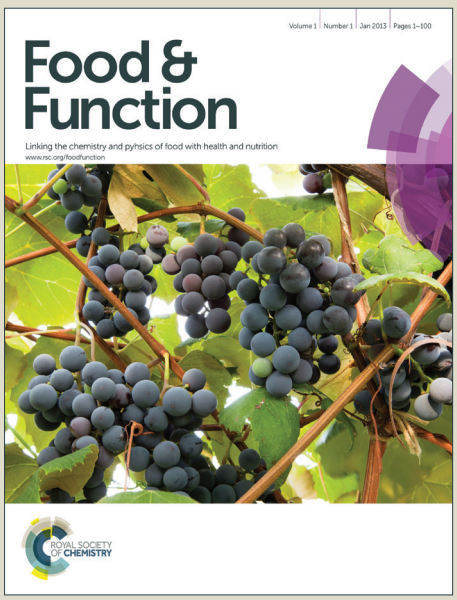
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Bioactive properties of commercialised pomegranate (*Punica granatum*) juice: antioxidant, antiproliferative and enzyme inhibiting activities

Francisco Les [1], Jose M. Prieto [2], Jose Miguel Arbonés-Mainar [3], Marta Sofía Valero [1], Víctor López [1 *]

[1] *Department of Pharmacy, Faculty of Health Sciences, San Jorge University, Villanueva de Gállego (Zaragoza), Spain.*

[2] *Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, London, UK.*

[3] *Adipocyte and Fat Biology Laboratory (AdipoFat), Unidad de Investigación Traslacional, Instituto Aragonés de Ciencias de la Salud (IACS), Hospital Universitario Miguel Servet, Zaragoza, Spain.*

*Corresponding author: Víctor López (ilopez@usj.es), Facultad de Ciencias de la Salud, Universidad San Jorge, Campus Universitario Villanueva de Gállego Autovía A-23 Zaragoza-Huesca Km. 299. 50.830 Villanueva de Gállego (Zaragoza).

ABSTRACT

Pomegranate juice and related products have long been used either in traditional medicine or as nutritional supplements claiming beneficial effects. Although there are several studies on this food plant, only few works have been performed with pomegranate juice or marketed products. The aim of this work is to evaluate the antioxidant effects of pomegranate juice on cellular models using hydrogen peroxide as an oxidizing agent or DPPH and superoxide radicals in cell free systems. The antiproliferative effects of the juice were measured on HeLa and PC-3 cells by the MTT assay and pharmacologically relevant enzymes (cyclooxygenases, xanthine oxidase, acetylcholinesterase and monoamine oxidase A) were selected for enzymatic inhibition assays. Pomegranate juice showed significant protective effects against hydrogen peroxide induced toxicity in the *Artemia salina* and HepG2 models; these effects may be attributed to radical scavenging properties of pomegranate as the juice was able to reduce DPPH and superoxide radicals. Moderate antiproliferative activities in HeLa and PC-3 cancer cells were observed. However, pomegranate juice was also able to inhibit COX-2 and MAO-A enzymes. This study reveals some mechanisms by which pomegranate juice may have interesting and beneficial effects in human health.

KEYWORDS: pomegranate juice, *Punica granatum*, ellagic acid, antioxidant, antiproliferative, COX-2, MAO-A

1. Introduction

Pomegranate, scientifically known as *Punica granatum* L. (Punicaceae), is a tree originally from the Himalayas. This species has been cultivated since antiquity in the Mediterranean and Southeast Asia, being also introduced in other areas such as tropical Africa and California[1]. It is a large-long lived tree, being able to reach three meters high with numerous branches. Its bark is grayish-green, bright green leaves and red flowers. The fruit is red and round, finishing in five triangular lobes, containing numerous seeds separated into groups by a membranous yellowish-white pericarp[2,3]. This fruit has been appreciated by numerous civilizations such as the Greek and Egyptian[4], and has been used in traditional medicine, especially in Ayurvedic medicine, for the treatment of various diseases such as diarrhea, diabetes, ulcers, parasitic infections or bleeding[5,6]. Medicinal plants and natural products have played an important role in drug discovery. They are relatively cheap and available, and their use depends, many times, on the ancestral experience. In developing countries, traditional medicinal plants remain very important in healthcare as they are used either as medicines or nutritional supplements[7].

The interest in this fruit as a nutritional or medicinal product and its therapeutic applications have increased significantly in recent years due to their potential beneficial effects on health, based on the presence of antioxidants, which may protect the human body from free radicals, oxidative processes and progression of many chronic diseases[8].

Beverages produced from fruit juices may be an interesting source of phytochemicals and antioxidants, contributing to prevent oxidation of biomolecules such as DNA, proteins, lipids and other cellular components[9,10]. Pomegranate can be eaten fresh or processed into wine, juice or extracts. Several studies have shown that pomegranate has one of the highest antioxidant activity compared to other juices and extracts such as red wine, red fruits juices, citrus and tea5[,11.12].

Studies on the composition of pomegranate show that the main components are polyphenols, highlighting the presence of punicalagins, ellagic acid, flavonoids and anthocyanins among others[13,14].

Most of its biological or pharmacological properties are attributed to this high levels of polyphenols contained in pomegranate seeds. Polyphenols possess important biological functions such as antioxidant, anti-mutagenic and anti-tumor activities[15,16].

Pomegranate can be considered as a functional food, and its juice may be a nutraceutical with a growing interest as an adjuvant in diseases such as atherosclerosis, whose development and progression is directly linked to oxidative processes in the cardiovascular system of the individual, being a risk factor for hypercholesterolemia, hypertension and diabetes. In addition, numerous other properties have been the focus of many studies, for instance, antimicrobial, anticancer, antiviral, antioxidant, antiproliferative, anti-parasitic or dermoprotective activities[17,18,19,20].

The aim of this study was to evaluate biological properties of a commercially available pure (100%, without additives) pomegranate organic juice, as many studies are performed with extracts made in the laboratory instead of registered and marketed beverages. The authors studied the antioxidant and protective effects of the juice in cellular and cell free systems, the antiproliferative effects in cancer cells (HeLa and PC-3) as well as its effects on enzymes with relevant pharmacological properties such as cyclooxygenases, xanthine oxidase, acetylcholinesterase and monoamine oxidase-A. These enzymes were selected because they are involved in inflammation, uric acid formation, dementia and depression respectively.

