

Influence of the Extractive Method of *Punica granatum* Peels in its Antibacterial and Antibiofilm Activities Against Gram-Negative and Gram-Positive Bacteria

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Abstract: The present study aimed to investigate the effect of the different types of solvents (water, ethanol and methanol) and extraction methods (ultrasound and maceration) on *Punica granatum* peels and to assess their antibacterial and antibiofilm activities. Our results revealed that the solvents and the extraction methods had a significant effect concerning the antimicrobial effects of *P. granatum* peels. The methanol extract produced by both the ultrasound and the maceration extraction techniques and the ethanol extract produced by the maceration extraction technique showed the most potent effect with the same bacteriostatic effect (MIC=25 mg/ml) on *Staphylococcus epidermidis* (CIP444). The antibacterial activities for all the *P. granatum* peels extracts were the same against *E. coli* (ATCC35218), with an MIC=25 mg/ml. The results showed that the methanol and the ethanol extracts produced by both the ultrasound and the maceration extraction techniques had an appreciable antibiofilm activity against *S. epidermidis* (CIP 444). These extracts showed the highest prevention capacity at a concentration of 12.5 mg/ml. In addition, the highest biofilm eradication activity was obtained using the ethanol extract with the ultrasound technique at a concentration of 25 mg/ml. We concluded that the *P. granatum* peels extracts have great potential as future natural antibacterial agents.

Keywords: *Punica granatum* Peels, Antibacterial Activity, Antibiofilm Activity

1. Introduction

Punica granatum, known as pomegranate, belongs to the family of Punicaceae. This plant is mainly found in the Middle East, India, China, United State, Mexico and throughout the Mediterranean region. The pomegranate

possesses different size, color and taste [1, 2]. Pomegranates were always considered as a sacred fruit that grow in the gardens of Paradise, in Hinduism, Persian, Greek, Chinese, Jewish, Christian and in Muslim culture [3]. Pomegranates were believed to increase fertility and to bring good luck since long time. In many countries and particularly in the

Mediterranean region, the pomegranate has been used extensively in the traditional medicine to treat many diseases. In the ancient Indian Ayurvedic tradition, the pomegranate was used as an antiparasitic agent and to treat ulcers and diarrhea [4]. In Greco-Arab Medicine, the pomegranate was used to treat diabetes [5]. The pomegranate fruit has been widely used as a traditional remedy against diarrhea, acidosis, microbial infections, helminth infection, snakebite, burns, leprosy, hemorrhage, dysentery, fever and respiratory pathologies [6-10]. Furthermore, the dried pomegranate peels are considered beneficial for the treatment of ulcers, colitis, diarrhea, headache, aphthae and dysentery [11]. In the Egyptian culture, the dried pomegranate peel was used to treat several disorders such as inflammation, cough, intestinal worms and infertility. The traditional importance of pomegranate as a medicinal plant is now being reinforced by data obtained by modern science. Pomegranate fruits can be also divided into three parts: the seeds which constitute about 3% of the fruit weight, the juice and the peel which includes the interior network of membranes comprising about 30% of the total fruit weight. Several studies have demonstrated that the different parts of pomegranate and especially the seeds are rich in polyphenols (including flavonoids and tannins) which have been proved to be responsible for its antioxidant properties [12]. The antioxidant activity of pomegranate juice is at least 20% higher than the other beverages like black apple juice, orange juice, cherry juice, cranberry juice, blueberry juice, grape juice, red wines and iced tea [13]. Pomegranate peel and juice contain high amounts of bioactive compounds especially phenolic compounds. The most common phenolic compounds present include flavonoids (anthocyanins and catechins) and hydrolyzable tannins (punicalin, pedunculagin, punicalagin, gallic and ellagic acid) [14]. These compounds are responsible for more than 90% of the antioxidant potential of the fruit [15, 16]. The hydrolyzable tannins, in addition to their antioxidant activity, possess antibacterial activities against several Gram-positive bacteria (*Staphylococcus aureus*), while a more modest protective effect was observed against Gram-negative bacteria, i.e., *Escherichia coli*, *Salmonella spp.*, *Shigella spp.* and *Vibrio cholera* [17, 18]. In fact, recent studies are focusing on the benefits for using natural herbal products for eliminating pathogenic bacteria that have resistance to antibiotics and to prevent the side effect of chemotherapeutic compounds.

