

See discussions, stats, and author profiles for this publication at: <http://www.researchgate.net/publication/276297584>

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

ARTICLE in FOOD & FUNCTION · JUNE 2015

Impact Factor: 2.79 · DOI: 10.1039/C5FO00426H

READS

95

5 AUTHORS, INCLUDING:



Jose Maria Prieto

University College London

64 PUBLICATIONS 1,182 CITATIONS

SEE PROFILE



Jose M Arbones-Mainar

Aragon Health Sciences Institute

37 PUBLICATIONS 763 CITATIONS

SEE PROFILE



Marta Sofia Valero

Universidad San Jorge

24 PUBLICATIONS 44 CITATIONS

SEE PROFILE



Victor Lopez

Universidad San Jorge

26 PUBLICATIONS 216 CITATIONS

SEE PROFILE

Food & Function

Accepted Manuscript



This article can be cited before page numbers have been issued, to do this please use: F. Les, J. M. Prieto, J. Arbonés-Mainar, M. Valero and V. López, *Food Funct.*, 2015, DOI: 10.1039/C5FO00426H.



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.

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

3

4 Francisco Les ¹, Jose M. Prieto ², Jose Miguel Arbonés-Mainar ³, Marta Sofia Valero ¹,
5 Víctor López ^{1*}

6

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

9 ² *Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy,*
10 *London, UK.*

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

14

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

18

19 **ABSTRACT**

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

36

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

39

40 1. Introduction

41 Pomegranate, scientifically known as *Punica granatum* L. (Punicaceae), is a tree
42 originally from the Himalayas. This species has been cultivated since antiquity in the
43 Mediterranean and Southeast Asia, being also introduced in other areas such as tropical
44 Africa and California¹. It is a large-long lived tree, being able to reach three meters high
45 with numerous branches. Its bark is grayish-green, bright green leaves and red flowers.
46 The fruit is red and round, finishing in five triangular lobes, containing numerous seeds
47 separated into groups by a membranous yellowish-white pericarp^{2,3}. This fruit has been
48 appreciated by numerous civilizations such as the Greek and Egyptian⁴, and has been
49 used in traditional medicine, especially in Ayurvedic medicine, for the treatment of
50 various diseases such as diarrhea, diabetes, ulcers, parasitic infections or bleeding^{5,6}.
51 Medicinal plants and natural products have played an important role in drug discovery.
52 They are relatively cheap and available, and their use depends, many times, on the
53 ancestral experience. In developing countries, traditional medicinal plants remain very
54 important in healthcare as they are used either as medicines or nutritional supplements⁷.

55 The interest in this fruit as a nutritional or medicinal product and its therapeutic
56 applications have increased significantly in recent years due to their potential beneficial
57 effects on health, based on the presence of antioxidants, which may protect the human
58 body from free radicals, oxidative processes and progression of many chronic diseases⁸.

59 Beverages produced from fruit juices may be an interesting source of phytochemicals
60 and antioxidants, contributing to prevent oxidation of biomolecules such as DNA,
61 proteins, lipids and other cellular components^{9,10}. Pomegranate can be eaten fresh or
62 processed into wine, juice or extracts. Several studies have shown that pomegranate has
63 one of the highest antioxidant activity compared to other juices and extracts such as red
64 wine, red fruits juices, citrus and tea^{5,11,12}.

65 Studies on the composition of pomegranate show that the main components are
66 polyphenols, highlighting the presence of punicalagins, ellagic acid, flavonoids and
67 anthocyanins among others^{13,14}.

