

The Anti-Bacterial Effect of *Punica granatum* Extracts Against Antibiotic Resistant *Pasteurella haemolytica*

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Background: Continuous researches on drugs that solve the problem of growing number of resistant bacteria are important.

Objectives: This research aims to investigate the effectiveness of anti-bacterial properties shown by the extracts prepared from different parts of *Punica granatum* L. (punicaceae), grown in Syria, against *Pasteurella haemolytica*, which are resistant to all studied antibiotics.

Materials and Methods: A total number of 504 samples of dead sheep lungs were investigated for detection of *P. haemolytica*, using blood agar, blue methylene, and biochemical tests (oxidase, catalase, indole, urease). Different parts of *P. granatum* (pericarp, leaves, flowers, seeds) were extracted by water, absolute alcohol, and ether using soxhlet device and rotary vacuum evaporator. Antibiotic susceptibility testing for *P. haemolytica* by Kirby-Bauer disk diffusion method was conducted, then the extracts susceptibility test against *P. haemolytica* was conducted.

Results: *P. haemolytica* has infected 20.63% of the total number of samples. The alcohol extracts prepared from different parts of *P. granatum* showed the high antibacterial effectiveness, the pericarp extract was the best, whereas the water and ether petroleum extracts had no antibacterial effectiveness against resistant *P. haemolytica*.

Conclusions: Ethanol extracts of *P. granatum* (pericarp, leaves, flowers, seeds) have antibacterial effects against *P. haemolytica* which has shown resistance to all studied antibiotics.

Keywords: *Punica granatum*; *Pasteurella haemolytica*; Resistant Bacteria; Antibiotics

1. Background

Punica granatum Linn (*pomegranate*) belonging to family of *Punicaceae*. The medicinal parts are the root, bark, fruits, peel of the fruit and the flowers (1). With regards to the popular therapeutic uses of pomegranate, it has known as an anti-diarrhea, antiparasitic agent for treatment of ulcers, diuretic, with antibacterial activity (2). It is widely used as an antipyretic analgesic in Chinese culture (3). Various parts of the *Pomegranate*, have been used for various medicinal purposes (1, 4). The fruit is good for dysentery, diarrhea and gastralgia. The extract from different parts of the fruit showed antibacterial activity (3). Although many studies have reported the antibacterial activity of pomegranate (5-7), but not enough studies have reported its effect on bacterial resistance, and had 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.

Pasteurella multocida and *Mannheimia (Pasteurella) haemolytica* are causative agents of several economically

significant veterinary diseases occurring in numerous species such as: cattle, buffaloes, sheep, goats, poultry, turkeys, rabbits, horses and camels. *M. haemolytica* is the causative agent of shipping fever or pneumonic pasteurellosis (8) and respiratory diseases in cattle, including bronchopneumonia in feedlot cattle. The species usually involved in these infections are *P. multocida* sp. *multocida* sp. *septica*, *P. canis*, *P. dagmatis*, and *P. stomatis*. Most human infections are associated with *Pasteurella* sp infection resulted from animal bites (9).

2. Objectives

Unfortunately, the resistance to antibiotics is increasing, and these bacteria showed resistance to many antibiotics (10-12). So in our investigation, we tried to discover the possibility of owning the plant capable to respond to these bacteria, and we believe that, this is the first study describing the antibacterial activity of *P. granatum* extracts against *P. haemolytica*, and we hope that its results to be a starting point in administering the plant extracts

Implication for health policy/practice/research/medical education:

Unfortunately, the resistance to antibiotics is increasing, so we tried in our investigation to discover the possibility of owning the plant the capacity to respond to *Pasteurella haemolytica*, a causative agent of several economically significant veterinary diseases, which were resistant to all studied antibiotics. We believe that, this is the first study describing the antibacterial activity by different parts of *Punica granatum* extracts against *P. haemolytica*, and we hope that its results to be a starting point in administering the plant extracts on infected animals.

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for infected animals.

3. Materials and Methods

3.1. Collection of Plant Specimens

Ripe fruits of pomegranate were collected from July to September 2010 from Damascus rural area (Duma), while the leaves and the flowers were collected in the early morning times from May to July 2010 from the mentioned location, which were identified by Damascus University. The peel were separated from the fruits, washed with cold 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 (13).

3.2. Preparing Plant Extracts

Plant parts were extracted separately by continuous extraction device (Soxhlet apparatus), based on the method described by Wang (14), for preparing plant extracts by organic solvents, 50 g of plant powder were placed by an electric mortar, inside the thimble-holder of Soxhlet apparatus, with 500 mL of each organic solvent (rate: 10:1 weight: 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 took 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 µm membrane filters (Whatman Co., UK) (13).

