Research Article

Evalution of Antibacterial Activity of *Portulaca oleracea* against *Escherichia Coli in vitro*

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Abstract | The study was conducted to find out the mechanism of action and the activity of *Portuluca oleracea* methanolic extract *in vitro* against *Escherichia coli*. The first step was involved collection and extraction of *P. oleracea* with absolute methanol in a Soxhlet apparatus and phytochemical analysis of extract. While in the second step, the antibacterial effects of the extract as compared with ciprofloxacin, as well as zone of inhibition, minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC), by using 4, 8, 16, 32, 64 and 128 mg/ml concentration of the extract, while 1.562, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400, 800 µg/ml concentration of Ciprofloxacin, on the other hand study mechanism of action of the extract against the same bacteria by Fe-Scanning Electron Microscopic(Fe-SEM). The extraction ratio was (15%) and the phytochemical analysis referred to the presence of alkaloids, flavonoids, steroids, carbohydrates, tannins, free amino acid and protein. While the gram staining, cultural characteristics, biochemical tests, Vitek 2 system and PCR tests referred to that bacteria was *E. Coli*. Bacteria was susceptible to the alcoholic extract of *P. oleracea* and gave an inhibitory zone 14-30 mm, while 15- 37 mm for ciprofloxacin. The MIC was 32 mg/ml for the extract and 25 µg/ml for the antibiotic. The MBC was 64 mg/ml for the extract and 50 µg/ml for ciprofloxacin. Fe-Scanning Electron Microscope results showed that surface of the bacteria was shrunk, rough, and leakage of the intracellular substances when treated with the MIC of the extract. From the foregoing results, we can be concluded that alcoholic extract of *P. oleracea* has an effect on *E. coli in vitro* by certain mechanism of action when compared with ciprofloxacin.

Keywords | Antibacterial Activity, *Portuluca oleracea*, *E coli,* Minimum inhibitory concentration, Zone of inhibition, Ciprofloxacin.

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INTRODUCTION

Escherichia coli probably one of the most commonly studied microorganisms and play a significant role in medicine, biological sciences, and industry (Atif et al., 2023). It is gram-negative, rod-shaped, non-spore forming, motile bacteria range in size from 2 micrometres in length to 0.6 micrometres in diameter and have a cell volume of 0.6 to 0.7 micrometres (Darnton et al., 2007). *E. coli* is a commensal bacterium that is classified as a member of the Enterobacteriaceae family (Tenaillon et al., 2010). It is an oxidase-negative facultative anaerobe with an optimal growth PH 6.0-7.0 and temperature of 37 °C. It can ferment glucose, lactose, and sucrose. However, certain laboratory strains of *E. coli* can proliferate at a temperature of 49 °C (Fotadar et al., 2005). It is found in the gastrointestinal tract of human and animals, but some species are known as pathogenic such as *E. coli O157:H7* (Khudhir,

