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Antibacterial power of Pomegranate extracts against Beta-Lactamase producing *Escherichia coli*

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Abstract. Herbs, as the pomegranate (*Punica granatum* L.)(*P. granatum*), has significant chemical constituents with distinct pharmacological properties. These chemicals confer neuroprotective, antioxidant, anticancer, anti-inflammatory, and antibacterial properties to the plant. Pomegranate has specific components that enable its pharmacological actions; one of the functions of pomegranate extracts is to deactivate what is called extended spectrum beta-lactamase (ESBL) that makes *Escherichia coli* (*E. coli*) resistant to standard antibiotics. Twenty *E. coli* strains identified as beta-lactamase producers, the strains isolated from urine samples collected from patients with symptomatic urinary tract infection (UTI) and molecularly characterised using 16S rDNA. The study evaluated the antibiotic sensitivity and antibacterial activities of acetone and ethanolic pomegranate leaf and peel extracts, assessing their antimicrobial susceptibility against nineteen antibiotics. The ehanolic peel (EP) and leave (EL) extracts showed inhibitory potential inhibition zones spanning (9.0-12.6-18.3 mm) against *E. coli* pathogen producing extended-spectrum beta-lactamase as compared with (10.2-15.3 mm) inhibition scale exhibited by acetone peel (AP) and leave (AL) extracts treatment. Pomegranate leaves and peel extracts contain bioactive compounds with antioxidant, antimicrobial, and other biological effects, and can be fractionated for the identification of new antibacterial bioactive compounds for the development of drugs against ESBL- *E. coli*, in addition to their synergy with antibiotics for combination therapy that may have effective management and treatment of multidrug-resistant infections such as urinary tract infection.

Keywords: *Punica granatum*, *E. coli*, beta-lactamase enzyme, antibacterial activities, antimicrobial assay, DNA sequencing.

1. INTRODUCTION

In addition to use plants as food, shelter, and building materials, plants were traditionally employed as medicinal herbs. Herbs were defined as those with medicinal properties (Kunle *et al*., 2012). The World Health Organization (WHO) defines herbal medicines as plants whose parts are used to treat and prevent illnesses in humans and animals (Msomi and Simelane 2019). They include active agents; either alone or in combination can be used in the treatment and management of chronic conditions, such as cancer and cardiovascular difficulties. Researchers have resorted to herbal drugs as alternatives to standard therapy methods as no bad side effects (Sutan *et al*., 2023). These unique natural constituents, herbal medication significantly contributed to disease management (Sutan *et al*., 2023; Kam and Neergheen 2022; Facciola and Stephen 1990). The usage of herbal medicines has grown in popularity and awareness around the world because to their inexpensive cost, little to non-existent side effects, and lack of bacterial resistance. In addition, these herbs are easily accessible as they are readily available. The therapeutic properties of *P. granatum* shrub are attributed to the extract from its numerous sections, which has been shown to be beneficial in treating and preventing the pathogenic *E. coli* strain (Stover and Mercure 2007). Pomegranate is a deciduous plant that grows between five and eight metres in height. It is a member of the Lythraceae family. Pomegranate was traditionally regarded as a symbol of life, fertility, health, wisdom, immortality, and longevity (Loizzo *et al*., 2019). Pomegranate is planted primarily in South Asia and the Middle East. Today, it is widely cultivated in dry and semiarid places due to its adaptability to harsh climates (Stover and Mercure 2007). Each anatomical component of the plant has a unique pharmacological and toxicological function. This includes the peel, seed, flower, juice, leaf, bark, and root. It is an edible fruit with a thick, reddish skin, a rounded hexagonal shape, and a 5 to 12 cm diameter. The fruit contains approximately 600 seeds, each wrapped in a water-filled aril (pulp) of varying hues ranging from white to deep purple to deep crimson. The fruit's edible portion, the seeds, can be utilised in baking, cooking, and beverage preparation (Loizzo *et al*., 2019). However, the Pomegranate has been widely utilised by numerous nations and civilizations for the treatment of numerous ailments (Kim and Choi 2009). Previous research indicates that the chemical constituents contained in Pomegranate seeds, bark, flowers, pericarp, and roots confer the plant with a variety of therapeutic effects. In addition, it has antioxidant, anti-atherosclerotic, anti-inflammatory, neuroprotective, anticancer, antimicrobial, antihypertensive, anti-teratogenic, stimulant, antidiabetic, antiviral, antifungal, anthelmintic, nephron-protective, wound-healing, and antiulcer properties.

In addition, its chemical ingredients help combat respiratory disorders and erectile dysfunction (Kim and Choi 2009). While Pomegranate seeds contain estrogenic chemicals, estradiol and oestrone, and the fruit has been widely used to treat microbial infections, acidosis, diarrhoea, haemorrhage, helminthic infection, respiratory ailments, and dysentery, they do not have estrogenic properties themselves (Ricci *et al*., 2006). Juice and dried pericarp of the fruit have been used to treat colic, headache, colitis menorrhagia, piles, oxyuriasis, diuretics, allergic dermatitis, acne, and oral illnesses (Berthe *et al*., 2013). *Escherichia coli* (*E. coli*) is a bacteria found in the intestines of animals (Denamur *et al*., 2021). The majority of *E. coli* strains are digestively helpful and harmless, some are dangerous and pathogenic (Khalid and Andreoli 2019). Pathogenic strains of *E. coli* bacteria are spread mostly by contaminated human-to-human contact, contaminated food or water consumption, and contaminated surfaces (Denamur *et al*., 2021; Al-Sarraj *et al*., 2021a; Alotibi *et al*., 2022). Infections of the urinary tract, gastrointestinal tract, and respiratory system are caused mainly by pathogenic *E. coli* strains. *E. coli* infections are characterised by moderate diarrhoea, abdominal discomfort, bloody diarrhoea, and renal failure (Qi *et al*., 2022). In order to treat or prevent *E. coli* infections, one must adhere to food safety and sanitary standards and practise proper hygiene.

Beta-lactams are the most often used antibiotics for the treatment of urinary tract infections (Flores-Mireles *et al*., 2015); nevertheless, the rise of multiple-drug resistant (MDR) bacteria, particularly beta-lactamase (ESBL) generating pathogens, is a huge concern to the global healthcare system (Shaikh *et al*., 2015; Hashem *et al*., 2017; Heidari *et al*., 2017; Houri *et al*., 2017; Motamedifar *et al*., 2015). The prevalence of *E. coli* bacteria that produce beta-lactamase enzymes has increased recently on a global scale (Pourakbari *et al*., 2012; Kazemian *et al*., 2016). It inhibits the effectiveness of certain beta-lactam antibiotics, such as penicillin and cephalosporins, by degrading them. By generating beta-lactamase enzymes, bacteria acquire antibiotic resistance. Enzymes degrade the structure of beta-lactam antibiotics, rendering their antibacterial effects inactive (Qi *et al*., 2022; Amanulla and Sundaram 2019). Therefore, treating bacterial infections caused by *E. coli* generating beta-lactamase becomes problematic. The proliferation of antibiotic-resistant bacteria has become a global health concern, compelling researchers to investigate

alternate preventative and therapeutic measures (Huang *et al*., 2019; Chauhan *et al*., 2020). The strategies include the creation of novel antibiotics, the use of natural chemicals such as plant extracts, and the improvement of sanitation practises to avoid the spread of infectious diseases (Amanulla and Sundaram 2019; Hashim and Pharma 2013). Leaves of *P. granatum* contain chemical substances with the ability to suppress the actions of *E. coli* strains producing beta-lactamase. With the correct concentration of the extract, pathogenic *E. coli* will eventually be incapable of degrading the structure of the antibiotics. Consequently, the molecules of the antibiotic continue to combat bacterial infections. This study comprehensively evaluates the antibacterial impacts of *P. granatum* leaves and peel extracts on *E. coli* extended spectrum β-Lactamase isolate and the antibiotic susceptibility patterns of *E. coli* isolate. Additionally, it provides a comparison between the concentrations of *P. granatum* extractions and seeks to find the most effective concentration and its synergy with antibiotics that may have potential for future treatment of multi-drug resistant infections.

2. MATERIALS AND METHOD

All of the chemicals and antibiotics employed were of analytical quality, purchased from Sigma-Aldrich, and were utilised without further purification.

2.1. Herbal material and microorganisms

The pomegranate plant used in this investigation was collected in Taif, Saudi Arabia. The Hematology department of the Faculty of Medicine at King Abdulaziz University in Jeddah, Saudi Arabia, reported urine samples from twenty *E. coli* isolates isolated from urine samples of twenty-one patients with urinary tract infections (UTIs) and one healthy control individual in June 2021. The bacteria were cultured in Luria-Bertani (LB) medium with 200 rpm shaking at 37 °C for twenty-four hours before being stored at 4 °C. Next, we cultivated bacterial isolates on LB agar overnight at 37 °C for 24 hours. After incubation, the cultures were inspected to determine if considerable growth had occurred. All collected strains were sequenced for the 16S rDNA gene to validate the culture-based identification of bacteria. The optical density of bacterial cultures was set to 600 nm (OD600) with sterile 0.1 M saline solution for evaluating Disk Diffusion, Antibiotic Susceptibility Assay, and the antibacterial activity of pomegranate leaves and peel extracts.

