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Research Article

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Phytochemical and antibacterial screening of *Punica granatum* L. bark of South-west Algeria

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ABSTRACT

For thousands of years, Punica granatum L. has been used in traditional medicine all over the world and predate the introduction of antibacterial drugs. The aim of the present study was to investigate the antibacterial activity of tannins , aqueous and methanolic extracts of Punica granatum L. bark obtained by extraction and maceration. The different extracts of Punica granatum L. bark have been tested for antibacterial activity against Gram-positive bacteria (Staphylococcus aureus, Listeria monocytogenes, Enterococcus faecalis, Bacillus stearothermophilus) and Gram-negative bacteria (Escherichia coli, Klebsiella pneumonia ,Pseudomonas aeruginosa) by disc diffusion method. The tannins and the methanolic extract showed the strong in vitro, tannins antibacterial activity against Listeria monocytogenes with zone inhibition of 25.80 mm. However, the results tests by disc diffusion method revealed the effectiveness of methanolic extract against Gram-positive bacteria (Staphylococcus aureus and Bacillus stearothermophilus) with diameter zone of inhibition varying with 23mm and 22 mm respectively.

Key words: *Punica granatum*, phytochemical screening, antibacterial, extract.

INTRODUCTION

The steadily increasing bacterial resistance to existing drugs is a serious problem in antibacterial therapy. *Staphylococcus aureus* is an example of bacterial resistance serial and is considered as the principal contaminant of clinical infections. Recently, the acceptance of traditional medicine as an alternative form for health care and the development of bacterial resistance to the available antibiotics has led authors to investigate the antibacterial activity of medicinal plants [27, 1, 12,15,8, 25]; Plants and plant derived agents have long history to clinical relevance as source of potential chemotherapeutic agents [6]. Thousands of plant species have been screened for their antimicrobial activity, but relatively few were found to be sufficiently active [22, 17] and non toxic to humans [9]. The tree of *Punica granatum* L. (Lythraceae) is extensively abundant in South-West of Algerian Sahara. The different parts of this plant such as flowers, seeds and bark have been employed against inflammatory and infectious pathologies.

The purpose of the present study was to investigate phytochemical compound and the antibacterial activity of bark extracts of *Punica granatum* against Gram-positive and Gram-negative bacteria. The extracts with the highest antibacterial effectiveness were chosen for subsequent use in pharmaceutical formulations.

EXPERIMENTAL SECTION

Plant material:
The plant used for the present study was collected in September 2014 in Bechar, a city in West Sahara Department, Algeria. The bark was separated from fruits and dried at room temperature for 12 days. The dried bark was milled to a fine powder in an electrical mill and stored in the dark at room temperature in closed containers until required.

Qualitative phytochemical screening
Each organ of plant (leaves, roots and fruits) was screened for the presence of key families of phytochemicals [24] [28].

Extraction of bark tannins:
50 g of each plant material were extracted with 1000 ml of acetone-water (700/300, v/v), and the mixture was stirred continuously for 72 h, at room temperature. Then, the mixture was filtered and evaporated under vacuum using a rotary evaporator (Büchi Rotavapor R-215) at 56 °C to remove acetone; the remaining solution was washed with 300 ml of dichloromethane to remove lipid soluble substances [3].

After the elimination of dichloromethane under reduced pressure, the aqueous phase was extracted with 300 ml of ethyl acetate. This process was repeated twice. Then, the organic phases (ethyl acetate) containing tannins were recovered and evaporated to dryness, and the resulting substance were referred to as aqueous extract. Next, the aqueous extract was weighed and dissolved in 30 ml of distilled water and stored in sterile flasks at 4 °C until use [32].

Preparation of plant extracts
Aqueous maceration:
10 g of each plant material were soaked in 100 ml of distilled water for 24 h at room temperature, and then the aqueous macerate was filtered through filter paper MN 640 of 125 mm in diameter, to remove fine particles [10] .This was repeated and then the aqueous macerate of each plant material was followed by a concentration in vacuum at 100 °C using rotary evaporator (Büchi Rotavapor R-215) [28].Next, it was collected in sterile flasks and stored at 4 °C.

Aqueous extraction:
10 g of each plant material were soaked in 100 ml of distilled water for 24 h at room temperature and then the extraction of the aqueous macerate was done in water bath at boiling temperature, under reflux for 1 h [10] .The obtained extract was filtered through filter paper MN 640 of 125 mm in diameter, to remove fine particles. This was repeated, and then the aqueous extract of each plant material was followed by concentration in vacuum at 100 °C using rotary.

