Evaluation of the Cytotoxicity, Antibacterial, Antioxidant, and Anti-inflammatory Effects of Different Extracts of *Punica granatum var. pleniflora*

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Abstract

Background: *Punica granatum var. pleniflora* (PGP) has been used for thousands of years as an effective agent to treat various types of diseases. However, there are a few new evidences addressing its therapeutic effects and related mechanisms. Therefore, the aim of this study was to investigate the cytotoxic, antioxidant, antibacterial, and anti-inflammatory effects of ethanolic (ET), dichloromethane (DM), and ethyl acetate (EA) extracts of PGP.

Methods: ET, DM, and EA extracts of PGP were first prepared using maceration method. Total phenolic content (TPC) of PGP was then assessed by the Folin-Ciocalteu assay, and its antioxidant capacity was determined by DPPH and FRAP methods. Furthermore, in-vitro antibacterial activity of the PGP extracts was performed. The effect of PGP on the viability of J774A.1, HUVECs, HT29, and MCF-7 cell lines was evaluated by the MTT assay. The anti-inflammatory effect of PGP was assessed in the lipopolysaccharide (LPS)-stimulated J774A.1 cell line using qRT-PCR method.

Results: EA extract contained the highest phenolic content (383.3 ± 9.1 mg gallic acid/g extract) and showed the highest antioxidant activity ($IC_{50} = 36.5 \pm 2.3$ µg/mL). PGP at concentration of 15 µg/mL significantly decreased the expression of COX-2 (ET) and iNOS (ET and EA) in J774A.1 cell. Also, EA showed the highest antibacterial activity. Furthermore, the PGP extracts decreased the viability of all tested cell lines in a concentration-dependent manner. As indicated by IC_{50}, EA demonstrated the lowest IC_{50} for all tested cell lines.

Conclusion: According to the results, antioxidant, anti-inflammatory, antibacterial, and cytotoxic effects of PGP might be driven by its phenolic compounds highly presented in the EA extract.

Introduction

For centuries, medicinal plants have been used as safe, cost-effective, and easy-to-access therapeutic agents for a variety of diseases so that recently the trend has been turned back to the use of herbal remedies instead of synthetic drugs (1). It has been shown that the adverse effects of herbal drugs are relatively less than alternative synthetic ones when they are used properly (2). Furthermore, the rates of death or hospitalization due to herbs are fewer than those related to synthetic drugs (1). Herbs and their derived natural compounds affect different cellular protective mechanisms, as well as anti-inflammatory, antibacterial, antioxidant, and anticancer effects (3-5). Hence, herbal compounds are considered as an interesting source for developing new remedies.

Punica granatum var. pleniflora (PGP) belongs to Punicaceae family and its therapeutic effects are attributed to its flowers, known as "Golnar-e-farsi" in traditional Persian medicine (TPM) (6). Traditionally, PGP has been used as an astringent, hemostatic, antibacterial, antifungal, antiviral, and as a remedy for different diseases (6,7). However, there are a few scientific evidences approving these traditional documents. For example, it has been used for the treatment of minor recurrent aphthous stomatitis (MiRAS) (8). In another study, the antibacterial activity of PGP was attributed to its phenolic and flavonoid contents (9). Moreover, PGP is considered as a safe and effective mouthwash in the treatment of gingivitis of patients with diabetes in comparison with chlorhexidine mouthwash (7).

In contrast to PGP, there are several evidences evaluating the therapeutic effects of *Punica granatum var. sativa* (PGS) or pomegranate in different in vitro and in vivo studies (10,11), indicating that PGS has antimicrobial, antioxidant, anticancer, and anti-inflammatory effects (10). A recent study by Prakash et al. (2011) has reported the existence of some unique phenolic compounds like punicalagin and punicalin in pericarp, leaf, and flower of PGS (12).

However, it seems that the mechanisms behind the therapeutic effect of PGP have been less investigated, therefore, the present study was conducted to investigate the cytotoxic, antibacterial, anti-inflammatory, and antioxidant effects of different fractions of PGP in in vitro experiments.

Materials and Methods

Plant material collection, extraction, and phytochemical analysis

The study protocols were approved by the Ethics Committee of Kerman University of Medical Sciences (Ethical code: IR.KMU.REC.1398.042). The flowers of PGP were collected from Kerman, Iran, in May/July 2017. The samples were authenticated by a professional herbalist using a voucher specimen (KF1634-1) deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences. Air-dried flowers were grounded by an electronic grinder and extracted with 80% ethanol using maceration method (13). The extract was dried in an oven, and then, a certain amount of dried extracts was suspended in ethanol (ET). The suspended extract was exposed to dichloromethane (DM) or ethyl acetate (EA) in order to fractionate compounds according to their polarity. Afterward, the extracts were dried separately in an oven at 45°C and stored at -20°C for further analysis.