2.2. Antibiotics

Amikacin AK 30mcg, Ampicillin AMP 10mcg, Cefrtriaxone CTR 30 mcg, Amoxyclav AMC (Amoxycillin+Clavulinic Acid) (Augmentin) 30mcg, Ceftazidime CAZ 30 mcg, Cefoperazone CPZ 75 mcg, Netilmicin NET 30 mcg, Cefuroxime CX.

2.3. Antibiotics sensitivity assay for bacteria

Twenty bacterial isolates were tested against eight distinct drugs to determine their susceptibility. The bacteria were cultured overnight in nutritional broth for activation. They were then determined by measuring optical density (OD) at 600 nm with a UV-visible spectrophotometer and subsequently standardised to 0.1 McFarland standards (3107 CFU/mL). Later, the bacterial suspension was dispersed on nutrient agar plates. The agar was then covered with antibiotic discs and incubated at 37 °C for 18-20 hrs. Later, the sizes of the zones of inhibition were measured and reported.

2.4. Preparing pomegranate extracts

The peel and leaves of the pomegranate were gathered by hand, separated, chopped into little pieces, then rinsed with tap water and then distilled water to eliminate any suspended soil and other particles. The samples were then air-dried in the shade at room temperature to preserve their freshness. An electric mill was used to grind the dried samples into a fine pomegranate powder. The powder was kept in airtight containers at 4 °C until usage. 50 g of the powder was soaked in 500 ml of ethanol and 500 ml of acetone for 5 minutes, and then the mixture was swirled continuously at 200 rpm for 72 hrs to dissolve. After that, filtration was performed in three stages. Initially, the fluid was filtered through numerous layers of gauze to remove the suspended plant fibres and particles. Afterward, it was filtered with Whitman No. 1 paper. Using a rotary evaporator, the filtrate was evaporated. The storage solution was made by dissolving 500 mg/ml of the extracted powder in 40 ml of 2 percent diluted Dimethyl Sulfoxide (DMSO).

2.5. Antibacterial activity of pomegranate extracts

Using the agar well diffusion method, the antibacterial activity of pomegranate leaf extract was determined. 20-25 ml of autoclaved Muller–Hinton agar media was poured into a sterile petri dish, which was then allowed to harden. Spread the bacterial suspensions on the plate

using a sterile cotton swab. Each plate includes five holes that were drilled with a 6mm cork borer and filled with 100µl of extracts of varying concentrations. As a negative control, 30 percent diluted DMSO without pomegranate leaf extract was used. The plate was left for 10 minutes to allow the chemicals to pre-diffuse into the agar. The plates were then incubated at 37 °C for 24 hours. The diameter of the inhibitory zone was then measured in millimetres.

2.6. Beta-Lactamase production assay

In order to determine the beta-lactamase synthesis of all examined *E. coli* isolates, broth samples were point-injected onto Mueller-Hinton agar (MHA) containing 1 percent starch and incubated at 37 degrees Celsius. After 24 hours of incubation, the plates were refilled with PBS containing potassium iodide, iodine, and penicillin. The creation of distinct colorless zones around the *E. coli* growth indicates the production of -lactamase enzymes, which convert Penicillin to penicilloic acid and iodine to iodide, as shown by the decolorization of the starch iodine complexes.

2.7. Molecular characterization of bacteria

DNA extraction and amplification of 16S rRNA genes

Total genomic DNA was extracted from 20 urine samples collected from patients with symptomatic urinary tract infection using a slightly modified version of the Azcárat-Peril and Raya (2001) protocol, in which 1 ml of pure bacteria culture grown overnight in NB was transferred to a 1.5 ml tube, and the tube was centrifuged at 10000 rpm, 4°C for 5 minutes. After discarding the supernatant, 200 µl of TES buffer is added and vortexed thoroughly. Then, 20µl of lysozyme (10 mg/ml) was added and thoroughly mixed using a vortex. Two hours were spent incubating the mixture at 37°C in a water bath. Each sample received 20µl of proteinase K (10 mg/ml), which was then vortexed and incubated at 37°C for two hours. The liquid was chilled for five minutes, then 250 µl of sodium acetate was added. After 5 minutes of centrifugation at 8000 rpm and 4°C, the top zone was carefully transferred to a new, clean Eppendorf tube. 250 µl of chloroform: isoamyl (24:1) was added to the mixture, which was then mixed by hand and centrifuged at 8000 rpm, 4°C for five minutes. The aqueous phase was then transferred to a fresh Eppendorf. Equal volume of isopropanol was added to the mixture, which was then stored overnight at -20 degrees Celsius. The following day, the solution was centrifuged at 10000 rpm for 5 minutes, after which the liquid zone was discarded and the pellet was allowed to dry at ambient temperature for 10 minutes before being suspended in 50 µl of distilled water. DNA from each bacterial isolate was served as template for amplification of the 16S rRNA genes using the following universal primers: 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3').

For the PCR, a total volume of 50µl was utilized, along with 2µlof 10 pmol of each appropriate primer and 25µl of Master Mix (GoTaq® Green Master Mix, 2X, Promega). Add 2l of DNA and use DEPEC-treated water to adjust the volume of the final PCR mixture to 50 µl. The 16S rRNA gene was amplified using a thermal cycler (applied biosystems TM Veriti TM 96-Well Thermal Cycler) under the following conditions: initial denaturation at 95 °C for 4 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing for 45 seconds, and extension at 72 °C for 60 seconds, and a final extension at 72 °C for 10 minutes.

2.8. Sequence analysis and phylogeny of E. coli strains

The consensus 16S rDNA amplicon sequences of *E. coli* isolates were modified and subjected to a BLAST search using the NCBI database http://blast.ncbi.nlm.nih.gov/Blast.cgi to assign presumptive identity with similar sequences. On the basis of sequence similarity measurements and inferences of phylogenetic trees, each bacterial isolate was assigned to its operational taxonomic unit (OTU). Where necessary, alignments were edited by hand. The pathogenic *E. coli* nucleotide sequences were submitted to GenBank for assignment of accession numbers. The acquired sequences were separated into distinct datasets in order to examine the evolutionary relationships between species and families. The NJ technique was employed to create phylogenetic trees, whereas MEGA software, version 10.0 (Tamura *et al.*, 2011) was utilised to undertake molecular evolutionary studies.

2.9. Statistical analysis

This study's statistical analysis was conducted using the SPSS software (Version 26.0). All analyses were conducted in triplicate using a one-way ANOVA analysis of variance with a significance level of p 0.05, and the results were represented as the mean standard deviation.

3. RESULTS

3.1. Molecular identification of the E. coli strains

All 20 bacterial isolates were identified molecularly by amplification of the 16S rRNA gene using universal primers. According to the PCR results, the 16S rRNA gene sequences of the selected isolates were amplified successfully from extracted template DNA. Using gel electrophoresis, the PCR products of the 16S rRNA genes of these isolates yielded bands with around 1500 bp (Figure 1).

DNA sequencing was used to identify the 20 examined pathogenic *E. coli* strains at the molecular level by analysing the 16S rRNA gene. The GenBank was accessed to obtain the accession numbers for all of the bacterium isolates listed in Table 1, and MEGA software was used to optimise the sequences that were obtained. Using the NCBI public database, nucleotide similarities between 16S rRNA gene sequences and those of GenBank-recognized species were analysed. The results indicated that the isolated microorganisms were *E. coli*. The sequences of the acquired *E. coli* strains were 99.2–100% comparable to those of their closely related isolates previously deposited in GenBank. The homologous sequences from the BLAST search were utilised to construct a phylogenetic tree, demonstrating their relationship to *E. coli* strains, as demonstrated in the Figure 2.

This Original Tree's evolutionary history was inferred using the UPGMA method (Sneath and Sokal 1973), and the 500-replicate bootstrap consensus tree (Felsenstein 1985) is considered to represent the evolutionary history of the species examined (Felsenstein 1985). The collapsed branches correspond to partitions that are replicated in less than half of bootstrap replicates. Next to each branch is the percentage of 500 replicate trees in which the relevant taxa clustered together in the bootstrap test (Felsenstein 1985). Using the Maximum Composite Likelihood approach (Tamura *et al*., 2004) and the amount of base substitutions per site, the evolutionary distances were estimated. This analysis used 40 nucleotide sequences, and for each sequence pair, all ambiguous locations were eliminated (pairwise deletion option). The final dataset contained 1030 locations in total. In MEGA X, evolutionary analyses were undertaken (Kumar *et al*. 2018).