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

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

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

87

88 **2. Materials and methods**

89 *2.1. Reagents and chemicals*

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

95

96 *2.2. Pomegranate juice lyophilization*

97 750 ml of Rabenhorst® pomegranate juice (PJ) were lyophilized using the VIRTIS
98 Genesis 25EL lyophilizer at -40°C (condenser at -80°C) for 288h, with previous vacuum
99 stage of 4 minutes until 113mTorr, and a posterior secondary drying phase of 36h with a

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

102

103 *2.3. Phytochemical analyses of lyophilized pomegranate juice*

104 *2.3.1. Polyphenol content*

105 Folin-Ciocalteu Assay was carried out with some modifications in order to adapt the
106 method to 96-well plates²¹. 9 µl of sample was mixed with 201.5 µl of Folin-Ciocalteu
107 reagent. After 5 min incubation at room temperature, 89.5 µl of 15% sodium carbonate
108 was added to the mixture and this was incubated again at room temperature in the dark
109 for 45 min. The blank wells were made with distilled water instead of Folin-Ciocalteu
110 reagent. Absorbance was measured at 752 nm in a microplate reader. The standard
111 curve was measured with different concentrations of gallic acid standard water solution:
112 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.0078125 mg/ml. The PJ water
113 solutions were 10, 5 and 2.5 mg/ml. The result was expressed µg of gallic acid per mg
114 of sample ± SD.

115 *2.3.2. HPLC-DAD analysis*

116 The phytochemical analysis of the lyophilized juice and the detection of the main
117 compounds were done by HPLC using an Agilent 1260 Infinity LC (column Eclipse
118 Plus C18 4.6 x 100 mm, 5 µm) coupled with a photodiode array detector, following a
119 described procedure with some modifications²². Elution was carried out at a flow rate of
120 1 ml/min using H₂O (solvent A) and acetonitrile (solvent B) from 0% to 100% of solvent
121 B in 50 min. Both solvents contained 0.5 % acetic acid. Detection was performed at 254
122 nm. The injection volume was 10 µl and the concentration of injected sample was 10
123 mg/ml. The presence of ellagic acid and punicalagins was confirmed by the same
124 retention times of standard acquired in Sigma.

125

126 *2.4. Protective effects of pomegranate juice in living organisms and cellular models*

127

128

129 *2.4.1. Protective effects of pomegranate juice against hydrogen peroxide induced*
130 *toxicity in Artemia salina*

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

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

144 *2.4.2. Protective effects of pomegranate juice against hydrogen peroxide induced*
145 *toxicity in HepG2 cells*

146 Cultures were grown in Minimum Essential Medium (MEM) supplemented with 10%
147 fetal bovine serum and 1% penicillin-streptomycin. Cultures were incubated in the
148 presence of 5% CO₂ at 37 °C and 100% relative humidified atmosphere. First of all, a
149 general cytotoxicity MTT assay was performed in order to detect non-cytotoxic doses of
150 pomegranate juice²⁵. Cells were seeded in 96-well microplates at a density of 7 x 10³
151 cells/ well and grown for 48 h at 37 °C. Cells were treated with different concentrations
152 of PJ (1-1000 µg/ml) and incubated for 24 hours. Cells were then treated with an MTT
153 solution and incubated for 3 hours. The MTT solution was removed, formazan crystals
154 were dissolved in DMSO and absorbance was read at 550 nm in a microplate reader.

155 The protective effect of PJ against toxicity induced by H₂O₂ in HepG2 cells was carried
156 out using the MTT assay. Cells were seeded as described above and treated with non-
157 cytotoxic concentrations of PJ (31.25, 15.62 and 3.90 µg/ml) for 24 h. HepG2 cells were
158 then exposed to DPBS containing 500 µM H₂O₂ for 1 hour and new medium was added

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

161

162 *2.5. Antioxidant activity in cell free systems*

163 *2.5.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity*

164 The capacity of the juice to scavenge DPPH free radicals was measured by a
165 colorimetric method²⁶. 150 μ l of a DPPH methanolic solution (0.04 mg/ml) were added
166 to 150 μ l of different concentrations of PJ dissolved in water at different concentrations.
167 Absorbance was measured at 517 nm after 30 min of reaction at room temperature in a
168 microplate reader. Controls contained all the reaction reagents except the samples.
169 Background interferences from solvents were deducted from the activities prior to
170 calculating radical scavenging capacity as follows: $RSC(\%) = [(Abs_{control} -$
171 $Abs_{sample})/Abs_{control}] \times 100$

