

Antioxidant and Anti-Inflammatory Effects of *Punica granatum* L. Seed Extract in Raw 264.7 Macrophages

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Abstract

This study was designed to investigate the antioxidant, cytotoxic and anti-inflammatory activity pomegranate seed extract. Pomegranate seed was extracted by different polarity solvents (1% HCl/Methanol, Methanol, Ethanol) with sequential shaking time and subjected to various antioxidants *in vitro* model systems for evaluating the antioxidant activity. The anti-inflammatory activity was evaluated using RAW264.7 macrophages induced by Lipopolysaccharide (LPS). The results showed that 1% HCl/Methanol Pomegranate Seed Extract (PSE) possessed highest total phenolic (99.73±0.62 mg/g DM) contents with maximum antioxidant activity. The selected 1% HCl/Methanol PSE (10µg/ml) showed the inhibitory effect on nitric oxide production induced by LPS in RAW 264.7 macrophages indicating potential anti-inflammatory activity. It was observed that PSE significantly reduced the level of TNF α , iNOS, IL6 and IL β mRNA and proteins of LPS stimulated RAW264.7 macrophages. Finally, the antioxidant and anti-inflammatory activity of PSE observed via different *in vitro* model systems.

Keywords: Anti-Inflammatory Activity; Antioxidant; Extract; Pomegranate; RAW264.7 Macrophages

Abbreviations

PSE	:	Pomegranate Seed Extract
LPS	:	Lipopolysaccharide
PSO	:	Pomegranate Seed Oil
TNF	:	Tumor Necrosis Factor
IL	:	Interleukin
INF	:	Interferon
NF- κ β	:	Nuclear Factor-K β
iNOS	:	Nitric Oxide Synthase
COX-2	:	Cyclooxygenase-2
TPC	:	Total Phenolic Contents

Introduction

Pomegranate (*Punica granatum* L.), one of the oldest

known fruit has been investigated for all of its parts i.e. leaf, peel, juice, seed with potential health benefits of bioactive constituents [1-3]. Pomegranate considers as a nutritionally superior quality fruit with pharmaceutical potential and there is a progressive increase in its cultivation area around the world. Pomegranate seeds (20% of whole fruit) considered as waste after processing, possess a lot of bioactive compounds, including 3,3-Di-O-methylelagic acid, 3,3,4-Tri-O-methylelagic acid, punicic acid, γ -tocopherol, hydroxybenzoic acids, ursolic acid, phenyl aliphatic glycosides as phenethylrutinoside [4-6]. Pomegranate seed oil has been investigated against various physiological disorders e.g. pomegranate seed oil (PSO) inhibit human PC-3 human prostate cancer cell phospholipase A2 expression *in-vitro*, inhibit prostaglandin biosynthesis [7]. Various studies showed that pomegranate seed extracts as waste possess the antioxidant activity, however, the bioactive compounds from seed residues have not been reported yet.

Inflammation considered as the main reason behind various physiological disorders e.g. atherosclerosis, rheumatoid arthritis with globally increasing prevalence. During the inflammation process, certain inflammation mediator e.g. pro-inflammatory cytokines (tumor necrosis factor (TNF), IL-6, IL-12, IL-18,

interleukin IL-1, interferon (INF) γ released. In the response of these pro-inflammatory mediators, anti-inflammatory cytokines (IL-4, IL-10, IL-13, IFN- α) released and antagonized the effect of the pro-inflammatory mediators. The transcription factor ‘nuclear factor- $\kappa\beta$ (NF- $\kappa\beta$)’ controls the expression of several genes that encode pro-inflammatory cytokines, chemokines, inducible enzymes such as cyclooxygenase-2 (COX-2), adhesion molecules, and growth factors [8]. Some of the inducible enzymes like nitric oxide synthase (iNOS), (COX-2) accelerate the generation of pro-inflammatory mediators. However, inflammation is primarily a protective process in the animal body against the allergens, microbe, and toxins, but in the case of chronic inflammation, the adverse response mechanism emerge to be flawed and the severe inflammation can be a cause of the beginning of various disorders [1].

In ancient times, inflammation and its related disorders treated with some plants or plant-based treatments including herbs, weeds, fruits and vegetable parts [9-12]. It has been demonstrated by experimental studies that different plant-based extracts and their chemical constituents possess the anti-inflammatory activity i.e. turmeric (*Curcuma longa*) extract showed anti-inflammatory and anti-atherosclerotic effects [12], ginger (*Zingiber zerumbet*) extract inhibit the production of nitric oxide (NO), prostaglandin E₂ (PGE₂) and iNOS production in cell culture, mulberry (*Morus alba*), strawberry (*Fragaria ananassa*) and loquat (*Eriobotrya japonica*) extracts lessened the IL6 and IL-1 β proinflammatory cytokines expression and also re-adjust the anti-inflammatory cytokines response in cell culture [13].

The objective of this study was to evaluate the defatted pomegranate seed residues of one of the best pomegranate varieties of Pakistan for its antioxidant potential including polyphenolic contents, DPPH and ABTS activity. After performing the above analysis, one of the best variety was selected from three varieties on the base of its antioxidant results and its anti-inflammatory was evaluated in LPS-stimulated RAW 264.7 murine macrophages using *in-vitro* model and characterize its effect on the nuclear translocation.