2. Materials and methods

2.1. Reagents and chemicals

All chemical reagents were acquired through Sigma-Aldrich (Spain). Pomegranate juice (Rabenhorst®) was acquired in a specialized shop. Authors selected this product because it was organic pomegranate juice, 100% pomegranate without additives. According to the manufacturer, the juice is obtained by expression, pasteurisation and bottled into glass bottles (batch and best before 04.03.2016; 11:57).

2.2. Pomegranate juice lyophilization

750 ml of Rabenhorst® pomegranate juice (PJ) were lyophilized using the VIRTIS Genesis 25EL lyophilizer at -40ºC (condenser at -80ºC) for 288h, with previous vacuum stage of 4 minutes until 113mTorr, and a posterior secondary drying phase of 36h with a

smooth transition of -40 to +40°C. A dried red powder was obtained and kept at -20 ºC before performing experiments.

2.3. Phytochemical analyses of lyophilized pomegranate juice

2.3.1. Polyphenol content

Folin-Ciocalteu Assay was carried out with some modifications in order to adapt the method to 96-well plates[21]. 9 µl of sample was mixed with 201.5 µl of Folin-Ciocalteu reagent. After 5 min incubation at room temperature, 89.5 µl of 15% sodium carbonate was added to the mixture and this was incubated again at room temperature in the dark for 45 min. The blank wells were made with distilled water instead of Folin-Ciocalteu reagent. Absorbance was measured at 752 nm in a microplate reader. The standard curve was measured with different concentrations of gallic acid standard water solution: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 mg/ml. The PJ water solutions were 10, 5 and 2.5 mg/ml. The result was expressed µg of gallic acid per mg of sample ± SD.

2.3.2. HPLC-DAD analysis

The phytochemical analysis of the lyophilized juice and the detection of the main compounds were done by HPLC using an Agilent 1260 Infinity LC (column Eclipse Plus C18 4.6 x 100 mm, 5 µm) coupled with a photodiode array detector, following a described procedure with some modifications[22]. Elution was carried out at a flow rate of 1 ml/min using H_20 (solvent A) and acetonitrile (solvent B) from 0% to 100% of solvent B in 50 min. Both solvents contained 0.5 % acetic acid. Detection was performed at 254 nm. The injection volume was 10 µl and the concentration of injected sample was 10 mg/ml. The presence of ellagic acid and punicalagins was confirmed by the same retention times of standard acquired in Sigma.

2.4. Protective effects of pomegranate juice in living organisms and cellular models

2.4.1. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity in Artemia salina

First of all, the toxicity of the juice was tested by the brine shrimp (*Artemia salina*) lethality assay[23,24]. Commercial dried cysts of brine shrimp were hatched in seawater with aeration for 72 hours. The lyophilized juice was dissolved in seawater and transferred to 6-well plates to obtain concentrations of 1, 10, 100, 1000 µg/ml in 5 ml sea water with 10 nauplii in each well. Control test wells were filled with 5 ml of seawater and 10 nauplii. After 24 h incubation at room temperature, the number of viable nauplii was counted. The percentage of mortality was calculated.

As pomegranate juice did not affect the viability of *Artemia salina* nauplii within the range 1-1000 µg/ml, the same experiment was performed but hydrogen peroxide was added at a concentration of 0.4 g/L in the wells containing pomegranate juice. Control wells without treatments and shrimps exposed hydrogen peroxide were also prepared. The viability of *Artemia salina* nauplii was studied every 24 hour for 3 consecutive days.

2.4.2. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity in HepG2 cells

Cultures were grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were incubated in the presence of 5% CO2 at 37 ºC and 100% relative humidified atmosphere. First of all, a general cytotoxicity MTT assay was performed in order to detect non-cytotoxic doses of pomegranate juice[25]. Cells were seeded in 96-well microplates at a density of 7 x 10^3 cells/ well and grown for 48 h at 37 ºC. Cells were treated with different concentrations of PJ (1-1000 µg/ml) and incubated for 24 hours. Cells were then treated with an MTT solution and incubated for 3 hours. The MTT solution was removed, formazan crystals were dissolved in DMSO and absorbance was read at 550 nm in a microplate reader.

The protective effect of PJ against toxicity induced by H_2O_2 in HepG2 cells was carried out using the MTT assay. Cells were seeded as described above and treated with non-cytotoxic concentrations of PJ (31.25, 15.62 and 3.90 µg/ml) for 24 h. HepG2 cells were then exposed to DPBS containing 500 µM H_2O_2 for 1 hour and new medium was added

to the cells. The MTT assay was performed 24 h after hydrogen peroxide exposure and cell survival was measured as described above.

2.5. Antioxidant activity in cell free systems

2.5.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity

The capacity of the juice to scavenge DPPH free radicals was measured by a colorimetric method[26]. 150 µl of a DPPH methanolic solution (0.04 mg/ml) were added to 150 µl of different concentrations of PJ dissolved in water at different concentrations. Absorbance was measured at 517 nm after 30 min of reaction at room temperature in a microplate reader. Controls contained all the reaction reagents except the samples. Background interferences from solvents were deducted from the activities prior to calculating radical scavenging capacity as follows: $RSC(\%)= [(Abs_{control}-Abs_{sample})/Abs_{control}]x100$

The DPPH radical scavenging capacity of ellagic acid was also measured in order to compare the activity of the juice with other compounds. Ellagic acid was dissolved in ethanol.

2.5.2. Superoxide radical scavenging activity

Superoxide radicals were generated by the xanthine/ xanthine oxidase (X/XO) system following a described procedure[27]. The reaction mixture in the wells contained: 240 µl of the following mixture (90 µM xanthine, 16 mM Na_2CO_3, 22.8 µM NBT in phosphate buffer pH 7.0) was mixed with 30 µl sample. The reaction was initiated by the addition of the enzyme (30 µl of xanthine oxidase 168 U/L) and the mixture was incubated for 2 min at 37 °C. Antioxidant activity was determined by monitoring the effect of the juice on the reduction of NBT to the blue chromogen formazan by the superoxide radical ($O_2^{\cdot -}$) at 560 nm: $RSC(\%)= [(Abs_{control}-Abs_{sample})/Abs_{control}]x100$

2.6. Antiproliferative activity in cancer cells

The antiproliferative effects of PJ were screened through the MTT assay using HeLa and PC-3 cells which are common models in screening techniques[25]. HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-

streptomycin-glutamine. PC-3 cells were grown in F-12K medium with 10% fetal bovine serum and 1% penicillin-streptomycin. Cultures were incubated in the presence of 5% CO_2 at 37 ºC and 100% relative humidified atmosphere. Cells were seeded in 96-well microplates at a density of 7 x 10^3 cells/well and grown for 24 h at 37 ºC. Cells were then treated with various concentrations of extract (0.001-1 mg/ml) for 72 h and a MTT solution was added and incubated for 3 h at 37 ºC. Cell survival was measured as reduction of MTT into formazan at 550 nm in a microplate reader. Three experiments were performed.