2021). About 39% and 30% of *E. coli* were sequentially isolated throughout the winter and summer seasons, temperature (both high and low) was significantly the most predisposing factors (Shareef, 2005). Extra intestinal pathogenic *E. coli* (ExPEC) is the term used to describe *E. coli* that can cause infections outside of the intestine system (Ahmed et al., 2019). According to recent data, the development of *E. coli* bacterial biofilms in a host generally appears to be mostly an intracellular process (Hanoun and Al-Samrraae, 2019). The second-generation group of nalidixic acid derivatives' strongest fluoroquinolone chemotherapeutic agent is ciprofloxacin (Majalekar and Shirote, 2020). It is frequently employed to treat infections that are primarily brought on by gram-negative and a few gram-positive bacteria and occur in both human and veterinary medicine (Sodhi and Singh, 2021). Eggs dipped in low concentrations of ciprofloxacin and enrofloxacin have minimal teratogenic and neurotoxic effects (Abdulhamza and Ibrahim, 2019). Ciprofloxacin binds to bacterial DNA gyrase with 100 times the affinity of mammalian DNA gyrase (Davis et al., 1996). In order to their broad spectrum of activity, the development of microbial tolerance to many antimicrobial drugs has become a well-known phenomenon, which is a big worry (Abdulridha and Ibrahim, 2018). Since synthetic medications are known to have a variety of harmful effects on human lives (Muraih et al., 2020). Plants have long been valued as a rich source of therapeutic and nutraceutical compounds (Pandey et al., 2011; Hasan, 2019). The use of herbal medicine is supported by scientific research, it is one of the promising solutions to the problem of the ongoing rise in bacterial resistance to antibiotics, the development of multi-resistant strains, and the treatment problems that result (Saliem and Abdulridha, 2022). In mild cases of infectious disorders, medicinal plants might be an alternate therapy option (Sarwat and Ahmad, 2012). They are also well-known for their antioxidant capabilities and have long been considered a rich source of antibacterial and anti-inflammatory agents (Hasan et al., 2014; Khalaf and Shawkat, 2023). Plant species are of significant interest for their therapeutic pharmacological qualities and their culinary uses, and they may serve as a source for novel, powerful antibiotics to which pathogenic strains are not resistant (Grimalt et al., 2018). The Portulacaceae family includes the wild plant purslane (*Portulaca oleracea*) (Jalali and Rahbardar, 2022). Purslane individuals exhibited an herb habit with branched shoot stems. The petiole is absent, the leaves are alternating, and the stems and leaves are glabrous (Al-Newani, 2019). Even though purslane is considered one of these unusual plants, it is common and well-liked in most places, including Iraq, China, Europe, and Mediterranean nations. It is also edible. More importantly, purslane is thought to have a variety of biological uses in the realms of food and medicine (Nemzer et al., 2020). The primary and secondary metabolites in *P. oleracea* include alkaloids, terpenes, coumarins, flavonoids, organic acids, and other ingredients (Syed et al., 2016). Which have strong antibacterial, anti-inflammatory, analgesic, anti-tumor, anti-oxidation, immune enhancement, and anti-cough effects (Du et al., 2017; Chen et al., 2019). Ethanolic extract from *P. oleracea* have antibacterial effect on enter toxigenic *E. coli,* with certain concentration can destroy the cell wall, damage the permeability and integrity permeability of the bacterial cell membrane, cause morphological changes, and inhibit the formation of *E. coli* biofilm with dose dependence (Jiang et al., 2021). The study was conducted to find out the mechanism of action and the activity of *Portuluca oleracea* methanolic extract *in vitro* against *Escherichia coli*. The first step was involved collection and extraction of *P. oleracea* with absolute methanol in a Soxhlet apparatus and phytochemical analysis of extract.

MATERIALS AND METHODS

Extraction And Phytochemical Screening Of Plant.

The sample (100 g) were extract in a Soxhlet extractor with methanol at 60°C for 6 hours. The extract will then have filtered and concentrated in rotary evaporator at 45°C. Finally, the extracts will have dried and kept in the dark at 4°C until further use (Ercisli et al., 2008). While the chemical analyzes were carried out on *Portulaca oleracea* methanolic extract using the standard methods of detection of major secondary metabolites.

Identification Of Bacteria

Using MacConkey agar and Eosin Methylene Blue (EMB) agar, microscopic identification and cultural characteristics were recorded on the medium. These characteristics included shape, size, and color, for the purpose of identifying *E. coli*. The culture media was prepared in accordance with the manufacturer's instructions, sterilized by autoclaving at 121 C°, 1.25 kg/cm2 for 15 min., and then subjected to biochemical characterization, including the Indole test, Catalase test, Methyl Red test, identification by the Vogues' Proskaur test, VITEK 2 System, and, finally, molecular characterization by PCR.

Preparation Of Standard Bacterial Suspension

Using the typical McFarland solution No. 0.5, it was possible for calculating viable average number of *E. coli* cells per ml of the stock suspension. By taking 1 ml of an overnight culture of an isolated bacterial suspension (nutrient broth), rinsing it with 9 ml of peptone water, and then 1 ml of suspension diluting it 10 times in serial doses, According to Baron et al. (1994), standard McFarland solution No. 0.5 was made as follows: 100 ml of distilled water were mixed with 1.175 g of barium chloride (BaCl2.2H2O) to create Solution A. Solution B was created by mixing

100 ml of distilled water with 1 cc of concentrated sulfuric acid (H2SO4). By adding 0.5 ml from solution A to 99.5 ml from solution B, the two solutions were combined. A rough estimate of 1.5 ×10^8 cells/ml was obtained using the prepared solution to compare the turbidity of the bacterial suspension (Quinn et al., 2004).

