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ARTICLE

Colon Cancer Chemopreventive Activities of Pomegranate Ellagitannins and Urolithins

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Pomegranate juice derived ellagitannins and their intestinal bacterial metabolites, urolithins, inhibited TCDD-induced CYP1-mediated EROD activity in vitro with IC_{50} values ranging from 56.7 μM for urolithin A to 74.8 μM for urolithin C. These compounds exhibited dose- and time-dependent decreases in cell proliferation and clonogenic efficiency of HT-29 cells. Inhibition of cell proliferation was mediated through cell cycle arrest in the G_0/G_1 and G_2/M stages of the cell cycle followed by induction of apoptosis. These results indicate that the ellagitannins and urolithins released in the colon upon consumption of pomegranate juice in considerable amounts could potentially curtail the risk of colon cancer development, by inhibiting cell proliferation and inducing apoptosis.

KEYWORDS: Ellagitannins; urolithins; pomegranate juice; colon cancer; chemoprevention

INTRODUCTION

Colon cancer is the most predominant digestive system related cancer. It is the fourth most common form of cancer occurring globally and is highest among all the cancer-related deaths in 2007 (*1*). Epidemiological evidence indicates that a diet rich in fat, meat, and fiber predisposes an individual to a risk of colon cancer (*2*−*4*). Related studies also signify that a diet rich in phytochemicals from fruit and vegetable sources reduces the risk of colon cancer (*5*, *6*). It was also reported that dietary supplements inhibit chemically induced colon carcinogenesis (*7*). The reports suggest that colon cancer is the most preventable form of cancer (*8*) and emphasize the importance of dietary modifications to reduce the risk of colon carcinogenesis. Polyphenols are the major non-nutrients in fruits, vegetables, nuts, and grains which elicit cancer chemopreventive properties (*9*). The principal mechanism by which they exert this activity is believed to be through a combination of their antioxidant, antiproliferative, and proapoptotic properties (*10*, *11*).

Pomegranate fruit is a rich source of polyphenols such as the flavonoid and gallo- and ellagitannin classes. Commercially available pomegranate juice, obtained by hydrostatic pressing of whole fruits, contains flavonols such as kaempferol and quercetin, flavones such as luteolin, anthocyanins such as cyanidin-3-glucoside, delphinidin-3-glucoside, pelargonidin-3,5-diglucoside, cyanidin 3,5-diglucoside, and delphinidin 3,5-diglucoside, and ellagitannins such as the punicalagins and punicalins, existing in solution as the α- and β-anomers and acyclic hydroxyaldehyde (*12*). The ellagitannins represent a significant portion of pomegranate juice polyphenols and coexist with ellagic acid, the major product of hydrolysis of this class of tannins (*13*, *14*). Pomegranate juice also contains variable amounts of gallic acid. A number of health-beneficial effects manifested by pomegranate juice consumption are attributed to the presence of ellagitannins. The ellagitannins are metabolized by intestinal bacteria into ellagic acid analogues called urolithins. The urolithins are systemically bioavailable and have been reported to accumulate in organs such as the colon, intestines, and prostate (*11*).

Chemical carcinogenesis induced by dietary carcinogens could also be modulated by modification of drug-metabolizing enzymes via inhibition of CYP1 enzymes and/or by inducing phase II conjugating enzymes (*15*). Pomegranate seed oil was reported to inhibit chemically induced colon carcinogenesis in rats (*7*). Ellagic acid inhibits CYP1 activation of procarcinogens and prevents cancer initiation (*16*). It is also known that ellagic acid induces phase II enzymes such as glutathione *S*-transferase (*17*). However, the ellagitannins and urolithins were not examined for their anticarcinogenic activity through inhibition of CYP1 and/or induction of phase II conjugating enzymes, a potential mechanism through which pomegranate juice consumption may prevent cancer formation.

The objective of this study was to investigate the colon cancer chemopreventive properties of relatively unexplored pomegranate ellagitannins and urolithins in HT-29 human colon cancer cells.

MATERIALS AND METHODS

Isolation and Identification of Pomegranate Juice Ellagitannins. Gallic acid, ellagic acid, gallagic acid, hexahydroxydiphenic acid, gallagyldilactone, and the punicalins and punicalagins were isolated as previously described (*13*, *18*). Their structures are shown in **Figure 1**.

Synthesis of Urolithins. Chemically stable synthetic urolithins were available from a previous study (*18*). The structures were confirmed by

