Original Article

POTENT ANTI-OXIDANT AND ANTI-INFLAMMATORY POTENTIALS OF *Punica granatum* LEAF AND FLOWER HYDROMETHANOLIC EXTRACTS *IN VITRO*

POTENCIAIS ANTIOXIDANTES E ANTI-INFLAMATÓRIOS POTENTES DE FOLHAS DE Punica granatum *E EXTRATOS HIDROMETANÓLICOS DE FLORES IN VITRO*

Ibtsam GHEITH[1,2]; Abubakr EL-MAHMOUDY[3*]

1. Department of Medical Laboratories Technology, Faculty of Applied Medical Sciences, Taibah University, 344 Medinah, Kingdom of Saudi Arabia; 2. Department of Biotechnology, Animal Health Research Institute, 11843 Dokki, Egypt; 3. Department of Pharmacology, Faculty of Veterinary Medicine, Benha University, 13736 Moshtohor, Egypt; *a.elmahmoudy@fvtm.bu.edu.eg

ABSTRACT: *Punica granatum* flower and leaf were extracted with hydromethanol 50% and evaluated for antioxidant activity using phosophomolybdenum, DPPH, reducing power, and $^{\cdot}OH$-scavenging assays; and for anti-inflammatory activity using HRBC-MS assay. Both extracts exhibited antioxidant activities indicated by DPPH decolorization and $^{\cdot}OH$-scavenging and increased Ferric reducing and phosphomolybdenum assays. Also, both extracts showed anti-inflammatory activities indicated by red blood cell membrane stabilization and reduced haemolysis-resultant haemoglobin. All results were concentration-dependent; and the leaf extract was more potent than that of the flower. The IC_{50} values for the flower and leaf extracts were 777.6 ± 1.48 & 656.4 ± 1.79; 184.3 ± 1.803 & 113.9 ± 2.001; 130.8 ± 1.66 & 81.4 ± 2.1; 132.4 ± 1.55 & 79.67 ± 0.03; and 126.1 ± 1.35 & 67.25 ± 1.28 µg/mL in phosophomolybdenum, DPPH, reducing power, $^{\cdot}OH$-scavenging and red blood cell-membrane stability assays, respectively. Phytochemical analysis revealed presence of tannins and flavonoids with results more obvious in the leaf extract. These active principle contents may account for the recorded antioxidant and anti-inflammatory activities of both extracts. These findings provide evidence for the possible beneficial applications of the *Punica granatum* leaf and flower extracts in stress-related disease conditions and for maintenance of normal health status and well-being as well.

KEYWORDS: *Punica granatum*. Extract. Antioxidant. Anti-inflammatory. Oxidative stress.

INTRODUCTION

Biological processes necessary for life are associated with continuous production and release of free radicals, which are physiologically scavenged by antioxidant defence mechanisms. Under normal conditions, both oxidant and antioxidant arms are in balance with benefit and no harm to the living body cells and organs. Reactive oxygen species (ROS) includes oxygen chemicals that take part in radical type reactions (i.e. gain or loss of electrons). These chemicals are either of non-radical ROS (do not have unpaired electrons) type as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3); or of true radical ROS type as superoxide anion radicals (O^{2-}) and hydroxyl radical species ($^{\cdot}OH$). Additionally, there are also reactive nitrogen species such as nitric oxide (NO) and nitrogen dioxide (NO_2) (NUNES et al., 2012). A third product coming from both oxygen and nitrogen radicals that is peroxynitrite ($ONOO^{-}$), which occurs at a limited rate (HALLIWELL, 2011). Among many processes, ROS may be generated in harmony through mitochondrial respiration, arachidonic acid metabolism, the activation of polymorphonuclear leukocytes, enzymatic functions, and iron or copper mediated catalysis (GUPTA; VERMA, 2010).

On the other hand, anti-oxidative defense molecules are either of enzymatic type such as catalase, superoxide dismutase, glutathione peroxidase; or of non-enzymatic type as glutathione, ascorbic acid (vitamin C) and tocopherol (vitamin E) (RAHMAN, 2007).

Physiologically, both oxidant and anti-oxidant sides act in harmony keeping a good health status of living being under aerobic conditions. However, if the production of free radicals exceeded the antioxidant capacity of a living system, the reactive oxygen and nitrogen species can react with lipids, proteins, and DNA causing structural and/or functional damage to the cell's components, enzymes and genetic material (BARREIROS et al., 2006), a pathological condition termed oxidative stress. The preluding role of oxidative stress in disease pathogenesis is well established in many acute and chronic disorders in human and animal beings, such as diabetes, atherosclerosis, aging,

Received: 24/03/16
Accepted: 05/11/16

immunosuppression and neurodegeneration (HARMAN, 2003).

Such situation has directed the interested personnel to the use of medicinal and dietary supplements for cellular protection particularly during disease. Previous studies on medicinal plants reviewed by (WYNN; FOUGÈRE, 2007) have indicated that some of them have good potentials as antioxidants including Ginger (*Zingiber officinale*), *Ginkgo biloba*, Grapeseed (*Vitis vinifera*), Green tea (*Camellia sinensis*), Reishi (*Ganoderma lucidum*), Rosemary (*Rosmarinus officinale*), Skullcap (*Scutellaria lateriflora*), Milk thistle (*Silybum marianum*), and Turmeric (*Curcuma longa*). The antioxidant potential of those plants was attributable to their content of one or more of active ingredients belong to phenolics, flavonoids and tannins (DZIAŁO et al., 2016).

Punica granatum, (*Punicaceae*) is a tree that is widely distributed all over the world including Middle East. The tree may grow up to 5 m in height. It has glossy, leathery leaves and bears red flowers at the branch tips. Pharmacologically, different types of *Punica* extracts from different parts have been proven to have various actions including antidiabetic (KHALIL, 2004), anticancer (LANSKY; NEWMAN, 2007), antiparasitic (FAHMY et al., 2009), anti-ulcerogenic (ALAM et al., 2010), antihypertensive (WAGHULDE et al., 2010), anti-inflammatory (LEE et al., 2010), and antimicrobial (MOORTHY et al., 2013).