The wide spectrum of health benefits of pomegranate peels have been attributed to its composition of a wide range of phytochemicals, which is why this study is focused on optimizing the extraction procedure of the bioactive compounds in order to obtain the extracts with the best antimicrobial activities.

2. Materials and Methods

2.1. Fruit Collection and Powder Preparation

Fresh fruits were collected from south Lebanon in 2016

from an altitude of 300 meters. After their collection, fruits were cleaned, washed with water, peeled and the peels were dried in the shade at room temperature and away from sunlight. Dried peels were then grinded to powders and were preserved in clean plastic containers and kept away from light, heat and moisture till use.

2.2. Preparation of Crude Extract by Maceration

15 g of the powdered peels of *P. granatum* were placed in different beakers, each containing 150 ml of one of the three different solvents, water, ethanol or methanol. The solution was macerated at room temperature for a period of at least 24 hrs with constant agitation until the soluble matter was dissolved. After the preparation of the macerate, the solution was filtered using 0.45 µm filter paper and then concentrated by a rotary evaporator at 40°C with reduced pressure. The obtained extracts were then stored at -20°C to be used in different tests [19].

2.3. Preparation of Crude Extracts by Ultrasound

1 g of the powdered peels of *P. granatum* dissolved in 50 ml of the different solvents (water, ethanol or methanol) was placed at 60°C in the ultrasound bath at 50 kHz with a power of 400 W for one hour. The obtained extracts were then filtered and evaporated to be stored at -20°C [20].

2.4. Antibacterial Testing

2.4.1. Bacterial Strain, Media, and Reagents

Two referenced strains were used in this study. One Gram-positive strain, *Staphylococcus epidermidis* (CIP444) and one Gram-negative strain, *Escherichia coli* (ATCC35218). Both strains were stored at -80°C in glycerol stocks and used as required. Brain heart infusion broth/agar (BHI/BHA), tryptic soya broth (TSB) and Mueller-Hinton broth (MHB) were purchased from HIMEDIA (Mumbai, India) and then prepared as indicated by the manufacturer.

2.4.2. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) Assays

MICs and MBCs were determined using the microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) [21]. Both bacterial strains were grown in TSB overnight and then bacterial suspensions of each were prepared using MHB at 5×10^5 CFU/ml to be used for inoculation. Briefly, 100 µl of each extract at an initial concentration (100 mg/ml) were taken to perform serial two-fold dilutions in MHB using a 96-well flat-bottom polystyrene tissue culture-treated microtiter plate (Corning® Costar® 3598; Corning, NY 14831, USA) then 100 µl of the previously prepared suspensions were inoculated into each well. Positive growth control lacking any plant extract and negative growth control lacking a bacterial inoculum were taken into account. The plates were then incubated at 37°C for 24 hrs. The MIC of each extract was taken as the lowest concentration with no visible growth in its corresponding well. Then, all wells with

no visible growth were plated on BHA to determine the MBC which is the lowest concentration able to reduce the initial bacterial inoculum by >99.9% (<5 CFU/plate). Tests were performed in triplicates and mean values were presented.

2.5. Antibiofilm Activity

2.5.1. Biofilm Formation

Assay for biofilm formation was performed in 96-well microtiter plates (polystyrene, flat bottom) as described previously [22]. Briefly, *S. epidermidis* (CIP444) was grown in TSB medium overnight at 37°C, then a bacterial suspension at a density of 5×10^5 CFU/ml was prepared in TSB supplemented with 0.25% glucose. 100 µl of this bacterial suspension were inoculated into each well of a sterile 96-well plate except for column 12 which was used as a negative control and filled only with sterile TSB medium; then, the plates were incubated for 24 hrs at 37°C. Their contents were then removed and the biofilms formed in the wells were fixed by incubating the plates at 80°C for 1 hr [23]. Then the microplates were kept to cool down at room temperature for 30 minutes then washed with distilled water to remove any non-adherent bacteria. Furthermore, 100µL of crystal violet (0.1%) were added to each well for staining then washed after 5 minutes. Finally, 100µL of distilled water was added and the optical density (OD) was measured at 570 nm using microplate reader (Tristar2 S LB 942, Berthold, Germany).

