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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

Osteosarcoma is the most common bone tumor with a high prevalence among children and adolescents. Polyphenols are widely investigated for their chemopreventive and chemotherapeutic proprieties. In the present study, we explored the pro-apoptotic effects of pearl millet, *Pennisetum glaucum*, phenolic compounds (PGPC) on osteosarcoma U-2OS cells. Our results show that PGPC induced U-2OS cells death, in a dose dependent manner, with an IC<sub>50</sub> of 80 µg/mL. Annexin-V and 7-AAD staining show that PGPC induced cell death mainly through caspase-dependent apoptosis as shown by a decrease in cell death when co-treated with pan-caspase inhibitor, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk). PGPC caused an increase in cytoplasmic calcium associated with caspases activation and poly (ADP-ribose) polymerase PARP cleavage. Western blot analysis revealed that PGPC upregulated p38 MAPK and SAPK/JNK activity and inhibited c-SRC/AKT pathway. Finally, cell cycle analysis shows that PGPC decreased CDK2 and increased cyclin E expression, resulting in U-2OS cells accumulation in S phase. These results demonstrate that PGPC induce p38 MAPK and SAPK/JNK activation, and attenuate AKT activation, leading to cell cycle arrest and apoptosis in osteosarcoma U-2OS cells.

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

**Abbreviations:** 3-MA, 3-Methyladenine; 5-FU, 5-Fluorouracil; AKT, Protein kinase B; Annexin V-APC/7-AAD, Annexin V-apoptosis -associated caspase/7-Aminoactinomycin D; 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 kinase-2; CDKs, cyclin-dependent kinases; CKIs, cyclin dependent kinase inhibitors; CQ,

chloroquine; c-Src, Proto-oncogene tyrosine-protein kinase; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; ERK1/2, p44/42 MAPK; Fura-2 AM, fura-2-acetoxymethylester; JAK 2, Janus kinase 2; LDH, lactate dehydrogenase; mTOR, mammalian target of rapamycin; p38 MAPK, p38 mitogen-activated protein kinases; p70 S6K, Ribosomal protein S6 kinase; p-CA, p-coumaric acid; PGPC, *Pennisetum glaucum* phenolic compounds, PI3-K, phospho-inositide 3-kinase; RuR, ruthenium red; SAPK/JNK, stress activated protein kinase/c-Jun N-terminal kinases; STAT3, Signal transducer and activator of transcription 3; TRAF2, tumor necrosis factor (TNF)-Receptor Associated Factor; TRAIL, tumor-necrosis-factor related apoptosis inducing ligand; XBP1, X box-binding protein 1; z-VAD-fmk, N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

## 1. Introduction

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

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

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

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

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

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

## 2. Materials and methods

### 2.1. Materials

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

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
$\beta$ -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

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## 2.2. Preparation of polyphenol-rich extract

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

## 2.3. Cell culture and viability assay

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

## 2.4. LDH cytotoxicity assay

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



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

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

## **2.5. Apoptosis Assay**

**U-2OS** cells (2x10<sup>5</sup>) were seeded in a 6-well plate. After 24 h, cells were pre-incubated or not with 50 µM N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) for 1 h. and then treated for 24 h with PGPC (60 µg/mL) and p-CA (12 500 µM). Cells were harvested into 15 mL Falcon tubes and washed twice with cold PBS. Subsequently, cell death was determined by APC-Annexin V and 7-amino-actinomycin D (7-AAD) staining with BioLegend's APC-Annexin V Apoptosis Detection Kit according to the manufacture's recommendations and analyzed by flow cytometry, on FACSCanto™ (BD Biosciences), and FlowJo software version 10 (Tree Star).

## **2.6. Measurement of free intracellular Ca<sup>2+</sup> concentrations; [Ca<sup>2+</sup>]<sub>i</sub>**

**U-2OS** cells were cultured on WillCo-dish wells with a glass bottom and loaded with Fura-2/AM (1 µM) for 60 minutes at 37°C in loading buffer that contained: 110 mM NaCl; 5.4 mM KCl; 25 mM NaHCO<sub>3</sub>; 0.8 mM MgCl<sub>2</sub>; 0.4 mM KH<sub>2</sub>PO<sub>4</sub>; 20 mM HEPES-Na; 0.33 mM Na<sub>2</sub>HPO<sub>4</sub>; 1.2 mM CaCl<sub>2</sub>, pH 7.4 as described by Dramane *et al.* (Dramane et al., 2012). The

