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Punicalin and ellagic acid from pomegranate peel extract facilitate apoptotic behaviour in the Hela cell line

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Abstract: Polyphenols may be an effective therapy for both the prevention and treatment of cancer. Previous studies have found that these compounds may inactive Hela cells, which may even be converted into a normal cells post-treatment. The present study extracted phenolic compounds from pomegranate peel, with the polyphenols then purified using different solvents and identified by means of high-performance liquid chromatography-tandem mass spectrometry (HPLC/MS). Once the phenolic compounds had been purified, we evaluated their cytotoxic effects on both the Hela and NIH-3T3 cell lines, on which an apoptosis assay was also carried out. Additionally, apoptosis assay was carried out on Hela and NIH-3T3. Lastly, the proteome profile was analysed via two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS). We isolated and then purified punicalagin and ellagic acid (EA) from pomegranate peel, with both compounds likely to have a cytotoxic effect on Hela and NIH-3T3. However, this effect depends on both concentration and exposure time. Results obtained using a Cayman commercial assay kit suggests that punicalin and EA regulate the apoptosis on the Hela and NIH-3T3 cell lines. Finally, we observed that polyphenols compounds regulate the expression of proteins related to apoptosis. In conclusion, punicalin and EA have a cytotoxic effect on Hela and, furthermore, reactive the apoptotic pathway in this cell.

Keywords: Polyphenols, punicalagin, cytotoxicity, apoptosis, proteomic analysis.

INTRODUCTION

Cancer remains a significant global public health problem in diverse countries (Duan *et al*., 2018). The development of cancer is associated with longevity, environmental factors, and lifestyles (Adhami *et al*., 2009), risk factors may cause aberrations in the cell signaling essentials for the typical development of the biological processes, thus causing cancer (Hejazi *et al*., 2018). Specifically, cervical cancer is the third or fourth most frequently diagnosed cancer in younger women and is the principal cause of cancer death (Abou-Hashem *et al*., 2019, Yang *et al*., 2018). Hispanic and Mexican women show higher cervical cancer incidence and mortality (Morales-Campos *et al*., 2018). Some of the aberrant signaling involved in the generation of cancer cells is found in the redox system, the Wnt/beta-catenin (Wingless/Integration) signaling pathway, and the apoptotic process. Currently, the cancer treatment is based on antineoplastic medication, antitumor antibiotics, natural products, and hormones (lmran Masood, 2016). However, cervical cancer (CC) treatments present low levels of efficiency and specificity, adverse consequences, great expense, patient´s relapse, and drug resistance (Tan *et al*., 2018, Susianti Susianti, 2018, Sanchez-Carranza *et al*., 2018).

Plants, fruits, and vegetables can contain pharmacologically active compounds or phytochemicals (Sanchez-Carranza *et al*., 2018, Susianti Susianti, 2018, Tan *et al*., 2018, Rached *et al*., 2018, Khwairakpam *et al*., 2018, Kumar N, 2018). Khwairakpam *et al*. (2018) show that pomegranate peel is rich in punicalagin, gallic acid (GA), EA and kaempferol (Khwairakpam *et al*., 2018, Kumar N, 2018), while Kumar *et al*. (2018) found that the peel is the part of pomegranate with the highest level of antioxidant activity (Rajagopal *et al*., 2018, Saeed *et al*., 2018). The high level of bioactive compounds in the pomegranate (*Punica granatum*) have different biological activities, including triggering apoptosis (Losada-Echeberria *et al*., 2017, Khwairakpam *et al*., 2018). Garcia Becerra *et al*. (2012) reported that pomegranate extracts exhibited cytotoxic effects on Hela cells (García-Becerra, 2012). Les *et al*. (2015) showed the dose-dependent antiproliferative effects on the Hela cell line

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(Les *et al*., 2015), while Gou *et al*. (2016) inhibited CC by promoting the expression of the IGFBP7 protein using EA taken from pomegranate (Guo *et al*., 2016). However, there are also report of crude aqueous pomegranate peel extracts displaying a lower level of cytotoxicity against the Hela cells (Zaynab, 2010); moreover, Pedriali *et al*. (2010) found that pomegranate seeds extracts present a null cytotoxic effect on Hela cells (Pedriali, 2010).