2.5.2. Minimal Biofilm Eradication Concentration (MBEC) Assay

After the fixation of the preformed biofilm as previously described, each well of the microtiter plate was filled with 100 µl of sterile physiological water for use as a diluent for our plant extracts. The serial 1:2 dilution was done with equal volume (100 µl) of the extract in the wells, and the plates were then incubated at 37°C for 18 hrs. The wells were then washed twice with saline water, filled with 100 µl of 0.1% crystal violet and incubated at room temperature for 5 min. The stain was then discarded, and the wells were washed 3 times with saline water. Finally, they were filled with 100 µl of physiological water and the OD₅₇₀ nm was measured. One microtiter plate column was skipped from treatment with the plant extracts and used as an untreated positive control. Tests were performed in quadruples. The Minimal Biofilm Eradication Concentration (MBEC) was defined as the lowest *P. granatum* peels concentration that strongly eradicates the pre-formed biofilm.

2.5.3. Minimal Biofilm Prevention Concentration (MBPC) Assay

One hundred microliters of MHB and 100 µL of extracts

were mixed in the first well column of 96-well microplates and a half serial dilution was done till the 10th well. Bacterial suspension was added as inoculum to each well to give a final concentration of 5×10^5 CFU/ml. Wells lacking any extract were used as positive control for biofilm formation. Wells without bacteria were used as negative control. The remaining steps for fixation, staining, and washing were done as previously described in part 2.4 and O. D. at 570 nm was measured. Minimal biofilm prevention concentration (MBPC) was defined as the lowest concentration exhibiting the highest significant biofilm formation prevention.

2.6. Statistical Analysis

Antibiofilm tests were performed in quadruple, and the results were presented as mean values ± standard errors of the mean (SEM). Differences in the biofilm eradication potentials were evaluated by Kruskal-Wallis test (unpaired non-parametric analysis of variance) followed by Dunn's multiple comparisons test. GraphPad Prism® version 7 (GraphPad Software Inc., CA, USA) was used for statistical analysis, considering * P < 0.05 significant, ** p < 0.01 highly significant and *** p < 0.001 very highly significant.

3. Results and Discussion

3.1. Antibacterial Effect of the Extractive Method of *Punica granatum* Peels

The antibacterial activity was determined according to the method specified above. Antibacterial activities were classified according to the ratio MBC/MIC. If the ratio of MBC/MIC > 4, the effect was considered as bacteriostatic while if the ratio of MBC/MIC < 4, the effect was considered as bactericidal. The results obtained showed that the water extract produced by both the ultrasound and the maceration extraction techniques and the ethanol extract produced by the ultrasound extraction technique have the same bacteriostatic effect on *S. epidermidis* (CIP444) with an MIC= 50 mg/ml (Table 1). Also, the methanol extract produced by both the ultrasound and the maceration extraction techniques and the ethanol extract produced by the maceration extraction technique were the most potent with the same bacteriostatic effect on *S. epidermidis* (CIP444), with an MIC= 25 mg/ml (Table 1). Regarding the MBC, only the methanol extract produced by both the ultrasound and the maceration extraction techniques and the ethanol extract produced by the maceration extraction technique were active with an MBC= 50 mg/ml for *S. epidermidis* (CIP444).

Table 1. MIC and MBC results for the extractive method of *P. granatum* peels against the 2 bacterial strains *S. epidermidis* (CIP444) and *E. coli* (ATCC35218).

Plant extracts	MIC (mg/mL)		MBC (mg/mL)	
	<i>S. epidermidis</i> (CIP444)	<i>E. coli</i> (ATCC35218)	<i>S. epidermidis</i> (CIP444)	<i>E. coli</i> (ATCC35218)
Water ultra	50	25	> IC	50
Water mac	50	25	> IC	50

