

## The Anti-Bacterial Activity of Various Parts of *Punica granatum* on Antibiotics Resistance *Escherichia coli*

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### ABSTRACT

**Objectives:** The aim of this study was to investigate the antibacterial effectiveness of the extracts which are prepared from different parts of Punica Granatum L (from the family Punicaceae) which grows in Syria against *Escherichia coli* type(1) which reveal resistance toward the studied antibiotics. This study has shown the presence of antibacterial effectiveness of the extracts prepared from different parts of Punica Granatum (L), whereas the studied antibiotics have not shown any antibacterial effectiveness.

**Materials and Methods:** Different parts of punica granatum (pericarp, leaves, flowers, seeds) were extracted by water, absolute alcohol, then ether using soxhlet device and rotary vacuum evaporator. 767 samples of dead calves (liver or intestines) were investigated for *Escherichia coli*, using blood agar, and biochemical tests (oxidase, catalase, indole). Antibiotic susceptibility testing for *E.coli* by Kirby-Bauer disk diffusion method was conducted. Then for stereotyping antibiotic resistance's *E.coli* API20E technique, and many selective culture media were used. At last extracts susceptibility testing for *E.coli* type(1) was studied.

**Results:** *E.coli* type(1) was 35.67% of the total number of samples. The studied antibiotics showed no antibacterial effectiveness against *E.coli* except Amikacin which had an acceptable effectiveness 10.34% of the total number of samples. However, The alcoholic extracts prepared from different parts of Punica granatum revealed different antibacterial activity against *E.coli* type(1), which affected calves, and had shown antibiotics resistance. Pericarp extract was the best. Whereas the water and ether petroleum extracts had no antibacterial effectiveness.

**Conclusion:** Ethanol extracts of punica granatum (pericarp, leaves, flowers, seeds) have antibacterial effect against *E.coli* type(1) which has shown resistance to all studied antibiotics.

**Keywords:** Punica Granatum, *E.coli* type(1), Punicaceae, resistance bacteria.

### INTRODUCTION

Cattle are a major reservoir for human infection, and outbreaks of disease are commonly linked either directly or indirectly with multiple vehicles. Sources of infection include direct contact with animals, meat, raw milk, cheese, and contaminated water source<sup>1</sup>. The natural habitat of *Escherichia coli* (*E.coli*) is the intestinal tract of humans and animals. It is therefore considered an indicator organism for fecal contamination of water and foods. *E.coli* is the most frequent causative pathogen in human bacterial infections. Pathogenic variants cause intestinal and extraintestinal infections, including gastroenteritis, urinary tract infection, meningitis, peritonitis, and septicemia<sup>2</sup>. Resistance in *E. coli* is consistently highest for antimicrobial agents that have been in use the longest time in human and veterinary medicine. The past 2 decades have witnessed major increases in emergence and spread of multidrug-resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins<sup>3</sup>. These bacteria showed resistance to many antibiotics<sup>4,5</sup>. Animal *E. coli* isolates showed an increasing resistance trend to 11 antimicrobial agents (ampicillin, sulfonamide, tetracycline, cephalothin,

trimethoprim/sulfamethoxazole, streptomycin, chloramphenicol, cefoxitin, gentamicin, amoxicillin/clavulanic acid, and kanamycin), and human *E. coli* isolates showed an increasing trend in resistance only to ampicillin, sulfonamide, and tetracycline<sup>3</sup>.

We tried in our investigation to discover the possibility of owning the plant the capacity to respond to these bacteria. And we believe that, this is the first study describing the antibacterial activity of *P. Granatum* extracts against *E. coli* type (1), and we hope that its results to be a starting point in administering the plant extracts on infected animals.

*Punica Granatum* Linn (Pomegranate) belonging to family Punicaceae. The medicinal parts are the root, the bark, the fruits, the peel of the fruit and the flowers<sup>6</sup>. Various parts of *Punica Granatum* L have been used for various medicinal purposes<sup>7</sup>. Although the global studies pointed to the impacts of the flowers, as decreases the blood glucose, reducing the cholesterol<sup>8</sup>, and anti-allergic effect<sup>9</sup>, but it did not showed the antibacterial effect.