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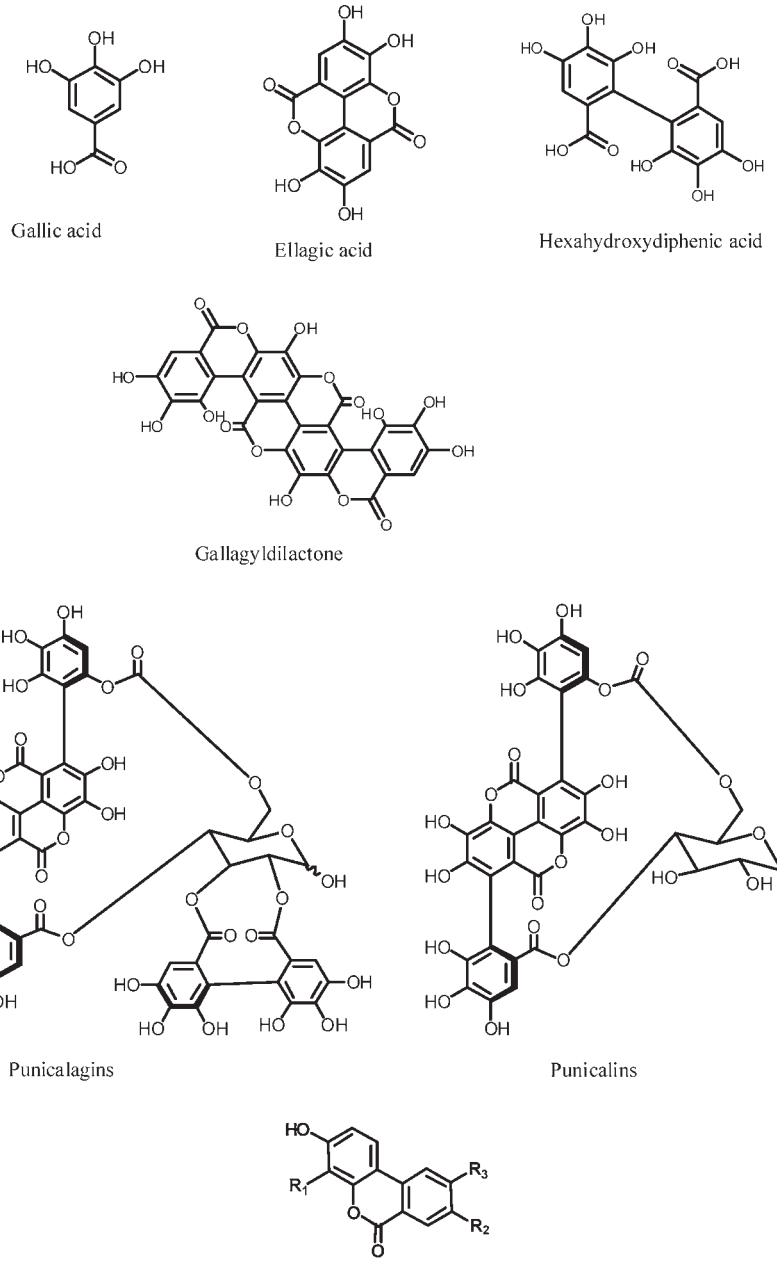

	R_1	R_2	R_3
Urolithin B (UB)	-H	-H	-H
Urolithin A (UA)	-H	-OH	-H
Urolithin C (UC)	-H	-OH	-OH
Urolithin D (UD)	-OH	-OH	-OH
8-*O*-Methylurolithin A (MUA)	-H	$-OCH_3$	-H
8,9-Di-*O*-methylurolithin C (DMUC)	-H	$-OCH_3$	$-OCH_3$
8,9-Di-*O*-methylurolithin D (DMUD)	-OH	$-OCH_3$	$-OCH_3$

Figure 1. Chemical structures of ellagitannins and urolithins.

their molecular mass and comparison of observed and reported ^{1}H NMR data (see **Figure 1** for structures).

Assay for Anticell Proliferative Activity. The pomegranate ellagitannins and their microbial metabolites were tested for their antiproliferative activity against a panel of cell lines that includes the five human cancerous cell lines BT-549 (ductal carcinoma, breast), HT-29 (colon carcinoma), KB (epidermal carcinoma, oral), SK-MEL (malignant melanoma), and SK-OV-3 (ovarian carcinoma) and the two noncancerous cell lines LLC-PK_{11} (porcine kidney proximal tubule) and Vero (African green monkey kidney fibroblasts). All cell lines were from the American Type Culture Collection (Manassas, VA). The assay was performed in 96-well microplates. Doxorubicin was used as a positive control. The number of viable cells was determined using the neutral red assay procedure (*19*).

Time-Dependent Anticell Proliferative Activity. The ellagitannins and urolithins exhibiting growth inhibition of HT-29 cells in the above assay were selected to evaluate their ability to inhibit cell proliferation with

regard to the time of incubation. The assay was performed in 96-well microplates as described above. The percent cell viability was determined at the end of 8, 12, 24, 48, and 72 h.

Clonogenic Efficiency Assay. A clonogenic efficiency assay of HT-29 cells was performed by seeding 100 cells per Petri dish (60 × 15 mm). The cells were allowed to adhere for 2 h, followed by addition of 25 and 50 μM of test compound. After an incubation time of 24 h the medium was removed, cells were washed with PBS, and 2 mL of fresh medium was added. After 3 days of incubation the medium was replaced with fresh medium and cells were allowed to grow and form colonies for 7 days. On the seventh day the colonies were washed, fixed with methanol, and stained with crystal violet. The colonies with a diameter of ≥20 μm were counted using a 10× optical microscope in 10 random fields. The experiment was repeated three times. The ability of the compounds to decrease clonogenic efficiency was determined by their capability to decrease colony formation compared to vehicle (DMSO 0.1%) control.

