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Phenolic and Flavonoids Analysis of Pomegranate Peel Extracts and their Antinflammatory and Antioxidant Activities

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ABSTRACT

An *in-vitro* evaluation of the anti-inflammatory and antioxidant activities of pomegranate peel extract from Palestine were investigated. In parallel, the total phenolic content (TPC) and the total flavonoids content (TFC) were measured. The antioxidant activities were determined spectrophotometrically by DPPH, FRAP, CUPRAC and the ABTS methods. The phenolic and flavonoid contents were separated and partially identified using HPLC and LC-MS. *In-vitro* inhibitory effect of the extract on production of Interlukin-6 (IL-6) and Tumor Necrosis Factor- α (TNF- α) by Lipopolysaccharide (LPS)-induced polymorphonuclear Cells (PMNCs) was evaluated. Pomegranate peel extract was found to have strong antiinflammatory activity as revealed by the reduction in the levels of IL-6 and TNF- α . It was also found that it is rich in phenolic and flavonoids that enhanced its reducing activity and free radical scavenging ability.

Keywords: Pomegranate peel, extract, anti-inflammatory, antioxidant activity, Phenolic compounds, flavonoids.

INTRODUCTION

Herbal plants contain active ingredients that help in recurring from many diseases including many infectious and chronic ones. Many of the active ingredients were reported to act as antiinflammatory, antimicrobial and free radicals scavenging agents. Such ingredients may include phenolics, anthocyanins, carotenoids, and thiols¹. The pomegranate tree (*Punica granatum L.*) is native in Palestine and has been used to treat dysentery, diarrhea, and intestinal parasites. Around half of the fruit weight belongs to its peel and the rest are its edible part consisting of seeds and arils parts²⁻⁴. Its peel was used traditionally for treatment of ulcer and inflammation, and it has been showed antioxidant and bacterial activities²⁻⁴. Several studies have demonstrated that the pomegranate peel is a good source of bioactive compounds such as catechin, ellagitannins, epicatechin, rutin, and many others. Such bioactive compounds are responsible for many biological activities as antimicrobial, antioxidant, antiinflammatory⁵⁻⁷. Additionally, other studies have shown the therapeutic effect of pomegranate fruit, juice, and peel for treatment of lung cancer, esophagus, breast, cardiovascular disorders and breast cancer. It has free radical scavenging activities which mostly related to its phytochemical components⁸⁻¹⁵.

Inflammation is part of a self-protection used by human body against pathogens and also helps in healing injured tissues. Lipopolysaccharide (LPS) is an endotoxin

released from many types of bacteria and cause systemic infection¹⁶. Tumor Necrosis Factor (TNF- α) and Interlukin-6 (IL-6) are pro-inflammatory cytokines that are released upon exposure to LPS from many cells as T cells, Monocytes, B-cells and others. Excessive release of such cytokines may lead to many inflammatory diseases as asthma, rheumatoid arthritis, atherosclerosis, and others¹⁷⁻¹⁹. Levels of IL-6 and TNF- α produced by LPS stimulated poly morpho nucleated cells are measured using Enzyme linked Immuno Sorbant Assay (ELISA). Antiinflammatory effect was examined by measuring the level of IL-6 and TNF- α by the mono nucleated white blood cells upon effect of LPS with different concentrations. Levels of the cytokines were measured using Enzyme Linked Immune Sorbent Assay method.

As part of our ongoing research about the medical effect of the plant extracts, antioxidant, antiinflammatory of pomegranate peel extracts were investigated along with their phenolic and flavonoids content as well as analysis of the active phenolic and flavonoid compounds present in the extracts by the use of HPLC-PDA and UHPLC-PDA-ESI-MS techniques.

MATERIALS AND METHODS

Plant Material and Extraction

Pomegranate fruit was purchased from Jenin market and then peeled off. The peel was dried in the shade at room temperature for 3 weeks then grinded to be like a powder.

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Table 1: Effects of *Pomegranate peel* extracts and LPS on PMNCs viability.

Contents	% Viability
PMNCs only	95.5±1.2
PMNCs with LPS	93.93±1.3
PMNCs with LPS and 300 µg/ml <i>Pomegranate peel</i> extract	90.1±1.0

Then, fifty grams of the above-mentioned powder was mixed with 500 ml of 96% Ethanol and kept on a shaker for 5 days. The extract was filtered through filter paper (Whatman blue ribbon No. 41). The filtrate solution was dried completely under vacuum using rotary evaporator (BUCHI Brand) at 50°C.