The present study was designed to assess and compare between the possible anti-oxidant and cell membrane stabilizing effects of the hydromethanolic extracts of leaves and flowers of *Punica granatum* using *in vitro* techniques. The extracts were also subjected to qualitative phytochemical analysis to determine the presence of flavonoids, phenolics, tannins and phlobatannins.

MATERIAL AND METHODS

Plant Material and extraction procedure

The leaves and flowers of *Punica granatum* (Figure 1) were collected from our local environment (University of Tripoli farm) and identified by a Botany specialist. Plant parts were refluxed in running tap water and then with bi-distilled water, shade dried at room temperature and coarsely crushed using a pestle and mortar. Extracts were prepared separately by macerating a weighed amount of the crushed plant parts (100 g) in a known volume (1 Litre) of water/organic solvent (bi-distilled water:absolute methanol, 50:50, *v/v*). Maceration continued for 72 hours in refrigerator (4 ˚C) with intermittent shaking. The hydro-methanolic extracts were then strained through muslin mesh and filtered through Whattman paper #1. The obtained filtrates were then concentrated using a shaking water bath at 56 °C in a wide-mouthed containers and the residues obtained (yields) were then lyophilized (LyoQuest-85, Telstar, Madrid, Spain), weighed and re-constituted by dissolving in measured amount of isosaline (NaCl 0.85%, *w/v*). Stock solutions of 1 mg/mL were prepared and then serially diluted to get 512, 256, 128, 64, 32, 16 and 8 µg/mL which were used for the antioxidant and anti-inflammatory activities testing. The method extraction was modified after (HARBORNE, 1998). Extracts for phytochemical testing were prepared appropriately according to the test applied (see below). Yield % was calculated as (= *100* × *Extracted residue / Original plant part weight*).

Figure 1: *Punnica granatum leaves and flowers (the collected plant material).*

Antioxidant activity

The antioxidant potential of the hydromethanolic extracts of leaves and flowers of *Punica granatum* was evaluated by the following assays:

***Phosphomolybdenum assay* (total antioxidant capacity)**

Total antioxidant capacity can be determined spectrophotometrically through the formation of phosphor-molybdenum(V) complex. The assay is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of a green phosphate-Mo(V) complex at acidic pH (PRIETO et al., 1999). An aliquot of 0.3 mL of *Punica* flower or leaf extract solutions (8 – 512 µg/mL) was mixed with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). A blank control and standard tubes were prepared by replacing the sample solution with normal saline and ascorbate (2 mM in saline), respectively. The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples had been cooled to room temperature, the absorbance of the mixtures for the blue color was measured at 695 nm against the blank. The antioxidant capacity was estimated in relation to the used standard using the following formula, where the increased absorbance indicates higher antioxidant activity:

$$Antioxidant\ \% = [(A_{sample} - A_{blank}) / (A_{standard} - A_{blank})] \times 100$$

DPPH scavenging activity

The principal of this assay was described by (BLOIS, 1958). The molecule 2,2`-diphenyl-1-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (A^H) that can donate a hydrogen atom, it gives rise to the reduced form with the loss of this violet color. In order to evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored. According to (MANZOCCO, 1998), the sample extract at a particular concentration (0.2 mL) was added to 2 mL of DPPH solution (0.5 mM in methanol). A blank control and standard tubes were prepared by replacing the sample solution with normal saline and ascorbate (2 mM in saline), respectively. After 30 min incubation at room temperature, the absorbance was measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the following equation, where the decrease of the degree of the violet color indicates higher antioxidant activity:

$$Antioxidant\ \% = [(A_{blank} - A_{sample}) / (A_{blank} - A_{standard})] \times 100$$

Ferric reducing antioxidant power assay (FRAP assay)

The principal of this test is based on the reducing power of an antioxidant upon Fe (III) to Fe (II) transformation in the presence of the an antioxidant (FEJES et al., 2000). The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm when mixed with with potassium ferricyanide, trichloro-acetic acid and ferric chloride. Increase in absorbance of the reaction mixture indicates the reducing power of the samples. In the method described by (OYAIZU, 1986), 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of $K_3Fe\ (CN)_6$ (1% w/v) were added to 1.0 mL of a tested extract sample dissolved in isosaline. A blank control and standard tubes were prepared by replacing the sample solution with isosaline and ascorbate (2 mM in saline), respectively. The resulting mixtures were incubated at 50 °C for 20 min, followed by the addition of 2.5 mL of trichloro-acetic acid (10% w/v). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of $FeCl_3$ (0.1%, w/v). The absorbance was then measured at 700 nm against the blank (calibrated as zero). The percent of reducing antioxidant activity was calculated in relation to the used standard using the following equation, where the increased absorbance indicates higher antioxidant activity:

$$Fe^{3+}\ Reducing\ \% = [(A_{sample} - A_{blank}) / (A_{standard} - A_{blank})] \times 100$$

Hydroxyl radical scavenging assay

The interaction of iron ions with hydrogen peroxide in biological systems can lead to formation of a highly reactive hydroxyl ($^{\cdot}OH$) tissue-damaging radical. This fact gave rise to the development of this method for assaying $^{\cdot}OH$ and related species in biological systems (GUTTERIDGE; HALLIWELL, 1988). The pentose sugar 2-deoxy-d-ribose is attacked by $^{\cdot}OH$ radicals to yield a mixture of products. On heating with thiobarbituric acid at low pH, some or all of these products react to form a pink chromogen that can be measured by its

absorbance at 532 nm; this chromogen is analogous to a thiobarbituric acid-malondialdehyde (TBA-MDA) adduct. Therefore, generation of a TBA-MDA adduct from deoxyribose was thus applied as a simple assay for $^{\cdot}OH$ generation in biological systems. If deoxyribose is incubated with H_2O_2 and an Fe^{2+}-EDTA complex (or an Fe^{3+}-EDTA complex in the presence of a reducing agent such as ascorbate), what is called Fenton reaction, the resulting deoxyribose degradation is inhibited by any added scavenger of $^{\cdot}OH$ to an extent that depends on the concentration of scavenger relative to deoxyribose. Hydroxyl radical scavenging activity was measured by the ability of the extracts of the different plant parts to scavenge the hydroxyl radicals generated by Fenton reaction as described below.