2.7. Inhibition of enzymes with relevant pharmacological properties

The following enzymes were selected because they are pharmacological targets for anti-inflammatory, anti-hyperuricemic, cognitive-enhancing or antidepressant drugs.

2.7.1. Inhibition of ciclooxygenases (COX-1 and COX-2) by enzyme immunoassay (EIA)

The capacity of PJ to inhibit COX-1 (ovine) and COX-2 (human recombinant) was measured in terms of prostaglandin production using a commercial kit (Cayman, item No. 560131). Authors followed kit instructions. PJ was tested at two different concentrations (0.4 and 0.2 in the reaction mixture).

2.7.2. Inhibition of xanthine oxidase (XO)

The effect of the juice on xanthine oxidase was also evaluated by measuring the formation of uric acid from xanthine at 295 nm after 2 min. The wells contained the same components as described above in the xanthine/xanthine oxidase system but the reaction mixture did not contain 22.8 μM NBT.

2.7.3. Inhibition of acetylcholinesterase (AChE)

The activity was measured using a 96-microplate reader based on Ellman's method.[28] Each well contained 25 μl of 15 mM ATCI in Millipore water, 125 μl of 3mM DTNB in buffer C (50 mM Tris–HCl, pH 8, 0.1 M NaCl, 0.02 M $MgCl_2$ 6 H_2O), 50 μl buffer B (50 mM Tris–HCl, pH 8, 0.1% Bovine Serum), 25 μl juice in buffer A (50 mM Tris–HCl, pH 8). The absorbance was read five times every 13 s for five times at 405 nm. Then, 25 μl 0.22 U/ml AChE were added and the absorbance was measured again eight times every 13 s at 405 nm.

2.7.4. Inhibition of monoamine oxidase A (MAO-A)

The bioassay was performed in a 96-well microplate (Olsen et al., 2008) [29]. Each well contained 50 µl juice (or appropriate solvent as control), 50 µl chromogenic solution (0.8 mM vanillic acid, 417 mM 4-aminoantipyrine and 4 U/ml horseradish peroxidase in potassium phosphate buffer pH 7.6), 100 µl 3 mM tyramine and 50 µl 8 U/ml MAO-A. Absorbance was read at 490 nm every 5 min for 30 min. Background interferences were deducted as the same way described above but without MAO enzyme. Data were analyzed using GraphPad to obtain IC_{50} values.

2.8. Statistical analysis

Results are expressed as mean ± standard error of experiments performed in triplicates. Data analysis was performed using GraphPad Prism version 5. ANOVA and appropriate post hoc tests were run with data depending on the type of experiments.

3. Results

3.1. Phytochemical analysis of the extract by HPLC and polyphenol content

Polyphenol content was measured by Folin-Ciocalteu method expressed as gallic acid equivalents (GAE). Our PJ contained 25.6 ± 0.9 µg GAE / mg of lyophilized pomegranate juice. *Punica granatum* juice was also analyzed by HPLC-DAD and two main peaks were detected at 254 nm. The main peaks at 1.1 min and 11.8 min were respectively identified as punicalagins and ellagic acid comparing retention times and UV-visible spectra with standards acquired in Sigma (Figure 1).

3.2. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity in Artemia salina

As shown in Figure 2, PJ increased survival of *Artemia salina* nauplii compared to 0.4 g/L hydrogen peroxide at 24, 48 and 72 hours. Hydrogen peroxide at 0.4 g/l induced significant toxicity at different times of the study; however, co-treatment of nauplii with

doses of 1 to 0.25 mg/ml enhances survival up to 80 - 100 % in the first 48h. At 72 h the percentage of *Artemia salina* survival decreases being significant only the doses of 1 mg/ml. PJ was not toxic in the range 0.001-1mg/ml (data not shown).

3.3. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity in HepG2 cells

Figure 3 shows that treating HepG2 cells with 500 μM of hydrogen peroxide for 1 hour reduced cell survival to 57.7 % compared to control. However, pre-incubation of cells with pomegranate juice at a dose of 31.25 μg/ml for 24 hours significantly increased cell viability by almost 20 % (percentage of cell survival was 78%). PJ was not toxic in the range 0.001-0.031 mg/ml in HepG2 cells. Cell viability of HepG2 was slightly reduced at higher doses (data not shown); for this reason hepatoprotective activity in HepG2 was screened at low non cytotoxic doses.

3.4. Antioxidant activity in cell free systems

The DPPH radicals scavenging effects of PJ and ellagic acid are shown in Figure 4. The antioxidant activity of PJ and ellagic acid is concentration dependent. IC_{50} values were also calculated using a nonlinear regression (one phase association) with GraphPad Prism. IC_{50} values were 23 μg/ml for PJ and 13 μg/mg for ellagic acid, which indicates that PJ antioxidant activity is at least in part due to the presence of this polyphenol in the juice.

Figure 5 shows the antioxidant effect of PJ and ellagic acid on superoxide radical, being concentration dependent. The procedure to calculate IC_{50} values was the same as DPPH method. IC_{50} values in this case were 8 μg/ml for PJ and 12 μg/mg for ellagic acid but significant differences between the juice and ellagic acid were not detected.

3.5. Antiproliferative activity in cancer cells

Pomegranate juice showed dose dependent antiproliferative effects in both HeLa (cervical cancer) and PC3 (prostate cancer) cells (Figure 6). Significant differences were detected at doses over 0.125 mg/ml in HeLa whereas statistically significant differences

in PC-3 cells were detected at lower doses (0.031 mg/ml), which indicate that this cell line seems to be more sensitive to pomegranate constituents. Cell viability was similar (close to 40 %) at the highest tested dose in both cell types.