Methods and Materials

Materials

Murine RAW264.7 macrophage was from DSMZ (Braunschweig, Germany). Supplements and cell culture media were purchased from PAA (Coelbe, Germany). Quantitative real-time Polymerase Chain Reaction (PCR) and RNA isolation reagents were from Bioline (Luckenwalde, Germany) and Quantace (London, UK). Primers were from MWG Biotech (Ebersberg, Germany). Western blotting reagents and materials were purchased from Bio-Rad (Bio-Rad Laboratories GmbH, Muenchen, Germany). Luciferase activity assay was obtained from Promega (Mannheim, Germany).

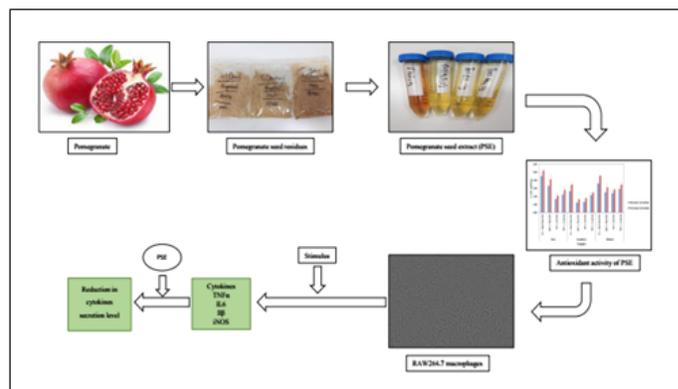
Antioxidant Extraction from Seed Residues

Optimization of extraction solvent

Preparation of extracts for the determination of antioxidant potential in pomegranate seed residues was optimized. The seed residues were extracted by using different solvents by one and two stages of extraction. The solvent able to extract the highest amount of antioxidant extract using a system combination of sequential extractions, different shaking time and different solvent polarities (1% HCl in methanol, 80% methanol, 70% ethanol, and 70% acetone) was used (Table 1).

Varieties	Solvents	Extraction time (Hours)
Desi	1% HCl in methanol	1 and 1+1
	80% methanol	
	70% ethanol	
	70% acetone	
Kandhari	1% HCl in methanol	
	80% methanol	
	70% ethanol	
	70% acetone	
Bedana	1% HCl in methanol	
	80% methanol	
	70% ethanol	
	70% acetone	

Table 1: Treatment plan for the extraction of antioxidant extract from pomegranate seed residues thro different solvents and extraction time.



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Antioxidant extraction

The selected solvents were used for the extraction of antioxidant

extract from seed residues of all three varieties, all the extraction was made in triplicate and in two stages. The selected solvents 1% HCl in methanol, 80% methanol, 70% ethanol, and 70% acetone were used for the extraction of antioxidant extract from pomegranate dried powder, the extraction was made in triplicate and in two stages. In the first stage, 0.5g samples extracted in a 10ml solvent in 15ml centrifuge tubes by shaking samples at a medium speed of Vortex-Genie 2 (Scientific Industries Inc. USA) then samples were centrifuged at 2800g in Heraeus Megafuge 16 R centrifuge (Thermo Fisher Scientific, USA) for 15 minutes and decanted. In the second stage, the samples residues from the stage one were re-extracted with 4ml solvent following the same procedure. These two aliquots were mixed and stored at 4°C in the dark for further study [14].

Total Phenolic Contents (TPC)

The TPC were quantified by using FolinCiocalteu reagent [15]. The 0.5 mL Folin-Ciocalteu reagent and 7.5 mL deionized water were mixed with 50 ml of all extract samples followed by incubation at room temperature for 10 minutes afterward addition of 20% sodium carbonate (w/v). The mixture kept in a water bath for 20 minutes at 40°C followed by cooled in an ice bath. The absorbance was measured by spectrophotometer at 765 wavelengths (U-2001, Hitachi Instruments Inc. Tokyo, Japan). TPC were calculated by gallic acid standard curve within the range of 10-100 µg/ml ($R^2 = 0.9900$). The results were expressed as Gallic Acid Equivalents (GAE) mg/g of oil. All samples were analyzed triplicate and results averaged.

Antioxidant Activity of Defatted Pomegranate Seed Extracts

DPPH Radical Scavenging Activity

DPPH radical scavenging activity of PSE was measured according to the method of GÜLÇİN (2010)[16]. Radical scavenging activity was calculated by the following formula.

$$\text{Reduction of absorbance (\%)} = [(AB - AA) / AB] \times 100$$

AB = absorbance of blank sample (t = 0 min)

AA = absorbance of tested extract solution (t = 15 min)

ABTS Radical Scavenging Assay

ABTS radical scavenging activity of PSE was measured according to the method of MILLER et al. (1996)[17]. Radical scavenging activity was calculated by the following formula.

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Where $\text{Abs}_{\text{control}}$ is the absorbance of ABTS radical + methanol and $\text{Abs}_{\text{sample}}$ is the absorbance of ABTS radical + sample extract/standard.