Methanol maceration:
10 g of each plant material were soaked in 100 ml of 50 % methanol for 24 h at room temperature, and then the ethanol macerate was filtered through filter paper MN 640 of 125 mm in diameter to remove fine particles [10] .This was repeated, and then the solvent was evaporated under vacuum and at 65 °C from each filtrate using a rotary evaporator (Büchi Rotavapor R-215) [30].The obtained extracts were collected in sterile flasks and stored at 4°C.

Methanol extraction:
10 g of each plant material were soaked in 100 ml of 50 % methanol for 24 h at room temperature and then the extraction of the solvent was done in water bath at boiling temperature, under reflux for 1 h The obtained extract was filtered through filter paper MN 640 of 125 mm in diameter to remove fine particles, this was repeated and then the solvent was evaporated under vacuum and at 65 °C from each filtrate using a rotary evaporator (Büchi Rotavapor R-215) [31].The obtained extracts were collected in sterile flasks and stored at 4 °C.

Bacterial strains and media
The antibacterial activity of different part extracts of *Punica granatum* were evaluated using the following strains bacteria, Gram-positive: *Listeria monocytogenes* (ATCC19115); Bacillus *stearothermophilus* (ATCC11778); *Staphylococcus aureus* (ATCC25923); Enterococcus faecalis(ATCC29212). Gram-negative bacteria were*:* *Klebsiella pneumonia* (ATCC4352); *Pseudomonas aeruginosa* (ATCC27853); *Escherichia coli* (ATCC25922). These bacterial strains were obtained from the Pasteur Institute, Algiers, Algeria. All strains were identified by the use of biochemical profiles according to the recommendations of the manual clinical microbiology [19]. All organisms were maintained in brain-heart infusion (BHI medium) containing 30(v/v) glycerol at -20°C. Before testing, the suspensions were transferred to trypticase soy agar supplemented with 5% of sheep blood and

aerobically grown overnight at 35°C. Individual colonies were isolated and suspended in5 ml of 0.9% Nacl solution. The inoculate were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard and diluted in CAMHB (Cation –adjusted Muller Hinton broth) in order to achieve the adequate inoculum in each case. The cell number in CAMHB was estimated using a serial dilution technique for each assay [20].

Antibacterial activity

Disk diffusion method

Petri dishes were prepared with 20 ml of base layer of Muller Hinton gelose medium and inoculated with 100 µl of each bacterial suspension (10^6 UFC) [29]. After drying in a sterile hood, 6 mm diameter disks soaked with different extract were placed at 35°C for 24 h. The antibacterial activity was expressed as the mean of inhibition diameters (mm) produced.

MIC determinations

The minimal inhibitory concentration (MIC) preventing visible bacterial growth measured by the different concentrations of extract of Muller Hinton agar media. Different volume of extract were prepared and added to 20 ml of Muller Hinton Agar media; after agitation , the select solution were transferred into a Petri plates which were incubated at 35°C for 24 h[1].

RESULTS AND DISCUSSION

Phytochemical screening:

The preliminary phytochemical screening of *Punica granatum* L. bark revealed the presence of various phytoconstituents in each dry peel. It showed the presence of saponins, alkaloids, flavonoids, tannins and sterols in *P. granatum* peels L. and the absent of unsaturated sterols terpenes and Steroids. The results of phytochemical screening of *P. granatum* L. bark are given in the **table 1.**

Table 1: Phytochemical Screening of *P. granatum* L.bark

Phytoconstituents	*P. granatum* L. bark
Saponins	+
Unsaturated sterols and terpenes	-
Sterols	+
Steroids	-
Alkaloids	+
Flavonoids	+
Tannins	+++

(+) and (-) refer to presence and absent amount, respectively.

Punica granatum is selected based on traditional medicine knowledge and random chosing from the local florae where it is reported that the bark, leaves, flowers; and fruit of pomegranate are used as phytotherapeutic agents [14] the phytochemestry of pomegranate has also been widely studied by some researechers ,where *P.granatum* L .fruit is found to be a rich source of polyphenolis compounds [13]

Our phytochemical screeninig revealed that *P.granatum* contain a various phytoconstituents such as saponins, alkaloids, flavonoids, sterols and tannins similary with our results [16] indicate that both flavonoids and tannins are more abundant in *P.granatum L. peels* .Also [23] indicate that its bark contain a wide variety of phytochemical compounds like gallotaninis, ellagicacid, gallagic acid and punicalins

Antibacterial activity

Disk diffusion method

The result of the disk diffusion method indicated that All the tested extracts showed some antibacterial activities The results of the disk diffusion test indicated that Tannins and methanol extracts of *Punica granatum* bark showed different degrees of growth inhibition, depending on the bacterial strains Results are presented in **Table 2**.