3.6. Inhibition of enzymes with relevant pharmacological properties

3.6.1. Inhibition of COX-1 and COX-2

As shown in Figure 7, concentrations of 0.4 and 0.2 mg/ml of PJ induced COX-2 inhibition of about 60% and 25% respectively. According to our date a dose-dependent effect is observed; However, PJ did not show activity on the COX-1 isoform (data not shown).

3.6.2. Inhibition of XO and AChE

The extract did not exert activity against these enzymes (data not shown).

3.6.3 Inhibition of MAO-A

Due to the fact that PJ showed a clear dose dependent MAO-A inhibition compared to other enzymes, the effects of ellagic acid and the selective MAO-A inhibitor clorgyline were studied. PJ, ellagic acid and clorgyline inhibition of MAO-A is shown in Figure 8. IC_{50} values were also calculated using a nonlinear regression with GraphPad Prism. IC_{50} were 69.5 µg/ml for PJ, 0.705 µg/ml for ellagic acid and 0.024 µg/ml for clorgyline.

4. Discussion

Pomegranate juices and products are widely considered as a natural source of different antioxidant compounds and some studies support these claims. The antioxidant activity of this fruit is generally attributed to phytochemicals of the polyphenol type[30].

In our phytochemical study, total polyphenols were 25.6 ± 0.9 µg GAE / mg of lyophilized pomegranate juice (approximately 3000 mg/L), highlighting the presence of ellagic acid and punicalagins. According to the HPLC-DAD analysis, the main

polyphenolic compound was ellagic acid, followed by punicalagins. This result demonstrates that the PJ used in this study may be a good source of phenolic compounds; however, other research works show different levels of polyphenols (from 144 to 10,086 mg GAE/ L) [31,32]. These differences may be due to the origin of the fruit, the juice manufacturing method or how polyphenols were quantified.

The protective effects of pomegranate juice (PJ) against toxicity induced by hydrogen peroxide were measured using living organisms such as *Artemia salina* and a cellular model based on HepG2 cells. In both cases, the juice showed significant differences versus cells or living organisms exposed to the oxidant agent. The authors performed experiments with the juice as a co-treatment with hydrogen peroxide in the case of *Artemia* and as a pretreatment in HepG2 cells with the aim of studying the protective effects against a common oxidant in this both situations. The highest protective effect was in the *Artemia salina* model of co-treatment, reaching an almost 100% survival of nauplii within 48 h. However, in HepG2 cells, the protective effect against hydrogen peroxide is 20% compared to control. This effect is consistent with other studies where oxidative stress was induced by tert-butyl hydroperoxide (t-BOOH) and treated with aqueous pomegranate seed extract[33]. In this case, the reduction of toxicity enhances 21 % when cells were pretreated with 100 μg/mg of the extract.

In the cell free systems procedures, PJ has shown great ability to reduce free radicals. The antioxidant activity of ellagic acid in cell free systems was also measured because this compound is considered to be bioavailable after oral ingestion of pomegranate juice and first pass metabolism[34]. The DPPH radical is widely used as a model to evaluate the antioxidant activity of compounds and extracts[35]. PJ has shown an ability to reduce DPPH radicals in a clear dose dependent mode of action, with an IC_{50} of 23 μg/ml. A recent study with pomegranate whole seed ethanolic extract (PSEE) showed antioxidant activity in the same range with an IC_{50} of 95.6 μg/ml[36]. In the DPPH method, IC_{50} of ellagic acid was lower than PJ, and therefore it may be considered that part of PJ activity was due to ellagic acid. However, in the xanthine oxidase system, IC_{50} values for ellagic acid and pomegranate were similar. These differences may be also due to the presence of other polyphenols, and also for the synergy of actions of these components. The xanthine oxidase system is a more relevant method of generating free radicals in biology as DPPH are artificial radicals that do not exist in physiological systems. As PJ

did not inhibit XO enzyme, we can conclude that the juice acts in this method only by capturing the superoxide radical generated by the reaction of this enzyme. This antioxidant activity is in accordance to other studies of XO and pomegranate juice[37].

In addition to the antioxidant activity, PJ has shown antiproliferative activity in cancer cells, referenced in several studies. In this study, authors evaluated the antiproliferative activity using the MTT assay in HeLa and PC-3 cells, which are common models in screening techniques. PJ showed dose dependent antiproliferative effects in both cell cultures. Cell viability was close to 40 % at the highest tested dose in both cell types. Other studies have reported better results in terms of antiproliferative or cytotoxic effects in cancer cell lines, where cell survival drops to 20% for both cells types too, with a treatment of pomegranate extract [36,38]. These differences may be explained due to the fact that many studies are performed with concentrated and purified extracts, where as our study was done with a commercially available pomegranate juice. In this sense, in a recent study, other authors obtained significant differences in proliferation of PC-3 cells between pomegranates peel extracts and seeds extracts, being almost four times higher the activity of the first extract[39]. These antiproliferative effects on tumor cells could be explained by the inhibition of protein kinase A, which is altered in some kind of cancers and dietary polyphenols may act as protein kinase A inhibitors[40].

Furthermore, our study reveals that PJ may also inhibits other enzymes with relevant pharmacological properties. The inhibition of cyclooxygenases was performed by an EIA procedure, having only significant differences on the inhibition of COX-2, which is a key enzyme for the conversion of arachidonic into prostaglandins, important inflammatory mediators. Among both isoenzymes, COX-2 is relevant in inflammatory processes, whereas COX-1 is believed to have more physiological effects. This is correlated with the studies where the extract of pomegranate fruit indicated a selective inhibition of COX-2[41,42].

Finally, PJ also showed inhibitory effects on MAO-A, which is a key enzyme in neurotransmitters metabolism, involved in deamination of catecholamines and serotonin; inhibition of MAO-A may lead to antidepressant and anxiolytic effects and pomegranate juice caused MAO-A inhibition in a dose dependent manner, which could be a mechanism involved in the antidepressant activity of pomegranate reported in mice in previous works[43,44,45].

As a conclusion, this study reveals that certain pomegranate products or beverages are an interesting source of phytochemicals with antioxidant, antiproliferative, antinflammatory or mood enhancing properties and therefore may have beneficial effects in human health. This work may help to elucidate mechanisms of action involved in properties that have been observed in previous animal or human studies performed with pomegranate products.