Phytochemical screening of the PGP extracts was done to confirm the existence of alkaloids, tannins, flavonoids,

saponins, anthraquinones, cardiac glycosides, steroids, and terpenoids (14).

Determination of total phenolic content (TPC) of PGP extracts

In Folin-Ciocalteu (F-C) assay, the reagent reacts with antioxidants to produce a blue complex that can be measured at 765 nm. The TPC of each extract was determined using F-C method as previously reported (15). Briefly, 100 µL of the extract or a standard solution of gallic acid was mixed with 500 µL of diluted F-C reagent (1:10 v/v). Afterwards, 400 µL of an aqueous solution of sodium carbonate (Na2CO3) (7.5% w/v) was added and the obtained mixture was incubated for 30 min in darkness at $25 \pm 2°C$. Then, 4000 µL of distilled water was added and the absorbance was measured at 765 nm using a multi-mode microplate reader (BioTek®, USA) against solvents as blank. The TPC of the fractions (mg gallic acid/g extract) was determined using the regression equation obtained from the standard calibration curve of gallic acid (150-500 µg/mL).

Evaluation of the antioxidant effect of the PGP extracts
α, α-diphenyl-β-picrylhydrazyl (DPPH) assay

DPPH assay can be used to evaluate antioxidant capacity. For example, when this free radical solution is reduced by an antioxidant, its color will change from violet to colorless. The free radical-scavenging activity of the fractions of PGP was assessed using DPPH assay. Different concentrations of the PGP extracts or DPPH as positive control (50 µl) were added to 0.004% w/v methanol solution of DPPH (150 µl). A blank was also performed using the same procedure to the solution without material. Then, the absorbance of color of samples and

blank was measured at 517 nm using a multi-mode microplate reader (BioTek®, USA) (5). Antioxidant activity of extracts was presented as IC_{50}, which is defined as the concentration of extracts cause a 50% reduction in the initial DPPH concentration. All measurements were performed three times.

Ferric reducing antioxidant power (FRAP) assay

In the FRAP assay, the colorless oxidized form of iron (Fe^{3+}) is converted to a blue-colored Fe^{2+} tri-pyridyl triazine (TPTZ). The assay measures the changes in the absorbance at a wavelength of 593 nm. As previously described, 5 µL of the sample was added to 295 µL of fresh FRAP reagent constituted with mixing of TPTZ, HCl, and $FeCl_3$ and warmed to $37°C$ prior to the use. The absorbance of the reaction mixture was then recorded at 593 nm after 10 min. The standard curve was constructed using $FeSO_4$ solution (100-1000 µmol/L). The results were expressed as µmol Fe(II)/g dry weight of extract and the assay was carried out in triplicate (16).

Evaluation of the antimicrobial activity
Bacteria

In the present study, *Staphylococcus aureus* (PTCC 25923), *Enterococcus faecalis* (PTCC 29212), *Streptococcus pyogenes* (PTCC 1447), and *Bacillus subtilis* (PTCC 1365) were used as gram-positive bacteria while Escherichia coli (PTCC 25922) and Serratia marcescens (PTCC 1053) were used as gram-negative bacteria.

Determination of minimum inhibitory concentration (MIC)

Two-fold dilutions of PGP fractions (0.2-8 mg/mL) were prepared to determine the MICs using broth microdilution

method (17). 2-(p-iodophenyl)-3-(p-nitrophenyl)-5phenyl tetrazolium chloride (INT) (Merck, Darmstadt, Germany) solution (10 μL of 0.6 mg/mL concentration) was used for the characterization of the microbial growth. Finally, the lowest concentration of the extracts that prevented bacterial growth was recorded as the MIC value.

Evaluation of the cytotoxicity effect of the PGP extracts

All materials for cell culture were obtained from Invitrogen (Carlsbad, CA, USA). The J774A.1 murine macrophage cell line, human umbilical vein endothelial cells (HUVECs), MCF-7 breast tumor cell line, and HT-29 human colorectal adenocarcinoma cell line were purchased from Iranian Biological Resource Center (Tehran, Iran). All cell lines were cultured in DMEM high glucose supplemented with 10% FBS and 1% (v/v) Penicillin/Streptomycin. They were maintained in a humidified chamber with a 5% CO_2 atmosphere at 37°C. Cells were seeded into the 96-well culture plates at a density of 10,000 cells per well for J774A.1, MCF-7, and HT-29, and 5,000 cells per well for HUVECs. Cells were treated with or without PGP extracts with different concentrations (7-500 μg/mL) for 24 hours (h). Afterward, MTT solution (Sigma, St Louis, MO, USA) was added and cells were incubated in darkness for 4 h. After adding dimethyl sulfoxide (DMSO), the

absorbance was measured at 570 nm using a microplate reader (Bio Tek ELX800, USA) (18).