Cell culture and cytotoxicity assays

Dulbecco's modified eagle medium was used for the cultivation of murine RAW264.7 macrophages with the addition of 100 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal bovine serum. Standard conditions (37°C and 5% CO₂) in humidified incubator were used for cell's growth with medium was replace with a new one after every two days. The 0.5×10^5 cells/cm² initial cell density was used for seeding cells for further experiments. RAW264.7 cells were incubated for 24 h with increasing concentrations of PSE (5, 10, 10, 30 µg/ml) to evaluate any toxic effect of pomegranate seed extract on cells viability. The neutral red assay was used to assess the cell viability in percent of solvent treated control cells. RAW264.7 cells were incubated with different concentrations (5, 10, 10, 30 µg/ml) of PSE and stimulated with (10 ng/ml; from *Salmonella enteritidis*, Sigma) for 2 up to 24 h for further experiments.

Nitrite Oxide Measurement

Griess reagent (1 g/L sulfanilamide and 0.1 g/L N-1-naphthylethylenediamine in 2.5% phosphoric acid solution) method was followed for the measurement of total nitrites in cell medium [18]. The RAW264.7 cells were incubated at 37°C for 12 h with or without LPS afterward 100 µL of each cell medium was mixed with an equal volume of Griess reagent with 10 minutes incubation at room temperature. The absorbance was read at 540 nm and compared with known standard solutions of NaNO₂.

Cytokine quantification

After 24 h of incubation of cells, the supernatant of cell cultures was diluted with an appropriate quantity of mouse TNFα Duo Set ELISA development kit (Rand D Systems, Wiesbaden, Germany) for the determination of tumor necrosis factor alpha (TNFα). Fluorescence-based Multiplex method (Bio-Rad) was used for determining the TNFα concentration in murine plasma samples.

RNA isolation and real-time PCR

The trizure reagent was used for the isolation of RNA and quantification of total RNA made by photometrically. Real-time qPCR (RT-qPCR) was conducted as a two-step method using the SensiMix Two-Step Kit. The Rotorgene 3000 (Corbett Life Science, Sydney, Australia) cyler was used for quantification of gene expression which was calculated by use of a standard curve. Standard tools (Spidey, Primer3, NCBI Blast) were used to design primers of TNFα, 1β, inducible nitric oxide synthase (iNOS) and IL6. Housekeeping gene β-actin was used for the expression of the target genes.

Western Blotting

Whole cell extracts were prepared by lysing the murine RAW264.7 macrophages with RIPA buffer (50 mmol/l Tris-

HCl, 150 mmol/l NaCl, 0.5% deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), and 1% NP-40; pH 7.4 with protease inhibitor cocktail, 1:100, by incubation on ice for 30 min and subsequent centrifugation at 12,000×g (4°C, 30 min). BCA assay (Pierce, Illinois, USA) was used for the determination of protein concentration in the supernatant of cell cultures. The 40 µg of protein was separated thro 12% SDS/polyacrylamide gel and transferred onto an immunoblot polyvinylidene fluoride membrane. Tris-buffered saline with 3% non-fat milk powder and 0.05% Tween-20 (TBS/T) at pH 7.4 used to block membrane for 2 h and probed with an anti-HO-1 antibody (Stressgen, Biotech, Canada) and β-actin (Santa Cruz Biotechnology, Heidelberg, Germany) at 4°C overnight. Afterward, those blocked membranes were incubated with conjugated secondary antibodies (1:4000) with horseradish peroxidase for 45 min. Chemiluminescence (ECL) reagent was used to visualize the specific bands on a ChemiDoc system and Quantity One programmer was used to quantify densitometrically. The predicted sizes for HO-1 and β-actin are 32 and 43 kDa, respectively, which were checked by the use of molecular weight markers.

The method of WAGNER et al. (2010)[19] was used for the isolation of nuclear fraction isolation. Protein contents were quantified in cytoplasmic and nuclear supernatants and anti-p65 (Santa Cruz) was used in western blotting for the detection of nuclear translocated p65 protein (75 kDa).

Statistical Analysis

The data was statistically analyzed by using SPSS Version 13.0 (Munich, Germany) and for normal distribution and equality of variance. Significant ranges were further compared using Duncan Multiple Range (DMRt) test. Data are expressed as mean±S. E. M. Significance level was established at p<05.

Results and Discussion

Antioxidant Activity

In current study, the total antioxidant potential of PSE was evaluated by various *in vitro* assays. The TPCof PSE for all three cultivars of pomegranate were compared as presented in Figures 1-4. The data showed the significant difference between varieties, solvents and extraction times for TPC. The highest extraction efficiency was observed in 1% HCl/MeOH solvent followed by 80% methanol while the lowest in 70% EtOH solvent. The TPC varied from 1.20±0.06-4.56±0.82 µMGAE/g in one-hour extraction and 1.45±0.09-5.12±0.91 µMGAE/g in two-hour extraction. The overall highest mean values of TPC were observed in Desi variety in 1% HCl/MeOH solvent while the lowest mean values were observed of Kandhari in 80% MeOH solvent (Figure 2). The Figure 3 represents the ABTS⁺ radical scavenging activity of PSE showed the highest value 15.82±1.09 µM Trolox/g for Desi in 1% HCl/MeOH and lowest as 5.80±0.50 µM Trolox/g for Kandhari in 70% EtOH in one hour extraction while highest as 17.85±1.25 µM Trolox/g for Desi in 1% HCl/MeOH and 5.80±0.90 µM Trolox/g for Kandhari in two hour extraction. The overall highest mean value for ABTS⁺ scavenging activity was observed in 1% HCl/MeOH for Desi and lowest in 70% EtOH for Kandhari variety. All of the solvent extracts of pomegranate seeds with different extraction times quenched the DPPH directly and showed the significant effect on the antioxidant activity as presented in Figure 4. The highest scavenging activity (1.83±0.09 and 1.85±0.10 µM Trolox/g) was observed in 1% HCl/methanol Desi extract while the lowest (1.05±0.08 and 1.02±0.05 µM Trolox/g) in 80% methanol Desi extract in one and two-hour extraction, respectively (Figure 4).