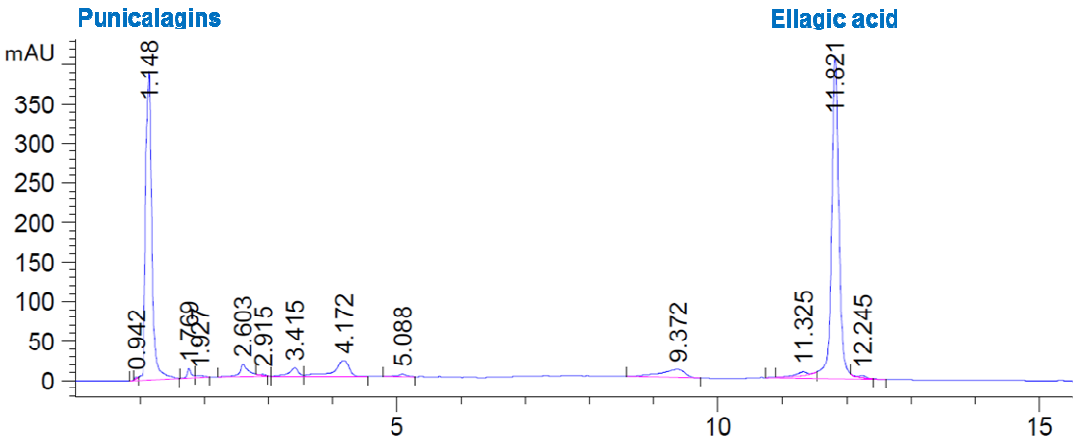
Acknowledgements

Andre Mazzari and Mukish Hanafi from University College London-School of Pharmacy are thanked for technical support with HepG2 and PC-3 cells. Dr. Olga Abián from Institute of Biocomputation and Physics of Complex Systems (BIFI) is thanked for providing HeLa cells. Miguel Ángel Céspedes from CITA-Aragón is gratefully acknowledged for lyophilization of the juice.

Conflict of interests

The authors declare no competing financial interests.

389 **Figures**

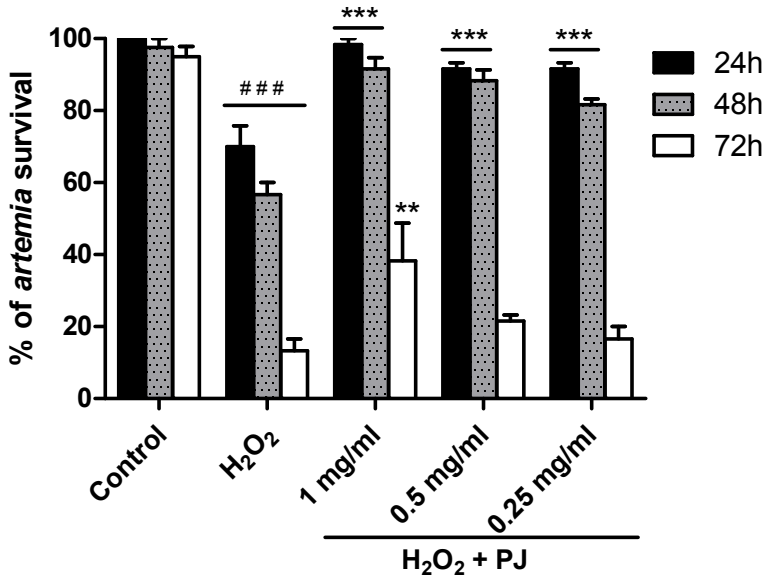


390

391 **Figure 1**. HPLC profile of pomegranate juice at 254 nm. Punicalagins (1.148 min) and
392 ellagin acid (11.821 min) were identified comparing retention times and UV-visible
393 spectra with standards analysed by the same method.

394

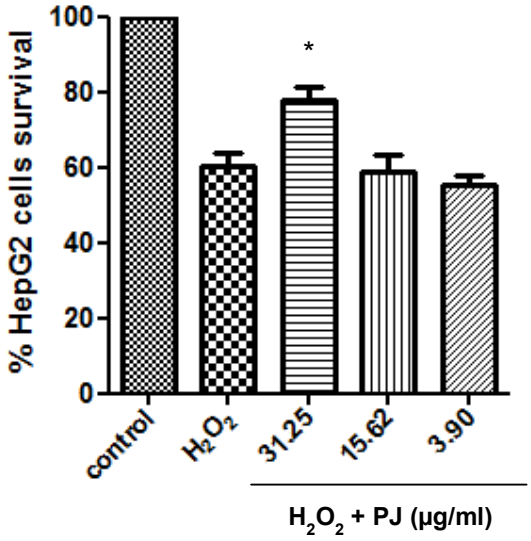
395



396

397 Figure 2. Protective effects of pomegranate juice (PJ) on hydrogen peroxide induced
398 toxicity in *Artemia salina.* ### Significant differences ($P < 0.001$) were observed between
399 control and H_2O_2 (0.4 g/l) samples at 24, 48 and 72 hours. *** Significant differences (P
400 < 0.001) also were observed between H_2O_2 and H_2O_2+PJ samples at 24 and 48 h. At 72
401 h only 1 mg/ml of PJ has protective effect with significant difference** ($P < 0.01$)
402 compared to H_2O_2 samples. Significant differences were calculated through ANOVA
403 and Dunnett´s Multiple Comparison Test.

404

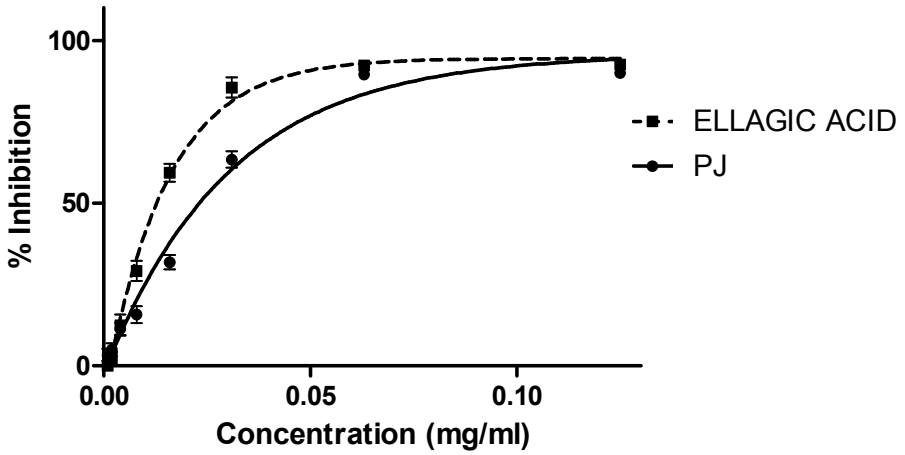


405

406 Figure 3. Protective effects of pomegranate juice (PJ) on hydrogen peroxide induced
407 toxicity in HepG2 cells. Results are expressed as % of cellular survival in terms of MTT
408 reduction. * $p < 0.05$ versus cells exposed to 500 mM hydrogen peroxide (ANOVA and
409 Newman Keuls Multiple comparison test). Concentration of pomegranate juice is
410 expressed in µg/ml.