The reaction mixtures of 1200 µl total volume consisted of the following chemical solutions prepared in de-aerated water (cooled boiled distilled water for 10 minutes) were mixed in screw capped centrifuge tubes: 100 µl of KH_2PO_4 120 mM (for final concentration in the mixture of 10 mM), 100 µl of 2-deoxyribose 33.6 mM (for final concentration in the mixture of 2.8 mM, pH 7.4), 100 µl of H_2O_2 33.6 mM (for final concentration of 2.8 mM), 200 µl of freshly premixed of EDTA 1.2 mM solution plus ferric chloride 300 µM (1:1; *v/v*; *i.e* 100 µl of each; for final concentrations of 100 and 25 µM, respectively), 600 µl of plant extract at a particular concentration (8~512 µg/mL) and finally 100 µl of 1.2 mM ascorbate (for final concentration of 100 µM) to trigger the reaction. The plant extract was replaced by 600 µl of tannin 1% (w/v) as standard or 600 µl of deaerated water as a blank control. All the tubes were mixed well, spinned and incubated for 1 h at 37 °C in water bath. After cooling, 1 mL of thiobarbituric acid (1%, *w/v*) and then 1 mL of trichloro-acetic acid (2.8%; *w/v*) were added. The reaction tubes were mixed well and then were heated for exactly 20 minutes in a boiling water bath; where a pink color was formed. After the mixture tubes have been cooled, equal volumes of l-butanol were added, shaken to mix the two layers and the absorbance at 532 nm was recorded against the blank. The scavenging activity on hydroxyl radical was calculated as follows:

$$^{\cdot}OH\ Scavenging\ \% = [(A_{blank} - A_{sample}) / (A_{blank} - A_{standard})] \times 100$$

In vitro anti-inflammatory activity

The anti-inflammatory potential of the hydromethanolic extracts of leaves and flowers of *Punica granatum* was evaluated *in vitro* by the Human red blood cell-Membrane stability (HRBC-MS) assay described by (TRNAVSKY et al., 1974) with minor modifications. Membrane stability of red blood cells incubated with the extract at escalating concentrations (8~512 µg/mL) was tested. Blood sample (10 mL) was collected from a healthy human (or sheep) donor not consuming any steroidal or non-steroidal anti-inflammatory drugs during the past two weeks and added to equal volume of isotonic normal saline (NaCl 0.85% in distilled water, pH 7.4). The fresh blood-saline solution mixture was centrifuged at 1120 *g* for 10 minutes and the supernatant was carefully pipetted out. The packed cells were re-suspended with equal volume of isotonic normal saline and centrifuged again. The process was repeated until the supernatant became clear. A 10% RBC suspension was then prepared with normal saline and used immediately for investigating the effect of the plant extracts on RBC membrane stability using hypotonic saline solution.

A 4.5 mL reaction mixture consisting of 2 mL hypotonic saline (NaCl 0.36 %, pH 7.4), 1 mL of sodium phosphate buffer (0.15 M, pH 7.4), 1 mL of plant extract (8~512 µg/mL) dissolved in normal saline and, finally, 0.5 mL of 10% of the freshly prepared RBC suspension. A standard control was made by including 1.0 mL of diclofenac potassium (100 µg/mL) in saline. A blank control was made with 1 mL of distilled water instead of plant extract. All of the mixtures were incubated at 37°C for 30 min. then centrifuged at 2520 *g* for 5 minutes. The supernatants (containing hemoglobin) were separated and the absorbance of the supernatants were read at 560 nm.

The percentage membrane stabilizing activity was determined using the following equation:

$$\%\ Membrane\ stability = [(A_{blank} - A_{sample}) / (A_{blank} - A_{standard})] \times 100$$

Phytochemical analysis

Detection of Tannins: About 2 g of the air-dried powder of the plant were extracted with 20 mL ethanol (50 %) by heating in water bath for 30 minutes at 70 °C and tested for the presence of tannins and/or other phenolic compounds using the following tests (RAMAKRISHNAN, 2004).

Ferric chloride test: A few drops of ferric chloride solution (1%) was added to 2 mL of the extract, the appearance of bluish or greenish black coloration indicates the presence of pyrogallol or catechol tannins, respectively.

Lead acetate test: Two mL of 10% lead acetate filtered clear solution were added to 2 mL of the extract. A bulky white precipitate indicates the presence of tannin and/or phenolic compounds.

Gelatin test: Equal amounts of the extract and 1% gelatin solution in sodium chloride (0.85%) were mixed in a clean dry test tube. Formation of white precipitate indicates the presence of tannins.

Hydrochloric acid test

100 mg of the dried plant part were boiled with 1% aqueous hydrochloric acid for 30 minutes; the deposition of a red precipitate indicated the presence of phlobatannins.

Vanillin test: Five mL of the alcoholic extract of the plant parts were mixed with 2 mL vanillin-hydrochloric acid solution. Formation of a precipitate indicates the presence of gallic acid.

Detection of Flavonoids

Shinoda's test: Two mL of 10% (1 g/10 mL; *w/v*) ethanolic extract of the plant parts were mixed with 0.5 ml of hydrochloric acid (10%) and a few mg of magnesium metal. Development of a reddish color indicates the presence of flavonoids (EVANS, 2002).

Lead Acetate test: Two mL of ethanolic extract were taken in a test tube and a few drops of clear lead acetate solution (10%) were added. Formation of yellow color precipitate indicates presence of flavonoids (RAAMAN, 2006).