Preparation Of Alamar Blue Reagent

By combining 337.5 mg of resazurin powder with 50 ml of sterile distilled water in a clean beaker, a resazurin solution was created. To guarantee homogeneity, the solution was mixed for an hour in a sterile vortex mixer. The resazurin solution was then kept in a brown bottle to prevent exposure to light since it is sensitive to light (Teh et al., 2017). The preparation process was carried out in the dark.

Antibacterial Activity Of The *Portulaca Oleracea* Methanolic Extract And Ciprofloxacin (Cip) *In Vitro*

According to Perez et al. (1990), the agar well diffusion method was used to evaluate the antibacterial activity of the prepared *P. Oleracea* extract and ciprofloxacin. Determination of the extract and ciprofloxacin activity by using the concentrations, 4, 8, 16, 32, 64 and 128 mg/ ml for the extract, while 1.562, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 µg/ ml for the antibiotic. 500 ml of sterile Mueller Hinton agar were combined with 5 ml of standardized *E. coli* bacterial stock suspension 1.5x 10^8 CFU /ml, and 25 ml of the resulting inoculated Mueller Hinton agar was put into each sterile petri dish. Ten minutes were given for the agar to set in order for it to solidify, then a 3 wells 6 mm in diameter were made using a sterile Pasteur pipette. After that, wells were filled with 100 microliters containing different concentration from extract, ciprofloxacin and the last one filled with distilled water, at room temperature for two hours, which allowed it to diffuse. For 24 hours, the plates were incubated at 37 °C, and five replicates were performed for each concentration of extract and ciprofloxacin. Zone of inhibition diameter surrounding each well was measured by ruler in millimeters against the tested organism by using zone meter.

Determination Of Minimum Inhibitory Concentration(Mic)And Minimum Bactericidal Concentration (Mbc) Of *Portulaca Oleracea* Methanolic Extract And Ciprofloxacin

A stock solution of *P. Oleracea* methanolic extract and ciprofloxacin were prepared in Mueller-Hinton broth, then make series dilution in different concentrations that ranges between 4- 128 mg/ ml for the extract, while ciprofloxacin ranges between 1.562- 800 µg/ ml, which were prepared in 96 well micro-titer plate with a U shaped bottom, 100 µl of 10^6 CFU/ml *E. coli* was inoculated into each well and incubated on 37 °C for 22 hours (CLSI, 2018). 200 µl of Muller-Hinton broth was introduced to the blank wells that were left without microorganisms as a negative control. Alamar blue was employed as an indicator to determine the bacterial growth after 22 hours because the resazurin-based solution serves as a cell health indicator by employing the reducing power of living cells to objectively measure viability. Alamar blue reagent's active component, resazurin, is a non-toxic, cell-permeable substance that is blue in colour and essentially non-fluorescent. Resazurin is transformed into the highly luminous chemical resorufin when it enters living cells (Teh et al., 2017).

Fe-Scanning Electron Microscope

Field- emission scanning electron microscope (Fe_ SEM) was used to observe the morphological changes according to the method as described by (Bajpai et al., 2013) with modifications. The bacterial sample was prepared for scanning electron microscope by prepare tube contained on 100 µl of the bacterial suspensions 1×10^8 CFU/ml that inoculated onto Mueller–Hinton broth (MHB) tube containing 32 mg/ ml of *Portulaca oleracea* methanolic extract. This tube was incubated at 37°C for 4 hours, and 600 µl of the aliquot was dispensed on glasses cover slid 1x1 cm. After treatments coverlids were washed with 0.1M of phosphate buffer saline. The washing technique was repeated 3 times and 1ml of glutaraldehyde 3% and paraformaldehyde 2% in 0.1M potassium phosphate buffer was added. Then, 3 washes with buffer solution were carried out. Dehydration was conducted with a gradual increased in the ethanol concentrations 50%, 60%, 70%, 80%,90% and 100%. After dehydration, the sample was dried in a silica desiccator for a 72 hours. before being analyzed by a scanning electron microscope (Ramage et al., 2001; Potts, 1997). All samples were sputter-coated with gold in an ion coater for 2 min, then underwent Fe-scanning electron microscopy microscopic inspections.

