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Polyphenols from *Pennisetum glaucum* grains induce MAP kinase phosphorylation and cell cycle arrest in human osteosarcoma cells

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Abstract

1 Osteosarcoma is the most common bone tumor with a high prevalence among children and
2 adolescents. Polyphenols are widely investigated for their chemopreventive and
3 chemotherapeutic proprieties. In the present study, we explored the pro-apoptotic effects of
4 pearl millet, *Pennisetum glaucum*, phenolic compounds (PGPC) on osteosarcoma U-2OS
5 cells. Our results show that PGPC induced U-2OS cells death, in a dose dependent manner,
6 with an IC₅₀ of 80 µg/mL. Annexin-V and 7-AAD staining show that PGPC induced cell
7 death mainly through caspase-dependent apoptosis as shown by a decrease in cell death when
8 co-treated with pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone
9 (z-VAD-fmk). PGPC caused an increase in cytoplasmic calcium associated with caspases
10 activation and poly (ADP-ribose) polymerase PARP cleavage. Western blot analysis revealed
11 that PGPC upregulated p38 MAPK and SAPK/JNK activity and inhibited c-SRC/AKT
12 pathway. Finally, cell cycle analysis shows that PGPC decreased CDK2 and increased cyclin
13 E expression, resulting in U-2OS cells accumulation in S phase. **These results demonstrate**
14 **that PGPC induce p38 MAPK and SAPK/JNK activation, and attenuate AKT activation,**
15 **leading to cell cycle arrest and apoptosis in osteosarcoma U-2OS cells.**

16

17 **Key words:** pearl millet; polyphenols; U-2OS cells; **cell signaling**; intracellular calcium; cell
18 cycle arrest; apoptosis

19 **Abbreviations:** 3-MA, 3-Methyladenine; 5-FU, 5-Fluorouracil; AKT, Protein kinase B;
20 Annexin V-APC/7-AAD, Annexin V-apoptosis -associated caspase/7-Aminoactinomycin D;
21 BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-*N,N,N,N'*-tetraacetic acid tetrakis (acetoxymethyl ester); cdc2, yeast Cyclin-dependent kinase-2; CDK2, human Cyclin-dependent
22 kinase-2; CDKs, cyclin-dependent kinases; CKIs, cyclin dependent kinase inhibitors; CQ,
23

24 chloroquine; c-Src, Proto-oncogene tyrosine-protein kinase; EGTA, ethylene glycol-bis(β -
25 aminoethyl ether)-N,N,N',N'-tetraacetic acid; ERK1/2, p44/42 MAPK; Fura-2 AM, fura-2-
26 acetoxymethylester; JAK 2, Janus kinase 2; LDH, lactate dehydrogenase ; mTOR,
27 mammalian target of rapamycin; p38 MAPK, p38 mitogen-activated protein kinases; p70
28 S6K, Ribosomal protein S6 kinase; p-CA, p-coumaric acid; PGPC, *Pennisetum glaucum*
29 phenolic compounds, PI3-K, phospho-inositide 3-kinase; RuR, ruthenium red; SAPK/JNK,
30 stress activated protein kinase/c-Jun N-terminal kinases; STAT3, Signal transducer and
31 activator of transcription 3; TRAF2, tumor necrosis factor (TNF)-Receptor Associated Factor;
32 TRAIL, tumor-necrosis-factor related apoptosis inducing ligand; XBPI, X box-bindingprotein
33 1; z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

34

35 1. Introduction

36 Osteosarcoma is the most common bone tumor with a high prevalence among children and
37 adolescents (Mirabello, Troisi, & Savage, 2009). Surgery and chemotherapy are the standard
38 of care for osteosarcoma (Luetke, Meyers, Lewis, & Juergens, 2014). However, besides
39 resistance or low response to chemotherapeutic agents, chemotherapy is known for its
40 toxicities including cardiomyopathy, encephalopathy, hearing loss, hemorrhagic cystitis and
41 hypomagnesemia (Allison et al., 2012; Bakhshi & Radhakrishnan, 2010; Cho et al., 2009;
42 Marina, Gebhardt, Teot, & Gorlick, 2004). Therefore, there is an increasing interest in diet-
43 derived agents for their potential chemopreventive properties. Epidemiological and preclinical
44 studies suggest that dietary polyphenols possess cancer chemopreventive properties with less
45 side effects and toxicities (Thomasset et al., 2007).

46 Impairment of programmed cell deaths and loss of normal cellular replication control are
47 fundamental defects in cancer mainly due to signaling pathway alterations (Ouyang et al.,

48 2012; Roskoski, 2016). The escape from apoptosis, a hallmark characteristic of cancer cells,
49 represents an important target for cancer prevention or therapy (Moore, Megaly, MacNeil,
50 Klentrou, & Tsiani, 2016). The serine/threonine kinase AKT, also known as protein kinase B
51 (PKB), is a proto-oncogene that regulates cancer cell division, growth, migration, survival,
52 and resistance to chemo and radio-therapy (Fruman & Rommel, 2014; Hanahan & Weinberg,
53 2011). AKT acts as an upstream component of cell cycle progression promoters, including
54 Signal transducer and activator of transcription 3 (STAT3) (Riemenschneider, Betensky,
55 Pasedag, & Louis, 2006), with direct regulation of apoptotic machinery (Downward, 2004).
56 p53 is a transcription factor that regulates the balance between pro-apoptotic and anti-
57 apoptotic genes, and the activation of this protein is required for apoptosis and cell cycle
58 arrest (Giono & Manfredi, 2006; Linke, Clarkin, Di Leonardo, Tsou, & Wahl, 1996; Vousden
59 & Prives, 2009).

60 Recently, many groups have demonstrated the anti-cancer properties of plant and food derived
61 polyphenols by regulation of phospho-inositide 3-kinase (PI3K)/AKT related pathways
62 (Banerjee, Kim, Krenek, Talcott, & Mertens-Talcott, 2015; Darvin et al., 2015; Moore et al.,
63 2016; T et al., 2016). Mango polyphenols, in part through the PI3K/AKT, have a
64 chemotherapeutic potential against breast cancer (Banerjee et al., 2015). Polyphenols from
65 Sorghum, a principal cereal food used besides millet in many countries, have been shown to
66 suppress colon cancer growth and pulmonary metastasis in animal models by co-targeting
67 Janus kinase 2 (JAK2)/STAT3 and PI3K/AKT/ mammalian target of rapamycin (mTOR)
68 pathways (Darvin et al., 2015). Ferulic acid, a ubiquitous phenolic acid in cereals including
69 millets, blocks cell cycle progression and induces apoptosis in 143B and MG63 osteosarcoma
70 cells via blockage of PI3K/AKT pathway (Wang et al., 2016).

71 We have previously shown that pearl millet (*Pennisetum glaucum*) grains are rich in
72 polyphenols. Moreover, p-coumaric and ferulic acids, which have been reported to exhibit

73 antitumor and antimetastatic activities, are the most abundant phenolic compounds (Nani et
74 al., 2015). In the present study we sought to investigate the mechanisms involved in the anti-
75 tumor activity of *Pennisetum glaucum* phenolic compounds (PGPC) against the U-2OS
76 osteosarcoma cell line.

77 **2. Materials and methods**

78 **2.1. Materials**

79 Grains of pearl millet were obtained from the region of Ouled Aïssa (174 km in the North of
80 Adrar city and 70 km to the North-West of Timimoun, Algeria). RPMI 1640 medium and L-
81 glutamine were purchased from Lonza Verviers SPRL (Verviers, Belgium). Fura-2 AM (fura-
82 2-acetoxymethylester) was procured from Life Technologies (France). Annexin V-APC/7-
83 AAD kit was purchased from Biolegend (France). Trypsin was purchased from Gibco (USA).
84 RNase-A was purchased from Euromedex (France) and propidium iodide from
85 Immunochemistry (USA). LDH kit was purchased from Abcam (France). All antibodies used
86 for Western blot analysis are listed in Table 1. All other chemicals were purchased from
87 Sigma (USA).

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97 Table 1: Primary antibodies used for western blot

| Antigen | dilution | Source/ Host | Catalog number | Company |
|---------------------------------------|-----------------|-------------------------|---------------------------|---------------------------|
| AKT | 1:1000 | Rabbit | 4685 | Cell Signaling Technology |
| Caspase-8 | 1:1000 | Mouse | 9746 | Cell Signaling Technology |
| Caspase-9 | 1:1000 | Mouse | 9508 | Cell Signaling Technology |
| cdc2 (POH1) | 1 :1000 | Mouse | 9116 | Cell Signaling Technology |
| CDK2 | 1 :1000 | Rabbit | 2546 | Cell Signaling Technology |
| Cleaved PARP (Asp214) | 1 :1000 | Rabbit | 9541 | Cell Signaling Technology |
| c-Src (SRC 2) | 1:500 | Rabbit | sc-18 | Santa Cruz Biotechnology |
| Cyclin A | 1:500 | Rabbit | sc-751 | Santa Cruz Biotechnology |
| Cyclin E | 1:500 | Rabbit | sc-481 | Santa Cruz Biotechnology |
| p38 MAPK | 1 :500 | Rabbit | 622401 | BioLegend |
| p53 | 1:1000 | Mouse | 2524 | Cell Signaling Technology |
| p70 S6K (Ribosomal protein S6 kinase) | 1 :1000 | Rabbit | 2708 | Cell Signaling Technology |
| Phospho -STAT3 (Ser 727) | 1:200 | Rabbit | sc-135649 | Santa Cruz Biotechnology |
| Phospho-AKT (Ser473) | 1 :1000 | Mouse | 4051 | Cell Signaling Technology |
| Phospho-AKT (Thr308) | 1:1000 | Rabbit | 9275 | Cell Signaling Technology |
| Phospho-p38 MAPK (Thr180/Tyr182) | 1:1000 | Rabbit | 4511 | Cell Signaling Technology |
| Phospho-p53 (Ser46) | 1:1000 | Rabbit | 2521 | Cell Signaling Technology |
| Phospho-p70 S6K (Thr389) | 1 :1000 | Rabbit | 9205 | Cell Signaling Technology |
| Phospho-SAPK/JNK (Thr183/Tyr185) | 1 :0000 | Mouse | 9255 | Cell Signaling Technology |
| SAPK/JNK | 1 :0000 | Rabbit | 9252 | Cell Signaling Technology |
| β -actin | 1:5000 | Mouse | sc-47778 | Santa Cruz Biotechnology |
| p44/42 MAPK (ERK1/2) | 1 :1000 | Rabbit | 9102 | Cell Signaling Technology |
| Phospho-p44/42 MAPK (ERK1/2) | 1 :1000 | Rabbit | 9100 | Cell Signaling Technology |

98

99

100

101 ***2.2. Preparation of polyphenol-rich extract***

102 The plant was recognized by a botanist (Pr Benabadji Nouri, Université Aboubekr Belkaïd,
103 Tlemcen) of the Herbarium Center of the Faculty of Pharmacy (Tlemcen) which contained the
104 voucher specimen (PM 1681). Phenolic extract from pearl millet was obtained as we have
105 previously described (Nani et al., 2015). The defatted extract was evaporated, to dryness,
106 under reduced pressure and then resuspended in PBS (Phosphate-buffered saline). Total
107 phenolic content in plant extract were determined by Folin-Ciocalteu method (Folin &
108 Ciocalteu, 1927).