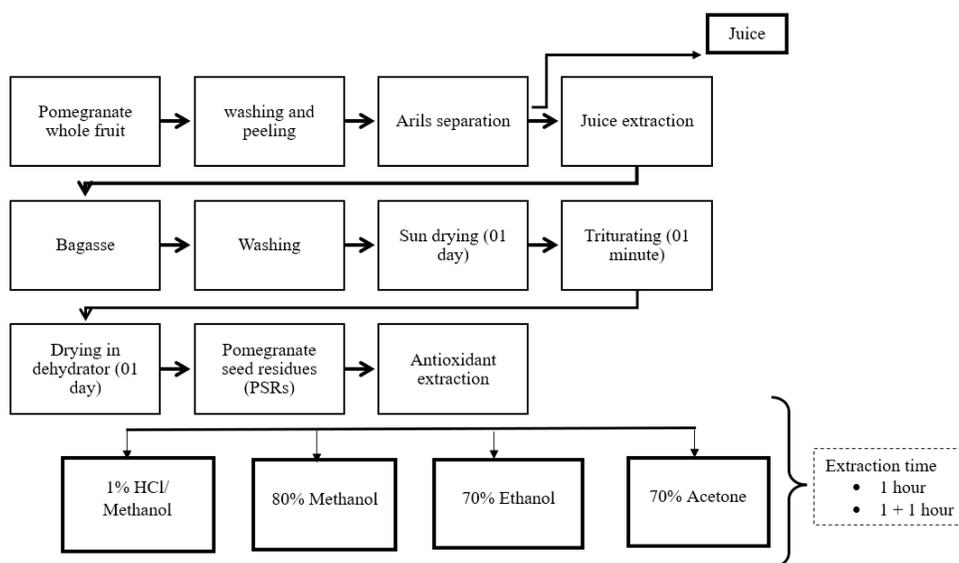


Figure 1: Flowchart of the process to obtain Pomegranate Seed Extract (PSE).

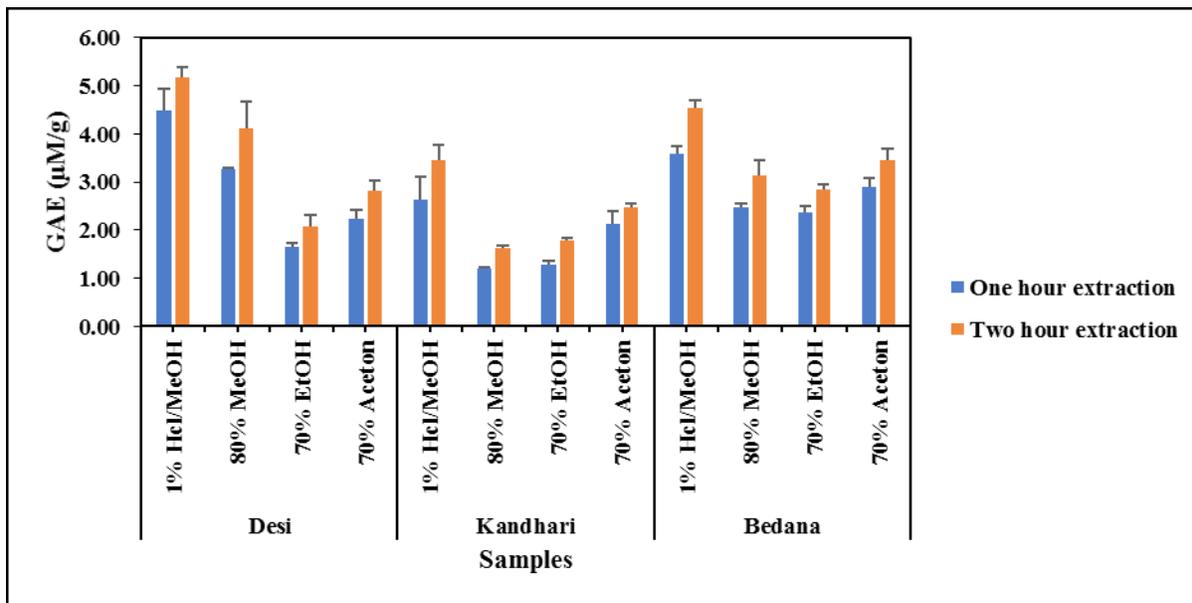


Figure 2: Total phenolic contents of pomegranate seeds residues. Results were expressed as GAE (mg/g) pomegranate seed residues. Tests were conducted in triplicate, with mean values shown and standard deviations depicted by the vertical bars.