3.3. Sampling Method

Samples of dead sheep were daily conducted to the morgue of the Central Laboratory of Veterinary in Damascus during 2010-2011. A total number of 504 lung biopsies samples were investigated. The samples were kept at 2 - 4°C and transported to the laboratory in sterile tubes, fitted with a strap closure, and card number includes name of the sample, place, and date of collection.

3.4. 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 lung), and passed on blood agar (HiMedia, India), and incubated for 20 - 24 hours at 35 - 37°C at

an aerobic culture incubator. It is worth mentioning that all samples were planted in two hours from the time of sampling. The bacteria were identified culturally, morphologically and biochemically.

Microscopic examination was conducted after 24 hours of incubation on blood agar plates using the Gram stain, immersion oil, methylene blue dye, light microscope. All of the following biochemical tests were conducted: oxidase, catalase, and indole. In addition, fermentation reactions of the following sugars: D-glucose, D-mannitol, lactose were conducted.

3.5. Bacterial Growth Inhibition Test by the Use of 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 (15).

The 5 mm diameter standard discs containing certain concentrations of many antibiotics (Bioanalyse), as follows: Amikacin (30 µg), Ampicillin (10 µg), Cephalexin (30 µg), Cephalothin (30 µg), Doxycyclin (30 µg), Cefadroxil (30 µg), Ciprofloxacin (5 µg), Clindamycin (2 µg), Chloramphenicol (30 µg), Erythromycin (15 µg), Gentamicin (10 µg), Norfloxacin (10 µg), Oxytetracycline (30 µg), Pefloxacin (5 µg), Oxacillin (1 µg), Enrofloxacin (5 µg), Tetracycline (30 µg) and Amoxicillin (25µg). the resistance breakpoints were defined by the national committee for Clinical Laboratory Standards (NCCLS, 2000) for Gram-negative bacteria (15).

4 - 5 colonies of bacteria were suspended (after pure isolation and identification) in the test tube containing 2 mL of physiological solution, mixed thoroughly until a turbid homogeneous was obtained. Sterile swab sticks immersed in suspension, and spread onto the surface of the Muller Hinton agar plates, and then the agar plates were partly covered with lids to dry before proceeding next step. The antibiotic discs were placed and gently pressed, by sterilized forceps, onto the middle of 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 an inhibition zone around the wells. The size of the inhibition zone was measured and the antibacterial activity expressed in millimeters according to the average diameter of the inhibition zone.

3.6. A Bacterial Growth Inhibition Test of Plant Extracts Using the Disk Diffusion Method

Sterile filter paper discs (5 mm) were soaked with 5mL of the diluted extracts (66 mg/mL) of pericarp, leaves, flowers, seeds in ethanol, water, and petroleum ether,

so each disc was impregnated with 0.33 mg / tablet. All extracts were filter-sterilized using a 0.45µm membrane filters (Whatman, Co., UK). Control disks also prepared with absolute ethanol, Water, and petroleum ether. The Disks were placed in Petri dishes containing Mueller Hinton agar and incubated for 16 hours at 37°C, after incubation, all growth inhibition zones were observed, and the diameters were 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 (14). After completing the work the petri dishes were eliminated through the autoclave.

4. Results

4.1. Identification of the Bacteria

Selected Bacteria samples gave us the following results:

Gram staining: Gram-negative, non-motile, rod-shaped bacteria, short chains organizing, compatible with reference (9). Methylene blue staining: bipolar staining, and mucous capsular membranes observed which were compatible with reference (9).

4.2. Colony and Cultural Characters

On standard agar plates of fastidious Gram-negative bacteria like blood agar, regular, smooth, convex, gray, nontransparent, circular, and small colonies with 0.5 – 2 mm diameter were appeared. These results complied with (9).

4.3. The Results of Biochemical Tests

The results were shown in Table 1, These results conformed with Selmi et al. findings (9). So in 104 (20.63%) of 504 samples, *P. haemolytica* were detected.

4.4. Antimicrobial Susceptibility Results Against *Pasteurella haemolytica*

Bacterial colonies were resistant to the studied antibiotics, since all the inhibition zone diameters were lower than the estimated values for each antibiotics, based on the NCCLS2000 criteria (15), and to the standard leaflet of antibiotic discs from the manufacturer.