Cell Cycle Analysis. To study the effect of pomegranate constituents on the HT-29 cell cycle, cells were seeded in a 24-well plate at a density of 10^5 cells per well and were allowed to adhere for 24 h. The cells were separately treated with 50 μg/mL of gallic acid and hexahydroxydiphenic acid, 100 μg/mL of ellagic acid, gallagyldilactone, punicalins, punicalagins, urolithin A, and urolithin B, and 5 μg/mL of podophyllotoxin (positive control) for 24 and 48 h. The cells were harvested and analyzed using a flow cytometer (*20*).

Evaluation of Apoptosis. *Assay for Caspase-3-like Activity*. Caspase-3 like activity induction in HT-29 cells by pomegranate constituents was measured using an EnzCheck Caspase-3 assay kit#2 (Invitrogen Corp.) using the manufacturer's protocol. This assay was also performed in the presence of the reversible aldehyde inhibitor (Ac-DEVD-CHO) of the caspase-3 enzyme, and only the Ac-DEVD-CHO sensitive portion of fluorescence was considered as true caspase-3-like activity. Camptothecin (5 μg/mL) was used as a positive control for induction of caspase-3-like activity. The fluorescence intensity was normalized using the amount of protein in each cell lysate.

DAPI Staining. This assay was used to determine whether the cell cycle arrest was related to induction of apoptosis. After treatment of cells with the test compounds at a concentration of 100 μM for 48 h, cells were collected by centrifuging at 2000*g* for 15 min, washed three times with cold PBS, and fixed in a solution of 3.7% formaldehyde for 10 min. Fixed cells were stained with 4 μg/mL DAPI for 15 min. The nuclear morphology of the cells was observed under a fluorescence microscope (*20*).

Annexin V and Propidium Iodide Staining. To distinguish between apoptotic and necrotic cell death, an Annexin V-FITC apoptosis detection kit [K101−100 Biovision] was used. Annexin V and propidium iodide staining was performed according to the maufacturer's protocol. The stained cells were analyzed using flow cytometry.

CYP1 Enzyme Inhibition Assay. To evaluate the effects of the test compounds on CYP1 activity, an ethoxy resorufin-*O*-deethylase (EROD) assay was conducted using HT-29 cells in a 48-well plate format. The cells were treated with TCDD for 24 h to induce CYP1 expression along with compounds for 24 h at concentrations ranging from 6.75 to 50 μM. The cells were also treated with DMSO, ellagitannins, and urolithins alone to evaluate their ability to induce CYP1 expression in the absence of TCDD (*21*).

Induction of Phase II Conjugating Enzymes Assay. The assays were performed according to the standard procedures described by Kirlin et al. (*22*).

Statistical Analysis. Data are given as the mean ± SEM. In the cell proliferation and CYP1 mediated EROD assays, the data was probit transformed and IC_{50} values were calculated. The other data was analyzed by global one way−ANOVA and post hoc Student−Newman−Keul tests. At least three independent experiments were carried out for evaluation of each of the variables. A P value of < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

It has been shown that a phytochemical-rich diet decreases the incidence of colon cancer (*23*, *24*). In this in vitro study we demonstrated that the pomegranate polyphenolic ellagitannins and urolithins exert a profound effect on the initiation and promotion stages of colon cancer development. Pomegranate juice consumption could potentially intercept ROS-mediated DNA damage and cancer initiation (*13*, *25*). Another pathway leading to cancer initiation is chemical carcinogen mediated DNA damage. Main dietary carcinogens comprise heterocyclic amines (HAs), polycyclic aromatic hydrocarbons (PAHs), and aromatic amines (AAs). Frying and/or broiling of meat and fish result in release of HAs through amino acid pyrolysis (*26*). Even common vegetable oils contain variable amounts of PAHs (*27*), while tobacco smoke liberates significant amounts of AAs and PAHs into the alimentary canal (*28*). These carcinogens are metabolically activated by CYP1 enzymes to mutagenic compounds, which initiate cancer (*29*). CYP1 enzymes are involved in activation of procarcinogens into cancer-inducing chemicals, while phase II enzymes such as quinone reductase and glutathione *S*-transferase are involved in detoxification of carcinogens by converting them into less toxic and water-soluble compounds, which are then eliminated from the body. It was shown that constitutively expressed CYP1A could exhibit 30 pmol/mg/min EROD activity in HT-29 cells (*30*). In our study CYP1-mediated EROD activity was not observed with basal CYP1 levels in HT-29 cells. However, a 24 h induction of HT-29 cells with TCDD, induced CYP1 expression which exhibited 17.3 ± 1.5 pmol/mg/min EROD activity. Ellagitannins and urolithins exhibited a significant dose-dependent inhibition of TCDD-induced CYP1 enzymes in HT-29 cells (**Figure 2**). The IC_{50}s for TCDD-induced CYP1 inhibition ranged from 56.7 ± 2.6 μM for urolithin A to 74.8 ± 2.29 μM for urolithin C. Gallic acid, HHDP, gallagyldilactone, and urolithin D exhibited no significant decrease in TCDD-induced CYP1 inhibition (**Table 2**).