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

175 *2.5.2. Superoxide radical scavenging activity*

176 Superoxide radicals were generated by the xanthine/ xanthine oxidase (X/XO) system
177 following a described procedure²⁷. The reaction mixture in the wells contained: 240 μ l
178 of the following mixture (90 μ M xanthine, 16 mM Na_2CO_3 , 22.8 μ M NBT in phosphate
179 buffer pH 7.0) was mixed with 30 μ l sample. The reaction was initiated by the addition
180 of the enzyme (30 μ l of xanthine oxidase 168 U/L) and the mixture was incubated for 2
181 min at 37 °C. Antioxidant activity was determined by monitoring the effect of the juice
182 on the reduction of NBT to the blue chromogen formazan by the superoxide radical
183 ($O_2^{\cdot -}$) at 560 nm: $RSC(\%) = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$

184

185 *2.6. Antiproliferative activity in cancer cells*

186 The antiproliferative effects of PJ were screened through the MTT assay using HeLa
187 and PC-3 cells which are common models in screening techniques²⁵. HeLa cells were
188 grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-

189 streptomycin-glutamine. PC-3 cells were grown in F-12K medium with 10% fetal
190 bovine serum and 1% penicillin-streptomycin. Cultures were incubated in the presence
191 of 5% CO₂ at 37 °C and 100% relative humidified atmosphere. Cells were seeded in 96-
192 well microplates at a density of 7 x 10³ cells/well and grown for 24 h at 37 °C. Cells
193 were then treated with various concentrations of extract (0.001-1 mg/ml) for 72 h and a
194 MTT solution was added and incubated for 3 h at 37 °C. Cell survival was measured as
195 reduction of MTT into formazan at 550 nm in a microplate reader. Three experiments
196 were performed.

197

198 *2.7. Inhibition of enzymes with relevant pharmacological properties*

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

201 *2.7.1. Inhibition of cyclooxygenases (COX-1 and COX-2) by enzyme immunoassay (EIA)*

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

206 *2.7.2. Inhibition of xanthine oxidase (XO)*

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

211 *2.7.3. Inhibition of acetylcholinesterase (AChE)*

212 The activity was measured using a 96-microplate reader based on Ellman's method.²⁸
213 Each well contained 25 μl of 15 mM ATCI in Millipore water, 125 μl of 3mM DTNB in
214 buffer C (50 mM Tris-HCl, pH 8, 0.1 M NaCl, 0.02 M MgCl₂ 6 H₂O), 50 μl buffer B
215 (50 mM Tris-HCl, pH 8, 0.1% Bovine Serum), 25 μl juice in buffer A (50 mM Tris-
216 HCl, pH 8). The absorbance was read five times every 13 s for five times at 405 nm.
217 Then, 25 μl 0.22 U/ml AChE were added and the absorbance was measured again eight
218 times every 13 s at 405 nm.

219 2.7.4. Inhibition of monoamine oxidase A (MAO-A)

220 The bioassay was performed in a 96-well microplate (Olsen et al., 2008)²⁹. Each well
221 contained 50 µl juice (or appropriate solvent as control), 50 µl chromogenic solution
222 (0.8 mM vanillic acid, 417 mM 4-aminoantipyrine and 4 U/ml horseradish peroxidase
223 in potassium phosphate buffer pH 7.6), 100 µl 3 mM tyramine and 50 µl 8 U/ml MAO-
224 A. Absorbance was read at 490 nm every 5 min for 30 min. Background interferences
225 were deducted as the same way described above but without MAO enzyme. Data were
226 analyzed using GraphPad to obtain IC₅₀ values.

