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ORIGINAL ARTICLE

Phytochemical composition and antimicrobial, and anti-quorum sensing activities of *Punica granatum* L. methanolic extract

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ABSTRACT

Background and Objectives: In this work, our aims were to investigate the antimicrobial resistance, and anti-quorum sensing activities of *Punica granatum* L. methanolic extract.
Materials and Methods: Antibacterial and antifungal activities were performed against thirteen bacteria and five fungal pathogens. Ultra-high performance liquid chromatography was used to identify the polyphenolic extract. The inhibition of pyocyanin production, proteolytic and elastolytic activity and swarming motility in *Pseudomonas aeruginosa* PAO1 test strain were estimated.
Results: The methanolic extract from *P. granatum* L. was dominated by chlorogenic acid (34.028 mg/g), rutin (26.05 mg/g), epicatechin (12.207 mg/g), gallic acid (11.157 mg/g), and caffeic acid 9.768 mg/g). Results showed antibacterial activities against almost all tested microorganisms with mean diameter of growth inhibition zone ranging from 6 ± 0 to 30 ± 0 mm for *Candida* species and from 6 ± 0 to 22.66 ± 0.57 for bacterial strains. The lowest minimal inhibitory concentrations were recorded for *Listeria monocytogenes* ATCC 19115 and *Salmonella enterica* CECT 529 (0.14 mg/ml, respectively). The anti-quorum sensing activity of methanolic extract against *P. aeruginosa* showed a significant inhibition of swarming motility and an attenuation in virulence factors like pyocyanin production at low concentrations.
Conclusion: The obtained results indicates that *P. granatum* L. extracts is a rich source of phenolic compounds and highlighted the possibilities uses of pomegranate to attenuate the expression of quorum sensing controlled factors in *P. aeruginosa* PAO1 strain.

Keywords: *Punica granatum* L; Antimicrobial activities; *Pseudomonas aeruginosa* PAO1; Swarming; Proteolytic activity; Elastolytic activity

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INTRODUCTION

Newly characterized plant extracts offer extraordinary opportunities for the discovery of new drugs. This has attracted the attention of many researchers to detect new medicinal plants. It has been demonstrated that several plant extracts offer important opportunities for new treatments discoveries (1). Thus, several scientific reports have proved that medicinal plants contain various compounds such as acids, peptides, alkaloids, essential oils, water-soluble compounds and such as phenols (2, 3). In recent years, several studies have shown that drug resistances are developed due to the use of commercial antimicrobial drugs used in the treatment of infectious diseases (4). Plant polyphenols have been considered as a source of natural antioxidants (5). These polyphenols are widely distributed and currently considered to be the most abundant secondary metabolites which are used against heart problems, headaches, colds, sores, and skin infections (6). Also, natural phytocompounds can offer a new source of antibacterial agents (6).

Punica granatum L. (pomegranate) has been reported to possess many properties and important medicinal characteristic (7). It is native in Asian countries, in African, including Algeria and Tunisia. It has been used in traditional medicine due to their capability as natural remedy against many ranges of pathogens. The pomegranate components, like fruit juice, peel, arils, flowers, and bark have been tested for antimicrobial activities. Studies have demonstrated antimicrobial proprieties in *P. granatum* and proved that the phytochemical activities are due to high source of ellagic acid, hydrolysable tannins, and anthocyanins (8). Numerous published articles showed that pomegranate constituents have anti-inflammatory, antimicrobial, antiviral, and antifungal effects. By *in vitro* and *in vivo* evidence, pomegranate proved anticancer activities such as breast, lung, prostate, colon, skin, and hepatocellular cell cancers (9, 10). The pharmacological properties of *P. granatum* indicate their powerful action as anticancer progression, including their interference with cancer cells proliferation, cell cycle arrest, metastasis, angiogenesis, and apoptosis. In the same context, Hora and his coworkers have proved that *P. granatum* components altered the breast, prostate, colon, and lung cancer cells growth in *in vitro* study (11). *Punica granatum* L. seed oil extract stop the proliferation of various tumor cell types in mice (11) and prevent carcinogenesis progression in a mouse mammary organ (12). In addition, anti-tumor role and the phytochemical components effects of pomegranate were demonstrated (13). Several phytochemical analyses indicated that *P. granatum* extracts possess antioxidant and inhibitory activities, including phenolic and flavonoid components (9). Many researchers have found that polyphenols from pomegranate have beneficial health and preventive activities. In this way, *in vitro* study showed that pomegranate peel extract has higher antioxidant activity than pulp extract (14). Other studies indicated that pomegranate seed oil, peel and juice have antioxidant action and inhibit pro-inflammatory enzymes including the cyclooxygenases and lipoxygenases (14-18). An antioxidant activity, polyphenolic content and phenolic specifications of methanol and aqueous extracts from pomegranate peel were revealed (19). These results indicated that pomegranate peel could be used as a possible source of natural antioxidant and anti-virulence agents. The present study aimed to evaluate the antimicrobial activities, and the anti-QS potential of the pomegranate methanolic extract against *Pseudomonas aeruginosa* PAO1.