Chemicals and reagents

2,4,6-tripyridyl- S-triazine (TPTZ), hydrochloric acid 37% (w/w), sodium hydroxide, ferric chloride trihydrate, ferrous sulfate heptahydrate, potassium persulphate, sodium acetate, sodium carbonate, sodium nitrite, aluminum chloride, methanol, folin-ciocalteu reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid, copper chloride, neocuproine, 99.9% ethanol, ammonium acetate, DPPH, methanol, ABTS (2,2-azino-di-(3-ethylbenzothiazine-sulphonic acid)), and potassium persulphate were all obtained from Sigma-Aldrich, Germany. All chemicals and reagents were of analytical grade. The acetonitrile and water solvents were of an HPLC grade from Sigma. Phenolic and flavonoids standards: Vanillic acid, Ferulic acid, Syringic acid, trans-cinnamic acid, Catechin, p-coumaric acid, Sinapic acid, 4-Hydroxyphenylacetic acid, Rutin hydrate, Caffeic acid, Quercetin, Gallic acid, 3,4-dihydroxyphenylacetic acid, chlorogenic acid, Taxifolin, Luteolin 7-glucoside, Apigenin 7-glucoside, Luteolin, Quercetin 3-D-galactose were from Sigma-Aldrich, Germany.

Reagents preparation

FRAP reagent was prepared according to Benzie and Strain (1999)²⁰ by the addition of 2.5 mL of a 10 mM tripydyltriazine (TPTZ) solution in 40 mM HCl plus 2.5 mL of 20mM FeCl₃.6H₂O and 25 mL of 0.3M acetate buffer at pH 3.6. Acetate buffer (0.3M) was prepared by dissolving 16.8 g of acetic acid and 0.8g of sodium hydroxide in 1000 mL of distilled water.

HPLC and UHPLC Instrumentation systems

The analytical HPLC is Waters Alliance (e2695 separations module), equipped with 2998 Photo diode Array (PDA). Data acquisition and control were carried out using Empower 3 chromatography data software (Waters, Germany). The chromatography was performed under reverse phase conditions using a TSQ Quantum Access MAX (Thermo Scientific, San Jose, CA, USA) which includes a Dionex Pump with degasser module, an Accela PDA detector and an Accela Autosampler.

Measurement of Antioxidant Activity

FRAP assay

The antioxidant activity of the extracts was determined using a modified method of the assay of ferric reducing/antioxidant power (FRAP) of Benzie and Strain (1999)²⁰. Freshly prepared FRAP reagent (3.0 mL) was

warmed at 37°C and mixed with 40 µl of the extract and the reaction mixtures were later incubated at 37°C. Absorbance at 593 nm was read with reference to a reagent blank containing distilled water which was also incubated at 37 °C for up to 1 hour instead of 4 min, which was the original time applied in FRAP assay. Aqueous solutions of known Fe⁺² concentrations in the range of 2-5 mM were used for calibration, and results were expressed as mmol Fe⁺² /g.

Cupric reducing antioxidant power (CUPRAC assay)

The cupric ion reducing antioxidant capacity of the extracts was determined according to the method of Apak et al. (2008)²¹. 100 µl of sample extract was mixed with 1ml each of 10 mM of copper chloride solution, 7.5 mM of neocuproine alcoholic solution (99.9% ethanol), and 1 M (pH 7.0) of ammonium acetate buffer solution, and 1 ml of distilled water to make final volume 4.1 ml. After 30 min, the absorbance was recorded at 450 nm against the reagent blank. Standard curve was prepared using different concentrations of Trolox. The results were expressed as µmol Trolox/g.

Free radical scavenging activity using DPPH (DPPH assay)

DPPH assay is based on the measurement of the scavenging ability of antioxidants towards the stable DPPH radical. A 3.9 mL aliquot of a 0.0634 mM of DPPH solution in methanol (95%) was added to 100 µl of each extract. The mixture was vortexed for 5-10 sec. The change in the absorbance of the sample extract was measured at 515 nm for 30 min till the absorbance reached a steady state. Methanol (95%) was used as a blank. Standard curve was prepared using different concentrations of Trolox. The results were expressed as µmol Trolox/g.