Evaluation of the anti-inflammatory activity of PGP

In order to evaluate the anti-inflammatory effect of the PGP extracts, J774A.1 cells were exposed to bacterial lipopolysaccharide (LPS) at a concentration of 5 μg/mL (5,19,20), and then, treated with different non-toxic concentrations of PGPs (ET and DM: 31 μg/ml and EA: 15 μg/ml). At the next step, non-treated and treated J774A.1 cell lines were used for the total RNA extraction using

Rnx-plus solution (CinnaGen, Tehran, Iran) according to the manufacturer's instructions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Complementary DNA was synthesized by TAKARA kits according to the manufacturer's recommendation. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out using an ABI system with a mixture of 6 μL Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, UK), cDNA template, and 1 μL specific primers (Table 1). The threshold cycle (Ct) was determined for 18s and the target genes (COX-2 and iNOS) of each sample. Subsequently, ΔCt was calculated and relative quantitation of each mRNA expression was normalized to the expression of the housekeeping gene 18s (21,22).

Table 1. Primer sequences for real-time quantitative PCR

Gene	Sense Primer(5'→ 3')	Anti-sense(5'→ 3')
COX2	5' AACCGCATTGCCTCTGAAT 3'	5' CATGTTCCAGGAGGATGGAG 3'
iNOS	5' CGAAACGCTTCACTTCCAA 3'	5' TGAGCCTATATTGCTGTGGCT 3'
18S	5' GTAACCCGTTGAACCCCATT 3'	5' CCATCCAATCGGTAGTAGCG 3'

iNOS: Inducible nitric oxide synthase
COX-2: Cyclooxygenase-2

Statistical analysis

Statistical analysis was done by GraphPad Prism version 8.0.0 (Graph Pad Software, San Diego, CA, USA). One-way ANOVA test was used for comparison of differences among multiple groups followed by Tukey's post-hoc test for exploring significantly different groups. Data were analyzed by unpaired student's t-test for comparison of differences between two groups.

Results

Phytochemical analysis

Phytochemical constituent of PGP is shown in Table 2. Tannin, flavonoid, and cardiac glycoside were observed in ET extract. Furthermore, EA fraction also contained tannin and flavonoid components.

Table 2. Phytochemical constituent of the PGP extracts

Phytochemical Constituent	Test Applied	ET	EA	DM
Alkaloid	Dragendroffs and Mayer reagent	-	-	-
Tannin	Ferric chloride test solution	+	+	-
Flavonoid	Lead acetate and dilute ammonia test	+	+	-
Saponin	Froth assay	-	-	-
Anthraquinone	Borntragers assay	-	-	-
Cardiac glycoside	Keller-killianis assay	+	-	-
Steroid	Liebermann-Burchard assay	-	-	-
Terpenoid	Salkowski's assay	-	-	-

+: Presence, -: Absence

TPC of PGP

The TPC of PGPs is shown in Table 3. The TPC of EA was about 383.3 ± 9.1 mg gallic acid/g extract, which is obviously higher than that of other extracts as shown in Table 3.

Table 3. Total phenolic content (TPC) of the PGP extracts

Extract Type	Gallic Acid (mg)/Extract (g)
Ethanolic	236.9 ± 6.7
Dichloromethane	130.6 ± 4.1
Ethyl acetate	383.3 ± 9.1

Antioxidant activity of PGP

Free radical-scavenging ability by the DPPH assay

As shown in Table 4, the highest scavenging activity belonged to EA fraction with IC_{50} value of 36.5 ± 2.3 µg/mL, while Butylated hydroxytoluene (BHT) exhibited the IC_{50} value of 53.8 ± 3.2 µg/mL.

Table 4. DPPH scavenging activity and IC50 values for different fractions of PGP

Extract Type	ET	DM	EA	BHT
IC_{50} (µg/mL)	$65`.1 \pm 2.03*$	$101.1 \pm 8.4****$	$36.51 \pm 2.31**$	53.8 ± 3.2

ET: Ethanolic extract of PGP
DM: Dichloromethane extract of PGP
EA: Ethyl acetate extract of PGP
BHT: Butylated hydroxytoluene (served as positive control)
All data are represented as mean $\pm$ SEM of three independent experiments.
(n=3; *$P<0.05$, ** $P<0.01$, **** $P<0.0001$ vs. BHT group)

FRAP assay

As shown in Table 5, EA fraction at all tested concentrations demonstrated significantly (P<0.01) higher level of antioxidant activity than Vitamin C. ET extract showed a similar pattern of antioxidant activity for Vitamin C.