changes in intracellular  $\text{Ca}^{2+}$  ( $\text{F}_{340}/\text{F}_{380}$ ) were monitored under a Nikon microscope (TiU) by using an S Fluor 40× oil immersion objective. The planes were taken at z intervals of 0.3  $\mu\text{m}$ , and NIS-Elements software was used to deconvolve the images. The microscope was equipped with an EM-CCD (Lucas) camera for real-time recording of 16-bit digital images. The dual excitation fluorescence imaging system was used for studies of individual cells. The changes in intracellular  $\text{Ca}^{2+}$  were expressed as  $\Delta$  ratio, which was calculated as the difference relative to the peak  $\text{F}_{340}/\text{F}_{380}$  ratio. The data were summarized from the large number of individual cells (20–40 cells in a single run, with 3–9 identical experiments done in at least 3 cell preparations). For experiments conducted in the absence of external calcium (0 %  $\text{Ca}^{2+}$ ),  $\text{CaCl}_2$  was replaced by 1 mM EGTA in the buffer. All test molecules were added in small volumes with no interruption in recordings.

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

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

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

Total RNA was prepared using trizol reagent (Invitrogen Life Technologies). 1  $\mu\text{g}$  of total RNA was reverse transcribed with superscript II RNase H-reverse transcriptase using oligo (dT) according to the manufacturer's instructions (Invitrogen Life Technologies, France).

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

## **2.9. Western Blot Analysis**

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

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

## **2.10. Statistical Analysis**

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

## **3. Results**

### **3.1. Cell Viability**

Trypan blue exclusion test showed that PGPC and p-CA significantly reduced U-2OS cell viability in a dose dependent-manner (Fig.1). IC<sub>50</sub> was estimated to be 80  $\mu$ g/mL for PGPC and around 17000  $\mu$ M for p-CA. Moreover, p-CA at 25000  $\mu$ M induced more than 80% of U-2OS cell death. However, 100  $\mu$ g/mL concentration of PGPC was required to achieve 75% of U-2OS cell death. The lactate dehydrogenase (LDH) assessment demonstrated that both p-Coumaric acid and PGPC induced U-2OS cell death in a dose-dependent manner as these treatments increased the membrane permeability, evidenced by high LDH levels, when compared with untreated cells (insert). We therefore chose less toxic doses of PGPC (*i.e.* 60  $\mu$ g/mL and 75 $\mu$ g/mL) and p-CA (12500  $\mu$ M) for all the subsequent experiments.

### 3.2. Apoptosis Assay

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

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

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

Since the inhibition of mitochondrial Ca<sup>2+</sup> uniporter exacerbated PGPC-induced cell death, in the subsequent experiment, cells, pre-incubated with RuR, were co-treated with z-VAD-fmk or with autophagy inhibitors, 3-Methyladenine (3-MA) and chloroquine (CQ).

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

### 3.3. Detection of Apoptosis-Related Proteins

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

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

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

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

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

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

### 3.6. Cell cycle distribution analysis

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

### 3.7. Cyclins and CDKs expression analysis

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

## 4. Discussion

Dietary polyphenols-rich food intake has been correlated with low cancer incidences. Moreover, several phenolic compounds have been proposed as potential anticancer agents alone or as adjuvants with classical chemotherapy (León-González, Auger, & Schini-Kerth, 2015). Dietary polyphenols exert their anti-carcinogenic activity by several mechanisms. Furthermore, depending on tissue or cell type, the effects of polyphenols could differ on 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 IC<sub>50</sub> 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



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

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

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

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

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

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

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

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

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

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

## **5. Conclusion**

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

## **Declare of interest**

The authors have declared no conflicts of interest.