227

228 2.8. Statistical analysis

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

232

233 3. Results

234

235 3.1. Phytochemical analysis of the extract by HPLC and polyphenol content

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

242

243 3.2. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity 244 in *Artemia salina*

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

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

251

252 *3.3. Protective effects of pomegranate juice against hydrogen peroxide induced toxicity* 253 *in HepG2 cells*

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

261

262 *3.4. Antioxidant activity in cell free systems*

263 The DPPH radicals scavenging effects of PJ and ellagic acid are shown in Figure 4. The
264 antioxidant activity of PJ and ellagic acid is concentration dependent. IC₅₀ values were
265 also calculated using a nonlinear regression (one phase association) with GraphPad
266 Prism. IC₅₀ values were 23 μ g/ml for PJ and 13 μ g/mg for ellagic acid, which indicates
267 that PJ antioxidant activity is at least in part due to the presence of this polyphenol in
268 the juice.

269 Figure 5 shows the antioxidant effect of PJ and ellagic acid on superoxide radical, being
270 concentration dependent. The procedure to calculate IC₅₀ values was the same as DPPH
271 method. IC₅₀ values in this case were 8 μ g/ml for PJ and 12 μ g/mg for ellagic acid but
272 significant differences between the juice and ellagic acid were not detected.

273

274 *3.5. Antiproliferative activity in cancer cells*

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

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

281

282 *3.6. Inhibition of enzymes with relevant pharmacological properties*

283

284 *3.6.1. Inhibition of COX-1 and COX-2*

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

289

290 *3.6.2. Inhibition of XO and AChE*

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

292

293 *3.6.3 Inhibition of MAO-A*

294 Due to the fact that PJ showed a clear dose dependent MAO-A inhibition compared to
295 other enzymes, the effects of ellagic acid and the selective MAO-A inhibitor clorgyline
296 were studied. PJ, ellagic acid and clorgyline inhibition of MAO-A is shown in Figure 8.
297 IC₅₀ values were also calculated using a nonlinear regression with GraphPad Prism. IC₅₀
298 were 69.5 µg/ml for PJ, 0.705 µg/ml for ellagic acid and 0.024 µg/ml for clorgyline.

299

300 **4. Discussion**

301 Pomegranate juices and products are widely considered as a natural source of different
302 antioxidant compounds and some studies support these claims. The antioxidant activity
303 of this fruit is generally attributed to phytochemicals of the polyphenol type³⁰.

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

307 polyphenolic compound was ellagic acid, followed by punicalagins. This result
308 demonstrates that the PJ used in this study may be a good source of phenolic
309 compounds; however, other research works show different levels of polyphenols (from
310 144 to 10,086 mg GAE/ L)^{31,32}. These differences may be due to the origin of the fruit,
311 the juice manufacturing method or how polyphenols were quantified.

312 The protective effects of pomegranate juice (PJ) against toxicity induced by hydrogen
313 peroxide were measured using living organisms such as *Artemia salina* and a cellular
314 model based on HepG2 cells. In both cases, the juice showed significant differences
315 versus cells or living organisms exposed to the oxidant agent. The authors performed
316 experiments with the juice as a co-treatment with hydrogen peroxide in the case of
317 *Artemia* and as a pretreatment in HepG2 cells with the aim of studying the protective
318 effects against a common oxidant in this both situations. The highest protective effect
319 was in the *Artemia salina* model of co-treatment, reaching an almost 100% survival of
320 nauplii within 48 h. However, in HepG2 cells, the protective effect against hydrogen
321 peroxide is 20% compared to control. This effect is consistent with other studies where
322 oxidative stress was induced by tert-butyl hydroperoxide (t-BOOH) and treated with
323 aqueous pomegranate seed extract³³. In this case, the reduction of toxicity enhances 21
324 % when cells were pretreated with 100 µg/mg of the extract.