Free radical scavenging activity using ABTS (ABTS assay)

A modified procedure using ABTS (2, 2-azino-di-(3-ethylbenzothiazine-sulphonic acid)) as described by Pellegrini et al. (1999)²² was used. The ABTS stock solution (7 mM) was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The working solution of ABTS⁺ was obtained by diluting the stock solution in 99.9% ethanol to give an absorption of 0.70 ± 0.02 at 734 nm. 200 µl sample extract was added to 1800 µl of ABTS⁺ solution and absorbance readings at 734 nm were taken at 30 °C exactly 10 min after initial mixing (A). The radical-scavenging activity of the test samples was expressed as Trolox equivalent antioxidant capacity TEAC (µmol Trolox/g sample).

Total phenolic content (Folin-Ciocalteu assay)

Total phenolics were determined using Folin-Ciocalteu reagents. *Z. spina-christi* plant extracts or gallic acid standard (40 µl) were mixed with 1.8 mL of Folin-Ciocalteu reagent (prediluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min, and then 1.2 mL of sodium bicarbonate (7.5%, w/v) was added to the mixture. After standing for 60 min at room temperature, absorbance was measured at 765 nm. Aqueous solutions of known gallic acid concentrations in

Table 2: Effect of pomegranate peel extract on production of IL-6 and TNF alpha by PMNCs.

	IL6 amount (pg/ml)		TNF alpha amount (pg/ml)	
	Average	STD	Average	STD
Cells only	106.7	1.2	64.3	1.7
Cells with LPS	640.3	2.1	987.3	5.2
Cells with LPS and 75 µ g extract	371	2.9	669	2.9
Cells with LPS and 150 µ g extract	160	2.9	314.3	3.3
Cells with LPS and 300 µ g extract	63.3	1.2	77.7	2.1

Table 3: Total phenolic content (TPC as mg Gallic acid/g DW*), total flavonoids contents (TFC as mg catechin/g DW), FRAP (mmol Fe²⁺/g DW), CUPRAC (µmol Trolox/g DW), DPPH (µmol Trolox/g DW), and ABTS (µmol Trolox/g DW) of pomegranate peel extract.

TPC** (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)	ABTS (µmol/g)
33.5± 1.5	8.3± 0.9	12.4 ± 0.4	3756 ±35	225 ± 6	46.2 ±1.5

* DW: Dry weight

** Results are expressed as an average of three samples.

the range of 10 - 500 mg/L were used for calibration. Results were expressed as mg gallic acid equivalents (GAE)/ g sample.

Total flavonoid content

The determination of total flavonoids was performed according to the colorimetric assay of Kim et al. (2003)²³. Distilled water (4 mL) was added to 1 mL of the extract in a test tube. Then, 0.3 mL of 5% sodium nitrite solution was added, followed by 0.3 mL of 10% aluminum chloride solution. Test tubes were incubated at ambient temperature for 5 minutes, and then 2 mL of 1 M sodium hydroxide were added to the mixture. Immediately, the volume of reaction mixture was made to 10 mL with distilled water. The mixture was thoroughly mixed using test tube shaker and the absorbance of the pink color developed was determined at 510 nm. Aqueous solutions of known catechin concentrations in the range of 50 - 100 mg/L were used for calibration and the results were expressed as mg catechin equivalents (CEQ)/ g sample.

Chromatographic conditions

The HPLC analytical experiments of the crude ethanol extracts were run on ODS column of Waters (XBridge, 4.6 ID x 150 mm, 5 µm) connected to guard column of Xbridge ODS, 20 mm x 4.6mm ID, 5 µm. The mobile phase is a mixture of 0.5% acetic acid solution (A) and acetonitrile (B) run in a linear gradient mode. A 100% (A) was descended to 70% (A) in 40 minutes then to 40% (A) in 20 minutes and then to 10% (A) in 2 minutes and stayed for 6 minutes and back to the initial conditions in 2 minutes. The HPLC system was equilibrated for 5 minutes with the initial acidic water mobile phase (100 % A) before injecting next sample. All the samples were filtered with a 0.45 µm PTFE filter. The PDA wavelengths range was from 210-500 nm. The flow rate was 1 ml/min. Injection volume was 20 µl and the column temperature was set at 25°C. Samples were filtered through 0.45 µm micro porous disposable filter.