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## Legends of figures

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

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

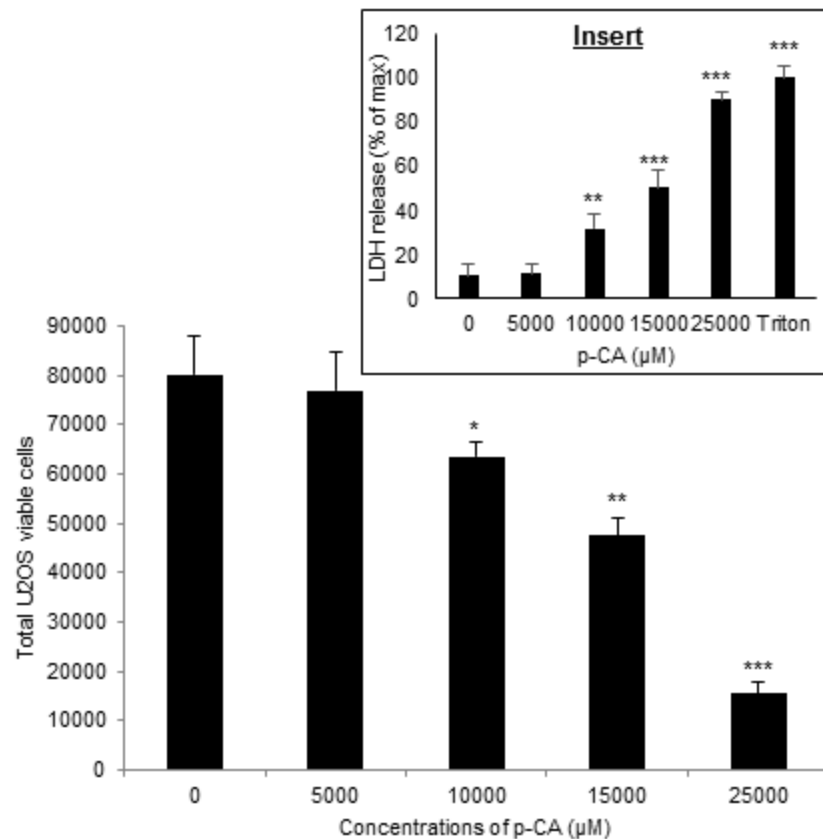
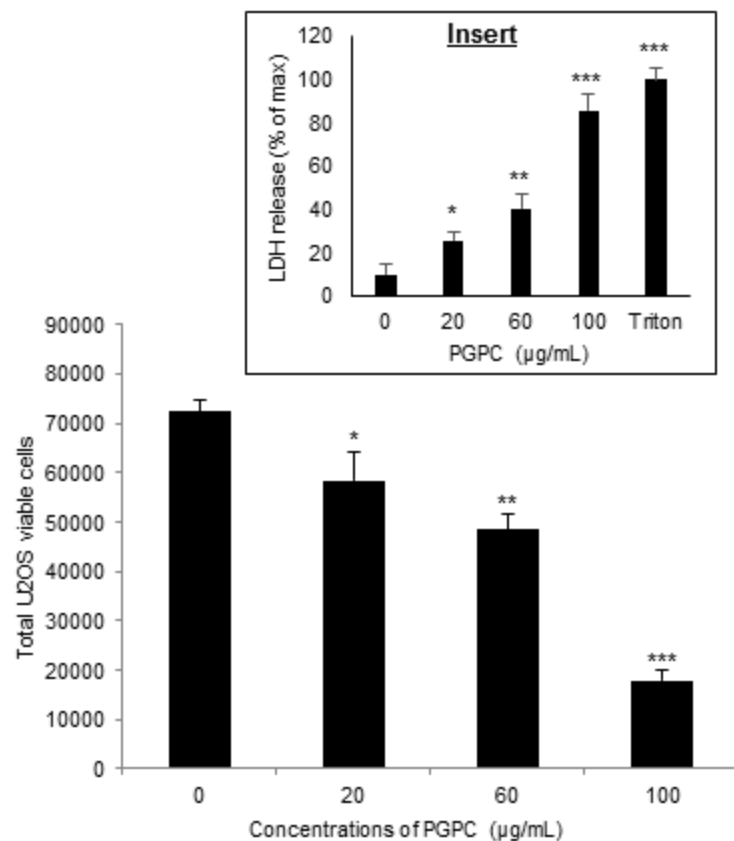
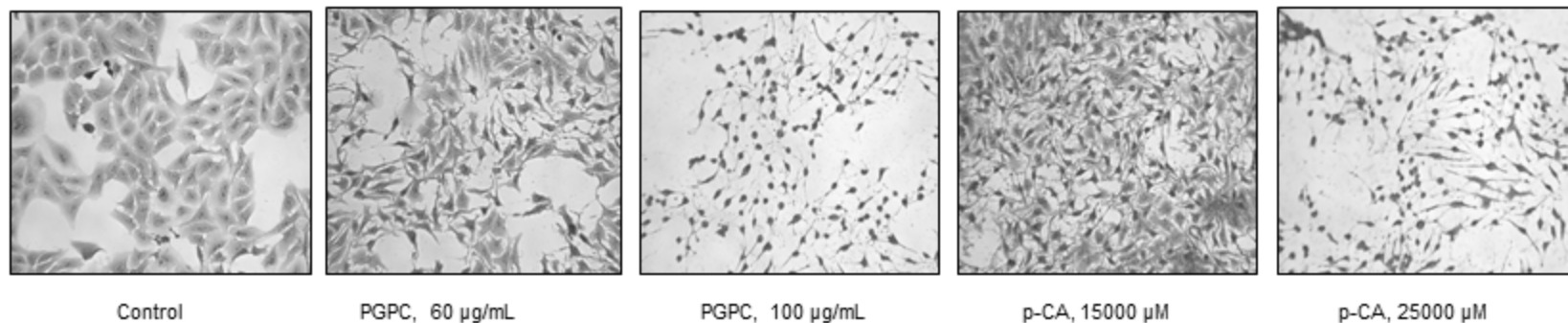
**Fig. 3.** Effects of PGPC on calcium-mediated apoptosis in U-2OS cells. (A) percentage of apoptotic cells (Annexin V positive) treated with PGPC (60  $\mu\text{g/mL}$ ) in the presence of one or combination of the following molecules: BAPTA-AM (5  $\mu\text{M}$ ), RuR (5  $\mu\text{M}$ ), zVAD (50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

