



In vivo and *in vitro* evaluation of antioxidant, antihemolytic, acute toxicity effects and high-performance liquid chromatography analysis of an aqueous extract of Moroccan *Punica granatum* L.

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ABSTRACT

Introduction: The use of pomegranates in Moroccan pharmacopeia is due to their healing and nutritional properties because of their richness in secondary molecules. The following study analyses the composition of the aqueous extract of *Punica granatum* peel and evaluates *in vivo* and *in vitro* antioxidant effects, hemolytic protection, and acute toxicity.

Methods: Quantification of the plant extract was realized by high-performance liquid chromatography (HPLC). The hemolytic assay was used for erythrocyte protection, while the *in vitro* antioxidant effect was evaluated by 2, 20-azinobis-(3-ethylbenzothiazoneline-6-sulphonic acid) (ABTS) and reducing ferric power (RFP) assays. The *in-vivo* antioxidant activity was tested by measuring levels of lipid peroxidation (LPO) in serum. The toxicological study was tested by oral administration of the extract to four groups of mice for 21 days, followed by a histopathological examination of the spleen.

Results: HPLC analysis showed the presence of some phenolic compounds such as coumarin, caffeic, gallic and syringic acids. The IC₅₀ of the antioxidant assays were 254.49 ± 62.17 µg/mL and 40.265 ± 2.9 µg/mL for ABTS and reducing power, respectively. Furthermore, the thiobarbituric acid reactive substances (TBARS) assay showed the lowest levels at 150 mg/mL concentration. All of the concentrations used for hemolytic protection did not exceed 15% of hemolysis. Moreover, the toxicity test showed no sign of mortality, signs of weakness, or weight loss; also the histopathological examination of the spleen tissues showed the absence of any damage.

Conclusion: The peel extract of *P. granatum* showed good potential and could be exploited as a natural antioxidant and antihemolytic remedy, leading to the development of new drugs.

Implication for health policy/practice/research/medical education:

The aqueous extract of Moroccan pomegranate peel showed valuable *in vivo* and *in vitro* antioxidant efficiency and a protective effect on erythrocytes hemolysis. More assays are requested to support the obtained effects. Biocompounds present in the aqueous extract of pomegranate might be used as a substitute for drugs to cure antioxidant trouble.

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Introduction

Reactive oxygen specie (ROS) remains a toxic by-product of normal oxygen metabolism and is involved in many pathologies such as atherosclerosis, an inflammatory disorder, cancer, and other diseases. This is due to the unsteadiness between the radical species production and

the cellular antioxidant capacities, this phenomenon is called oxidative stress. ROS and membrane stabilization contribute to regulating the inflammatory response under the control of the antioxidant cellular system (1). The excessive and exaggerated activation of ROS production influence the role of the erythrocyte membrane by altering

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its fluidity and protein profile (2). The use of chemical drugs has proven to be effective in eliminating ROS. Still, over time they have shown undesirable side effects on the human body, hence the need for an orientation to herbal medicine known for these medicinal benefits. Recently, research on plants and their compositions has increased, and this is because of their characteristics, among these searches the phytochemical characterization, the study of toxicity, and all the biological properties generated by these plants. The use of the medicinal plant in the treatment of oxidative stress is promising due to biomolecules such as polyphenols. These components are known for their antioxidant properties and help fight against ROS, anti-inflammatory, and antihemolytic (3). *Punica granatum* L. is considered the oldest fruit species cultivated in Morocco. This species, widely cultivated in the country, seems ubiquitous given its great adaptive potential to environmental conditions (soils and climates). In recent decades, the pomegranate tree has become increasingly important, and its cultivation has gone from being traditional to developing into commercial orchards. The beneficial effects of *P. granatum* extracts are related to bioactive compounds, particularly polyphenols, tannins, and flavonoids, the used area plants, and the extraction methods (4). According to the literature, the wealth of these substances gives *P. granatum* a huge scale of biological properties such as anti-inflammatory, antioxidant, and antihemolytic activities (5).

The main of the following study is to identify the chemical composition of the aqueous extract using the high-performance liquid chromatography (HPLC) method and to evaluate the antihemolytic effect on erythrocytes, the acute toxicity in Wistar rats, and *in vitro* and *in vivo* antioxidant effects of *P. granatum*.

Materials and Methods

Chemicals and reagents

All the reagents and the chemicals products were bought from Sigma-Aldrich-Co Morocco.

