Medicinal & Aromatic Plants
ISSN: 2167-0412

Reguieg and Hammadi, Med Aromat Plants (Los Angel) 2017, 6:1
DOI: 10.4172/2167-0412.1000282

Research Article Open Access

Enhancement of the Bark of *Punica granatum* Fruit through the Phytochemical and Antimicrobial Activity Studies

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Abstract

In our days, the use of medicinal plants in phytotherapy has received a great interest in biomedical research and becomes as important as chemotherapy. This research focuses on the phytochemical study and determination of polyphenolic compounds of various phenolic extracts from the bark of the pomegranate on one hand, and evaluation of antibacterial activity of the other. Phytochemical analysis of the extracts showed the presence of groups of biologically active compounds, including flavonoids, phenols and polyphenols, and tannins with total polyphenol values ranging from 270.5 µg EAG/mg to 284.61 µg EAG/mg, That the flavonoid content varies between 16.83 µg EQ/mg and 38.47 µg EQ/mg. Very interesting bactericidal properties of the phenolic extracts of the pomegranate bark have been found on the bacteria. At the end of the antimicrobial tests, we found that this extract has a very high activity with diameters of the zones of inhibition varying between 12 and 34 mm. MICs of the extract varied between 0.01 and 0.31 mg/ml. This work showed antibacterial activity against five bacteria can contribute to the fight against infectious diseases.

Keywords: *Punica granatum*; Phytochemical analysis; Antibacterial activity; Phenolic extract

Introduction

The plant kingdom is an inexhaustible source of new molecules that can be used directly as an active ingredient or as a guide molecule for the development of new antioxidants. The search for new drugs of natural origin is an important research focus at the global level.

The pomegranate is a native shrub from western Asia and Mediterranean Europe that has a rich history of traditional use of medicine [1]. Recently become of great interest to scientists who engage themselves in pharmaceutical research, nutrition, and the development of new drug because of these multiple distinctive officinale parts and multiple bioactivity such as, diabetes, antiviral, antibacterial and antioxidant [2]. These plants are endowed with a very wide range of biological activities thanks to their active secondary metabolites such as phenolic compounds which have the advantage of being of a great diversity of chemical structure [3]. Our work is part of a contribution to a valorization of this shrub and to discover certain constituents of this plant that possess biological activities. To this end, our study encompasses two aspects, the first of which is phytochemical based mainly on the extraction, screening and quantification of phenolic compounds. The second aspect devoted to *in vitro* evaluation the antimicrobial activity of these phenolic extracts.

Materials and Methods

Plant material

The fruits of pomegranate (*Punica granatum*) are harvested at maturity in the month of October (2015), in the region of Mascara. The epicarp of these fruits is isolated, dried in the open air and protected from light, and then ground by means of a mortar until a fine powder is obtained for the preparation of Various extracts.

Preparation of phenolic extracts

Ten grams of the plant powder of *Punica granatum* are macerated for 24 hours at room temperature, in a solvent-water mixture (70:30 V/V) or in 100 ml of distilled water, and then filtered on Whatman paper, The extraction is repeated several times with renewal of the solvent. The solvent is removed from the filtrate by rotary evaporation in a rotavapor (BÜCHI). The extraction series makes it possible to obtain three extracts; Aqueous, ethanolic and methanolic extract. The extracts are dried and stored until later use.

Phytochemical screening

The method for detecting the various families of compounds consists of precipitation or staining by specific reagents. Indeed, these reactions result in the appearance of turbidity, flocculation or color change which informs us about the nature of the families existing in the plants.

Phytochemical determination

Determination of total phenolic compounds: The total polyphenols were assayed using the Folin-Ciocalteu colorimetric reagent [4]. The concentration of the total polyphenols is calculated from the regression equation of the calibration range established with the standard gallic acid and expressed in micrograms of gallic acid equivalents per milligram of extract.

Determination of flavonoids: The method of aluminum trichloride is used to quantify flavonoids in phenolic extracts [5]. The concentration of flavonoids in the extracts is calculated from the calibration range established with quercetin and expressed in micrograms of equivalents of Quercetin per milligram of extract.