109 ***2.3. Cell culture and viability assay***

110 **U-2OS** osteosarcoma cells were obtained from the American Type Culture Collection
111 (ATCC, Rockville, USA). **Cells were maintained in RPMI 1640 Medium supplemented with**
112 **10% heat inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100**
113 **µg/ml), L-glutamine (2 mM).** The cell viability was investigated by the trypan blue exclusion
114 test. Briefly, **U-2OS** osteosarcoma cells were seeded into 24-well plate (3×10^4 cells/well) and
115 incubated for 24 h in a humidified chamber containing 95 % air and 5 % CO₂. Cells were then
116 treated with increasing concentrations of PGPC (from 20 to 100 µg/mL) and p-coumaric acid
117 (p-CA, from 5000 to 25000 µM). After 24 h, cells were trypsinized and diluted in 0.5% trypan
118 blue. Cells were then counted with a Malassez hemocytometer under the light microscope
119 “Nikon”.

120 ***2.4. LDH cytotoxicity assay***

121 **The lactate dehydrogenase (LDH) cytotoxicity assay was performed to measure cell**
122 **membrane integrity and the release of lactate dehydrogenase (LDH) from the cytosol of**
123 **damaged cells into the supernatant by using a commercial kit (Abcam, France), according to**
124 **the manufacturer’s instruction. Briefly, 1×10^4 cells/100 µL were seeded in in 96-well plates.**

125 After 24 h incubation at 37°C in a CO₂ humidified incubator cells were treated with several
126 concentrations of PGPC or p-CA in triplicate well (test samples). Controls included in each
127 experiment were as follow: 1) background control (medium only or various concentrations of
128 PGPC or P-CA); this background values has to be subtracted from their corresponding values,
129 2) cells without treatment (low control) to measure the spontaneous release of LDH by cells,
130 3) maximum LDH release (high control) where Triton x-100 (1%) was used to disrupt the
131 cytoplasmic membrane. At the end of incubation, 50 µL of supernatant was taken, and
132 LDH activity was assessed by measuring absorbance at 490 nm with a microplate reader.

133 Cytotoxicity (%) was calculated as follow: (test samples – low control /high control - low
134 Control) x 100.

135 **2.5. Apoptosis Assay**

136 **U-2OS** cells (2x10⁵) were seeded in a 6-well plate. After 24 h, cells were pre-incubated or not
137 with 50 µM N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) for 1 h.
138 and then treated for 24 h with PGPC (60 µg/mL) and p-CA (12 500 µM). Cells were
139 harvested into 15 mL Falcon tubes and washed twice with cold PBS. Subsequently, cell death
140 was determined by APC-Annexin V and 7-amino-actinomycin D (7-AAD) staining with
141 BioLegend's APC-Annexin V Apoptosis Detection Kit according to the manufacture's
142 recommendations and analyzed by flow cytometry, on FACSCanto™ (BD Biosciences), and
143 FlowJo software version 10 (Tree Star).

144 **2.6. Measurement of free intracellular Ca²⁺ concentrations; [Ca²⁺]_i**

145 **U-2OS** cells were cultured on WillCo-dish wells with a glass bottom and loaded with Fura-
146 2/AM (1 µM) for 60 minutes at 37°C in loading buffer that contained: 110 mM NaCl; 5.4 mM
147 KCl; 25 mM NaHCO₃; 0.8 mM MgCl₂; 0.4 mM KH₂PO₄; 20 mM HEPES-Na; 0.33 mM
148 Na₂HPO₄; 1.2 mM CaCl₂, pH 7.4 as described by Dramane *et al.* (Dramane et al., 2012). The

149 changes in intracellular Ca^{2+} ($\text{F}_{340}/\text{F}_{380}$) were monitored under a Nikon microscope (TiU) by
150 using an S Fluor 40× oil immersion objective. The planes were taken at z intervals of 0.3 μm ,
151 and NIS-Elements software was used to deconvolve the images. The microscope was
152 equipped with an EM-CCD (Lucas) camera for real-time recording of 16-bit digital images.
153 The dual excitation fluorescence imaging system was used for studies of individual cells. The
154 changes in intracellular Ca^{2+} were expressed as Δ ratio, which was calculated as the difference
155 relative to the peak $\text{F}_{340}/\text{F}_{380}$ ratio. The data were summarized from the large number of
156 individual cells (20–40 cells in a single run, with 3–9 identical experiments done in at least 3
157 cell preparations). For experiments conducted in the absence of external calcium (0 % Ca^{2+}),
158 CaCl_2 was replaced by 1 mM EGTA in the buffer. All test molecules were added in small
159 volumes with no interruption in recordings.

160 **2.7. Cell cycle analysis by propidium iodide staining**

161 **U-2OS** cells were seeded in a 6-well plate (2×10^5 cells/well). After 24 h, cells were treated
162 with PGPC (60 $\mu\text{g}/\text{mL}$) and p-CA (12 500 μM), which were combined or not with 5-
163 fluorouracil (5-FU, 2.5 μM) for another 24 h. Cells were harvested and fixed in 70 % ethanol
164 for the overnight. Cells were washed twice, stained with propidium iodide, and treated with
165 RNase-A for 1 h in darkness at 37 °C. Cells were assessed for their DNA contents by
166 cytometry on FACS Canto, and data were analyzed using ModFit LT software version 3.3.11.

167

168 **2.8. RT-PCR quantification assay**

169 Total RNA was prepared using trizol reagent (Invitrogen Life Technologies). 1 μg of total
170 RNA was reverse transcribed with superscript II RNase H-reverse transcriptase using oligo
171 (dT) according to the manufacturer's instructions (Invitrogen Life Technologies, France).

172 Real-time PCR was performed using the icycler iQ™ Real Time Detection system (Applied).
173 Amplification reactions were performed using SYBR Green I detection 7500 Fast Real-Time
174 PCR System (Applied Biosystems) with the following primers as previously described
175 (Minville-Walz et al., 2010) : CHOP: sense 5'-ACA CAG ATG AAA ATG GGG GTA CCT-
176 3' and antisense 5'-AGA AGC AGG ATC AAG AGT GGT CCT-3'; BAK sense 5'-TTA
177 CCG CCA TCA GCA GGA ACA-3' and antisense: 5'-ATG GGA CCA TTG CCC AAG
178 TTC-3'; BAX sense 5'-ACC AGC TCT GAG CAG ATC ATG G-3' and antisense: 5'-CCT
179 CTT CCA GAT GGT GAG CGA-3'; Bcl-2 sense 5'-GTC ATG TCT GTC GAG AGC GT-3'
180 and antisense 5'-ACA GTT CCA CCA AGG CAT CC-3' ; XBP1 sense 5'-AAA CAG AGT
181 AGC AGC TCA GAC TGC-3' and antisense 5'-TCC TTC TGG GTA GAC CTC TGG GAG-
182 3'; β -actin: 5'-CTG GTG CCT GGG GCG-3' and 5'-AGC CTC GCC TTT GCC GA-3'.
183 CHOP, BAK, BAX, Bcl-2, and XBP1 expressions were normalized to β -actin and calculated
184 using the $2^{-\Delta\Delta C_t}$ method.

185 **2.9. Western Blot Analysis**

186 **U-2OS** cells (2×10^6) were seeded in Petri dishes and treated with two concentrations of
187 PGPC (60 and 75 $\mu\text{g}/\text{mL}$) for 24 h. Cells were lysed in 200 μL RIPA lysis buffer (Thermo
188 Fisher Scientific Inc., Rockford, IL, USA). Proteins concentration was determined by
189 Bicinchoninic acid (BCA) Assay Kit (Sigma-Aldrich, USA). Subsequently, proteins (80 μg)
190 were separated on a 12% polyacrylamide gel and electroblotted on a PVDF membrane. The
191 membrane was saturated during 1 h in 5% bovine serum albumin (w/v) in Tris Buffered
192 Saline containing 0.1% Tween-20 (TBS-T) and then incubated with primary antibodies: anti-
193 phospho-AKT (Ser473), anti-phospho-AKT (Thr308), anti-AKT, anti-Caspase-8, anti-
194 caspase-9, anti-cleaved PARP, anti-cdc2, anti-CDK2, anti-cyclin A, anti-cyclin E, anti-c-Src,
195 anti-phospho-p70 S6K, anti-p70 S6K, anti-phospho-p44/42 MAPK, anti-p44/42 MAPK, anti-
196 phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-SAPK/JNK, anti- SAPK/JNK, anti-

197 phospho-p53, anti-p53, and anti-phospho-STAT3 at 4°C overnight. β -actin was used as an
198 internal control. Primary antibodies were then detected either with anti-rabbit IgG, HRP-
199 linked antibody or anti-mouse IgG2b-HRP secondary antibody. Blots were visualized using
200 an ECL Kit (Merck Millipore) on Bio-Rad Chemi-Doc XRS⁺ system. Densitometric analysis
201 was performed on Bio-Rad Image Lab Software (version 4.1).

202 **2.10. Statistical Analysis**

203 Results were expressed as mean \pm SD (standard error deviation) for a given number of
204 experiments (n). Data were analyzed by using Statistica (4.1 version, Statsoft, Paris, France).
205 The significance of differences between mean values was determined by one-way ANOVA,
206 followed by Fisher's least-significant-difference (LSD) test. Differences with $p < 0.05$ were
207 considered to be significant.

208 **3. Results**

209 **3.1. Cell Viability**

210 Trypan blue exclusion test showed that PGPC and p-CA significantly reduced U-2OS cell
211 viability in a dose dependent-manner (Fig.1). IC₅₀ was estimated to be 80 μ g/mL for PGPC
212 and around 17000 μ M for p-CA. Moreover, p-CA at 25000 μ M induced more than 80% of U-
213 OS cell death. However, 100 μ g/mL concentration of PGPC was required to achieve 75% of
214 U-2OS cell death. The lactate dehydrogenase (LDH) assessment demonstrated that both p-
215 Coumaric acid and PGPC induced U-2OS cell death in a dose-dependent manner as these
216 treatments increased the membrane permeability, evidenced by high LDH levels, when
217 compared with untreated cells (insert). We therefore chose less toxic doses of PGPC (*i.e.* 60
218 μ g/mL and 75 μ g/mL) and p-CA (12500 μ M) for all the subsequent experiments.

219

220 **3.2. Apoptosis Assay**

221 We used Annexin-V/7-AAD staining to investigate the cell death induced by PGPC and p-
222 CA. Interestingly, our findings demonstrate that PGPC and to a lesser extent p-CA induced
223 cell death mainly via apoptosis as shown by a significant decrease in cell death when cells
224 were co-treated with z-VAD-fmk, a pan-caspase inhibitor (Fig. 2).

225 We further explored the involvement of calcium in the apoptosis induced by PGPG. In this
226 regard, **U-2OS** cells were treated with PGPC (60 µg/mL) in presence of BAPTA-AM, a
227 cytoplasmic calcium chelator, (5 µM) or ruthenium red (RuR), an inhibitor of mitochondrial
228 Ca²⁺ uptake, (5 µM).

229 As expected, we observed that co-treatment with BAPTA-AM impaired **U-2OS** cell death
230 induced by PGPC, but surprisingly, RuR potentiated PGPC-induced cell death as 64 % of
231 cells were Annexin V positive (Fig. 3).