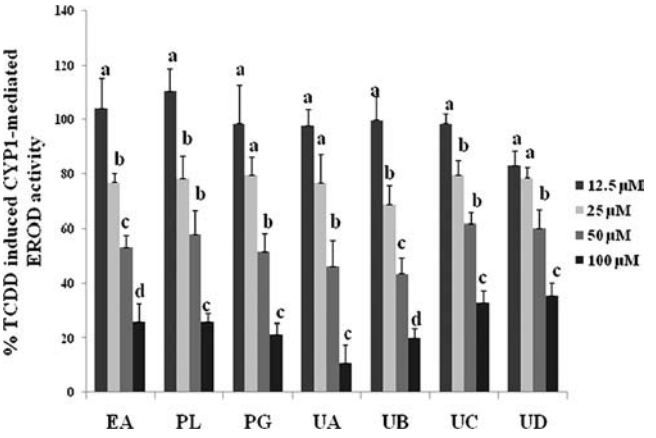

Figure 2. Effects of increasing concentrations of pomegranate EA, PL, PG, UA, UB (12.5, 25, 50, and 100 μM) on the TCDD (0.5×10^{-9} nM)-induced CYP1-dependent EROD activity in intact HT-29 human colon cancer cell lines. The compounds were added 24 h prior to EROD measurement. In the same treatment group, bars with different letters are statistically different.

Following the initiation stage, the second important step in cancer development is cancer progression. In this step the cells with damaged DNA accrue more genetic flaws and forfeit the normal cell cycle, resulting in excessive proliferation and tumor formation (*31*). Thus, further investigations were performed to explore the effects of ellagitannins/urolithins on the progressive stage of cancer. In order to evaluate the antiproliferative activity of the pomegranate constituents, they were tested against five cancerous and two noncancerous cell lines. Our objective was to examine if the test compounds possessed a selective antiproliferative activity against HT-29 colon cancer cells. Ellagitannins and urolithins exerted a dose- and time-dependent cell growth

Table 1. Antiproliferation Activity of Pomegranate Constituents[a]

	IC_{50} of antiproliferation activity, μM ± SEM						
chemical constituent	BT-549	HT-29	KB	LLC-PK_{11}	SK-MEL	SK-OV-3	Vero
GA	160 ± 8.8	235 ± 9.4	332 ± 4.1	341 ± 7.6	70.5 ± 8.8	105 ± 8.2	NA
HHDP	88 ± 9.5	123 ± 9.3	294 ± 8.5	168 ± 4.1	147 ± 9.2	44 ± 4.7	256 ± 3.7
EA	198 ± 19.8	462 ± 46.2	300 ± 3.3	372 ± 3.7	132 ± 13.2	222 ± 12	NA
GD	NA	315 ± 1.4	NA	NA	197 ± 7.4	NA	NA
PL	80 ± 11.5	196 ± 3.5	153 ± 5.2	NA	122 ± 5	136 ± 1.7	NA
PG	47 ± 7.9	135 ± 3.9	156 ± 2.6	NA	89 ± 14.3	104 ± 2.0	NA
UA	363 ± 5.5	378 ± 6.5	264 ± 2.8	NA	122 ± 7	NA	NA
UB	359 ± 2.4	357 ± 8.1	425 ± 5.2	NA	190 ± 10.3	NA	NA
UC	348 ± 9.7	353 ± 6.9	455 ± 10.2	NA	77 ± 5.1	NA	NA
UD	NA	316 ± 7.5	NA	NA	402 ± 9.8	NA	NA

[a] The effects of gallic acid (GA), hexahydroxydiphenic acid (HHDP), ellagic acid (EA), gallagyldilactone (GD), punicalins (PL), punicalagins (PG), urolithin A (UA), urolithin B (UB), urolithin C (UC), and urolithin D (UD) were studied in the proliferation of five cancerous and two non-cancer cell lines for 48 h. IC_{50} values are given as mean ± SEM of results of three independent experiments conducted in triplicate. NA: Not active.

Table 2. Inhibition of TCDD-Induced CYP1-Mediated EROD Activity[a]

chemical constituent	IC_{50}, μM ± SEM
urolithin A (UA)	56.7 ± 2.6
urolithin B (UB)	58.6 ± 4.6
urolithin C (UC)	74.8 ± 2.2
urolithin D (UD)	76.3 ± 3.3
ellagic acid (EA)	67.9 ± 2.6
punicalins (PL)	65.4 ± 5.2
punicalagins (PG)	64.0 ± 3.4

[a] HT-29 cells were analyzed for their CYP1-mediated EROD activity after cotreatment of TCDD with the test compounds. IC_{50} values are given as mean ± SEM of at least three independent experiments conducted in triplicate.