Determination of flavones and flavonols: The method used for the estimation of flavonol levels is that described by Kostova [6]. The concentration of flavonoids in the extracts is calculated from

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Received January 13, 2017; **Accepted** January 23, 2017; **Published** January 28, 2017

Citation: Reguieg YA, Hammadi K (2017) Enhancement of the Bark of *Punica granatum* Fruit through the Phytochemical and Antimicrobial Activity Studies. Med Aromat Plants (Los Angel) 6: 282. doi: 10.4172/2167-0412.1000282

the calibration range established with quercetin and expressed in micrograms of equivalents of quercetin per Milligram of extract.

Determination of condensed tannins compounds: The amounts of condensed tannins are estimated using the acidic vanillin method [7]. The concentration of tannins is estimated in milligrams of catechin equivalents per milligram of extract.

Sensitivity of strains to antibiotics

The aim of carrying out an antibiogram is to predict the sensitivity of a germ to one or more antibiotics in an essentially therapeutic perspective.

Antibacterial activity by diffusion method in solid medium

The antibacterial activity of the extracts was determined by the diffusion method in agar medium standardized by (NCLLS) [8]. NCCLS: (National committe for clinical laboratory standards)

Preparation of concentrations: The extracts were taken up with sterile distilled water. Serial dilutions of 1/2 to 1/8 were then carried out to obtain concentrations of 100 to 12.5 mg/ml.

Insemination: Within 15 minutes after adjusting the turbidity of the inoculum suspension (0.5 Mc Farland), a swab was dipped into the suspension and the entire surface of the agar (Mueller Hinton Agar) was plated three times. The sterile discs impregnated with the increasing concentrations of extracts at a rate of 10 µl per disc were deposited sterile using a forceps on the surface of the agar. The dishes were incubated for 24 h at 37°C. in a normal atmosphere.

The antibacterial activity was determined by measuring with a rule the diameter of the inhibition zone, determined by the different concentrations of the various extracts around the disc.

Determination of the MIC (Minimum Inhibitory Concentration)

MICs are determined by the standardized method of micro-dilution in liquid medium. The study is made of a plastic microplate with 96 then (08 rows and 12 then numbered from 01 to 12) in Muller Hinton broth. With a final density inoculum located above, the microplate are incubated from 18 h to 24 h. The dilutions of the sample were cascaded in the wells of the highest concentration (10 mg/ml) to the lowest (0.004 mg/ml), the dilutions of the extracts were carried out in DMSO. The MIC corresponds to the first dilution where growth is negative (no visible culture) [9], kinetics are evaluated by measuring the absorbance of the microplate at 620 nm at t_0 and after 2 h, 4 h, 8 h, 16 h and 24 h.

Statistical study

All tests were performed in duplicate or triplicate. Results are presented as mean ± standard deviation of two or three independent determinations. All statistical analyses were carried out by Graphpad prism 5 using analysis of variance (ANOVA) and differences among the means were determined for significance at $p \leq 0.05$ using least significant.

Results and Discussion

Extraction yield

The extraction method must allow the complete extraction of the compounds of interest and must avoid their chemical modification. Water, aqueous mixtures of ethanol are generally used for extraction [10].

Compounds	Results
Flavonoides	+++
Tanins	+++ (galic)
Saponosides	++
Anthocyanosides	++
Anthracénosides	+++
Coumarines	-
Reducing compounds	+++
Heterosides	-
Alcaloides	-
Starch	-

(+++) Strongly present; (++) Moderately present; (+) Weakly present; (-) Negative test

Table 1: Results of phytochemical tests carried out on phenolic extracts of *Punica grantlum*.

The solubility of the phenolic compounds depends on their degree of polymerization, the interaction with the other constituents and the type of solvent used. Methanol has been recommended and frequently used for the extraction of phenolic compounds [11]. 70% aqueous methanol is twice as effective as pure methanol for the extraction of phenolic compounds [12]. The extraction efficiency reading shows that the yield obtained by the methanol extract is important with a value of 28.5%.

Phytochemical analysis

The phytochemical analysis carried out on various extracts of the epicarp of the fruit of *Punica granatum* gave the results mentioned in Table 1. The method of detection of the various families of compounds consists of precipitation or staining with specific reagents. Indeed, these reactions result in the appearance of turbidity, flocculation or color change which informs us about the nature of the families existing in the plants.