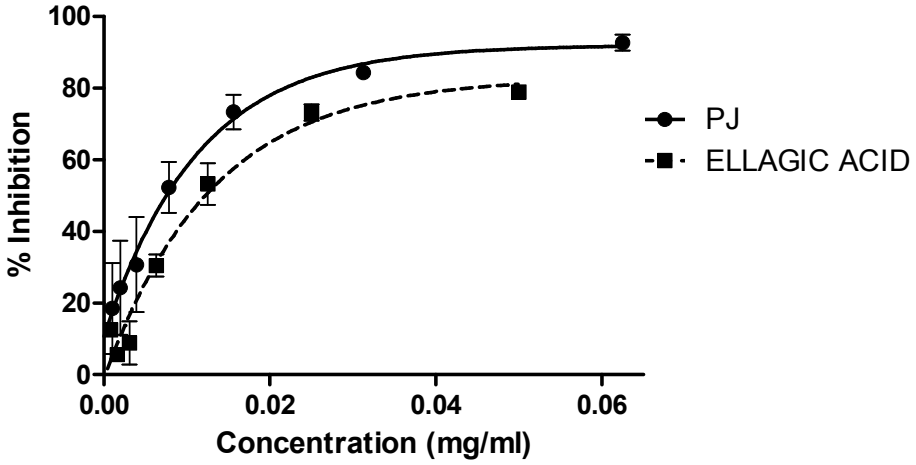
411

412



413

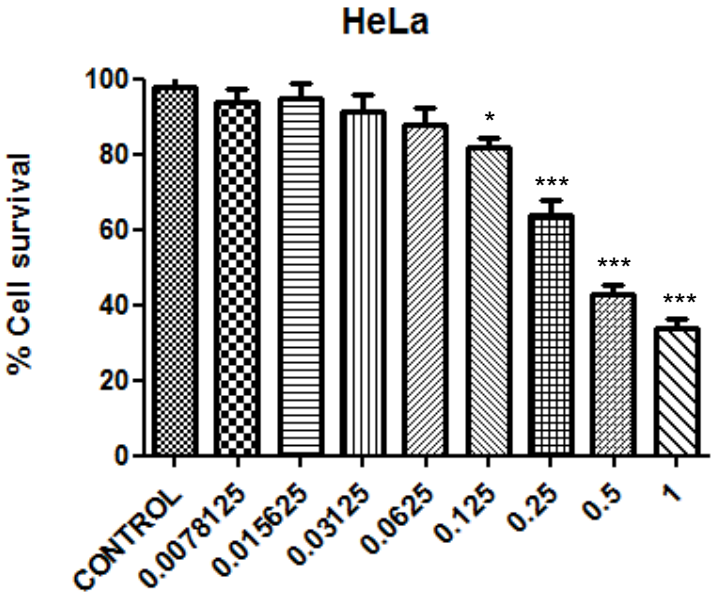
414 Figure 4. Antioxidant activity of pomegranate juice (PJ) and ellagic acid against DPPH
415 radicals. IC_{50} values were calculated by non linear regression (23 µg/ml for PJ and 13
416 µg/mg for ellagic acid).



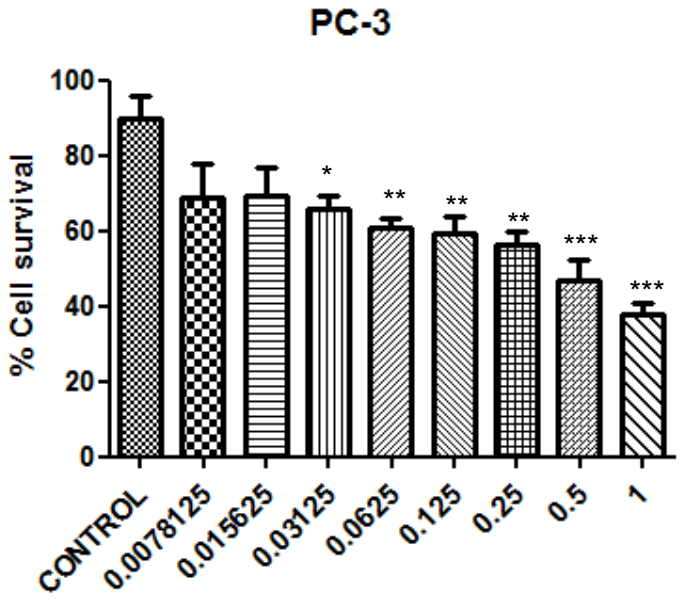
417

418 Figure 5. Antioxidant activity of pomegranate juice (PJ) and ellagic acid against
419 superoxide radicals generated by the xanthine/xathine oxidase method. IC_{50} values were
420 calculated by non-linear regression (8 µg/ml for PJ and 12 µg/mg for ellagic acid).
421 There were no significant diferences between IC_{50} values of pomegranate juice (PJ) and
422 ellagic acid (Student t test)

423



424



425

426 Figure 6. Antiproliferative effects of pomegranate juice on HeLa (human cervix
427 adenocarcinoma) and PC-3 (human prostate cancer) cells expressed as % of cell
428 survival (% MTT reduction). * $p < 0.05$, ** $p < 0.01$ versus control (non treated cells).
429 Significant differences were calculated through ANOVA and Dunnett´s Multiple
430 Comparison Test. Concentrations of pomegranate juice extract on the X axis are
431 expressed as mg/ml.