Table 5. Comparative evaluation of antioxidant activity via FRAP assay

		Samples			
		ET	DM	EA	Vitamin C
	1000	4.2 ± 0.4	1.7 ± 0.4***	5.8 ± 0.4**	4.2 ± 0.4
	500	3.5 ± 0.3	1.2 ± 0.1***	4.7 ± 0.3**	3.3 ± 0.6
Concentrations (µg/mL)	250	2.4 ± 0.1	0.7 ± 0.1***	3.2 ± 0.1***	2.2 ± 0.3
	125	1.5 ± 0.1	0.5 ± 0.1**	2.0 ± 0.2***	1.2 ± 0.2
	62	0.9 ± 0.0	0.31 ± 0.0**	1.2 ± 0.1**	0.8 ± 0.2

ET: Ethanolic extract of PGP
DM: Dichloromethane extract of PGP
EA: Ethyl acetate extract of PGP
All data are represented as mean ± SEM of three independent experiments
(n=3; *P<0.05, ** P<0.01, **** P<0.0001 vs. Vitamin C group as a positive control)

Antibacterial activity of PGP

As shown in Table 6, among all tested extracts, EA had the lowest MIC for all types of bacteria, especially *E. faecalis*, while DM had the highest MIC, except for *B. subtilis*.

Table 6. Minimum inhibitory concentration (mg/mL) of PGP fractions

Microorganism / Extract Type	*S. aureus*	*B. subtilis*	*E. coli*	*E. feacalis*	*S. marcescens*	*S. pyogenes*
ET	8	4	8	8	>8	8
DM	8	4	>8	>8	>8	8
EA	2	2	2	1	2	2

ET: Ethanolic extract of PGP
DM: Dichloromethane extract of PGP
EA: Ethyl acetate extract of PGP

Cytotoxic effect of PGP

The cytotoxic effect of different concentrations of PGP extracts (7-500 µg/ml) on normal and cancerous cells was evaluated using MTT assay. As shown in Figure 1, PGP extracts reduced the viability of all cell lines including MCF-7, HT-29, HUVECs, and J774A.1 in a concentration-dependent manner. As indicated by the IC_{50} values (Table 7), it seems that the ET and EA extracts had a much higher effect on cancerous cell lines including MCF-7 and HT-29. Moreover, EA demonstrated the lowest IC_{50} for all cell lines.

Table 7. IC$_{50}$ values for PGP extracts

Cell line	IC$_{50}$ (µg/ml) ± SEM		
	ET	**EA**	**DM**
MCF-7	348.9 ± 28.1	199.0 ± 24.6	420.5 ± 10.9
HT-29	438.3 ± 18.8	327.5 ± 61.1	500 µg/ml≥
HUVECs	500 µg/ml≥	500 µg/ml≥	500 µg/ml≥
J774A.1	500 µg/ml≥	444.8 ± 45.6	500 µg/ml≥

ET: Ethanolic extract of PGP

DM: Dichloromethane extract of PGP
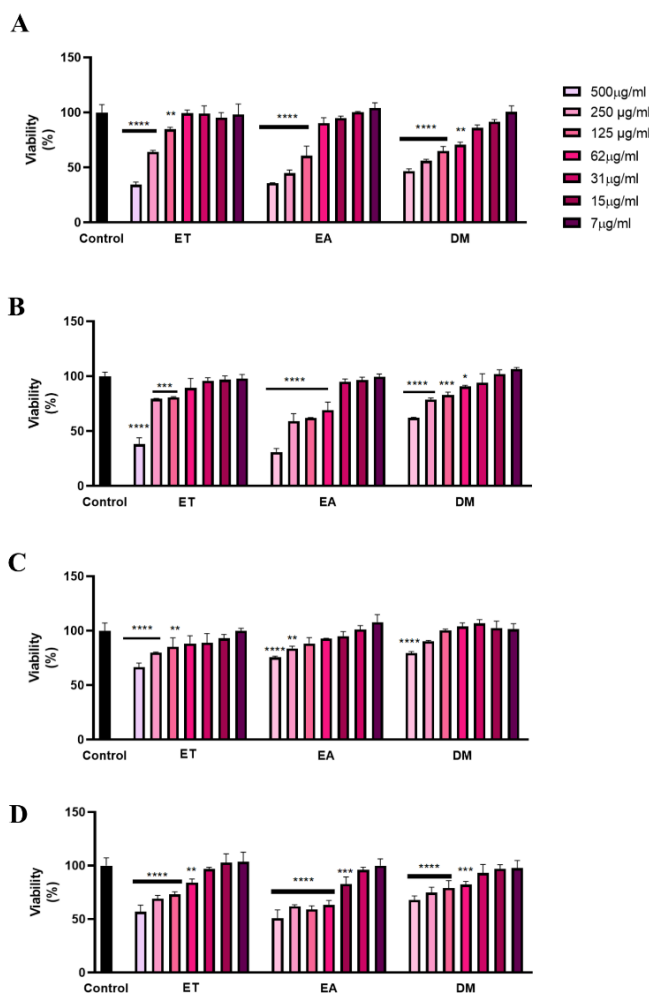
EA: Ethyl acetate extract of PGP

