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Phytochemical analysis, antibacterial and antioxidant activities of pomegranate (*Punica granatum* L.) peel extracts

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Abstract

The pomegranate and their derivative parts are rich source of phytochemicals with multiple beneficial biological effects. This study aims to extract the phenolic compound from pomegranate (*Punica granatum L.*) peel using methanol and ethanol in order to assess their antioxidant and antibacterial activities. The phenolic compounds and flavonoids content were measured using colorimetric methods. The antioxidant activity was determined by ferric reducing antioxidant power (FRAP). Furthermore, the antibacterial activity of peel extracts was tested on Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram negative bacteria (*Escherichia coli*) using the agar diffusion and microbroth dilution methods. The results have shown that the pomegranate peel of both extracts contained a similar amount of phenolic compounds and flavonoids. However, the antioxidant activity was significantly ($p<0.05$) higher in ethanolic extract with EC_{50} value of 58.42± 0.21µg/mL compared to methanolic extract (EC_{50} = 80±1µg/mL). Both extracts exhibited a good antibacterial effect against *Staphylococcus aureus* and *Bacillus subtilis* with minimal inhibitory concentration (MIC) values ranging from 0.97 to 3.9 mg/mL and minimal bactericidal concentration (MBC) values ranging from 7.81 to 62.5 mg/mL. According to these findings, the pomegranate peel extracts have an important antibacterial and antioxidant properties and may be used as an alternative to antibiotics in the treatment of infections and in the prevention of pathologies associated with oxidative stress.

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Introduction

The increasing of antibiotics resistant microbes and the undesirable effect of synthetic drugs led researchers to focus on alternative solution derived from medicinal herb (Patel *et al.*, 2011). Many studies have reported the antimicrobial and antioxidative properties of medicinal plants and their components (Kusuma *et al.*, 2014). The therapeutic properties of these natural products are due to the presence of wide variety of secondary metabolites, like polyphenols. These biomolecules, with therapeutic virtues, have received considerable attention because of their diverse biological function.

The pomegranate fruit (*Punica granatum*. L) is one of the oldest fruits, it is cultivated mainly in the Mediterranean region, has been used for several centuries in traditional medicine for a wide variety of diseases such as parasitic and microbial infections, ulcers, diarrhea and cancers (Kim *et al.*, 2002 ; Reddy *et al.*, 2007; Johanningsmeier and Harris, 2011). Pomegranate can be eaten fresh or made into fruit juice, jellies and jams; however the consumption of this fruit generates considerable quantities of by-products. Indeed, pomegranate peels are frequently rejected without recovery. Thus, pomegranate peel contains important phytochemical compounds such as tannins and anthocyanins (Gil *et al.*, 2000; Zaouay *et al.*, 2012). These bioactive compounds posses different biological activities such as scavenging of free radicals, inhibiting microbial growth and reducing the risk of cardiovascular, cerebrovascular diseases and certain cancers (Mena *et al.*, 2011; Zhu and Liu, 2013; Romeo *et al* ., 2015). Pomegranate peel can be considered as natural products which have become widely used for medical and food applications, therefore, several studies have interested to find pomegranate peel valorization methods and to determine their therapeutic benefits. It is necessary to pay particular attention to the process and type of solvent extraction to determine the extract with optimal efficiency which makes it possible to restore all the molecular complexity of this plant. In this context, the objective of this study is to determine the phytochemical antibacterial and antioxidant activities of pomegranate peel extracts.

Materials and methods

Chemicals

Folin-Ciocalteu, Gallic acid, Resorcinol, Rutin, Syringic acid, Quercetin and Ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other solvents and chemicals were of analytical grade.

Plant material

The pomegranate (*Punica granatum* L.) fruit type used in this study is known as "*Séfri*"; it comes from the region of Mostaganem (35°55'59.999"N and 0°4'59.999"E in Algeria).The pomegranate peels were dried in dark at room temperature, then ground with a mechanical grinder (Pulverisette, Fritsch, Germany).

Bacterial strains

The strains used to evaluate antibacterial activity are: *Escherichia coli* ATCC 10536, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 10876. They were kindly provided by the microbiology unit of the SAIDAL group, Media (Algeria).