325 In the cell free systems procedures, PJ has shown great ability to reduce free radicals.
326 The antioxidant activity of ellagic acid in cell free systems was also measured because
327 this compound is considered to be bioavailable after oral ingestion of pomegranate juice
328 and first pass metabolism³⁴. The DPPH radical is widely used as a model to evaluate the
329 antioxidant activity of compounds and extracts³⁵. PJ has shown an ability to reduce
330 DPPH radicals in a clear dose dependent mode of action, with an IC₅₀ of 23 µg/ml. A
331 recent study with pomegranate whole seed ethanolic extract (PSEE) showed antioxidant
332 activity in the same range with an IC₅₀ of 95.6 µg/ml³⁶. In the DPPH method, IC₅₀ of
333 ellagic acid was lower than PJ, and therefore it may be considered that part of PJ
334 activity was due to ellagic acid. However, in the xanthine oxidase system, IC₅₀ values
335 for ellagic acid and pomegranate were similar. These differences may be also due to the
336 presence of other polyphenols, and also for the synergy of actions of these components.
337 The xanthine oxidase system is a more relevant method of generating free radicals in
338 biology as DPPH are artificial radicals that do not exist in physiological systems. As PJ

339 did not inhibit XO enzyme, we can conclude that the juice acts in this method only by
340 capturing the superoxide radical generated by the reaction of this enzyme. This
341 antioxidant activity is in accordance to other studies of XO and pomegranate juice³⁷.

342 In addition to the antioxidant activity, PJ has shown antiproliferative activity in cancer
343 cells, referenced in several studies. In this study, authors evaluated the antiproliferative
344 activity using the MTT assay in HeLa and PC-3 cells, which are common models in
345 screening techniques. PJ showed dose dependent antiproliferative effects in both cell
346 cultures. Cell viability was close to 40 % at the highest tested dose in both cell types.
347 Other studies have reported better results in terms of antiproliferative or cytotoxic
348 effects in cancer cell lines, where cell survival drops to 20% for both cells types too,
349 with a treatment of pomegranate extract^{36,38}. These differences may be explained due to
350 the fact that many studies are performed with concentrated and purified extracts, where
351 as our study was done with a commercially available pomegranate juice. In this sense,
352 in a recent study, other authors obtained significant differences in proliferation of PC-3
353 cells between pomegranates peel extracts and seeds extracts, being almost four times
354 higher the activity of the first extract³⁹. These antiproliferative effects on tumor cells
355 could be explained by the inhibition of protein kinase A, which is altered in some kind
356 of cancers and dietary polyphenols may act as protein kinase A inhibitors⁴⁰.

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

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

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

377

378

379 **Acknowledgements**

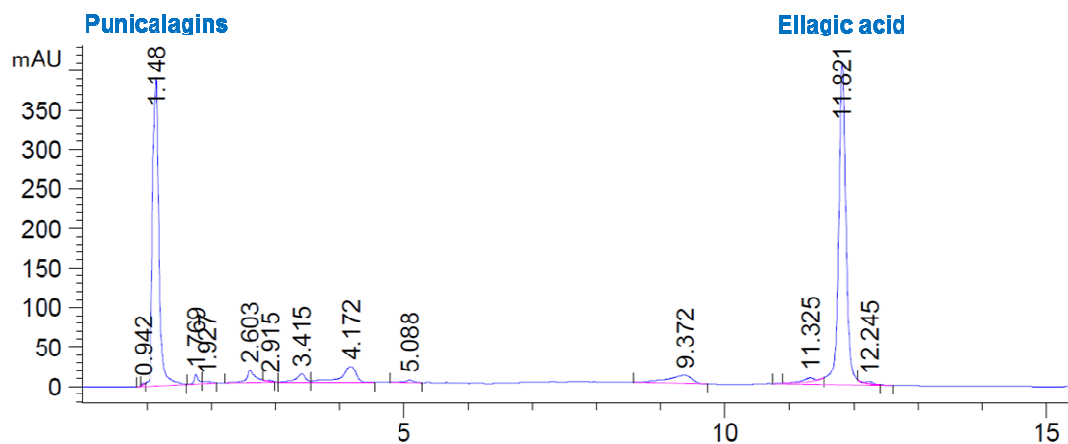
380 Andre Mazzari and Mukish Hanafi from University College London-School of
381 Pharmacy are thanked for technical support with HepG2 and PC-3 cells. Dr. Olga Abián
382 from Institute of Biocomputation and Physics of Complex Systems (BIFI) is thanked for
383 providing HeLa cells. Miguel Ángel Céspedes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