Plant collection and identification

Punica granatum is available in the market as a seasonal fruit in Morocco. The peel was dried and crushed to powder for final use. The identification of pomegranate fruit plant was done by Professor Khyati Najat, a plant specialist (Department of Biology, Faculty of Sciences Ain Chock, Hassan II University of Casablanca). A voucher specimen of the plant-dried peels was deposited at the biology department under voucher number PG-04102019 for *P. granatum* L.

Preparation of plant extracts

The dried powder of the *P. granatum* peel was extracted by decoction method. Fifty grams of the powder peel was mixed with distilled water (Ratio 1:10; w/v) brought to 70°C and keep stirring until cool. The suspension was

centrifuged for 10 minutes at 489 g and filtered with paper (Whatman No. 1), and then the solvent was eliminated by a rotary evaporator. The yield of aqueous extracts was 38%. The sample was prepared in sterile phosphate buffered saline (PBS) and stored at -20°C until used.

Animals

Adult rats and mice were used. All these animals came from the breeding of the Faculty of Sciences, University Hassan II; Morocco, animals were preserved in cages in an environmental (24°C, 12 hours:12 hours dark/light cycle), and the animals had access to drinking water and food at will. The experiments on animals respected the approval of the Ain chock sciences faculty ethical committee.

HPLC analysis

The used HPLC in this study is Shimadzu, furnished with detector type SPD-20A UV/Vis, and the LC-Solution data processing station was used to identify the compounds of the aqueous extract *P. granatum*. The elution of the extract was carried on column type (RP-C18) with an isocratic elution for the mobile phase water and acetonitrile was used (88,12 respectively), and measured at 285 nm. Sample and polyphenol standards were injected at a volume of 20 µL. The elution flow rate was fixed at 1 mL/min as mentioned by (6). Synthetic polyphenols like Gallic, vanillin, ferulic acids, quercetin, and catechin from (Sigma Chemical Company, USA) were used as standards. The comparison of the retention time and a standard was used to determine the compounds.

Antioxidant activity

The evaluation of the antioxidant effect of the aqueous extract of *P. granatum*, *in vitro* and *in vivo* assays was carried out as below:

In vitro assays

ABTS assay

ABTS assay was carried out using the method of Dudonné et al (7). Briefly, 7.4 mM of ABTS (2, 20-azino-bis-(3-ethylbenzothiazolone-6-sulphonic acid) was treated with 2.45 mM of potassium persulfate ($K_2S_2O_8$) and incubated 18 hours in the dark to release free radicals (ABTS⁺). The solution was diluted in ethanol (1:50, V/V) to obtain a solution with an absorbance of 0.7 at 734 nm. Aqueous extract of *P. granatum* and ascorbic acid at the chosen concentrations were added to 1 mL of ABTS⁺ to react with it and incubated for 10 minutes in a dark environment. Trolox was used as a positive control. The absorbance was determined at 734 nm. Ethanol was used as a blank:

$$\%ABTS = \frac{Abs\ control - Abs\ sample}{Abs\ control} \times 100$$

Where Abs control is the absorbance of ABTS⁺ and Abs sample is the absorbance of samples.

Reducing ferric power (RFP) assay

The RFP of the aqueous extract of *P. granatum* was determined by the method of Oyaizu (1986). Briefly, 1 mL of extract at different concentrations was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide ($K_3[Fe(CN)_6]$) (1% in water) and was incubated at 50°C for 20 minutes. 2.5 mL of trichloroacetic acid (10% in water) was added to the mixture and then centrifuged for 10 minutes. 2.5 mL of supernatant was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1% in water). The concentration of extract providing 0.5 of absorbance corresponds to the effective concentration (EC_{50}). Ascorbic acid was used as a standard. The absorbance of all samples was measured at 700 nm.

In vivo assay

Twelve albino Wistar mice were divided into four groups with three animals each. Group 1 served as the control and received 0.4 mL of distilled water. Groups 2, 3, and 4 received the same volume at different concentrations of 50 mg/kg, 100 mg/kg, and 150 mg/kg of the aqueous extract of *P. granatum*, respectively. The animals received daily 0.4 mL extract by oral gastric for 21 days (8). The blood was collected using a blood collection plain tube twenty-four hours after the last treatment and used in the determination of lipid peroxidation (LPO). All the experiments were carried out in triplicate.