inhibition. It was also observed that these constituents were selective in inhibiting proliferation of cancerous cells, in comparison to noncancerous cells (**Table 1**). Though our particular interest was in colon cancer cells, the test compounds did not exhibit selectivity toward HT-29 colon cancer cells as compared to other cancer cells as shown in **Table 1**. Gallic acid exhibited antiproliferation of HT-29 cells with an IC_{50} of 235 μM, while hexahydroxydiphenic acid (HHDP), a dimer of gallic acid, inhibited HT-29 cell proliferation with an IC_{50} of 123 μM. Ellagic acid was less effective with an IC_{50} value of 462 μM. Punicalagins, punicalins, and gallagyldilactone, the major representatives of pomegranate ellagitannins, exhibited IC_{50} values of 135, 196, and 315 μM, respectively, against HT-29 cell lines. The urolithins exhibited antiproliferative activity against colon cancer cell lines with IC_{50} values in the 316−378 μM range.

It was previously reported that ellagitannins and their metabolites accumulate in the colon and their complete metabolism can take place over several days (*32*, *33*). Therefore, the time-dependent antiproliferative activity of the compounds listed in **Table 1** were studied in HT-29 colon cancer cells. None of the molecules influenced proliferation between 0 and 12 h. However, gallic acid and HHDP exhibited a profound antiproliferative activity between 12 and 24 h. Ellagic acid, gallagyldilactone, punicalins, punicalagins, and urolithins exhibited significant antiproliferation between 24 and 48 h and a maximum effect at 72 h. There was no significant difference in the IC_{50} values of the compounds in time- and dose-dependent antiproliferative assays.

In order to further evaluate the antiproliferation activity, a clonogenic assay was performed. Ellagitannins and urolithins cause reproductive death in HT-29 cells and exhibit a dose-dependent decrease in colony size and number (**Figure 3**). Urolithins A and B were potent inhibitors of clonogenic efficiency (85% decrease) at 50 μM, while ellagic acid exhibited the least

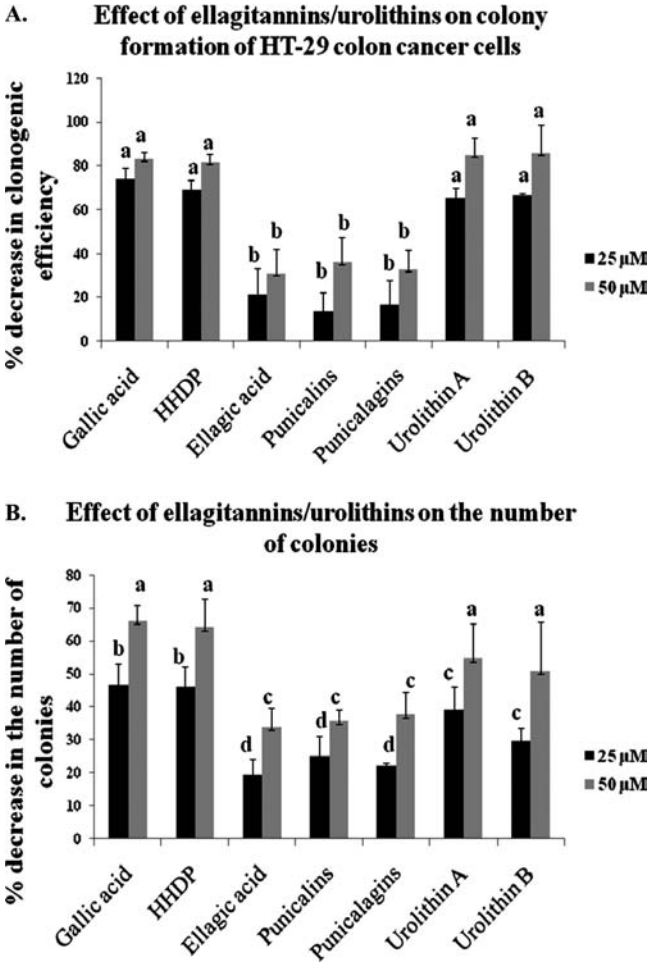

Figure 3. Effects of increasing concentrations of GA, HHDP, EA, PL, PG, UA, UB (25 and 50 μM) on (**A**) clonogenic efficiency and (**B**) number of colonies of HT-29 human colon cancer cells. Each data point represents mean ± SEM of three individual experiments. In the same treatment groups, bars with different letters are statistically different: $P < 0.05$.

potency (32% decrease) at 50 μM (**Figure 3A**). The pomegranate constituents also decreased the number of total colonies formed per plate, which ranged from 66% for 50 μM of gallic acid to 33% for 25 μM of ellagic acid (**Figure 3B**). All the above results indicate that these constituents curb the progression of cancer development.