Extraction of phenolic compounds

A 10 g of powder was macerated in 125 mL of methanol or ethanol for 24 h at room temperature and in the dark. The extracts were filtered through Wattman n°1 filter paper. The filtrate obtained was evaporated using a rotary evaporator in order to obtain a dry extract. The extracts were kept at -20 °C (Hadrich *et al.*, 2014).

Phytochemical analysis

Phenolic compounds determination

The total phenolic compounds were estimated according to the Folin-Ciocalteu method (Singleton *et al.*, 1999). Briefly, 250 µL of the extract was added to 250 µL of Folin Ciocalteu reagent (0.2 N), the mixture was incubated at room temperature for 2 min before 500 µL of Na2CO3 (7.5%, w/v) were added. The reaction mixture was then incubated for 30 min at room temperature and in dark. The absorbance was

measured at 760 nm against a blank without extract using a spectrophotometer (Shimadzu Corporation, Japan). The content of phenolic compounds is expressed as mg Gallic Acid Equivalent per g of dry matter (mg EAG / g DM) by referring to the calibration curve for gallic acid.

Flavonoids contents

The content of the total flavonoids was determined by the method of Bahorun *et al.* (1996). A 1mL of the extract solution was added to 1mL of $AlCl_3$ (2% w/v), after 10 min of incubation, the absorbance was read at 430 nm using a UV-visible spectrophotometer. Total flavonoid content is calculated from the calibration curve performed by quercetin. The results are expressed as mg quercetine equivalent per g dry matter (mg EQ / g DM).

Analysis of phenolic compounds by HPLC

The analysis of phenolic compounds in pomegranate peel extracts was carried out using a high performance liquid chromatography (HPLC) Agilent YL9100 system equipped with a UV detector (254 nm). The separation was performed with a reverse phase column (Zorbax eclipse×DB-C18), 15 cm long and 4.6 mm internal diameter, using a flow rate of 1 mL / min, and a mobile phase: acidified water /acetic acid 1% (A) and methanol 100% (B), at a temperature of 25°C and detection at 254 nm. The gradient elution started with 95% of solvent A and 5% of solvent B and increase of solvent B to 95%, after 55 min.

Phenolic compounds of each pomegranate peel extract sample were identified by comparing their retention times (Rt) with those of the pure standards injected in the same conditions.

Antioxidant activity evaluation

Ferric reducing antioxidant power (FRAP) assay

The reducing power of iron (Fe^{3+}) in the extract was determined according to the method described by Yen and Duh, (1993). 1 mL of the extract at different concentrations were mixed with 2.5 mL of phosphate buffer solution (0.2 M , pH 6.6) and 2.5 mL of a potassium ferricyanide solution $K_3Fe(CN)6$ (1%, w/v). The mixtures were incubated at 50°C for 20 min, After incubation, 2.5 mL of trichloroacetic acid (10%,w/v) was added to stop the reaction. Finally, 1 mL of upper layer was mixed with 1mL of distilled water and 0.5 mL of ferric chloride $FeCl_3$ (1%, w/v), the absorbance was measured at 700 nm against a blank. An increase in absorbance of the reaction corresponds to an increase in the reducing power of the tested extract. The reducing potential of the extract and of the standards is expressed by the effective concentration values at 50% (EC_{50}).

Evaluation of the antibacterial activity

Diffusion method agar

Bacterial suspension was prepared in sterile physiological water (0.9%) for each strain. The turbidity of this suspension has been adjusted to 0.5 Mac Farland. This inoculum was spread on the surface of Mueller-Hinton agar plate. Sterile filter discs (6 mm in diameter) were impregnated with 20 µL of each extract solution then were deposited on the surface of the inoculated agar. The plates were incubated at 37°C for 24 h (Adesokan *et al.*, 2007). Tetracycline and chloramphenicol antibiotic discs (Merck, Germany) served as positive controls and discs impregnated with dimethyl sulfoxide (DMSO) served as negative controls. Antibacterial activity was determined by measuring the diameter of the inhibition zone around each disc.