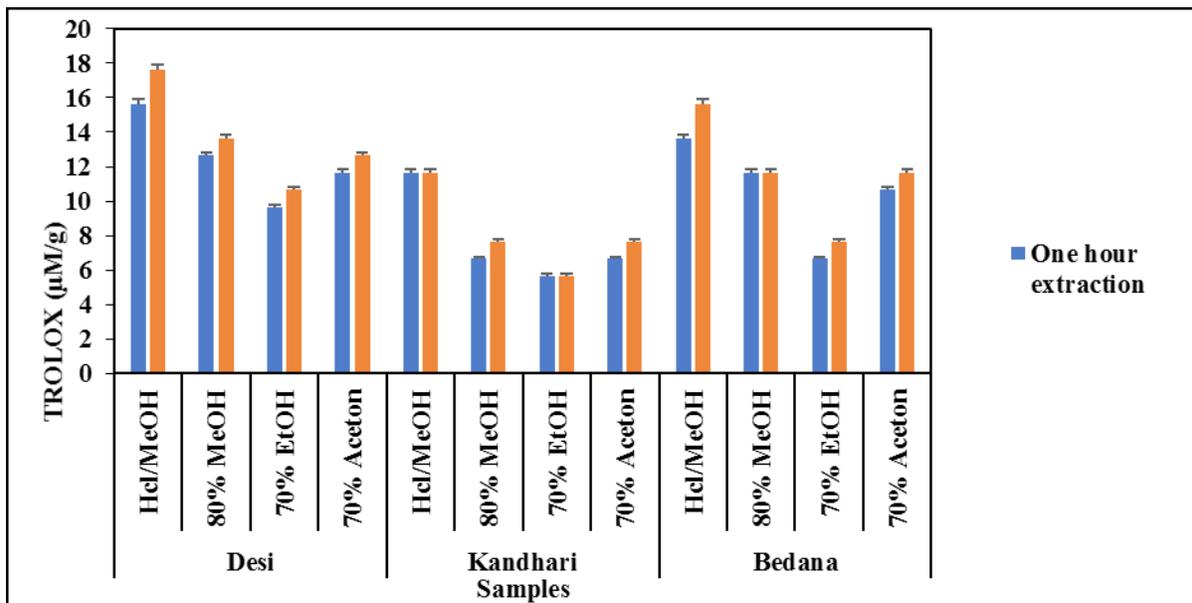


Figure 3: ABTS⁺ scavenging ability of pomegranate seeds residues. Results were expressed as µmol trolox equivalents/g pomegranate seed residues. Tests were conducted in triplicate, with mean values shown and standard deviations depicted by the vertical bars.

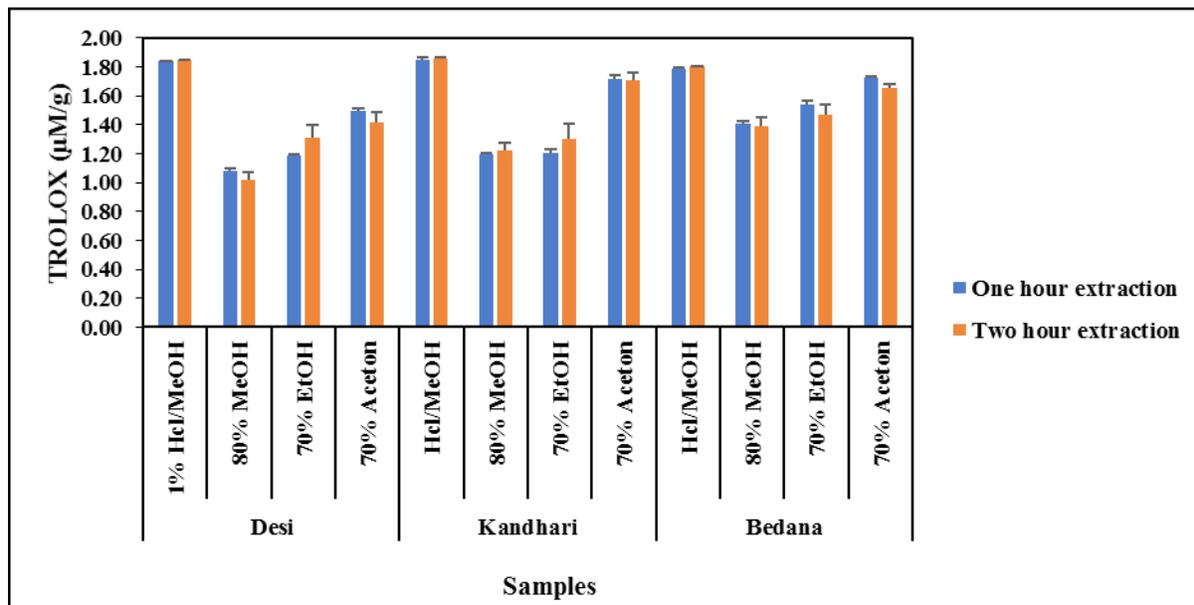


Figure 4: DPPH. scavenging ability of pomegranate seeds residues. Results are expressed as µmol/g trolox of pomegranate seed residues. Tests were conducted in triplicate, with mean values shown and standard deviations depicted by the vertical bars.