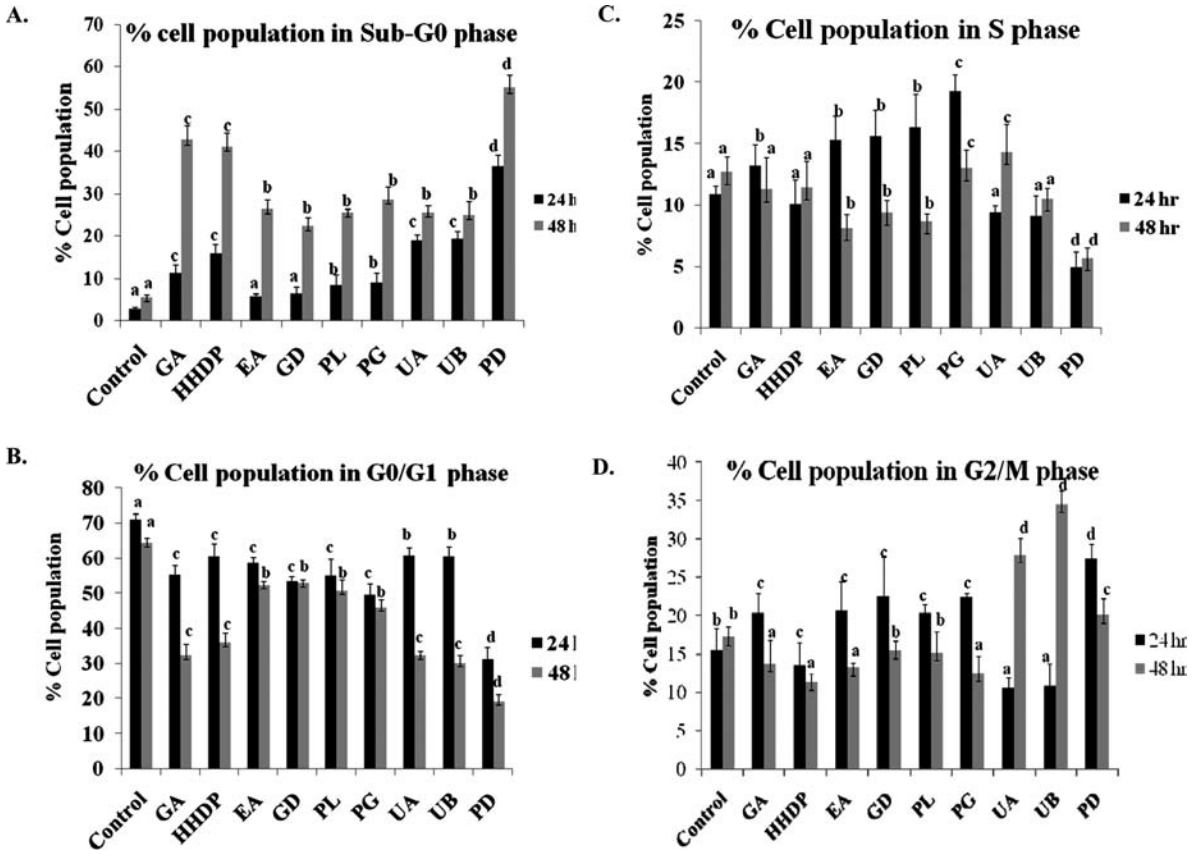

Figure 4. Effects of 50 μg/mL of GA and HHDP and 100 μg/mL of EA, PL, PG, UA, and UB on (**A**) sub-G_0 phase, (**B**) G_0/G_1 phase, (**C**) S phase, and (**D**) G_2/M phase of the HT-29 cell cycle. Each data point represents mean ± SEM of three individual experiments. In the treatment groups bars with different letters for same phase are statistically different: $P < 0.05$.

During normal cell proliferation at the checkpoints of the G_1/S and G_2/M phase, damaged DNA is corrected and regulated, but in cancerous cells the regulatory mechanisms deteriorate, resulting in propagation of defective cells. Hence, one of the mechanisms through which proliferation of cancerous cells could be inhibited is through cell cycle disruption. Further investigations were done to study the distribution of cells in each phase of the cell cycle upon treatment with ellagitannins and urolithins. A time-dependent disruption of the cell cycle was observed in HT-29 cells upon incubation with ellagitannins and urolithins for 24 and 48 h, respectively. Results (**Figure 4**) suggest that ellagitannins exhibit a significant reduction of cells in the G_0/G_1 phase ($P < 0.001$), while there was no significant increase in cells in the S and G_2/M phases during the first 24 h followed by a significant decrease after 48 h treatment. The decrease of cell numbers in the S and G_2/M phases between 24 and 48 h treatments is due to cell death, resulting in a corresponding increase of cells in the sub-G_0 phase. Urolithins A ($P < 0.001$) and B ($P < 0.01$) significantly lowered the G_0/G_1 cells upon 48 h treatment with a corresponding increase of cells in the G_2/M and sub-G_0 phases. Thus, all the tested compounds affected the cell cycle at different stages in a time-dependent fashion, leading to a significant accumulation of cells in the sub-G_0 phase (dead cells).