Alkaline reagent test: Two mL of aqueous solution of the extract were treated with 10% ammonium hydroxide solution; yellow fluorescence indicates the presence of flavonoids (RAAMAN, 2006).

Statistical analysis

Data are expressed as mean ± SEM from three separate observations. Each observation was calculated as % of the activity of the corresponding standard. For *in vitro* antioxidant assays one way ANOVA test followed by Tukey's test was used to calculate significance. A probability of $P \leq 0.05$ was considered significant from controls. IC_{50} of each extract was calculated for each assay considering the top value is that of the given standard and was set as 100%. All procedure of statistical analysis and graphing were done using the GraphPad Prism version 6 software (CA, USA).

RESULTS

The leaves and flowers of *Punica granatum* (Figure 1) were collected and extracted (see Experimental section). The yield % from the extracted 100 grams of the chopped *Punica granatum* flowers and leaves were 14.3 and 34.7%, respectively (Table 1).

Table 1. Extraction yields and phytochemical analysis test results of the flower and leaf extracts of *Punica granatum*

	Flower extract	**Leaf extract**
Extraction yield (%)	14.3	34.7
$FeCl_3$ test	+	++
Lead acetate test	++	+++
Gelatin test	+/-	+
Hydrochloric acid test	+/-	+
Vanilin test	-	-
Shinoda's test	+	++
Lead acetate test	++	+++
Alkaline reagent test	+	++

Data of the present study revealed that *Punica granatum* flower as well as leaf hydromethanolic extracts, exhibited potent total antioxidant activities indicated by phosphomolybdenum assay. The effects were concentration-dependent with IC_{50} values of 777.6 ± 1.48 and 656.4 ± 1.79 µg/mL, respectively (Figure 2 A, B).

With regard to DPPH radical-scavenging activity, Figure 3 (A, B) shows that the DPPH radical-scavenging activities of the both *Punica granatum* extracts were concentration-dependent with IC_{50} values of 184.3 ± 1.803 and 113.9 ± 2.001 µg/mL, respectively.

Flower and leaf extracts exhibited potent reducing powers on Ferric ions indicated by FRAP assay. The reducing activities were concentration-dependent, with IC_{50} values of 130.8 ± 1.66 and 81.4 ± 2.1 µg/mL, respectively (Figure 4A, B).

Hydroxyl free radicals were scavenged by both flower and leaf extracts indicated by deoxy-d-ribose degradation assay. The scavenging activities

were concentration-dependent, with IC_{50} values of 132.4 ± 1.55 and 79.67 ± 0.03 µg/mL, respectively (Figure 5A, B).

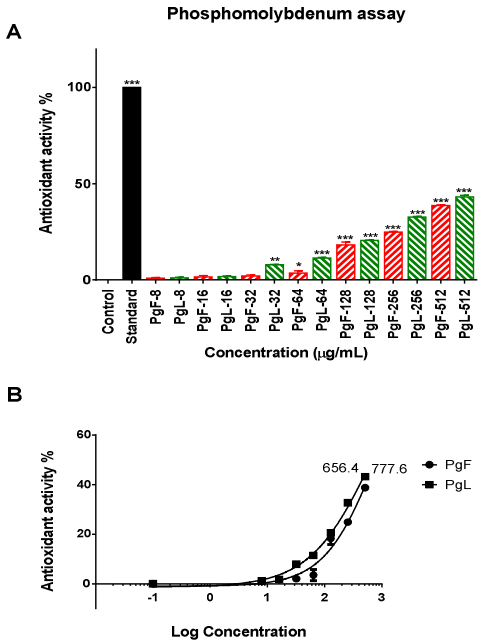

Figure 2. Bar (A) and line (B) graphs showing the % increase and IC_{50} of the phospho-molybdenum complex formation by different concentrations of *Punica granatum* flower (PgF) and leaf (PgL) hydromethanolic extracts (8~512 µg/mL); values are means ± SEM of triplicates; *,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively, compared to that without extract (ANOVA followed by Tukey's post-hoc test).

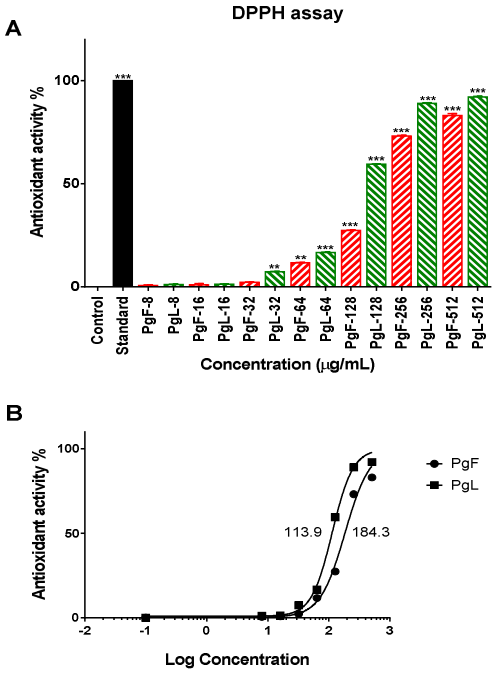

Figure 3. Bar (A) and line (B) graphs showing the % decrease and IC_{50} in the DPPH radical scavenging assay by different concentrations of *Punica granatum* flower (PgF) and leaf (PgL) hydromethanolic extracts (8~512 µg/mL); values are means ± SEM of triplicates; *,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively, compared to that without extract (ANOVA followed by Tukey's post-hoc test).

