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**Pomegranate inhibits neuroinflammation and amyloidogenesis in
IL-1 β -stimulated SK-N-SH cells**

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Abstract

Purpose: Pomegranate fruit, *Punica granatum L.* (Punicaceae) and its constituents have been shown to inhibit inflammation. In this study we aimed to assess the effects of freeze-dried pomegranate (PWE) on PGE₂ production in IL-1 β stimulated SK-N-SH cells.

Methods: An enzyme immuno assay (EIA) was used to measure prostaglandin E₂ (PGE₂) production from supernatants of IL-1 β stimulated SK-N-SH cells. Expression of COX-2, phospho-I κ B and phospho-IKK proteins were evaluated, while NF- κ B reporter gene assay was carried out in TNF α -stimulated HEK293 cells to determine the effect of PWE on NF- κ B transactivation. Levels of BACE-1 and A β in SK-N-SH cells stimulated with IL-1 β were measured with an in cell ELISA.

Results: PWE (25-200 μ g/ml) dose dependently reduced COX-2 dependent PGE₂ production in SK-N-SH cells stimulated with IL-1 β . Phosphorylation of I κ B and IKK were significantly ($p < 0.001$) inhibited by PWE (50- 200 μ g/ml). Our studies also show that PWE (50-200 μ g/ml) significantly ($p < 0.01$) inhibited NF- κ B transactivation in TNF α -stimulated HEK293 cells. Furthermore PWE inhibited BACE-1 and A β expression in SK-N-SH cells treated with IL-1 β .

Conclusions: Taken together, our study demonstrates that pomegranate inhibits inflammation, as well as amyloidogenesis in IL-1 β -stimulated SK-N-SH cells. We propose that pomegranate is a potential nutritional strategy in slowing the progression of neurodegenerative disorders like Alzheimer's disease.

Keywords

Pomegranate; Neuroinflammation; Amyloidogenesis; Neurons; Interleukin-1 β

1 **Introduction**

2 Alzheimer's disease (AD) is still the most common cause of dementia accounting for
3 50% to 75% of all cases [1], especially in the elderly [2]. As the population of the
4 European continent continues to age, it is predicted that AD will continue to be a
5 major public health problem. Consequently, there is need to identify and develop
6 therapeutic strategies aimed at delaying progression of AD.

7 Neurodegeneration in AD is linked to the accumulation of senile plaques which
8 consist of small peptides, known as amyloid- β ($A\beta$), and intracellular neurofibrillary
9 tangles, consisting of aggregates of hyperphosphorylated tau protein [3].

10 Neuroinflammation is a process which principally involves activation of astrocytes
11 and microglia by inflammatory mediators in AD [4, 5]. However, in spite of the widely-
12 reported roles of microglia and astrocytes in neuroinflammation, it has been
13 suggested that PGE_2 produced in neurons may contribute to the self-propagating
14 processes involved in AD. For instance, Hoshino et al. showed that PGE_2 stimulates
15 the production of $A\beta$ in cultured human neuroblastoma (SH-SY-5Y) cells [6]. Also,
16 reports have demonstrated elevated levels of PGE_2 and COX-2 in the brains of AD
17 patients [7, 8]. Inhibition of PGE_2 production and COX-2 expression therefore
18 provides a critical target for reducing the contributions of neurons to the self-
19 perpetuating cycle of neuroinflammation.

20 The production of COX-2 and other inflammatory factors is regulated by the
21 transcription factor, nuclear factor-kappa B (NF- κ B), which has been shown to be
22 widely expressed in the brain. Evidences have been put forward that NF- κ B
23 signalling pathways may be activated in AD brains [9]. These have been supported
24 by reports demonstrating that $A\beta$ peptides could activate NF- κ B in neurons [10].
25 NF κ B pathway therefore provides an important target in the understanding of
26 mechanisms involved in modulating inflammation in the neurons.

27 Accumulation of extracellular $A\beta$ plaques in neurons is one of the important
28 pathological hallmarks in AD. Also, the beta-site amyloid precursor protein cleaving
29 enzyme1 (BACE-1) play a key role in the processing of $A\beta$ and its aggregation
30 through catalysing amyloid precursor protein (APP) [11]. Various studies have
31 demonstrated that the transcription of BACE-1 is controlled by NF- κ B, and thus $A\beta$

32 production in neurons [12, 13 14, 15]. In this regard targeting BACE-1 and A β
33 production could be a potential strategy in slowing down the progression of AD.