Cytotoxic Effect of Pomegranate Extract on Cell Viability

Cell viability of RAW264.7 macrophages was assured by incubating the cells with increasing concentration (0-30µg/ml) of PSE for 24 hours to assure that the pomegranate seed extract may not exert any toxic effect on cells and examined thro neutral red assay. As presented in Figure 5, pomegranate extract affects the cell viability significantly from 77-96%. A decreasing trend in cell viability was observed with increasing concentration of the extract. However, a less cell viability (87%) observed in the 5µg/ml extract while the highest cell viability (96%) at 10µg/ml extract. Based on these results, 10µg/ml pomegranate extract was selected for further study.

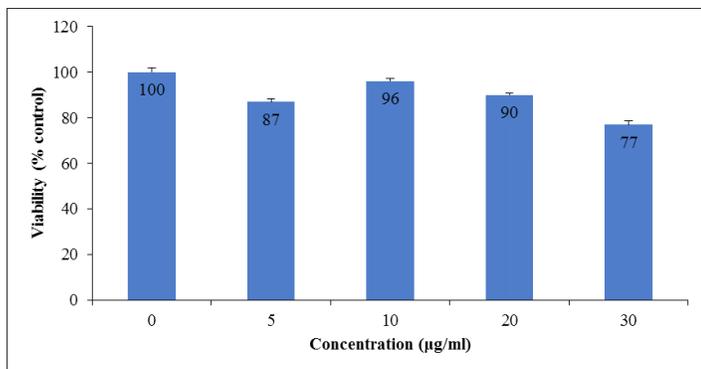


Figure 5: Effect of different concentrations of pomegranate seed extract on cell viability of RAW264.7 macrophages. Data are mean±S.E.M. of three independent experiments.

Effect of Pomegranate Seed Extract on Nitric Oxide (NO) Production

To evaluate the effect of PSE on the NO production in LPS activated RAW264.7 macrophages, Griess reaction method was used, known as the index for the measurement of NO in cell culture. It measures the nitrite accumulated in cell medium during incubation. It was observed that a markedly increased production of NO noticed in LPS stimulated cells while PSE inhibited the NO production after 12 hours stimulation in different concentrations. However, an increase was observed in NO production with increasing concentration of PSE. The highest inhibition of NO production observed in PSE (10µg/ml) as presented in Figure 6.

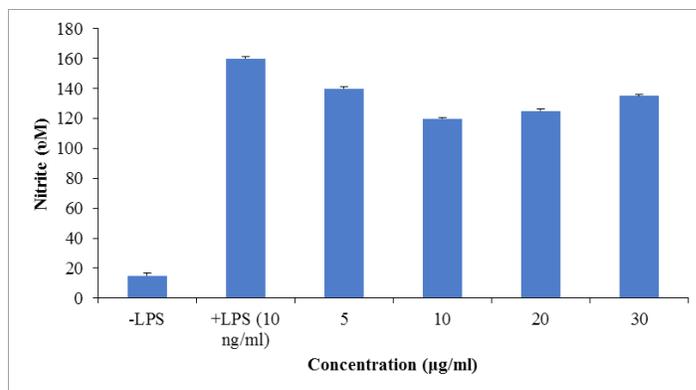


Figure 6: Effect of different concentrations of pomegranate seed extract on nitric oxide (NO) production in RAW264.7 macrophages induced by LPS. Data are mean±S.E.M. of three independent experiments.

Effect of Pomegranate Seed Extract on Cytokines Expression and Its Secretion Level

RAW264.7 cells were incubated by PSE (10µg/ml) and then stimulated with LPS for 24 hours. The TNFα mRNA level measured after 2 hours while secretion level measured after 24 hours of incubation. It was observed that PSE reduced the both TNFα mRNA level and TNFα secretion as shown in (Figure 7a,b). The PSE (10µg/ml) significantly reduced the level of iNOS, IL6 and ILβ mRNA and proteins of LPS stimulated RAW264.7 macrophages (Figure 8).

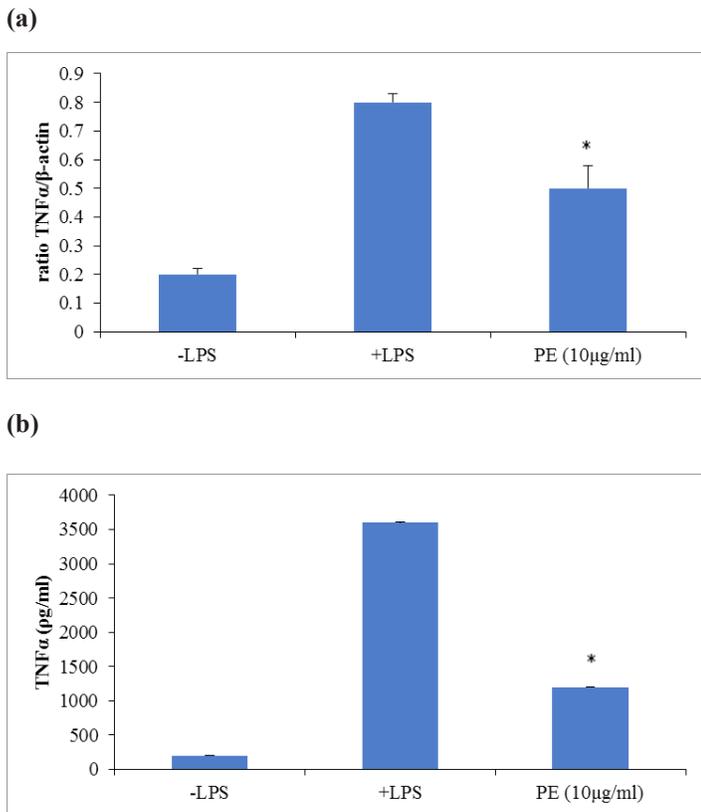


Figure 7: Effect of pomegranate extract (10µg/ml) on (a) TNFα mRNA levels and (b) TNFα secretion in RAW264.7 macrophages stimulated by LPS. Data are mean±S.E.M. of three independent experiments.