385

386 **Conflict of interests**

387 The authors declare no competing financial interests.

388

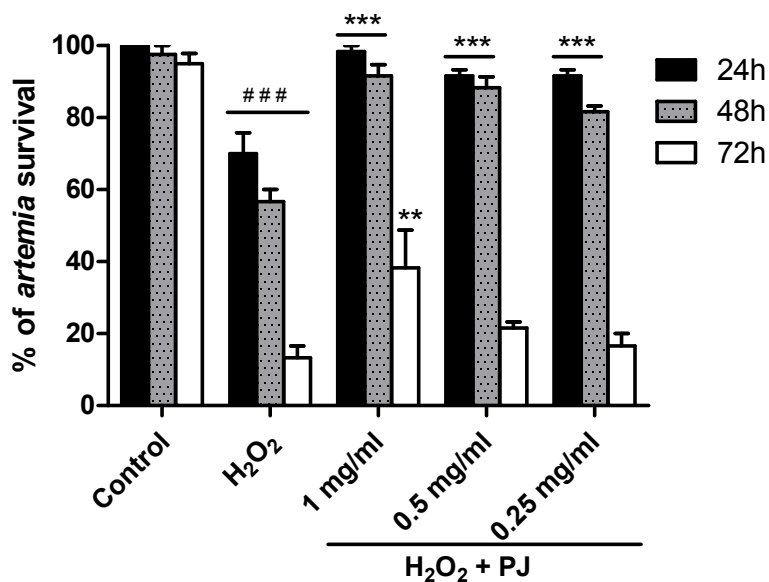
389 **Figures**

390

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

394

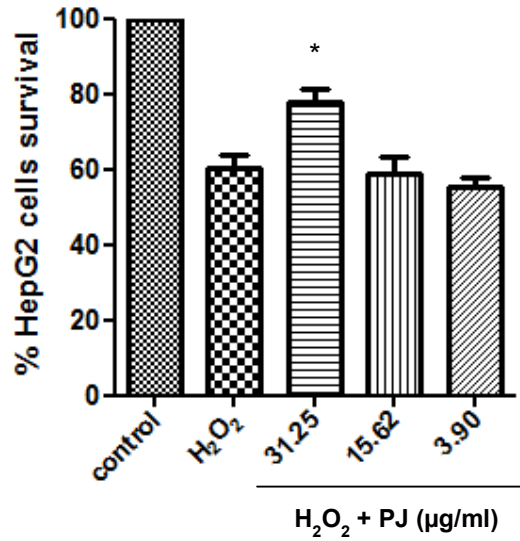
395



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 H₂O₂ (0.4 g/l) samples at 24, 48 and 72 hours. *** Significant differences (P
 400 < 0.001) also were observed between H₂O₂ and H₂O₂+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 H₂O₂ samples. Significant differences were calculated through ANOVA
 403 and Dunnett's Multiple Comparison Test.

404

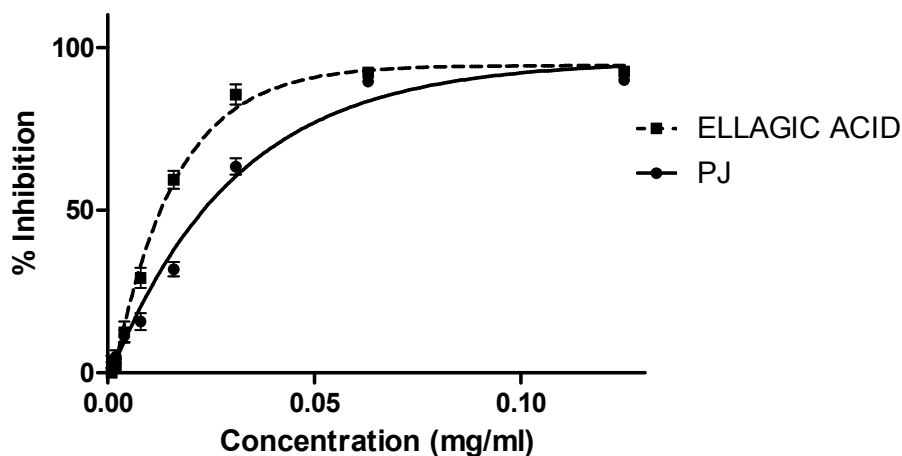


405

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 $\mu\text{g/ml}$.