Statistical Analysis

The Statistical Analysis System (SAS) (2018) programmer was used to analyze the data and find the influence of various factors on the research parameters. In this study, a significant comparison of means was made using the least significant difference (LSD) test (ANOVA).

RESULTS AND DISCUSSION

Extraction of *P. Oleracea* with absolute methanol gave a dark blue color pasty extract with extraction ratio of 15%. The result was in agreement with Ahmed et al. (2022) who found that the percentage of Methanol extraction of 1.5 kg purslane has produced 225.75 g, which is equal to 15% extract that was extracted by using a Soxhlet apparatus. The near similarity in yield percentage may be attributed to the same solvent which was used in the present extraction.

Phytochemical Screening

The results of phytochemical screening as seen in Table 1 agreed with those obtained by Wasnik and Tumane (2014) which they found many types of chemical compounds present in this plant, including alkaloids, terpenoids, organic acids, coumarins, flavonoids, volatile oil and polysaccharides. Also these results were in agreement with (Dhole et al., 2011) who they found that methanolic extract of *P. oleracea* L. showed the presence of saponins, glycosides, alkaloids, flavonoids, phenolic substance, steroids, di and tri-terpenes and tannins.

Table 1: Chemical component of *P. oleracea* methanolic extract.

Results	Constituent
+	Alkaloids
+	Flavonoids
+	Steroids
+	Carbohydrates
-	Terpenoids
+	Tannins
-	Quinones
+	Free amino acids
+	Total protein

+ Presence, - Absence

Table 2: Results of some biochemical tests for *E. coli*.

Biochemical tests	Results
Catalase	+
Indole production	+
Methyl Red	+
Voges-Proskauer	-

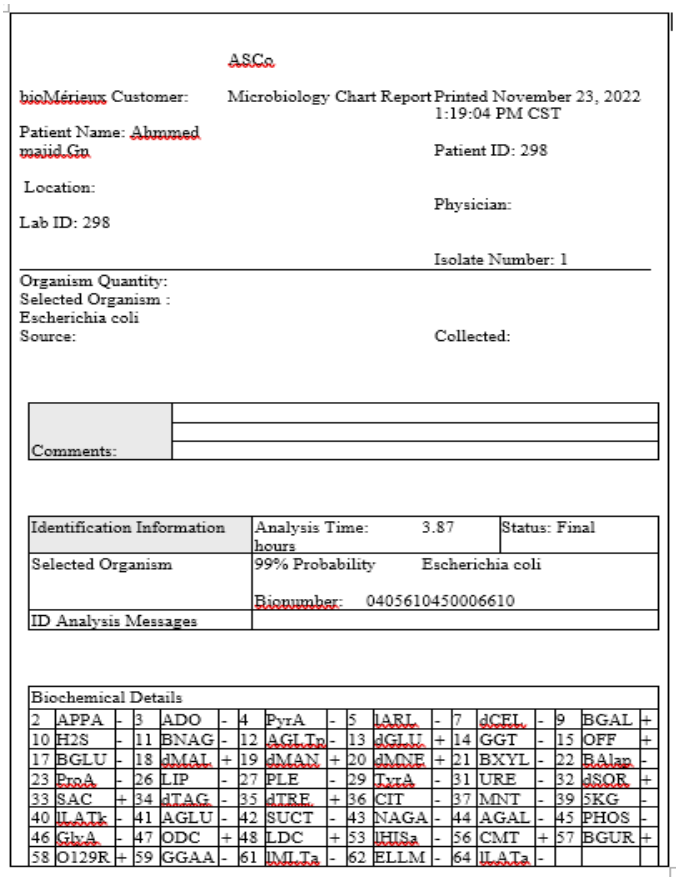
Bacterial Identification

Microscopic Examination appeared as gram negative, pleomorphic rods and non-spore forming under light microscope, Biochemical tests were positive for catalase, indole and methyl red test (MR), while they were negative for Voges – Proskaure (VP), these results illustrated in Table 2. Culturing on agar medium results in a pink colonial appearance on MacConkey agar, while on eosin methylene blue agar colonies appear as green metallic sheen with a dark center which agreed with (Johnson et al., 2013), identification of by VITEK® 2 System had been achieving an excellent level of identification with 99% of probability based on the manufacturers technical datasheet as displaced in Figure 1 and lastly result for PCR test as seen in Figure 2. All these results indicated the isolated bacteria was *Escherichia coli*.