MATERIALS AND METHODS

Biological material. The antibacterial activity of pomegranate extracts was evaluated against twelve bacterial strains frequently isolated from human infections and food origin. The bacteria tested were as follow: *Staphylococcus aureus* ATCC 43300, *Salmonella enterica* subsp. *enterica* CECT 443, *Listeria monocytogenes* CECT 933, *Vibrio parahaemolyticus* CECT 511, *Shigella flexneri* CECT4804, *Vibrio vulnificus* CECT 529, *Listeria monocytogenes* ATCC 19115, *Vibrio alginolyticus* ATCC 33787, *Staphylococcus aureus* ATCC 6858, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 12224, and *Vibrio alginolyticus* (Strain 2). Additionally, four yeast strains belonging to *Candida* genus (commonly associated with cutaneous, oral, digestive candidiasis and vaginal candidiasis) were tested including: *C. albicans* ATCC 2019, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 06-085 and *C. Krusei* ATCC 6285. The bacteria and fungi strains were stored at -80°C in glycerol broth (10% glycerol) (Biolife, Milano, Italy) until the use.

Plant material sampling. The fresh peels parts of wild pomegranate were collected from Kalaa Sghira, Ennagr (Tunisia). An initial quality evaluation of the plant material was carried out to validate its authenticity and to ensure quality, using techniques adopted from the WHO Guidelines on Herbal Quality Control. The authentication work was done by a botanist from the High Institute of Biotechnology-University of Monastir (Monastir, Tunisia), where the plant material was deposited. The pomegranate fruit was washed with tap water followed by distilled water and the peels were immediately separated from the grains, frozen in liquid nitrogen and stored at -20°C for 3 days. For the experiment, Pomegranate small pieces were air-dried for 15 days at room temperature. Then, 40 grams of peelings were cut into small pieces, minced well in a pestle and a mortar. The air-dried samples were ground to a mesh size of 1 mm with blender (Moulinex Blender Blendforce, France).

Methanolic extract. Ten grams of grounded peels were added to 100 ml of methanol. After 24 h of maceration with continuous stirring at 200 rev/min, the mixture was filtered using Whatman filter paper. This operation is repeated four times in order to increase the yield. At the end of extraction, the obtained fractions were collected in a vial and then were evaporated using a rotary evaporator (RE-52C, China).

Determination of fraction yield. The yield was determined after estimating the weight of the dry extract after evaporation following the equation 1 (Eq.1).

Yield % = (m1 * 100)/m0; Eq. 1

Where, m1 is the quantity of the dry extract in gram and m0 that of the dry plant material.

UP-HPLC analysis. To obtain a profile of polyphenolic composition of methanolic extract, an Ultra-high performance liquid chromatography (UHPLC) analysis was carried out as described by Maja et al. (20).

Antimicrobial activity. Antimicrobial and antifungal activities of the peel's extracts were carried out according to the method described by Snoussi et al. (21). After preparing the bacterial suspensions in physiological water, bacterial and fungal charges were adjusted (OD_{600} = 0.1 for bacteria and OD_{540} = 0.4 for yeast). Petri dishes containing Mueller-Hinton agar media were flooded with suspension using the swabbing method recommended by the French Society of Antibiogram in 2016 (EUCAST-SFM, 2016). The reading was taken after incubation at 37°C for 24 h. The appearance of a halo around each disc reflects the strains growth inhibition.

For the susceptibility test, ampicillin (10 mg/ml) for bacteria and amphotericin B against yeasts (10 mg/ml). The plates are incubated at 37°C for 24 to 48 h. Each diameter of growth inhibition zone was represented by the average of three measurements expressed in millimeter.