Determination of the LPO level in serum

The using method to quantify the production of malondialdehyde (MDA) and the level of thiobarbituric acid reactive substance (TBARS) was carried out as described by Draper and Hadley with slight modifications (9,10). Fifty microliters of serum were deproteinized after adding 1 mL of trichloroacetic acid (14%) and 1 mL of thiobarbituric acid (0.6%). To complete the reaction the mixture was heated for 30 minutes in a water bath. The sample stayed on the ice for 5 minutes and was centrifuged at (2000 g) for 10 minutes. The absorbance was measured at 535 nm with a UV spectrophotometer. The concentration of TBARS was calculated using the molar extinction coefficient of MDA (1.56×10^5 mol/L/cm) using the following formula:

$A = \Sigma CL$, where A = absorbance, Σ = molar coefficient, C = concentration, and L = path length.

The expression of the results was in nmol/mg of protein.

Protein content assay

The protein quantity in the serum was determined according to Bradford's method (11).

Hemolytic assay

The evaluation of the hemolytic activity was conducted using methods described by Bulmus et al and Bendiar

et al (12,13). To begin all the volunteers were aware of the purpose of the sample, the collection of the human blood was conducted in ethylene diamine tetra-acetic acid (EDTA) tubes, followed by centrifugation at 2500 rpm at 15 minutes and plasma was collected to purify human red blood cells (HRBCs), series of three washes using a solution of NaCl (0.9%) was proceeded.

The pellet containing the HRBCs was mixed in 100 mM phosphate buffer solution (PBS) with a dilution (1:10, V/V) to have a final concentration of one hundred million HRBCs per 200 μ L.

Then, 200 μ L of HRBCs were mixed with 800 μ L of PBS at the chosen pH, and 50 μ L of different concentrations (50, 100, 200, 400, and 800 mg/mL) of aqueous extract of *P. granatum* were added. Then the sample was centrifuged for 5 minutes at 13500 g after 1 hour of incubation at 37°C. The absorbance of the collected supernatant was measured at 540 nm. A sample containing the HRBCs incubated with Triton-X 100 (1%) was used as the positive control, and PBS alone was used as the negative control.

The inhibitory activity of the extract was calculated using the following equation and expressed as a percent of hemolytic activity:

$$\% \text{hemolytic activity} = \frac{(Abs_{sample} - Abs_{Cn})}{(Abs_{Cp} - Abs_{Cn})} \times 100$$

Cn: negative control, Cp: Positive control, and Abs: Absorbance

Determination of acute toxicity

Twelve albino Wistar rats weighing 180–200 g, were kept at room temperature, relative humidity, and exposure to the natural light-dark cycle. The rats were divided into 4 groups containing 3 rats per group. Group 1 was used as a control. Groups 2, 3, and 4 received 0.4 mL of different concentrations of the extract by oral administration at 50, 100, and 150 mg/kg of body weight, respectively. The groups were observed during the experiences for other toxic signs or death observation. Observation continued for 21 days, then the animals were sacrificed, and the liver was taken to be used for histopathological studies (14). All the experiments were carried out in triplicate.

Histopathological examination of the liver

After the collection of livers from treated and untreated groups, they were dissected into small slices and fixed in buffered formaldehyde solution at 10%. The sections were dehydrated after a stint in a bath with an evolving concentration of ethanol, then the sample was immersed in paraffin. The staining of the sections of tissue was processed with the hematoxylin-eosin method, and all the slides were examined and validated by a pathologist (15).

Results

HPLC analysis

Analysis of *P. granatum* compounds was conducted using

the HPLC method. The identification of compounds was carried out by matching retention times of standards and extract; results are presented in Figure 1. The results showed the presence of gallic acid (1), caffeic acid (2), syringic acid (3), and coumarin (4) in the aqueous extract of *P. granatum*.

Antioxidant assays

In vitro assays

The antioxidant activity was measured using ABTS and RFP assays. The obtained results are exposed in Table 1. The results revealed that the aqueous extract of *P. granatum* was able to neutralize ABTS radical and reduce potassium ferricyanide from the form (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}) form. The value of IC_{50} of the sample was $254.49 \pm 62.17 \mu\text{g/mL}$, while for Trolox was $1.99 \pm 0.053 \mu\text{g/mL}$, and EC_{50} of the sample was $40.265 \pm 2.9 \mu\text{g/mL}$, while for ascorbic acid was $59.87 \pm 1.8 \mu\text{g/mL}$.