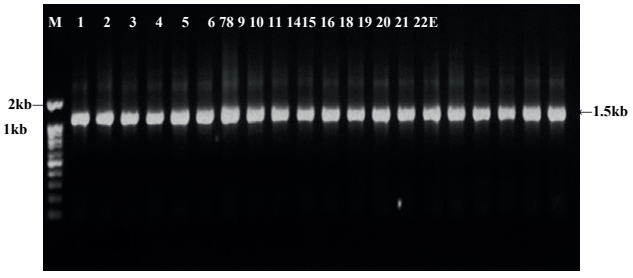

Figure 1. Agarose gel electrophoresis of PCR products for 16s rDNA gene. Lane M: 2kb DNA size marker, Lanes 1-E: 16S gene amplicon of 20 *E. coli* isolates from clinical samples.

Table 1. GenBank accession numbers and β-cell lactamase assay of different *E. coli* strains under study.

Ser.	Bacterial strains	*β*-cell lactamase	Accession No.
1	*E. coli* strain WFGM S1	Positive	OR472881
2	*E. coli* strain WFGM S2	Positive	OR472882
3	*E. coli* strain WFGM S3	Positive	OR472883
4	*E. coli* strain WFGM S4	Positive	OR472884
5	*E. coli* strain WFGM S5	Positive	OR472885
6	*E. coli* strain WFGM S6	Positive	OR472886
7	*E. coli* strain WFGM S7	Positive	OR472887
8	*E. coli* strain WFGM S8	Positive	OR472888
9	*E. coli* strain WFGM S9	Positive	OR472889
10	*E. coli* strain WFGM S10	Positive	OR472890
11	*E. coli* strain WFGM S11	Positive	OR472891
12	*E. coli* strain WFGM S14	Positive	OR472892
13	*E. coli* strain WFGM S15	Positive	OR472893
14	*E. coli* strain WFGM S16	Positive	OR472894
15	*E. coli* strain WFGM S18	Positive	OR472895
16	*E. coli* strain WFGM S19	Positive	OR472896
1	*E. coli* strain WFGM S20	Positive	OR472897
18	*E. coli* strain WFGM S21	Positive	OR472898
19	*E. coli* strain WFGM S22	Positive	OR472899
Control	*E. coli* strain WFGM SE	Positive	OR472900

3.2. Antibiotic sensitivity assay of ESBL E. coli strains

In the present investigation, the beta-lactamase enzyme assay results indicated that all twenty studied *E. coli* strains produced beta-lactamase, and the positive result was observed as an inhibitory zone surrounding the bacterial colonies. The disc diffusion susceptibility test was carried out to determine the sensitivity or resistance of pathogenic microorganisms to various antimicrobial agents. On Mueller-Hinton agar, the pathogenic bacteria were cultivated in the presence of antimicrobial-impregnated filter paper discs. The presence or absence of bacterial growth around the discs is regarded an indirect sign of the compound's ability to inhibit the organism. Table 2 illustrates the antibiotic resistance characteristics of the pathogenic bacterial strains (Figure 3A, 3B). In order to evaluate the antibiotic resistance patterns of the examined bacterial strains, the Antibiot-

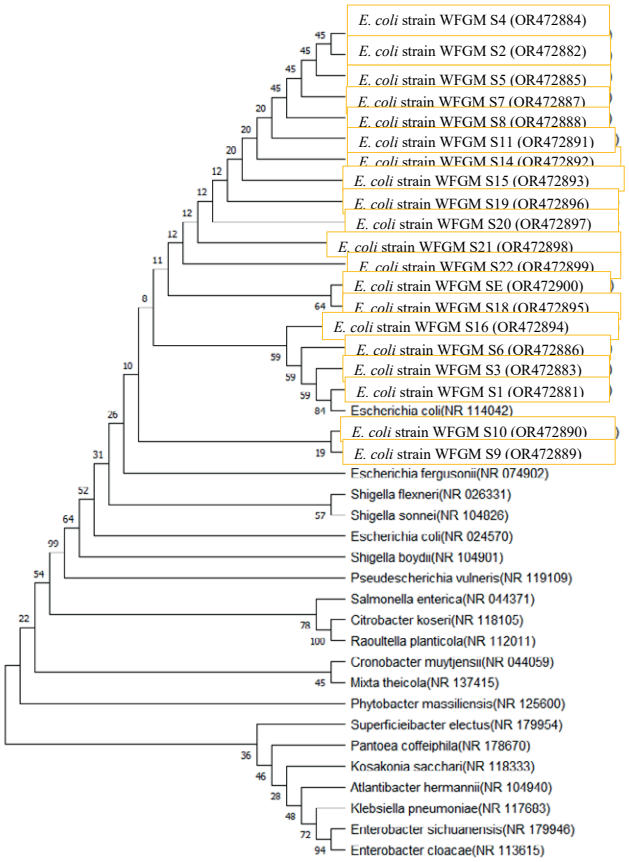

Figure 2. Phylogeny analysis of twenty *E. coli* strains from 16S region sequences compared with different related bacterial isolates obtained from GenBank.

ic-disk assay was developed utilising various antibiotics. The majority of the bacteria exhibited both antibiotic resistance and sensitivity, according to the results. Also, antibiotics susceptibility testing data revealed that the highest resistant pattern ranged between (44-61 percent) was found in 8 isolates (S11, S18, S3, S22, S7, S15, S16 and S20) from 19 (44 percent) examined bacteria, which were resistant to (9-11) antibiotics, followed by resistant pattern ranged between (39-44 percent) was observed in 7 isolates (S4, S5, S8, S14, S6, S14, S21, S6, S The resistance patterns of *E. coli* strains S9 and SE (control) are modest (5 percent and 6 percent, respectively). All examined *E. coli* isolates exhibited resistance to at least one of the antimicrobials examined. In particular, 95% of *E. coli* was resistant to CAZ, whereas 90% of same bacteria were resistant to AMP and CN. And 85% showed resistance to CXM and CFM. Eighty percent of the strains were resistant to AMC, and with a moderate susceptibility of 55%, 50% were resistant to CTX and COT, respectively. Additionally, 30% of the studied *E. coli* isolates were resistant to CIP, whereas 15% were resistant to CPZ. Only 10% of the examined *E. coli* bacteria were resistant to CTR and K, while 5% were resistant to GEN. None of the tested isolates were resistant to AK, NET, OF, or LE.

3.3. Antibacterial activity of pomegranate leaves and peels extracts

The antibacterial activity of pomegranate leaves and peels was evaluated against 20 *E. coli* isolates using the well diffusion technique. The results revealed significant heterogeneity in the zone of inhibition's size. To extract the active compounds from the powdered peel and leaves of pomegranate used in this investigation, two organic solvents (80% ethanol and 80% acetone) were employed. The antibacterial activity of the examined extracts in 2 percent DMSO was dose-dependent, ranging from 100 mg/ml to 500 mg/ml, and the inhibitory zone scale expanded as the concentration of the test extract increased.

The data in (Table 3) and (Figure 4) indicate that the ethanolic extracts 100-500mg/ml of pomegranate peels (EP) extract. Most the investigated strains showed resistance with absence of inhibition zone at 100mg/ml, compared with a strong antibacterial activity against the tested clinical *E. coli* strains, and showed maximum inhibition scale (9.0- 18.3 mm) as the EP extract concentration increased from 200 mg/ml to 500 mg/ml, followed by the inhibitory zone range (8.3-15.9 mm) at a dosage of 400 mg/ml EP extract, and inhibitory zone range (8.1-15.8 mm) at a dosage of 300 mg/ml EP extract. The more susceptible clinical *E. coli* strains to the (9.0- 18.3 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EP extract were strains S16, S20, S3, S4, S6, S11, and S12 strains.

By regarding the antibacterial activity of acetone extraction of pomegranate peels (AP) at a dosage of 100-500 mg/ml as data represented in (Table 4) and (Figure 5). Most the investigated strains showed resistance with absence of inhibition zone at 100mg/ml and 200 mg/ml AP extract, and by increasing AP extract concentration from 200 mg/ml to 500 mg/ml demonstrated a potent antibacterial activity with a (9.07-15.3 mm) inhibitory zone ranges between tested clinical *E. coli* strains, followed by the inhibitory zone range (9.04-14.4 mm) at a dosage of 400 mg/ml AP extract, and inhibitory zone range (9.07-14.0 mm) at a dosage of 300 mg/ml AP extract. The more susceptible clinical *E. coli* strains to the (9.0-15.3 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EP extract were strains S9, S20, S7, S8, and S10 strains.