Figure 1. Evaluation of the effects of PGP total extracts and its fractions on the viability of (A) MCF-7, (B) HT-29, (C) HUVECs, and (D) J774A.1 cell lines by MTT assay. The viability percentage of all cell lines was shown after treatment with different concentrations of PGP total extract and its fractions. The results are presented as mean ± SEM (n=6; *P<0.05 (a), **P<0.01 (b), ***P<0.001 (c) vs. control).

Anti-inflammatory activity of PGP

As shown in Figure 2, the LPS-treated cell had a remarkable higher expression of both COX-2 (P<0.01) and iNOS (P<0.001) genes compared to the control group. The expression of COX-2 (P<0.05) and iNOS (P<0.01) was significantly decreased by ET extract at concentration of 31 µg/ml. EA fraction had a tendency to decrease the expression of COX-2, but this decrease was not significant. However, it significantly reduced the expression of iNOS at concentration of 15 µg/ml (P<0.05).
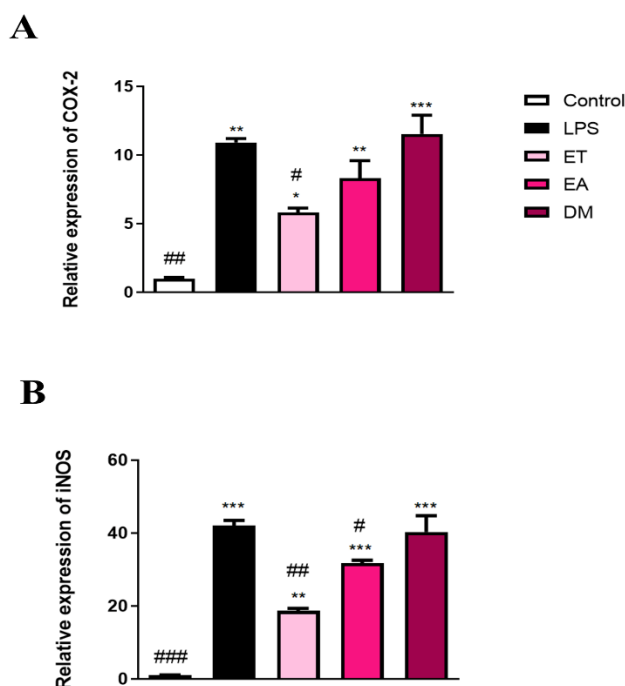

Figure 2. The effect of PGP total extract and its fractions on the expression levels of COX2 (A) and iNOS (B) genes in the LPS-treated J774A.1 cell line. LPS significantly increased the expression level of both inflammatory genes. PGP total extract and EA fraction reduced the expression of inflammatory genes. All data are represented as mean ± SEM of three independent experiments (n=3; *P<0.05 (a), **P<0.01 (b), ***P<0.001 (c) vs. control and #P<0.05 (a), ##P<0.01 (b), ###P<0.001 (c) vs. LPS group).

Discussion

Nowadays, herbal remedies are widely preferred by many people all around the world (23) due to their lower side effects, availability, and affordability (24). Furthermore, herbs as a source of many effective metabolites can be a good substitute for synthetic drugs (25). The results of the present study revealed that different extracts of PGP had radical-scavenging activity at a wide range of concentrations from 62 µg/mL to 1000 µg/mL. The EA extract with IC_{50} of 36.5 ± 2.3 µg/mL showed the highest scavenging activity. To the best of our knowledge, only a study by Gavanji et al. (2014) has assessed the antioxidant activity of total hydro-alcoholic extract of PGP by DPPH assay (8). Their results showed IC_{50} of 87.4 ± 2.8 for hydro-alcoholic extract, which is comparable to the results of this study indicated IC_{50} of 65.1 ± 2.8 for ET extract.