In vivo assay

In vivo antioxidant activity of the aqueous extract of *P. granatum* was evaluated with the determination of LPO in serum, by analyzing the levels of TBARS and MDA production. The results (Figure 2) showed a decreasing activity of TBARS levels compared to the untreated group. This decrease was dose-dependent ($P < 0.0001$), and the lowest TBARS level was 0.5 mmol/mg of proteins obtained at the concentration of 150 mg/kg.

Hemolytic assay

This assay aimed to assess whether *P. granatum* aqueous extract had a hemolytic effect on HRBC *in vitro* or not. The obtained results are shown in Figure 3. The results showed that the aqueous extract had low lysis even with pH variation and for all concentrations compared to the control, which had 100% of hemolysis. It suggests that *P. granatum* extract does not impact the HRBCs hemolysis in both pHs.

Table 1. IC_{50} and EC_{50} values of ABTS and RFP antioxidant activity of aqueous extract of *Punica granatum*

Treatments	Antioxidant assays	
	ABTS	RFP
	$\text{IC}_{50} (\mu\text{g/mL})$	$\text{EC}_{50} (\mu\text{g/mL})$
<i>P. granatum</i> aqueous extract	$254.49 \pm 62.17^*$	$40.265 \pm 2.9^{***}$
Ascorbic acid	-	$59.87 \pm 1.8^{**}$
Trolox	$1.99 \pm 0.053^*$	-

ABTS: 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); RFP: Reducing ferric power; IC_{50} : Inhibition concentration 50; EC_{50} : Effective concentration 50.

The results are presented in mean \pm standard deviation (SD) ($n = 3$), * $P < 0.002$ Trolox compared to *P. granatum* aqueous extracts; ** $P < 0.0001$ Ascorbic acid vs *P. granatum* aqueous extract by *t* test.

Determination of acute toxicity

During the test period, no mortality in the rats was observed neither in the untreated nor treated groups, after administration of all doses used during the tests. No signs of weakness, fur loss, or a decrease in diet were noticed during the test period.

Histopathological examination of the liver

The results of the histopathological examination are presented in Figure 4. The results showed that there was no difference between untreated and treated tissues. The spleen section tissue of treated rats with the different doses showed normal hepatic cells with prominent cytoplasm and was well-preserved. Some vacuolization post-mortem was observed after 21 days of administration.

Discussion

Pomegranate is a fruit with beneficial effects on health due to its richness in natural compounds like phenolic acids and flavonoids, which can inhibit oxidation (16). The HPLC analysis of the aqueous extract of *P. granatum* revealed the presence of four compounds. According to

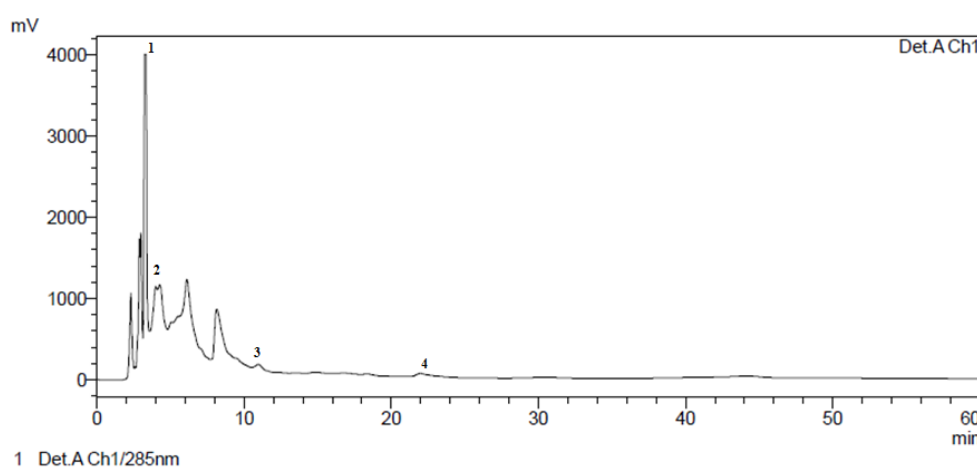


Figure 1. Chromatogram of major compounds from *Punica granatum* peels aqueous extract obtained with high-performance liquid chromatography (HPLC). Peaks 1, 2, 3, and 4 correspond to gallic acid, caffeic acid, syringic acid, and coumarin, respectively.