Table 2. Resistance (%) or susceptibility of the ESBL *E. coli* strains used in the present study to different antibiotics

Strains	Resistance or inhibitory zone (mm) by antibiotics																		Antibiotic resistance (%)
	AMP	AK	AMC	CTX	CIP	CXM	CPZ	CAZ	GEN	NET	OF	NX	CFM	CTR	CN	K	LE	COT	
S1	7	21	R	11	25	22	10	9	17	23	23	20	R	12	R	18	26	29	22
S2	R	26	8*	30*	R	14*	27*	R	25*	16	14*	R	15*	28*	R	22*	22*	30*	28
S3	R	53	R	R	9	R	8	R	12	21	13	R	R	8	R	R	18	R	56
S4	R	37	R	10	R	R	14	R	11	21	11	14	R	16	R	13	16	31	39
S5	R	42	R	9	R	R	13	R	20*	19	13	R	R	13	R	14	18	25*	44
S6	R	42	R	R	25	R	11	R	19	23	25	20	R	9	R	19	27	R	44
S7	R	47	R	R	16*	R	9	R	17*	15	25	12*	R	8	R	R	30	R	50
S8	R	42	R	8	8	R	12	R	20	23	13	R	R	12	R	19	10	8	44
S9	11*	5	15*	29*	32	17*	28*	R	19*	22	32*	27*	16*	29*	17*	19*	30	31	6
S10	R	26	9	11	25	R	12	R	22*	20	24	19	R	15	R	18*	22	22	28
S11	R	58	R	R	R	R	8	R	12	20	8	R	R	R	R	15	8	R	61
S14	R	42	R	9	R	R	11	R	18	25	10	R	R	11	R	18	10	26	44
S15	R	47	R	R	20	R	R	R	17	21	21	18	R	9	R	17	25	R	50
S16	R	47	R	R	10	R	8	R	17	20	12	R	R	8	R	16	12	R	50
S18	R	58	R	R	R	R	R	R	19*	21	10	7	R	R	R	11	11	R	61
S19	R	37	R	R	25	R	8*	R	18	21	28	20	R	16	R	18	22	25	39
S20	R	47	R	R	22	R	R	R	17	21	25	24	R	8	R	17	25	R	50
S21	R	37	R	R	22	R	8	R	18	21	23	19	R	12	R	18	22	24	39
S22	R	53	R	R	9	R	9	R	R	17	8	R	R	9	R	15	11	R	56
SE	R	23	8	29	34	9	25	R	18	23	31	26	10	27	9	18	31	25	11

*R: Resistance. Antibiotic compounds used; AMP: Ampicillin, AK: Amikacin, AMC: Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin), CTX: Cephotaxime, CIP: Ciprofloxacin, CXM: Cefuroxime, CPZ: Cefoperazone, CAZ: Ceftazidime, GEN: Gentamicin, NET: Netilmicin, OF: Ofloxacin, NX: Norfloxacin, CFM: Cefixime, CTR: Cefrtriaxone, CN: Cephalexin, K: Kanamycin, LE: Levofloxacin, COT: Co-Trimoxazole.

The antibacterial activity of ethanolic extraction of pomegranate leaves (EL) at a dosage of 100-500 mg/ml, and the bioactivity data against clinical *E. coli* strains represented in (Table 5) and (Figure 6). Most the investigated strains showed resistance, and no antibacterial activity was identified at 100mg/ml and 200 mg/ml EL extract, and by increasing EL extract concentration from 200 mg/ml to 500 mg/ml, the antibacterial activity significantly increased (8.5-12.6 mm) inhibitory zone ranges between tested clinical *E. coli* strains, followed by the inhibitory zone range (8.1-10.0 mm) at a dosage of 400 mg/ml EL extract, and inhibitory zone range (7.6-9.2mm) at a dosage of 300 mg/ml EL extract. The more susceptible clinical *E. coli* strains to the (8.0- 12.6 mm) as the concentration increased from 200 mg/ml to 500 mg/ml EL extract were strains S5, S2, S4, S7, S8, and S9 strains.

The antibacterial activity of 100-500 mg/ml acetone extraction of pomegranate leaves (AL) against clinical *E. coli* strains as demonstrated in (Table 6) and (Figure 7). The smallest concentrations (100mg/ml, 200mg/ml, 300 mg/ml AL extract showed high antibacterial resistance or a little antibacterial activity was identified, increasing AL extract concentration from 300 mg/ml to 500 mg/ml exhibited inhibitory zones spanning (7.8-10.2 mm) ranges between tested clinical *E. coli* strains, followed with inhibitory scale (8.0-9.5 mm) at a dosage of 400 mg/ml AL extract, and smallest inhibitory scale (7.8-8.1 mm) at a dosage of 300 mg/ml AL extract. The more susceptible clinical *E. coli* strains to the (8.7.8- 110.2mm) as the concentration increased from 200 mg/ml to 500 mg/ml AL extract were strains S6, S7, S8, S2, S6, and S17 strains.

4. DISCUSSION

Due to its numerous health benefits, *P. granatum* is not only a tasty fruit but also a therapeutic herb. Its leaves are abundant in phytochemicals with antibacterial characteristics (Stover and Mercure 2007; Fernández-Mazarrasa *et al*. 2009; Al-Sarraj 2021b). In this work, the researcher examined the antibacterial activity of pomegranate leaf extract against *E. coli* strains resistant to antibiotics. According to the data the inhibitory zone in different investigated bacterial strains were ranged

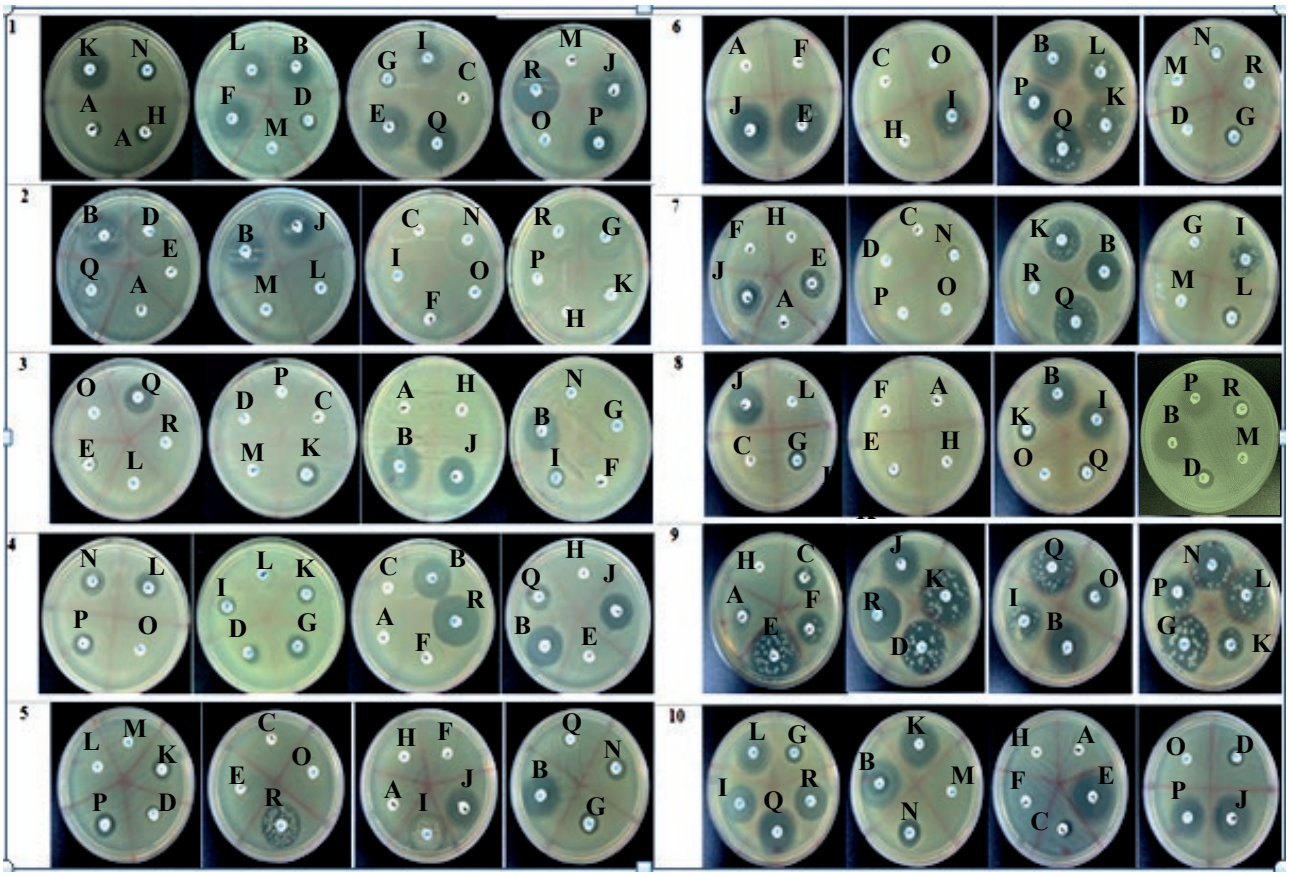

Figure 3A. Antibiotic resistance profiles of ten (1-10) *E. coli* pathogenic bacterial strains used in these studies. Antibiotic compounds used; Ampicillin (A), Amikacin (B), Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin) (C), Cephotaxime (D), Ciprofloxacin (E), Cefuroxime (F), Cefoperazone (G), Ceftazidime (H), Gentamicin (I), Netilmicin (J), Ofloxacin (K), Norfloxacin (L), Cefixime (M), Cefrtriaxone (N), Cephalexin (O), Kanamycin (P), Levofloxacin (Q), Co-Trimoxazole (R).