Table 3: *In vitro* antibacterial activity (zone of inhibition mm.) for different concentrations of *Portulaca oleracea* mehanolic extract against *E. Coli* compared with distilled water.

P. Oleracea Conc. (mg/ml)	Zone of inhibition(mm) M±SE	
	Extract	D.W.
128	30.20 ±0.25Aa	0.00 ±0.0 Ba
64	25.40 ±0.22Ab	0.00 ±0.0 Ba
32	14.70 ±0.21Ac	0.00 ±0.0 Ba
16	4.40 ±0.22Ad	0.00 ±0.0 Ba
8	0.20 ±0.13Ae	0.00 ±0.0 Ba
4	0.00 ±0.00Ae	0.00 ±0.0 Ba
LSD value	0.547 *	0.00 NS

Means having with the different small letters in same column and capital letters in same raw differed significantly, * (P≤0.05).



ASCo

bioMérieux Customer: Microbiology Chart Report Printed November 23, 2022 1:19:04 PM CST

Patient Name: Ahmmed maiid.Gn — Patient ID: 298

Location: — Physician:

Lab ID: 298 — Isolate Number: 1

Organism Quantity:
Selected Organism :
Escherichia coli
Source: — Collected:

Comments:

Identification Information	Analysis Time: 3.87 hours	Status: Final
Selected Organism	99% Probability	Escherichia coli
	Bionumber: 0405610450006610	
ID Analysis Messages		

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	-	41	AGLU	-	42	SUCT	-	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	-	64	ILATa	-			

Figure 1: Report of Identification of *E. coli* by VITEK ®2 System.

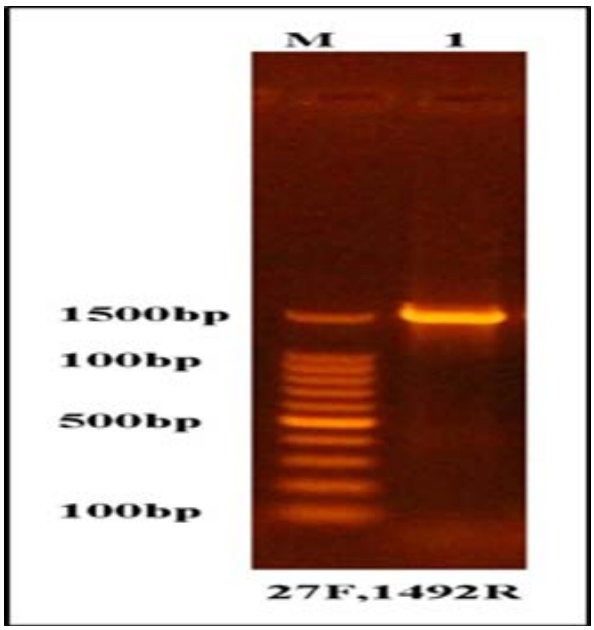


Figure 2: Results of the amplification of 16s RNA gene of the bacteria were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 1 resemble 1500bp PCR products.