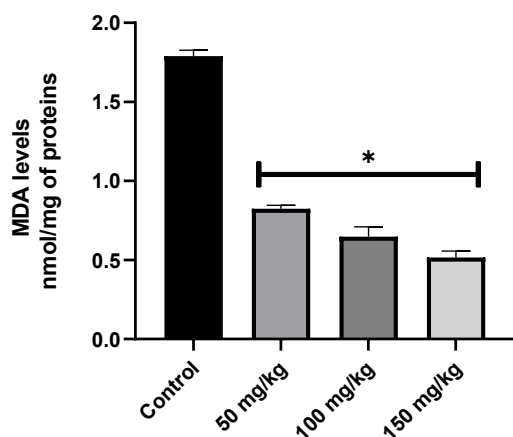


Figure 2. Levels of thiobarbituric acid reactive substances (TBARS) for the treated rat with three concentrations of *Punica granatum* aqueous extract. All results were represented as mean ($n = 3$) \pm SD for the level of malondialdehyde (MDA). * $P < 0.001$ compared to control (untreated rats) by ordinary one-way ANOVA test.

Middha et al, the HPLC analysis showed the presence of two major phenolic compounds (gallic and ellagic acids) (17). The same results were obtained by Chidambara et al by analyzing the methanolic extract of pomegranate peel, which showed the presence of gallic and ellagic acids as the major components (18). He et al confirmed the presence of catechin, caffeic acid, and kaempferol 3-O-rutinoside, and reported that the peel contained more phenolic compounds than the seed (19). The absence of flavonol compounds in our extract was noticed. Wang et al identified the HPLC profile of pomegranate peel extract and found the presence of punicalagin and ellagic acid by comparing with standard compounds (20). Li

et al identified pomegranate juice by HPLC profile and found the following compounds: punicalagin, catechin, epicatechin, rutin, ellagic, chlorogenic, gallic, and caffeic acids (21).

The most generally laboring assays to evaluate the capacity of plant extract antioxidants are distributed into two main categories: those that measure free radical scavenging ability, including RFP and ABTS assays, and those that evaluate LPO, like TBARS assay. The ABTS assay depends on reducing the cation radical of ABTS⁺ and recording the color change from blue to green (21). The RFP assay is simple and quick, consisting of reducing yellow colored ferric tripyridyltriazine complex (Fe³⁺-TPTZ) to a ferrous complex (Fe²⁺-TPTZ) with a blue color, to confirm the presence of the phenolic compounds (22). In the TBARS method, the formation of a pink chromophore with TBA means the decomposition of lipid hydro-peroxide which leads to measuring the MDA (23, 24). In plants, hydroxyl groups in phenolic and flavonoid compounds give them an important antioxidant activity (23). The evaluation of *in vitro* and *in vivo* antioxidant effects of the aqueous extract of *P. granatum* has seemed to neutralize ABTS and reduce Fe³⁺ with different IC₅₀, so whenever IC₅₀ is low, the antioxidant activity of the samples is more potent (25). The results showed that for the ABTS assay the aqueous extract of *P. granatum* presented a low antioxidant activity compared to Trolox in this study ($P < 0.002$), while for the RFP assay, the aqueous extract was on the same level compared to the ascorbic acid. According to the literature, numerous studies were established to evaluate the antioxidant effect of *P. granatum*. Mayasankaravalli et al reported that the aqueous and ethanolic extracts of *P. granatum* had IC_{50s}