With regard to the popular therapeutic uses of pomegranate, it has known as an anti-diarrhea, antiparasitic agent, ulcers, diuretic, and an antibacterial

Table 1: Antibacterial activity of Ethanol extract of studied parts against *E. coli* type(1).

<i>Punicagranatum</i>	inhibitory zone diameters of plant extracts (mm) (mean $\pm$ standard deviation)	Percentage of sensitive bacteria %
Control/5 $\mu$ m	0	0
Pericarp	23.99 $\pm$ 0.74	88.2
Seeds	21.01 $\pm$ 0.76	65.77
Flowers	15.05 $\pm$ 0.69	91.21
Leaves	10.02 $\pm$ 0.77	54

Figure 1: Inhibition zones of seeds extracts against *E.coli* type(1)Figure 2: Inhibition zones of pericarp extracts against *E.coli* type(1)Figure 3: Shows no effect of studied antibiotics against *E.coli*

activity<sup>10</sup>. The pharmacological functions of pomegranate include antioxidation, anti-tumour anti-hepatotoxicity, anti-lipoperoxidation, and anti-inflammatory<sup>11</sup>. Although many studies have Reported the antibacterial activity of pomegranate<sup>11,12</sup>, but it did not revealed enough studies about its effect on bacterial resistance, and did not determined the most effective part of the plant in dealing with bacteria, whether peel of the fruit, leaves, flowers, or seeds of the pomegranate.

## MATERIALS AND METHODS

Collection of plant material: Leaves and the flowers of pomegranate were collected in the early morning hours during the period from March to April, while the ripe fruits were collected during the period from July to September, from Damascus rural area, which were identified by Damascus University.

The peel were separated from the fruits, washed with cold water, distilled water, then dried with hot air at a temperature not exceeding 60°C in shadow. Then were crushed properly by metal mortar in order to obtain fine homogeneous powder, kept in paper bags with free humidity conditions, ready to prepare extract<sup>13</sup>.

Preparing plant extracts: Plant parts were extracted separately by continuous extraction device (Soxhlet apparatus), adopted method described by Wang<sup>14</sup> for preparing plant extracts by organic solvents. 50g of plant powder were placed by an electric mortar, inside the thimble-holder of Soxhlet apparatus, with 500 ml of each organic solvent (rate: 1:10weight: volume). Three different polar solvents have been selected to extract the components of the plants, which are respectively: water, absolute ethanol, petroleum ether. Extraction period was 4 hours, until the used solvent comes out of thimble colorless. Then to concentrate the extracts the ethanol, and petroleum ether extracts were dried using rotary vacuum evaporator at a temperature not exceeding 40°C, while the aqueous extract was dried using freeze dryer. The thick layer of the bottom was stored in sterile bottles at 4°C for

further experiments. All extracts were filter-sterilized using a 0.45  $\mu$ m membrane filters (whatman, Co., UK)<sup>13</sup>. Sampling method: Samples of dead calves (liver or intestines) were submitted daily to the morgue of the Central Laboratory of Veterinary. The 767 samples were preserved in sterile boxes, fitted with a strap closure, and card number includes name of the sample, number, and date of collection.

Cultured and identification Methods of pathological sample: The following information was registered on the bottom of the petri plates: the number, name of the sample, place, and date of collection. Then the platinum rod after sterilization by flame lamp was planted within the sample (biopsy liver and eggs), and passed on blood agar (HiMedia, India), and incubated for 20-24 hours at 35-37°C at an aerobic culture incubator. All the samples were planted in two hours from the time of sampling.

*Escherichia coli* are selected after the following steps: The bacteria were identified culturally, morphologically and biochemically:

Microscopic examination: Microscopic examination was carried out after 24 hours of incubation on blood agar plates using the gram stain, immersion oil, light microscope.

Biochemical tests: All of the following biochemical tests were conducted: oxidase, catalase, and indole.

A bacterial growth inhibition test to antibiotics by the disk diffusion method: The antimicrobial susceptibility testing was carried out on blood agar by disc diffusion method (Kirby-Bauer Disk Diffusion Susceptibility Test Protocol) using the following antimicrobial substances (Becton Dickinson, Microbiology Systems, MD, USA) as described by National Committee For Clinical Laboratory Standards<sup>15</sup>.