Determination of the minimal inhibitory concentration (MIC)

Microdilution assay was determined according to the method described by Klančnik *et al.* (2010). Two-fold serially diluted pomegranate peel extracts were prepared in sterile Mueller-Hinton broth. 95 µL of each solution were deposited in the wells of a 96-well microplate plate, and then 5 µL of each bacterial suspension was added to the wells. The final volume in each well was 100 µL. The wells containing the Muller Hinton broth alone were used as a negative control, while the wells containing the Mueller Hinton inoculated with the each bacterium—and without extract were used as positive controls. The microplates thus prepared were incubated for 18 h at

37°C. At the end of this incubation the bacterial growth was visualized by adding 20 µL of 2,3,5-tripheny-tetrazolium chloride (Sigma-Aldrich). Bacterial growth was indicated by red colour while clear wells recorded inhibition growth.The MIC was recorded from the lowest concentration of the extract that inhibits the visible bacterial growth. The minimal bactericidal concentration (MBC) was determined as the lowest extract concentration that killed 99% of bacteria in the initial inoculums within 24 h.

Statistical analysis

The results obtained were expressed as means± standard deviation (SD). The statistical analysis of the data was carried out using the STATISTICA software (version 6.1, Stat soft, Tulsa. OK, USA). The comparison of the means was carried out via ANOVA with a factor, followed by the tukey Test. A value of $p < 0.05$ was used as the significance level.

Results and discussion

Yield extraction, quantification of phenolic compounds and flavonoids

The yield extraction obtained from the pomegranate peel using methanol was a high yield of 41 ± 1.73% (w/w) compared to the ethanolic extract with an average of 31 ± 2.47% (w /w).These results are approximately similar to those obtained by Shiban *et al.* (2012) where the yield was 45%. Another studies indicated that methanolic extract represents yield ranging from 31.5 to 48.2% (Li *et al.*, 2006; Zaki *et al.*, 2015).

Table 1. Diameters of inhibition zones (mm) of pomegranate peel extracts and antibiotic discs against the tested bacteria.

PPEE (mg /mL)	Bacteria		
	B. subtillis	*S. aureus*	*E.coli*
250	14.17 ± 0.29^{a}	15.33± 0.58^{b}	11 ± 1^{c}
125	12± 1^{a}	12.67± 0.58^{a}	8.67±0.58^{b}
62.5	10±1^{a}	10.67± 0.58^{a}	7.67 ± 0.58^{b}
PPME (mg/mL)			
250	19.67±2.52^{a}	20 ±1.73^{a}	13± 1^{b}
125	14.33 ± 0.57^{a} 4	17.33± 0.58^{b}	11.33 ± 0.58^{c}
62.5	10.33± 1^{a}	12± 1^{a}	9.33±1.15^{b}
Antibiotics			
Chloramphenicol (30µg/mL)	33.66	30	21
Tetracycline (20µg/mL)	28.33	19	15 1115151515.33

The values represented are the mean ±SD. PPEE: Pomegranate peel ethanolic extract; PPME: Pomegranate peel Methanolic extract; Different letters (a,b,c) indicate significant differences ($p<0.05$).

These variations observed between the yields are may be due to the type of solvent in fact, it affects the extraction; it is clear that there is an affinity between the extraction solvent and the extracted compounds, as well as its biological activity (Lee *et al.*, 2003;Ghasemzadeh *et al.*, *2011*). Furthermore, It is established that variations in extraction yields could be attributed not only to the difference in solvent polarity, which plays a key role in increasing the solubility of phenolic compounds; but also the polarity of the phenolic compounds which constitute the extract (Felhi *et al.*, 2017). The quantitative analysis of the phenolic and flavonoid contents of the pomegranate peel extracts was determined from the linear regression equations of each calibration curve expressed successively in mg EGA/g DM and in µgEQ/g DM respectively. Thus, the results obtained are illustrated in Fig 1. and 2. In this study, the total phenolic contents were in order to 379.61 ± 31.71 mg EAG / g DM for methanolic extract and 381.51 ± 32.39 mg EAG / g DM for the ethanolic extract. This quantification showed no significant difference between the methanol and ethanol pomegranate peel extracts.