Regarding Folin-Ciocalteu, the results also demonstrated that ET, EA, and DM extracts of PGP contained 236.9 ± 6.7, 383.3 ± 9.1, and 130.6 ± 4.1 mg gallic acid/g of extract, respectively. A previous study showed that the total hydro-alcoholic extract of PGP contained 258.1 ± 9.1 mg/mL gallic acid/g of extract (8), which is consistent with the result of this study. Another study demonstrated that the TPC of PGP ranged from 3.8 to 18.1 mg gallic acid/g of dry powder. Also, the chloroform and methanolic fractions had the lowest and highest TPC, respectively. Similar to the results of the present study, they proved the existence of more flavonoid compounds in both ET and EA extracts (9).

In the present study, PGP and its fractions also showed an inhibitory effect against both gram-positive and gram-negative bacteria. MIC ranged from 1 mg/mL to more than 8 mg/mL for different fractions. *B. subtilis*, a gram-positive bacterium showed the highest sensitivity to the extracts and *S. marcescens*, a gram-negative bacterium, showed the highest resistance against different extracts. The EA fraction had the highest antibacterial effect compared to other extracts with MIC of 2 mg/mL for all tested bacteria except for *E. fecalis* (MIC: 1 mg/mL). Although there is no report on the antibacterial effect of the PGP extracts, the difference between antibacterial activity of fractions of other herbs was supported by the previous reports (26-27). Njeru et al. (2015) have reported that EA and DM fractions of *Premna resinosa* had better anti-bacterial activity than petro ether and methanol fractions (26). In the present study, DM extract had the lowest antibacterial activity among all extracts, which is consistent with the results of a study by Bazzaz et al. (2011) on *Scutellaria litwinowii* (27). They showed that EA and methanolic extracts had greater anti-bacterial activity compared to DM extract at

concentration of 6.5 to 100 mg/mL. In contrast, DM fraction of *Barringtonia asiatica* has been reported to have an antibacterial activity (28). This discrepancy may be related to the type of bacterial spices used or plants. According to the results of phytochemical analysis of PGP in this study, there was high levels of tannin and flavonoid in EA fraction. These components might be responsible for its antibacterial activity.

The results of this study showed that PGP extracts decreased the viability of all four cell lines in a concentration-dependent manner. However, the IC_{50} of PGP reported in the present study, is higher than the IC_{50} of pomegranate, as one of the most studied members in this family (29). For example, Bekir et al. (2013) showed that the cytotoxicity effect of pomegranate leaves extract on MCF-7 was dependent on the type of solvent, so that among five derived extracts, methanolic, ET, and EA extracts, exhibited promising results with the IC_{50} values less than 50 mg/L. In contrast, DM and hexane extracts had low IC_{50} value (> 100 mg/L) (30). The present study revealed that EA extract had the best cytotoxic effect compared to the ET and DM extracts. The previous study suggested that the higher TPCs of pomegranate presented in polar extracts predominantly contributed to their higher cytotoxic activity (30). In another study, the MTT test was applied to evaluate the cytotoxic effect of ET extract of pomegranate HepG2 and Caco cell lines. All tested extracts reduced cell viability in a dose-dependent manner with IC_{50} values less than 50 mg/L (31).

The present study also investigated the effect of PGP extracts on the expression of COX-2 and iNOS as two important inflammatory mediators (32), in the LPS-treated J774A.1 cell line. The expression of these genes was evaluated

using qRT-PCR and the results showed that ET extract at concentration of 31 μg/mL remarkably reduced the expression of COX-2 and iNOS in the

LPS-treated J774A.1. Moreover, ET at concentration of 31 μg/mL significantly reduced the expression of iNOS in the LPS-treated J774A.1 cell line. It was shown that anti-inflammatory effects may be related to the content of flavonoid compounds (33,34). Hämäläinen et al. (2007) investigated the anti-inflammatory effect of 36 natural flavonoids on the LPS-treated macrophages. They showed that these compounds inhibited the production of iNOS, nitric oxide, and NFκB factor (35). Likewise, in a previous study by Jung et al. (2006), pomegranate reduced the expression of TNF-α in the LPS-stimulated microglial cells (36). Although EA extract had a higher flavonoid contents and better antioxidant activity compared to ET, its anti-inflammatory activity was lower. The reason for this discrepancy is that in the TPC and antioxidant power measurements, an equal amount of the PGP extracts was chosen. However, for inflammatory test, the first

non-toxic concentration was selected. As a limitation of the present study, it was better to evaluate a similar non-toxic concentration of PGP extracts in this study.