232 Since the inhibition of mitochondrial Ca²⁺ uniporter exacerbated PGPC-induced cell death, in
233 the subsequent experiment, cells, pre-incubated with RuR, were co-treated with z-VAD-fmk
234 or with autophagy inhibitors, 3-Methyladenine (3-MA) and chloroquine (CQ).

235 Again, z-VAD-fmk attenuated cell death caused by co-treatment with RuR, but 3-MA and CQ
236 failed to impair the already observed cell death (Fig. 3).

237 **3.3. Detection of Apoptosis-Related Proteins**

238 Fig. 4A shows that PGPC treatment induced p53 phosphorylation in **U-2OS** cells. Thus,
239 PGPC induced caspase-8 and caspase-9, which is an essential downstream component of p53,
240 activation. Indeed, PGPC triggered pro-caspase-8 and pro-caspase-9 cleavage into their active
241 fragments. Furthermore, PGPC induced PARP cleavage, a downstream substrate of both
242 caspase-8 and caspase-9.

243 The activation of pro-apoptotic proteins (XBP1, BAX / BAK, and CHOP) and the inactivation
244 of the anti-apoptotic protein Bcl-2 play key roles in endoplasmic reticulum (ER)-dependent
245 apoptosis. Our results showed that PGPC treatment increased transcription of XBP1, BAX /
246 BAK, and CHOP mRNA expression and decreased Bcl-2 mRNA expression in **U-2OS** cancer
247 cells (Fig. **4B**).

248 ***3.4. Effect of PGPC on intracellular Ca^{2+} in U-2OS cells***

249 PGPC induced a sustained increase in $[Ca^{2+}]_i$ in **U-2OS** cells. In order to assess the origin of
250 Ca^{2+} mobilized by phenolic compounds, we conducted experiments in the absence (0 % Ca^{2+})
251 and presence (100 % Ca^{2+}) of Ca^{2+} in the extracellular medium. Fig. **5** shows that the PGPC
252 induced significant increases in $[Ca^{2+}]_i$ in 0% and in 100 % Ca^{2+} medium, suggesting that
253 PGPC induced a Ca^{2+} influx from extracellular medium and also mobilized calcium release
254 from intracellular stores.

255

256 ***3.5. Effect of PGPC on AKT/MAPK pathways in U-2OS cells***

257 We assessed the effect of PGPC on AKT activation in **U-2OS** osteosarcoma cells. PGPC
258 treatment resulted in a significant decrease of AKT phosphorylation in Thr308 and ser 473
259 residues which were associated with proto-oncogene tyrosine-protein kinase (c-SRC), an
260 AKT upstream effector, decrease. Regarding AKT downstream substrates, PGPC treatment
261 negatively regulated P70 S6K and STAT3 phosphorylation (Fig. **6A**). In addition to a
262 significant increase in p38 MAPK phosphorylation, SAPK/JNK was also phosphorylated in
263 response to PGPC treatment, however, PGPC did not affect ERK1/2 MAPK activation (Fig.
264 **6B**).

265

266

267 **3.6. Cell cycle distribution analysis**

268 We further analyzed the effect of PGPC on cell cycle progression by using propidium iodide
269 staining. We observed that PGPC (60 µg/mL) and to a greater extent p-CA (12500 µM)
270 induced cell cycle arrest of **U-2OS** cells in the S phase (Fig. 7A). As shown in Fig. 7B, the
271 repartition of cell populations between different phases of cell cycle in **U-2OS** was as follow:
272 49.53% in G1, 28.37% in S and 22.09% in G2/M. PGPC (60 µg/mL) treatment increased S
273 population to 41.37% and p-CA to 42.18%. A small concentration of 5-FU, 2.5 µM, induced
274 cell cycle arrest in G1 phase. However 5-FUco-treated with PGPC or p-CA induced cell cycle
275 arrest in S phase with a significant decrease of G2/M phase.

276

277 **3.7. Cyclins and CDKs expression analysis**

278 Western blot analyses shows that PGPC induced a significant decrease in human cyclin-
279 dependent kinase-2 (CDK2) associated with cyclin E increase when compared to untreated
280 cells. However, no effects were noticed on cyclin-dependent kinase cdc2/CDK1, and cyclinA
281 expression in **U-2OS** cells (Fig. 7C).

282 **4. Discussion**

283 Dietary polyphenols-rich food intake has been correlated with low cancer incidences.
284 Moreover, several phenolic compounds have been proposed as potential anticancer agents
285 alone or as **adjuvants** with classical chemotherapy (León-González, Auger, & Schini-Kerth,
286 2015). Dietary polyphenols exert their anti-carcinogenic activity by several mechanisms.
287 **Furthermore**, depending on tissue or cell type, the effects of polyphenols could differ on
288 whether they are used at high or low doses (Ramos, 2008).

289 The aim of this study was to investigate the effects of *Pennisetum glaucum* phenolic
290 compounds (PGPC) on human osteosarcoma U-2OS cells, which express wild type p53
291 (Grossel, Baker, & Hinds, 1999), *in vitro*.

292 We have shown previously through HPLC analysis that that p-coumaric acid and ferulic acid
293 are the most abundant phenolic compounds in *Pennisetum glaucum* grains (Nani et al., 2015).
294 In addition, gallic acid, chlorogenic acid, caffeic acid, quercetin, and apigenin were detected
295 in these grains (Nani et al., 2015; Shahidi & Chandrasekara, 2013). In the present study, our
296 results indicate that PGPC and, to a lesser extent, p-CA induced U-2OS cell death, in a dose
297 dependent manner, with an IC₅₀ of 80 µg/mL and 17000 µM, respectively. Next, we have
298 investigated the type of the observed cell death. PGPC and p-CA increased LDH release from
299 U-2OS cells suggesting that U-2OS cells underwent an apoptosis in response to PGPC and p-
300 CA treatments. Interestingly, Annexin V/7-AAD staining revealed that PGPC and p-CA
301 induced cell death mainly via apoptosis as evidenced by a significant impairment of cell death
302 when co-treated with z-VAD-fmk. The apoptotic effects of food-derived polyphenols and
303 purified phenolic compounds have been extensively researched (Darvin et al., 2015;
304 Jaganathan, Supriyanto, & Mandal, 2013; Moore et al., 2016; Mouria et al., 2002).
305 Polyphenols obtained from sorghum, a principal cereal food used besides millet in many
306 countries, induced apoptosis in HCT-116 and HCT-15 colon cancer cells (Darvin et al., 2015).
307 Thus, p-CA has been reported to exert apoptotic effects in HCT-15 colon cancer cells
308 (Jaganathan et al., 2013). Other study reported that ferulic acid, the second most abundant
309 phenolic acid in *Pennisetum glaucum*, promoted apoptosis in osteosarcoma cells (T et al.,
310 2016).

311 Since calcium was shown to play a crucial role in apoptosis (Nicotera & Orrenius, 1998), in
312 subsequent experiment, U-2OS cells were pre-incubated with either BAPTA-AM (5 µM) or
313 RuR (5 µM) for 1 h, and then treated with PGPC for 24 h. BAPTA-AM co-treatment

314 significantly curtailed PGPC-induced cell death, but RuR potentiated PGPC-induced cell
315 death. Apoptosis is the major type of cell death that occurs in response to irreparable DNA
316 damage. Conversely, conventional apoptosis blockage can promote other programmed cell
317 death pathways, *i.e.* autophagy and necroptosis, although the boundary between apoptosis and
318 autophagy has never been completely clear (Goodall et al., 2016; Lockshin & Zakeri, 2004;
319 Ouyang et al., 2012).

320 To further investigate whether there was a switch to autophagy programmed cell death when
321 mitochondrial calcium uptake was inhibited, cells were pre-incubated with RuR (5 μ M), and
322 then co-treated with z-VAD-fmk (50 μ M) or 3-MA (5 mM) or CQ (50 μ M). Results showed
323 that z-VAD-fmk impaired the cell death induced by RuR-co-treatment. In contrast, we did not
324 notice any significant effect of 3-MA and CQ on the cell death induced by RuR-co-treatment.
325 Taken together, these findings suggest that the inhibition of mitochondrial-dependent
326 apoptosis might exacerbate an alternative mitochondrial-independent apoptosis pathway
327 rather than induce autophagy.

328 The extrinsic, or death-receptor pathway, and intrinsic, or mitochondrial pathway are the
329 most described pathways of the apoptosis machinery (Eum & Lee, 2011). In most human
330 cancers, the p53 –tumor suppressor– protein or pathway is mutated or inactivated (Vogelstein,
331 Lane, & Levine, 2000). We and others have shown that p53 can mediate caspase-dependent
332 apoptosis, and many stimuli, including polyphenols, trigger apoptosis through the activation
333 of initiator caspases (caspase-8 and caspase-9) (Ghanemi et al., 2017; Hajiaghaalipour,
334 Kanthimathi, Sanusi, & Rajarajeswaran, 2015; Torikin, Lavoie, Kaplan, & Yeger, 2005). In
335 the current study, western blot analysis showed that PGPC treatment enhanced p53 expression
336 and activation associated with both caspase-8 and caspase-9 cleavage into their active

337 substrates. Indeed, PARP, a downstream substrate of caspase-8 and caspase-9, was also
338 cleaved in response to PGPC treatment.

339 A new intrinsic pathway of apoptosis involving the endoplasmic reticulum (ER) stress has
340 been described (Li, Guo, Tang, Jiang, & Chen, 2014; Rodriguez, Rojas-rivera, & Hetz, 2011).
341 In response to ER stress, inositol-requiring enzyme 1 (IRE1) mediates a pathway known as
342 unfolded protein response (UPR) in which gene expression is altered (Li et al., 2014).
343 However, severe ER stress can lead to **programmed cell death. Indeed, ER stress can activate**
344 **the pro-apoptotic Jun N-terminal Kinase (JNK) through IRE1-dependent tumor necrosis**
345 **factor (TNF)-Receptor Associated Factor (TRAF2) recruitment (Deegan, Saveljeva, Gorman,**
346 **& Samali, 2013).** The latter could abolish the mTOR-mediated oncogenic pathway (Ding et
347 al., 2007). Moreover, alteration in calcium homeostasis, splicing of X box-bindingprotein 1
348 (XBP1) mRNA, and increased CHOP expression are usually described in ER stress-mediated
349 apoptosis (Rodriguez et al., 2011). Thus, p38 MAPK has been reported to downregulate Bcl-2
350 survival protein via CHOP, a highly stress-inducible gene, activation (Szegezdi, Logue,
351 Gorman, & Samali, 2006). We have previously demonstrated that PEOL induced intrinsic
352 **apoptosis** mediated by ROS generation, increase of mRNA CHOP expression and
353 phosphorylation of eukaryotic initiation factor 2alpha (eIF2 α) in HCT 116 and HCT8 human
354 colorectal cancer cells (Zerriouh et al., 2017). In the present study, PGPC treatment increased
355 mRNA expression of Bax, XBP1, and CHOP in **U-2OS** osteosarcoma cells, **whereas, Bcl-2**
356 **mRNA expression has decreased in response to PGPC treatment.** Consistent with our
357 observations, resveratrol induced cell cycle **arrest** via UPR in chronic myelogenous leukemia
358 cells (K562). **In addition to eIF2alpha phosphorylation,** transcriptional induction of CHOP
359 and ER stress-specific XBP1 splicing were observed when K562 cells were treated with
360 resveratrol (Liu et al., 2010). Overall, these findings suggest that PGPC induced both caspase-
361 dependent and ER stress-mediated apoptosis in **U-2OS** osteosarcoma cells.