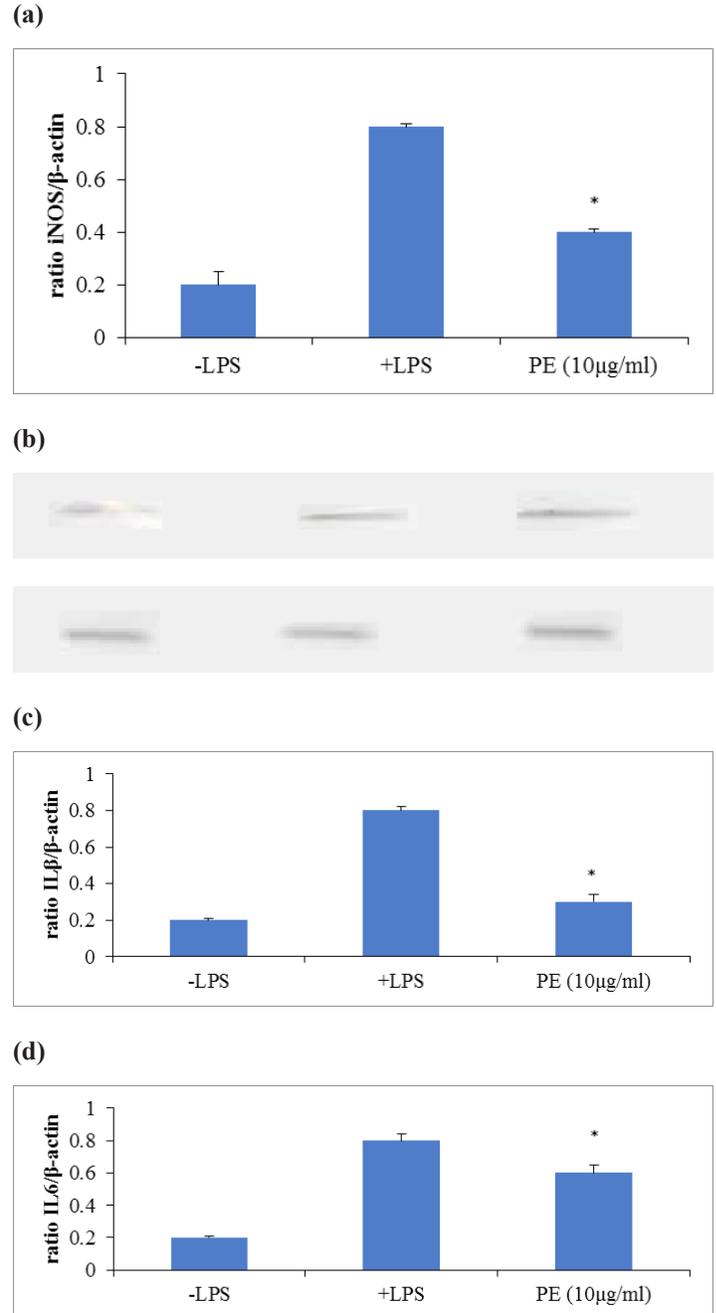


Figure 8: Effect of pomegranate extract (10µg/ml) on (a, c, d) iNOS, ILβ and IL6 mRNA levels and (b) iNOS protein levels in RAW264.7 macrophages stimulated by LPS with 6 h incubation determined by real time PCR and after 24 h incubation by western blotting. Data are mean±S.E.M.

Effects of PSE on Nfkb Nuclear Translocation

Western blot analysis was performed using the nuclear extract of cell culture to study the effect of PSE (10 μ g/ml) after the LPS activation of RAW264.7 macrophages on the translocation of the stimulated p65 sub-unit in the nucleus. It was observed that p65 induced noticeably in RAW264.7 macrophages nucleus but decreased in the presence of PSE (10 μ g/ml) as presented in Figure 9.



Figure 9: Effect of PSE on p65 translocation in LPS-stimulated RAW 264.7 macrophages. Shown is a representative western blot.

Discussion

From centuries, pomegranate is known as a therapeutic agent against various health issues due to the polyphenolic enriched composition as reported in different studies. It is believed that pomegranate possesses the anti-inflammatory, anti-oxidative, and anti-cancer activity [20]. The phenolic fraction of pomegranate seed composed of hydrolysable tannins (punigluconin, punicalin, pedunculagin, punicalagin) [21]. Different fractions of pomegranate i.e. peel, bark, leaves, flower etc. were studied for their anti-inflammatory activity. In this study, pomegranate seed extract was used to evaluate its anti-inflammatory potential. The NO production was considered to be related to the pathogenesis of several diseases including inflammation.