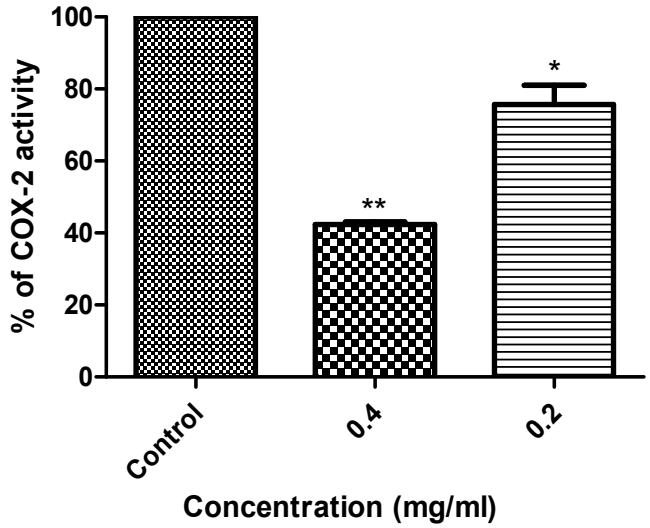


Figure 7. Inhibition of COX-2 by pomegranate juice. Significant differences compared to control exist for 0.4 mg/ml ($P < 0.01$) and 0.2 mg/ml ($P < 0.05$). Significant differences were calculated through ANOVA and Dunnett´s Multiple Comparison Test.

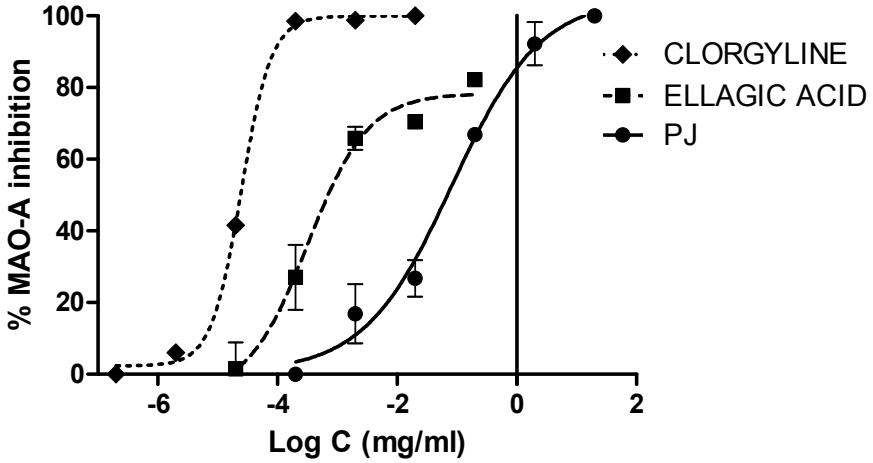


Figure 8. MAO-A inhibition profile of pomegranate juice (PJ), ellagic acid and the selective inhibitor clorgyline. Data and IC_{50} values were calculated using non-linear regression representing log C inhibitor in X axis and percentage of enzyme inhibition on Y axis.

442 **References**

[1] Salaheddin ME, Kader AA. Post-harvest physiology and storage behaviour of pomegranate fruits. Scientia Horticulturae. 1984, 24(3-4):287-298.
[2] Usanmaz S, Kahramanoğlu I, Yılmaz N. Yield and Pomological Characteristics of Three Pomegranate (Punica granatum L) Cultivars: Wonderful, Acco and Herskovitz. American Journal of Agriculture and Forestry. 2014, 2(3):61-65.
[3] Mir MM, Umar I, Mir SA, Rehman MU, Rather GH, Banday SA. Quality evaluation of pomegranate crop – a review. International journal of agriculture and biology. 2012, 14(4):658-667.
[4] Aboelsoud NH. Herbal medicine in ancient Egypt. Journal of Medicinal Plants Research. 2010, 4(2):82–86.
[5] Jurenka JS. Therapeutic applications of pomegranate (Punica granatum L.): a review. Alternative medicine review. 2008, 13(2):128-44.
[6] Bhandari PR. Pomegranate (Punica granatum L). Ancient seeds for modern cure? Review of potential therapeutic applications. International Journal of Nutrition, Pharmacology, Neurological Diseases. 2012, 2:171-84
[7] Klimpel S, Abdel-Ghaffar F, Al-Rasheid KAS et al. The effects of different plant extracts on nematodes. Parasitology Research. 2011, 108(4):1047–1054.
[8] Prior RL, Cao G. Antioxidant phytochemicals in fruits and vegetables: Diet and health implications. HortScience. 2000, 35(4):589-592.
[9] Block G, Patterson B, Subar A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. Nutrition and Cancer. 1992, 18(1):1-29.
[10] Arts IC, Jacobs DR, Harnack LJ, Gross M, Folsom AR. Dietary catechins in relation to coronary heart disease death among postmenopausal women. Epidemiology. 2001, 12(6):668-675.
[11] Elfalleh W, Hannachi H, Tlili N, Yahia Y, Nasri N, Ferchichi A. Total Phenolic Contents and Antioxidant Activity of Pomegranate (Punica granatum L.) Peel Extracts. Journal of Medicinal Plants Research. 2012, 6:4724-4730.
[12] Gil MI, Tomas-Barberan FA, Hess-Pierce B, Holcroft DM, Kader AA. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. Journal of Agricultural and Food Chemistry. 2000, 48(10):4581-4589.
[13] Nawwar MAM, Hussein SAH, Merfort I. NMR spectral analysis of polyphenols from Punica granatum. The International Journal of Plant Biochemistry. 1994, 36(3):793–798.
[14] González ME, Moreno DA, García-Viguera C. A new drink rich in healthy bioactives combining lemon and pomegranate juices. Food Chemistry, 2009; 115:1364-1372.
[15] Balasundram N, Sundram K, Sammar S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. Food Chemistry. 2006, 68:191-203.
[16] Othman A, Ismail A, Ghani AN, Adenan I. Antioxidant capacity and phenolic content of cocoa beans. Food Chemistry. 2007, 100:1523-1530.