between (7.6-18.3 mm). Concentration 200 mg/ml of ethanolic and acetone-extracted leaves resulted in formation a small scale of inhibition zone 7.6-8.5 mm) The 500 mg/ml concentration produced the greatest inhibition zone scale in ethanolic and acetone extraction of peels ranging between (9.0-18.3 mm), overall, at different concentrations (100 g/l - 500 mg/ml) pomegranate peel and leaves, the ethanol extracts of peel and leaves were more active than the acetone peel and leave extracts. The specifics are as follows. The leaf extract inhibited the proliferation and growth of *E. coli* that was resistant to antibiotics by breaking the cell membrane of the bacteria, so allowing antibiotics to enter the bacteria. The extract contains bioactive chemicals, including as tannins, alkaloids, and flavonoids, which generate antibacterial substances (Di Sotto *et al*., 2019; Alamshani *et al*., 2023; Zam and Khaddour 2017). The chemicals damage the bacterial cell membranes, leading in the release of the cellular contents and the eventual death of the bacterium. According to mass spectrometry research, *P. granatum* includes *oligomeric ellagitannins* consisting of up to 5 core glucose units, which are the most potent antibacterial chemicals in pomegranate.

In addition, multiple prior researches have demonstrated that the aqueous extract of pomegranate leaves inhibits the growth of uropathogenic *E. coli*. In addition, the extract exhibited a minimum inhibitory concentration (MIC) value of 0.6 mg/ml, resulting in an eighty percent reduction in the *E. coli* adhesion index. Additionally, the extract exhibited a minimum bactericidal concentration (MBC) of 1.2 mg/ml (Jam *et al*., 2022; Church *et al*., 2007). Thus, the peel extract inhibited *E. coli* biofilm formation and decreased bacterial adhesion capacity (Alamshani *et al*., 2023). Solvent extraction is a method used to extract antioxidant chemicals from plants. The research outcome is determined by the type and amount of solvent utilised during the extraction procedure (Ellatif *et al*., 2021; Ellatif *et al*., 2022a; Ellatif

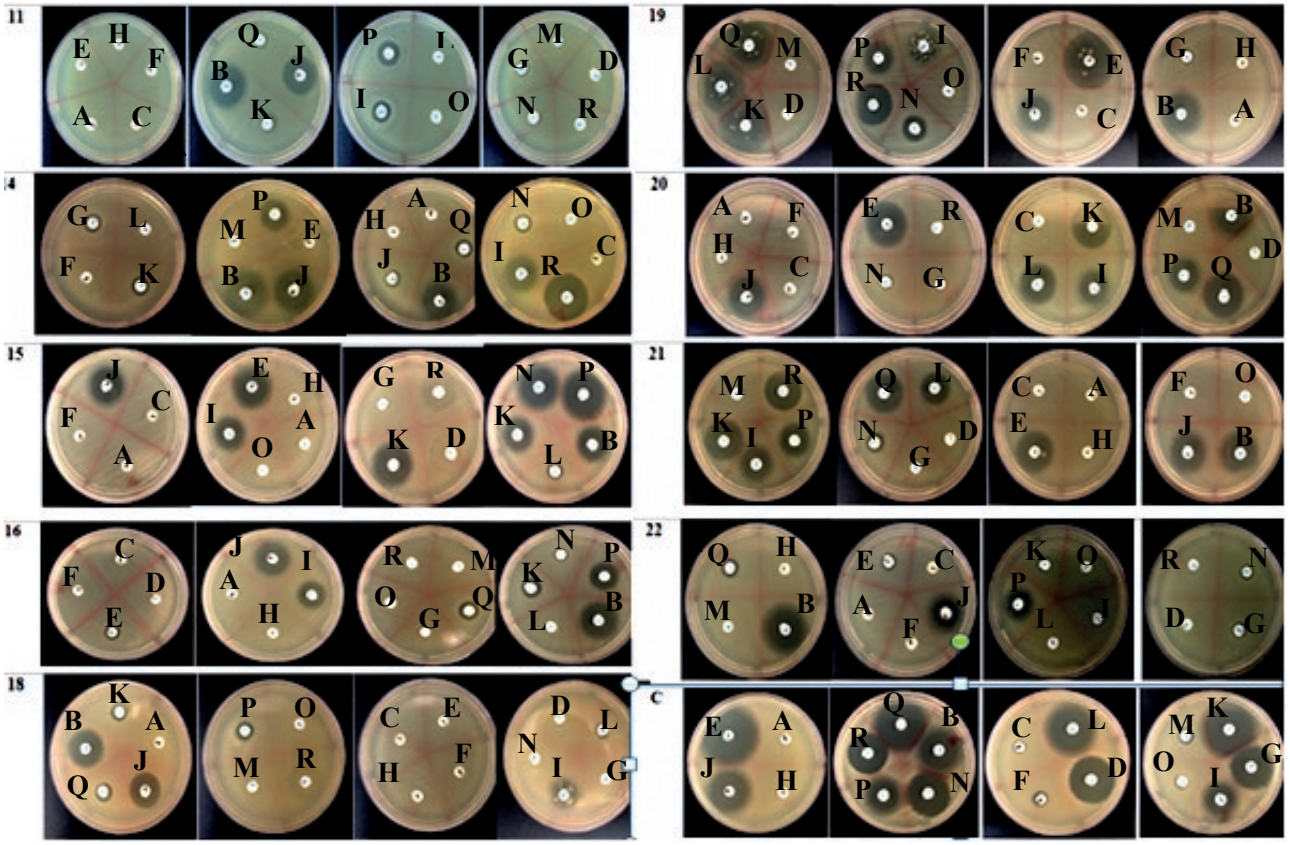

Figure 3B. Antibiotic resistance profiles of ten (11, 14, 15, 16, 18- 22 and C) *E. coli* pathogenic bacterial strains used in these studies. Antibiotic compounds used; Ampicillin (A), Amikacin (B), Amoxyclav (Amoxycillin+Clavulinic Acid) (Augmentin) (C), Cephotaxime (D), Ciprofloxacin (E), Cefuroxime (F), Cefoperazone (G), Ceftazidime (H), Gentamicin (I), Netilmicin (J), Ofloxacin (K), Norfloxacin (L), Cefixime (M), Cefrtriaxone (N), Cephalexin (O), Kanamycin (P), Levofloxacin (Q), Co-Trimoxazole (R).

et al., 2022b). For such experiments, aqueous solvents such as acetate, ethanol, ethyl acetate, and methanol are advised. Due to their polarity, ethanol and methanol have been widely utilised to extract antioxidant components from numerous plants and plant-based meals. Moreover, the two solvents are compatible with the human body (Sutan *et al*., 2023; Park *et al*., 2011). The plant was extracted using ethanol and acetone in this investigation. Mueller-Hinton agar is a regularly used solid culture medium for assessing the antibiotic susceptibility of microorganisms. Its composition (beef extract, casein hydrolysate, and starch) allows for precise testing of the susceptibility of microorganisms to antibiotics. Due to its low concentration of magnesium and calcium ions, Mueller-Hinton agar can be used for antimicrobial susceptibility testing. The low ion concentration inhibits the medium from reacting with certain antibiotics and guarantees that the medications' effects on the bacteria are not obscured. In this work, Mueller-Hinton agar was utilised to reveal *E. coli*'s susceptibility to the employed antibiotics. McConkey Agar is a differential and selective culture medium used to isolate and differentiate gram-negative bacteria, namely members of the Enterobacteriaceae family. It is composed of bile salts, neutral red indicators, crystal violet dye, peptones, and lactose (Erylmaz *et al*., 2010; Ho *et al*., These components promote the growth of Gram-negative bacteria while inhibiting the growth of Gram-positive bacteria. Its uniqueness rests on the capacity of bacteria to ferment lactose, hence creating acid and lowering the pH of the medium. This often results in a shift in the colour of the colonies, from pink to red (Phillips and Garda *et al*. 019; George *et al*., 2008; Giri *et al*., 2021). Effective culture medium because *E. coli* is an example of an enteric bacteria (Alamshani *et al*., 2023).

The results of the investigation reveal that the majority of strains demonstrated both antibiotic resistance and sensitivity. The most effective antibiotics against the pathogen *E. coli* are therefore Amikacin, Netilmicin, Ofloxacin, Cefrtriaxone, and Levofloxacin.