The 18 antibiotics and concentration ranges tested were as follows: amikacin (30  $\mu$ g), ampicillin (10  $\mu$ g), Cephalexin (30  $\mu$ g), cephalothin (30  $\mu$ g), Doxycycline (30  $\mu$ g), Cefadroxil (30  $\mu$ g), ciprofloxacin (5  $\mu$ g), clindamycin (2  $\mu$ g), chloramphenicol (30  $\mu$ g), erythromycin (15  $\mu$ g),

Table 2: Amikacin susceptibility results against *E. coli* type(1)

classes	$x_i$	$f_i$	$f_i \cdot x_i$	$(\sim - x_i)^2$	$f_i (\sim - x_i)^2$
- 22	22.5	27	607.5	$(1.48)^2$	59.14
- 23	23.5	111	2608.5	$(0.48)^2$	25.57
- 24	24.5	114	2793	$(- 0.52)^2$	30.83
25 - 26	25.5	22	561	$(- 1.52)^2$	50.83
Total		274	9570		
Mean	$\sim = 23.98$		Stander Division	$\dagger = 0.78$	

gentamicin (10 µg), Norfloxacin (10 µg), Oxytetracycline (30 µg), Pefloxacin (5 µg), Oxacillin (1 µg), Enrofloxacin (5 µg), tetracycline (30 µg) and Amoxicillin(25 µg).

The resistance breakpoints were those defined by the National Committee for Clinical Laboratory Standards (NCCLS, 2000) for Gram-negative bacteria<sup>15</sup>.

5colonies of bacterial were Suspended (after pure isolation and identification) to the test tube in 2 ml of physiological solution, Mixed thoroughly until aturbid homogeneous obtained. Sterile swab sticks immersed in suspension, and spread onto the surface of the blood agar plates, then the agar plates were covered partly with lids to dry before proceeding to the next step. Forceps was sterilized by flame lamp for placing the antibiotics with gently pressed onto the middle plates (Forceps was sterilized after each antibiotic), finally the agar plates were covered and incubated in aerobic incubator at 37° C for 24 hours.

The plates were observed for the presence of inhibition of bacterial growth that was indicated by a clear zone around the wells. The size of the zones of inhibition was measured and the antibacterial activity expressed in terms of the average diameter of the zone inhibition in millimeters.

Analytical Profile Index technique (API20E): This technique was performed according to the manufacturer's instructions Bio Merieux, France:

Antibiotics resistant bacterial colonies were suspended in 0.85% sterile saline to achieve a homogeneous bacterial suspension. However, the bacterial colonies, which were susceptibility to the antibiotic, were abandoned. The honey-combed of the incubation box were filled with distilled water. The bacterial suspension was distributed into the tubes of the strip (some were completely filled, and some were partially filled, while others were added a paraffin oil), then were incubated for 24 hours at 37 ° C, after that the appropriate reagents are added. The result was recorded on the result sheet back to the documentation accompanying by the manufacturer.

All of the following tests were conducted: oxidase, catalase, indole.

23 differential biochemical tests were performed by API20E system (Analytical Profile Index) manufactured by Bio Merieux, France:

a- Investigate the effectiveness of enzymes: 2 - nitrophenyl- D-galactopyranoside, Arginine dihydrolase, oxidase.

b- Decarboxylation Interactionsof amino acids: L-lysine, L-omithine.

c- Deaminase interaction of the amino acid: L-tryptophan.

d- Fermentation reactions of the following sugars: D-glucose, D-mannitol, Inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, Amygdaline Arabinose.

e- Production reactions: indole, Asitoen.

f- Reactions produce gases: hydrogen sulphide, nitrogen, nitrogen oxide.

g- Study the interactions of Recipes: gelatin diluted, citrate use, hydrolysis of urea (urease).

Microbial selective cultures for stereotyping resistance *E. coli*: The selective media according to the method of 16, were McCoonky agar, XLD agar, Salmonella – shigella agar, bisimuth sulfate agar, citrate, and potassium cianor. Where the bacteria from nutrient broth was cultured, then incubated for 40-48 hours at 35-37 °C. The API20E system was also used to support the identification process.