[17] Faria A, Calhau C. The bioactivity of pomegranate: impact on health and disease. Critical reviews in food science and nutrition. 2011, 51(7):626-34.
[18] Loren DJ, Seeram NP, Schulman RN, Holtzman DM. Maternal dietary supplementation with pomegranate juice is neuroprotective in an animal model of neonatal hypoxic-ischemic brain injury. Pediatric Research. 2005, 57:864-858.
[19] Bhowmik D, Gopinath H, Kumar BP, Duraivel S, Aravind G, Kumar KPS. Medicinal uses of Punica granatum and its health benefits. Journal of Pharmacognosy and Phytochemistry. 2013, 1(5):28-35.
[20] Machado TB, Leal CR, Amaral AC, Santos KR, Silva MG, Kuster RM. Antimicrobial ellagitannin of Punica granatum fruits. Journal of the Brazilian Chemical Society. 2002; 13:606-610.
[21] Singleton VL. Citation classic—colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Current Contents/Agriculture, Biology & Environmental Sciences. 1985, 48:18-18.
[22] Kim MS, Choi CS. HPLC analysis and in vitro study of the extract from Punica granatum peel. Rapid Communication in Photoscience. 2013, 2(1):28-30.
[23] Michael AS, Thompson CG, Abramovitz M. Artemia salina as a test organism for bioassay. Science. 1956, 123:464.
[24] Mayer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, Mclaughlin JL. Brine shrimp: a convenient bioassay for active plant constituents. Planta Medica. 1982, 45:31–34.
[25] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983, 65:55–63.
[26] Lopez V, Akerreta S, Casanova E, Garcia-Mina JM, Cavero RY, Calvo MI. In vitro antioxidant and anti-rhizopus activities of Lamiaceae herbal extracts. Plant Foods for Human Nutrition. 2007, 62:151–155.
[27] Rodríguez-Chávez JL, Coballase-Urrutia E, Nieto-Camacho A, Delgado-Lamas G. Antioxidant Capacity of "Mexican Arnica" Heterotheca inuloides Cass Natural Products and Some Derivatives: Their Anti-Inflammatory Evaluation and Effect on C. elegans Life Span. Oxidative Medicine and Cellular Longevity. 2015, 843237.
[28] Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. (2001) Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. Journal of chromatography. A. 2001, 27;915(1-2):217-23.
[29] Olsen HT, Stafford GI, van Staden J, Christensen SB, Jäger AK. Isolation of the MAO-inhibitor naringenin from Mentha aquatica L. Journal of Ethnopharmacology. 2008, 22;117(3):500-2.
[30] Lansky EP, Newman RA. Punica granatum (pomegranate) and its potential for prevention and treatment of inflammation and cancer. Journal of Ethnopharmacology. 2007, 109:177-206.

[31] Tezcan F, Gultekin-Ozguven M, Diken T, et al. Antioxidant activity and total phenolic, organic acid and sugar content in commercial pomegranate juices. Food Chemistry. 2009, 115(3):873-877.
[32] Ozgen M, Durgac C, Serce S, et al. Chemical and antioxidant properties of pomegranate cultivars grown in the Mediterranean region of Turkey. Food Chemistry. 2008, 111(3):703-706.
[33] Navarro M, Amigo-Benavent M, Mesias M, Baeza G, Gökmen V, Bravo L, Morales FJ. An aqueous pomegranate seed extract ameliorates oxidative stress of human hepatoma HepG2 cells. Journal of the Science of Food and Agriculture. 2014, 94(8):1622-7.
[34] Mertens-Talcott SU, Jilma-Stohlawetz P, Rios J, Hingorani L, Derendorf H. Absorption, metabolism, and antioxidant effects of pomegranate (Punica granatum l.) polyphenols after ingestion of a standardized extract in healthy human volunteers. Journal of Agricultural and Food Chemistry. 2006, 54(23):8956-61.
[35] Moon JK, Shibamoto T. Antioxidant assays for plant and food components. Journal of Agricultural and Food Chemistry. 2009, 11;57(5):1655-66.
[36] Lucci P, Pacetti D, Loizzo MR, Frega NG. Punica granatum cv. Dente di Cavallo seed ethanolic extract: antioxidant and antiproliferative activities. Food Chemistry. 2015, 15;167:475-83.
[37] Sestili P, Martinelli C, Ricci D, Fraternale D, Bucchini A, Giamperi L, Curcio R, Piccoli G, Stocchi V. Cytoprotective effect of preparations from various parts of Punica granatum L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems. Pharmacological research: the official journal of the Italian Pharmacological Society. 2007, 56(1):18-26.
[38] Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. Proceedings of the National Academy of Sciences of the United States of America. 2005, 11;102(41):14813-8.
[39] Sineh Sepehr K, Baradaran B, Mazandarani M, Khori V, Shahneh FZ. Studies on the Cytotoxic Activities of Punica granatum L. var. spinosa (Apple Punice) Extract on Prostate Cell Line by Induction of Apoptosis. ISRN Pharmaceutics. 2012, 547942.
[40] Moskaug JØ, Borge GI, Fagervoll AM, Paur I, Carlsen H, Blomhoff R. Dietary polyphenols identified as intracellular protein kinase A inhibitors. European Journal of Nutrition. 2008, 47(8):460-9.
[41] Shukla M, Gupta K, Rasheed Z, Khan KA, Haqqi TM. Bioavailable constituents/metabolites of pomegranate (Punica granatum L) preferentially inhibit COX2 activity ex vivo and IL-1beta-induced PGE2 production in human chondrocytes in vitro. Journal of inflammation. 2008, 13;5:9.
[42] Viladomiu M, Hontecillas R, Lu P, Bassaganya-Riera J. Preventive and Prophylactic Mechanisms of Action of Pomegranate Bioactive Constituents. Evidence-Based Complementary and Alternative Medicine. 2013, 2013:18.
[43] Naveen S, Siddalingaswamy M, Singsit D, Khanum F. Anti-depressive effect of polyphenols and omega-3 fatty acid from pomegranate peel and flax seed in mice

exposed to chronic mild stress. Psychiatry and Clinical Neurosciences. 2013, 67(7):501-8.

[44] Kumar S, Maheshwari KK, Singh V. Central nervous system activity of acute administration of ethanol extract of Punica granatum L. seeds in mice. Indian journal of experimental biology. 2008, 46(12):811-6.

[45] Mori-Okamoto J, Otawara-Hamamoto Y, Yamato H, Yoshimura H. Pomegranate extract improves a depressive state and bone properties in menopausal syndrome model ovariectomized mice. Journal of Ethnopharmacology. 2004, 92(1):93-101.