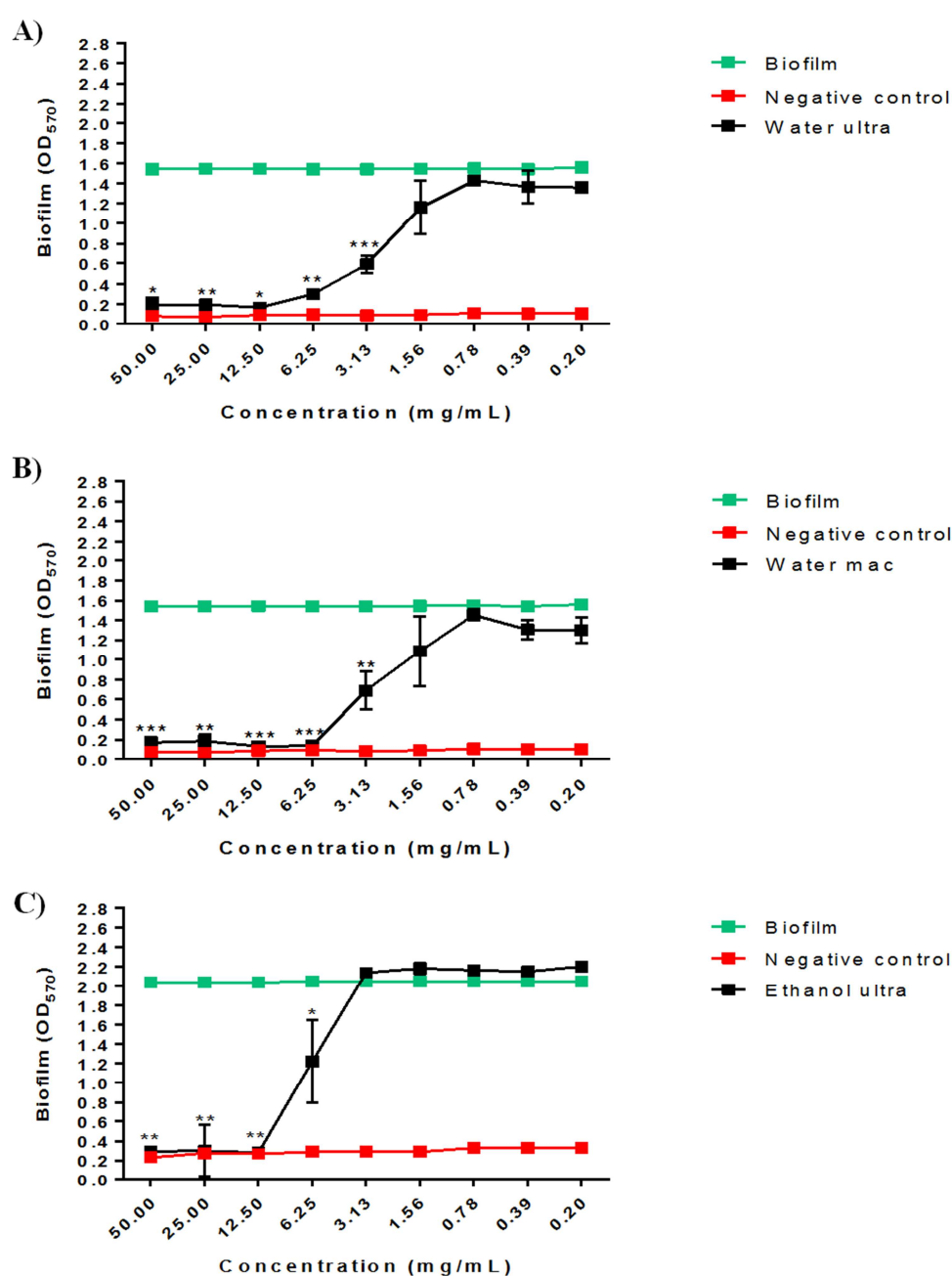
Plant extracts	MIC (mg/mL)		MBC (mg/mL)	
	<i>S. epidermidis</i> (CIP444)	<i>E. coli</i> (ATCC35218)	<i>S. epidermidis</i> (CIP444)	<i>E. coli</i> (ATCC35218)
Methol ultra	25	25	50	50
Methol mac	25	25	50	50
Ethanol ultra	50	25	> IC	50
Ethanol mac	25	25	50	50

Ultra: ultrasound, mac: maceration, MIC: minimal inhibitory concentration, MBC: minimal bactericidal concentration, IC: initial concentration.

For the other extracts, no MBC was obtained for *S. epidermidis* (CIP444) at the used concentration therefore the symbol (>) was used to indicate that a higher concentration might be needed to achieve an effect. While, MBC and MIC values for all the *P. granatum* peels extracts were the same using *E. coli* (ATCC35218), with an MIC= 25

mg/ml and an MBC= 50 mg/ml. In these results, it can be concluded that the different extraction method don't have any bacteriostatic effect or bactericidal effect on *E. coli* (ATCC35218), while the bacteriostatic is more efficient with methanol extract (mac or ultra) and with ethanol extract (mac).