MIC determination

As Table 3 shows, the MICs are more or less important depending on the type of bacteria studied. Tannins show the best antibacterial activity screw all bacterial strains tested.

Table 2: Antibacterial Activity of the Tannins, Aqueous and metanolic Extract of *P. granatum* L. bark by Disc Diffusion Method

Bacterial strains	T	A.M	A.E	M.M	M.E
Gram-negative bacteria					
Klebsiella pneumonia (ATCC4352)	24,0	19,63	20,8	22,4	23,3
Escherichia coli (ATCC25922)	23	17,42	19,4	21,6	22
Pseudomonas aeruginosa (ATCC27853)	21,8	17,80	18,5	20,6	22
Gram-positive bacteria					
Listeria monocytogen (ATCC19115)	25,8	22,42	22,9	23,2	21
Bacillus sterothermophillus (ATCC11778)	22,3	20,60	21	22,8	23
Staphylococcus aureus (ATCC25923)	20,9	19, 35	21,5	20,30	22
Enterococcus faecalis (ATCC29212)	21,8	19	21,2	19,45	20,4

T*:tannins* ***A.M****:Aqueous maceration* ***A .E*** *: Aqueous extraction* ***M.M*** *Methanol maceration* ***M.E:*** *Methanol extraction*

Table 3: the MICs of the Tannins Aqueous and metanolic Extract of *P. granatum* L. bark

Bacterial strains	T	A.M	A.E	M.M	M.E
Gram-negative bacteria					
Klebsiella pneumonia (ATCC4352)	5,30	7,25	6,25	7,36	6,10
Escherichia coli (ATCC25922)	5,62	7,85	6,85	7,55	6,25
Pseudomonas aeruginosa (ATCC27853)	5,70	7,10	7,10	7,80	6,00
Gram-positive bacteria					
Listeria monocytogen (ATCC19115)	5,30	7,20	6,20	7,60	6,35
Bacillussterothermophillus(ATCC11778)	5,45	7,70	6,70	7,50	6,20
Staphylococcus aureus (ATCC25923)	5,65	7,80	6,80	7,68	6,55
Enterococcus faecalis (ATCC29212	5,80	7,90	6,90	7,55	6,80

T*:tannins* ***A.M****:Aqueous maceration* ***A .E*** *: Aqueous extraction* ***MM*** *Methanol maceration* ***ME****: Methanol extraction*

Tannins and methanol extraction extracts showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the diameter of inhibition zone, 25.80-24.50 mm with the MIC obtained is 5.30 mg/ml and 23.3-23 mm respectively with the MIC obtained is 6,10 mg/ml;.6,30 mg/ml). Tannins showed the highest antibacterial activity against some strains, such as Listeria monocytogen (25.80mm), and Klebsiella pneumonia (24.5mm).

These results are in agreement with those reported by [5]. They found that *Punica granatum* bark have to be *in vitro* antibacterial activity. These results suggest that the inhibitory effect exhibited by the macerate and decoctate crude extracts of *Punica granatum* bark may be attributable to the tannins that represent 28% of bark constituents [4]. This family of compounds has been found *in vitro* to have various pharmacological properties such as antioxidant, antimicrobial and anti-inflammatory [5] .Furthermore, the observed antibacterial activity may be due also to other secondary metabolites like phenolic compounds and saponins. In general, we observed that the ethanol decoctate extract was more efficacy than aqueous extract because ethanol allowed to extract well the less polar compounds such as terpenic derives [7] [18] .The obtained results might be considered sufficient to further studies for the isolation and identification of the active principles and to the evaluation of possible synergism among extract components for their antimicrobial activity. Investigations are in progress to determine the degree of toxicity of these extracts.

The reason for different sensitivity between Gram- positive and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms. Gram- negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da [21]. The Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier [26].

CONCLUSION

Extracts of *Punica granatum* L. bark in this study demonstrated a broad-spectrum of activity against both gram positive and gram negative bacteria with different diameter zone of inhibition. The broad-spectrum antibacterial activities of the plant extract, possibly due to the secondary metabolites such as tannins, phenolic compounds or saponins that were abundant in this plant. This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity. Further studies should be undertaken to elucidate the exact mechanism of action by which extracts exert their antibacterial effect.

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