The UHPLC chromatographic separations were run on a Kinetex™ (Phenomenex, Torrance, CA, USA) through C8 column (2.6 µm particle size, 100 Å pore size, 100 x 2.1 mm), protected by a SecurityGuard™ (Phenomenex, Torrance, CA, USA) cartridge C8 column (2.1 mm ID). The injection volume was 10 µL, the oven temperature

was maintained at 35°C. The chromatographic separation was achieved using the same HPLC linear gradient program using formic acid instead of acetic acid at a constant flow rate of 0.4 mL/min over a total run time of 70 min. The samples were detected by a TSQ Quantum Access Max mass spectrometer using Electron Spray ionization (ESI) and full scan acquisition. Air was produced (SF 2 FF compressor, Atlas Copco, Belgium). Purified nitrogen was used as source and exhaust gases.

Samples of the crude extracts were prepared at a concentration of 5 mg/ml by dissolving 50 mg of crude extract in 10 ml of respective solvent (water, 80% ethanol, or 100% ethanol).

Cell Culture

Polymorphonuclear cells were isolated from a freshly transfused 5 ml whole blood. The blood was mixed with an equal amount of phosphate buffered saline (PBs) in a 1:1 ratio in a sterile condition. Three ml of Histopaque were pipetted in 15 ml conical tube. The mixture of the PBS and the blood was added gently to the tube and then spun for 20 min at 400 G. The PMNCs were aspirated and then washed 3 times with 10 ml PBS in a 12 ml conical tube at 100 G for 10 minutes. Supernatant was discarded and the cells were isolated and used in our study.

The isolated PMNCs were cultured in Roswell Park Memorial Institute (RPMI) medium that was enriched with 100-µg ml⁻¹ streptomycin, 100 Uml⁻¹ penicillin, and with heat-inactivated 10% Fetal Bovine Serum (FBS). The mixtures were incubated into 12-well tray at 37°C in 5% CO₂ incubator for 24 hours. One ml of the mixture contains 1 million of the cells and was incubated in one well. Different concentrations of the pomegranate extract were added to lipo poly saccharide (1 µg/ well) stimulated cells.

Cytotoxicity Test

The trypan blue exclusion test was used to evaluate the cytotoxicity of the pomegranate extract as illustrated by Avelar-Freitas et al. 2014¹⁴. Trypan blue dye of an acid azo exclusion medium was prepared in 0.4 % dilution, then mixed with an equal amount of the cell suspension with 300 µ gram/1 ml of the pomegranate extract. After 2 minutes, Hemocytometer was used to count the cells.

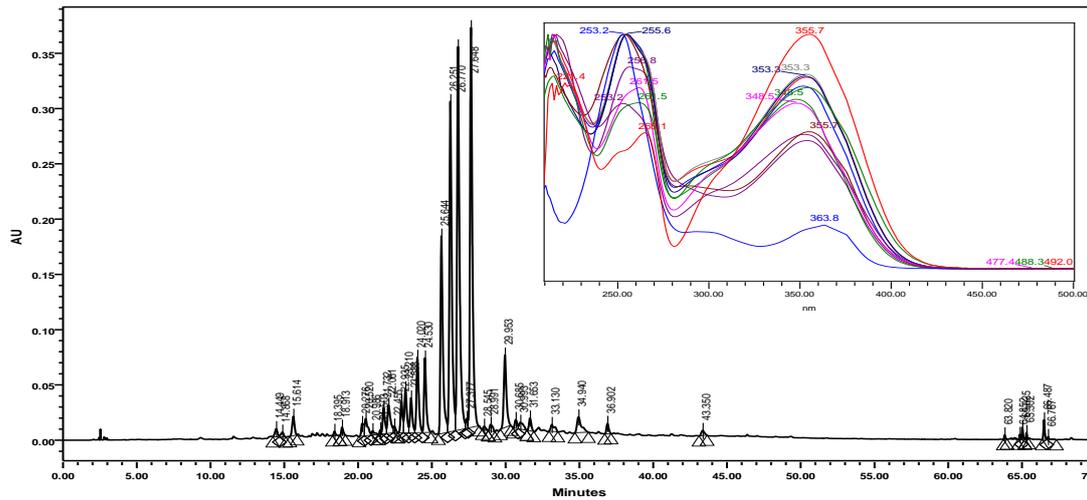


Figure 1: HPLC-PDA chromatograms of crude Pomegranate peel ethanol extract at 350 nm.