Determination of MICs and MBCs values in liquid medium. The determination of MIC and MBC values was performed by the microdilution technique. MBC was determined by sub-culturing the samples having a value of lesser or equal to MIC value on the appropriate media namely Mueller Hinton (MH) for bacteria and Sabouraud Chloramphenicol agar (SC) for *Candida* strains. The highest dilution (lesser concentration) that yielded no single bacterial colony was taken as MBC value. Each extract was diluted in 5% dimethyl sulfoxide (DMSO, Kemika, Zagreb, Croatia) and subjected to serial dilution starting from 150 mg/ml. The 96-well plates (round bottom) were prepared in the same way that each well contains 95 µl of Sabouraud or MH broth, 5 µl of microbial suspension and 100 µl of dilution of the extract (300 mg/ml) to be tested. The negative control well contains 195 µl of enrichment broth without extract and 5 µl of suspension of the microorganism to be tested. After incubation at 37°C for 18 to 24 h, the lowest concentration, at which there was no turbidity, was also regarded as MIC value of the extract. MBC/MFC values are estimated by the concentration of the extract which totally inhibits the growth of bacteria or yeast, and this concentration was subsequently confirmed on MH or SC agar after incubation 24 h at 37°C.

Anti-quorum sensing activity. To evaluate the possibility of attenuation of the virulence factors controlled by the QS system in the PAO1 strain, swarming mobility, elastase and protease production were tested.

Swarming motility assays. This assay was evaluated in modified Lauria Bertani media containing 0.5% (w/v) agar-agar as previously described (23).

Five microliters of an overnight culture of the tested *P. aeruginosa* PAO1 bacteria (OD_{600} = 0.1) were point inoculated into the center of swarming medium plates containing of 1% peptone, 0.5% NaCl, 0.5% agar and 0.5% of filter-sterilized D-glucose with various concentrations of pomegranate peels methanolic extract (0.5, 1, 2.5 and 10 mg/ml). The plate without the extract was maintained as control. The plates were incubated upright in upright position at 37°C for 18 h, and the swarming migration was recorded by following swarm fronts of the bacterial cells expressed in millimeter. All samples and controls were tested in triplicate.

Elastase assay. The estimation of the effects of *P. granatum* extracts on the elastolytic activity of *P. aeruginosa* PAO1 was established based on protocols published by Vasavi et al. (24). Elastin Congo red (ECR; Sigma, St. Louis, USA) assay was used to measure elastase activity. PAO1 was grown in Luria-Bertani broth (LB; Sigma Aldrich, USA) at 37°C and the different concentrations of methanolic extracts (0.5; 1; 2.5; 5 and10 mg/ml). PAO1 culture was centrifuged and 100 µl supernatant was added to 900 µl of Elastin Congo Red buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of elastin Congo red and incubated with shaking at 37°C for 3 h. After centrifugation for 15 min at 13,000 rpm, 200 µl of the supernatant were recovered in 96-well flat-bottom ELISA plates. The optical density was measured at 495nm using a specific ELISA reader (France).

Protease production. The inhibition of proteolytic activity was assessed according to the protocol proposed by Dong et al. (25). For the experiment, 100 µl of culture supernatant were added to 900 µl of Elastin Congo Red buffer containing 3 mg of azocasein (Sigma, St. Louis, USA) and incubated for 30 min at 37°C. Trichloroacetic acid (10%, 100 µl; Sigma, USA) were added to all reaction tubes. After 30 min, the optical density of the mixture was determined at 440 nm in 96-well flat bottom ELISA plates using a plate reader (France). Cell-free broth medium alone and with extract were used as negative controls for both the assays.

Statistical analysis. All the analyses were run in triplicate and averaged. All values are expressed as mean ± standard deviation.

RESULTS

Phytochemical composition. UP-HPLC technique (Fig. 1, Table 1) showed that the tested methanolic extract from pomegranate peels was dominated by several phenolic acids mainly chlorogenic acid (34.028 mg/g), rutin (26.05 mg/g), epicatechin (12.207 mg/g), gallic acid (11.157 mg/g), and caffeic acid 9.768 mg/g).