362 Increase in **cytoplasmic** (or intracellular) calcium, $[Ca^{2+}]_i$, is described to accompany or to
363 initiate apoptosis (Nicotera & Orrenius, 1998). We examined the action of PGPC on the
364 increase in $[Ca^{2+}]_i$ in **U-2OS** cells. In the presence of 100 % Ca^{2+} PGPC induced high
365 increases in $[Ca^{2+}]_i$, suggesting that Ca^{2+} influx plays a major role in $[Ca^{2+}]_i$ increase evoked
366 by this extract. In contrast, in calcium-free medium (0 % Ca^{2+}), we also noticed increases in
367 $[Ca^{2+}]_i$, suggesting that PGPC mobilize calcium also from internal stores. The kinetic study of
368 PGPC-induced Ca^{2+} mobilization showed that these compounds produced a sustained increase
369 in $[Ca^{2+}]_i$. We have previously demonstrated such increase in $[Ca^{2+}]_i$ associated with
370 **antiproliferative** effect of polyphenols (Aires et al., 2004; Nani et al., 2015; Zeriouh et al.,
371 2017).

372 AKT signaling is one of the most common aberrant pathways in cancer cells. AKT has
373 emerged as a primary downstream mediator of PI3K signaling, and multiple downstream
374 mediators of AKT, including mammalian target of rapamycin (mTOR) and transcription
375 factor STAT3 have been suggested (Nishimoto, 2000; Nitulescu et al., 2016;
376 Riemenschneider et al., 2006). In fact, through up-regulation of mTOR signaling with direct
377 inhibition of pro-apoptotic proteins, AKT, enhances proliferation and inhibits apoptosis of
378 tumor cells (Fruman & Rommel, 2014; Osaki, Oshimura, & Ito, 2004). Indeed, it has been
379 reported that AKT could promote p53 tumor **suppressor** inactivation and degradation
380 (Downward, 2004). Consequently, AKT inhibitors have been gaining ground as anticancer
381 agents nearly two decades ago (Nitulescu et al., 2016). It has been shown that PI3K/AKT
382 signal transduction pathway and STAT3 activation can be **triggered** by Src (Díaz-Montero,
383 Wygant, & McIntyre, 2006; Fossey et al., 2009; Hingorani, Zhang, Gorlick, & Kolb, 2009).
384 This latter is a non-receptor tyrosine kinase that is activated in multiple cancers, including
385 osteosarcoma (Díaz-Montero et al., 2006). PI3K/AKT signaling promotes the proliferation
386 and migration of osteosarcoma cells (Xu, Wang, & Xu, 2013). One report indicates that Src

387 activation resulted in an increase of AKT phosphorylation at Ser473 in human osteosarcoma
388 SAOSp cells (Díaz-Montero et al., 2006). A recent study has focused on the inhibition of Src
389 and mTOR kinases as a potential therapy of osteosarcoma (Botter, Neri, & Fuchs, 2014).
390 Moreover, it has been demonstrated that STAT3, which is strongly associated with the
391 prognosis of osteosarcoma, was phosphorylated in a subset of human osteosarcoma tissues
392 and cell lines (Fossey et al., 2009; Zhou et al., 2014). In our study, interestingly, PGPC
393 **treatment** curtailed c-SRC expression, AKT phosphorylation (at Ser 473 and Thr 308), and
394 STAT3 phosphorylation, suggesting that PGPC exerted an anti-proliferative activity via the
395 downregulation of c-SRC/AKT/STAT3 pathway in **U-2OS** osteosarcoma cells. In line with
396 our results, many researches have shown that polyphenols can regulate **both** upstream **and**
397 downstream effectors of AKT (Darvin et al., 2015; Wang et al., 2016; Yu et al., 2001). Ferulic
398 acid, the second major phenolic acid in *Pennisetum glaucum*, was shown to attenuate AKT
399 phosphorylation in osteosarcoma cells (Wang et al., 2016). Sorghum polyphenols dose-
400 **dependently** decreased STAT3 phosphorylation in HCT-116 and HCT-15 colon cancer cells
401 (Darvin et al., 2015). Thus, resveratrol **diminished** c-Src activity in Hela cells (Yu et al.,
402 2001).

403 AKT activation is thought, also, to abolish SAPK/JNK and p38 MAPK (also referred as p38),
404 which have been considered as putative tumor **suppressors**, activation in response to various
405 stresses (**Jiang et al., 2017; Su et al., 2014; Wagner & Nebreda, 2009**). p38, can phosphorylate
406 p53 in several residues, including Ser46, which are all involved in the onset of apoptosis
407 (Huang, Ma, Maxiner, Sun, & Dong, 1999; Perfettini et al., 2005; Zechner, Thuerlauf,
408 Hanford, McDonough, & Glembotski, 1997). We, therefore, wanted to assess the effects of
409 PGPC on MAPKs pathways. Interestingly, PGPC treatment significantly increased
410 SAPK/JNK and p38 but not ERK activity. This is consistent with results demonstrating the
411 anticancer proprieties of polyphenols, such as quercetin and resveratrol, through the

412 modulation of SAPK/JNK and p38 activity (Kim, Kim, Choi, & Son, 2016; She, Bode, Ma,
413 Chen, & Dong, 2001). Quercetin sensitized pancreatic cancer cells to **tumor-necrosis-factor**
414 **related apoptosis inducing ligand** (TRAIL)-induced apoptosis through SAPK/JNK activation
415 (Kim et al., 2016). Reseveratrol induced p38-mediated phosphorylation of p53 in JB6 Cl 41
416 cells (She et al., 2001).

417
418 P70 S6K, one of the crucial targets of mTOR, can promote cell proliferation, in part by
419 inactivation of the death promoter Bad (Harada, Andersen, Mann, Terada, & Korsmeyer,
420 2001). Therefore, we further explored the effect of PGPC on P70 S6K. Interestingly, PGPC
421 seemed effective in inhibition of p70S6K activation, suggesting that PGPC treatment
422 abolished AKT/mTOR/p70S6K pathway in **U-2OS** osteosarcoma cells. Moreover, previous
423 studies have revealed that polyphenols suppressed cell proliferation through the inhibition of
424 AKT/mTOR/p70S6K (Castillo-Pichardo & Dharmawardhane, 2012; Moore et al., 2016).

425
426 Cancer cells are known for their hallmark ability to replicate indefinitely and escape
427 programmed cell death (Pentimalli & Giordano, 2009). It has been reported that proteolysis of
428 p53 and cyclin dependent kinase inhibitors (CKIs), such as p21 and p27, were correlated with
429 overexpression of cyclins and loss of CKIs expression in many cancer cells (Rastogi &
430 Mishra, 2012). Indeed, one report suggested that p53 is required for maintenance of S-phase
431 arrest (Giono & Manfredi, 2006). Therefore, challenging efforts aim to conceive therapeutic
432 approaches based on cyclin-dependent kinases(CDKs) inhibition (Malumbres & Barbacid,
433 2009). In the current study, we showed that both PGPC and p-CA induced cell cycle arrest in
434 S phase, while 5-FU at low dose (2.5 μ M) blocked cell progression in G1 phase. However, 5-
435 FU combination with PGPC or p-CA caused **U-2OS** cells accumulation in S phase, suggesting
436 that PGPC and p-CA do not share common targets with 5-FU. Regarding cyclins and CDKs
437 involved in S phase entry and progression, CDK2 expression was significantly decreased, and

438 cyclin E was significantly increased in response to PGPC treatment. Nonetheless, PGPC did
439 not seem to affect cdc2/CDK1 and cyclin A expression.

440 Previous studies have demonstrated that polyphenols caused cells accumulation in S phase by
441 decreasing CDK2 and/or increasing cyclin E expression (Chou et al., 2010; Larrosa, **Tomàs-**
442 **Barberà**n, & **Espín**, 2003). Another study highlighted the inhibitory effect of ferulic acid on
443 CDK2 expression in osteosarcoma cells (Wang et al., 2016).

444 **5. Conclusion**

445 From our findings, we can state that PGPC downregulates AKT upstream and downstream
446 effectors which are associated with p38 and SAPK/JNK up-regulation, and increased
447 $[Ca^{2+}]_i$, resulting in cell cycle arrest and caspase-dependent apoptosis in osteosarcoma **U-2OS**
448 cells. Overall, this study encourages the exploration of pearl millet polyphenols as a
449 promising candidate for osteosarcoma prevention and treatment. Nonetheless, anti-tumor
450 effects in animal models remain to be investigated.

451

452 **Declare of interest**

453 The authors have declared no conflicts of interest.

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462

463 **References**

464 Aires, V., Adote, S., Hichami, A., Moutairou, K., Boustani, E. S., & Khan, N. A. (2004).
465 Modulation of intracellular calcium concentrations and T cell activation by prickly pear

466 polyphenols. *Mol Cell Biochem*, 260(1-2), 103–110. Retrieved from
467 <http://www.ncbi.nlm.nih.gov/pubmed/15228091>

468 Allison, D. C., Carney, S. C., Ahlmann, E. R., Hendifar, A., Chawla, S., Fedenko, A.,
469 Angeles, C., & Menendez, L. R. (2012). A meta-analysis of osteosarcoma outcomes in
470 the modern medical era. *Sarcoma*, 2012. <http://doi.org/10.1155/2012/704872>

471 Bakhshi, S., & Radhakrishnan, V. (2010). Prognostic markers in osteosarcoma. *Expert Review*
472 *of Anticancer Therapy*, 10(2), 271–287.

473 Banerjee, N., Kim, H., Krenek, K., Talcott, S. T., & Mertens-Talcott, S. U. (2015). Mango
474 polyphenolics suppressed tumor growth in breast cancer xenografts in mice: role of the
475 PI3K/AKT pathway and associated microRNAs. *Nutrition Research*, 35(8), 744–751.
476 <http://doi.org/10.1016/j.nutres.2015.06.002>

477 Botter, S. M., Neri, D., & Fuchs, B. (2014). Recent advances in osteosarcoma. *Current*
478 *Opinion in Pharmacology*, 16, 15–23.

479 Castillo-Pichardo, L., & Dharmawardhane, S. F. (2012). Grape Polyphenols Inhibit
480 Akt/Mammalian Target of Rapamycin Signaling and Potentiate the Effects of Gefitinib
481 in Breast Cancer. *Nutrition and Cancer*, 64(7), 1058–1069.
482 <http://doi.org/10.1080/01635581.2012.716898>

483 Cho, I. J., Chang, H. J., Lee, K. E., Won, H. S., Choi, M. Y., Nam, E. M., Mun, Y-C., Lee, S.
484 N., & Seong, C.-M. (2009). A case of Wernicke's encephalopathy following
485 fluorouracil-based chemotherapy. *Journal of Korean Medical Science*, 24(4), 747–750.