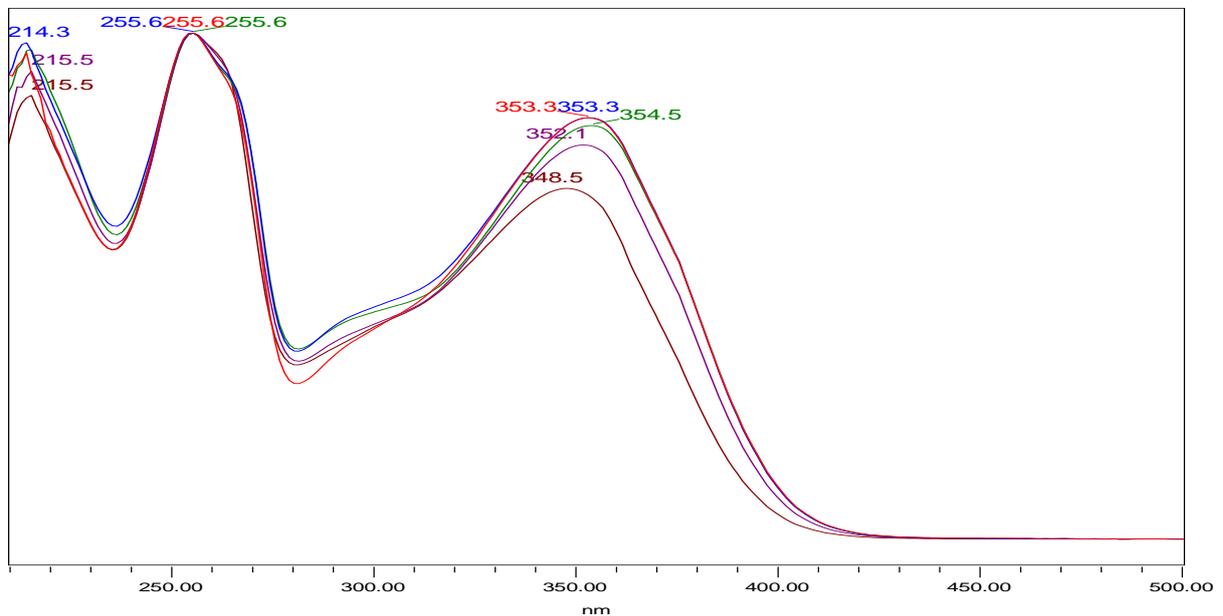


Figure 2: Overlaid UV-Vis spectra of the major five peaks of Pomegranate peel ethanol extract eluted between 25.64-29.95 minutes.

Viable cells were unstained and dead cells were stained blue.

Immunoassay for TNF-alpha and IL-6:

Enzyme Linked Immunoassay was employed to measure IL-6 and TNF-alpha according to manufacturer's instructions.

Statistical Analysis

The means of TNF- α and IL-6 concentrations were compared using Paired samples t test applying SPSS version 19. Differences with P less than 0.05 were considered significant.

RESULTS AND DISCUSSIONS

Cytotoxicity of the extracts

Pomegranate peel extracts at concentration of 300 $\mu\text{g}/\text{ml}$ and LPS at concentration of 1 $\mu\text{g}/\text{ml}$ have no obvious

effect on the viability of the PMNCs when compared with the control group. Table 1 represents such results. Such results indicate that any possible reduction in the cytokines' production is not due to the PMNCs death.

Anti-inflammatory activities of the plant extracts

LPS-stimulated PMNCs after 24h, TNF- α , and IL-6 levels increased significantly. However when it was treated with pomegranate peel extracts with concentrations ranged from 75-300 $\mu\text{g}/\text{ml}$, TNF- α and IL-6 in cell culture medium were all significantly reduced and the greater the reduction in their concentration, the stronger the anti-inflammatory effect of the extract (Table 2). Such results make the pomegranate peel extracts a good source candidate for anti-inflammatory drugs.

Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Pomegranate peel extract was found to be rich with phenolic and flavonoids with TPC and TFC of 33.5 and 8.3 mg/g, respectively, Table 3.

Antioxidant activity

There are two types of typical assays used to evaluate the AA of plant extracts. The first category measures the potential of plant extracts to reduce ions or oxidants (by acting as reducing agents) like ferric ion, cupric ion. The main two assays of this category are FRAP (measures the reduction potential of ferric to ferrous ion), and CUPRAC (measures the reduction of cupric to cuprous ion). The second category of AA measures the ability of plant extracts to scavenge free radicals. DPPH and ABTS assays (where DPPH and ABTS are stable free radicals) are the two main examples of this category. These assays are quick and simple, and their reactions are reproducible and linearly related to the concentration of the antioxidant(s) present.