Antibacterial screening. Antimicrobial activities of the methanolic extract from pomegranate peels was determined by two methods: (i) the disc diffusion assay for the determination of the diameters of bacterial growth inhibition (GIZ) and (ii) microdilution method for the determination of MICs and MBC/MFCs values. In fact, the inhibition of bacteria and yeast growth usually results in the appearance of a halo around each disc soaked by the extract to be tested. Thus, the diameter of the observed halo reflects the effectiveness of the extract and the sensitivity of the strains compared to the activity of ampicillin and amphotericin B. Preliminary antimicrobial screening assay of the pomegranate extracts was shown with methanolic extract and gave relatively wide inhibition zones against the test strains compared with the positive control. The antibacterial activity of the pomegranate extracts was recorded in Table 2. The results of GIZ reveal that the antimicrobial activity of the extract differs from strain to another and were directly related to the concentration of the tested extract. We notice that, for the yeasts and positive Gram bacteria tested, slightly difference in GIZ when the concentration of the pomegranate extract increases from 50 to 300 mg/ml. But at the same condition, GIZ increases after applying the extract against negative Gram bacteria. In fact, disc diffusion technique showed higher activity of the extract against the yeasts (inhibition diameters ranged from 6 to 30 mm) as compared to Gram-negative bacteria (inhibition diameters ranged from 6 to 22.66 mm) and Gram-positive bacteria (inhibition diameters of 6-10 mm). However, the methanolic extract of pomegranate showed an extreme inhibitory effect against *C. parapsilosis* ATCC 22019 (29.66 ± 0.57 mm) and *C. krusei* ATCC 6285 (30 ± 0 mm) tested at 300 mg/ml. Using the MBC/MIC and MFC/MIC ratios (Table 3), our results showed that the tested extract exhibited bacteriostatic and fungistatic activity against all bacteria, and fungal strains tested respectively.

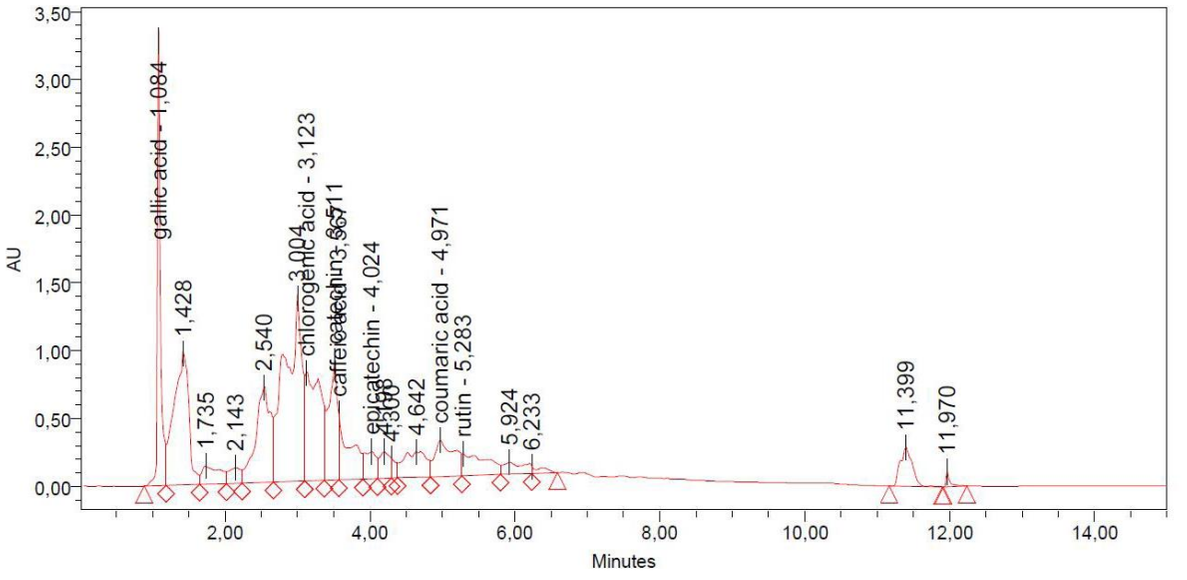

Fig. 1. Ultra-high performance liquid chromatography (UP-HPLC) of the methanolic extract of pomegranate (peels).

Table 1. Phytochemical composition of *P. granatum* methanolic extract by using UP-HPLC technique.