486 Chou, C.-C., Yang, J.-S., Lu, H.-F., Ip, S.-W., Lo, C., Wu, C.-C., Lin, J-P., Tang, N-Y.,
487 Chung, J-G., Chou, M-J., Teng, Y-H., & Chen, D.-R. (2010). Quercetin-mediated cell
488 cycle arrest and apoptosis involving activation of a caspase cascade through the
489 mitochondrial pathway in human breast cancer MCF-7 cells. *Archives of Pharmacal*
490 *Research*, 33(8), 1181–1191. <http://doi.org/10.1007/s12272-010-0808-y>

491 Darwin, P., Joung, Y. H., Nipin, S. P., Kang, D. Y., Byun, H. J., Hwang, D. Y., Cho, K. H.,
492 Park, K. D., Lee, H. K., & Yang, Y. M. (2015). Sorghum polyphenol suppresses the
493 growth as well as metastasis of colon cancer xenografts through co-targeting
494 jak2/STAT3 and PI3K/Akt/mTOR pathways. *Journal of Functional Foods*, *15*, 193–206.
495 <http://doi.org/10.1016/j.jff.2015.03.020>

496 Deegan, S., Saveljeva, S., Gorman, A. M., & Samali, A. (2013). Stress-induced self-
497 cannibalism: on the regulation of autophagy by endoplasmic reticulum stress. *Cellular*
498 *and Molecular Life Sciences*, *70*(14), 2425–2441.

499 Díaz-Montero, C. M., Wygant, J. N., & McIntyre, B. W. (2006). PI3-K/Akt-mediated anoikis
500 resistance of human osteosarcoma cells requires Src activation. *European Journal of*
501 *Cancer*, *42*(10), 1491–1500. <http://doi.org/10.1016/j.ejca.2006.03.007>

502 Ding, W.-X., Ni, H.-M., Gao, W., Yoshimori, T., Stolz, D. B., Ron, D., & Yin, X.-M. (2007).
503 Linking of autophagy to ubiquitin-proteasome system is important for the regulation of
504 endoplasmic reticulum stress and cell viability. *The American Journal of Pathology*,
505 *171*(2), 513–524.

506 Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Seminars in Cell and Developmental*
507 *Biology*, *15*(2), 177–182. <http://doi.org/10.1016/j.semcdb.2004.01.002>

508 Dramane, G., Abdoul-Azize, S., Hichami, A., Vögtle, T., Akpona, S., Chouabe, C., Sadou, H.,
509 Nieswandt, B., Besnard, P., & Khan, N. A. (2012). STIM1 regulates calcium signaling in
510 taste bud cells and preference for fat in mice. *Journal of Clinical Investigation*, *122*(6),
511 2267–2282. <http://doi.org/10.1172/JCI59953>

512 Eum, K. H., & Lee, M. (2011). Crosstalk between autophagy and apoptosis in the regulation
513 of paclitaxel-induced cell death in v-Ha-ras-transformed fibroblasts. *Molecular and*
514 *Cellular Biochemistry*, *348*(1-2), 61–68. <http://doi.org/10.1007/s11010-010-0638-8>

515 Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. *J.*

516 *Biol. Chem*, 73(2), 627–650.

517 Fossey, S. L., Liao, A. T., McCleese, J. K., Bear, M. D., Lin, J., Li, P.-K., Kisseberth, W. C.,
518 & London, C. A. (2009). Characterization of STAT3 activation and expression in canine
519 and human osteosarcoma. *BMC Cancer*, 9, 81. <http://doi.org/10.1186/1471-2407-9-81>

520 Fruman, D. A., & Rommel, C. (2014). PI3K and cancer: lessons, challenges and
521 opportunities. *Nature Reviews Drug Discovery*, 13(2), 140.

522 Ghanemi, F. Z., Belarbi, M., Fluckiger, A., Nani, A., Dumont, A., De Rosny, Aboura, I.,
523 Khan, A. S., Murtaza, B., Benammar, C., Lahfa, B. F., Patoli, D., Delmas, D., Rébé, C.,
524 Apetoh, L., Khan, N. A., Ghringhelli, F., Rialland, M., & Hichami, A. (2017). Carob leaf
525 polyphenols trigger intrinsic apoptotic pathway and induce cell cycle arrest in colon
526 cancer cells. *Journal of Functional Foods*, 33, 112–121.

527 Giono, L. E., & Manfredi, J. J. (2006). The p53 tumor suppressor participates in multiple cell
528 cycle checkpoints. *Journal of Cellular Physiology*, 209(1), 13–20.

529 Goodall, M. L., Fitzwalter, B. E., Zahedi, S., Wu, M., Rodriguez, D., Mulcahy-Levy, J. M.,
530 Green, D. R., Morgan, M., Cramer, S. D. & Thorburn, A. (2016). The Autophagy
531 Machinery Controls Cell Death Switching between Apoptosis and Necroptosis.
532 *Developmental Cell*, 37(4), 337–349. <http://doi.org/10.1016/j.devcel.2016.04.018>

533 Grossel, M. J., Baker, G. L., & Hinds, P. W. (1999). Cdk6 can shorten G1 phase dependent
534 upon the N-terminal INK4 interaction domain. *Journal of Biological Chemistry*, 274(42),
535 29960–29967.

536 Hajiaghaalipour, F., Kanthimathi, M. S., Sanusi, J., & Rajarajeswaran, J. (2015). White tea
537 (*Camellia sinensis*) inhibits proliferation of the colon cancer cell line, HT-29, activates
538 caspases and protects DNA of normal cells against oxidative damage. *Food Chemistry*,
539 169, 401–410. <http://doi.org/10.1016/j.foodchem.2014.07.005>

540 Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*,

541 144(5), 646–674.

542 Harada, H., Andersen, J. S., Mann, M., Terada, N., & Korsmeyer, S. J. (2001). p70S6 kinase
543 signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD.
544 *Proceedings of the National Academy of Sciences of the United States of America*,
545 98(17), 9666–70. <http://doi.org/10.1073/pnas.171301998>

546 Hingorani, P., Zhang, W., Gorlick, R., & Kolb, E. A. (2009). Inhibition of Src
547 phosphorylation alters metastatic potential of osteosarcoma in vitro but not in vivo.
548 *Clinical Cancer Research*, 15(10), 3416–3422. [http://doi.org/10.1158/1078-0432.CCR-](http://doi.org/10.1158/1078-0432.CCR-08-1657)
549 08-1657

550 Huang, C., Ma, W. Y., Maxiner, A., Sun, Y., & Dong, Z. (1999). p38 kinase mediates UV-
551 induced phosphorylation of p53 protein at serine 389. *Journal of Biological Chemistry*,
552 274(18), 12229–12235. <http://doi.org/10.1074/jbc.274.18.12229>

553 Jaganathan, S. K., Supriyanto, E., & Mandal, M. (2013). Events associated with apoptotic
554 effect of p -Coumaric acid in HCT-15 colon cancer cells. *World Journal of*
555 *Gastroenterology*, 19(43), 7726–7734. <http://doi.org/10.3748/wjg.v19.i43.7726>

556 Jiang, X., Zhu, X., Xu, H., Zhao, Z., Li, S., Li, S., Cai, J.-H., & Cao, J. (2017). Diallyl
557 trisulfide suppresses tumor growth through the attenuation of Nrf2/Akt and activation of
558 p38/JNK and potentiates cisplatin efficacy in gastric cancer treatment. *Acta*
559 *Pharmacologica Sinica*, 38(7), 1048.

560 Kim, J. H., Kim, M. J., Choi, K.-C., & Son, J. (2016). Quercetin sensitizes pancreatic cancer
561 cells to TRAIL-induced apoptosis through JNK-mediated cFLIP turnover. *The*
562 *International Journal of Biochemistry & Cell Biology*, 78, 327–334.
563 <http://doi.org/10.1016/j.biocel.2016.07.033>

564 Larrosa, M., Tomás-Barberán, F. A., & Espín, J. C. (2003). Grape polyphenol resveratrol and
565 the related molecule 4-hydroxystilbene induce growth inhibition, apoptosis, S-phase

566 arrest, and upregulation of cyclins A, E, and B1 in human SK-Mel-28 melanoma cells.
567 *Journal of Agricultural and Food Chemistry*, 51(16), 4576–4584.
568 <http://doi.org/10.1021/jf030073c>

569 León-González, A. J., Auger, C., & Schini-Kerth, V. B. (2015). Pro-oxidant activity of
570 polyphenols and its implication on cancer chemoprevention and chemotherapy.
571 *Biochemical Pharmacology*, 98(3), 371–380. <http://doi.org/10.1016/j.bcp.2015.07.017>

572 Li, Y., Guo, Y., Tang, J., Jiang, J., & Chen, Z. (2014). New insights into the roles of CHOP-
573 induced apoptosis in ER stress Structure and Properties of C / EBP Homologous Protein
574 Roles of CHOP in ER Stress-Mediated Apoptosis. *Acta Biochimica et Biophysica Sinica*,
575 46(8), 629–640. <http://doi.org/10.1093/abbs/gmu048.Review>

576 Linke, S. P., Clarkin, K. C., Di Leonardo, A., Tsou, A., & Wahl, G. M. (1996). A reversible,
577 p53-dependent G₀/G₁ cell cycle arrest induced by ribonucleotide depletion in the
578 absence of detectable DNA damage. *Genes & Development*, 10, 934–937.
579 <http://doi.org/10.1101/gad.10.8.934>

580 Liu, B.-Q., Gao, Y.-Y., Niu, X.-F., Xie, J.-S., Meng, X., Guan, Y., & Wang, H.-Q. (2010).
581 Implication of unfolded protein response in resveratrol-induced inhibition of K562 cell
582 proliferation. *Biochemical and Biophysical Research Communications*, 391(1), 778–782.

583 Lockshin, R. A., & Zakeri, Z. (2004). Apoptosis, autophagy, and more. *International Journal*
584 *of Biochemistry and Cell Biology*, 36(12), 2405–2419.
585 <http://doi.org/10.1016/j.biocel.2004.04.011>

586 Luetke, A., Meyers, P. A., Lewis, I., & Juergens, H. (2014). Osteosarcoma treatment - Where
587 do we stand? A state of the art review. *Cancer Treatment Reviews*, 40(4), 523–532.
588 <http://doi.org/10.1016/j.ctrv.2013.11.006>

589 Malumbres, M., & Barbacid, M. (2009). Cell cycle, CDKs and cancer: a changing paradigm.
590 *Nature Reviews. Cancer*, 9(3), 153–166. <http://doi.org/10.1038/nrc2602>

591 Marina, N., Gebhardt, M., Teot, L., & Gorlick, R. (2004). Biology and therapeutic advances
592 for pediatric osteosarcoma. *The Oncologist*, *9*(4), 422–441.

593 Minville-Walz, M., Pierre, A.-S., Pichon, L., Bellenger, S., Fèvre, C., Bellenger, J., Fèvre, C.,
594 Bellenger, J., Tessier, C., Narce, M., & Rialland, M. (2010). Inhibition of stearyl-CoA
595 desaturase 1 expression induces CHOP-dependent cell death in human cancer cells. *PLoS*
596 *One*, *5*(12), e14363.

