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Evaluation of Antibacterial Activity of *Laurus nobilis* Leaves Extract against *Escherichia coli* Isolates

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Abstract: The aim of this study is to evaluating the antibacterial activity of *Laurus nobilis* leaves extract on *E. coli* isolates. Maceration and Soxhlet apparatus were used to prepare aqueous and methanolic extracts; total phenolic content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were conducted to determine the active compounds in the extracts. The results showed that both *Laurus nobilis* methanolic and aqueous extracts have a noticeable effect on scavenging free radicals. Free radical scavenging activity. The total phenolic contents were 28.60 ±0.12 and 16.58 ±0.11mg/g in 50 mg/ml, in methanolic and aqueous extracts respectively. The antibacterial activity of *Laurus nobilis* leaves extracts showed that the methanolic extract was more effective than aqueous extract in concentration 64mg/ml. Moreover, the result of the minimum inhibitory concentration (MIC) showed that the methanolic extract on *E. coli* isolates was 16 mg/ml, while the MIC values of aqueous extract were 64 and 128 mg/ml.

Keywords*:* *Laurus nobilis*, antibacterial activity, DPPH, MIC, Total phenol.

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Introduction

Laurel (*Laurus nobilis* L.) is an aromatic plant and evergreen tree which belongs to the family of *Lauraceae*; it is one of the most widely used culinary spices in all Western countries and Asian countries, and as an ornamental plant throughout Europe and America (1). Plants have been well documented for their medicinal uses for thousands of years and traditional medicines are still a major part of habitual treatments of different maladies in different parts of the world. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. Plants are considered as one of the main sources of biologically active materials (2). The plant's leaves and berries are commonly used as a spice aroma and enhancer for foods especially for meats, sauces and soups. Besides its special aroma, it is also used to cure diseases all over the world. Some compounds of this plant such as essential oils and organic acids have shown strong antibacterial activity against some foodborne pathogen microorganism besides spoilage bacteria (3,4). *Escherichia coli* are gram-negative bacilli of the family Enterobacteriaceae. They are facultative anaerobes and nonsporulating. *E. coli* are normal inhabitants of the human large intestine. Most strains are harmless, but some strains acquire bacteriophage or plasmid DNA-

encoding enterotoxins or invasion factors and become pathogenic. These virulent strains are responsible for diarrheal infections worldwide, as well as neonatal meningitis, septicemia, and urinary tract infections (UTIs). The source of *E coli* and other gram-negative bacterial pathogens in neonatal infections is often through the maternal genital tract. Hospital acquisition of gram-negative organisms through person-to-person transmission from nursery personnel or environmental sites can occur (5).

Materials and methods

Chemical reagents

The chemical reagents Absolute methanol was purchased from (BDH, England). Folin Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), Resazurin dye, Muller-Hinton agar and broth (Himedia, India).

Collection of plant

Laraus nobilis were collected from the local Iraqi markets, identified as (*laraus nobilis L*) by the specialist, Department of Biology, College of Science, University of Baghdad. The leaves were washed with water and dried at room temperature, and ground using a grinder, then stored at 4^{o}C for further analysis.

Preparation of aqueous extract

The aqueous extract was prepared according to (6), 250 grams of *laraurs nobilis* leaves macerated in 2000 ml of distilled water for 72 hours, after extraction, the mixture was filtered through Whatman No.1 paper. The filtrate evaporated to dryness under vacuum at 50^{o}C by a rotary evaporator to eliminate water. The resulting extract stored in amber glass vials at 4^{o}C until analyzed.

Preparation of methanolic extract

The methanolic extract was prepared according to (7) by using Soxhlet apparatus. 250 gm of *laraus nobilis* leaves were put in a thimble and 250 gm of *L. nobilis* leaves were put in a thimble and 1700 ml of 70% methanol and 300 ml of distilled water was added within 40-60 ^{o}C for 6 hours. The solution was filtered through a filter paper Whitman No.1 and evaporated to dryness under vacuum at 40^{o} C by a rotary evaporator to get rid of methanol; the extract was stored in amber glass vials at 4^{o}C until analyzed.

Determination of total phenolic contents

Total phenolic content of *Laurus nobilis* extracts were determined spectrophotometrically using the Folin-Ciocalteu method described by (8), 2 ml of Folin-Ciocalteu reagent (diluted 10 times) was mixed with 1.6 ml of 7.5% sodium carbonate solution and 0.4 ml of *Laurus nobilis* extracts. The volume was completed to 5 ml by adding distilled water. The tubes were covered with parafilm for 30 min. at room temperature, and then the absorbance was read at 760 nm spectrophotometrically.