According to the Table 1, phytochemical tests reveal that the fruit epicarp of *Punica granatum* is very rich in tannins, flavonoids, anthracenosides and reducing compounds, whereas anthocyanosides and saponosides are moderately present. Alkaloids, Steroids, coumarins and starch were negative in this analysis.

Determination of total polyphenols, flavonoids, flavones and condensed tannins

Phenolic compounds such as phenolic acids, flavonoids and tannins are considered major contributors to the antioxidant capacity of plants [13]. These compounds also possess various biological activities such as anti-inflammatory, antibacterial, antiviral, antiallergic, antithrombotic and vasodilating activities which can be related to their anti-oxidative activity [14]. For this reason, the analyzes of total polyphenols, flavonoids and tannins of *Punica granatum* were carried out in this study.

The polyphenol content was estimated using the Folin-Ciocalteu colorimetric method. This is one of the oldest methods designed to determine the content of polyphenols, medicinal plants and foods. Gallic acid is the standard (Figure 2) most often used in the Folin-Ciocalteu method [15].

The content of the total polyphenols of different extracts is determined by reference to the calibration curve established with the standard gallic acid standard (5-200 µg/ml) and expressed in micrograms of gallic acid equivalents per milligram of extract (µg GAE/mg) by the standard equation: y=0.01x+0.05 (Figure 1).

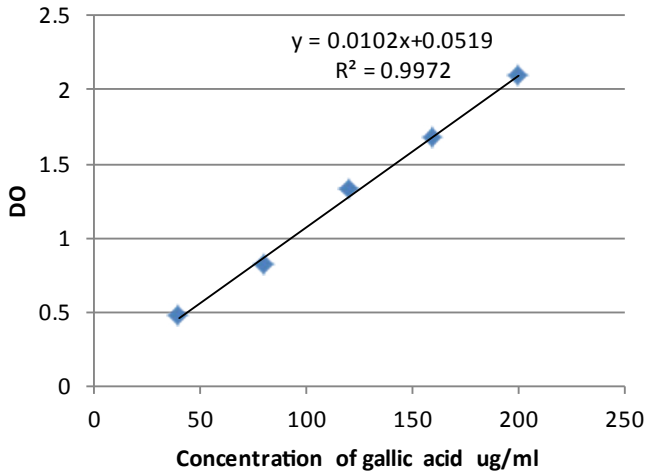


Figure 1: Calibration line for gallic acid for the determination of total polyphenols.

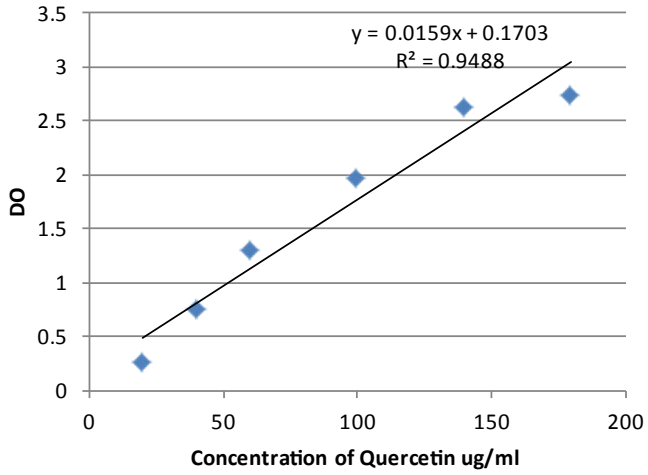


Figure 2: Calibration line for quercitin for the determination of flavonoids.

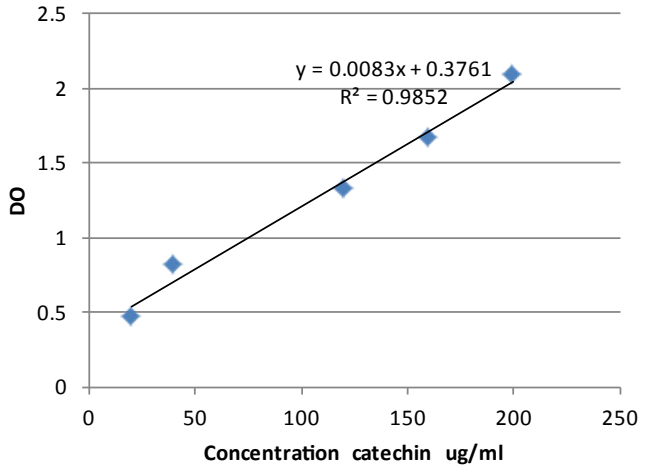


Figure 3: Catechin calibration line for the determination of condensed tannins.