Table 2. Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of pomegranate peel extracts against the tested bacteria.

Bacteria	PPEE (mg/mL)		PPME (mg/mL)	
	MIC	MBC	MIC	MBC
Bacillus subtillis	3.9	62.5	3.9	31.25
Staphylococcus aureus	3.90	31.25	0.97	7.81
E.coli	7.81	62.5	7.81	62.5

PPEE: Pomegranate peel ethanolic extract; PPME: Pomegranate peel Methanolic extract.

The flavonoid contents of the methanolic and ethanolic extracts were found to be similar and equal to 50.95 ± 11.57 and 53.64 ± 12.31 mg EQ / g DM, respectively. This result is in agreement with that reported by Hadrich *et al.* (2014). These authors noted that the total phenolic compounds in the extracts obtained after 24 h of maceration varied from 0 to 290.10 ± 0.57 mg EAG / g DM. Another study showed that the phenolic content of the methanolic extract from the pomegranate peel was around 274 mg EAG/ g and 56.4 mg RE /g of flavonoids (Shiban *et al.*, 2012).

Table 3. Efficient concentration 50 (EC_{50}) of different pomegranate peel extracts and standard antioxidants in reducing power.

	EC_{50} (µg/mL)
PPEE	58.42± 0.21[a]
PPME	80 ± 1.00[b]
Gallic acid	17.56± 0.44[c]
Quercetin	35.76± 0.84[d]
Ascorbic acid	44.20± 0.84[e]

The values represented are the mean ±SD. PPEE : Pomegranate peel ethanolic extract ; PPME :Pomegranate peel Methanolic extract ; Different letters (a,b,c,d,e) indicate significant differences ($p<0.05$).

HPLC analysis

The HPLC chromatograms of pomegranate peel ethanolic and methanolic extracts are shown in Figure 3 .The polyphenols profile of the pomegranate peel extracts were similar, however peak area of individual compounds varied. The results of the present study revealed that the Gallic acid, Resorcinol, Punicalagin, Rutin, Syringic acid and Quercetin were most abundant phenolic compounds in both extracts. Several studies have shown the presence of different phenolic compounds in pomegranate peel extracts. The presence of gallic acid, chlorogenic, caffeic acid tannic acid and Rutin pomegranate peel extracts was reported by Shaban *et al.* (2013); while Cai *et al.* (2004) and Ahmed *et al.* (2018) highlighted the presence of vannilic acid and quercetin. Gullon *et al.* (2016) also reported the presence of punicalagin and ellagic acid as main bioactive compounds in pomegranate peel.

The difference in the content of phenolic and flavonoid compounds is well documented. This can be explained by the fact that the type of solvents, the methods and the extraction time can significantly influence the content of phenolic and flavonoids in different extracts (Grujic *et al.*, 2012).

Antioxidant activity of pomegranate peel extracts

Ferric Reducing Antioxidant Power (FRAP)

The results of the reducing power of pomegranate peel extracts were proportional to the increase in the concentration of the extracts (Fig. 4). Indeed, the ethanolic extract showed a higher reducing power in comparison with the methanolic extract (58.42 ± 0.22 *vs* 180 ± 5.29 µg/mL). However, this iron-reducing power remains lower compared to the reducing power

of standard antioxidants such as gallic acid and ascorbic acid. This is noted by EC_{50} (Gallic acid: 17.56 ± 0.45; ascorbic acid: 44.20 ± 0.84 µg / mL) (Table 3). This high reducing power of the pomegranate peel extract may be due to the presence of certain bioactive compounds such as ellagitannins, punicalin, punicalagin and numerous piperidine alkaloids (Gullon *et al.*, 2016).