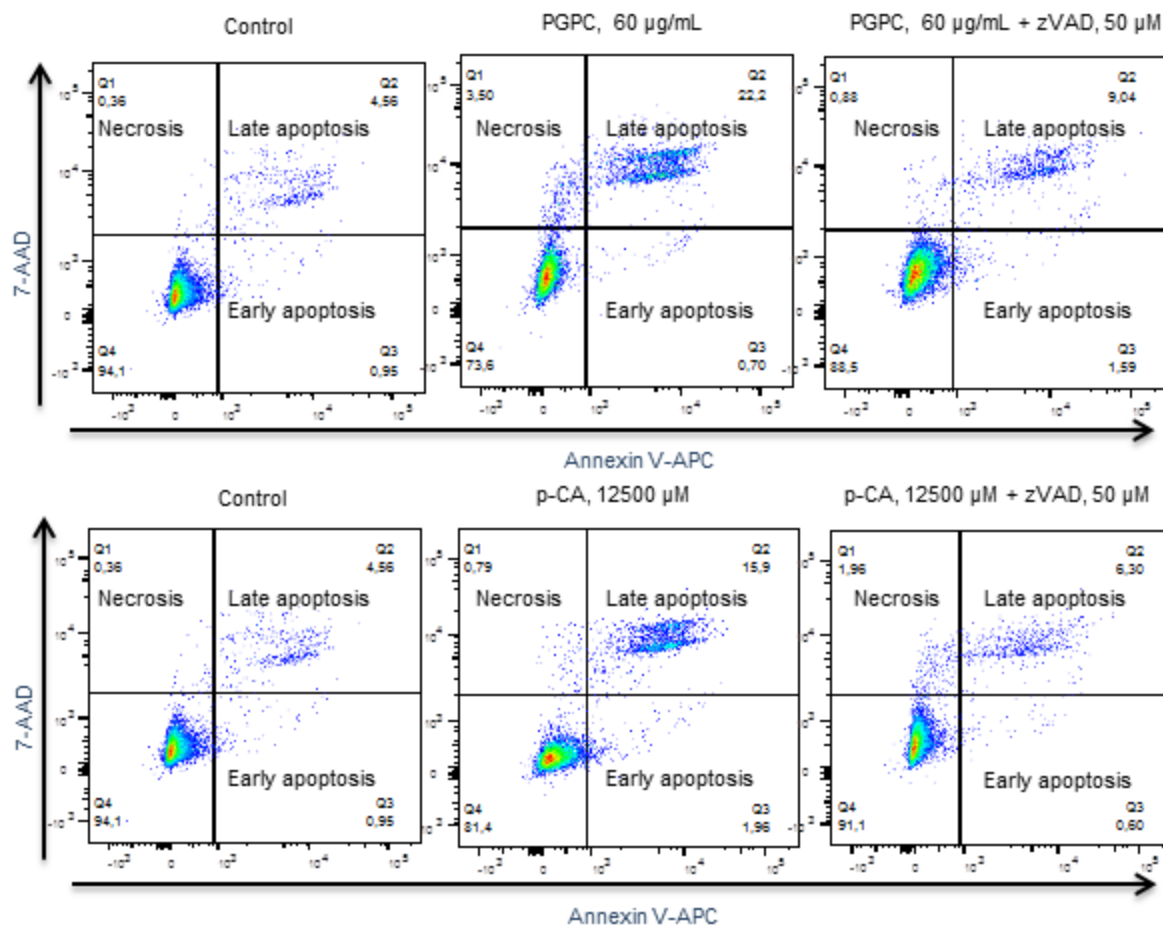
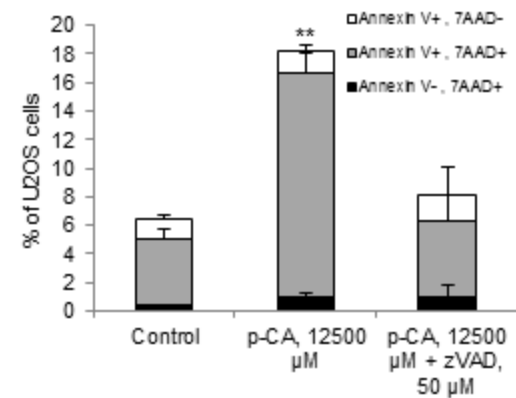
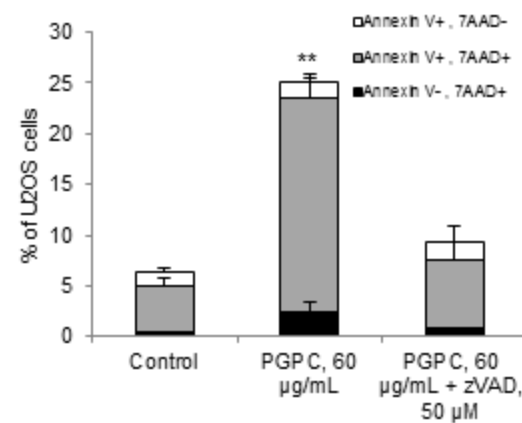
**Fig. 4.** Effect of PGPC on apoptosis-related proteins. “A” represents up-regulation of p-53, caspase-8 and -9 and PARP activity in PGPC-treated U-2OS cells for 24 h, as assessed by western blotting analysis.  $\beta$ -actin was used as the internal protein control. “B” represents ER-mediated apoptosis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