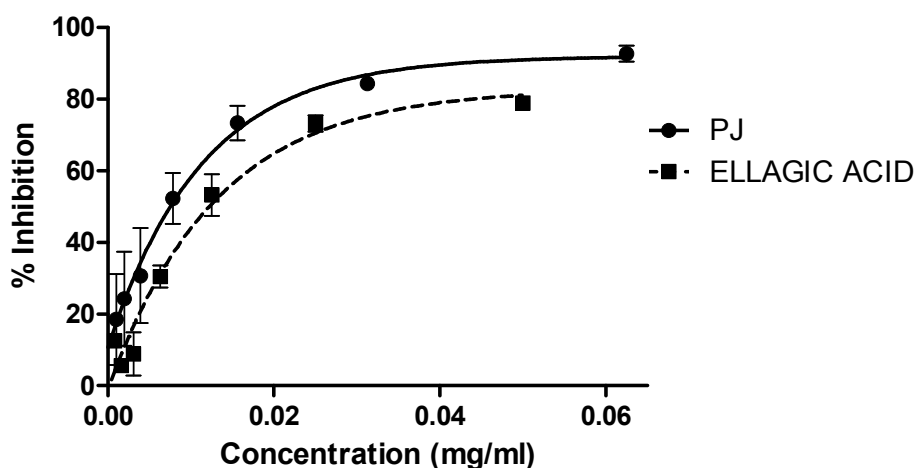
411

412



413

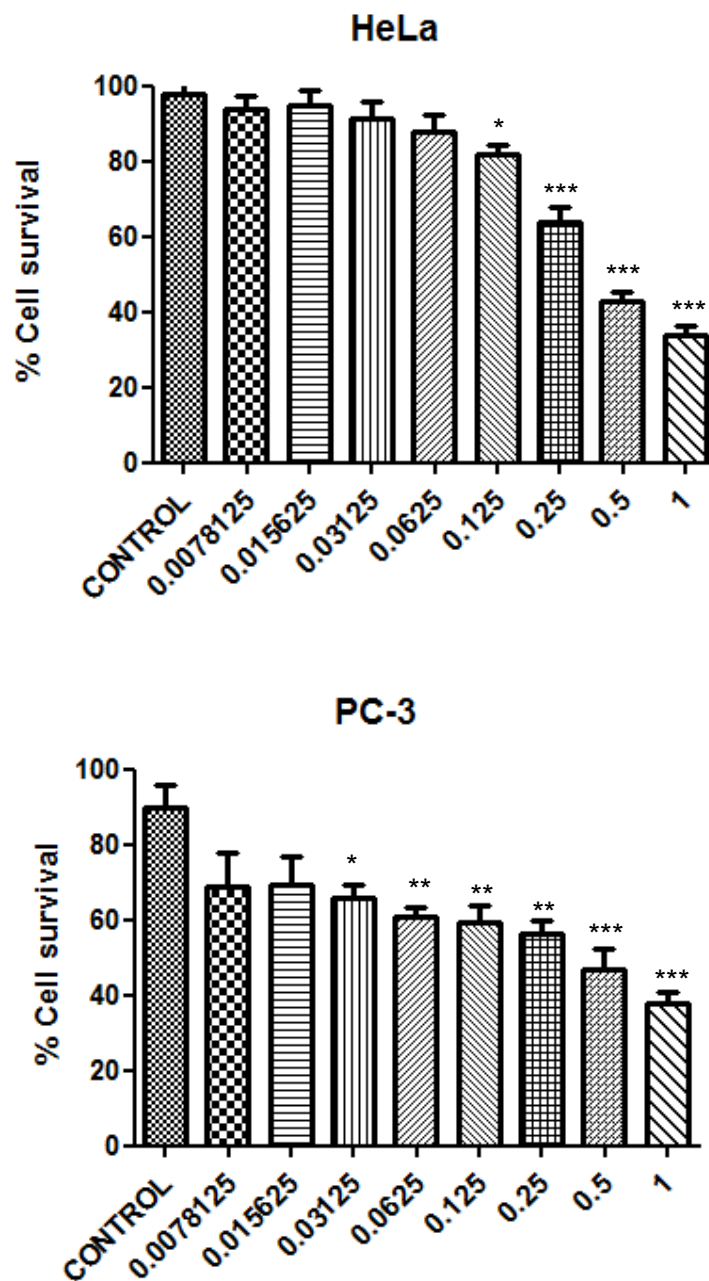
414 Figure 4. Antioxidant activity of pomegranate juice (PJ) and ellagic acid against DPPH
415 radicals. IC₅₀ values were calculated by non linear regression (23 μ g/ml for PJ and 13
416 μ g/mg for ellagic acid).