597 Mirabello, L., Troisi, R. J., & Savage, S. A. (2009). Osteosarcoma incidence and survival
598 rates from 1973 to 2004: Data from the surveillance, epidemiology, and end results
599 program. *Cancer*, *115*(7), 1531–1543. <http://doi.org/10.1002/cncr.24121>

600 Moore, J., Megaly, M., MacNeil, A. J., Klentrou, P., & Tsiani, E. (2016). Rosemary extract
601 reduces Akt/mTOR/p70S6K activation and inhibits proliferation and survival of A549
602 human lung cancer cells. *Biomedicine & Pharmacotherapy*, *83*, 725–732.
603 <http://doi.org/10.1016/j.biopha.2016.07.043>

604 Mouria, M., Gukovskaya, A. S., Jung, Y., Buechler, P., Hines, O. J., Reber, H. A., & Pandol,
605 S. J. (2002). Food-derived polyphenols inhibit pancreatic cancer growth through
606 mitochondrial cytochrome c release and apoptosis. *International Journal of Cancer*,
607 *98*(5), 761–769. <http://doi.org/10.1002/ijc.10202>

608 Nani, A., Belarbi, M., Ksouri-Megdiche, W., Abdoul-Azize, S., Benammar, C., Ghiringhelli,
609 F., Hichami, A., & Khan, N. A. (2015). Effects of polyphenols and lipids from
610 Pennisetum glaucum grains on T-cell activation: modulation of Ca²⁺ and ERK1/ERK2
611 signaling. *BMC Complementary and Alternative Medicine*, *15*(1), 426.
612 <http://doi.org/10.1186/s12906-015-0946-3>

613 Nicotera, P., & Orrenius, S. (1998). The role of calcium in apoptosis. *Cell Calcium*, *23*(2),
614 173–180.

615 Nishimoto, T. (2000). Upstream and downstream of ran GTPase. *Biological Chemistry*,

616 381(5-6), 397–405. <http://doi.org/10.1515/BC.2000.052>

617 Nitulescu, G. M., Margina, D., Juzenas, P., Peng, Q., Olaru, O. T., Saloustros, E., Fenga, C.,
618 Spandidos, D. A., Libra, M., & Tsatsakis, A. M. (2016). Akt inhibitors in cancer
619 treatment: The long journey from drug discovery to clinical use (Review). *International*
620 *Journal of Oncology*, 48(3), 869–885. <http://doi.org/10.3892/ijo.2015.3306>

621 Osaki, M., Oshimura, M., & Ito, H. (2004). PI3K-Akt pathway: its functions and alterations in
622 human cancer. *Apoptosis*, 9(6), 667–676.
623 <http://doi.org/10.1023/B:APPT.0000045801.15585.dd>

624 Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012).
625 Programmed cell death pathways in cancer: A review of apoptosis, autophagy and
626 programmed necrosis. *Cell Proliferation*, 45(6), 487–498. [http://doi.org/10.1111/j.1365-](http://doi.org/10.1111/j.1365-2184.2012.00845.x)
627 [2184.2012.00845.x](http://doi.org/10.1111/j.1365-2184.2012.00845.x)

628 Pentimalli, F., & Giordano, A. (2009). Promises and drawbacks of targeting cell cycle kinases
629 in cancer. *Discovery Medicine*, 8(43), 177–180.

630 Perfettini, J.-L., Castedo, M., Nardacci, R., Ciccocanti, F., Boya, P., Roumier, T., Larochette,
631 Nathanael, Piacentini, M., & Kroemer, G. (2005). Essential role of p53 phosphorylation
632 by p38 MAPK in apoptosis induction by the HIV-1 envelope. *The Journal of*
633 *Experimental Medicine*, 201(2), 279–89. <http://doi.org/10.1084/jem.20041502>

634 Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and
635 signalling pathways. *Molecular Nutrition and Food Research*, 52(5), 507–526.
636 <http://doi.org/10.1002/mnfr.200700326>

637 Rastogi, N., & Mishra, D. P. (2012). Therapeutic targeting of cancer cell cycle using
638 proteasome inhibitors. *Cell Division*, 7, 26. <http://doi.org/10.1186/1747-1028-7-26>

639 Riemenschneider, M. J., Betensky, R. A., Pasedag, S. M., & Louis, D. N. (2006). AKT
640 activation in human glioblastomas enhances proliferation via TSC2 and S6 kinase

641 signaling. *Cancer Research*, 66(11), 5618–5623. <http://doi.org/10.1158/0008->
642 5472.CAN-06-0364

643 Rodriguez, D., Rojas-rivera, D., & Hetz, C. (2011). Biochimica et Biophysica Acta
644 Integrating stress signals at the endoplasmic reticulum: The BCL-2 protein family
645 rheostat ☆. *BBA - Molecular Cell Research*, 1813(4), 564–574.
646 <http://doi.org/10.1016/j.bbamcr.2010.11.012>

647 Roskoski, R. (2016). Cyclin-dependent protein kinase inhibitors including palbociclib as
648 anticancer drugs. *Pharmacological Research*, 107, 249–275.
649 <http://doi.org/10.1016/j.phrs.2016.03.012>

650 Shahidi, F., & Chandrasekara, A. (2013). Millet grain phenolics and their role in disease risk
651 reduction and health promotion: A review. *Journal of Functional Foods*, 5(2), 570–581.

652 She, Q.-B., Bode, A. M., Ma, W.-Y., Chen, N.-Y., & Dong, Z. (2001). Resveratrol-induced
653 activation of p53 and apoptosis is mediated by extracellular- signal-regulated protein
654 kinases and p38 kinase. *Cancer Res.*, 61(4), 1604–1610.

655 Su, C.-C., Chen, J. Y.-F., Din, Z.-H., Su, J.-H., Yang, Z.-Y., Chen, Y.-J., & Wu, Y.-J. (2014).
656 13-acetoxysarcocrassolide induces apoptosis on human gastric carcinoma cells through
657 mitochondria-related apoptotic pathways: p38/JNK activation and PI3K/AKT
658 suppression. *Marine Drugs*, 12(10), 5295–5315.

659 Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic
660 reticulum stress-induced apoptosis. *EMBO Reports*, 7(9), 880–885.

661 T, W., X, G., R, J., H, L., W, D., & Kuang, G. (2016). Ferulic acid inhibits proliferation and
662 promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma cell. *American*
663 *Journal of Translational Research*, 8(2), 968–980.

664 Thomasset, S. C., Berry, D. P., Garcea, G., Marczylo, T., Steward, W. P., & Gescher, A. J.
665 (2007). Dietary polyphenolic phytochemicals - Promising cancer chemopreventive

666 agents in humans? A review of their clinical properties. *International Journal of Cancer*,
667 *120*(3), 451–458. <http://doi.org/10.1002/ijc.22419>

668 Torkin, R., Lavoie, J.-F., Kaplan, D. R., & Yeger, H. (2005). Induction of caspase-dependent,
669 p53-mediated apoptosis by apigenin in human neuroblastoma. *Molecular Cancer*
670 *Therapeutics*, *4*(1), 1–11. Retrieved from
671 PM:15657348\http://www.ncbi.nlm.nih.gov/pubmed/15657348

672 Vogelstein, B., Lane, D., & Levine, a J. (2000). Surfing the p53 network. *Nature*, *408*(6810),
673 307–310. <http://doi.org/10.1038/35042675>

674 Vousden, K. H., & Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53.
675 *Cell*, *137*(3), 413–431. <http://doi.org/10.1016/j.cell.2009.04.037>

676 Wagner, E. F., & Nebreda, A. R. (2009). Signal integration by JNK and p38 MAPK pathways
677 in cancer development. *Nature Reviews. Cancer*, *9*, 537–549.
678 <http://doi.org/10.1038/nrc2694>

679 Wang, T., Gong, X., Jiang, R., Li, H., Du, W., & Kuang, G. (2016). Ferulic acid inhibits
680 proliferation and promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma
681 cell. *American Journal of Translational Research*, *8*(2), 968.

682 Xu, X., Wang, B., & Xu, Y. (2013). Expression of lysyl oxidase in human osteosarcoma and
683 its clinical significance: a tumor suppressive role of LOX in human osteosarcoma cells.
684 *International Journal of Oncology*, *43*(5), 1578–1586.

685 Yu, R., Hebbar, V., Kim, D. W., Mandlekar, S., Pezzuto, J. M., & Kong, A. N. (2001).
686 Resveratrol inhibits phorbol ester and UV-induced activator protein 1 activation by
687 interfering with mitogen-activated protein kinase pathways. *Mol Pharmacol*, *60*(1), 217–
688 224. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11408617>

689 Zechner, D., Thuerauf, D. J., Hanford, D. S., McDonough, P. M., & Glembotski, C. C.
690 (1997). A Role for the p38 Mitogen-activated Protein Kinase Pathway in Myocardial

691 Cell Growth, Sarcomeric Organization, and Cardiac-specific Gene Expression. *The*
692 *Journal of Cell Biology*, 139(1), 115–127. <http://doi.org/10.1083/jcb.139.1.115>

693 Zeriouh, W., Nani, A., Belarbi, M., Dumont, A., de Rosny, C., Aboura, I., **Ghanemi, F. Z.,**
694 **Murtaza, B., Patoli, D., Apetoh, L. Rébé, C. Delmas, D. Khan, N.A. Ghiringhelli, F.**
695 **Rialland, M., & Hichami, A.** (2017). Correction: Phenolic extract from oleaster (*Olea*
696 *europaea* var. *Sylvestris*) leaves reduces colon cancer growth and induces caspase-
697 dependent apoptosis in colon cancer cells via the mitochondrial apoptotic pathway. *PloS*
698 *One*, 12(4), e0176574.

699 Zhou, W., Hao, M., Du, X., Chen, K., Wang, G., & Yang, J. (2014). Advances in targeted
700 therapy for osteosarcoma. *Discovery Medicine*, 17(96), 301–307.

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716 Legends of figures

717 **Fig. 1.** Effects of PGPC and p-CA on cell viability. (A) The cells (seeded at the initial cell
718 density of 3×10^4 cells/well) were incubated with different concentrations of PGPC or p-CA
719 for 24 h as described in Experimental Section. Cell viability was assessed by trypan blue
720 exclusion test. Cell numbers were determined by a hemocytometer. Inserts show the release of
721 LDH into cell culture, measured from supernatant, in response to PGPC (left panel) and p-CA
722 (right panel) treatments. Triton X-100 (1%) was used as a high control. Data represent the
723 mean \pm S.D. of three independent experiments performed in triplicate ($n = 3$). *, **, and ***
724 represent $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively as compared to untreated
725 cells. (B) U-2OS cells morphology after 24 h of incubation with PGPC (60 and 100 $\mu\text{g}/\text{mL}$)
726 and p-CA (15000 and 25000 μM), as assessed by light inverted microscopy (magnification
727 $\times 200$).

728

729 **Fig. 2.** Effects of PGPC and p-CA on induction of apoptosis in U-2OS cells. (A) Annexin-
730 V/7-AAD staining cytograms of U-2OS cells pre-incubated or not with z-VAD-fmk, and then
731 treated with PGPC (60 $\mu\text{g}/\text{mL}$) or p-CA (12500 μM) for 24h. Cytograms are representative of
732 three independent experiments. (B) Rate of early or late apoptosis and necrosis of U-2OS cell
733 lines. Annexin V+, 7AAD+ values were considered statistically significant if $p < 0.01$ (**)
734 when compared with untreated cells.