As described above, polyphenols are known to regulate different pathways, suggesting that polyphenols protect Hela cells against reactive oxygen species (ROS) and induce apoptosis via oxidative stress (Biswal *et al*., 2017, You *et al*., 2010, Rached *et al*., 2018). However, polyphenols increase pro-oxidant effects depending on the presence of iron or H_2O_2 in the medium (You *et al*., 2010). Current researches report the down-regulation of inflammatory markers, such as heat shock proteins 70 and 90 (HSP 70-90), the nuclear factor kappa beta (NF-κβ), and the Phosphatidylinositol-3-kinase/Protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway (Khwairakpam *et al*., 2018, Danciu *et al*., 2018, Guo *et al*., 2016, Singh *et al*., 2018). Specifically, punicalin inhibits cell proliferation, induces cell arrest at the G1/S phase, and triggers apoptosis via the Bax over-expressed and the Bcl-2 inhibition (Guo *et al*., 2016, Khwairakpam *et al*., 2018). The aim of the present study was to evaluate the cytotoxic effect and apoptotic activity on Hela and NIH-3T3 cells exposed to one pomegranate extract. Phenolic compounds of pomegranate peel were purified and characterized by means of the HPLC/MS method. The cytotoxicity in Hela and NIH-3T3 as promoted by polyphenols was subsequently measured, with apoptosis assays conducted on the cell lines and the proteome profile of Hela and NIH-3T3 analyzed via 2-DE and LC/MS/MS. Our analyses found that punicalin and EA are present as a significant component in the pomegranate extract. The cytotoxic effect of polyphenols on Hela and NIH-3T3 was concentration and time-dependent. Punicalin and EA were found to regulate apoptosis in Hela and NIH-3T3 cell lines with the protein profile both up- and down-regulating proteins associated with apoptosis and metastasis.

MATERIALS AND METHODS

Plant material and preparation

Red pomegranate peels (*Punica granatum*) were used for the extraction of polyphenols, wherein it was dehydrated at 60ºC and subsequently pulverized in a commercial grinder (Wang Z, 2011). The powder recovered was screened to ensure distinct particle sizes (whole powder, 149, 425 and 841μm).

Polyphenols extraction and quantification

100 g powder (whole powder, 149, 425 and 841μm) per 500mL water was used for the polyphenol extraction. The blind was heated at 60ºC for 30 min with manual agitation, after which the supernatants were filtered and centrifuged at 800g for 15 min. The extracts obtained were preserved at -4ºC (Ascacio-Valdés *et al*., 2010), with the hydrolyzed tannins quantified via the Folin-Ciocalteu method and the condensed tannins quantified via the HCl-Butanol method.

Separation of extracts and polyphenols tracking

The aqueous pomegranate extracts were fractionated by dispersion, using hexane (Hx) and ethyl acetate (EtAcO) in a stirring system at room temperature. Separation was performed in a separatory funnel containing a first layer of Hx, while the organic phase was recovered in a rotary evaporator. Phenolic hydroxyl test was used to track polyphenols, 50μL of the fractions obtained were introduced to 50μL of FeCl3; with a blue-to-black coloration indicating the presence of polyphenols in the sample. GA was used as positive control and ethanol as a negative control.

Chromatographic separation in an Amberlite XAD-16 column

The aqueous extract of pomegranate peels was purified using the Amberlite XAD-16 resin (Ascacio-Valdés *et al*., 2010), with the polyphenols recovered using a stove at 60ºC for 24h and were stored in amber containers.

Identification of polyphenol by Reverse Phase High-Performance Liquid Chromatography (RP/HPLC/ESI/MS)

The analysis required for polyphenol identification was conducted, as indicated by Ascacio *et al*. (2010). Further analysis was performed to identify the polyphenols, following the methodology proposed by (Hernández-Hernández *et al*., 2019, Ascacio-Valdés *et al*., 2010).

Polyphenols solution preparation

A solution of pomegranate polyphenols (PGP) was prepared at 2,000 ppm as an initial concentration. First, powder polyphenols were diluted in 1mL of absolute ethanol and diluted in DMEM medium, the solutions were filtered and sterilized using a 0.45 μm sterile Millipore filter (Ascacio-Valdés *et al*., 2010).

Cell culture

NIH/3T3 and Hela cells were kindly donated by School of Medicine of the Autonomous University of Nuevo Leon, Medicine School. These cell lines were kept at 37°C in a 5% CO_2 atmosphere (Govea-Salas *et al*., 2016).