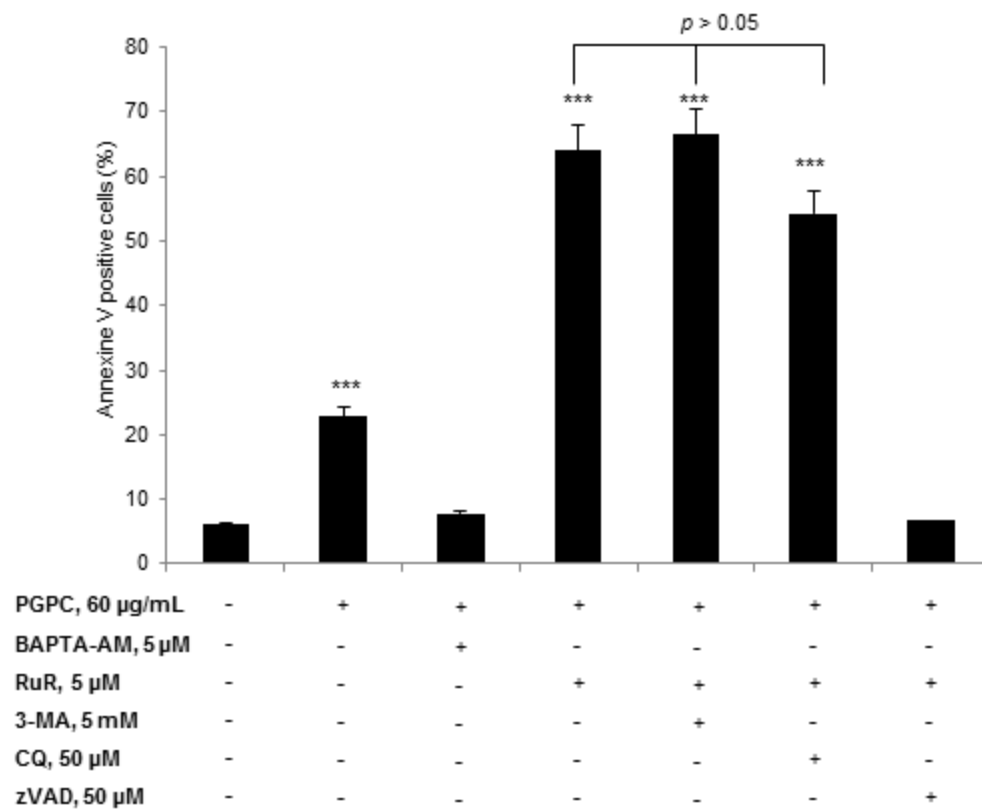
**Fig. 5.**  $\text{Ca}^{2+}$  signaling modulation by PGPC in U-2OS cells. The colored time-lapse changes in the increases in  $[\text{Ca}^{2+}]_i$  were recorded in PGPC-treated U-2OS cells in 100 %  $\text{Ca}^{2+}$ -buffer (A) or 0 %  $\text{Ca}^{2+}$ -buffer (B). Figure C shows the single traces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