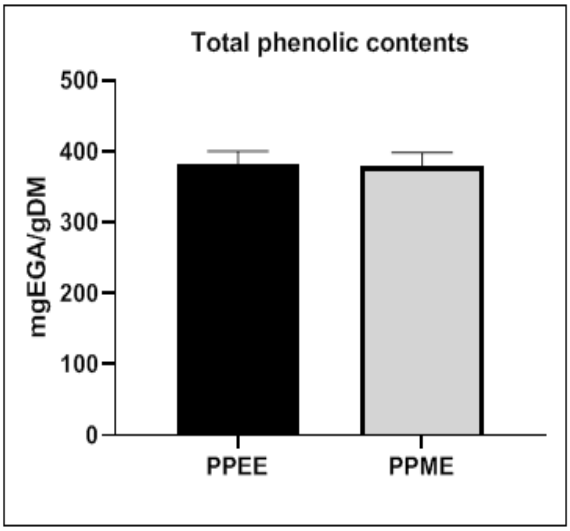

Fig. 1. Total phenolic contents of pomegranate peel extracts.

The results are expressed as means ± SD. PPEE: Pomegranate peel ethanolic extract; PPME: Pomegranate peel methanolic extract; mg GAE /gDM: mg Gallic acid equivalent/g dry matter.

Antibacterial activity

The obtained result highlighted that the ethanolic and methanolic extracts have an inhibitory effect on the growth of all tested bacteria (*E.coli, B. subtilis* and *S. aureus*) as shown in Table 1. The diameters of the inhibition zones were ranging from 10 to 20 mm for the methanolic extract and 10 to 15.33 mm for the ethanolic extract. Indeed the Gram positive bacteria *S. aureus* and *B. subtilis* were the most sensitive in comparison with the Gram negative bacteria *E. coli*, and this could be related to the difference in the wall structure between Gram positive and negative bacteria (Ali-Shtayeh *et al.*, 1998).

These results are in agreement with those reported by Malviya *et al.* (2014), who showed that *S. aureus* is sensitive to the methanolic and ethanolic pomegranate peel extracts with diameter inhibition zones of 24 ± 0.53 mm and 20 ± 0.31mm, respectively; unlike *E. coli* which has been shown to be resistant to these same compounds (7 mm) (Naziri *et al.*, 2012). Thus, the resistance of bacteria to these extracts can be attributed to the nature and the composition of lipopolysaccharides of the cell wall of these strains.

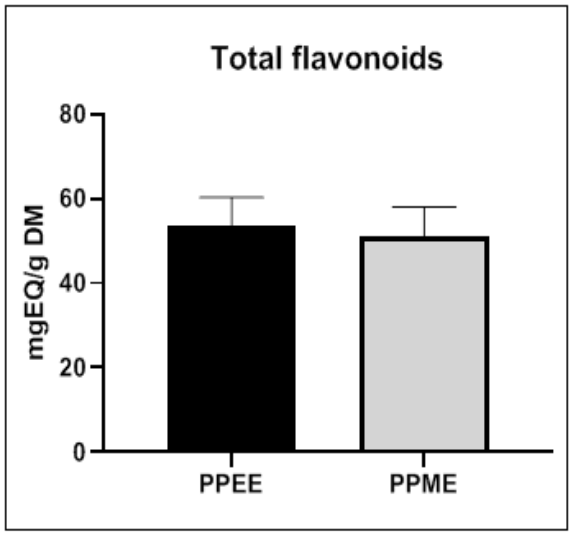

Fig. 2. Total flavonoids contents of pomegranate peel extracts.

The results are expressed as means ± SD. PPEE: Pomegranate peel ethanolic extract; PPME: Pomegranate peel methanolic extract; mg QE/gDM: mg Quercetin equivalent/g dry matter;

Determination of the minimal inhibitory concentrations (MIC)

The results of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) evaluated by the microdilution method suggest that both extracts of pomegranate peel exert different degrees of antibacterial activity (Table 2). The most powerful inhibitory effect of methanolic extract was observed against *S. aureus* with MIC of 0.97 mg/mL followed by *B. subtilis and E. coli* with MIC of 3.9 and 7.81 mg/mL, respectively. Whereas, the strains of *S. aureus and B. subtilis* were sensitive to the ethanolic extract, in particular with an MIC of 3.9 mg/mL and MBC ranging from 31.5 to 62.5 mg/mL, respectively; in comparison with *E. coli,* where the MIC and MBC obtained were around 7.81 and 62.5 mg/mL, respectively.