34 Pomegranate fruit (*Punica granatum* L.) is widely consumed for its broad spectrum of
35 nutritional and health benefits. Pomegranate contains polyphenols and tannins,
36 which have been shown to be responsible for most of its nutritional benefits. Extracts
37 and bioactive constituents of pomegranate fruit have been shown to suppress
38 inflammation. Components such as punicalagin and punicalin have been shown to
39 reduce nitric oxide and PGE₂ production in intestinal cells [16, 17]. *In vitro* and *in vivo*
40 studies showed that pomegranate produced significant reduction in egg albumin-
41 induced hind paw inflammation following intraperitoneal and intracerebroventricular
42 administrations in rats, reduction in carrageenan-induced paw oedema, and NO
43 production and iNOS expression in RAW 264.7 cells [18, 19]. Recently, we showed
44 that one of the bioactive components of pomegranate, punicalagin inhibited
45 neuroinflammation in LPS-activated microglia [20]. In spite of accumulating evidence
46 showing that inflammation in neurons contribute to the pathology in AD, it is not
47 currently known if pomegranate or its constituents produce any direct effect on these
48 cells. In this study, we have evaluated the activity of freeze-dried pomegranate juice
49 on PGE₂ production in IL-1 β -stimulated SK-N-SH cells. In light of the importance of
50 neuroinflammation to amyloidogenesis, we also investigated whether pomegranate
51 could inhibit BACE-1 and A β protein expression in IL-1 β -activated neuronal cells.

52 **Materials and methods**

53 **Materials**

54 Pomegranate juice (POM Wonderful LLC, Los Angeles, CA) was freeze-dried to a
55 solid sample (PWE) which was then reconstituted in sterile water and stored at -
56 20°C. Pomegranate juice used in this study was made from fruit skins, which has
57 been standardised to ellagitannins, as punicalagins (80-85%) and free ellagic acid
58 (1.3%) as determined by high-performance liquid chromatography [21].

59 **Cell culture**

60 The human neuroblastoma (SK-N-SH) cells were obtained from the HPA Culture
61 Collection (Salisbury, UK) and were grown in MEM-Eagle's medium (Life

62 Technologies, UK). Medium was supplemented with 10% foetal bovine serum
63 (Sigma, UK), 2 mM L-glutamine, 1 mM sodium pyruvate, 40 units/ml
64 penicillin/streptomycin (Sigma, UK). Confluent monolayers were passaged routinely
65 by trypsinisation. Cultures were grown at 37°C in 5% CO₂ until 80% confluence, and
66 the medium was to serum free MEM the day before treatment.
67 HEK293 cells were obtained from the HPA Culture Collection (Salisbury, UK) and
68 were grown in MEM-Eagle's medium (Life Technologies, UK). Medium was
69 supplemented with 10% foetal bovine serum (Sigma, UK), 2 mM L-glutamine, 1 mM
70 sodium pyruvate, 40 units/ml penicillin/streptomycin (Sigma, UK). Confluent
71 monolayers were passaged routinely by trypsinisation. Cultures were grown at 37°C
72 in 5% CO₂ until 80% confluence.

73 **PGE₂ measurement**

74 Quantification of PGE₂ accumulation was carried out in SK-N-SH cells by seeding in
75 96-well plates (2 × 10⁵/well), cultured for 48 h, and incubated with or without IL-1β
76 (10 U/ml) in the absence or presence of PWE (25-200 μg/ml) for 24 h. PGE₂
77 concentration was assessed in cell supernatants with a commercially available kit
78 (Arbor Assays, Ann Arbor, MI, USA), followed by measurement at 450 nm with a
79 microplate reader. Experiments were performed at least three times and in triplicate.

80 **Sandwich ELISA for COX-2, phospho-IκBα and phospho-IKKα**

81 Protein expressions of COX-2, phospho-IκBα and phospho-IKKα were determined
82 using an ELISA for human COX-2, phospho-IκBα and phospho-IKKα. Cultured SK-
83 N-SH cells were stimulated with IL-1β (10 U/ml) in the presence or absence of PWE
84 (25-200 μg/ml) for 24 h (COX-2), or 5 min (phospho-IκBα and phospho-IKKα). At the
85 end of the experiments, cells were washed with phosphate-buffered saline (PBS)
86 and lysed with 400 μl cell lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM
87 Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-
88 glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin) and incubated on ice for 5 min.
89 The cells were then scraped and centrifuged at 13,500 rpm. Cell lysates were
90 collected and measured for levels of COX-2, phospho-IκBα and phospho-IKKα,
91 using a PathScan[®] sandwich ELISA kit (Cell Signalling Technology), according to the
92 manufacturer's instructions.