MTT viability assay

The Hela and NIH-3T3 cells were cultured in accordance with methods reported in previous studies (Govea-Salas *et al*., 2016, Pedriali, 2010). A 96 well plate was prepared with 10^4 cell/well at a final volume of 100μL and were incubated for 24h at 37ºC and 5% CO_2. After this

incubation period, the cells were exposed to PGPs at different concentrations (100, 500, and 1000 ppm) for 24, 48 and 72h in DMEM medium at a final volume of 200µL. Once the exposed cells had been recovered, 200µL of 0.83mg/mL MTT was added. After four hours, the MTT reagent was replaced by 200µL of DMSO and incubated for 25min to solubilize the formazan crystals formed. Absorbance was measured at 590nm and the viability percentage for each treatment was calculated. The cytotoxic effect of the treatments was expressed in lethal concentration (LC_{50}). The assay was performed in triplicate, with untreated cells used as a negative control and 5% DMSO treatment as a positive control (Govea-Salas *et al*., 2016, Pedriali, 2010). T student analysis was applied to determine the statistically significant differences.

Apoptotic cell death assay

A total of $5x10^4$ Hela cells were incubated in Lab-Tek™ Chambered Coverglass (Thermo Scientific) and were treated with PGP at different concentrations (380, 500, and 662) for 48h. The protocol of the Cayman Apoptotic Blebs Assay Kit® was then followed, where, according to said specifications, dead cells were colored red, cells that had died due to apoptosis were colored orange and the apoptotic blebs were colored green. The apoptosis assay results showed that the nuclei of untreated cells were colored blue via the addition of DAPI (4',6-diamidino-2-phenylindole) (4a). The dead cells were colored red (4b) via the application of DMSO, cells that had died due to apoptotic damage were colored orange (4c).

Protein analysis

Protein extraction

Hela cells ($5x10^5$ cells/ well) were incubated in six-well plates and treated with PGP at concentrations of 380 and 662 ppm for 48h. Treated cells were scraped from the plate and placed in Eppendorf tubes and centrifuged at 600g for 5min, with the supernatant then removed. The protein pellet was dissolved in 50µL of RIPA lysis buffer and frozen at -80ºC until use. The cells were thawed and centrifuged at 10,000 rpm for 5 min at 4ºC the supernatant was recovered, and the protein concentration was quantified.

Protein quantification

This was by means of the Bradford reaction and the Pierce BCA protein assay kit®.

SDS-PAGE Protein Electrophoresis

Sample preparation

Protein samples were dialyzed using a prewashed cellulose membrane stirred overnight at 4ºC, with the sample then mixed with three equal parts of absolute ethanol and left overnight at -20ºC for precipitation. These samples were centrifuged at 13,000g for 20 min at 4ºC and the supernatant was discarded. The protein pellet was dissolved in 50µL Milli-Q water and heated (if required), while stirred at 50ºC.

Electrophoresis

The BIO-RAD Mini-protean electrophoresis kit® was used for this analysis, wherein 1D electrophoresis used a protein concentration of 100µg/mL, while the cellular proteins were separated according to molecular weight, in a range of 14 to 212 kDa at 80-100 volts for 1.5-2h. The 2D electrophoresis conducted used 200µg/mL of protein. This process comprised three steps: firstly, BIO-RAD strips were rehydrated with protein samples for 12 h; after this, the isoelectric focusing was performed at 12, 000 volts and 50ºC for 9h to separate the proteins based on isoelectric points in a pH range of 3-10 and finally the electrophoresis process described above was then carried out. In both 1D and 2D electrophoresis processes, the gels obtained were stained with Coomassie blue to visualize the proteins of interest. The resolution of the spots was improved with a second stained using a silver solution, with the spots then cut out, still encased in the gel, and digested with trypsin for further sequencing analysis and identification (Govea-Salas *et al*., 2016, Andrej Shevchenko, 1996).