3.2. *Punica Granatum* Peels Extracts Inhibits Biofilm Formation and Eradicates the Preformed Biofilm



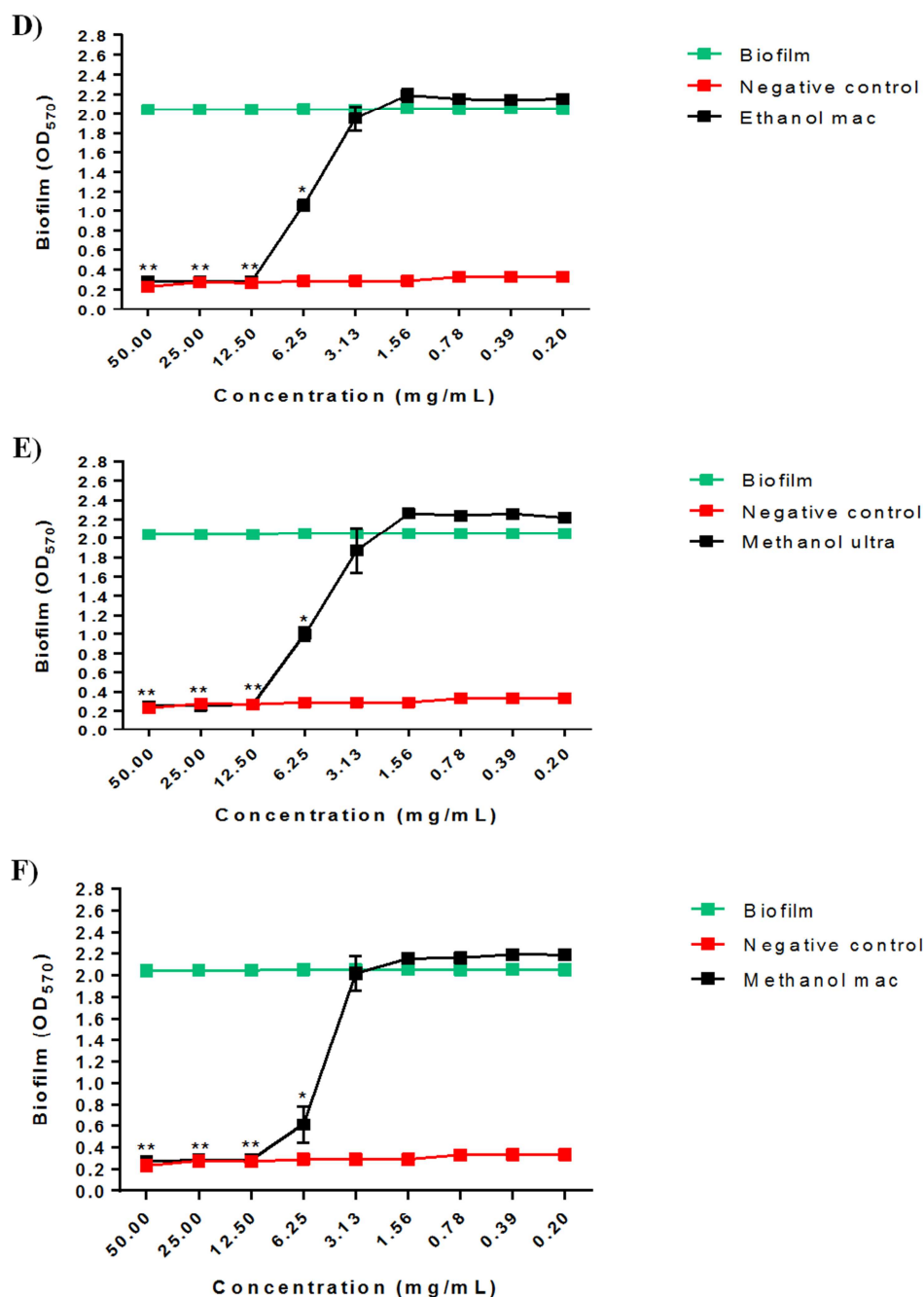


Figure 1. Minimal Biofilm Prevention Concentration (MBPC) assays for the extractive method of *P. granatum* peels against *S. epidermidis* biofilm formation studied spectrophotometrically. A, F. *S. epidermidis* (CIP444) was incubated with different concentrations of different solvents and extraction methods using *P. granatum* plant peels (0-50 mg/ml) for 18 hrs. (A) Water extract produced by ultrasound extraction technique; (B) Water extract produced by maceration extraction technique; (C) Ethanol extract produced by ultrasound extraction technique; (D) Ethanol extract produced by maceration extraction technique; (E) Methanol extract produced by ultrasound extraction technique; (F) Methanol extract produced by maceration extraction technique. Error bars show the standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. MBPC and MBEC results for the extractive method of *P. granatum* peels against *S. epidermidis* (CIP444).