Evaluation of the antioxidant activity (DPPH assay)

According to (9), the antioxidant activity of the prepared *L. nobilis* methanolic and aqueous leaves extracts was conducted. Five ml of a freshly prepared 0.001 % of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was mixed with 50 µl of different concentrations (0.312, 0.625, 1.25, 2.5,

5 and 10) mg/ml, these were prepared by dissolving 0.01 gram of the *L. nobilis* extract in distilled water. Then, the volume was completed into 10 ml to make the working solution 50 mg/ml. Serial two-fold dilutions of the *L. nobilis* extract were prepared to make the concentrations 10- 0.312 mg/ml.The absorbance of each dilution, after 30 minutes, was measured at 517 nm. Butylated hydroxytoluene (BHT) and vitamin C were used as a positive control. All tests were performed in triplicate. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated.

Bacterial isolates

Escherichia coli isolates obtained from student from Institute of Genetic Engineering and Biotechnology and they were diagnosed, and for further diagnosis of E. *coli* isolates the vitek-2 system had been utilized in this study.

Antibiotic susceptibility test

Kirby-Bauer method was followed as described by World Health Organization (10), to carry out the antibiotic's susceptibility test for 10 different antibiotics. The bacterial suspension was prepared by picking 1-2 isolated colonies of bacteria from the original culture and introduced into a test tube containing 4 ml of normal saline to produce a bacterial suspension of moderate turbidity compared with the standard turbidity solution. This approximately equals to $1.5x10^8$ CFU/ml. By a sterile cotton swab, a portion of bacterial suspension was transferred and carefully and evenly spread on Mueller- Hinton agar medium, and then it was left for 10 min. Thereafter the antimicrobial discs were placed on the agar with a sterile forceps pressed firmly to ensure contact with the agar. Later the plates were inverted and incubated at 37ºC for 18-24 hours. Inhibition zones developed around the discs were measured by a millimeter (mm) using a metric ruler according to Clinical Laboratories Standards Institute (11).

Disc diffusion method

Disc diffusion method for antibacterial activity was carried out according to the standard method by (12) to assess the presence of antibacterial activities of the *Laurus nobilis* methanolic and aqueous extracts. The bacterial culture (which have been adjusted to 0.5 McFarland standard), was used to inoculums Muller Hinton agar plates. A stock solution of plant extract was prepared by dissolving 4 g of the extracts with 10 ml of their respective solvents (distilled water and dimethyl sulfoxide (DMSO) for an aqueous and methanolic extract respectively) to produce a final concentration of 400 mg/ml. The stock solution was then diluted to concentrations of 100, 200 and 400 mg/ml of extract. 20 μl of each dilution was impregnated into sterile blank discs 6mm in diameter, all discs placed on the Mueller Hinton agar surface. The plates were incubated at 37°C for 18 to 24 hours. After the incubation, the antibacterial activity was evaluated by measuring the diameter of the inhibition zone around the discs.

Determination of MIC of *Laurus nobilis* extracts

The broth microdilution method was used to determine the (MIC) of the *Laurus nobilis* extracts using the 96-well microtiter plate. The working solution of the plant extracts was

prepared at 512 mg/ml in broth and serial two-fold dilutions of extract were prepared directly on the plate to make the concentrations 512-4 mg/ml for each the methanolic and aqueous extracts. 200 µl of the prepared *Laurus nobilis* methanolic and aqueous extracts were introduced into the first wells in row A. Rows B-H in columns had 100 µl of the broth alone. Twofold serial dilutions using micropipette were done systematically down the columns (from rows A-H). 100 µl was removed from the starting concentrations in row A and transferred to the next row with the 100µl broth, properly mixed, and the procedure was repeated up to the last row (H) where the last 100µl was discarded. This brings the final volume in all the test wells with the extracts to 100 µl except the column which had 200 µl of the broth that served as sterility control. 100µl of the 1×10^6 CFU/ ml bacterial inoculum was transferred into all the wells except the negative control . Microtiter plates were incubated at 37^oC for 18-20 hrs. After incubation, 20 µl of resazurin dye was added to all the wells and incubated for 30 minutes to observe any color changes. The Minimum Inhibitory Concentrations were determined visually in broth micro dilutions as the lowest concentrations of the extracts at which no color changed from blue to pink in the resazurin broth assay (13).