93 **Transient transfection and luciferase reporter gene assay**

94 In order to determine the effect of PWE on the transactivation of NF- κ B, a luciferase
95 reporter gene assay was carried out. HEK293 cells were seeded out at a
96 concentration of 4×10^5 cells/ml. Twenty-four hours later, cells were transfected with
97 a Cignal[®] NF- κ B Reporter (luc) (SABiosciences), using TransIT[®]-LT1 transfection
98 reagent (Mirus Bio LLC) and incubated for a further 16 h at 37 °C in 5% CO₂. Twenty-
99 four hours later, transfected HEK293 cells were stimulated with TNF α (1 ng/ml) in the
100 presence or absence of PWE (25-200 μ g/ml) for 6 h. NF- κ B-mediated gene
101 expression was measured with ONE-Glo luciferase assay kit (Promega,
102 Southampton, UK) according to the manufacturer's instructions.

103 **In Cell ELISA for BACE1 and A β**

104 In Cell ELISA is used for quantitative protein analysis directly in adherent cell
105 cultures, and was used to measure BACE-1 and A β protein expression following
106 stimulation of SK-N-SH cells with IL-1 β , as described earlier [22]. The protocol was
107 based on the MaxDiscovery In Cell ELISA kit (Bio Scientific, Texas). SK-N-SH cells
108 were seeded out in a 96-well plate (2.5×10^5 cells/ml). At 80% confluence, cells were
109 pre-treated with PWE (25-200 μ g/ml) 30 min before stimulation with IL-1 β (10 U/ml)
110 for 24 h. At the end of stimulation, cells were washed with 100 μ l PBS, fixed and
111 permeabilised. Primary antibodies (rabbit anti-BACE-1 or rabbit anti-A β) were diluted
112 1:100 and added to each sample well and incubated at room temperature for 1 h.
113 This was followed by incubation with HRP-conjugated anti-rabbit IgG antibody at
114 room temperature for 1 h. TMB solution was added, followed by stop solution and
115 the plate read at 450 nm using a Tecan F50 microplate reader. GAPDH was used as
116 internal control.

117 **Determination of cell viability**

118 Viability of SK-N-SH cells treated with IL-1 β (10 U/ml) in the presence or absence of
119 PWE (25-200 μ g/ml) was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-
120 diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well plates ($2 \times$
121 10^5 cells/ml) and incubated for 48 h. Thereafter, cells were pre-treated with PWE
122 (25-200 μ g/ml) prior to stimulation with IL-1 β (10 U/ml). Twenty-four hours after
123 stimulation, culture medium was replaced with MTT solution (5 mg/ml) and incubated

124 for 4 h at 37°C in 5% CO₂. Thereafter 150 µl of MTT solution was replaced with
125 DMSO and mixed thoroughly on a plate shaker and read at 540 nm.

126 Cell viability was also measured using the Lactate dehydrogenase (LDH) assay [23].
127 LDH is a cytosolic enzyme that is an indicator of cellular toxicity. When the plasma
128 membrane is damaged, LDH is released into cell culture media. Cells were seeded
129 in 96-well plates (2 × 10⁵ cells/ml) and incubated for 48 h. Thereafter, cells were pre-
130 treated with PWE (25-200 µg/ml) prior to stimulation with IL-1β (10 U/ml). Cells were
131 then lysed and supernatants collected for LDH assay. LDH levels in supernatants
132 were determined using the CytoTox 96® non-radioactive cytotoxicity assay kit
133 (Promega, Southampton).

134 **Statistical analysis**

135 Values of all experiments were represented as mean ±SEM of at least
136 3 experiments. Values were compared using t-test (two groups) or one-way ANOVA
137 with post-hoc Student Newman–Keuls test (multiple comparisons). Levels of
138 significance were set at *p < 0.05, **p<0.01, ***p<0.001.