Table 3. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate peels ethanol extracts against *E. coli* strains. Data are expressed as the mean ± SD

Strains	Pomegranate peels ethanol extracts (mg/ml)				
	100	200	300	400	500
1	R	R	8.6±0.01^{e}	14.6±0.12^{c}	16±0.24^{b}
2	R	R	14.3±0.21^{c}	15±0.14^{b}	15.8±0.13^{b}
3	R	9.1±0.12^{e}	14.1±0.12^{c}	15.90.12^{b}	17.2±0.21^{a}
4	R	R	13.2±0.11^{c}	15.7±0.14^{b}	17.1±0.15^{a}
5	R	8.2±0.14^{e}	9.6±0.04^{e}	10.3±0.1^{d}	11.6±0.02
6	R	R	9.3±0.025^{e}	10.2±0.012^{d}	11.1±0.11
7	R	R	8.1±0.014^{e}	10.3±0.14^{d}	12.3±0.14
8	9.0±0.02^{e}	11.3±0.13^{d}	13.3±0.11^{c}	14.6±0.012^{c}	15.6±0.12^{b}
9	R	R	R	8.4±0.02^{e}	10.0±0.01^{d}
10	R	10.6±0.11^{d}	12.1±0.07^{d}	13.6±0.04^{c}	14.6±0.1
11	R	R	12.2±0.10^{d}	14.05±0.12^{c}	15.2±0.09^{b}
12	R	8.0±0.01^{e}	10.1±0.011^{d}	14.3±0.13^{c}	15.6±0.14^{b}
13	R	R	R	R	R
14	R	R	R	R	R
15	R	R	R	R	9.0±0.05^{e}
16	R	R	15.8±0.12^{b}	14.2±0.011^{c}	18.3±0.24^{a}
17	R	R	R	R	9.2±0.10^{e}
18	R	10.1±0.05^{d}	11.2±0.1^{d}	12.02±0.10^{d}	13.3±0.12^{c}
19	R	R	R	8.3±0.04^{e}	9.1±0.01
20	R	R	9.1±0.05^{e}	12.3±0.013^{d}	16.1±0.13^{b}

*R: Resistance.

Table 4. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate peels acetone extracts against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	8.0±0.021^{d}	9.1±0.014^{d}	10.2±0.015^{c}	13.2±0.02^{b}
2	R	R	R	11.1±0.012^{c}	14.3±0.2^{a}
3	R	R	11.2±0.01^{c}	12.6±0.014^{b}	13.4±0.14^{b}
4	R	R	R	9.04±0.016^{d}	9.2±0.025^{d}
5	R	9.3±0.04^{d}	9.07±0.015^{d}	10.2±0.017^{c}	11.1±0.1^{c}
6	R	R	R	R	R
7	9.1±0.14^{d}	10.20.25^{c}	12.2±0.03^{b}	14.0±0.23^{a}	15.1±0.023^{a}
8	R	9.1±0.18^{d}	10.4±0.02^{c}	14.0±0.018^{a}	15.0±0.17^{a}
9	9.2±0.21^{d}	12.3±0.16^{b}	14.0±0.021^{a}	14.4±0.17^{a}	15.3±0.014^{a}
10	R	12.2±0.12^{b}	13.2±0.22^{b}	14.1±0.3^{a}	14.6±0.1^{a}
11	R	R	R	R	R
12	R	10.1±0.13^{c}	11.8±0.16^{c}	12.2±0.21^{b}	13.4±0.012
13	R	R	R	12.1±0.16^{b}	11.12±0.014^{c}
14	R	R	11.1±0.015^{c}	13.2±0.24^{b}	14.11±0.13^{a}
15	R	R	R	R	8.1±0.01^{d}
16	R	R	11.7±0.02^{c}	12.4±0.12^{b}	15.1±0.024^{a}
17	R	8.0±0.02^{d}	9.1±0.04^{d}	11.05±0.02^{c}	12.2±0.016^{b}
18	8.1±0.012^{d}	9.1±0.012^{d}	10.2±0.02^{c}	10.7±0.011^{c}	3±0.02^{b}
19	R	R	R	R	R
20	R	8.0±0.02^{d}	12.1±0.12^{b}	14.3±0.15^{a}	15.2±0.17^{a}

*R: Resistance.

These antibiotics were chosen because the drugs' microbial membrane was protected from damage by the bacteria injected. According to the findings, the pomegranate component extract has the greatest ability to inhibit the growth of pathogenic microorganisms (Alamshani *et al*., 2023). Additionally, the higher the concentration of the leaf extract, the simpler it is to alter the structural membrane of the *E. coli*-causing pathogen. This extract can be used to replace or even enhance the performance of standard antibiotics. Antimicrobial activity was greatest in the ethanolic extract. This is in contrast to acetone.

In conclusion, *P. granatum* leaves and peels have a substantial effect on *E. coli* that is resistant to antibiotics. The study indicated that the leaf extract had antibacterial properties that suppress the growth of *E. coli* strains resistant to antibiotics. The active chemicals in the extract eliminate antibiotic resistance by destroying the integrity of the bacteria. When *P. granatum* leaf extract is mixed with standard antibiotics, synergistic effects are produced. The combo eradicates antibiotic-resistant *E. coli* bacteria more effectively than conventional antibiotics alone. These findings demonstrate the potential of *P. granatum* leaves as a natural alternative and treatment for conventional antibiotics in the management and treatment of antibiotic-resistant bacterial illnesses. To determine the precise mechanisms of action, optimise the extraction procedures, and assess the long-term efficacy and safety of *P. granatum* leaf extracts, additional research is required. However, the antibacterial properties of *P. granatum* have permitted the development of innovative, safe, and successful therapeutic techniques against *E. coli* resistant to antibiotics. tannins and alkaloids metabolites in the leaves and peels of pomegranate are considered antimicrobial (Joshi *et al*., 2019; Shaygannia *et al*. 2016; Wu and Tian 2017; Warsi and Sulistyani 2018; Joshi and Nair 1960). Alkaline groups in alkaloids interact with amino acid groups in cells, resulting in structural and chemical changes that are damaging to the cell (Johan *et al*., 2020; Seeram *et al*., 2005; Nurdin *et al*., 2019). Antibacterial flavonoids in pomegranate inhibit bacterial growth by inhibiting the DNA gyrase mechanism, resulting in intracellular leakage (Shaygannia *et al*., 2016). Queercetin, a flavonoid found in pomegranate, may kill microorganisms by increasing membrane permeability and hurting the potential of bacteria within membranes (Khan *et al*. 2012).