Table 1. The Biochemical Tests Results for *P. haemolytica*

Test	Result
Oxidase	positive
Catalase	positive
Indol	negative
D-mannitol	positive
D-glucose	positive
Lactose	positive
Urease	negative

4.5. Antibacterial Effect of Plant Extracts

As shown in Table 2, the extracts from different parts of the plant (pericarp, leaves, flowers, seeds) had antibacterial activity against *P. haemolytica*, with the inhibition zone ranging from 10 to 25 mm. The results indicated the presence of a 24 mm inhibition zone of ethanol extract of pericarp (the distinctive zones of inhibition), while the ethanol extract of leaves were 14 mm, 12.3 mm for flowers, and 9.8 mm for seeds extracts. The most active extracts were those obtained from the flowers of *P. granatum* (98.5%). The organic solvent ether, and water extract from all parts of the plants were not active against *P. haemolytica* (without any inhibition zone).

Table 2. Antibacterial Activity of Different Extracts of Studied Plants Against *P. haemolytica*

<i>P. granatum</i>	Inhibition Zone of Plant Extracts mm, Mean ± SD			Sensitive Bacteria, %
	Water Extract	Ether Petroleum Extract	Ethanol Extract	
Control/5 µm	0	0	0	0
Pericarp	0	0	24 ± 1.2	97.6
Leaves	0	0	14 ± 0.9	96.4
Flowers	0	0	12.3 ± 1.1	98.5
Seeds	0	0	9.8 ± 0.87	95.3

5. Discussion

M. haemolytica serotype 1 is the primary causative agent responsible for bovine pneumonic manheimiosis, also known as shipping fever in cattle. The bacterium produces a variety of virulence factors, foremost of which is the exotoxin leukotoxin (16). The outer membrane proteins and capsular material including hyaluronic acid of serotype A have been characterized and are probably related

with virulence by interacting with host immune system (9). The present study showed that the mortality rate in sheeps with *P. haemolytica* infection was 20.63%, nearly 12% in the results of Cecile study (12).

P. haemolytica was resistant to 18 tested antibiotics. The increasing resistance in other studies against this type of bacteria of traditional antibiotics such as Chloramphenicol (12), Penicillin, Cephalotin, Enrofloxacin (17), Tetracycline (18), Oxytetracycline, Doxycycline (19), Erythromycin,

and Penicillin (20), has confirmed our findings. However, several studies demonstrated the Ciprofloxacin effectiveness against this bacteria (21), Amikacin, and Gentamicin (20), of which we have not examined in our study.

That directs us that there is an urgent need to develop alternative antimicrobial drugs against this type of bacteria, so we used *P. granatum* with different extracts. The results showed that *P. granatum* possesses strong antibacterial activities against Gram-negative *P. haemolytica*, and this is reported for the first time. Previous studies have also demonstrated that *P. granatum* L. has the ability to inhibit the activity of *Staphylococcus aureus* (Gram positive), *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Salmonella typhi* (Gram negative) organisms (3, 4). Although this study (4) showed that the methanol and water extracts of the *P. granatum* L. leaves, peel have antibacterial activity, but our study showed an opposite result for water extract. Both aqueous and ether petroleum extracts of different parts of the plant (pericarp, leaves, flowers, seeds) did not have antibacterial effect, while ethanol extracts produced disparate inhibition zone for *P. haemolytica*, that was ranging from 10 to 25 mm.

Although the previous studies pointed out the impacts of flowers, in decreasing the blood glucose (22), and reducing the cholesterol or triglyceride serum level (23), which have analgesic (24), and anti-allergic effect (25), but it did not show the antibacterial effects, while our study proved it in the alcoholic extract which had caused 12.3 mm inhibition zone. In the present study, the ethanol extracts of the seeds had antibacterial activity, which was the lowest effect comparative to other studied parts. This result supports the findings of Al-Zoreky study (26), and Abdollahzadeh (27). While the difference was in the water extract which had antimicrobial effects (28, 29).

It was found that *P. granatum* peels contain tannins, anthocynins, flavonoids, pectins, three estrogen compounds luteolin, quercetin and kaempferol (4). *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 indicate the presence of some metabolic toxins or broad-spectrum antibiotic compounds (3). The tannins makes it useful as an astringent for sore throats, diarrhea and dysentery, but the drugs containing tannins and alkaloids, are anthelmintic and amoeboid (30). *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 infections (30). Peels of *P. granatum* L. also include a 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 be due to phenolic compounds which could increase the concentration of organic acids (31).

The ethanol extracts of the *P. granatum* revealed differ-

ent antibacterial activity against *P. haemolytica*, which affected sheep, and had shown completely antibiotics resistance. While the water and ether extracts had no antibacterial effectiveness, the antibacterial effect may be caused by many compounds.

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Authors' Contribution

50% for each one .

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