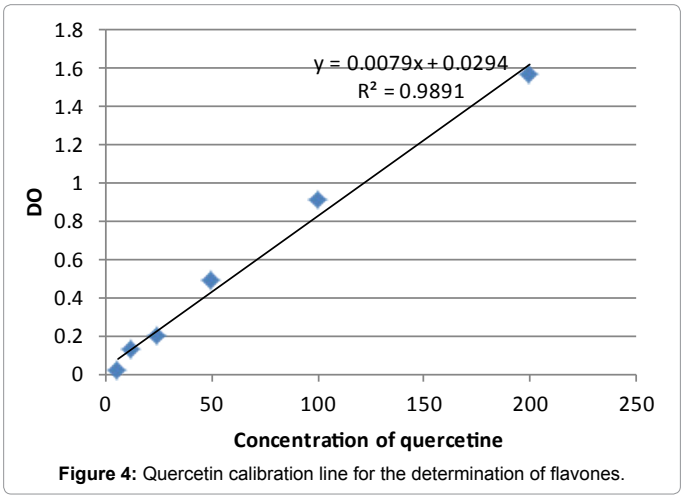


Figure 4: Quercetin calibration line for the determination of flavones.

The concentration of flavonoids in the extracts is calculated from the calibration range established with quercetin (2-14 µg/ml) and expressed in micrograms of equivalent of quercetin per milligram of extract (µg EQ/mg) by the equation: y=0.01x+0.17 (Figure 2).

The concentration of taninns in the extracts is calculated from the calibration range established with catechin and expressed in micrograms of catechin equivalent per milligram of extract (µg QE/mg) by the equation: y=0.008x+0.137 (Figure 3).

The concentration of flavones in the extracts is calculated from the calibration range established with quercetin (2-14 µg/ml) and expressed in micrograms of equivalents of quercetin per milligram of extract (µg EQ/mg) by the equation: y=0.007x+0.02 (Figure 4).

In the light of these calibration curves, the total phenolic content, flavonoids, flavones and condensed tannins of the *Punica granatum* epicarp are represented in the Figure 5.

According to the results illustrated above, it can be seen that the polyphenol content depends on the solvent polarity. Our results show that the extracts are rich in polyphenols with total polyphenol values ranging from 194.96 µg EAG/mg to 336.14 µg EAG/mg, while the flavonoid content ranges from 9.54 µg EQ/mg to 27 µg EQ/mg while The values of falavones and condensed tannins ranging from 19.96 µg EQ/mg to 39.01 µg EG/mg and from 81.86 µg EC/mg to 153 µg EC/mg respectively.

Generally, all plants of the family *Punicaceae* are known for their phenolic compounds [16]. This is in accordance with our results. The extracts are mixtures of several compounds, with different functional groups, polarities and chemical behaviors. This chemical complexity of the extracts could lead to scattered results according to the test used [17].

Generally and according to the scientific literature, the polyphenolic content varies qualitatively and quantitatively according to several factors: climatic and environmental factors: the geographical area, drought, soil, aggression and disease etc. [18]. The genetic make-up, the period of harvest and the stage of development of the plant [19], the type of polyphenol, the health of the fruit, the method of harvesting and storage [20] and the extraction method and the method can also influence the estimation of total polyphenol content [21]. It has been shown that total polyphenol contents are high when the plant environment is inadequate, in which case the plant promotes

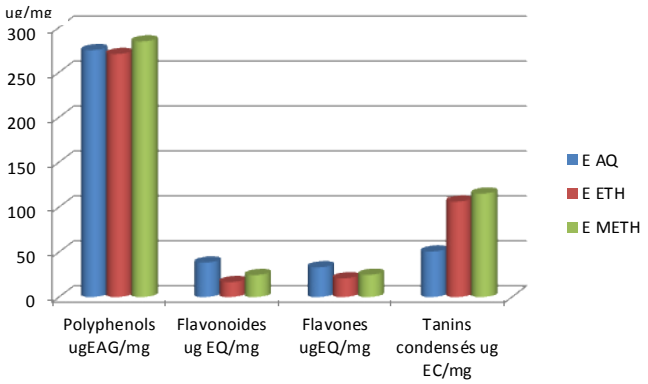


Figure 5: Content of total polyphenols, flavonoids, flavones and condensed tannins of extracts of *Punica granatum* (µg/mg extract).