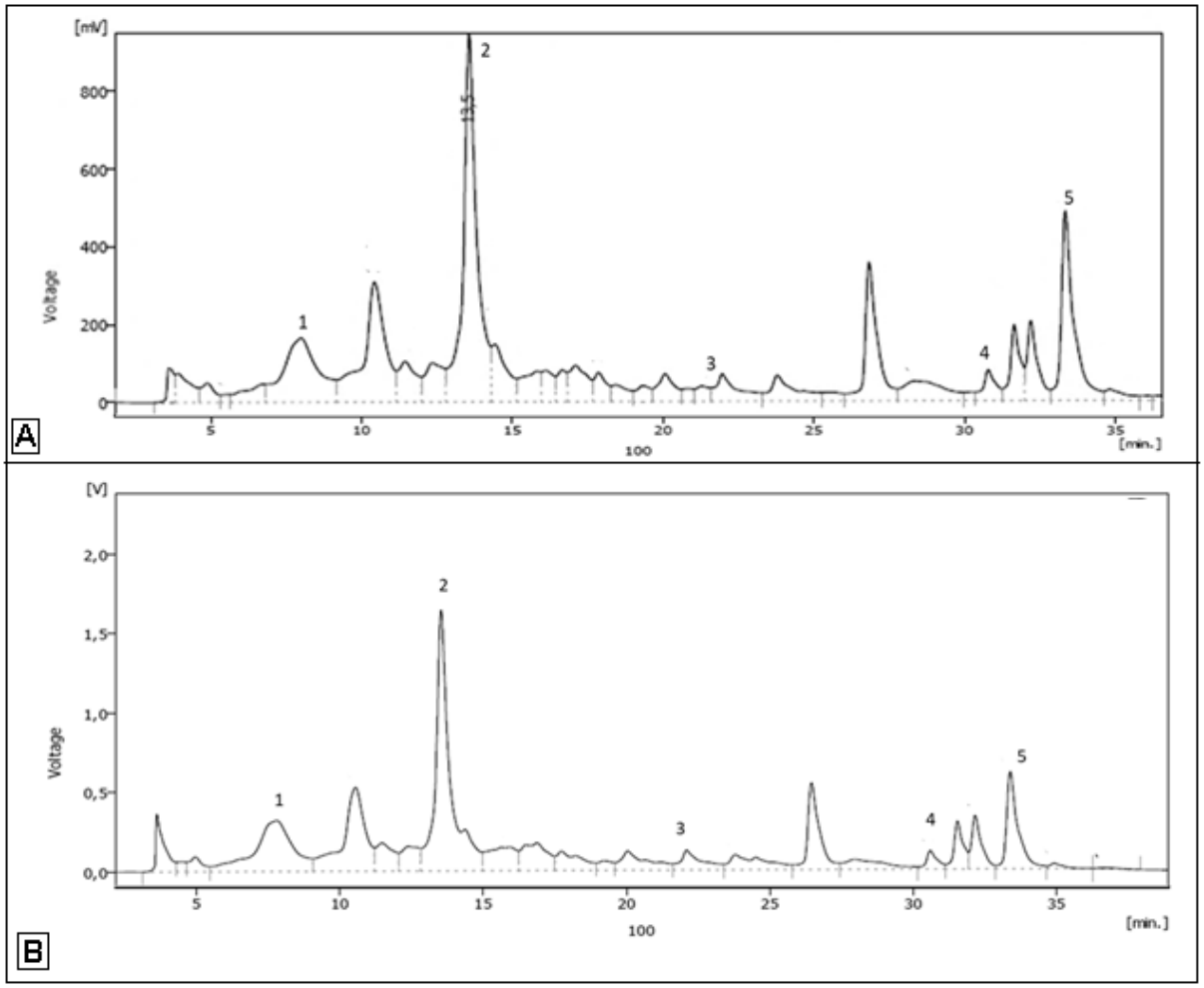

Fig. 3. HPLC chromatograms of pomegranate peel extracts A) Ethanolic extract B) Methanolic extract. 1: Gallic acid; 2: Punicalagin; 3: Syringic acid; 4: Rutin; 5: Quercetin.