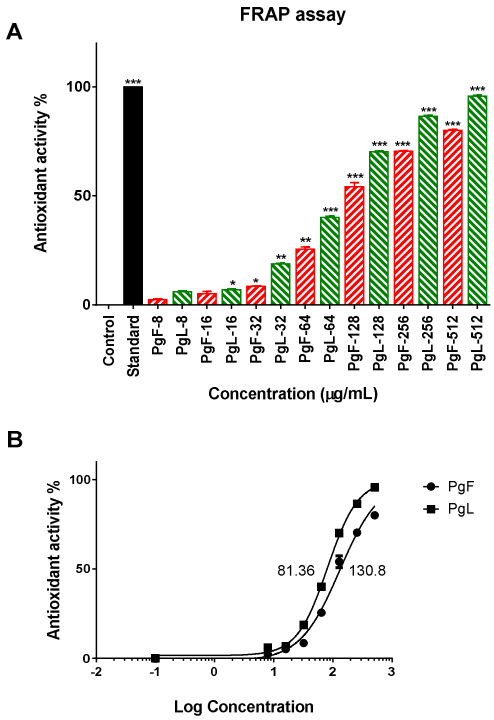

Figure 4. Bar (A) and line (B) graphs showing the % increase and IC_{50} in the Fe^{3+} reduction to Fe^{2+} assay by different concentrations of *Punica granatum* flower (PgF) and leaf (PgL) hydromethanolic extracts (8~512 µg/mL); values are means ± SEM of triplicates; *,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively, compared to that without extract (ANOVA followed by Tukey's post-hoc test).

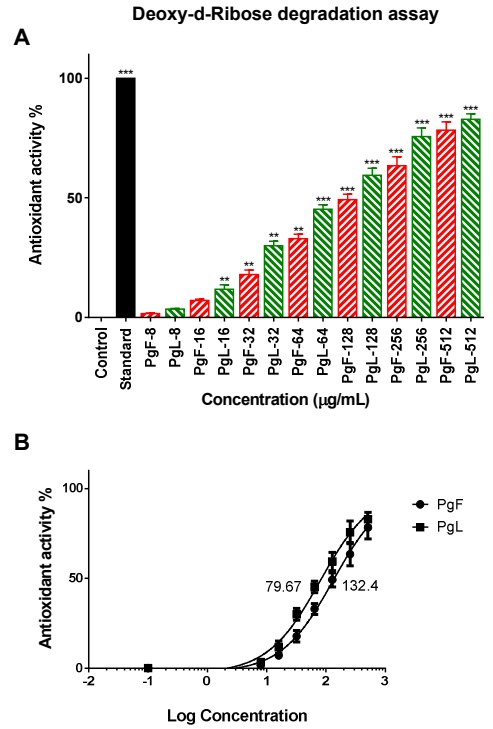

Figure 5. Bar (A) and line (B) graphs showing the % increase and IC_{50} in the deoxy-d-ribose degradation assay by different concentrations of *Punica granatum* flower (PgF) and leaf (PgL) hydromethanolic extracts (8~512 µg/mL); values are means ± SEM of triplicates; *,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively, compared to that without extract (ANOVA followed by Tukey's post-hoc test).

As indicated by HRBC-MS assay, flower and leaf extracts exhibited potent anti-inflammatory activities on red blood cell membranes that exceeded the potency of the standard used at high concentrations. The anti-inflammatory activities were concentration-dependent, with hemolysis IC_{50} values of 126.1± 1.35 and 67.25± 1.28 µg/mL, respectively (Figure 6A, B).

Phytochemical analysis revealed presence of tannin- and flavonoid-compounds with more obvious results in case of leaf extract, (Table 1).

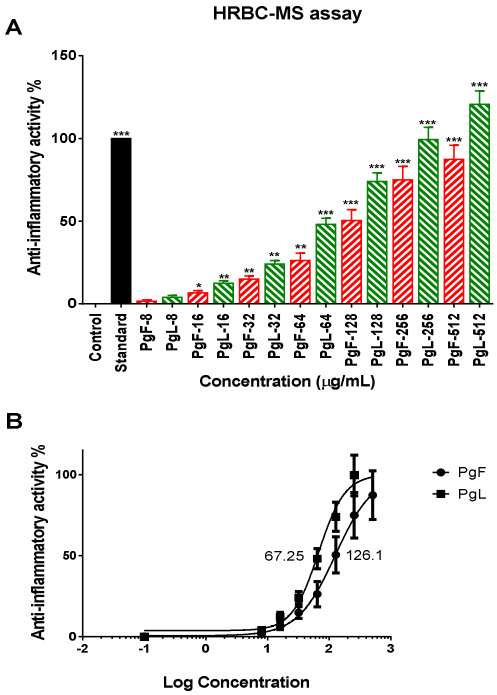

Figure 6. Bar (A) and line (B) graphs showing the % increase and IC_{50} in the HRBC membrane stability assay by different concentrations of *Punica granatum* flower (PgF) and leaf (PgL) hydromethanolic extracts (8~512 µg/mL); values are means ± SEM of triplicates; *,**,***Significant at $P < 0.05$, 0.01 and 0.001, respectively, compared to that without extract (ANOVA followed by Tukey's post-hoc test).

DISCUSSION

Substantial evidence indicated the key roles for the ROS and other oxidants in causing numerous disorders and diseases (D'AUTRÉAUX; TOLEDANO, 2007). All ROS types, at high concentrations, react readily with proteins, lipids, carbohydrates, and nucleic acids, inducing irreversible functional alterations or even complete damage to the exposed cells.

Oxidative stress is associated with the general aging process and cell death, affecting all organ systems (KRAUSE, 2007) and ROS have a part in many age-associated diseases, including Alzheimer's disease (QIN et al., 2006), Parkinson's disease (ZHANG et al., 2000), and almost all cardiovascular diseases, including hypertension, atherosclerosis, ischemic heart disease, cardiomyopathies and congestive heart failure (DHALLA et al., 2000) as well as cancer.

Normal antioxidant defenses may not always sufficient to scavenge extra radicals and to maintain the proper ROS balance especially in case of persistent long-term exposure to the source of the radicals. Major sources of ROS include cellular respiration and metabolic processes, also generated by radiation and smoking (BHATTACHARYYA et al., 2014).