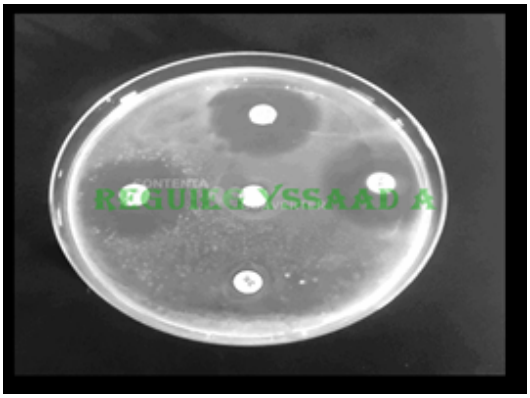


Figure 6: Antibiogram of *Pseudomonas aeruginosa*.

Figure 7: Antibiogram of *Escherichia coli*.

the synthesis of secondary metabolites in order to adapt and survive [22,23].

Sensitivity of strains to different antibiotics

The interpretation of the antibiogram (sensitive, intermediate and resistant) was done in accordance with the recommendations of French society [24].

Antimicrobial activity of phenolic extracts

The antimicrobial activity of the phenolic extracts of *Punica granatum* is evaluated on 5 bacterial strains of reference. This activity is evaluated by the aromatogram method, the antimicrobial power of the phenolic extracts is obtained by measuring the diameter of the inhibition zone in mm. The diameter of the inhibition zone differs from one bacterium to another and from one extract to another. It appears that all microbial strains tested are inhibited at least by one of the extracts, confirming the broad spectrum of the antimicrobial activity of this fruit. As has been reported in the literature, we considered that an extract has a bacteriostatic action if its inhibition diameter is greater than 12 mm [25]. According to our results, the extracts have a very diversified and variable activity, they attack the strains tested with a different intensity according to the concentration, the type of extract and the microbial strains it is for these reasons that they are presented in the form of Table.

The evaluation of the antimicrobial activity showed great heterogeneity in the results. Methanolic extract of *Punica granatum* showed the best effect in this method with the largest zone of inhibition recorded against *staphylococcus aureus* of 34 ± 0.01 mm, it is more than the diameter given by the action of the antibiotics.

This is proved by the study of Negi et al., On an ecotype of *Punica granatum* or the methanol extract had an interesting inhibitory activity [26]. The antibacterial effect of the phenolic extracts of *Punica granatum* was noted with respect to the five strains tested with inhibition diameters of 11 ± 0.03 to 34 ± 0.01 mm. This effect remains more inhibitory than that found by antibiotics.

These results corroborate those of Reddy et al. Who demonstrated that pomegranate extracts have significant antimicrobial activity against *E. coli* (Figure 7), *Pseudomonas aeruginosa* (Figure 6) and *S. aureus* [27] those of Al-Zoreky which showed that extracts of Pomegranate bark constitute a potent growth inhibitor of *S. aureus* and *E. coli* [28] and those of Choi et al., who studied the *in vivo* and *in vitro* effect of the application of various d Extracts of pomegranate bark to inhibit the growth of Salmonella [29].

This antimicrobial activity of this extract is due, at least partially, to the presence of the polyphenols. This is confirmed by other research that has attributed antimicrobial activity to the presence of polyphenols. According to the results of the antibacterial tests, it is found that the bacterial strains exhibit a high sensitivity. And the latter is linked to the composition of the bacteria's membrane (Gram-positive and Gram-negative) and the major component of the extract. By way of comparison between the antibacterial effect of the antibiotics and the effect of the extracts of *Punica granatum*. It is observed that the diameters of the zones of inhibition in the case of the extracts are greater than for the antibiotics. The results of the evaluation of the bactericidal effects of plant extracts are given in the Tables 2-8 or are included on the one hand the minimum bactericidal concentrations (mg/ml) of all the extracts and on the other hand the values of the ratio between The MIC and the BMC.