139 **Results**

140 **PWE reduced PGE₂ production by inhibiting cyclooxygenase-2 (COX-2) protein** 141 **expression in IL-1β-activated SK-N-SH cells.**

142 In the presence of IL-1β (10 U/ml), there was a marked increase (***p<0.01) in PGE₂
143 production in supernatants of SK-N-SH cells, when compared to unstimulated cells.
144 However, treatment with PWE (25-200 µg/ml) for 30 min prior to stimulation with IL-
145 1β resulted in significant reduction (***p<0.001) in PGE₂ production, in comparison
146 with IL-1β control (Fig. 1). PGE₂ is synthesised through the enzymatic activity of
147 COX-2. Consequently, we sought to determine whether the effect of PWE on PGE₂
148 was mediated through inhibition of the activities of this enzyme. Interestingly,
149 experiments showed that PWE (25-200 µg/ml) produced significant reduction
150 (***p<0.001) in COX-2 protein levels in IL-1β-stimulated SK-N-SH cells (Fig. 2).

151 **PWE inhibited NF-κB dependent reporter gene expression in TNFα activated** 152 **HEK293 cells.**

153 In order to determine the effect of PWE on the transactivation of NF- κ B, a luciferase
154 reporter gene assay was carried out. We observed that stimulation of transfected
155 cells with TNF α (1 ng/ml) resulted in activation of the NF- κ B-driven luciferase
156 expression (Fig. 3). Pre-incubation with PWE (25 μ g/ml) did not affect luciferase
157 expression. However, pre-treatment with 50, 100 and 200 μ g/ml of PWE resulted in
158 significant ($p < 0.01$) and concentration-dependent inhibition of NF- κ B-driven
159 luciferase expression, demonstrating that PWE suppresses NF- κ B-dependent gene
160 expression in general.

161 **PWE inhibited IL-1 β dependent I κ B and IKK phosphorylation in SK-N-SH cells.**

162 Based on our observation that PWE inhibited NF- κ B-mediated gene expression in
163 general, we sought to investigate its effect on IKK and I κ B phosphorylation following
164 stimulation with IL-1 β (10 U/ml). Using a sandwich ELISA kit, we observed that IL-1 β
165 treatment resulted in phosphorylation of IKK and I κ B in IL-1 β treated cells, compared
166 with unstimulated cells. These were significantly inhibited by pre-treatment with 50,
167 100 and 200 μ g/ml of PWE (Fig. 4 and 5).

168 **Pre-treatment of SK-N-SH cells with PWE resulted in inhibition of BACE-1 and** 169 **A β proteins**

170 Exposure of the cells to IL-1 β resulted in a marked increase in both BACE-1 and A β
171 proteins (Fig. 6 and 7). However, pre-treatment with PWE (50, 100 and 200 μ g/ml)
172 significantly reduced the levels of BACE-1 and A β proteins.

173 **PWE did not affect the viability of SK-N-SH cells**

174 In order to show that PWE did not affect viability of SK-N-SH cells at concentrations
175 used in this experiment, an MTT assay was performed. Results showed that
176 treatment with PWE (25-200 μ g/ml) did not have significant effect on the viability of
177 the cells (Fig. 8a). LDH assay also showed that concentrations of PWE used for
178 pharmacological investigations did not affect viability (Fig. 8b). These results suggest
179 that the observed effects of PWE were not due to cytotoxicity as a result of
180 decreased live cells.

181 **Discussion**

182 Studies on the role of neuroinflammation in AD have focused mainly on the activity of
183 the microglia. However, studies have shown that inflammation in neurons also
184 contribute to the self-perpetuating processes leading to neuronal loss. For example,
185 studies have shown that PGE₂ is able to stimulate A β production in SH-SY5Y cells
186 [6]. Furthermore, IL-18 has been shown to increase BACE-1 expression in
187 differentiated SH-SY5Y cells [24]. We therefore investigated whether pomegranate
188 could affect IL-1 β -induced PGE₂ production, as well as BACE-1 and A β production in
189 SK-N-SH cells.

190 Studies have shown that elevated levels of COX-2 and its metabolic product PGE₂
191 was observed in AD brains, while COX-2 inhibitors markedly reduce the risk of AD
192 [25]. Recent *in vivo* studies also show that long term treatment of APP transgenic
193 mice with NSAIDs significantly diminished inflammatory factors and its dependant A β
194 deposition [2626]. Our results show that pomegranate significantly inhibited COX-2-
195 mediated PGE₂ production in IL-1 β -stimulated SK-N-SH cells, suggesting that
196 pomegranate could reduce the toxic effects of PGE₂ overproduction in neurons.