Cell cycle analyses signify that gallic acid, HHDP, ellagic acid, gallagyldilactone, punicalins, and punicalagins caused a cell cycle arrest at the S phase. These results are in accordance with previous studies (*14*), where it was shown that punicalagins and ellagic acid downregulated the cyclin-dependent kinases A and B1, which are required for progress of cells from the S to the G_2/M phase. Urolithins A and B caused a cell cycle arrest in the G_2/M phase. The results are in accordance with a recent study (*34*), where it was shown that urolithins A and B changed the expression profile of MAPK genes (growth factor receptors, oncogenes, tumor suppression genes) and cell cycle genes (CCNB1, CCNB1/P1) in Caco-2 cells, at concentrations achievable in the lumen. A significant accumulation of dead cells (sub-G_0 phase) increased in a time-dependent manner with all the treatments. Further studies were performed to determine the nature (apoptotic and/or necrotic) of cells accumulated in the sub-G_0 phase.

Suppression of apoptosis is another pathway by which cancer progresses, because the excessively proliferating cells forbid apoptosis and prolong their survival. Thus, any compound inducing apoptosis in cancerous cells will prevent proliferation and tumor formation. The caspase-3 induction is a biomarker in cells undergoing apoptosis. The HT-29 cells treated with ellagitannins/urolithins were assessed for their caspase-3 like activity. Any induction in caspase-3 activity in HT-29 cells caused by the pomegranate constituents indicates their apoptotic activity. All the ellagitannins and urolithins induced caspase-3 like activity in a dose-dependent manner (**Figure 5A**). The highest induction (197% compared to control cells) was observed in HT-29 cells treated with 100 μM punicalagins, and the lowest induction (42%) was observed for 25 μM HHDP. A 2−4-fold increase in caspase-3 like activity was observed with an increase in the dose from 25 to 100 μM. The results indicate that apoptosis is one of the major pathways activated by ellagitannins and urolithins in HT-29 cancer cells, leading to cell death and growth inhibition. Induction of apoptosis was further confirmed by DAPI staining in HT-29 cells (**Figure 4B**). The cells treated with ellagitannins and urolithins exhibited condensed nuclear DNA, which indicates apoptosis. Annexin-v and propidium iodide staining analyses (**Figure 5C**) indicated that all the test compounds caused apoptosis