417

418 Figure 5. Antioxidant activity of pomegranate juice (PJ) and ellagic acid against
419 superoxide radicals generated by the xanthine/xathine oxidase method. IC₅₀ values were
420 calculated by non-linear regression (8 μ g/ml for PJ and 12 μ g/mg for ellagic acid).
421 There were no significant differences between IC₅₀ values of pomegranate juice (PJ) and
422 ellagic acid (Student t test)

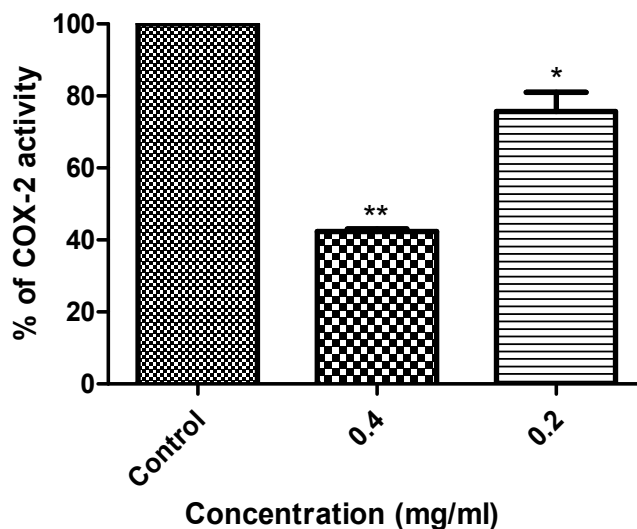
423



424

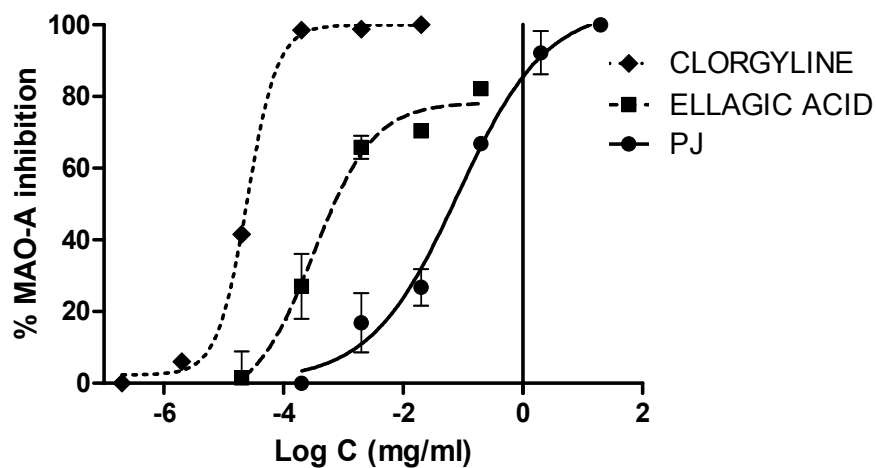
425

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.



432

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



436

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

441

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, Caverro 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, Fraternali 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, Singit 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.