Epidemiological studies support the relationship between oxidative state and global health; where high consumption of foods rich in antioxidants is associated with lower disease rates and preventive protection (WANNAMETHEE et al., 2006). The evidence has brought the attention of scientists and the general public to an appreciation of antioxidants for prevention and treatment of diseases, and maintenance of human health.

Antioxidants inhibit or delay the oxidation of cellular components by limiting either the initiation, or the propagation of the oxidizing chain reactions. They either natural or synthetic; natural antioxidants include phenolic compounds (tocopherols, flavonoids), nitrogen compounds (alkaloids, amino acids), carotenoids and ascorbic acid; while synthetic antioxidants are phenolic structures with various degrees of alkyl substitution such as butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) and propyl gallate (VELIOGLU et al., 1998).

Because of the toxicity of synthetic chemical antioxidants, studies have begun to investigate the potential of plant products to serve as antioxidants for protection against ROS and other free radicals. Phenolics, flavonoids, tannins present in various herbal extracts have been reported to be radical scavengers and inhibitors to lipid peroxidation (NUNES *et al.*, 2012).

In the present study, *Punica granatum* flower and leaf extracts have been evaluated regarding their tannin and flavonoid contents and thus their possible antioxidant and anti-inflammatory activities using some of the available screening techniques *in vitro*. The antioxidant capacity of *Punica granatum* flower and leaf extracts was measured spectrophotometrically through phospho-molybdenum complex formation assay. The method is based on the reduction of Mo(VI) to Mo (V) by adding an antioxidant with the subsequent formation of green phosphate/Mo(V) compounds that could be measured at 695 nm at which maximum absorption occurs. The present study demonstrated that *Punica granatum* flower and leaf extracts exhibited antioxidant capacity indicated by this assay. The potency of the leaf extract was higher than that of the flower. More than one study have shown that many flavonoid and related polyphenols present in medicinal plants contribute significantly to the MO(VI) scavenging and phosphor-MO(V) formation activity of medicinal plants (SHARIFIFAR et al., 2009). Positive test results for flavonoids and tannins recorded in the present study may give *Punica granatum* this property.

The electron donation ability of any substance can be measured by DPPH violet-colored solution bleaching as mentioned earlier in the methods section. The principal is based on scavenging of DPPH through the addition of an antioxidant that decolorizes the DPPH solution. The degree of color bleaching is proportional to the concentration and potency of the added antioxidant. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under investigation. In the present study flower and leaf hydromethanolic extracts of *Punica granatum* showed significant inhibition percentages that were positively correlated with the strength of precipitates of phytochemical tests; where the leaf extract showed more obvious DPPH scavenging capacity. This result may suggest that *Punica granatum* extracts contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage; and these constituents are concentrated in the leaves more than flowers.

In FRAP assay, the yellow-orange color of the test solution changes to bluish-green depending on the reducing power of the sample under investigation. Previous reports suggested that the reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain (GORDON, 1990). The active constituents present in the flower and leaf extracts of *Punica granatum* were able to exhibit concentration-dependent increase in the bluish-green coloration indicating conversion of the ferri-cyanide complex to the ferrous form, and thus proved to have reducing power. Again the effect was more evident with the leaf extract than that of flower, though both of them were highly effective.

Hydroxyl radical is one of the most serious reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell (HALLIWELL; GUTTERIDGE, 1981). The hydroxyl radical is regarded as a detrimental species in pathophysiological processes and capable of damaging any molecule of the biological system and, thus, contributes to carcinogenesis, mutagenesis and cytotoxicity (BABU et al., 2001). As mentioned in the methods section, hydroxyl radicals were produced by the reaction of H_2O_2 and the ferrous that would react with 2-deoxy-d-ribose. The reaction was stopped by adding TBA reagent that would give a pink color if the malonaldehyde was formed as the result of the reaction between the radical and 2-deox-d-yribose. Hydroxyl radical scavenging capacity of an extract is directly proportional to its antioxidant activity which is depicted by the lower intensity of pink color. Both extracts of *Punica granatum* when added to the reaction mixture actively scavenged the hydroxyl radicals and prevented the degradation of 2-deoxy-d-ribose. However, the effect of leaf extract was more obvious than that of the flower indicating higher antioxidant potential. This finding is supported by the higher flavonoid and tannin

content in the leaf extract indicated by phytochemical screening tests.

The results of the effect of *Punica granatum* extracts on RBC membrane stability were found to be very promising. Both extracts exhibited concentration-dependent membrane stabilizing effect indicated by inhibiting hypotonic saline-induced lysis of RBCs. The percentage of membrane stabilization produced by the hydromethanolic extract of *Punica granatum* leaves was 120.6 % at concentration of 512 µg/mL compared to the used standard drug Diclofenac potassium. The erythrocytic cell membrane is analogous to that of lysosomes (CHOU, 1997) and, thus, its stabilization implies that the extract may as well stabilize the lysosomal membranes. Stabilization of lysosomal membranes is important in limiting the inflammatory response by inhibiting the release of intra-lysosomal inflammatory mediators (MURUGESH et al., 1981).

IC_{50} values of radical scavenging, reducing as well as anti-inflammatory activities of the *Punica granatum* leaf extract were always lower than those of the flower extract indicating the higher potency of the former.

CONCLUSION

Punica granatum flower and leaf extracts have considerable potentials of free radical scavenging, reducing and antioxidant activities which are higher in the leaf extract; and these potentials are attributed to the tannin and flavonoid constituents present in the leaves and flowers. The leaves of *Punica granatum*, thus, can be a good source of natural antioxidant and anti-inflammatory remedies.