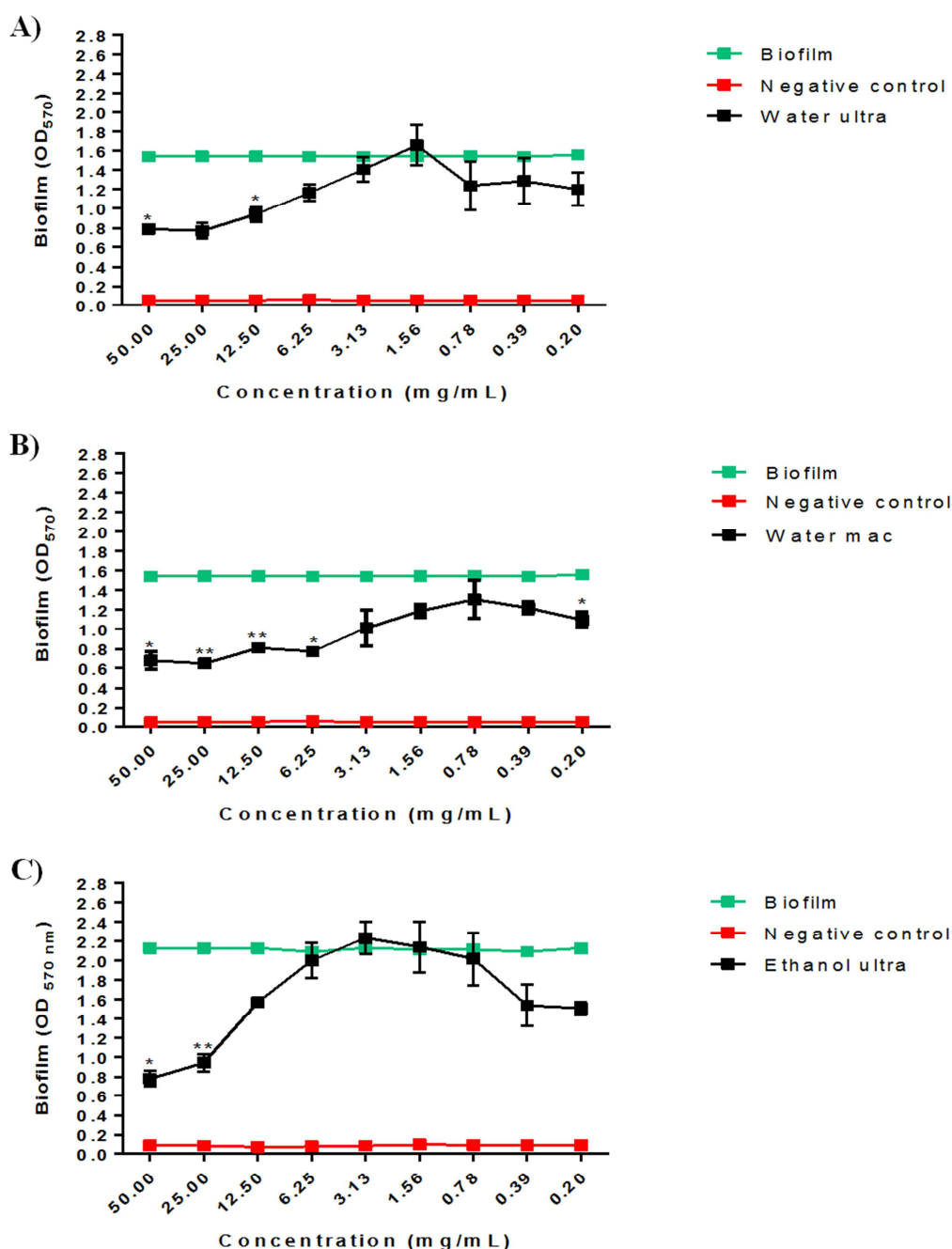
Plant extract	Initial concentration (mg/ml)	Prevention		Eradication	
		MBPC (mg/m)	MBPC (%)	MBEC (mg/ml)	MBEC (%)
Water ultra	100	12.5	94	25	44
Water mac	100	6.25	96	25	54
Methol ultra	100	12.5	100	6.25	55
Methol mac	100	12.5	100	12.5	40
Ethanol ultra	100	12.5	100	25	63
Ethanol mac	100	12.5	100	12.5	38

Ultra: ultrasound, mac: maceration, MBPC: Minimal Biofilm Prevention Concentration. MBEC: Minimal Biofilm Eradication Concentration.