According to the Table 9 the MIC/MBC ratio does not exceed 8 for all the tests, we can conclude that the bacteria tested do not exhibit a tolerance to phenolic extracts *Punica granatum*. On all strains, a concentration ranging from 0.07 to 2.5 mg/ml is capable of killing more than 90% of the initial bacterial population. For methanolic extract, MBCs range from 0.07 mg/ml to Gram- (Enterobacteriaceae) bacteria and 1.25 mg/ml against Gram+ bacteria.

This increased sensitivity is confirmed by the MBC values which are closer to those of the MICs for all extracts. According to Canillac and Mourey, when the MIC/MBC ratio is less than or equal to 4, the antibacterial agent is considered to be bactericidal [30]. We find that the

The bacterial strains	Diameter of the inhibition zone* (mm)				
	Gentamycin	Tétracyclin	Colistin	Aztreonam	Pénicillin G
Klebseila pneumoniae	12 ± 0.06	14 ± 0.03	19 ± 0.04	20 ± 0.08	16 ± 0.06
Pseudomonas aeruginosa	10 ± 0.02	9 ± 0.03	20 ± 0.06	20 ± 0.02	21 ± 0.08
Staphylococcus aureus	12 ± 0.03	21 ± 0.06	11 ± 0.01	21 ± 0.03	15 ± 0.02
Streptococcus intermedius	14 ± 0.02	18 ± 0.04	12 ± 0.06	25 ± 0.08	19 ± 0.07
Escherichia coli	13 ± 0.06	14 ± 0.08	17 ± 0.03	21 ± 0.01	22 ± 0.02

Table 2: Diameter of the antibiotic inhibition zone.

Bacterial strains	Diameter of the inhibition zone* (mm) concentrations of the Aqueous extract (mg/ml)			
	12.5	25	50	100
Klebseila pneumoniae	11 ± 0.03	14 ± 0.001	19 ± 0.07	21 ± 0.02
Pseudomonas aeruginosa	13 ± 0.06	15 ± 0.02	17 ± 0.01	19 ± 0.06
Staphylococcus aureus	17 ± 0.001	21 ± 0.03	26 ± 0.06	29 ± 0.01
Streptococcus intermedius	14 ± 0.02	18 ± 0.07	25 ± 0.001	29 ± 0.03
Escherichia coli	13 ± 0.01	15 ± 0.03	18 ± 0.02	21 ± 0.07

(*) Diameter of the inhibition zone produced around the discs by the addition of 15 µL of extract (Diameter of the disc is included) the values represent the average of 3 measurements ± SD.

Table 3: Diameter of the inhibition zone of the Aqueous extract.

Bacterial strains	Diameter of the inhibition zone* (mm) concentrations of the ethanol extract (mg/ml)			
	12.5	25	50	100
Klebseila pneumoniae	12 ± 0.06	13 ± 0.01	18 ± 0.02	22 ± 0.07
Pseudomonas aeruginosa	13 ± 0.03	16 ± 0.001	17 ± 0.03	19 ± 0.01
Staphylococcus aureus	17 ± 0.001	20 ± 0.07	25 ± 0.06	29 ± 0.03
Streptococcus intermedius	14 ± 0.07	19 ± 0.03	26 ± 0.02	29 ± 0.01
Escherichia coli	13 ± 0.03	16 ± 0.06	18 ± 0.001	22 ± 0.02

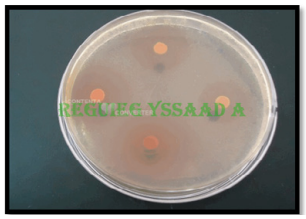
Diameter of the inhibition zone produced around the discs by the addition of 15 µL of extract (the diameter of the disc is included) the values represent the average of 3 measurements ± SD.

Table 4: Diameter of the inhibition zone of the ethanol extract.