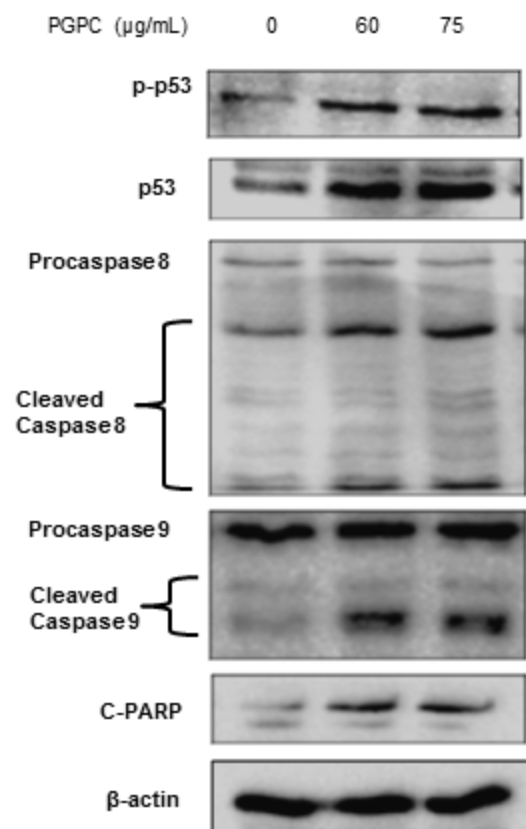
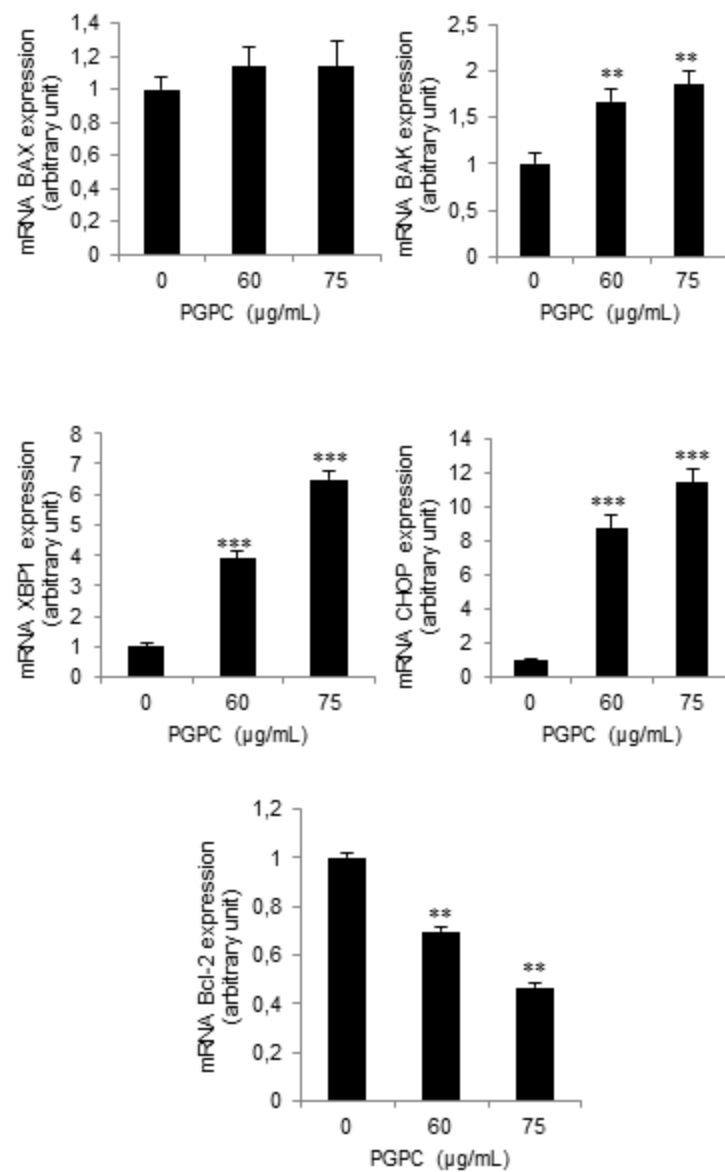
**Fig. 6.** Effect of PGPC on ATK (A) and MAPKs pathway (B) in U-2OS cells, as assessed by western blotting.  $\beta$ -actin was used as the internal protein control.