Conclusion

The present study provided more in-depth information about the possible mechanisms behind the therapeutic effects of PGP. The results of this study proved that PGP can inhibit the expression of mediators engaged in inflammation and oxidative stress and had anti-bacterial effects especially against *B. subtilis*. It can be concluded that the effects of PGP extracts are highly related to its flavonoid contents. Nonetheless, additional in vitro and in vivo studies should be performed to understand unknown therapeutic aspects of PGP and its constituent.

References

1. Karimi A, Majlesi M, Rafieian-Kopaei M. Herbal versus synthetic drugs; beliefs and facts. J Nephropharmacol 2015; 4(1):27-30.

2. Calixto J. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res 2000; 33(2):179-89.

3. Sitarek P, Rijo P, Garcia C, Skała E, Kalemba D, Białas AJ, et al. Antibacterial, anti-inflammatory, antioxidant, and antiproliferative properties of essential oils from hairy and normal roots of Leonurus sibiricus L. and their chemical composition. Oxid Med Cell Longev 2017; 2017:7384061.

4. Mehrzadi S, Bahrami N, Mehrabani M, Motevalian M, Mansouri E, Goudarzi M. Ellagic acid: a promising protective remedy against testicular toxicity induced by arsenic. Biomedicine & Pharmacotherapy 2018; 103:1464-72.

5. Raeiszadeh M, Esmaeili-Tarzi M, Bahrampour-Juybari K, Nematollahi-mahani SN, Pardakhty A, Nematollahi MH, et al. Evaluation the effect of Myrtus communis L. extract on several underlying mechanisms involved in wound healing: An in vitro study. South African Journal of Botany 2018; 118:144-50.

6. Gavanji S, Larki B, Bakhtari A. Comparative analysis of extract of Punica granatum var.

pleniflora (Golnar-e-farsi) to some antibiotics on pathogens. Journal of Essential Oil Bearing Plants 2015; 18(1):168-78.

7. Sedigh-Rahimabadi M, Fani M, Rostami-Chijan M, Zarshenas MM, Shams M. A traditional mouthwash (Punica granatum var pleniflora) for controlling gingivitis of diabetic patients: a double-blind randomized controlled clinical trial. J Evid Based Complementary Altern Med 2017; 22(1):59-67.

8. Gavanji S, Larki B, Bakhtari A. The effect of extract of Punica granatum var. pleniflora for treatment of minor recurrent aphthous stomatitis. Integr Med Res 2014; 3(2):83-90.

9. Mahboubi A, Asgarpanah J, Sadaghiyani PN, Faizi M. Total phenolic and flavonoid content and antibacterial activity of Punica granatum L. var. pleniflora flowers (Golnar) against bacterial strains causing foodborne diseases. BMC Complement Altern Med 2015; 15:366.

10. Miguel MG, Neves MA, Antunes MD. pomegranate (punica granatum l.): a medicinal plant with myriad biological properties-a short review. Journal of Medicinal Plants Research 2010; 4(25):2836-47.

11. Arun N, Singh DP. Punica granatum: a review on pharmacological and therapeutic properties. IJPSR 2012; 3(5):1240-45.

12. Prakash CV, Prakash I. Bioactive chemical constituents from pomegranate (Punica granatum) juice, seed and peel-a review. International Journal of Research in Chemistry and Environment 2011; 1(1):1-18.

13. Rameshk M, Sharififar F, Mehrabani M, Pardakhty A, Farsinejad A, Mehrabani M. Proliferation and in vitro wound healing effects of the microniosomes containing narcissus tazetta l. bulb extract on primary human fibroblasts (HDFs). Daru 2018; 26(1):31-42.

14. Sofowora A. Medicinal Plants and Traditional Medicine in Africa. Wiley; 1982.

15. Raeiszadeh M, Pardakhty A, Sharififar F, Mehrabani M, Nejat-mehrab-kermani H, Mehrabani M. Phytoniosome: a novel drug delivery for Myrtle extract. Iran J Pharm Res 2018; 17(3):804-17.

16. Bahrampour-Juybari K, Ebrahimi G, Momeni-Moghaddam MA, Asadikaram G, Torkzadeh-Mahani M, Akbari M, Mirzamohammadi S, Karimi A, Nematollahi MH, et al. Evaluation of serum arsenic and its effects on antioxidant alterations in relapsing-remitting multiple sclerosis patients. Mult Scler Relat Disord 2018; 19(1):79-84.

17. Raeiszadeh M, Pardakhty A, Sharififar F, Farsinejad A, Mehrabani M, Hosseini-Nave H, et al. Development, physicochemical characterization, and antimicrobial evaluation of niosomal myrtle essential oil. Res Pharm Sci 2018; 13(3):250-61.