Results and discussion

Total phenolic content of *Laurus nobilis* leaves extracts

Numerous phenolic compounds have been studied for their biological properties and benefits to human health(14). The total phenolic contents of the aqueous and methanolic *Laurus nobilis* leaves extracts were evaluated by using Folin-Ciocalteu reagent. The results showed that the total phenolic content of the *Laurus nobilis* extracts increased gradually with increases of the concentration, with significant differences ($P\leq0.01$). The highest values were 28.60 ±0.12 and 16.58 ±0.11mg/g in 50 mg/ml in both aqueous and methanolic extracts respectively as shown in Table (1). The number of phenols in the *Laurus nobilis* L. extracts varies between 21±0.72 and 494.86±3.62 mg equivalent to gallic acid/gram (15). Another study by (16) reported the total phenolic content of alcoholic *Laurus nobilis* extract was 71.2±2.5.

Table (1): Total phenolic content of *Laurus nobilis* leaves extract

Concentration (mg\ml)	Methanolic extract (mg\g)	Aqueous extract (mg\g)	LSD value
1.25	15.43 ±0.13	5.42 ±0.05	0.607 **
2.5	22.76 ±0.12	9.39 ±0.02	0.588 **
5	28.60 ±0.12	16.58 ±0.11	0.692 **
LSD value	0.445 **	0.236 **	---
**** (P≤0.01).**			

Evaluation of the antioxidant activity (DPPH assay)

The antioxidant activity of the *Laurus nobilis* methanolic extract was evaluated by 1,1-diphenyl-2-picryl-hydrazil (DPPH) free radical-scavenging method. The relatively stable DPPH radical had been used widely to test the ability of compounds to act as free radical scavengers or

hydrogen donors. In addition, this capability was used to evaluate antioxidant activity (17). *Laurus nobilis* extracts had free radical scavenging activity; this was evident in a concentration-dependent manner with significant differences (P≤0.01) between concentrations. The scavenging activity increased gradually with extract concentrations. The radical scavenging activity of each extract was compared at concentrations of (0.312, 0.625, 1.25, 2.5, 5 and 10) mg/ml. BHT and vitamin C were used as control. The results showed that the free radical scavenging activity of the methanolic extract was (92.44 ±0.08) in 10 mg/ml and it was higher than the aqueous extract (87.13 ±0.02) in the same concentration. It was approached with the natural antioxidant (vitamin C) and artificial antioxidant (BHT) which were (97.20 ±0.06 and 93.11 ±0.12) respectively as shown in Table (2).

Table (2): Radical scavenging activity of *L. nobilis* leaves extract

Conc. (mg/ml)	Aqueous extract	Methanolic extract	BHT	Vit. C	LSD value
0.312	17.82 ±0.03	31.36 ±0.11	30.18 ±0.08	82.44 ±0.23	0.597 **
0.625	25.45 ±0.21	54.55 ±0.22	52.15 ±0.11	91.42 ±0.12	0.703 **
1.25	35.72 ±0.13	67.48 ±0.21	75.43 ±0.29	94.26 ±0.02	0.792 **
2.5	54.61 ±0.16	87.64 ±0.14	88.61 ±0.14	96.81 ±0.01	0.831 **
5	74.83 ±0.11	90.78 ±0.02	91.69 ±0.11	96.93 ±0.02	0.788 **
10	87.13 ±0.02	92.44 ±0.08	93.11 ±0.12	97.20 ±0.06	0.847 **
LSD value	0.398 **	0.461 **	0.497 **	0.342 **	---
** (P≤0.01).					

The table above illustrates a significant ($p \leq 0.01$) decrease in the concentration of DPPH radicals due to the scavenging of the both *Laurus nobilis* extracts and standards , The methanolic extract of *Laurus nobilis* showed a significantly stronger DPPH scavenging activity than the water *Laurus nobilis* extract ($p \leq 0.01$).The strength of the scavenging activity of aqueous and Methanolic extracts of *Laurus nobilis* and standards on the DPPH radical followed the order of BHT > methanolic extract > aqueous extract >Vit C, respectively. These results indicated that both *Laurus nobilis* extracts have a noticeable effect on scavenging free radicals. Free radical scavenging activity also increased with increasing concentration and that was agreement with (18) they found that the both Laurus *nobilis* extracts had effective reducing power, DPPH· free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities.

Antibiotic susceptibility test

The antibiotic susceptibility test revealed that the resistance of *E. coli* isolates was 40% - 100% for the antibiotic. The highest resistance percentage was found toward Amikacin, Imipenem, Ticarcillin and Ceftriaxone as shown in Table (3). Due to the increase in resistant isolates, there is a paramount need to develop new and innovative antimicrobial agents. Therefore, researchers are looking for new leads in the discovery of better alternatives against multidrug resistant microbial strains. Among the potential sources of new agents, plants have long been investigated owing to their popular use as remedies for diverse infectious diseases because they contain many

bioactive compounds that could be interest in therapeutics (19).