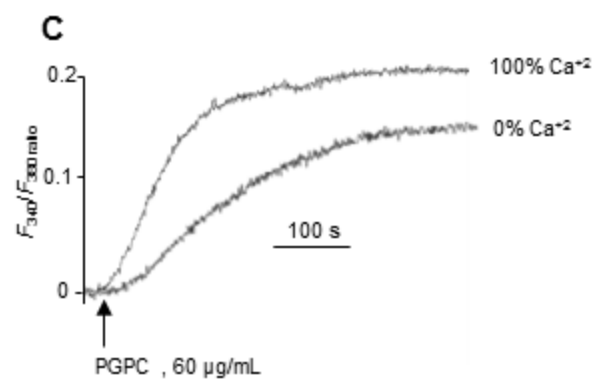
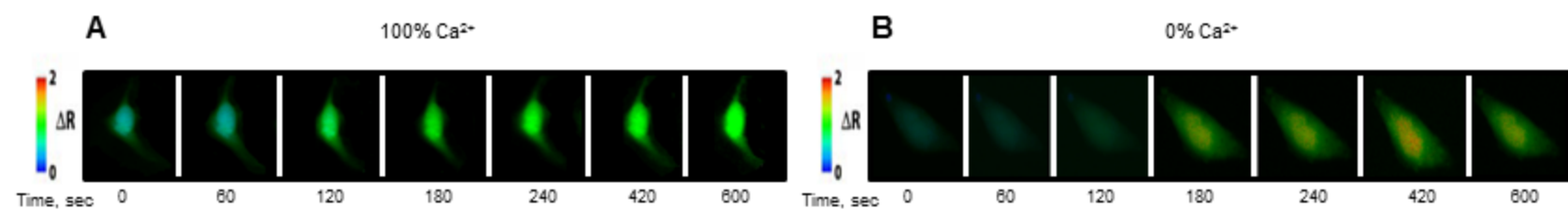
**Fig. 7.** Effect of PGPC on cell cycle progression. (A) Cell cycle representative profile of U-2OS in untreated or treated cells with PGPC (60  $\mu\text{g}/\text{mL}$ ) or p-CA (12500  $\mu\text{M}$ ) for 24 h then labeled with PI and analyzed by FACSCanto. (B) Statistical analysis of G1, S and G2/M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

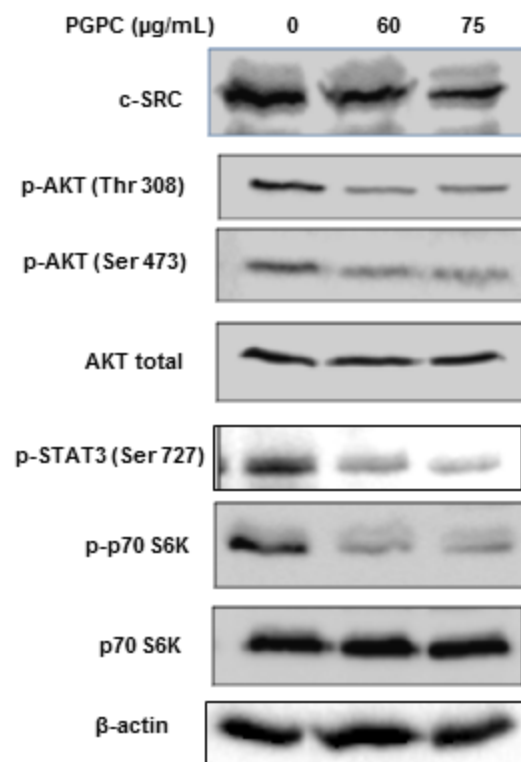
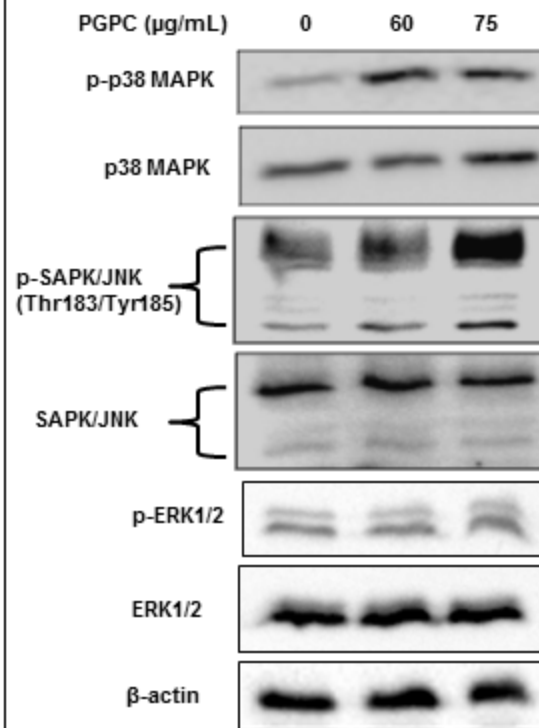
**A****B****U2OS cells**