197 NF- κ B plays a crucial role in regulating the transcription of a wide variety of genes
198 and during neuroinflammation and neurodegeneration. In the cytoplasm NF- κ B is
199 coupled with I κ B, an inhibitory protein which stays inactive. On activation, I κ B
200 undergoes phosphorylation by IKK, resulting in the liberation of NF- κ B. This free
201 NF- κ B translocates into the nucleus and binds to the promoter region of respective
202 genes such as COX-2. Furthermore, NF- κ B activation has been shown to control the
203 transcription of the BACE-1 and APP genes in neurons [13]. To investigate the effect
204 of pomegranate on NF- κ B-mediated gene expression in general, a reporter gene
205 assay was carried out. Results show that pomegranate significantly inhibited NF- κ B-
206 driven luciferase expression in TNF α stimulated HEK293 cells, suggesting that this
207 compound is able to attenuate NF- κ B mediated gene expression. To gain a better
208 understanding on the modulatory action of pomegranate on NF- κ B signalling
209 pathway we studied its activity on upstream protein targets. Results show that
210 pomegranate blocked phosphorylation of I κ B and IKK in SK-N-SH neuronal cells
211 stimulated with IL-1 β ; this outcome might suggest that pomegranate acts through
212 interference with NF- κ B pathway in neurons. These results were consistent with the
213 outcome of studies conducted by Romier-Crouzet et al in human intestinal cells.

214 They reported that polyphenolic aqueous extract of pomegranate significantly
215 suppressed NF- κ B mediated NO, PGE₂, IL-8 production in IL-1 β activated Caco-2
216 cells [27]. Our results also reflect the results of studies conducted by Ahmed et al
217 where it was shown that pomegranate inhibited NF- κ B in IL-1 β -activated human
218 chondrocytes [28].

219 In AD brains, A β is generated through proteolysis of APP by β -secretase enzymes.
220 Several studies have identified BACE-1 as an important β -secretase enzyme which
221 effectively cleaves membrane bound APP [29]. *In vivo* studies in transgenic mice
222 have also revealed that BACE-1 is highly involved in A β plaque formation, and
223 employing BACE-1 blockers has completely reversed A β production [30]. As BACE-1
224 transcription has been shown to be controlled by NF- κ B [13], and since we have
225 shown that pomegranate inhibits NF- κ B signalling in SK-N-SH cells, we investigated
226 whether pomegranate would block BACE-1 protein in IL-1 β -stimulated SK-N-SH
227 cells. Expectedly, IL-1 β induced marked increase in BACE-1 expression in these
228 cells and this increase was significantly blocked with pomegranate pre-treatment.
229 Interestingly, pomegranate also inhibited A β protein induced by IL-1 β , suggesting
230 that its effect is probably mediated through the observed interference with BACE-1
231 enzymatic activity.

232 These *in vitro* evidence of the potential nutritional benefits of pomegranate in AD
233 does not prove bioavailability or *in vivo* biological activity of pomegranate
234 polyphenols following oral intake in humans. However, bioavailability studies have
235 shown that bioactive polyphenols in pomegranate are absorbed from the
236 gastrointestinal tract. Lei et al. reported that punicalagin and ellagic acid reached a
237 plasma concentration of 30 μ g/ml and 213 ng/ml, respectively following oral
238 administration in rats [31]. A study in rabbits reported that pomegranate constituents
239 become bioavailable 2 hours after oral ingestion of concentrated pomegranate
240 extract, ellagic acid reaching a plasma value of 247 ng/ml [32]. In a human study,
241 Seeram et al. administered 180 ml of pomegranate juice containing 25 mg ellagic
242 acid and 318 mg ellagitannins to a human subject. Results of this study showed that
243 ellagic acid was detected in human plasma at a concentration of 31.9 ng/ml 1 hour
244 after ingestion [33]. Furthermore, ellagitannins in pomegranate have been shown to
245 be metabolised by gut bacteria into urolithins that readily enter systemic circulation.
246 These metabolites appeared in human systemic circulation within a few hours of

247 consumption of pomegranate products, reaching maximum concentrations between
248 24 and 48 hours [34].