18. Nematollahi MH, Pardakhty A, Torkzadeh-Mahanai M, Mehrabani M, Asadikaram G. Changes in physical and chemical properties of niosome membrane induced by cholesterol: a promising approach for niosome bilayer intervention. RSC Advances 2017; 7(78):49463-72.

19. Zhang Y, Liu C, Dong B, Ma X, Hou L, Cao X, et al. Anti-inflammatory activity and mechanism of surfactin in lipopolysaccharide-activated macrophages. Inflammation 2015; 38(2):756-64.

20. Seghatoleslam A, Hemmati M, Ebadat S, Movahedi B, Mostafavi-Pour Z. Macrophage Immune Response Suppression by Recombinant Mycobacterium tuberculosis Antigens, the ESAT-6, CFP-10, and ESAT-6/CFP-10 Fusion Proteins. Iranian Journal of Medical Sciences 2016; 41(4):296-304.

21. Pham TN, Jeong SY, Kim DH, Park YH, Lee JS, Lee KW, et al. Protective mechanisms of avocado

oil extract against ototoxicity. Nutrients 2020; 12(4):947-52.

22. Verbout NG, Benedito L, Williams AS, Kasahara DI, Wurmbrand AP, Si H, et al. Impact of adiponectin overexpression on allergic airways responses in mice. J Allergy (Cairo) 2013; 2013: 349520.

23. Halberstein RA. Medicinal plants: historical and cross-cultural usage patterns. Ann Epidemiol 2005; 15(9):686-99.

24. George P. Concerns regarding the safety and toxicity of medicinal plants-An overview. Journal of Applied Pharmaceutical Science 2011; 1(6):40-4.

25. Kumar S, Bajwa B, Kuldeep S, Kalia A. Anti-inflammatory activity of herbal plants: a review. Int J Adv Pharm Biol Chem 2013; 2(2):272-81.

26. Njeru SN, Obonyo MA, Nyambati SO, Ngari SM. Antimicrobial and cytotoxicity properties of the crude extracts and fractions of Premna resinosa (Hochst.) Schauer (Compositae): Kenyan traditional medicinal plant. BMC Complement Altern Med 2015; 15:295.

27. Bazzaz BSF, Khayat MH, Emami SA, Asili J, Sahebkar A, Neishabory EJ. Antioxidant and antimicrobial activity of methanol, dichloromethane, and ethyl acetate extracts of Scutellaria litwinowii. ScienceAsia 2011; 37(4):327-34.

28. Umaru IJ, Badruddin FA, Umaru HA. Phytochemical screening of essential oils and antibacterial activity and antioxidant properties of Barringtonia asiatica (L) leaf extract. Biochemistry Research International 2019; 2019:7143989.

29. Kalaycıoğlu Z, Erim FB. Total phenolic contents, antioxidant activities, and bioactive ingredients of juices from pomegranate cultivars worldwide. Food Chem 2017; 221:496-507.

30. Bekir J, Mars M, Souchard JP, Bouajila J. Assessment of antioxidant, anti-inflammatory, anti-cholinesterase and cytotoxic activities of pomegranate (Punica granatum) leaves. Food Chem Toxicol 2013; 55:470-5.

31. El-Awady MA, Awad NS, El-Tarras AE. Evaluation of the anticancer activities of pomegranate (Punica granatum) and harmal (Rhazya stricta) plants grown in Saudi arabia. Int J Curr Microbiol App Sci 2015; 4(5):1158-67.

32. Kwon DJ, Ju SM, Youn GS, Choi SY, Park J. Suppression of iNOS and COX-2 expression by flavokawain A via blockade of NF-κB and AP-1 activation in RAW 264.7 macrophages. Food Chem Toxicol 2013; 58:479-86.

33. Raso GM, Meli R, Di Carlo G, Pacilio M, Di Carlo R. Inhibition of inducible nitric oxide synthase and cyclooxygenase-2 expression by flavonoids in macrophage J774A. 1. Life Sci 2001; 68(8):921-31.

34. Fu Y, Chen J, Li YJ, Zheng YF, Li P. Antioxidant and anti-inflammatory activities of six flavonoids separated from licorice. Food Chem 2013; 141(2):1063-71.

35. Hämäläinen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007; 2007:45673.

36. Jung KH, Kim MJ, Ha E, Uhm YK, Kim HK, Chung JH, et al. Suppressive effect of Punica granatum on the production of tumor necrosis factor (Tnf) in BV2 microglial cells. Biol Pharm Bull 2006; 29(6):1258-61.