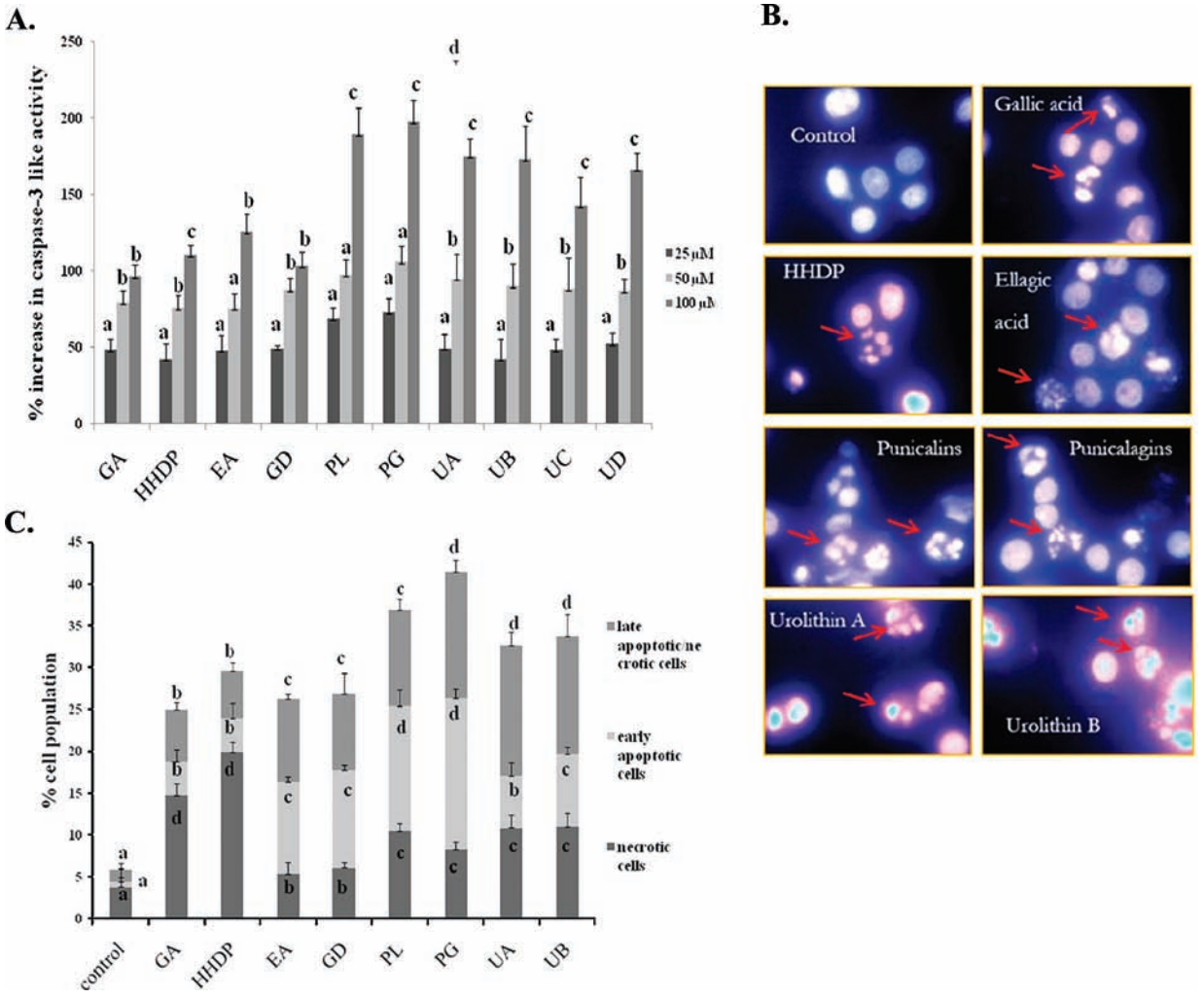

Figure 5. (**A**) Effect of increasing concentrations of GA, HHDP, EA, PL, PG, UA, UB, UC, and UD (25, 50, and 100 μM) on caspase-3 like activity in HT-29 human colon cancer cells. In the same treatment groups bars with different letters are statistically different: $P < 0.05$. (**B**) Effect of 48 h treatment of HT-29 cells with GA, HHDP, EA, PL, PG, UA, and UB (100 μM). The arrows indicate condensed/fragmented DNA, suggesting apoptotic death of the cells. The control indicates HT-29 cells treated with DMSO. (**C**) Effect of 100 μM of GA, HHDP, EA, PL, PG, UA, and UB on the nature of HT-29 cell death upon 48 h treatment. In the treatment groups bars with different letters for the same kind of cell death are statistically different: $P < 0.05$.

upon 24 h incubation. HHDP (50 μM) caused necrosis in the highest number of cells; however, treatment with punicalagins (100 μM) resulted in the highest amounts of early and late apoptotic/necrotic death in HT-29 cells. Our investigation suggests that pomegranate constituents lower the proliferation of colon cancer cells by causing cell cycle arrest and inducing apoptosis.

Pomegranate juice is a rich source of ellagitannins, the most abundant being the punicalagins, reaching maximum levels of >2 g/L juice (variable with manufacturer). We observed that pomegranate ellagitannins inhibit HT-29 cell proliferation by arresting the cell cycle and inducing apoptosis. The ellagitannins and urolithins exerted antiproliferative activity with IC_{50} values ranging from 100 to 500 μM and inhibited clonogenic efficiency and CYP1-mediated carcinogen bioactivation at concentrations ranging from 25 to 100 μM. An in vitro gastrointestinal digestion study of pomegranate juice indicated that at least 29% of the polyphenolic content (292 mg/L gallic acid equivalents) could reach the colon intact (*35*). It was also described that, apart from ellagic acid, pomegranate ellagitannins are systemically not bioavailable. Instead, these ellagitannins are metabolized by colonic microflora into the urolithins. The urolithins exhibit a tissue-specific accumulation with higher affinity toward the prostate, intestines, and colon (*11*). Our studies involving seven different urolithins showed that they inhibit CYP1 activity, increase clonogenic efficiency and proliferative activity, induce apoptotic activity, and increase cell cycle arrest in HT-29 colon cancer cells. Though the actual amount of urolithins that accumulates in the colon is not known, it is plausible that continuous consumption of pomegranate juice could provide a sufficient concentration of urolithins to inhibit colon cancer development. Cancers of the alimentary canal represent a special case, because the cancerous cells come in direct contact with large amounts of food-related phytochemicals. Pomegranate ellagitannins, though systemically not available, can exert their cancer chemopreventive effects on the colon epithelium through direct contact. The intestinal bacterial metabolites of these ellagitannins also contribute to colon cancer chemoprevention. Therefore, the greater the oral intake of pomegranate ellagitannins, the lesser the incidence of colon cancer should be. In order to evaluate the safety of higher consumption of pomegranate ellagitannin content, toxicological studies performed indicated that an average intake of 400−1200 mg/day of punicalagins did not exert any toxicological effects in rats (*36*). Even in human subjects, consumption of POMx capsules providing 435−870 mg of gallic acid equivalents of ellagitannins was shown to be safe. It was also

seen that the plasma antioxidant potency increased significantly after consumption of POMx supplementation (610 mg of gallic acid equivalents) (*25*).

In conclusion, the results of our study indicate that not only ellagic acid and punicalagins but also other ellagitannins present in pomegranate juice can potentially contribute to colon cancer chemoprevention. It was also observed that the urolithins may exert an anticancer-like effect, preventing the incidence of colon cancer. The colon cancer chemopreventive ability of the ellagitannins and urolithins may be attributed to their ability to inhibit the initiation and proliferation stages of cancer development. This study highlights the pharmacological importance of ellagitannins, and the results may serve to improve the guidelines for an appropriate formulation of commercial pomegranate juice.

ABBREVIATIONS USED

CYP, cytochrome P450; PAH, polycyclic aromatic hydrocarbons; HHDP, hexahydroxydiphenic acid; EROD, ethoxyresorufin-*O*-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HA, heterocyclic amines; AA, aromatic amines; MAPK, mitogen-activated protein kinase.

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