ARN extraction and RT-qPCR condition

The RNA extraction of Hela cancer cells and 3T3 cells was carried out as indicated Govea-Salas *et al*. (2016). The RT-qPCR also was carried out as indicated Govea-Salas *et al*., 2016 and Abou-Hasem *et al*., 2019. 200ng of cDNA was amplified by qPCR to quantify the levels of Caspase-3 and Bcl-2 gene. GADPH was used as an internal control. Table 1 mentions the list of primers employed. The PCR consisted of initial denaturation at 95°C for 2 min, followed by 40 reaction cycles (15 s at 95°C, 60 s at 60°C and 60s at 72°C). The relative quantification of target gene expression, normalized to an endogenous control and relative to a calibrator, was expressed as $2^{-\Delta\Delta ct}$ (fold) (Abou-Hashem *et al*., 2019, Govea-Salas *et al*., 2016).

RESULTS

Quantification and identification of polyphenols

The present study obtained a low yield in terms of total polyphenols using both full and 149µm particle sizes, and using an 841 µm particle size, obtained a similar yield to that obtained with a 425µm particle size. The polyphenols present in the powder prepared using the 425µm pomegranate particle size was both condensed (CE) and hydrolyzable (GAE) tannins, with, specifically, 34 mg/g (6.8 gL-1) of GAE obtained from the first extraction (table 2). Table 2 also shows increased PGP levels post-purification, GAE = 532.98 mgg-1 and CE = 471.81 mgg-1. The RP/HPLC/ESI/MS analysis revealed that punicalin, punicalagin and EA and GA are the main compounds of aqueous pomegranate extract (table 3).

Cell viability assay

Our results show that cell viability in NIH-3T3 and Hela cells exposed to PGP decreased. Similar cell death was found for both NIH-3T3 and Hela cells treated with 500 ppm, with no statistical difference. The results obtained with an adjusted PGP concentration revealed that, at 48 h, the LC50 required for NIH-3T3 cells was 750-1000 mg/L of PGP (fig. 1), while the LC50 required for the Hela cells was 375 mg/L of the same compound (fig. 2). Both assays demonstrated that cell death increases in function of concentration and exposure time (fig. 1). The value calculated via the T student test was 0.022.

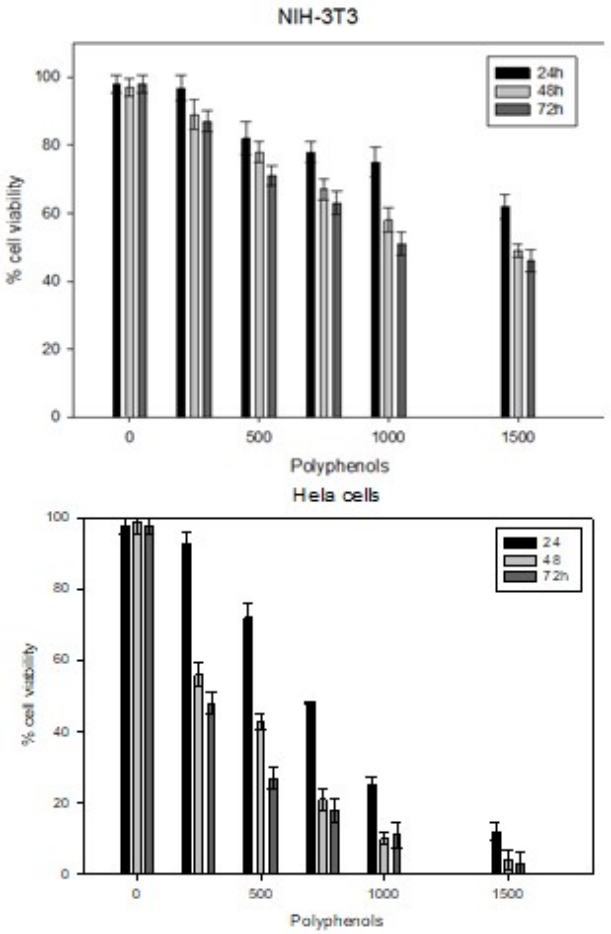

Fig. 1: LC_{50}. NIH-3T3 and Hela cells increase cell death as a function of concentration and exposure time. Statistical analysis showed an $\alpha<0.05$.

Apoptotic cell death assay

To confirm our previous results, we analyzed the apoptotic state of NIH-3T3, and Hela cells treated with different polyphenols, with cell death by apoptosis indicated by the orange-colored nuclei observed in both cell lines treated with PGP (fig. 3). However, the Hela cells presented a higher death rate due to apoptosis than the NIH-3T3 cells. Cellular damage increased in a dose-dependent manner in the treated cells, as was expected given the previous results of the cytotoxicity assay.