Phytocompound	Punica granatum methanolic extract (mg/g)
Gallic acid	11.157
Chlorogenic acid	34.028
Catechin	4.985
Epicatechin	12.207
Caffeic acid	9.768
Coumaric acid	2.213
Rutin	26.05

Determination of MIC and MBC values. The results obtained highlighted that the MIC and MBC values obtained with the methanolic pomegranate extract (Table 3). In fact, the results obtained showed that the lowest MIC value obtained for the four tested *Candida* species was recorded for *C. parapsilosis* and *C. krusei*, respectively (MIC= 4.68 mg/ml). While, for bacterial strains, the lowest MICs values were obtained against *S. enterica* CECT 443 and *L. monocytogenes* ATCC 19115 (MIC = 4.68 mg/ml, respectively). Low concentrations of pomegranate extract were needed to kill almost all tested bacteria (MBCs values ranged from 0.58 to 37.5 mg/ml) as compared to *Candida* strains (MFCs values ranged from 37.5 to >150 mg/ml).

Anti-quorum sensing activity: swarming motility assay. Fig. 2 summarizes the effect of different concentrations from pomegranate methanolic extract on the motility activity of *P. aeruginosa* PAO1. Results demonstrated an increasing efficiency in reducing the diffusion diameter of *P. aeruginosa* PAO1 colonies on semi-solid agar in a concentration dependent manner. In fact, at 10 mg/ml of *P. granatum* extract, the motility of *P. aeruginosa* PAO1 strain was completely inhibited as compared to the control strain without the extract. At 5 mg/ml, we noticed the complete inhibition of the production of pyocyanin by *P. aeruginosa* PAO1 strain (Fig. 3).

Elastase assay. Fig. 4. showed that the methanolic fraction of pomegranate was able to significantly increase elastase production in the PAO1 strain at high concentrations of methanolic extract.

Protease production. Fig. 5. showed that the extract tested was able to considerably increase of the production of protease by the PAO1 strain depending on the concentration of the extract used.

DISCUSSION

Study of antimicrobial activity. Currently, infectious diseases are a major cause of morbidity and mortality. The control of microorganisms, particularly multi-resistant pathogens, has become increasingly important in the development of effective treatment for microbial diseases. Therefore, plants appear

Table 2. Evaluation of antimicrobial activity of pomegranate methanolic against a large collection of *Candida* and bacterial strains by the determination of the inhibition zone (expressed in mm ± Standard deviation).

***Candida* species tested**	**Pomegranate methanolic extract**			**Ampicillin**
	50 mg/ml	**150 mg/ml**	**300 mg/ml**	**(10 mg/ml)**
Candida albicans ATCC 2019	6 ± 0	6 ± 0	6 ± 0	6 ± 0
Candida parapsilosis ATCC 22019	28.66 ± 1.15	29.33 ± 0.57	29.66 ± 0.57	11.33 ± 1.15
Candida tropicalis ATCC 06-085	6 ± 0	6 ± 0	6 ± 0	15 ± 0
Candida Krusei ATCC 6285	30 ± 0	30 ± 0	30 ± 0	16 ± 0
Gram+ and Gram- strains tested	Pomegranate methanolic extract			Amphotericin B
	50 mg/ml	150 mg/ml	300 mg/ml	(10 mg/ml)
Staphylococcus aureus ATCC 6858	18 ± 0	18.33 ± 0.57	18 ± 0	50.66 ± 1.15
Staphylococcus aureus ATCC 43300	15.33 ± 0.57	17.33 ± 1.15	18.66 ± 1.15	37.33 ± 1.15
Listeria monocytogenes CEC T933	6 ± 0	6 ± 0	6 ± 0	18.33 ± 0.57
Listeria monocytogenes ATCC 19115	10 ± 0.57	10 ± 0.57	10 ± 0	21 ± 1.15
Pseudomonas aeruginosa ATCC 27853	20 ± 0	23.67 ± 0.57	26 ± 0	6 ± 0
Escherichia coli ATCC 12224	22.66 ± 0.57	24.33 ± 0.57	27.33 ± 0.57	30 ± 0
Vibrio alginolyticus ATCC 33787	20 ± 0	22.66 ± 1.15	24 ± 0	28 ± 0
Vibrio alginolyticus (Strain 2)	6 ± 0	6 ± 0	12 ± 0	20 ± 0
Vibrio parahaemolyticus CECT 511	14.33 ± 0.57	16.66 ± 0.57	20.67 ± 0.57	19 ± 0
Vibrio vulnificus CECT 529	16.67 ± 1.15	22 ± 0	22 ± 0	7 ± 0
Salmonella enterica CECT 443	6 ± 0	6 ± 0	20 ± 0	37 ± 0
Shigella flexneri CECT4804	6 ± 0	6 ± 0	6 ± 0	90 ± 0

Table 3. Determination of MICs and MBCs/MFCs values by using the microdilution assay.

