* and *Proteus mirabilis*. Mumtaz *et al.* (31) showed that multidrug resistant and ESBL-producing gram-negative bacteria are the major cause of urinary tract infection. The prevalence of ESBLs among clinical isolates varies in different countries and in different hospitals, and patterns are rapidly changing over time.

The highest rates of ESBL-producing bacterial infection in patients may be due to various factors such as their length of hospital stay, severity of illness, compromised immunity, and urinary catheterization (32). Many clinical laboratories are not fully aware of the importance of ESBL and how to detect them; laboratories may also lack the resources to curb the spread of these resistance mechanisms. This lack of understanding or resources is responsible for a continuing failure to respond appropriately to prevent the rapid worldwide dissemination of pathogens possessing *β-lactamase* (33).

Plasmid profiling

On the basis of gel electrophoresis, the plasmid copies were found to vary between two and five bands (Figure 3). E7 is represented by lane 2, E8 by lane 3, E9 by lane 4, E10 by lane 5, and E3 by lane 6 that have two bands. Plasmid size ranged from 2.5 kb to 10 kb (1-kb ladder).

In this study, we found a large number of plasmids having different molecular weights. All the isolates with 10 kb showed multidrug resistance. Clinical isolates of *E. coli* are known to harbor plasmids of different molecular sizes ranged from 10 kb to 2.5 kb Danbara *et al.* (34) have also reported the plasmid sizes between 3.9 kb and 50 kb in the *E. coli* strain isolated from patients suffering from traveler's diarrhea. Also Jan *et al.* (35) reported that 76 *E. coli* isolates possessed single-sized plasmids, whereas others possessed plasmids of different sizes ranged from 2.3 kb to 26 kb.

The antimicrobial activity of *L. sativum* on *E. coli* isolates

Antimicrobial activities of the extracts obtained from the *L. sativum* plant using different solvents against the tested organisms are shown in Table 4. The result revealed that the selected extracts showed antibacterial activity with varying magnitudes.

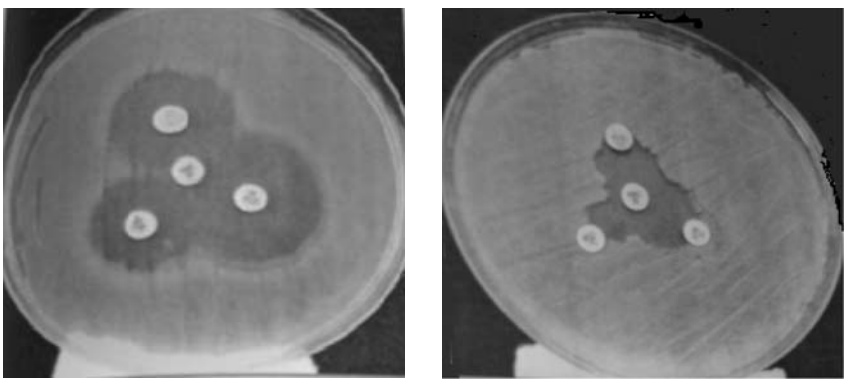

Figure 2: Double-disk diffusion test used for the detection of ESBL production in gram-negative bacteria.

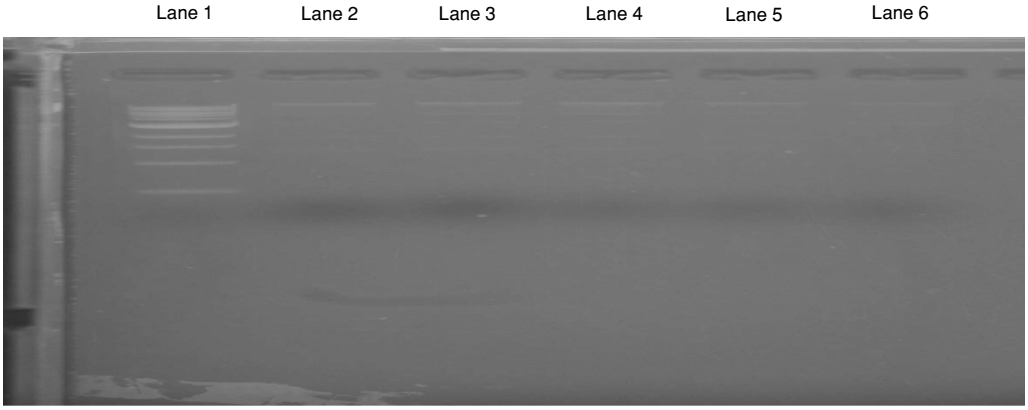

Figure 3: Plasmid profile of E. coli isolates. Lane 1: ladder (1 kb), Lane 2: E7, Lane 3: E8, Lane 4: E9, Lane 5: E10, Lane 6: E3