As demonstrated in Table 2 and Figures 1 and 2, all the extracts were able to prevent the formation of *S. epidermidis* (CIP444) biofilm. Their prevention capabilities were proportional to their concentrations. The results obtained showed that methanol and ethanol extracts produced by ultrasound and maceration extraction techniques had visually appreciable antibiofilm activity against the established *S. epidermidis* (CIP444), which had the highest prevention capacity of 100% at a concentration of 12.5 mg/ml (Figure 1). Whereas the water extracts produced by both the ultrasound and the maceration extraction techniques showed relatively lower biofilm prevention capabilities than those of methanol and ethanol extracts, which had a prevention

capacities of 94% and 96% at the concentrations of 12.5 and 6.25 mg/mL, respectively (Figure 1).

In contrast, it was noticed that the eradication activities of water, ethanol and methanol extracts by ultrasound and maceration extraction techniques against *S. epidermidis* (CIP444) for a biofilm production were lower than the prevention efficiencies (Figure 2). The biofilm eradication capacities for water (ultrasound and maceration extraction), methanol (ultrasound and maceration extraction) and ethanol (ultrasound and maceration extraction) extracts were ranging between 44%, 54%, 55%, 40%, 63%, 38%, respectively at the concentrations of 25, 25, 6.25, 12.5, 25, 12.5 mg/ml, respectively.