Table (3): Antibiotic sensitive test of *E. coli* isolates

Antibiotic / Isolate	CS	AK	AUG	FEP	IM1	TOB	CIP	TC	TMP	CRO	Percentage of Resistance
E_1	S	R	R	R	R	S	S	R	R	R	70 %
E_2	S	S	R	R	R	S	R	R	R	R	70 %
E_3	R	I	R	R	R	S	S	R	R	R	70 %
E_4	R	I	R	R	R	R	R	R	R	R	90 %
E_5	R	I	R	R	R	S	R	R	R	R	80 %
E_6	R	I	R	R	R	S	S	R	R	R	70 %
E_7	R	R	R	R	R	S	S	R	S	R	70 %
E_8	R	R	R	R	R	R	R	R	R	R	100 %
E_9	R	S	R	R	R	R	R	R	R	R	90 %
E_{10}	R	R	R	I	R	S	S	R	R	R	70 %
Resistance rate	80%	40%	100%	90%	100%	30%	50%	100%	90%	100%	

(*E*): *E. coli*, **(R):** Resistant, **(S):** Sensitive, **(I):** intermediate, (**CS):** Colistin**,** (**AK):** Amikacin**, (AUG):** Amoxicillin-Clavulanic acid**, (FEP):** Cefepime**,** (**IM1):** Imipenem**,** (**TOB):** Tobramycin**,** (**CIP):** Ciprofloxacin**,** (**TC):** Ticarcillin**,** (**TMP):** Trimethoprim**,** (**CRO):** Ceftriaxone.

Antibacterial activity of *Laurus nobilis*

The antibacterial activity of *Laurus nobilis* leaves extracts was evaluated by disk-diffusion method on *E. coli* isolates. The result showed that the methanolic extract was more effective than aqueous extract in concentration 100mg/ml with a significant difference ($P \leq 0.01$) as seen in Table (4).

Table (4): Antibacterial activity of *Laurus nobilis* methanolic and aqueous extracts on *E. coli isolate*

No. of Isolate	Methanolic extract			Aqueous extract			LSD value
	100 mg/ml	200 mg/ml	400 mg/ml	100 mg/ml	200 mg/ml	400 mg/ml	
E_1	13.33 ±0.33	18.33 ±0.33	22.33 ±0.33	10.33 ±0.33	12.66 ±0.33	17.67 ±0.33	2.07 **
E_2	12.67 ±0.67	16.33 ±0.33	20.66 ±0.33	10.67 ±0.33	12.33 ±0.33	16.33 ±0.33	1.84 **
E_3	13.33 ±0.33	16.33 ±0.33	21.67 ±0.33	10.67 ±0.66	12.67 ±0.33	17.33 ±0.33	1.91 **
E_4	9.33 ±0.33	12.33 ±0.67	16.33 ±0.33	7.66 ±0.33	9.66 ±0.33	12.67 ±0.33	1.68 **
E_5	12.33 ±0.33	17.67 ±0.33	23.33 ±0.67	10.33 ±0.33	13.67 ±0.33	17.66 ±0.33	1.98 **
E_6	13.33 ±0.33	17.33 ±0.33	22.33 ±0.67	10.33 ±0.66	14.33 ±0.33	18.67 ±0.33	2.14 **
E_7	12.67 ±0.33	17.66 ±0.33	22.33 ±0.33	9.66 ±0.33	13.67 ±0.33	17.67 ±0.33	1.84 **
E_8	10.33 ±0.33	13.67 ±0.33	17.66 ±0.66	8.66 ±0.33	11.33 ±0.33	14.33 ±0.33	1.79 **
E_9	9.66 ±0.33	14.33 ±0.67	17.66 ±0.33	8.33 ±0.33	11.66 ±0.33	14.33 ±0.33	1.95 **
E_{10}	13.67 ±0.33	18.33 ±0.67	23.67 ±0.33	10.33 ±0.33	14.67 ±0.33	18.67 ±0.33	1.78 **
LSD value	1.21 **	1.36 **	1.36 **	1.24 **	0.983 **	0.983 **	---
** ($P \leq 0.01$).							