Expression of apoptotic genes

To confirm the apoptosis mediated by bioactive compounds, we analyzed caspase 3 and Bcl-2 expression in Hela and NIH-3T3. The results demonstrated in the treated cancer cells that ellagitannin activated caspase 3 and inhibited Bcl-2 in a dose-dependent manner. Caspase 3 increased four times its expression concerning the GADPH. In NIH-3T3 treated cells, caspase 3 and Bcl-2 expression level was similar and non-significant difference was remarkable (fig. 4).

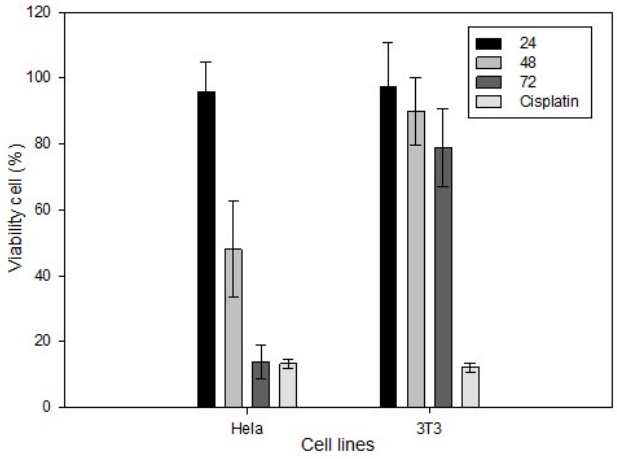

Fig. 2: Viability of Hela and NIH-3T3 cell lines. The Hela and NIH-3T3 were exposed to 375 ppm of polyphenols. The Hela cells received major damage to the 72h, however, the selected treatment of 375 ppm x 48h, because at this point, it was reached the LC_{50}. Statistical analysis showed an $\alpha < 0.05$.

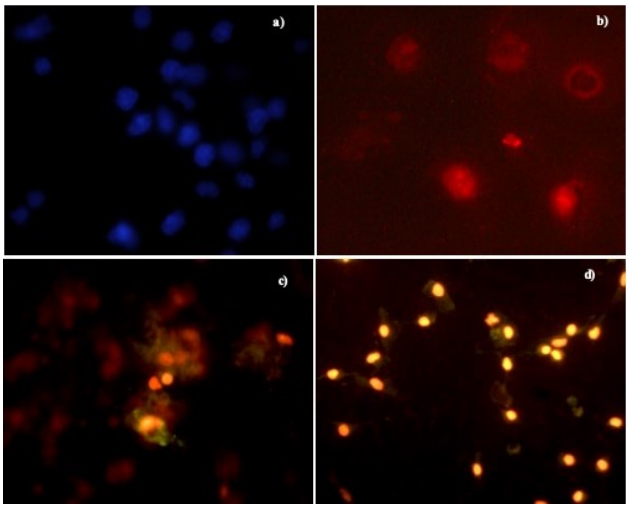

Fig. 3: Apoptotic cell death assay. (A): negative control, untreated cells; (B): positive control, cell death with 5% DMSO; (C): NIH-3T3 PG 661.41 ppm treatment; (D): Hela PN 375±0.26 ppm treatment.

Protein analysis. 2D-SDS PAGE electrophoresis

The last experiment conducted in the present research revealed a different level of protein expression in the

proteome of the treated cell (fig. 5). The proteins CDC42, E3 ubiquitin, Bcl-2, and JK were up-regulated in the untreated cell; however, these same proteins were down-regulated in the cell treated with PGP (table 4). This result was consistent with the apoptotic cell death assay, although it was observed that none of the proteins related to cell growth, proliferation, tumor growth and metastasis were overexpressed in the treated cells. These results were confirmed via amino acid sequences obtained by means of LC/MS/MS.