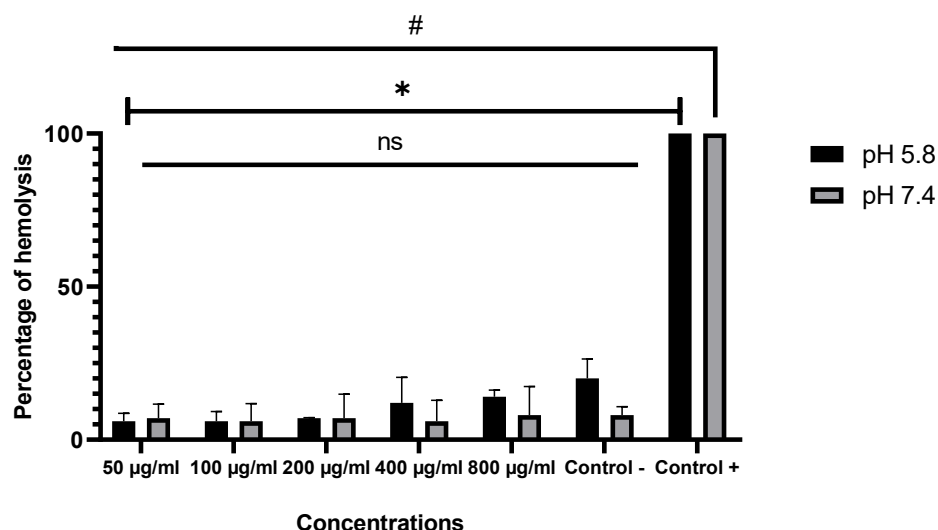


Figure 3. Hemolytic protection effect of *Punica granatum* aqueous extract using the concentration range between 50 and 800 µg/mL with a pH variation. Results are mean \pm SD ($n=3$). * $P < 0.001$ for all PG-AE concentrations compared to the control group at pH 5.8; # $P < 0.001$ for all PG-AE concentrations compared to the control group at pH 7; ns $P = 0.119$ for all PG-AE concentrations in a medium at pH 5.8 compared to all PG-AE concentrations in the medium at pH 7 by Ordinary one-way ANOVA test. PG-AE: *Punica granatum* aqueous extract.

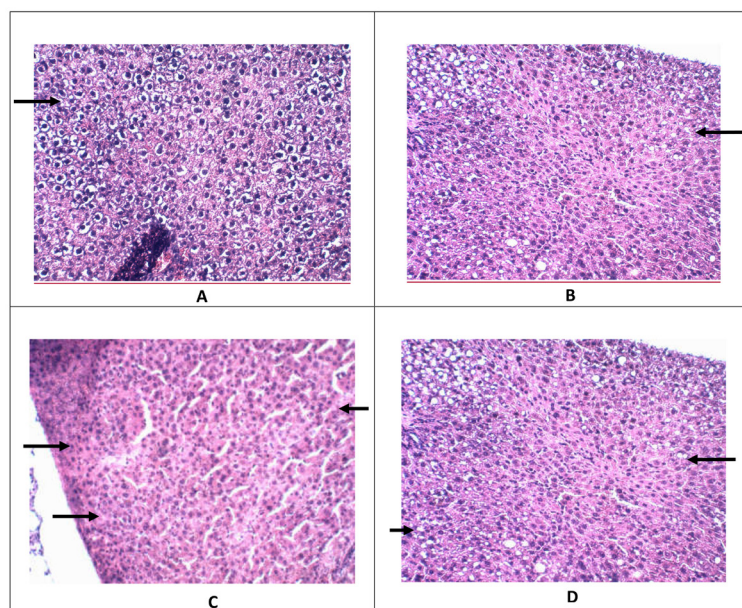


Figure 4. Histological observation of liver tissues after acute treatment with the aqueous extract of *Punica granatum*. (A) Untreated rats; (B) group treated with 50 mg/kg; (C) group treated with 100 mg/kg; (D) group treated with 150 mg/kg. The three treated groups and untreated group showed a normal architecture without lesions on hepatic cells as shown with arrows.

value of 471.70 $\mu\text{g/mL}$ and 509.16 $\mu\text{g/mL}$, respectively (26). Also to confirm that the peel part of the plant had a good antioxidant effect, Derakhshan et al revealed that the percentage of antioxidant activity using the β -carotene bleaching test of peel was higher than seed and juice extracts with the percentages of $58 \pm 10.52\%$, $54 \pm 12.5\%$, and $9 \pm 3.02\%$, respectively (16). Kaur et al studied the antioxidant effect of *P. granatum* flower extract by the RFP method, they found an EC_{50} of 75 $\mu\text{g/mL}$ lower value than our peel extract results (27). After analyzing these results, we can assume that the peel extract has a strong potential antioxidant compared to other parts of the pomegranate. The LPO was quantified by TBARS assay. Our results revealed a decrease in the TBARS levels compared to the control, with a dose dependent-manner ($P < 0.0001$). Bhaskar et al studied the effect of flower ethanolic and aqueous extracts of *P. granatum*. They demonstrated that the administration of these extracts remarkably reduced the levels of TBARS, increased the levels of the enzymic antioxidant, and reduced glutathione (28). Benchagra et al studied the LPO effect of hydroalcoholic extract of peel and seed part of a variety named Sefri of *P. granatum*. They showed that the two extracts decreased LPO compared to the control (29). According to Zaid et al, a pomegranate fruit extract decreased the level of LPO in human immortalized (human keratinocyte cell lines) HaCaT. This extract reduced peroxide accumulation by scavenging ROS and inhibited LPO due to its richness in polyphenols (30). Chidambara et al reported that using pomegranate peel methanolic extract at a concentration of 50 mg/kg showed an anti-lipid peroxidative effect with a percentage of inhibition of 54% compared to the control (18).