***Candida* species tested**	**Methanolic extract**		**Interpretation**
	MIC (mg/ml)	**MFC (mg/ml)**	**MFC/MIC ratio**
Candida albicans ATCC 2019	9.37	>150	>4; Fungistatic
Candida parapsilosis ATCC 22019	4.68	37.5	>4; Fungistatic
Candida tropicalis ATCC 06-085	18.75	>150	>4; Fungistatic
Candida Krusei ATCC 6285	4.68	>75	>4; Fungistatic
Gram+ and Gram- bacteria tested	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC ratio
Staphylococcus aureus ATCC 6858	0.58	2.34	>4; Bacteriostatic
Staphylococcus aureus ATCC 43300	1.17	2.34	>4; Bacteriostatic
Listeria monocytogenes CEC T933	4.68	37.5	>4; Bacteriostatic
Listeria monocytogenes ATCC 19115	0.14	0.58	≤4; Bactericidal
Pseudomonas aeruginosa ATCC 27853	9.37	18.75	≤4; Bactericidal
Escherichia coli ATCC 12224	2.34	9.37	≤4; Bactericidal
Vibrio alginolyticus ATCC 33787	4.68	18.75	>4; Bacteriostatic
Vibrio alginolyticus (Strain 2)	2.34	18.75	>4; Bacteriostatic
Vibrio parahaemolyticus CECT 511	4.68	37.5	>4; Bacteriostatic
Vibrio vulnificus CECT 529	9.37	37.5	≤4; Bactericidal
Salmonella enterica CECT 443	0.14	0.58	≤4; Bactericidal
Shigella flexneri CECT4804	9.37	37.5	≤4; Bactericidal

MIC: minimal inhibitory concentration (mg/ml). MBC: minimum bactericidal concentration (mg/ml), MFC: Minimal Fungicidal Concentration.

Concentration of *P. granatum* methanolic extract in (mg/ml)	C	0.5	1	2.5	5	10
IZ± SD (mm)	9 ± 0.57	8 ± 1.08	5.7 ± 1.15	3 ± 0	3 ± 0	0

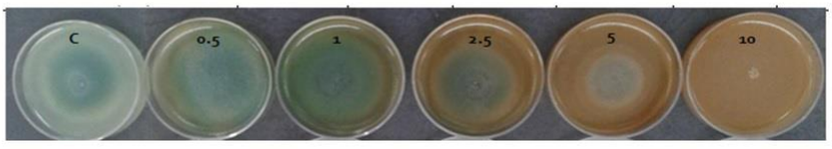

Fig. 2. Effect of the extract tested on the spread of *P. aeruginosa* PAO1 strain in semi-solid agar.

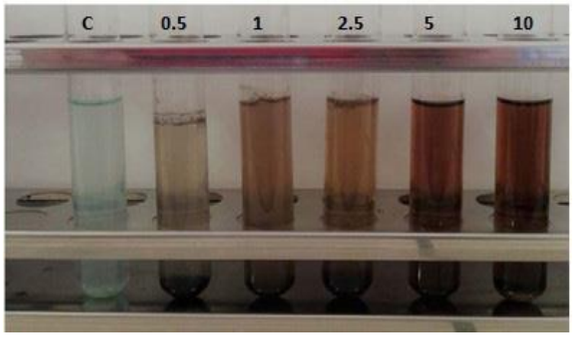

Fig. 3. Inhibition of pyocyanin production by *P. aeruginosa* PAO1 at different concentrations of *P. granatum* methanolic extract.