The antioxidant activity of *L. nobilis* aqueous and methanolic extracts may be due to flavonoids derivatives (e.g., Apigenin, Caffeic acid, Gallic acid, Rosemaric acid, Rutin, Syringic acid and kaempferol)) present in the extracts Flavonoids subgroups are the most common, and almost ubiquitous, throughout the plant kingdom (20). The antimicrobial effects of flavonoids related to the interactions with the cell membrane; (21) showed that flavonol quercetin, rutin (quercetin-3-O-rhamnoglucoside, compound) and tiliroside decreased the bilayer thickness; furthermore rutin disrupted the lipid monolayer structure. Furthermore**,** (22) reported that the mode of action of killing bacteria by flavonoids was found to be an oxidative burst by the generation of reactive oxygen species (ROS) that cause alteration in the membrane permeability and membrane damage. In this study, the antimicrobial activity seems to be related with the amount of flavonoids present in the methanolic and aqueous extracts, which is the main component of *Laurus nobilis* leaves extracts.

Determination of the (MIC) of the *Laurus nobilis* leaves extracts

Broth microdilution method was used to determine the MIC of the plant extracts using the 96-well microtiter plate. A method using the oxidation-reduction colorimetric indicator resazurin has been proposed for the determination of the MIC of the antimicrobial agents against *E. coli*. Resazurin, which is blue in its oxidized state, turns pink when reduced by viable cells and can easily be detected with the naked eyes and the MIC determined even without the aid of a spectrophotometer (23). The result of the MIC showed that the methanolic extract was more effective than aqueous extract. The MIC values of the methanolic extract on all *E. coli* isolates were 16 mg\ml except isolate No.4,8,9 which were 64, 32, 32 mg\ml, while the MIC values of aqueous extract were 64 mg\ml except the isolates No. 4, 8 and 9 which were 128 mg\ml, as shown in Figure (1).

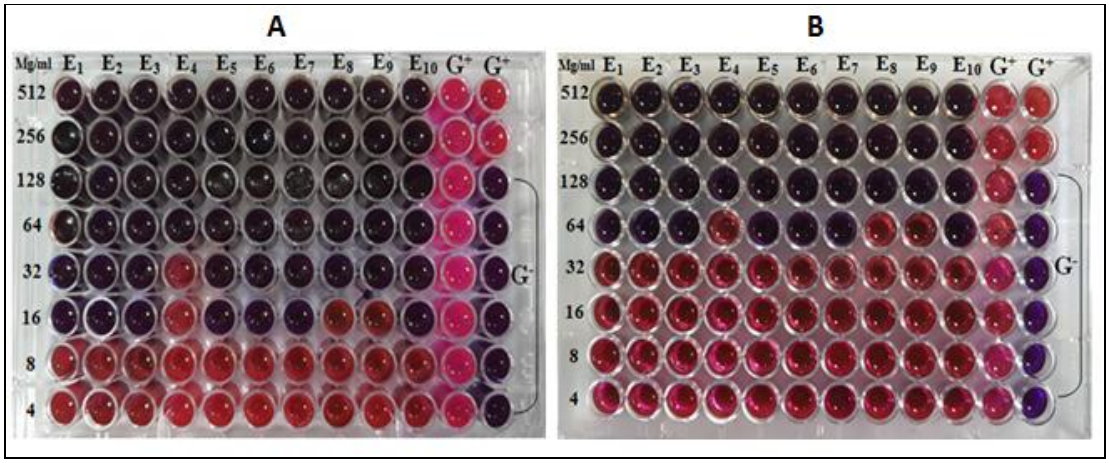

Figure (1): MIC of *Laurus nobilis* (A) methanolic leaves extract, (B) aqueous leaves extract.
(E): *E. coli* isolate, **(G^+):** Control positive (Bacteria + Media), **(G^-):** Control negative (Media only)

The result of this study was agreement with (24). They reported that the ethanol extract of *L. nobilis* leaves proved antibacterial activity against *E.*

coli with MIC: 22.2 mg/mL, so that is considered a kind of drug development substance for multidrug resistant bacteria. Another study by (25) reported that the *L. nobilis* Methanolic extracts were better inhibitors of *E. coli* growth than the aqueous extracts and they explain that the differences in bioactivity might be related to the higher phenolic contents in methanolic extracts.

Conclusion

The antimicrobial activity of *Laurus nobilis* leaves extracts related with the flavonoid's derivatives (Apigenin, Caffeic acid, Gallic acid, Rosemaric acid, Rutin, Syringic acid and kaempferol) present in the methanolic and aqueous extracts. The methanolic extract was more effective than aqueous extract as antibacterial activity against *E. coli* isolates which were more resistant to commonly used antibiotics.

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