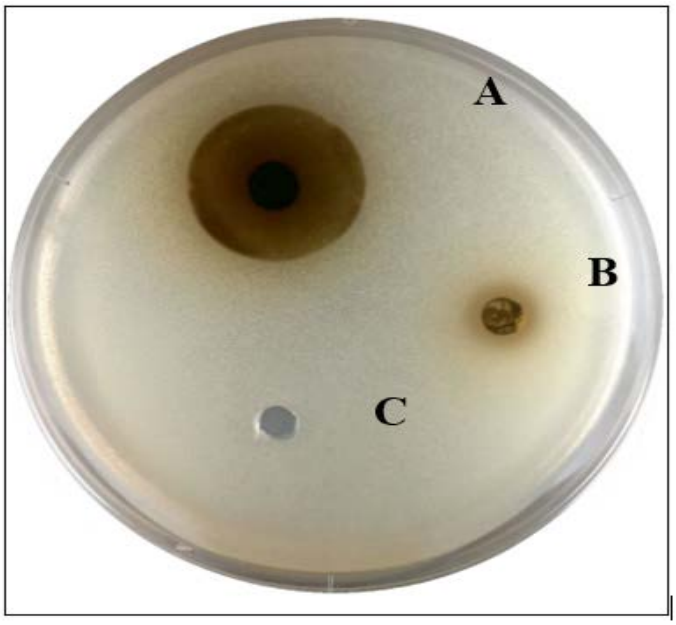


Figure 3: Susceptibility of *E. coli* to different concentrations of the extract in comparison with distilled water A (128 mg/ ml), B (32 mg/ ml) and C (D.W).

Antibacterial Activity Of *P. Oleracea* Methanolic Extract Against *E. Coli*

Agar well diffusion assay was used to determine the antibacterial properties of *P. oleracea* methanolic extract against *E. coli*. This method showed that *P. oleracea* methanolic extract produced inhibition zone sizes between 14- 28 mm. As shown in Table 3, the size of the inhibitory zones varied depending on the extract concentrations and proportionally grew larger as the concentration of *P. oleracea* methanolic extract rose. The results showed that *E. coli* was susceptible to 32-128mg/ml concentrations of the extract as we seen in Figure 3. There was a significant (P≤0.05) increasing in the zone of inhibition with the increasing of extract concentrations. Distilled water which was used as solvent for *P. oleracea* methanolic extract also used as control and didn't give any noticed zone of inhibition. Extract of *P. oleracea* against *E. coli* was 26 mm, which the concentration was 100 mg/ml, while the highest zone of inhibition for our studying was 30 mm for 128 mg/ml concentration of extract. Also (Murtaza et al., 2020) reported that (30 mg/ml) for the same plant with methanolic extraction gave 15 mm zone of inhibition against the same bacteria.

Antibacterial Activity Of Ciprofloxacin (Cip) Against *E. Coli*

In the agar well diffusion assay, ciprofloxacin was used at various concentrations 1.562, 3.125,6.5,12.5, 25, 50, 100, 200, 400, and 800 µg/ ml, zones of inhibition against *E. coli* resulted in varying degrees. The zone of inhibition differed with the ciprofloxacin concentrations, with an increase in ciprofloxacin concentrations, the size of the inhibition zone increased proportionally as displaced in Table 4 and Figure 4. Results revealed that *E. coli* susceptibility to 50-800µg/ml concentrations of ciprofloxacin (Rakhmawatie et al., 2022). In that used concentrations significantly (P≤0.05) there was an increase in diameter of zone of inhibition against *E. coli*. Distilled water was used as a solvent for ciprofloxacin during *in-vitro* tests as a control, it did not produce any detectable zones of inhibition.

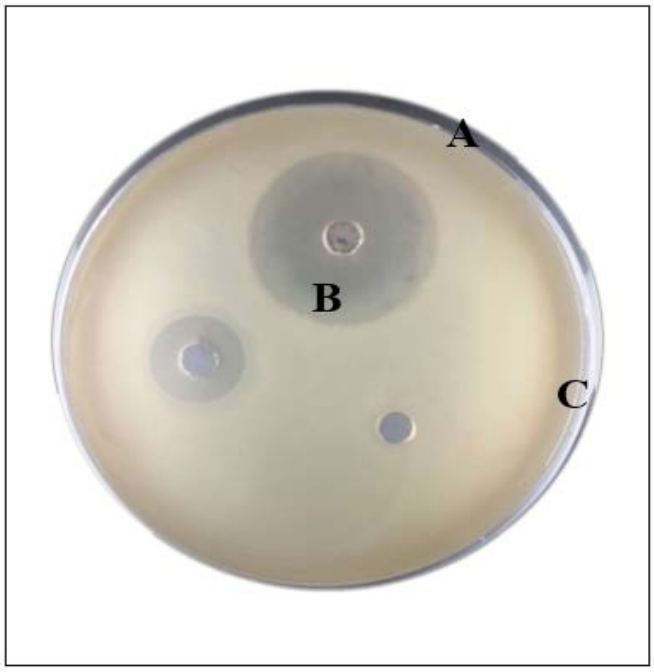


Figure 4: Susceptibility of *E. coli* to different concentrations of ciprofloxacin, in comparison with distilled water A (800µg/ml), B (50µg/ml) and C (D.W).