735

736 **Fig. 3.** Effects of PGPC on calcium-mediated apoptosis in U-2OS cells. (A) percentage of
737 apoptotic cells (Annexin V positive) treated with PGPC (60 $\mu\text{g}/\text{mL}$) in the presence of one or
738 combination of the following molecules: BAPTA-AM (5 μM), RuR (5 μM), zVAD (50 μM),
739 3-MA (5 mM), CQ (50 μM). *, ** and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$
740 respectively as compared to untreated cells.

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743 **Fig. 4.** Effect of PGPC on apoptosis-related proteins. “A” represents up-regulation of p-53,
744 caspase-8 and -9 and PARP activity in PGPC-treated U-2OS cells for 24 h, as assessed by
745 western blotting analysis. β -actin was used as the internal protein control. “B” represents ER-
746 mediated apoptosis proteins expression in response to U-2OS cells treatment with PGPC, as
747 assessed by qRT PCR.

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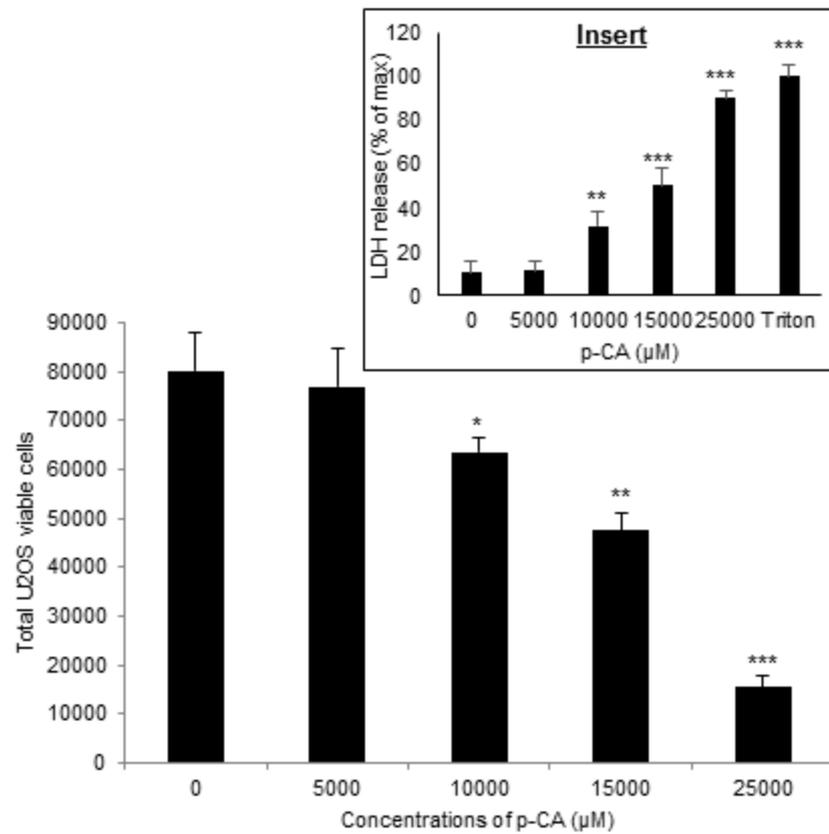
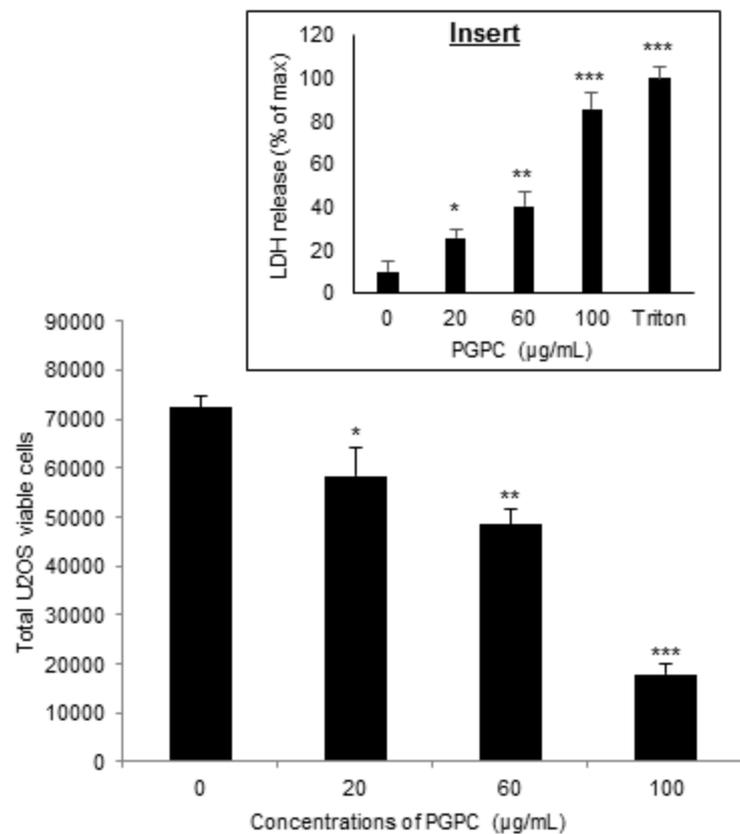
749 **Fig. 5.** Ca^{2+} signaling modulation by PGPC in U-2OS cells. The colored time-lapse changes
750 in the increases in $[\text{Ca}^{2+}]_i$ were recorded in PGPC-treated U-2OS cells in 100 % Ca^{2+} -buffer
751 (A) or 0 % Ca^{2+} -buffer (B). Figure C shows the single traces of observations which were
752 reproduced independently (n = 3). The arrow head indicates the time when PGPC was added
753 into the wells without interruptions in recordings.

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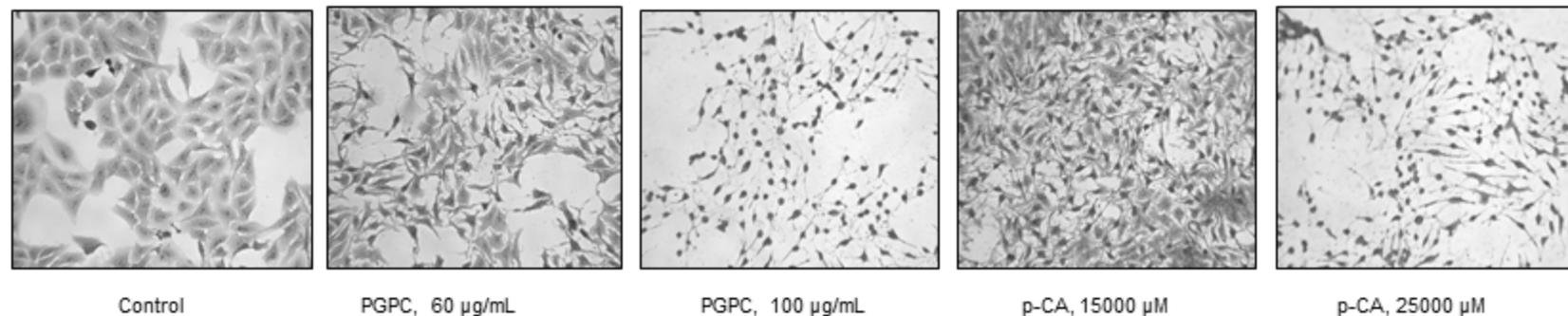
755 **Fig. 6.** Effect of PGPC on ATK (A) and MAPKs pathway (B) in U-2OS cells, as assessed by
756 western blotting. β -actin was used as the internal protein control.

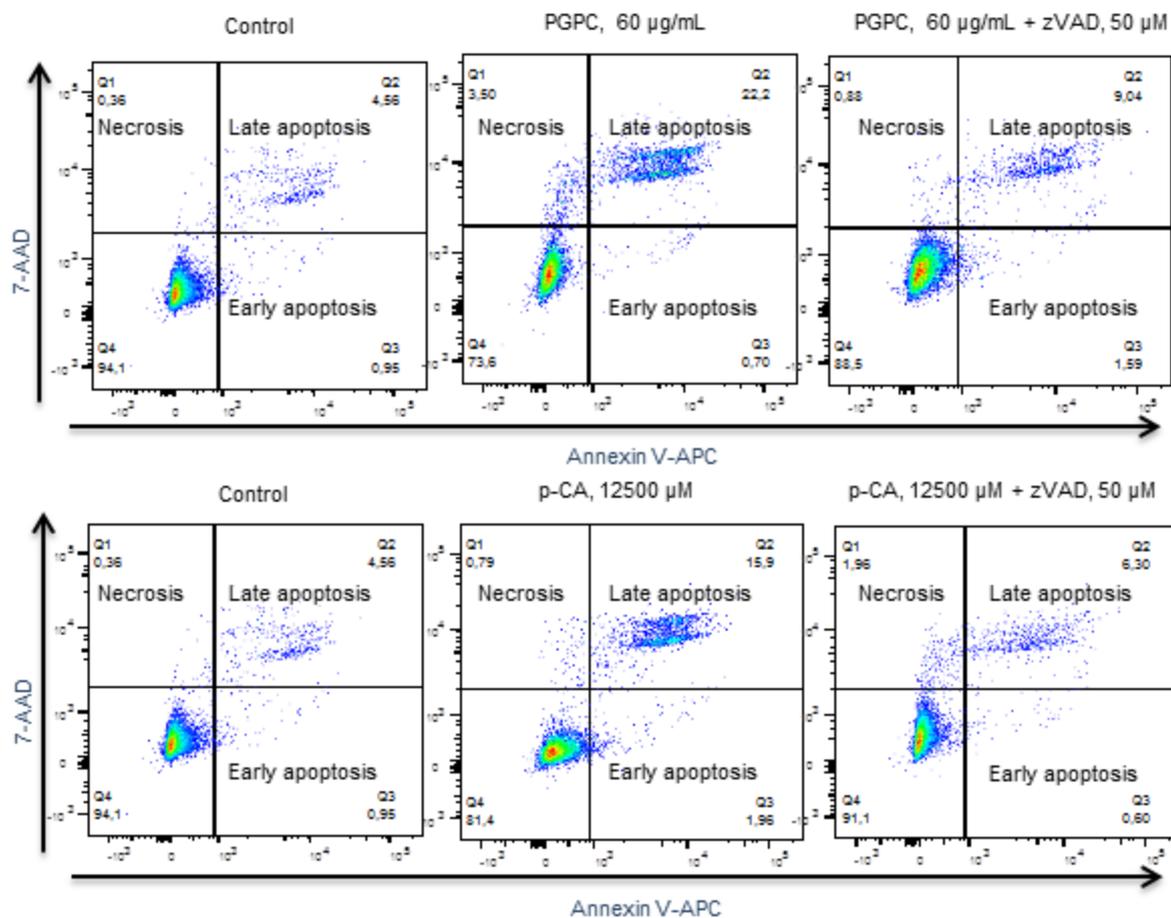
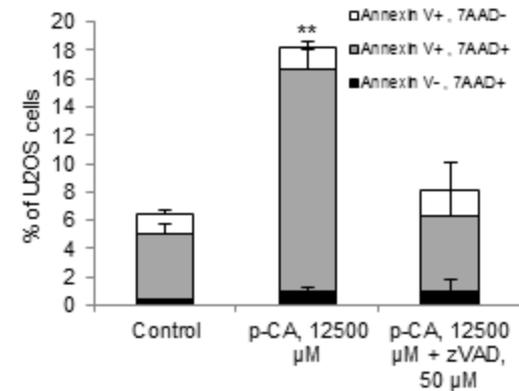
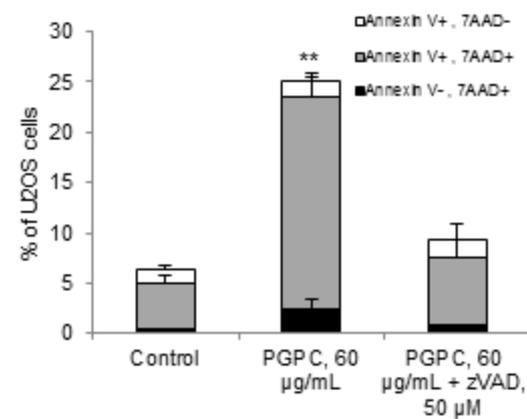
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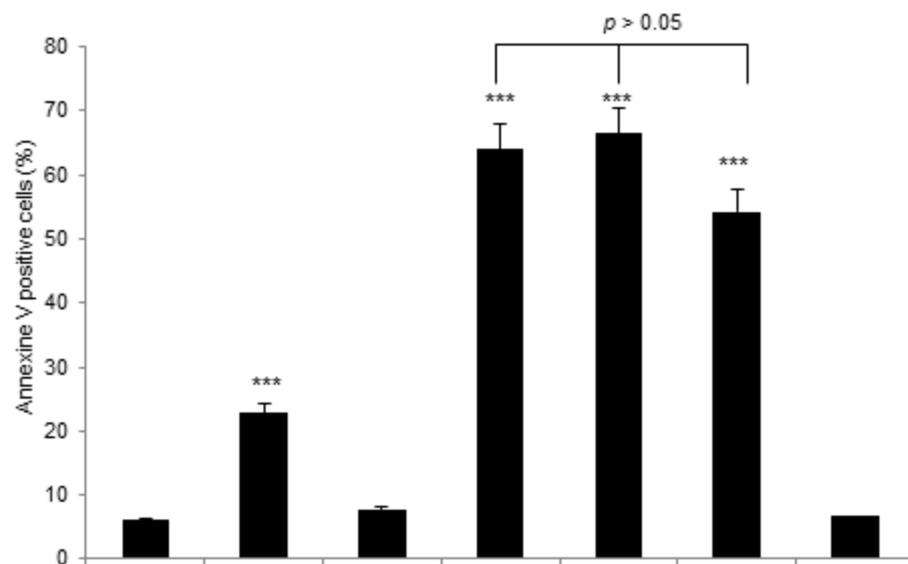
758 **Fig. 7.** Effect of PGPC on cell cycle progression. (A) Cell cycle representative profile of U-
759 2OS in untreated or treated cells with PGPC (60 $\mu\text{g}/\text{mL}$) or p-CA (12500 μM) for 24 h then
760 labeled with PI and analyzed by FACSCanto. (B) Statistical analysis of G1, S and G2/M
761 phases of cell cycle in untreated and treated cells with PGPC (60 $\mu\text{g}/\text{mL}$) or p-CA (12500
762 μM) co-treated or not with 5-FU (2,5 μM). § represents $p < 0.05$ in S phase as compared to
763 control group, * represents $p < 0.05$ in S phase as compared to 5-fu treated group. (C) Effects
764 of PGPC on S phase cyclins and CDKs, as determined by western blotting analysis. β -actin
765 was used as the internal protein control.