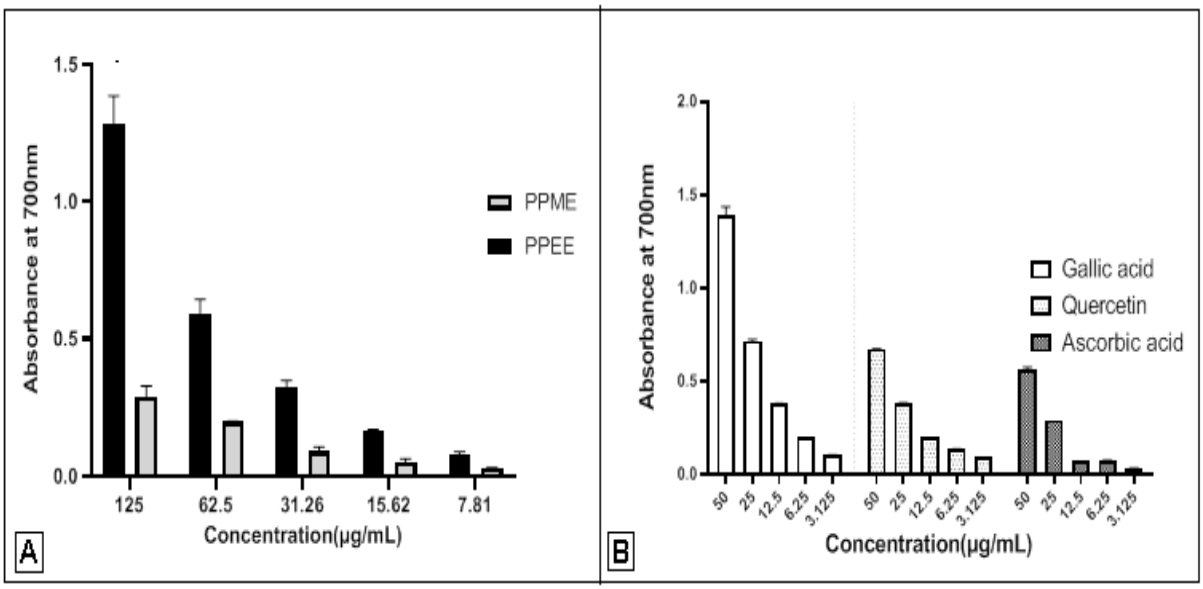

Fig. 4. Reducing power activity of pomegranate peel extracts, gallic acid, quercetin and ascorbic acid evaluated by FRAP method.

The results are expressed as means ± SD. PPEE: Pomegranate peel ethanolic extract; PPME: Pomegranate peel methanolic extract.

These findings are in agreement with those of Naziri *et al.* (2012), who reported that the methanolic extract of the pomegranate peel exerts an inhibitory effect on Gram positive bacteria compared to those of Gram negative bacteria. Therefore, the MIC of the extract of the pomegranate peel determined by the method of dilution against *E. coli* and *S. aureus* were 31.3 mg /mL and 7.8 mg /mL, respectively. This is in agreement with the results of Naz *et al.* (2007).These authors have investigated the effect of different extracts of pomegranate on six bacterial species: *S aureus*, *E.coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *B subtilis*, *Salmonella typhi*, and have shown that both extracts exert an antibacterial activity against all tested bacteria. The antimicrobial activity of pomegranate peel extract is believed to be due to its phytochemical composition, and in particular, to the nature of its major phenolic compounds. It can also be attributed to one or more molecules, present in low proportion (s) in the extract (Mphahlele *et al.*, 2016).

Conclusion

This study has shown that the yield of pomegranate peel extract was higher than that of the ethanolic one. However, similar amounts of phenolic compounds and flavonoids were found in both extracts.

The antioxidant activity was significantly ($p<0.05$) higher in ethanolic extract. Both extracts exhibited a strong antibacterial activity against Gram positive bacteria (*S. aureus* and *Bacillus subtilis)* than against the Gram negative (*E. coli*). These extracts are promising agents for the prevention of pathologies associated with oxidative stress.

Further studies such as identification of bioactive molecules of pomegranate peel extracts are needed to determine the effectiveness of each molecule in the antioxidant and antibacterial activities.

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