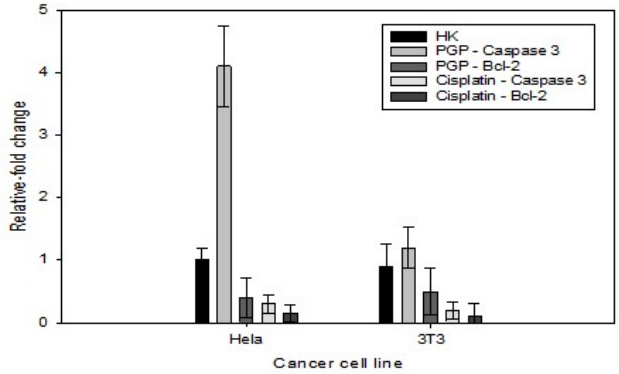

Fig. 4: Expression of apoptotic genes. Our results showed that caspase 3 increased the expression after treatment with ellagitannin compounds. Also, caspase 3 was four times more expressed than control GADPH (α = <0.05). In contrast, ellagitannin inhibited Bcl-2 expression.

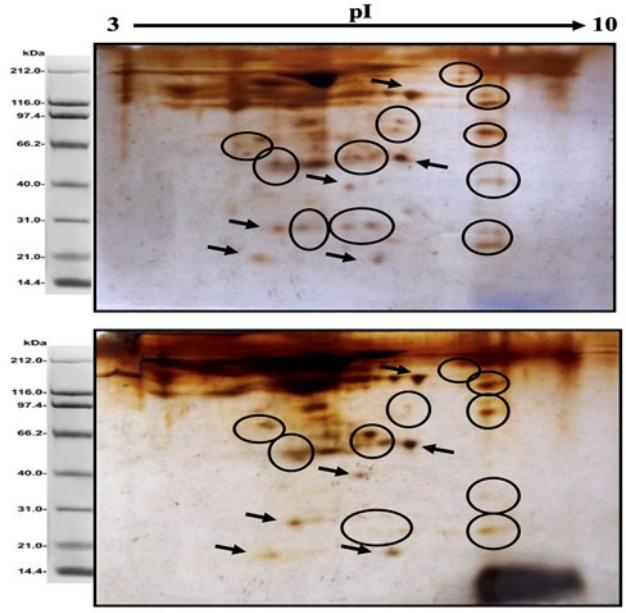

Fig. 5: Two-dimensional electrophoresis of proteins extracted from Hela. Hela cells without treatment are presented above. Presented below are the Hela cells treated with 360 ppm of PGP at 48 h. The arrow represents similar proteins, and the circles represent up-regulated and down-regulated proteins.

DISCUSSION

The present study found that particle size, time, and temperature influenced the polyphenols extraction. When the particle size is small, the diffusion distance between solute and solvent is shorter, increasing the concentration gradient and, thus, the extraction rate increased, too (Wang Z, 2011). Kossah *et al*. (2010) obtained high concentrations of phenolic compounds using small particle size and reducing the extraction time from 5h to 1h the extraction time (Kossah R, 2010). The present study identified the condensed and hydrolyzable tannins in both whole and aqueous pomegranate extracts, with the first extraction was obtained 34mg/g (6.8gL-1) of GAE. Les *et al* (2015) reported 25.6gL-1 of GAE, while Hadjadj *et al*. (2018) recovered between 158 to 170mgg-1 of GAE (Les *et al*., 2015, Hadjadj S, 2018). Differences in the product recovered depend on both extraction sources and conditions (Zarei *et al*., 2011, Wang Z, 2011), while soluble compounds such as sugar can interfere in initial quantification (Callemien D, 2006). After purification, an increase in the final GAE (532.98) and CE (471.81) concentrations were observed. Frejnagel (2017) reported obtaining phenol compounds for green tea (611mgg-1), honeysuckle (633mgg-1) and chokeberry (714 mgg-1) extracts after purification with Amberlite XAD-16 resin (S, 2017). Veljkovic *et al*. (2016) noticed that pomegranate contains 101 compounds, and 50% of them showed anti-cancer properties (Veljkovic V, 2016). Punicalin and EA were identified as principal components in the pomegranate samples. Several studies reported EA, punicalin and punicalagin as the main bioactive components in different pomegranate extracts (Singh *et al*., 2018, Veljkovic V, 2016, Les *et al*., 2015, Abid *et al*., 2017, Saeed *et al*., 2018). *Punica granatum* peel has more quantity of antioxidant compounds than other parts of pomegranate (Kumar N, 2018). In pomegranate peels, the main constituents are punicalagin and EA (Deng *et al*., 2017).