The present study results are in agreement with the previous studies on pomegranate polyphenol contents. It was observed in this study that highest TPC were detected in 1% HCl/MeOH as compared to previous studies in which the highest TPC were extracted by 80% MeOH [22,23]. The higher extraction yield in 1% HCl/MeOH might be due to some compounds in pomegranate seeds that are more soluble in methanol with HCl as compare to individual organic solvent. The HCl may help to some phenolic compounds to hydrolyze from seed residues that remained attached in other solvents. In past, methanolic HCl has not been used in any study to extract phenolic compounds from pomegranate. So, the methanolic HCl could be a better solvent for the extraction of phenolic compounds from plant-based foods in future studies. Data presented that pomegranate seed residue is potential source of bioactive compounds and showing higher radical scavenging activity. However, the TPC found in Pakistani pomegranate cultivar is higher as compared to previously studied pomegranate cultivars. JING et al. (2012) [23] investigated the TPC in four China-grown cultivars

and found the highest phenolic contents in the range of 1.29 to 2.17 mg GAE/g DW. In another study, the TPC were observed between 1.20 to 1.70 mg GAE/g DW in Georgia-grown pomegranates [24]. VIUDA-MARTOS et al. (2011) [25] found a higher amount of TPC in whole fruit bagasse (10 mg GAE/g DW) as compared to aril bagasse (4.62 mg GAE/g DW) of pomegranate. The total anthocyanins found in different cultivars of Oman are in the range of 5.56 mg 100 g⁻¹ and 30.11 mg 100 g⁻¹[26]. It was studied that the red pomegranate has more anthocyanin contents as compared to the white ones with higher antioxidant activity and also the anthocyanin contents are negatively related to the fruit weight [27]. ELBANDY&ASHOUSH (2012) [28] observed 14.76 mg/g DW and 98.2 % DPPH radical scavenging activity in defatted pomegranate seed flour. The results are in agreement with the findings of JING et al. (2012) [23], they found the 50% acetone as a better solvent for antioxidant extraction from pomegranate seeds. VIUDA-MARTOS et al. (2011) [25] used methanol as an extraction solvent for antioxidants and observed the counterpart values for ABTS scavenging activity in pomegranate bagasse. The variation in the antioxidant activity may be due to many factors as the antioxidant mechanism is a one and not a single method can judge the main mechanism behind the specific compounds [29]. However, our present results are not in counterpart with the findings of JING et al. (2012) [23]. The defatted pomegranate seed extract showed the higher DPPH scavenging activity with 80% methanol as compared to other solvents. Besides, imperative relationships were found between the red colour intensity *a** and some bioactive compounds like anthocyanins, phenolic contents and/or antioxidant substances recommending the lavishness of red juices in anthocyanins, phenolic contents and antioxidant components [30].

The antioxidant activity of PSE might be due to the polyphenolic fraction present in seed part. A lot of studies demonstrated the inhibitory impact of PSE on the enzymes required in the era of generation of inflammatory mediators for example, Nitric Oxide (NO), leukotrienes and prostanoids. Similarly, polyphenol e.g. punicalagin, punigluconin, punicalin inhibit NO generation and the interpretation of inducible NO synthase (iNOS) in the mouse macrophage like cell lines RAW 264.7. In this study, RAW264.7 macrophages were used to assess the anti-inflammatory activity induced by LPS and cytotoxic consequence of PSE assessed by using neutral red assay to assure that cytotoxic effect of extract did not affect the anti-inflammatory activity of cells while NO production quantified by Griess reagent. Results obtained from our study showed that PSE is a potent anti-inflammatory agent and the first time reported study about the anti-inflammatory effect of pomegranate seed extract. In last few years, various studies were conducted on anti-inflammatory activity of different parts of pomegranate fruit including flower, leaves, roots, peel etc. The anti-inflammatory activity of pomegranate seed extract is considered may be due to the phytochemicals present in its seed portion

including phenolics and flavonoids that can play an important role as an anti-inflammatory agent. Phytochemicals are responsible for anti-inflammatory activity via inhibiting NO production and iNOS expression [31]. OSKOUËIAN et al. (2011)[32] also reported that phenolic compounds have antioxidant activity and act as free radical to scavenge the NO. The inhibition of cytokines TNF α , iNOS, IL6 and IL β in the RAW 264.7 cells might be due to the NO scavenging activity of phytochemicals of pomegranate seed extract as reported by KAZŁOWSKA et al. (2010)[33] that phenolic and flavonoid compounds help to inhibit cytokines expression by suppressing NO production. In spite of the fact that the understanding about the action of activity of flavonoids responsible for their mitigating and immuno-modulatory activity is as yet restricted, distinctive processes influencing cell signaling have been explored. The most broadly examined has been the NF- κ B pathway.

This study dealt with the antioxidant and anti-inflammatory activity of PSE. In this study, PSE of different solvents possessed significant antioxidant activity with highest antioxidant activity in 1% HCl/MeOH solvent. PSE showed the promising antioxidant and anti-inflammatory activity that might be due to the bioactive compounds of pomegranate seed. Cytotoxic results showed that PSE did not exhibit a toxic effect on RAW264.7 macrophages. It inhibits the NO production in RAW264.7 macrophages with a reduction in the cytokines expression and its secretion level. PSE showed the NO inhibitory and cell protective effect against LPS-stimulated cytotoxicity [34].

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Conflict of Interest

The authors declare that they have no conflict interests.

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