Table 4: *In vitro* antibacterial activity (zone of inhibition mm.) for different concentrations of ciprofloxacin against *E. Coli* compared with distilled water.

Ciprofloxacin Conc. (µg/ml)	Zone of growth inhibition(mm) M±SE	
	Ciprofloxacin	D.W.
800	37.00 ±0.29 Aa	0.00 ±0.0 Ba
400	31.10 ±0.27 Ab	0.00 ±0.0 Ba
200	26.90 ±0.27 Ac	0.00 ±0.0 Ba
100	21.20 ±0.39 Ad	0.00 ±0.0 Ba
50	15.80 ±0.25 Ae	0.00 ±0.0 Ba
25	12.32 ±0.09 Af	0.00 ±0.0 Ba
12.5	2.35 ±0.08 Ag	0.00 ±0.0 Ba
6.25	0.30 ±0.15 Ah	0.00 ±0.0 Ba
3.125	0.00 ±0.00 Ah	0.00 ±0.0 Ba
LSD value	0.659 *	0.00 NS

Means having with the different small letters in same column and capital letters in same raw differed significantly, * (P≤0.05).

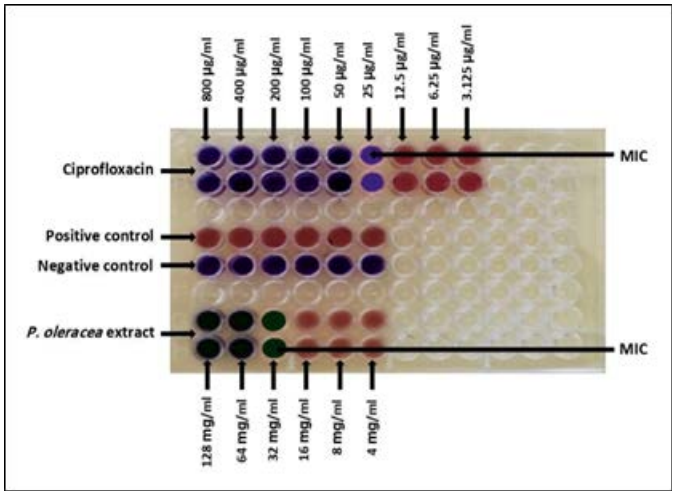


Figure 5: Checkerboard assay for minimum inhibitory concentration of the extract and ciprofloxacin against *E. coli* (Blue: no growth, red: growth).

Minimum Inhibitory Concentration And Minimum Bactericidal Concentrations Of *P. Oleracea* Methanolic Extract And Ciprofloxacin

Determine the minimum inhibitory concentration (MIC) of ciprofloxacin and *P. oleracea* methanolic extract using the VITEK 2 system and the PCR analysis that verified the identification of the *E. coli* isolate. The results demonstrated that *P. oleracea* methanolic extract at a concentration of 32 mg/ml was effective against *E. coli*. Figure 5 from the micro-dilution assay test results The same bacteria (*E. coli*) was similarly suppressed by ciprofloxacin at a dosage of 25 g/ml. According to Figures 6 and 7, the MBC values for the extract were 64 mg/ml, and the antibiotic was 50 g/ml. Because of the resazurin-based solution may quantitatively assess viability by utilizing the reducing power of living cells as a cell health indication (Veiga et al., 2019), after 22 hours' visual readings performed by observing the bacterial growth, alamar blue was used as an indicator. Resazurin, the active ingredient in alamar blue reagent, is a blue, cell-permeable, non-fluorescent chemical that is non-toxic. When resazurin reaches living cells, it changes into the extremely luminescent chemical resorufin (Teh et al., 2017).