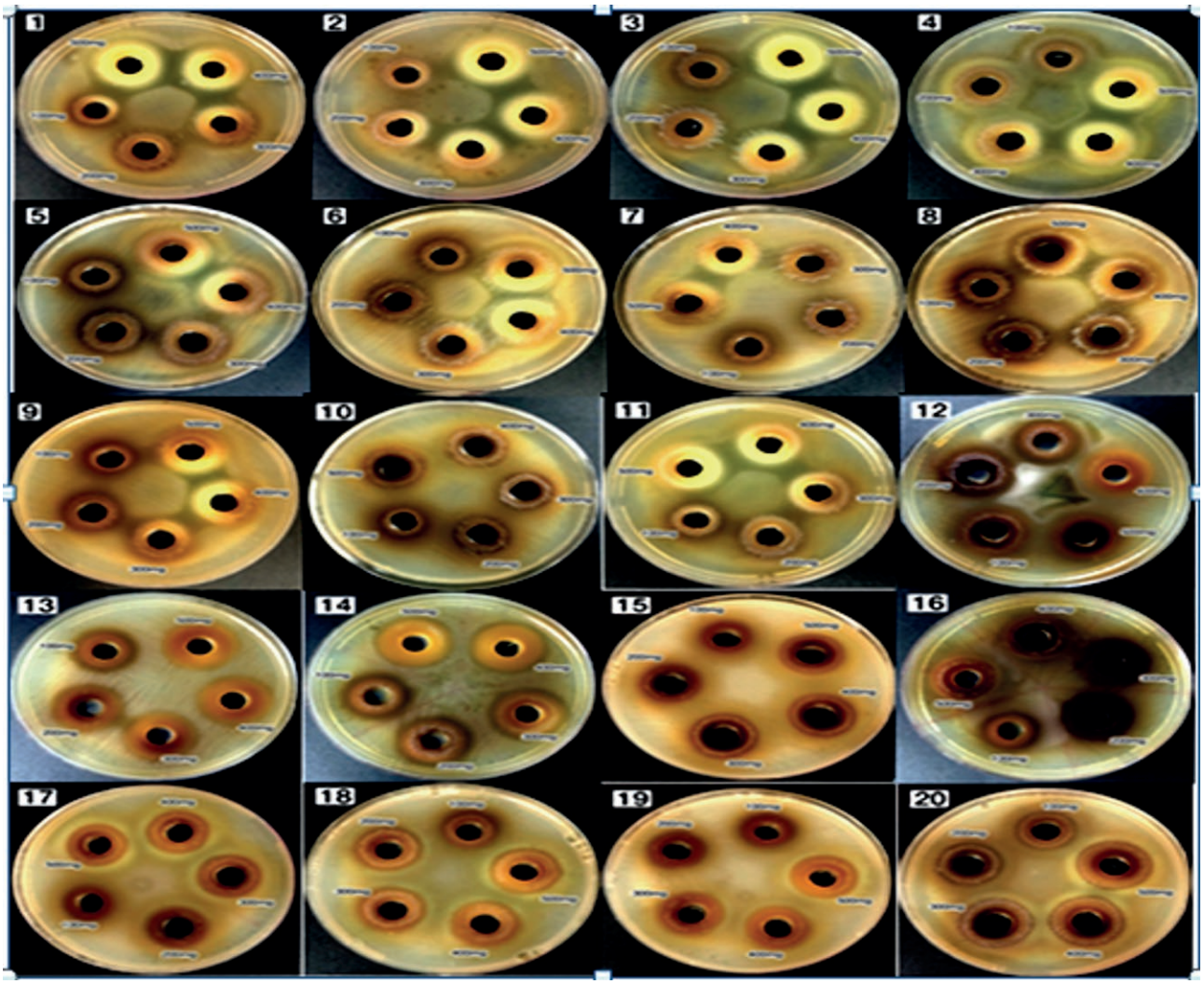

Figure 4. The inhibition zone diameters of different concentrations (100mg-500mg) of Pomegranate peels ethanol extracts against *E. coli* strains.

5. CONCLUSION

As a result of the global problem with MDR bacteria, scientists have been attempting to develop novel bioactive compounds derived from natural resources that can be exploited as safe phytotherapy. According to the findings of this study, pomegranate peels and leaf extract can suppress the growth of Extended-Spectrum Beta-Lactamase -*E. coli* pathogenic strains at doses 100 and 200 mg/ml with highest significant antibacterial activity. Accordingly, the ethanolic and acetone extracts exhibited the best antibacterial activity and may be used to enhance or even replace antibiotics with less cytotoxic effect.

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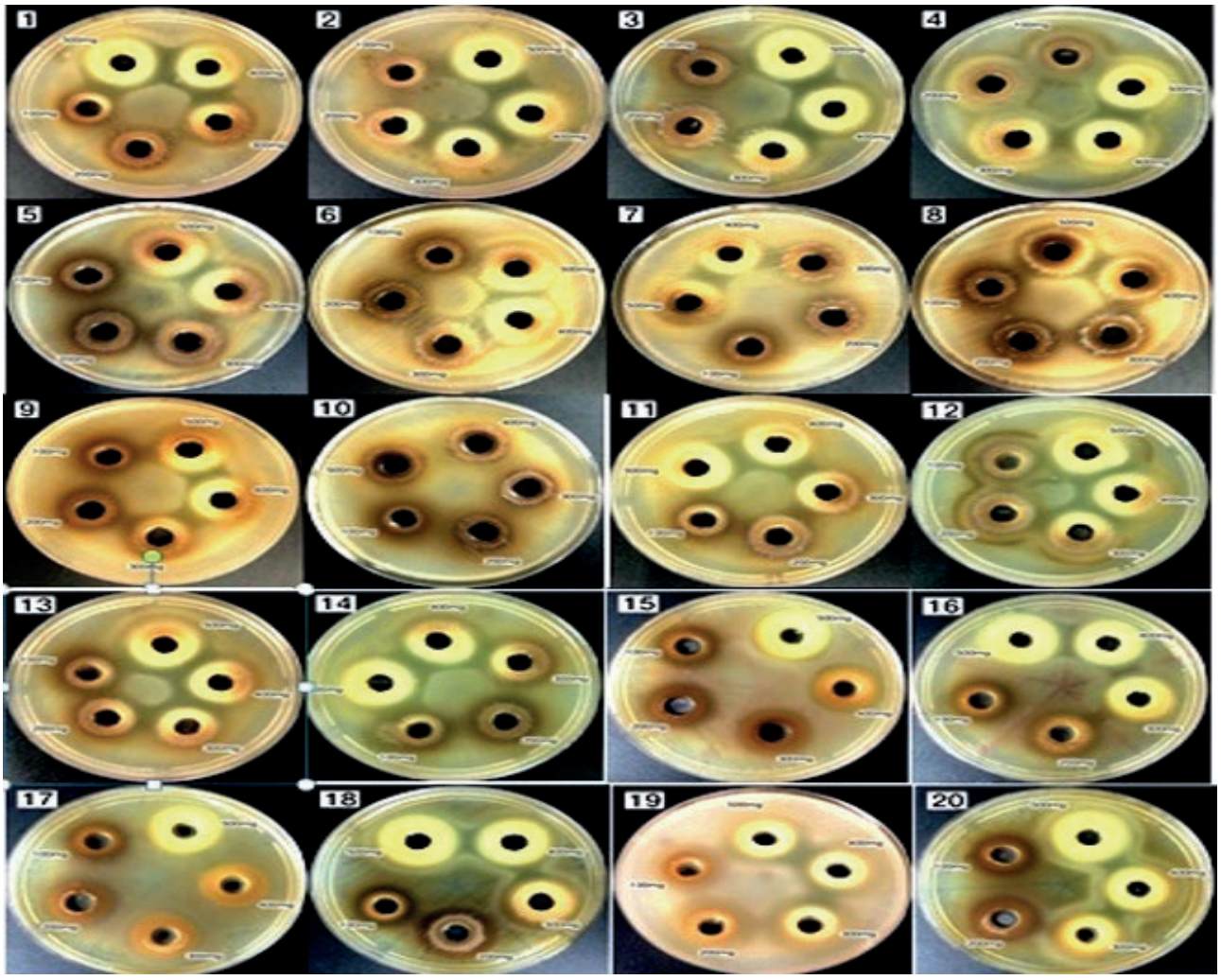

Figure 5. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate peels acetone extracts against *E. coli* strains.

AUTHOR CONTRIBUTIONS

All the authors contributed to the study's conception and design. The Faisal Al-Sarraj, Raed Albiheyri and Noor M. Bataweel authors were Identify ultimate objective of study and designed the required analysis and bio-assays. Wafa H. Alamshani, Majid Al-Zahrani, Tahani M. Alqahtani, Mashail A. Alghamdi, Nada Nass and Thamer Bouback authors were involved in funding acquisition, prepare the chemical, kits and performed the practical part of this work. Ibrahim Alotibi, Mohammed A. Al-Matary and Bayan H. Sajer authors were participate in study analysis, obtained raw data collection, supervision, and perform the statistical analysis. Faisal Al-Sarraj, Raed Albi, M. Attallah and Wafa H. Alamshani authors were shared in paper writing the manuscript body, create a strong conclusion and language editing

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Table 5. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate leaves ethanol extracts (EL) against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	R	R	R	8.0±0.011^{d}
2	R	8.0±0.010^{d}	9.0±0.1^{c}	10.0±0.02^{b}	11.6±0.04^{a}
3	R	R	7.8±0.03^{d}	8.0±0.011	8.4±0.012^{d}
4	7.6±0.01^{d}	8.0±0.03^{d}	8.6±0.07^{d}	9.3±0.012	10.0±0.03^{b}
5	8.0±0.02^{d}	8.5±0.013^{d}	9.2±0.02^{c}	9.6±0.011	10.0±0.02^{b}
6	R	R	R	8.1±0.015^{c}	8.7±0.012^{d}
7	R	R	8.7±0.01^{c}	9.8±0.2^{c}	12.6±0.025^{a}
8	R	R	8.0±0.02^{d}	9.3±0.1^{c}	12.0±0.017^{a}
9	R	R	7.6±0.02^{d}	R	8.5±0.011^{d}
10	R	R	R	R	9.0±0.013^{c}
11	R	R	R	R	R
12	R	R	8.3±0.011^{c}	8.6±0.012^{d}	9.0±0.014^{c}
13	R	R	R	R	R
14	R	R	R	R	R
15	R	R	R	R	R
16	R	R	R	R	R
17	R	R	R	8	R
18	R	R	R	R	R
19	R	R	R	R	R
20	R	R	R	R	R

*R: Resistance.

Table 6. The inhibition zone diameters (mm) of different concentrations (100-500 mg) of Pomegranate leaves acetone extracts (AL) against *E. coli* strains. Data are expressed as the mean ± SD.