249 To provide benefits in CNS diseases like AD, pomegranate polyphenols must
250 permeate the blood-brain barrier (BBB). It is not yet clear if biologically-active levels
251 of these compounds could be detected in the CNS following oral administration.
252 However, a study by Farbood et al [35] showed that oral administration of 100 mg/kg
253 ellagic acid for seven days prevented cognitive and long-term potentiation deficits in
254 rats. The outcome of this study suggests that ellagic acid permeated the BBB to act
255 in the CNS.

256 Our study did not establish if the concentrations of pomegranate used in the
257 experiments contain quantities of ellagic acid and ellagitannins which reflect levels
258 which have been detected in plasma. However, we can conclude that bioactive
259 polyphenols in pomegranate could be absorbed from the gastrointestinal tract.

260 In conclusion, we have provided further data showing that pomegranate inhibits
261 induced inflammation in SK-N-SH cells. It appears that the effects of pomegranate
262 on inflammatory processes in SK-N-SH cells results in a reduction of BACE-1 and
263 the neurotoxic A β . We propose that pomegranate is a potential nutritional strategy in
264 slowing the progression of neuroinflammatory diseases such as AD, possibly
265 through its anti-inflammatory effect. Further pharmacokinetic studies in animals and
266 humans are needed to confirm whether pomegranate polyphenols permeate the
267 BBB and reach biologically-active levels in the brain.

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272 **Conflict of interest**

273 On behalf of all authors, the corresponding author states that there is no conflict of
274 interest.

References

1. Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. *Lancet* 368:387-403.
2. Castellani RK, Rolston RK, Smith MA (2010) Alzheimer disease. *Dis Mon* 56:484-546.
3. Selkoe DJ (2001) Alzheimer's disease. Genes, proteins, and therapy. *Physiol Rev* 81:741-766.
4. Messmer K, Reynolds GP (2005) An *in vitro* model of inflammatory neurodegeneration and its neuroprotection. *Neurosci Lett* 388:39-44.
5. Chen CH, Zhou W, Liu S, Deng Y, Cai F, Tone M, Tone Y, Tong Y, Song W (2012) Increased NF- κ B signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *Int J Neuropsychopharmacol* 15:77-90.
6. Hoshino T, Nakaya T, Homan T, Tanaka K, Sugimoto Y, Araki W, Narita M, Narumiya S, Suzuki T, Mizushima T (2007). Involvement of prostaglandin E₂ in production of amyloid-peptides both *in vitro* and *in vivo*. *J Biol Chem* 282:32676-32688.
7. Yasojima K, Schwab C, McGeer EG, McGeer PL (1999) Distribution of cyclooxygenase-1 and cyclooxygenase-2 mRNAs and proteins in human brain and peripheral organs. *Brain Res* 830:226-236.
8. Montine TJ, Sidell KR, Crews BC, Markesbery WR, Marnett LJ, Roberts LJ 2nd, Morrow JD (1999) Elevated CSF prostaglandin E₂ levels in patients with probable AD. *Neurology* 53:1495-1498.
9. Paris D, Patel N, Quadros A, Linan M, Bakshi P, Ait-Ghezala G, Mullan M (2007). Inhibition of Abeta production by NF-kappaB inhibitors. *Neurosci Lett* 415:11-16.
10. Bales KR, Du Y, Dodel RC, Yan GM, Hamilton-Byrd E, Paul SM (1998) The NF-kappaB/Rel family of proteins mediates Abeta-induced neurotoxicity and glial activation. *Brain Res Mol Brain Res* 57:63-72.
11. Marwarha G, Raza S, Meiers C, Ghribi O (2014) Leptin attenuates BACE1 expression and amyloid- β genesis via the activation of SIRT1 signaling pathway. *Biochim Biophys Acta* 1842:1587-1595.
12. Bourne KZ, Ferrari DC, Lange-Dohna C, Rossner S, Wood TG, Perez-Polo JR (2007) Differential regulation of BACE1 promoter activity by NFB in neurons and glia upon exposure to Ab peptides. *J Neurosci Res* 85:1194-1204.