Table 4 showed that the MIC of watery extract of *L. sativum* was 4% for the chosen isolates while the MIC of ethanolic extract was 5% for isolates No. 7, 8, and 10. For isolate No. 9, the MIC was 6%. However, the MIC of methanolic extract was 5% for all chosen isolates. The MIC was determined as the lowest concentration of extract inhibiting the visible growth of the organism on the agar plate.

Hussain *et al.* (20) investigated the antimicrobial activity of the watery extract for *L. sativum*. They found high activity of the watery extract against Proteus vulgaris, the inhibition zone was 13 mm.

Prakash *et al.* (36) investigated for MIC and MBC of the methanolic extract of the *L. sativum* seed against some Gram negative bacteria like E. coli, Pseudomonas and Klebsiella, were the MIC value ranged from 1.56 mg/ml to 25.0 mg/ml, whereas the MBC value ranged from 6.25 mg/ml to 25.0 mg/ml. Table 5 recognized the phytochemical screening of *L. sativum*, indicating the presence of alkaloids, flavonoids, tannis, phenols, and saponin in both watery and methanolic extracts and the absence of saponin in the ethanolic extract.

Same compounds were extracted by Hasan (20) and Bhasin *et al.* (37), which thus support our results. The possibility of the tested medicinal plant extracts acting as antimicrobial agents (as growth inhibitor) may be attributed to the presence of active compounds, such as alkaloids, flavonoids, etc., in them. These compounds are known to be biologically active. Tannins have been found to form irreversible complexes with proline-rich proteins resulting in the inhibition of the cell protein synthesis. This activity was exhibited against

Table 4: The MIC of aqueous, ethanolic, and methanolic extracts on E. coli isolates.

Watery extraction conc.	No. of isolates 7	8	9	10	Ethanolic extraction conc.	No. of isolates 7	8	9	10	Methanolic extraction conc.	No. of isolates 7	8	9	10
1%	R	R	R	R	1%	R	R	R	R	1%	R	R	R	R
2%	R	R	R	R	2%	R	R	R	R	2%	R	R	R	R
3%	R	R	R	R	3%	R	R	R	R	3%	R	R	R	R
4%	S	S	S	S	4%	R	R	R	R	4%	R	R	R	R
5%	S	S	S	S	5%	S	S	R	S	5%	S	S	S	S
6%	S	S	S	S	6%	S	S	S	S	6%	S	S	S	S
7%	S	S	S	S	7%	S	S	S	S	7%	S	S	S	S
8%	S	S	S	S	8%	S	S	S	S	8%	S	S	S	S
9%	S	S	S	S	9%	S	S	S	S	9%	S	S	S	S
10%	S	S	S	S	10%	S	S	S	S	10%	S	S	S	S

Table 5: Phytochemical screening of L. sativum.

Plant extract types	Compound detected				
	Alkaloids	Flavonoids	Tannis	Phenols	Saponins
Aqueous extract	+	+	+	+	+
Ethanolic extract	+	+	+	+	-
Methanolic extract	+	+	+	+	+

(+): present, (-): absent.

the test organisms with different concentrations of the plant extracts in this study. Apart from exhibiting the antimicrobial activity, tannins also react with proteins to provide the typical tanning effect (11). The mechanism of polyphenols toxicity against microbes probably pertains to inhibition of hydrolytic enzymes (38) or other interactions to inactivate microbial adhesions, cell envelope transport proteins, non-specific interactions with carbohydrates, and so on (39).

Ozcelik *et al.* (40) used six flavonoids against ESBL-producing microorganisms and found that all showed in vitro antimicrobial activity of 32.64 g/ml. Thus the mode of antimicrobial action may be related to their ability to inactivate microbial adhesion, cell envelop transport proteins, and so on. They also complex with polysaccharides.

In conclusion, the three used extracts of *L. sativum* have antibacterial activity against multidrug resistant *E. coli* isolates, and the watery extract was more potent than the alcoholic (ethanolic and methanolic) extracts as a MIC.

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