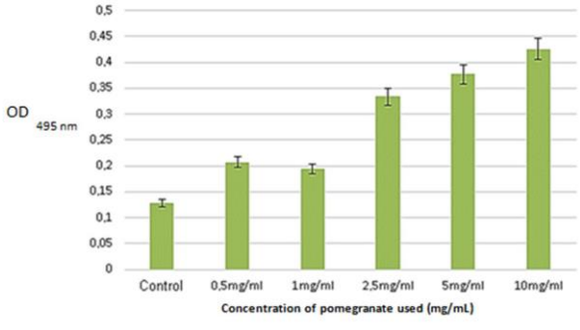

Fig. 4. Evaluation of the effect of different *P. granatum* methanolic extract on the elastase activity of *P. aeruginosa* PAO1.

to be a potential source of antimicrobial agents given their richness in alkaloids, anthraquinones, saponins, tannins and polyphenols (26). From this context, our study aims to evaluate the effectiveness of pomegranate extract as therapeutic agents against strains harmful to health. To get this goal, we worked on 16 references bacterial strains that are generally associated with human infections, four of them are *Candida* yeasts that are commonly associated with skin, oral, digestive and vaginal candidiasis.

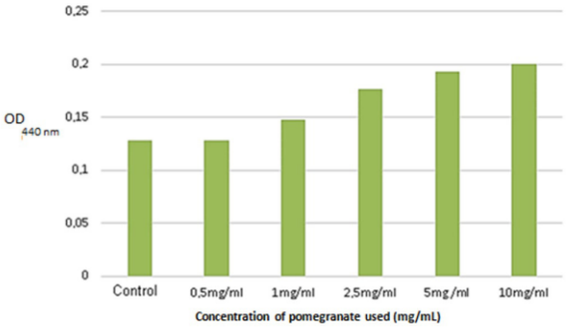

Fig. 5. Evaluation of the effect of different concentrations from *P. granatum* methanolic extract on the protease activity of *P. aeruginosa* PAO1.

Based on the results obtained by the methanolic extract of pomegranate, the diameters of the GIZ, showed an antimicrobial activity that was sometimes more effective than the antibiotic. The inhibition zones are 26 mm for PAO1 and 27 mm for *E. coli*. However, it was moderately effective in the genus *S. enterica*, *S. aureus* ATCC 6852 and 43300 with an average GIZ of 18-22 mm. On the same approach, our methanolic pomegranate extract showed a greater inhibitory effect against Gram negative bacteria (with average GIZ = 26 ± 1.57 mm) compared to Gram positive bacteria (with average GIZ = 17.06 ± 0.57 mm). Our results were in agreement with those reported by Joshi et al. (27) who proved that the hydroalcoholic extract of pomegranate peel is very effective against two Gram-negative bacteria: PAO1 and *Chromobacterium violaceum*. In addition, methanolic pomegranate extract (300 mg/ml) was very active against two yeasts: *C. parapsilosis* and *C. krusei* with GIZs of 29.66 mm and 30 mm, respectively. Da silva et al. (28) reported that pomegranate contains a chitin binding lectin which is responsible for the antifungal activity against *C. albicans* and *C. krusei*. These potent antibacterial properties of the methanolic pomegranate extract could be attributed

to its richness in secondary phytochemical metabolite and it was active with different degrees on all the bacteria and yeasts tested.

According to the MBC/MIC ratio, it was found that the obtained ratio is ≤ 2 of PAO1 and *S. aureus* ATCC 43300 (MRSA). A bactericidal effect is noted, and the value of the MBC is very close to MIC. As for the other strains, this ratio was greater than 2 therefore; it is not a bactericidal effect. Our results are in agreement with the work published by Al-Zoreky (9), who showed the presence of antimicrobial activity using pomegranate extract. Thus, he noted that the methanolic extract of pomegranate was a potent inhibitor of *L. monocytogenes, S. aureus* ATCC 6538, *E. coli, Yersinia enterocolitica, Bacillus subtilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. In the same way, Elmanama et al. (29) showed that the pomegranate extracts possessed bacteriostatic and bactericidal action against *Micrococcus luteus, Serratia marcescens* and *Streptococcus mutans* with MICs ranging from 1.25 to 50 g/ml and MBCs ranging from 18 to 100 g/ml. In addition, the same extract inhibited the growth of *Aeromonas* sp., *Enterococcus faecalis, E. coli, S. enterica serovar, S. aureus, S. epidermidis, S. saprophyticus, P. aeruginosa* and *C. albicans* with MICs ranging from 3 to 50 g/ml.