The toxic effect of PGP on HeLa and NIH-3T3 cell lines depends on both concentration and exposure time. Les *et al*. (2015) determined the LC50 at 0.5mgml^{-1} for 72h of exposure in Hela cells treated with pomegranate peels extract (Les *et al*., 2015). Da Silva Veloso *et al*. (2020), worked with two different varieties of pomegranate and they obtained an EC50 at 91µgml^{-1} for Mollar de Elche ethanolic extract and 153 µgml^{-1} for Purple Queen ethanolic extract (da Silva Veloso *et al*., 2020). Prior studies observed that cancer cells lines treated with pomegranate phenolic compounds (100-300µg/ml) by 48-72h increases the apoptosis via B-cell lymphoma-2 (Bcl-2) inhibition (Singh *et al*., 2018). On the other hand, our study detected an apoptotic effect on the Hela cell line via PGP treatment. Liu *et al*. (2019) reported the condensation and fragmentation of nuclei and formation of apoptotic bodies, in the Hela cell line treated

Table 1: List of primers. Set of primers related to the expression of apoptotic genes.

Gen	Sequence
Caspase-3	Forward: 5′-TTTGTTTGTGTGCTTCTGAGCC-3′
	Reverse: 5′-ATTCTGTTGCCACCTTTCGG-3′
Bcl-2	Forward: 5′-AGATGTCCAGCCAGCTGCACCTGAC-3′
	Reverse: 5′- AGATAGGCACCCAGGGTGATGCAAGCT-3′
GADPH	Forward 5'- GTGTTCCTACCCCCAATGTGT-3'
	Reverse 5'-ATTGTCATACCAGGAAATGAGCTT-3'
	5'-FAM-CTGCACGACACTCATAC-NFQ-3' (TaqMan)

Table 2: Total polyphenol contents (mgg^{-1}) of pomegranate extracts. *EP: Pomegranate Extract; EP-W: EP-Whole extract; EP-A: Final Aqueous extract; PGP: Pomegranate polyphenols; GAE: GA equivalents; CE: Catechin equivalents.

	EP-W	EP-A	PGP
Hydrolysable tannins (GAE)	34.0 ± 1.92	64.03 ± 0.31	532.98 ± 2.06
Condensed tannins (CE)	17.3 ± 2.03	11.89 ± 0.86	471.81 ± 18.18

Table 3: Identification of pomegranate polyphenols via RP/HPLC/ESI/MS.

RT (min)	Id	Compounds	[M-H](*m/z*)	MS^2(*m/z*)	Group
2.84	1	Punicalin a	781	601, 721	Ellagitannin
11.07	2	Punicalin B	781	601, 721	Ellagitannin
22.39	3	Punicalin a	1083	601, 781	Ellagitannin
24.58	4	Punicalin B	1083	601, 781	Ellagitannin
25.54	5	Ellagic acid derivative	799	271, 391, 451, 262	Ellagitannin
26.89	6	Pedunculagin I	783	479, 781, 301, 299	Ellagitannin
28.50	7	Galloyl-HHDP-hexoside	633	301.249, 302, 463	Ellagitannin
29.85	8	Ellagic acid hexoside	463	301, 300, 302	Ellagitannin
31.28	9	Granatin B	951	933, 934,915, 301	Ellagitannin
33.21	10	Ellagic acid	301	301, 229, 185	Ellagitannin

Table 4: Spots (peptides) identified via PDQuest and LC/MS/MS. ^represents PDQuest identified spots. -represents spots were identified via LC/MS/MS. *represents spots were identified by both, PDQuest and LC/MS/MS.