13. Buggia-Prevot V, Sevalle J, Rossner S, Checler F (2008) NF κ B-dependent control of BACE1 promoter transactivation by Abeta42. *J Biol Chem* 283:10037-10047.
14. Guglielmotto M, Aragno M, Tamagno E, Vercellinatto I, Visentin S, Medana C, Catalano MG, Smith MA, Perry G, Danni O, Boccuzzi G, Tabaton M (2012) AGEs/RAGE complex upregulates BACE1 via NF- κ B pathway activation. *Neurobiol Aging* 33:196.e13-27.
15. Camandola S, Poli G, Mattson MP (2000) The lipid peroxidation product 4-hydroxy-2, 3-nonenal inhibits constitutive and inducible activity of nuclear factor kappa B in neurons. *Brain Res Mol Brain Res* 85:53-60.
16. Lee SI, Kim BS, Kim KS, Lee S, Shin KS, Lim JS (2008) Immunosuppressive activity of punicalagin via inhibition of NFAT activation. *Biochem Biophys Res Commun* 371:799-803.
17. Romier, B., Van De Walle J, During A, Larondelle Y, Schneider YJ (2008) Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr* 100:542-551.
18. Oucharif, A., Khalki H, Chaib S, Mountassir M, Aboufatima R, Farouk L, Benharraf A, Chait A (2012) Comparative study of the anti-inflammatory and antinociceptive effects of two varieties of *Punica granatum*. *Pharm Biol* 50:429-438.
19. Lee CJ, Chen LG, Liang WL, Wang CC (2010) Anti-inflammatory effects of *Punica granatum* Linne *in vitro* and *in vivo*. *Food Chem* 118:315-322.
20. Olajide OA, Kumar A, Velagapudi R, Okorji U, Fiebich BL (2014) Punicalagin inhibits neuroinflammation in LPS-activated rat primary microglia. *Mol Nutr Food Res* 58:1843-1851.
21. Seeram NP, Lee R, Hardy ML, Heber D (2005) Large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry. *Sep Purif Tech* 41:49-55.
22. Olajide OA, Velagapudi R, Okorji U, Sarker S, Fiebich BL (2014) *Picralima nitida* seeds suppress PGE2 production by interfering with multiple signalling pathways in IL-1 β -stimulated SK-N-SH neuronal cells. *J Ethnopharmacol* 152: 377-383.
23. Decker T, Lohmann-Matthes ML (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular

- cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods*. 115: 61-69.
24. Sutinen EM, Pirttilä T, Anderson G, Salminen A, Ojala JO (2012) Pro-inflammatory interleukin-18 increases Alzheimer's disease-associated amyloid- β production in human neuron-like cells. *J Neuroinflammation* 9:199.
25. Pasinetti GM, Aisen PS (1998) Cyclooxygenase-2 expression is increased in frontal cortex of Alzheimer's disease brain. *Neuroscience* 87:319-324.
26. Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Ashe KH, Frautschy SA, Cole GM (2000) Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. *J Neurosci*. 20:5709-5714.
27. Romier-Crouzet B, Van De Walle J, During A, Joly A, Rousseau C, Henry O, Larondelle Y, Schneider YJ (2009) Inhibition of inflammatory mediators by polyphenolic plant extracts in human intestinal Caco-2 cells. *Food Chem Toxicol* 47:1221-1230.
28. Ahmed S, Wang N, Hafeez BB, Cheruvu VK, Haqqi TM (2005) *Punica granatum* L. extract inhibits IL-1 β -induced expression of matrix metalloproteinases by inhibiting the activation of MAP kinases and NF- κ B in human chondrocytes in vitro. *J Nutr* 135:2096-2102.
29. Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, Frigon N, Hong J, Jacobson-Croak K, Jewett N, Keim P, Knops J, Lieberburg I, Power M, Tan H, Tatsuno G, Tung J, Schenk D, Seubert P, Suomensari SM, Wang S, Walker D, Zhao J, McConlogue L, John V (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* 402:537-540.
30. Luo Y., Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R. (2001). Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nat Neurosci* 4:231-232.
31. Lei F, Xing DM, Xiang L, Zhao YN, Wang W, Zhang LJ, Du LJ. (2003) Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract.
32. Shukla M, Gupta K, Rasheed Z, Khan KA, Haqqi TM. (2008) 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*. J Inflamm (Lond) 5:9.