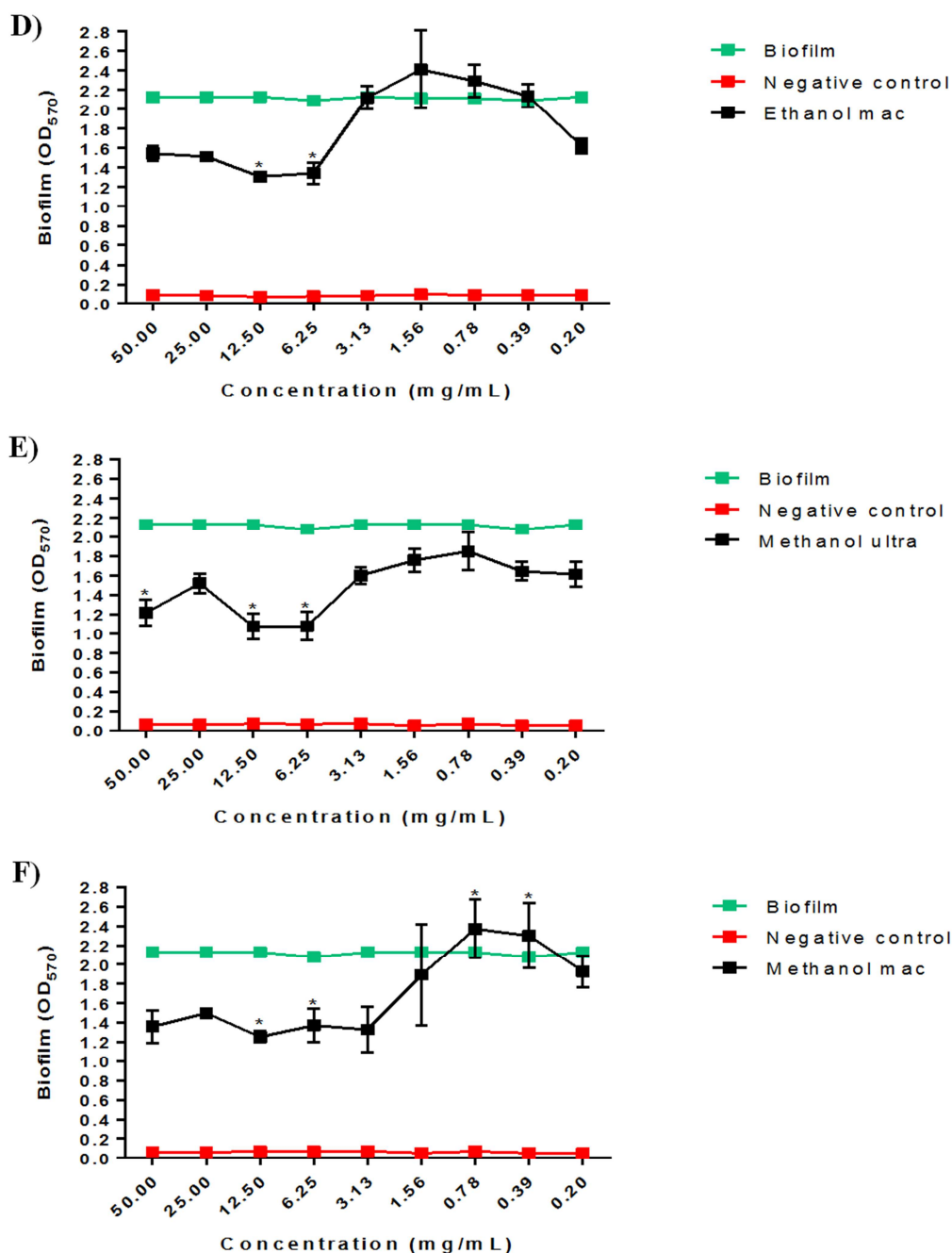


Figure 2. Minimal Biofilm Eradication Concentration (MBPC) assays for the extractive method of *P. granatum* peels against a preformed *S. epidermidis* biofilm studied spectrophotometrically. A, F. *S. epidermidis* (CIP444) biofilm was incubated in different concentrations of different solvents and extraction methods of *P. granatum* plant peels (0-50 mg/ml) for 18 hrs. (A) Water extract produced by ultrasound extraction technique; (B) Water extract produced by maceration extraction technique; (C) Ethanol extract produced by ultrasound extraction technique; (D) Ethanol extract produced by maceration extraction technique; (E) Methanol extract produced by ultrasound extraction technique; (F) Methanol extract produced by maceration extraction technique. Error bars showed the standard deviation. * $P < 0.05$, ** $P < 0.01$.

In India and Asia, pomegranate peels have been used traditionally for centuries to cure various diseases related to bacterial infection, such as diarrhea, dysentery, urinary infections and stomach illness [24]. Numerous *in vitro* and *in vivo* studies demonstrated the antimicrobial activity of pomegranate extracts [4, 25, 26, 27, 28]. Dahham *et al.* [29] showed that the peel extract showed the highest antimicrobial activity compared to other extracts (seed, juice and whole fruit extract). Among the selected bacterial activities, the highest

antibacterial activity was recorded against *Staphylococcus aureus*. The punicalagin and ellagic acid in pomegranate extracts were responsible for their antimicrobial properties [30, 31]. These bioactive compounds play a role in the disruption of the biofilm by the alteration the surface charges of bacteria [32] and by inducing astringency [33]. Endo *et al.* [34] demonstrated that the combination of pomegranate extracts with antibiotics could help to combat drug-resistant pathogens. The pomegranate peels leading to cell lysis of *Staphylococcus*

aureus and *Escherichia coli* by their ability to precipitate membrane proteins and inhibit enzymes such as glycosyl transferases [26, 34, 35, 36, 37, 38, 39]. A recent study showed that the antibiofilm activity of the peel powder of pomegranate was markedly greater than that of the seed powder [40]. An important study indicated that the most potent antibacterial activity of pomegranate peel extracts against the selected bacteria are those extracted with acetone, methanol and water, respectively [41]. Another study confirmed also that the antibacterial activity of methanol extracts of PP is more effective than water extracts [29].

4. Conclusion

The aim of this work was to determine the best extraction method for pomegranate peel to obtain the highest antimicrobial capacity. The extract in methanol (by ultrasound and maceration) and the extract in ethanol (by maceration), showed an antibacterial activity against *S. epidermidis* (CIP444), comparable to each other but higher than the extract in ethanol (by ultrasound) or water. The antibacterial activities for all the pomegranate peel extracts were the same against the bacterial strain *E. coli* (ATCC35218). Also, it was demonstrated that ethanol and methanol extracts have higher antibiofilm activities against *S. epidermidis* (CIP444) than the water extract. Apart from inhibiting the formation of biofilm, the highest biofilm eradication activity for the pre-formed biofilms was obtained in ethanol extract with the ultrasound technique. Finally, this study demonstrated that the good extraction method is important to give a best antimicrobial activity.

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Conflict of Interest

All the authors do not have any possible conflicts of interest.

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