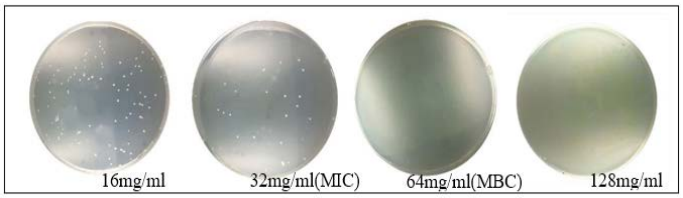


Figure 6: Minimum bactericidal concentrations of the extract against *E. coli* (64mg/ml).

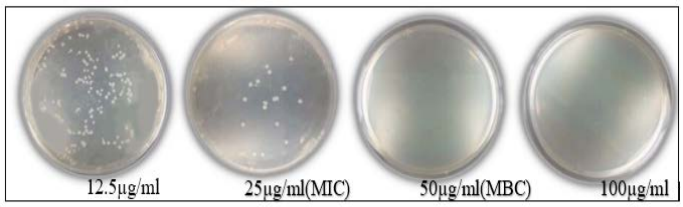


Figure 7: Minimum bactericidal concentrations of ciprofloxacin against *E. coli* (50µg/ml).

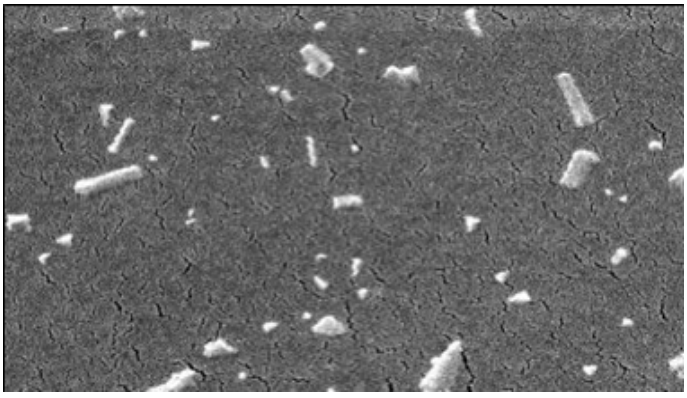


Figure 8: Identify mechanism of action of *P. oleracea* methanolic extract by Scanning Electron Microscope (SEM). Destruction of cell and alteration in the shape of the bacterial cells, shrinkage, and mass accumulation of cell debris.

Fe-Scanning Electron Microscope (Sem)

The field- emission scanning electron microscopy (Fe-

SEM) analysis is employed to characterize the size, shape, morphology of the bacteria after treated with antibacterial agents, Fe-SEM analysis was passed down to look at how the surface of *E. coli* cells that had been exposed to *P. oleracea* extract looked and how well the cell membrane held together. The result of Fe-SEM showed that bacterial cell walls when treated with MIC of *P. oleracea* extract represent destruction of cell and alteration in the shape of the bacterial cells, shrinkage, and mass accumulation of cell debris as we see in Figure 8. This result was in agreement with (Jiang et al., 2021), which they found that the surface of the bacteria was shrunk and rough, and the leakage of the intracellular substances was detected in cells after treated with *P. oleracea* methanolic extract at MIC concentration.

CONCLUSION AND RECOMMENDATIONS

Alcoholic extract from *P. oleracea* had antibacterial effect on *E. coli in vitro* study in comparison with ciprofloxacin, as well as the minimum inhibitory concentration(MIC) for the extract was 32 mg/ml, while the minimum bactericidal concentration (MBC) for the extract was 64 mg/ ml. We suggested study the effect of this extract on other gram negative bacteria.

ACKNOWLEDGMENTS

I want to express my gratitude to the faculty and staff of the Physiology and Pharmacology Department of Baghdad University's College of Veterinary Medicine for their flexibility, wise counsel, and professional thoughts throughout my studies.

NOVELTY STATEMENT

The novelty of the study is focused on therapeutic effect of *Portulaca oleracea* methanolic extract against *E. coli in vitro.*

AUTHORS CONTRIBUTION

Each author made an equal contribution.

CONFLICT OF INTEREST

There are no stated conflicts of interest by the authors.

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