33. Seeram NP, Lee R, Heber D. (2004) Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. Clin Chim Acta. 348: 63-68.
34. Bialonska D, Kasimsetty SG, Khan SI, Ferreira D. (2009) Urolithins, intestinal microbial metabolites of Pomegranate ellagitannins, exhibit potent antioxidant activity in a cell-based assay. J Agric Food Chem. 57: 10181-10186.
35. Farbood Y, Sarkaki A, Dianat M, Khodadadi A, Haddad MK, Mashhadizadeh S. (2015) Ellagic acid prevents cognitive and hippocampal long-term potentiation deficits and brain inflammation in rat with traumatic brain injury. Life Sci. 124:120-127.

Figure legends

Figure 1

PWE reduced PGE₂ production in IL-1 β -stimulated SK-N-SH cells. Cells were stimulated with IL-1 β (10 U/ml) in the presence or absence of PWE (25-200 μ g/ml) pre-incubated for 30 min. After 24 h, supernatants were collected for PGE₂ measurement. All values are expressed as mean \pm SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p < 0.05, **p<0.01, ***p<0.001 in comparison with IL-1 β control.

Figure 2

PWE inhibited COX-2 protein expression in IL-1 β -stimulated SK-N-SH cells. Cells were stimulated with IL-1 β (10 U/ml) in the presence or absence of PWE (25-200 μ g/ml) pre-incubated for 30 min. After 24 h, COX-2 protein expression was determined using PathScan[®] sandwich ELISA. All values are expressed as mean \pm SEM for at least 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p < 0.05, **p<0.01, ***p<0.001 in comparison with IL-1 β control.

Figure 3

TNF α -induced NF- κ B-dependent gene expression in HEK293 cells was inhibited by PWE. Transfected cells were incubated with different concentrations of PWE followed by stimulation with TNF α (1 ng/ml) for an additional 6 h. Luminescence was then measured. All values are expressed as mean \pm SEM for 3 independent experiments performed in triplicates. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, *p < 0.05, **p<0.01, ***p<0.001 in comparison with TNF α control.

Figure 4

PWE inhibited IL-1 β -induced I κ B phosphorylation in SK-N-SH cells. Cells were stimulated with IL-1 β (10 U/ml) in the presence or absence of PWE (25-200 μ g/ml) pre-treated for 30 min. After 5 min, p-I κ B α protein expression was determined using PathScan[®] sandwich ELISA. All values are expressed as mean \pm SEM for 3 independent experiments. Optical densities were measured at 450 nm with a micro

plate reader. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with IL-1 β control.

Figure 5

PWE inhibited IL-1 β -induced IKK phosphorylation in SK-N-SH cells. Cells were stimulated with IL-1 β (10 U/ml) in the presence or absence of PWE (25-200 μ g/ml) for 5 min. At the end of incubation period, p-IKK α protein expression was determined using PathScan[®] sandwich ELISA. All values are expressed as mean \pm SEM for 3 independent experiments. Optical densities were measured at 450 nm with a micro plate reader. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with IL-1 β control.

Figure 6

PWE inhibited IL-1 β -induced BACE-1 in SK-N-SH cells. Cells were pre-treated with PWE (25-200 μ g/ml) 30 min before stimulation with IL-1 β (10 U/ml) for 24 h. At the end of stimulation, levels of BACE-1 were determined using MaxDiscovery In Cell ELISA kit. All values are expressed as mean \pm SEM for 3 independent experiments. Optical densities were measured at 450 nm with a micro plate reader. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with IL-1 β control. GAPDH was used as internal control.

Figure 7

PWE attenuated IL-1 β -induced A β production in SK-N-SH cells. Cells were pre-treated with PWE (25-200 μ g/ml) 30 min before stimulation with IL-1 β (10 U/ml) for 24 h. At the end of stimulation, A β production was determined using MaxDiscovery In Cell ELISA kit. All values are expressed as mean \pm SEM for 3 independent experiments. Optical densities were measured at 450 nm with a micro plate reader. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ in comparison with IL-1 β control. GAPDH was used as internal control.

Figure 8

Pre-treatment with PWE (25-200 $\mu\text{g/ml}$) did not affect the viability of SK-N-SH cells stimulated with IL-1 β (10 U/ml). Cells were pre-incubated for 30 min with PWE (25-200 $\mu\text{g/ml}$) in the presence or absence of IL-1 β for 24 h. At the end of the incubation period, MTT and LDH assays were carried out on cells. All values are expressed as mean \pm SEM for 3 independent experiments.