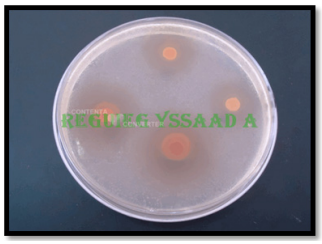
Bacterial strains	Diameter of the inhibition zone*(mm) concentrations of the methanol extract (mg/ml)			
	12.5	25	50	100
Klebseila pneumoniae	13 ± 0.03	16 ± 0.06	19 ± 0.02	23 ± 0.06
Pseudomonas aeruginosa	14 ± 0.001	15 ± 0.03	18 ± 0.001	21 ± 0.03
Staphylococcus aureus	17 ± 0.07	20 ± 0.02	29 ± 0.01	34 ± 0.01
Streptococcus intermedius	15 ± 0.03	19 ± 0.06	25 ± 0.07	30 ± 0.001
Escherichia coli	13 ± 0.06	16 ± 0.001	19 ± 0.03	24 ± 0.06

(*) Diameter of the inhibition zone produced around the discs by the addition of 15 µl of extract (the diameter of the disc is included) the values represent the average of 3 measurements ± SD.

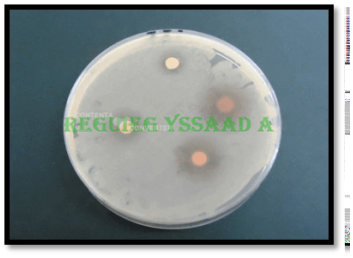
Table 5: Diameter of the inhibition zone of the methanol extract.



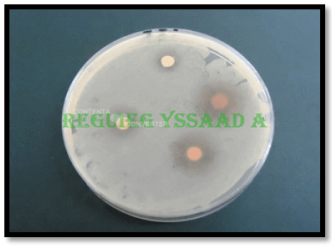
Sensitivity of *Staphylococcus aureus* to methanol extract



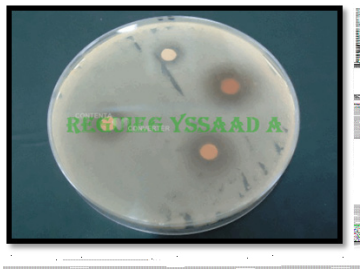
Sensitivity of *Staphylococcus aureus* to ethanol extract



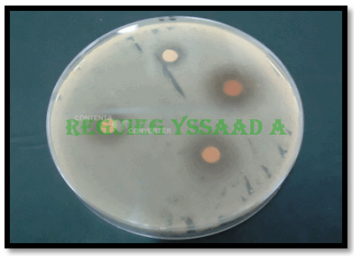
Sensitivity of *Klebseila pneumoniae* to methanol extract



Sensitivity of *Klebseila pneumoniae* to aqueous extract



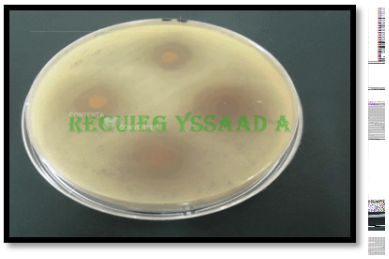
Sensitivity *Streptococcus intermedius* to aqueous extract



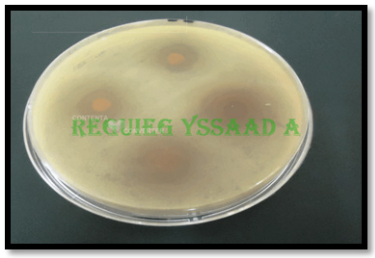
Sensitivity *Escherichia coli* to aqueous extract

Sensitivity *Streptococcus intermedius* to methanol extract

Sensitivity *Escherichia coli* to methanol extract



Sensitivity *Pseudomonas aeruginosa* to methanol extract



Sensitivity *Pseudomonas aeruginosa* to aqueous extract

Table 6: Sensitivity of the tested strains to phenolic extracts.

Bacterial strains	Aqueous extract in mg/ml MIC	MBI	MIC/MBC	Interpretation
Klebseila pneumoniae	0.312	2.5	8	Strong inhibition
Pseudomonas aeruginosa	0.625	1.25	2	Moderate Inhibition
Staphylococcus aureus	0.07	0.312	2	Strong inhibition
Streptococcus intermedius	0.156	0.625	4	Strong inhibition
Escherichia coli	0.156	0.625	4	Strong inhibition

Table 7: Minimum bactericidal concentrations of aqueous extract and MIC/MBI ratio.