RESUMO: A flor e a folha de Punica granatum foram extraídas com hidrometanol 50% e avaliadas quanto à atividade antioxidante usando ensaios de fosfomolibdênio, DPPH, poder redutor e de eliminação de OH; E para atividade anti-inflamatória utilizando ensaio de HRBC-MS. Ambos os extratos exibiram actividades antioxidantes indicadas por descoloração do DPPH e por eliminação de OH e aumento nos ensaios de redução férrica e fosfomolibdênio. Além disso, ambos os extratos mostraram atividades anti-inflamatórias indicadas pela estabilização da membrana dos glóbulos vermelhos e redução da hemoglobina resultante da hemólise. Todos os resultados foram dependentes da concentração; E o extrato foliar era mais potente que o da flor. Os valores de IC50 para os extractos de flores e folhas foram de 777,6 ± 1,48 & 656,4 ± 1,79; 184,3 ± 1,803 e 113,9 ± 2,001; 130,8 ± 1,66 & 81,4 ± 2,1; 132,4 ± 1,55 & 79,67 ± 0,03; E 126,1 ± 1,35 & 67,25 ± 1,28 µg / mL em ensaios de fosfomolibdênio, DPPH, poder redutor, eliminação de OH e de estabilidade de membrana de glóbulos vermelhos, respectivamente. A análise fitoquímica revelou presença de taninos e flavonóides com resultados mais evidentes no extrato foliar. Estes conteúdos de princípio activo podem explicar as actividades antioxidantes e anti-inflamatórias registadas de ambos os extractos. Estes resultados fornecem evidências para as possíveis aplicações benéficas da folha de Punica granatum e extratos de flores em condições de doença relacionadas ao estresse e para a manutenção do estado de saúde normal e bem-estar também.

PALAVRAS-CHAVE: Punica granatum. Extrato. Antioxidante. Anti-inflamatório. Estresse oxidativo.

REFERENCES

ALAM, M. S.; ALAM, M. A.; AHMAD, S.; NAJMI, A. K.; ASIF, M.; JAHANGIR, T. Protective effects of Punica granatum in experimentally-induced gastric ulcers. **Toxicol. Mech. Methods.,** v. 20, n. 9, p. 572-578, 2010. https://doi.org/10.3109/15376516.2010.508079

BABU, B.; SHYLESH, B.; PADIKKALA, J. Antioxidant and hepatoprotective effect of Acanthus ilicifolius. **Fitoterapia,** v. 72, n. 3, p. 272-277, 2001. https://doi.org/10.1016/S0367-326X(00)00300-2

BARREIROS, A. L.; DAVID, J. M.; DAVID, J. P. Oxidative stress: relations between the formation of reactive species and the organism's defense. **Química Nova,** v. 29, n. 1, p. 113-123, 2006. https://doi.org/10.1590/S0100-40422006000100021

BHATTACHARYYA, A.; CHATTOPADHYAY, R.; MITRA, S.; CROWE, S. E. Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. **Physiol. Rev.,** v. 94, n. 2, p. 329-354, 2014. https://doi.org/10.1152/physrev.00040.2012

BLOIS, M. S. Antioxidant determinations by the use of a stable free radical. **Nature,** v. 181, p. Nature 181, 1199 - 1200, 1958. https://doi.org/10.1038/1811199a0

CHOU, C. T. The Antiinflammatory Effect of an Extract of Tripterygium wilfordii Hook F on Adjuvant-induced Paw Oedema in Rats and Inflammatory Mediators Release. **Phytother. Res.,** v. 11, n. 2, p. 152-154, 1997. https://doi.org/10.1002/(SICI)1099-1573(199703)11:2<152::AID-PTR45>3.0.CO;2-L

D'AUTRÉAUX, B.; TOLEDANO, M. B. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. **Nat. Rev. Mol. Cell Biol.,** v. 8, n. 10, p. 813-824, 2007. https://doi.org/10.1038/nrm2256

DHALLA, N. S.; TEMSAH, R. M.; NETTICADAN, T. Role of oxidative stress in cardiovascular diseases. **J. Hypertens.,** v. 18, n. 6, p. 655-673, 2000. https://doi.org/10.1097/00004872-200018060-00002

DZIAŁO, M.; MIERZIAK, J.; KORZUN, U.; PREISNER, M.; SZOPA, J.; KULMA, A. The Potential of Plant Phenolics in Prevention and Therapy of Skin Disorders. **International journal of molecular sciences,** v. 17, n. 2, p. 160, 2016. https://doi.org/10.3390/ijms17020160

EVANS, W. **Trease and Evans. Pharmacognosy, WB Saunders**. 15. Edinburgh, London: 2002.

FAHMY, Z. H.; EL-SHENNAWY, A. M.; EL-KOMY, W.; ALI, E.; HAMID, S. S. A. Potential antiparasitic activity of pomegranate extracts against schistosomules and mature worms of Schistosoma mansoni: in vitro and in vivo study. **Austr. J. Basic App. Sci.,** v. 3, n. 4, p. 4634-4643, 2009.

FEJES, S.; BLÁZOVICS, A.; LUGASI, A.; LEMBERKOVICS, É.; PETRI, G.; KÉRY, Á. In vitro antioxidant activity of Anthriscus cerefolium L.(Hoffm.) extracts. **J. Ethnopharmacol.,** v. 69, n. 3, p. 259-265, 2000. https://doi.org/10.1016/S0378-8741(99)00171-3

GORDON, M. The mechanism of antioxidant action in vitro. In: (Ed.). **Food antioxidants**: Springer, 1990. p. 1-18. https://doi.org/10.1007/978-94-009-0753-9_1

GUPTA, V.; VERMA, A. **Comprehensive Bioactive Natural Products, Vol. 4**: Stadium Press LCC, Houston, TX, USA 2010.

GUTTERIDGE, J.; HALLIWELL, B. The deoxyribose assay: an assay both for'free'hydroxyl radical and for site-specific hydroxyl radical production. **Biochem. J.,** v. 253, n. 3, p. 932, 1988. https://doi.org/10.1042/bj2530932

HALLIWELL, B. Free radicals and antioxidants–quo vadis? **Trends Pharmacol. Sci.,** v. 32, n. 3, p. 125-130, 2011. https://doi.org/10.1016/j.tips.2010.12.002

HALLIWELL, B.; GUTTERIDGE, J. M. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. **FEBS Lett.,** v. 128, n. 2, p. 347-352, 1981. https://doi.org/10.1016/0014-5793(81)80114-7

HARBORNE, J. **Phytochemical Methods, A guide to modern Techniques of plant analysis**. 3. London: Chapman & Hall, 1998.