A**B**

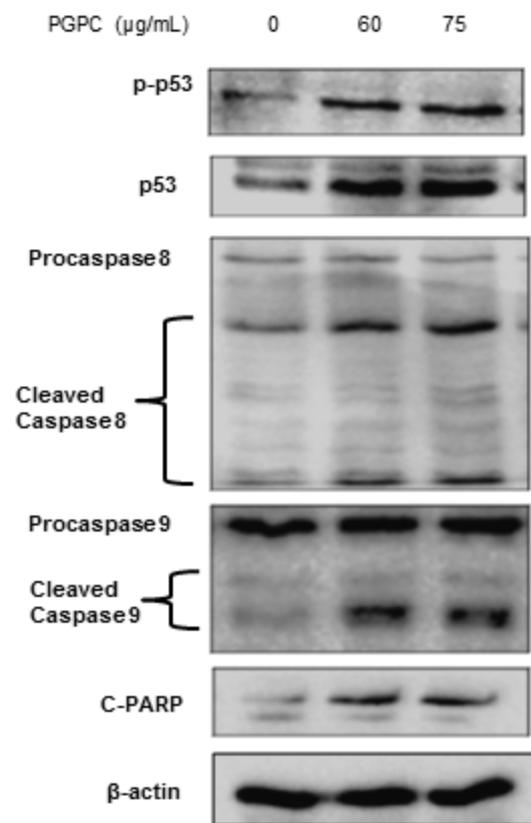
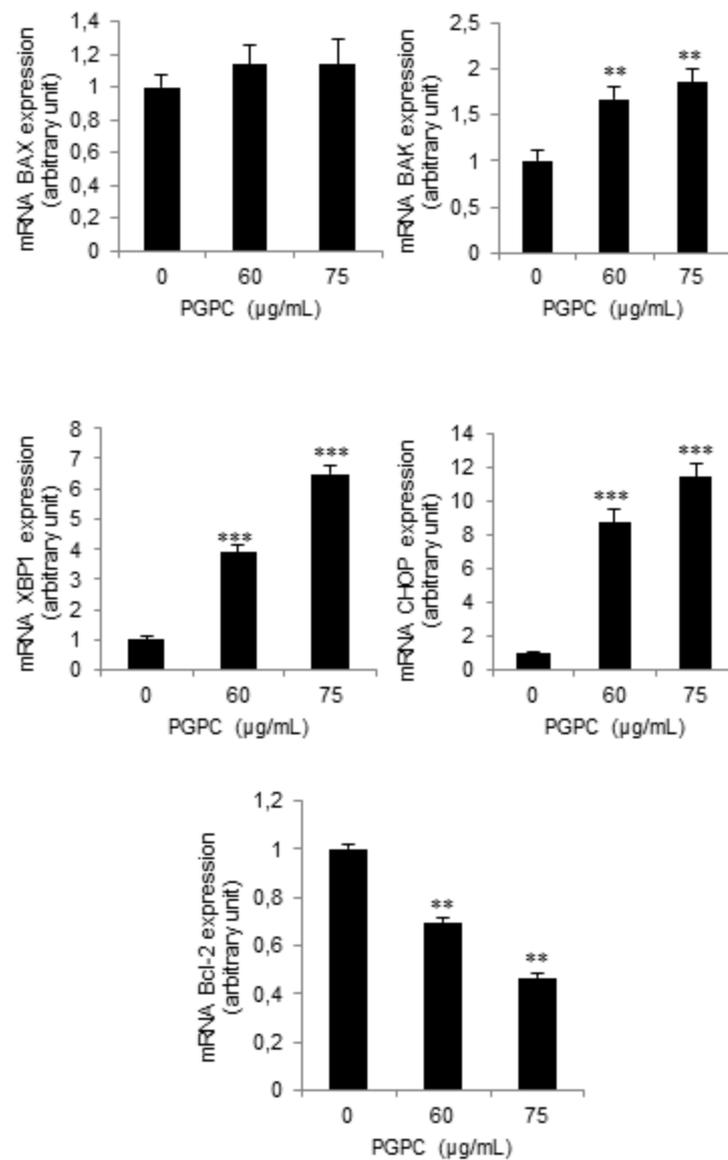
U2OS cells

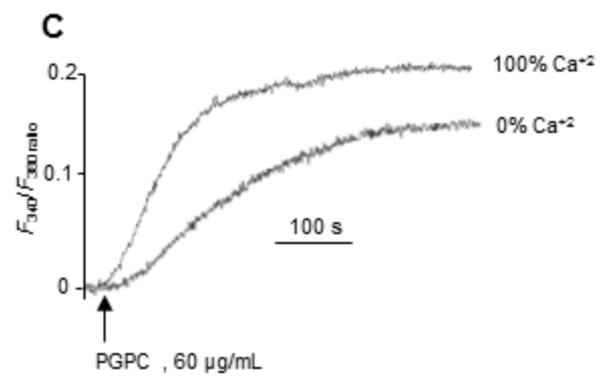
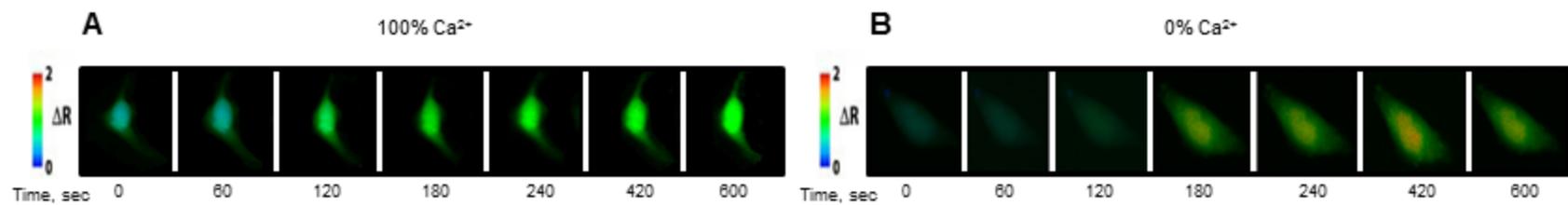


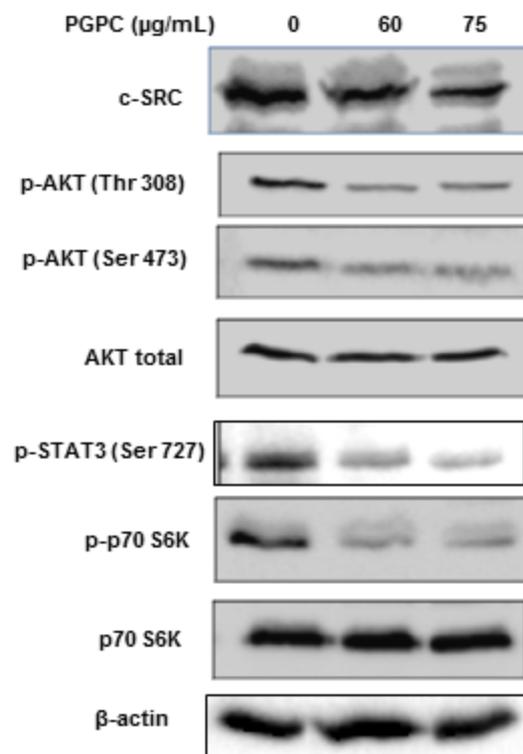
A**B**



| | | | | | | | |
|----------------------------------|---|---|---|---|---|---|---|
| PGPC, 60 $\mu\text{g}/\text{mL}$ | - | + | + | + | + | + | + |
| BAPTA-AM, 5 μM | - | - | + | - | - | - | - |
| RuR, 5 μM | - | - | - | + | + | + | + |
| 3-MA, 5 mM | - | - | - | - | + | - | - |
| CQ, 50 μM | - | - | - | - | - | + | - |
| zVAD, 50 μM | - | - | - | - | - | - | + |

A**B**



A**B**