Reducing potential of plant extracts

FRAP assay (Ferric reducing-antioxidant power)

This method measures the ability of antioxidants to reduce ferric iron. It is based on the reduction of the yellow complex of ferric iron and 2,3,5-triphenyl-1,3,4-triazole-2-azoniacyclopenta-1,4-diene chloride (TPTZ) to the blue ferrous form by electron-donating substances (such as phenolic compounds) at low pH. This reduction is monitored by measuring the change in absorption at 593 nm. Antioxidant assay was conducted by the method developed by (Benzie and Strain 1999). FRAP values can be obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe^{2+} .

The antioxidant test based on FRAP assay of *pomegranate peel* extract was found to be 12.4 ± 0.4 mmol Fe^{2+} /g of dry material.

Cupric reducing antioxidant power (CUPRAC)

Although FRAP antioxidant assay has been very popular among researchers, CUPRAC assay is a relatively new assay (Apak et al. 2008). It utilizes the copper(II)-neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidizing agent and is based on the cupric reducing ability of reducing compounds to cuprous.

The antioxidant test based on CUPRAC assay of *pomegranate peel* extract was found to be 3756 ± 35 μ mol Trolox /g of dry material.

Free radical scavenging ability of plant extracts

Free radical scavenging activity using DPPH

DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various samples (Sakanaka et al. 2005²⁴). It is a stable free radical with a characteristic absorption at 517 nm that was used to study the radical-scavenging effects of extracts. As antioxidants donate protons to this radical, the absorption decreases. Antioxidants, on interaction with DPPH, either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character (Naik et al. 2003)²⁵. The color changed from purple to yellow and the absorbance at wavelength 517 nm decreased.

DPPH assay is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with

hydrogen donors including phenolics. The bleaching of DPPH solution increases linearly with increasing amount of extract in a given volume.

DPPH antioxidant activity of pomegranate peel extract was found to be 225 ± 6 μ mol Trolox/g (Table 1).

Free radical scavenging activity using ABTS

A modified procedure using ABTS (2,2-azino-di-(3-ethylbenzothiazoline-sulphonic acid) was used. The ABTS^{•+} stock solution (7 mM) was prepared through reaction of 7 mM ABTS and 2.45 mM of potassium persulphate as the oxidant agent. The working solution of ABTS^{•+} was obtained by diluting the stock solution in ethanol to give an absorption of 0.70 ± 0.02 at $\lambda = 734$ nm. Sample extract (100 μ l) was added to 900 μ l of ABTS^{•+} solution and absorbance readings at 734 nm were taken at 30 °C exactly 10 min after initial mixing.

The ABTS assay measures the relative antioxidant ability of extracts to scavenge the radical-cation ABTS^{•+} produced by the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate.

ABTS antioxidant activity of *pomegranate peel* extract was found to be 46.2 ± 1.5 μ mol Trolox/g (Table 1).

HPLC-PDA profiles of pomegranate peel extract

Pomegranate peel was extracted in ethanol solvent and concentrated. The chromatogram of 10 μ l extract at 350 nm is shown in figure 1 along with overlaid UV-Vis spectra. The first eluted peaks at 14.45, 14.86 and 15.61 share the same wavelength maximum of 226.6 nm and a shoulder at 274 nm. The peaks from 18.39 to 24.53 minutes were also shared similar spectral pattern of two maxima's of around 265 nm and 355 nm respectively. Moreover, the UV-Vis spectra of the major eluted peaks between 25.64 to 29.95 minutes is shown in figure 2. The first maximum wavelengths were at 255.6 nm and the second maxima was between 348-354 nm. These absorption patterns characteristics are of typical phenolic and flavonoid compounds.

Using very similar conditions to the analytical HPLC-PDA, a full scan of the ethanol extract was examined using UHPLC-MS in the positive and negative electrospray ionization modes between the range of 200-1200 Da. Formic acid was used instead of acetic acid at 0.1% concentration. The MS results revealed the presence of some phenolic and flavonoids which were previously identified using different spectroscopic methods.

CONCLUSIONS

Pomegranate peel extract is rich in phenolic and flavonoids contents and presented an excellent DPPH and ABTS radical scavenging activities. It also showed excellent reducing abilities as represented by FRAP and CUPRAC assays. Pomegranate peel extract also showed a strong reduction in the production of IL-6 and TNF- α from the LPS-induced PMNCs, indicating strong antiinflammatory effects. Such results make extracts of *pomegranate peels* promising source of antiinflammatory candidates to be used in the pharmacological industry.

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