A bacterial growth inhibition test of plant extracts aginast *E. colitype* (1) by the disk diffusion method: Bacterial suspension was emulsified with Agar-agar medium to test the susceptibility of plant extracts. Sterile filter paper discs (5 mm) were soaked with 5mcl of the diluted extracts (66 mg/ml) of pericarp, leaves, flowers ,seeds in ethanol, water ,and petroleum ether, so that each disc was impregnated with 0.33 mg / tablet. Control disks also prepared with absolute ethanol, Water, and petroleum ether. The Disks were placed in Petri dishes containing agar agar and incubated for 16 hours at 37 °C.

After incubation, all dishes were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters with a ruler. Results were expressed as the percentage of inhibition of bacterial growth, determined by comparing it with Control disks, and standard susceptibility disks<sup>12</sup>.

## STATISTICAL ANALYSIS

The data are presented as mean ( $\mu$ ) and standard deviation ( ).

## RESULTS

Identification of the bacteria: Bacteria samples that gave us the following results were selected:

Staining with a gram stain: Gram-negative, non-motile, straight rods with round edges, no capsule, and non sporing bacteria that form compatible with reference<sup>16,17</sup>.

The results of biochemical tests: The results were shown in Table (1), these results were depended according to<sup>17</sup>.

Biochemical confirmation of the strains was performed and *E. coli* was defined as oxidase negative, indole positive, catalase positive.

Antimicrobial susceptibility results against *E. coli*: Bacterial colonies showed resistant to the studied antibiotics, where all the diameter zones of inhibition were zero, except for Amikacin was 24 mm. The percentage was 80(10.43%) out of the total number, based on the criteria of NCCLS200015, and to the standard's leaflet of

Table 3: Antibacterial activity of pericarp ethanol extract against *E. coli* type(1).

classes	$x_i$	$f_i$	$f_i \cdot x_i$	$(\sim - x_i)^2$	$f_i (\sim - x_i)^2$
- 22	22.5	25	562.5	( 1.49) <sup>2</sup>	55.50
- 23	23.5	105	2467.5	(0.49) <sup>2</sup>	25.21
- 24	24.5	128	3136	( -0.51) <sup>2</sup>	33.29
25 - 26	25.5	16	408	( -1.51) <sup>2</sup>	36.48
Total		274	6574		150.48
Mean	$\sim = 23.99$			Stander Division	$\dagger = 0.74$

Table 4: Antibacterial activity of seeds ethanol extract against *E. coli* type (1).

classes	$x_i$	$f_i$	$f_i \cdot x_i$	$(\sim - x_i)^2$	$f_i (\sim - x_i)^2$
- 19	19.5	21	409.5	( 1.51) <sup>2</sup>	47.88
- 20	20.5	115	2357.5	( 0.51) <sup>2</sup>	29.91
- 21	21.5	114	2451	( -0.49) <sup>2</sup>	27.37
22 - 23	22.5	24	540	( -1.49) <sup>2</sup>	53.28
Total		274	5758		158.44
Mean	$\sim = 21.01$			Stander Division	$\dagger = 0.76$

Table 5: Antibacterial activity of flowers ethanol extract against *E. coli* type(1).

classes	$x_i$	$f_i$	$f_i \cdot x_i$	$(\sim - x_i)^2$	$f_i (\sim - x_i)^2$
- 13	13.5	13	175.5	( 1.55) <sup>2</sup>	31.23
- 14	14.5	117	1696.5	(0.55) <sup>2</sup>	35.39
- 15	15.5	125	1937.5	( -0.45) <sup>2</sup>	25.31
16 - 17	16.5	19	313.5	( -1.45) <sup>2</sup>	39.95
Total		274	4123		131.88
Mean	$\sim = 15.05$			Stander Division	$\dagger = 0.69$