PDQuest		LC/MS/MS	
Code	Name	Code	Name
Q07912	*Activated CDC42 kinase 1 (ACK-1)	Q07912	*Tyrosine kinase non-receptor protein 2 (TNK2)
Q00987	*E3 ubiquitin-protein ligase (MSL-2)	Q00987	*Similar to E3 ubiquitin-protein ligase (MSL-2)
Q075474	*GSK-3-binding protein (FRAT-2)	Q92837	*Proto-oncogene FRAT Family
Q6R5N8	*Toll-like receptor 13 (TLR-13)	Q9Y2C9	*Toll-like receptor Family
Q96N16	*Janus kinase and microtubule-interacting protein 1 (JAKMIP-1)	P27448	*GABA-B receptor-binding protein Isoform 1 of MAP kinase 3 (MARK3)
Q9HD23	^Magnesium transporter MRS2 homolog, mitochondrial (MRS-2)	Q9H0T7 P80192 Q8N9B8	-Ras-related protein Rab-17 (RAB17) -Isoform 1 of Mitogen-activated protein kinase 9 (MAP3K9) -Isoform 2 of Ras-GEF (RASGEF1A)
Q14568	*Putative heat shock protein HSP 90	Q14568	*HSP90AA2 Putative heat shock protein HSP 90-alpha A2
P07237	*Protein disulfide- isomerase A4 (PDIA4)	P07237	*Protein disulfide-isomerase (P4HB)
		P78417 P13667 P04179 Q07817	-Glutathione S-transferase omega-1 (GSTO1) -Superoxide dismutase [Mn], mitochondrial (SOD2) -Cytochrome b-c1 complex subunit 2, mitochondrial - ICE-like apoptotic protease 6

with flavones (Liu *et al*., 2019). Also, we analyzed the expression of Bcl-2 and Caspase 3 in the same cancer cell line and NIH-3T3. It is still well-documented that, in Hela treated with active compounds, the pro-apoptotic genes increased their expression, while Bcl-2 is inhibited (Singh *et al*., 2018). Similar results had been observed in prostate cancer cell line (PC-3) and Breast cancer cell line (MCF-7) treated with polyphenolic compounds (Deng *et al*., 2017, Singh *et al*., 2018). Previously, Guo *et al*. (2016) indicated the down-expression of Akt/mTOR in Hela cancer cell line treated with EA (Guo *et al*., 2016).

The down-regulated proteins identified in the present study were Cdc42-associated kinase 1 (ACK-1), Bcl-2. Frequently rearranged in advanced T-cell lymphomas (Frat-1), Janus Kinase and microtubule interacting protein 1 (JAKMIP1) and MSL-2. Also, all of them are involved in cell growth and proliferation, and differentiation, as well as apoptosis (table 4). ACK-1 is over-expressed in different types of cancers, including ovarian cancer (Zhao *et al*., 2018). Bcl-2 controls the mitochondrial suicide program apoptosis negatively (Edlich 2018; Timucin *et al*., 2019; Hejazi *et al*., 2018) reported the downregulation of Bcl-2 in HepG2, Hela and MCF-7 cells treated with different fractions of *Curculigo orchioides* (Hejazi *et al*., 2018). Frat is a regulator of Wnt/β-catenin; however, the Frat-1 overexpression is associated with different human cancers (van der Wal T, 2020). Previous studies demonstrated mutations on JAKMIP1, and its up-expression enhances the β-catenin accumulation in Hela cells and endometrial cancer patients (Okai *et al*., 2013, Chang *et al*., 2017). Finally, E3-ligase (MSL-2), is associated with HPV-E6, and induces the nuclear export of p53 and its degradation, enabling viral replication in a host cell (Liu *et al*., 2020).

Other down-regulated proteins are related to the reactive oxygen/nitrogen species such as protein disulfide isomerase (PDI), Glutathione S-transferase omega-1 (GSTO1), and Superoxide dismutase [Mn], mitochondrial (MnSOD2). Also, Shen *et al*. (2010) identified the fusion of GST and ACK (Shen *et al*., 2011), while Zamani *et al*. (2018) found an association, in Iranian women, between the GSTO1 polymorphisms A140D and HPV serotypes 6, 16, 18 infections and CC (Zamani *et al*., 2018, Manupati *et al*., 2019). Soma *et al*. (2017) reported that the PDI family could be a risk factor in epithelial ovarian cancer (Soma Samanta, 2017). Although prior reports have established the potential of MnSOD such as an antioxidant (Zou *et al*., 2017), Hart *et al*. (2015) observed that increased MnSOD levels also increase oxidative damage, thus showing that MnSOD carries out pro-oxidant activity in human cancers (Ganini *et al*., 2015).

CONCLUSION

Punicalin and EA have properties anti-cancer, precisely, against Hela cancer cell line. These compounds can activate apoptosis such as main via to decrease cancer. Also, punicalin and EA inhibiting other signaling pathways, including Wnt/b-catenin and Akt/mTOR, or oxidative stress. We demonstrate, the regulation of the proteins that turn-on/off those pathways above describes. However, further studies about genes/proteins regulated by the active compounds in cancer cell lines are necessary.

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