HARMAN, D. The free radical theory of aging. **Antioxidants and Redox Signaling,** v. 5, n. 5, p. 557-561, 2003. https://doi.org/10.1089/152308603770310202

KHALIL, E. A. Antidiabetic effect of an aqueous extract of pomegranate (Punica granatum L.) peels in normal and alloxan diabetic rats. **Egy. J. Hosp. Med.,** v. 16, n. 1, p. 92-99, 2004.

KRAUSE, K.-H. Aging: a revisited theory based on free radicals generated by NOX family NADPH oxidases. **Exp. Gerontol.,** v. 42, n. 4, p. 256-262, 2007. https://doi.org/10.1016/j.exger.2006.10.011

LANSKY, E. P.; NEWMAN, R. A. Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. **J. Ethnopharmacol.,** v. 109, n. 2, p. 177-206, 2007. https://doi.org/10.1016/j.jep.2006.09.006

LEE, C.-J.; CHEN, L.-G.; LIANG, W.-L.; WANG, C.-C. Anti-inflammatory effects of Punica granatum Linne invitro and in vivo. **Food Chem.,** v. 118, n. 2, p. 315-322, 2010. https://doi.org/10.1016/j.foodchem.2009.04.123

MANZOCCO, L., ANESE, M., NICOLI, M.C. Antioxidant properties of tea extracts as affected by processing. **Lebens-mittel-Wissenschaft Und-Technologie,** v. 31, p. 694–698, 1998. https://doi.org/10.1006/fstl.1998.0491

MOORTHY, K.; PUNITHA, T.; VINODHINI, R.; SURESHKUMAR, B. T.; VIJAYALAKSHMI, P.; THAJUDDIN, N. Antimicrobial activity and qualitative phytochemical analysis of Punica granatum Linn.(PERICARP). **J. Med. Plant. Res.,** v. 7, p. 474-479, 2013.

MURUGESH, N.; VEMBAR, S.; DAMODARAN, C. Studies on erythrocyte membrane IV: in vitro haemolytic activity of oleander extract. **Toxicol. Lett.,** v. 8, n. 1, p. 33-38, 1981. https://doi.org/10.1016/0378-4274(81)90134-X

NUNES, X. P.; SILVA, F. S.; ALMEIDA, J. R. G. D. S.; BARBOSA FILHO, J. M.; DE LIMA, J. T.; DE ARAÚJO RIBEIRO, L. A.; JÚNIOR, L. J. Q. **Biological oxidations and antioxidant activity of natural products**. Rijeka, Croatia: INTECH, 2012.

OYAIZU, M. Studies on products of browning reaction--antioxidative activities of products of browning reaction prepared from glucosamine. **Jap. J. Nutr.,** v. 44, n. 6, p. 307-315, 1986. https://doi.org/10.5264/eiyogakuzashi.44.307

PRIETO, P.; PINEDA, M.; AGUILAR, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. **Anal. Biochem.,** v. 269, n. 2, p. 337-341, 1999. https://doi.org/10.1006/abio.1999.4019

QIN, B.; CARTIER, L.; DUBOIS-DAUPHIN, M.; LI, B.; SERRANDER, L.; KRAUSE, K.-H. A key role for the microglial NADPH oxidase in APP-dependent killing of neurons. **Neurobiol. Aging,** v. 27, n. 11, p. 1577-1587, 2006. https://doi.org/10.1016/j.neurobiolaging.2005.09.036

RAAMAN, N. **Phytochemical techniques**. New India Publishing, 2006.

RAHMAN, K. Studies on free radicals, antioxidants, and co-factors. **Clinical interventions in aging,** v. 2, n. 2, p. 219, 2007.

RAMAKRISHNAN, S. **Textbook of Medical Biochemistry**. Orient Longman, 2004.

SHARIFIFAR, F.; DEHGHN-NUDEH, G.; MIRTAJALDINI, M. Major flavonoids with antioxidant activity from Teucrium polium L. **Food Chem.,** v. 112, n. 4, p. 885-888, 2009. https://doi.org/10.1016/j.foodchem.2008.06.064

TRNAVSKY, K.; SCHERRER, R.; WHITEHOUSE, M. Drug effects on connective tissue metabolism. **Anti-inflamm. Agents,** v. 2, p. 303-326, 1974.

VELIOGLU, Y.; MAZZA, G.; GAO, L.; OOMAH, B. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. **Journal of agricultural and food chemistry,** v. 46, n. 10, p. 4113-4117, 1998. https://doi.org/10.1021/jf9801973

WAGHULDE, H.; MOHAN, M.; KASTURE, S.; BALARAMAN, R. Punica granatum attenuates angiotensin-II induced hypertension in wistar rats. **Int. J. Pharm. Tech. Res.,** v. 2, n. 1, p. 60-67, 2010.

WANNAMETHEE, S. G.; LOWE, G. D.; RUMLEY, A.; BRUCKDORFER, K. R.; WHINCUP, P. H. Associations of vitamin C status, fruit and vegetable intakes, and markers of inflammation and hemostasis. **Am. J. Clin. Nutr.,** v. 83, n. 3, p. 567-574, 2006.

WYNN, S. G.; FOUGÈRE, B. J. Veterinary herbal medicine: a systems-based approach. In: (Ed.). **Veterinary herbal medicine**: Mosby/Elsevier, New York, 2007. p.291. https://doi.org/10.1016/b978-0-323-02998-8.50024-x

ZHANG, Y.; DAWSON, V. L.; DAWSON, T. M. Oxidative stress and genetics in the pathogenesis of Parkinson's disease. **Neurobiol. Dis.,** v. 7, n. 4, p. 240-250, 2000. https://doi.org/10.1006/nbdi.2000.0319