Strains	Pomegranate peels acetone extracts (mg/ml)				
	100	200	300	400	500
1	R	R	R	R	R
2	R	7.6±0.012^{c}	8.1±0.02^{c}	8.4±0.015^{c}	9.2±0.014^{b}
3	R	R	R	R	R
4	R	R	R	R	R
5	R	R	R	R	8.1±0.011^{a}
6	R	R	8.0±0.014^{c}	9.0±0.025^{b}	10.1±0.12^{a}
7	R	R	R	9.5±0.04^{b}	10.2±0.015^{a}
8	R	R	8.0±0.012^{c}	9.3±0.12^{b}	10.1±0.21^{a}
9	R	R	R	R	9.2±0.013^{b}
10	R	R	R	R	8.0±0.01^{c}
11	R	R	R	R	R
12	R	R	8	8	8.0±0.013^{c}
13	R	R	R	R	8.0±0.01^{c}
14	R	R	R	R	8.1±0.012^{c}
15	R	R	R	R	R
16	R	R	R	R	R
17	R	R	7.8±0.011^{c}	8.0±0.012^{c}	8.6±0.011
18	R	R	R	R	8.0±0.01^{c}
19	R	R	R	R	8.1±0.12^{c}
20	R	R	R	R	R

*R: Resistance.

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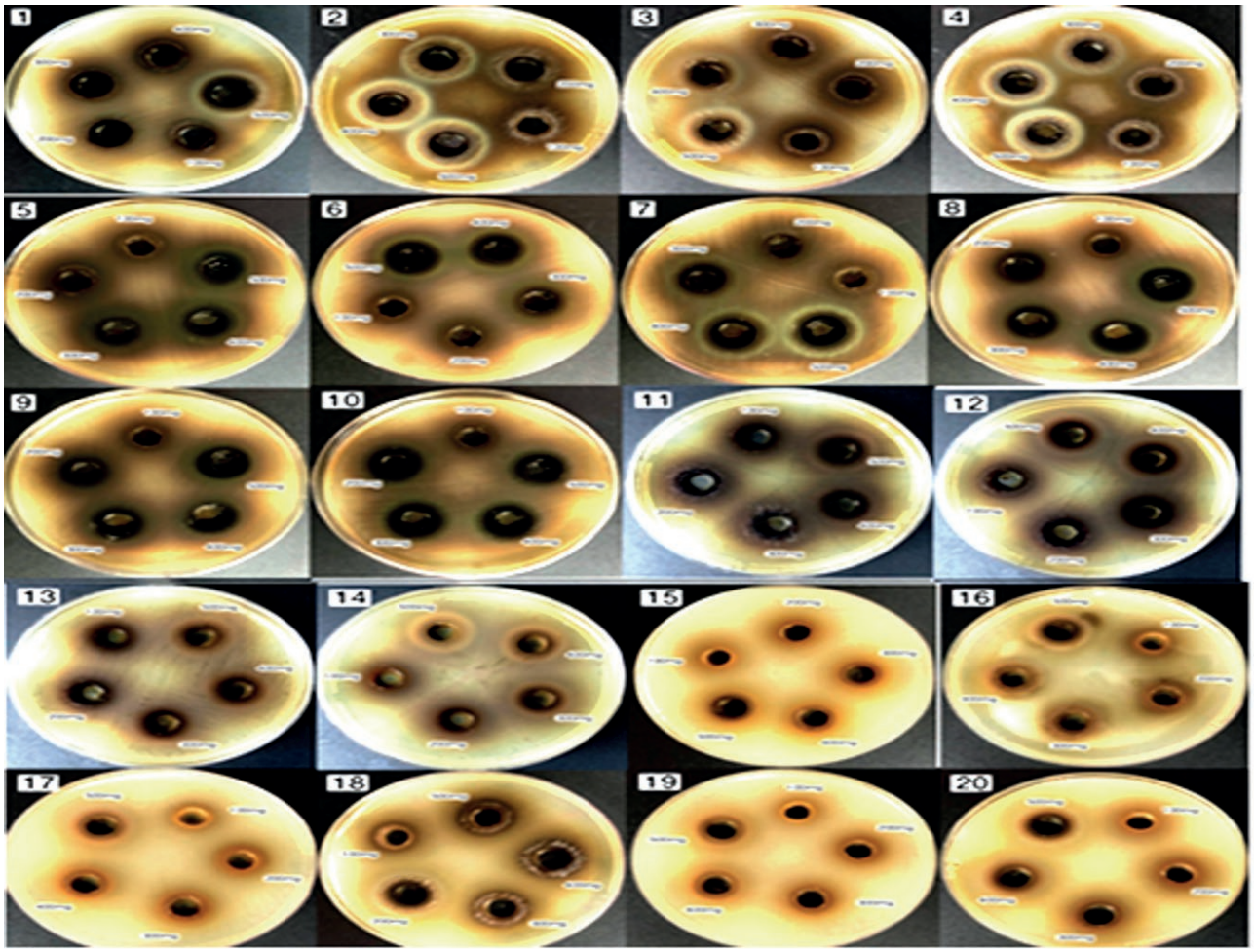

Figure 6. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate leaves ethanol extracts against *E. coli* strains.

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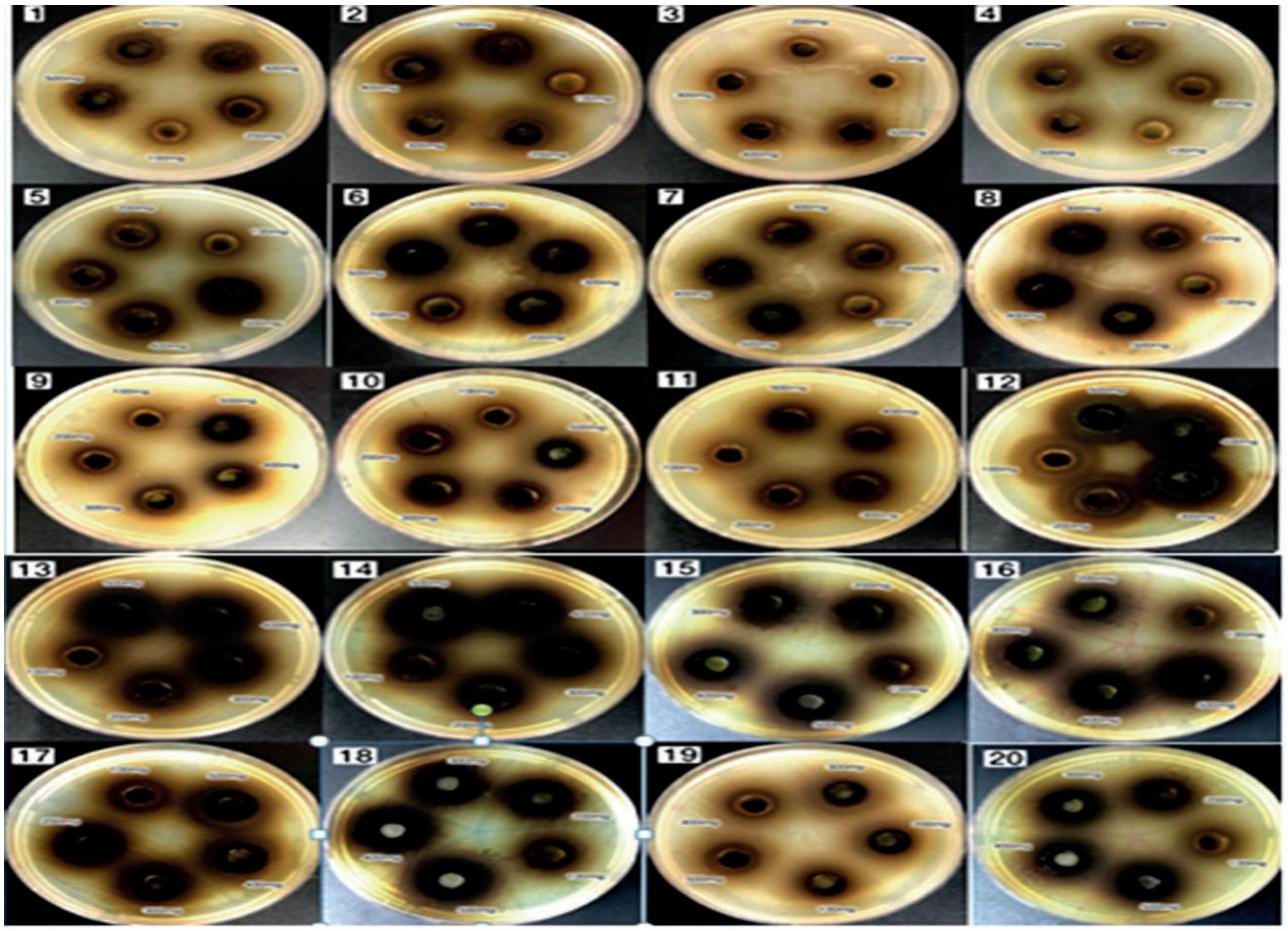

Figure 7. The inhibition zone diameters of different concentrations (100-500 mg) of Pomegranate leaves acetone extracts against *E. coli* strains.

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