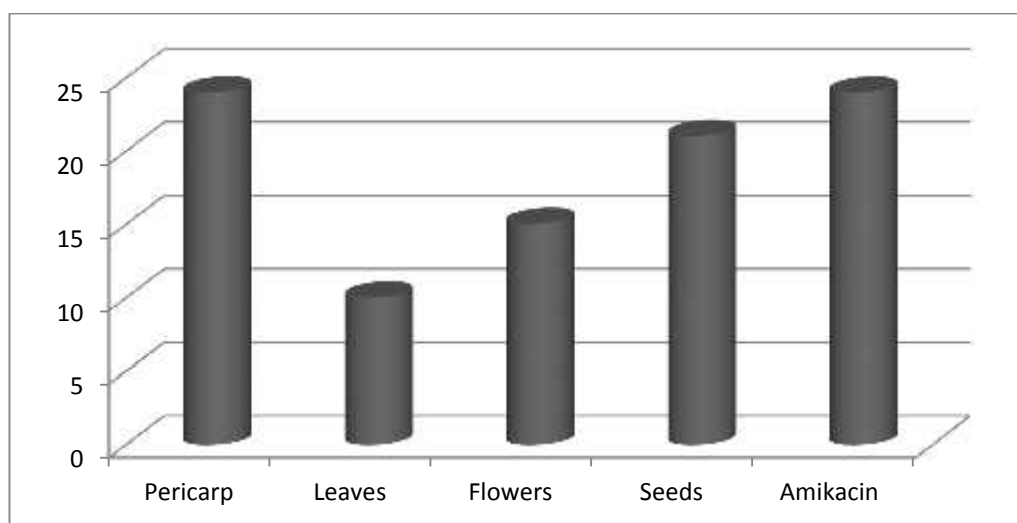


Figure 4: Mean of inhibition diameter of plants extract and Amikacin against *E. coli* type (1).

antibiotic discs from the manufacturer. These colonies were selected to test the plant extracts.

The results of Analytical Profile Index technique (API20E): According to the data contained in the annex with these tests the results of these tests were depended. The results of the biochemical analysis (API20e) after the end of the incubation period, according to the method used in the directory of the test facilities, were: 5144512 as a result of this reading on *E. coli* type(1). So as a result 274(35.67%) out of total number 767 samples were gotten of *E. coli* type(1).

Colonial and cultural characters: These results were depended according to<sup>16,17</sup>.

- Growth on blood agar: circular colonies, white, and smooth with a fecal odor.
- Growth on McCoonky agar: the growth of red colonies.
- Growth on XLD agar (xylose lysine desoxycholate agar) european pharmacopoeia: yellow colonies with wet strength, and with zone of yellow precipitation around the colonies, These results were depended according.
- Growth on the bisimuth sulfate agar (BIS): brown colonies.
- Growth on the citrate medium: not grow

Table 6: Antibacterial activity of leaves ethanol extract against *E. coli* type (1).

classes	$x_i$	$f_i$	$f_i \cdot x_i$	$(\sim - x_i)^2$	$f_i (\sim - x_i)^2$
- 8	8.5	27	229.5	(1.52) <sup>2</sup>	62.38
- 9	9.5	98	931	(0.52) <sup>2</sup>	26.50
- 10	10.5	129	1354.5	(-0.48) <sup>2</sup>	29.72
11 -12	11.5	20	230	(-1.48) <sup>2</sup>	43.81
Total		274	2745		162.41
Mean	$\sim = 10.02$		Stander Division	$\dagger = 0.77$	

- Growth on potassium cyanide medium: did not grow

The results of Antibacterial Efficacy of plant extracts against *E. coli* type(1): The organic solvent petroleum Ether, and water extract from all parts of the plants were not active against *E. coli* type I (1) (diameters of zone of inhibition were zero). As shown in Table (1), the extracts from different parts of the plant studied (pericarp, leaves, flowers, seeds) showed antibacterial activity against *E. coli* type(1), with the diameters of zone of inhibition ranging between 8 and 26 mm. Mean inhibition zones indicate that pomegranate pericarp and seeds extracts exert a powerful effect on *E. coli* type(1). The results of the statistical analysis: The results were shown in Tables (2-6).

## DISCUSSION

Much attention has been focused on the possible that some strains of *E. coli* are associated with deaths in babies and in young calves due to various forms of gastro-enteritis. The present study showed that the incidence ratio of dead calves (liver or intestines) with *Escherichia coli* was 35.67% out of the total number of samples, nearly to the infection rates 20.3% of France calves faecal samples<sup>18</sup>. Regarding the efficiency of the antibiotics, *E. coli* was resistant to all tested antibiotics except Amikacin which was 10.43% out of the total number of samples, as verified by Malik<sup>4</sup>. Our results agree with those that have reported the resistance to two types of *E. coli* to many different antibiotics such as Neomycin, Streptomycin, Lincomycin, Enrofloxacin, Tetracycline<sup>19</sup>. As well as others study proved the resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, trimethoprim, and nalidixic acid<sup>5,20,21</sup>. The resistance of Gram-negative bacteria against antibacterial substances may be due to outer phospholipidic membrane carrying the structural lipopolysaccharide components, which make it impermeable to lipophilic solutes, and porins constitute a selective barrier to the hydrophilic solutes<sup>22</sup>.