**A****B**

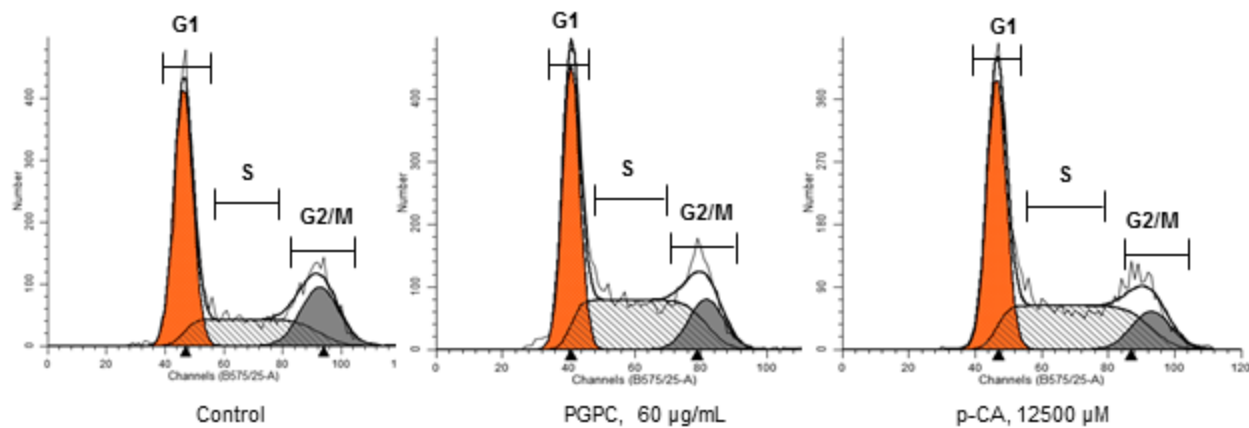
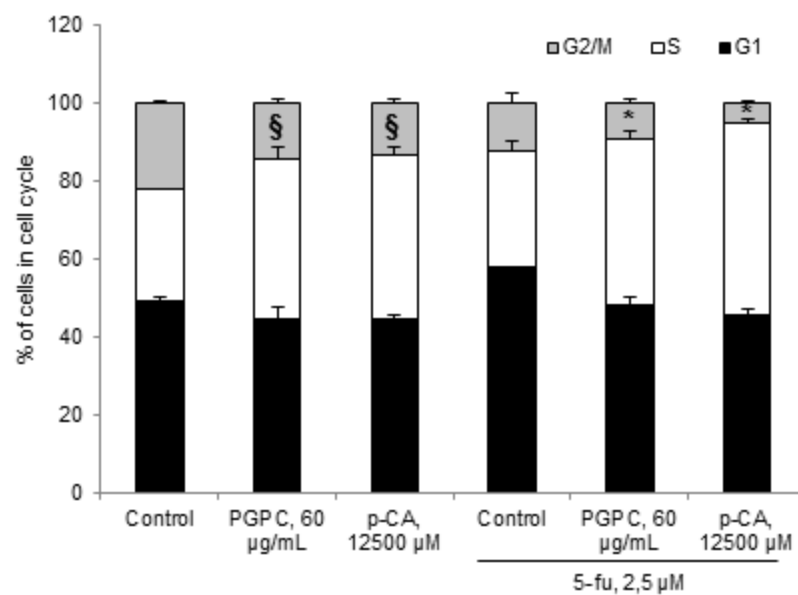


**A****B**



**A****B**



**A****B****C**