Bacterial strains	Ethanol Extract in mg/ml MIC	MBC	MIC/MBC	Interpretation
Klebseila pneumoniae	0.312	0.625	2	Strong inhibition
Pseudomonas aeruginosa	0.156	0.625	4	Strong inhibition
Staphylococcus aureus	0.156	0.312	2	Strong inhibition
Streptococcus intermedius	0.156	0.312	2	Strong inhibition
Escherichia coli	0.625	1.25	2	Moderate Inhibition

Table 8: Minimum Bactericidal Concentrations of Ethanol Extract and CMB/MIC ratio.

action of the methanolic extract of *Punica granatum* is bactericidal. However, for the aqueous extract the action varies according to the germ.

View the qualitative and quantitative phytochemical results of the phenolic extracts of *Punica granatum* found previously and the presence of the tannins in these extracts which bind to proteins rich in proline and which can interfere with the synthesis of the proteins of the bacterial walls, this is proposed as a mechanism explaining the antibacterial effect of this extract [31]. Flavonoids also react and can form complexes with soluble proteins and cell walls of bacteria, the saponins present also exert an antibacterial effect which can be attributed to its ability to cause leakage of proteins as well as certain enzymes of the cell.

Overall the inhibitory action is noted in Gram+ bacteria than Gram- bacteria. The differences in susceptibility of Gram-negative bacteria and Gram-positive bacteria indicated by the presence of antimicrobial substances seen to be related to the structure and composition of their cell walls. Indeed Gram-positive bacteria have a more permeable outer layer rich in peptidoglycan while Gram-negative bacteria have a more rigid outer barrier phospholipids.

The results of the antibacterial activity revealed the efficacy of all the extracts against all of the strains tested. The antibacterial activity

of plant extracts is due to the various chemical agents present in these extracts, including flavonoids and tannins as well as other free phenolic compounds or hydroxyl groups which are classified as highly active antibiotic compounds. Richness of *Punica granatum* in tannins [32] and according to Cowan to suggest that the antimicrobial properties of tannins could be related to their ability to inactivate microbial adhesion, synthesis of certain enzymes and membrane proteins of microorganisms by the complex with polysaccharides, their ability to bind to substrates such as minerals, vitamins and carbohydrates, making them unavailable for microorganisms and their ability to modify the morphology of microorganisms [33]. On the other hand, since the fractions of grenades contain a wide range of flavonoids in particular anthocyanins [34] could exert antibacterial effects since they are potent *in vitro* inhibitors of DNA gyrase.

On the other hand, the mechanism of growth perturbation is explained by the action of phenolic compounds on the membrane. These extracts will cause loss of the selective permeability of the cell membrane by changing these physical properties.

Conclusion

In our days, the use of medicinal plants in phytotherapy has received a great interest in biomedical research and becomes as important as chemotherapy. This renewed interest comes from an inexhaustible source on the one hand of the fact that medicinal plants represent bioactive natural substances and compounds and on the other hand the need for the search for a better medication by a softer therapy without side effects.

In this work, we investigated the antimicrobial effects of the various extracts of the fruit of *Punica granatum*, a plant widely used in traditional medicine throughout the world.

However, *Punica granatum* reveals an immense richness of phenolic compounds, especially total polyphenols, flavonoids, flavones and condensed tannins in the four *Punica granatum* extracts with values ranging from 194.96 µg EAG/mg to 336.14 µg EAG/mg for total polyphenols, Whereas the flavonoid content varies between 9.54 µg EQ/mg and 27 µg EQ/mg while the condensed tannin and falavone values ranging from 19.96 µg EQ/mg to 39.01 µg EG/mg and 81.86 µg EC/mg to 153 Ug EC/mg respectively.

Moreover, according to the results of the antibioaromatogram, all our phenolic extracts have proved an antimicrobial effect on all the strains tested with a strong inhibiting power like antibiotics or more times.

Given that *Punica granatum* is characterized by a fairly large reservoir of secondary metabolites with particular therapeutic and pharmacological characteristics that need to be exploited by subsequent research.

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