In fact, Kalemba and Kunicka (30) claimed that the variability of the sensitivity of a microorganism depend on the properties of the microorganism, on the extract and its content of anthocyanins, tannins, and flavonoids. Similarly, Sindi et al. (31) show that endogenous factors are linked to the anatomical and physiological characteristics of each plant and to the biosynthetic pathways of volatile substances, which could be modified according to the different plant tissues or according to the seasons but could also be influenced by DNA adaptation. As for exogenous factors, these can affect certain genes responsible for the formation of these secondary metabolites. Similarly, the increase in GIZ values was very clear with the highly extract concentration. This allows us to say that the extract acts at concentration-dependent doses. The antimicrobial activity is proportional to the extract concentration. However, this obviously remains to be verified because there are many extracts which can be toxic in high doses or even in high concentrations whose activity does not change.

Anti-quorum sensing activity. PAO1 has been widely described in nosocomial infections. It is characterized by its resistance and its pathogenicity linked to a set of virulence factors in addition to its biofilm production. This gives it greater resistance to antibiotics through a regulatory system called QS, allowing it to regulate its gene expression as well as communicate with other bacteria. For more detailed, the activation of this mechanism is only possible when the AHL level reaches a critical threshold. At this point, acyl homoserine lactone binds to specific regulatory proteins. The complex thus formed activates the transcription of several virulence factors such as elastase, protease, pyocyanin, besides the swarming mechanism. In this regard, we have been interested in this work to prove anti-QS activities in the PAO1 model and to evaluate the ability of our extracts to inhibit these virulence factors described above. The evaluation of anti-swarming activity on semi-solid medium showed that the pomegranate extracts influenced significantly PAO1 mobility and pyocyanin production. This is reflected by the inhibition of the extension area and from the disappearance in the green coloration of the pyocyanin pigment to a specific concentration. Indeed, the inhibition of this area shows, on the one hand, a destructive effect of the bacteria mobility, and on the other hand, this concentration could affect the associative life between bacteria and become more sensitive. Our results are consistent with the work published by Elmanama et al. (29) which has shown that *punica* exhibit anti-swarming activity and is able to reduce pyocyanin production. In the same context, Zhou et al. (34) proved that 1-4-amino-2-hydroxyphenylethanone which is extracted from pomegranate leaf has a strong anti-QS effect. This extract was able to inhibit the secretion of acyl-homoserine lactones and virulence factors in PAO1. In the same context, other study proves that pomegranate contains punicalagin, a type of phenolic compound able to inhibit the biofilm formation of *Salmonella* (35). The pomegranate peel extract has also the role of the secondary messenger suppressor gene, *ydiV* that may affect the virulence activity of *Salmonella* according to Mahadwar et al. (36). In addition, the study of Yang et al. (37) shows that pomegranate rind has an anti-QS activity against *Chromobacterium violaceum* and an anti-biofilm formation against *E. coli*. With regard to anti-enzymatic activity, it appears that our extracts have failed to inhibit the enzymes production. Indeed, according to the results obtained, pomegranate extract was able to significantly increase the pro-

duction of elastin and protease by PAO1. However, Elmanama et al. (29) showed that pomegranate was able to reduce the production of these two factors with a percentage of 58.3%. This difference in results may be due to changes in the genomic and in plants protein expression or even of the tested strain. These results are coherent with the study of Khemakhem et al. (38). They confirmed that in pomegranate, the physicochemical characterization of three Tunisian varieties (Jebali, Testouri and Gabsi) showed enormous differences in component levels, especially the phenolic compounds. For example, polyphenol content of Gabsi ecotype was roughly 3 and 23 times higher than that of Jebali and Testouri ecotypes, respectively.

CONCLUSION

In conclusion, our results showed the richness of the tested methanolic extract from *P. granatum* in phenolic compounds, and mainly chlorogenic acid and rutin. In addition, a promising antibacterial and antifungal activities were recorded against pathogenic Gram-positive, Gram- negative, and *Candida* species. This extract was able to interfere with the quorum sensing system in *P. aeruginosa* PAO1 strain by inhibiting its motility and the production of pyocyanin. While the production of elastase and protease enzymes in PAO1 were enhanced at high concentration of pomegranate methanolic extract. Further experiments are needed to elucidate the mechanism of action of the identified compounds in pomegranate extract against the quorum sensing system in *P. aeruginosa* strain.

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