According to the literature, ROS's first target during oxidative stress is erythrocytes due to the presence of both oxygen transport associated with redox-active hemoglobin and a high concentration of polyunsaturated fatty acids (31). The obtained results in this study showed that the aqueous extract exhibited no hemolytic effect on HRBCs even with the variation of the concentrations of the extract or the pH medium; that's why we suggested that this observed protective effect could be owing to the presence of phenolic and flavonoids compounds (32).

The elimination of LPO or changing the conformity of the erythrocytes membrane under the effect of the phenolic and flavonoid compounds could explain the anti-hemolytic effect of our extract. A study by Costa et al revealed that the presence of phenol and flavonoid compounds could inactivate free radicals, leading to a decrease in oxidative hemolytic activity (33). Chaudhuri et al have shown that flavonoids interacted with the HRBCs membrane by decreasing the fluidity, thus decreasing the diffusion of ROS into the membrane (34) by the observed anti-hemolytic effect of *P. granatum* aqueous extract.

We studied the acute toxicity of *P. granatum* aqueous extract, and no mortality was reported in rats after the administration of all doses mentioned above. According to Vidal et al, the lethal dose 50 (LD_{50}) of *P. granatum* hydroethanolic extract administered to mice was 731.1 mg/kg (14). Bassiri et al also reported that administering low doses of *P. granatum* peel extract between 0.5, 1.9, and 7.5 mg/kg in BALB/c mice did not cause any animal death. No toxic signs or symptoms were observed (35). Cerda et al noticed that there was no toxic effect on the hematological or biochemical parameters and the obtained results of the histopathological analysis of the liver confirmed the

absence of any toxicity. Also, no differences were observed between the control groups (36). The histopathological analysis of our results showed normal hepatic cells after treatment with our extract. Kaur et al had pretreated mice with pomegranate flower extract followed by ferric nitrilotriacetate (Fe-NTA) and the histopathological observation revealed a normal hepatic architecture with fewer pathological changes. The result suggested that *P. granatum* flower extract exerted a protective effect against Fe-NTA-induced hepatic damage (26). According to Vidal et al, the histopathological studies of mice treated with *P. granatum* hydroethanolic extract presented slight congestion of the liver (14). Vale et al showed that the treatment with *P. granatum* fruit peel didn't induce any serious lesions in the organs compared to the control (37). Chidambara et al reported that the administration of the peel extract followed by exposure to carbon tetrachloride showed no effect on the liver and suggested that the peel extract possesses a protective effect (18).

Conclusion

The aqueous extract of pomegranate peel contained different active compounds, such as gallic, caffeic, syringic acids, and coumarin. The extract had a low level of toxicity with the used concentration and good potential for antioxidants *in vivo* and *in vitro*. The extract demonstrated the ability to protect RBCs against hemolysis. The peel extract of *P. granatum* needs further research to analyze our molecules signaling mechanisms, and targets, which can be therapeutic candidates.

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Authors' contributions

All authors contributed to the study design, experiments, data analysis, and interpretation. IE, FD and RS contributed to the conceptualization of the study. IE, ML, RE and AT established the methodology. Then the formal analysis and investigation have been insured by IE and OE, IE and OE wrote the draft. The final manuscript was wrote, reviewed, and edited by IE, FD, EM, SR. Whereas, FD and SR supervised the progress of the study.

Conflict of interests

None to be declared.

Ethical considerations

All authors inspected this study for compliance with ethical issues of plagiarism, data fabrication, falsification, or redundancy related to the manuscript, and the ethical committee of the biology department at the faculty of

science Ain chock Casablanca confirmed the study (TS-PG-LIB-021).

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