That lead us that there is an urgent need to develop alternative antimicrobial drugs against this type of bacteria

The results showed that ethanolic extracts possesses strong antibacterial activity directed against Gram-negative *Escherichia coli* type(1), and this is the first time reported. This study showed that *Punica Granatum* water extract weren't equally active as organic extracts against. Both aqueous and ether petroleum extracts from different parts of the plant studied (pericarp, leaves, flowers, seeds) did not have antibacterial effect, while ethanol extracts produced disparate zones of inhibition against *Escherichia coli* type(1). Of the parts studied, the most active extracts were those obtained from Pericarp as seeds of *Punica*

*Granatum*. The results indicated the presence of zone of inhibition of 24 mm diameter of ethanol extract of Pericarp as seeds, and 15 mm of ethanol extract of flowers as leaves. The activity of ethanol extracts from *Punica Granatum* was similar to Amikacin.

Previous studies have also demonstrated the similar activity that extracts of the whole fruit were highly active against *Micrococcus pyogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli*<sup>12</sup>. Mathabe et al.<sup>23</sup> showed that methanol, ethanol, acetone and water extracts obtained from pomegranate were active and effective against the tested many microorganisms such as *E. coli*. Duman et al.<sup>24</sup> also reported the in vitro antibacterial activity of extracts obtained from six pomegranate cultivators against *E. coli*. As well as the antimicrobial activity of pomegranate seeds<sup>25</sup>, and the leaf extracts<sup>26</sup>.

Global studies pointed to the impacts of the flowers, but it did not showed the antibacterial effect, while our study proved it in the alcoholic extract which had produced 15mm diameter zones of inhibition.

Although this study<sup>27</sup> showed that the methanol and water extracts of the *Punica Granatum* leaf, and peel have the antibacterial activity, but our study showed the opposite for water extract. These findings exhibited some differences with Orak<sup>22</sup> who stated that water extract from pomegranate peel showed positive antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella Enteritidis*.

Phytochemical screening on the ethanolic extracts of *Punica Granatum* pericarp demonstrated that they contain flavonoid, sterols, triterpenes, phenols, and tannins<sup>28</sup>.

*P. Granatum* contains large amount of tannins (25%) and the antibacterial activity may be indicating the presence of some secondary metabolites. The antibacterial activity may be the indicative of the presence of some metabolic toxins or broad-spectrum antibiotic compounds. The tannins in the drug make it useful as an astringent for sore throats, diarrhea and dysentery. The drug which contains tannins and alkaloids, is anthelmintic and amoeboid.

Pomegranate fruit peel compounds Tannins, Piperidine alkaloids and Pelletierin triggers like strychnine, a raised stimulant reflex, which can escalate to tetanus and is effective against diverse tapeworms, ring worms and nematodes<sup>6</sup>. Peels of *Punica Granatum* L. also include wide variety of phytochemical compounds, e.g., gallotannins, ellagic acid, gallic acid, punicalins, punicalagins, and this fruit is found to be a rich source of polyphenolic compounds. The antibacterial effect may return to phenolic compounds which could be increasing with the presence of organic acids<sup>29</sup>.

Methanolic extract of pomegranate fruit has pelargonidin-3-galactose, cyanidin-3-glucose, gallic acid, quercetin, and myricetin. All these compounds exhibited substantial activity against species of coryne bacteria, staphylococci, streptococci, *Bacillus subtilis*, *Shigella*, *Salmonella*, *Vibrio cholera*, and *Escherichia coli*. The gallic acid showed the highest antibacterial activity. The antibacterial activity of all pure compounds was attributed to their phenolic structure<sup>30</sup>.

## CONCLUSION

The ethanol extracts of the *Punica Granatum* revealed different antibacterial activity against *Escherichia coli* type (1), which affected clones, and had shown completely antibiotics resistance to all studied antibiotic except Amikacin. While the water and ether petroleum extracts had no antibacterial effectiveness. And the antibacterial effect may back to many compounds.

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