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1 **In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic**
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3 **polyphenols as found in pomegranate juice**

4

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15 Running Title: Antiproliferative pomegranate polyphenols

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**Abstract**

Pomegranate (*Punica granatum* L.) fruits are widely consumed as juice (PJ). The potent antioxidant and anti-atherosclerotic activities of PJ are attributed to its polyphenols including punicalagin, the major fruit ellagitannin, and ellagic acid (EA). Punicalagin is the major antioxidant polyphenol ingredient in PJ. Punicalagin, EA, a standardized total pomegranate tannin extract (TPT) and PJ were evaluated for in vitro antiproliferative, apoptotic and antioxidant activities. Punicalagin, EA and TPT were evaluated for antiproliferative activity at 12.5-100 μg/mL on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, 22Rv1) tumor cells. Punicalagin, EA and TPT were evaluated at 100 μg/mL concentrations for apoptotic effects and at 10 μg/mL concentrations for antioxidant properties. However, to evaluate the synergistic and/or additive contributions from other PJ phytochemicals, PJ was tested at concentrations normalized to deliver equivalent amounts of punicalagin (w/w). Apoptotic effects were evaluated against the HT-29 and HCT116 colon cancer cell lines. Antioxidant effects were evaluated using inhibition of lipid peroxidation and Trolox Equivalent Antioxidant Capacity (TEAC) assays. PJ showed greatest antiproliferative activity against all cell lines by inhibiting proliferation from 30-100%. At 100 μg/mL, PJ, EA, punicalagin and TPT induced apoptosis in HT-29 colon cells. However, in the HCT116 colon cells, EA, punicalagin and TPT but not PJ induced apoptosis. The trend in antioxidant activity was PJ>TPT>punicalagin>EA. The superior bioactivity of PJ compared to its purified polyphenols illustrated the multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients.

*Keywords*: Pomegranates; Punicalagin; Ellagic Acid; Antiproliferative; Apoptosis; Antioxidant

## 1. Introduction

Epidemiological studies suggest that a reduced risk of cancer is associated with the consumption of a phytochemical rich diet that includes fruits and vegetables [1]. Fresh and processed fruits and food products contain high levels of a diverse range of phytochemicals of which polyphenols including hydrolysable tannins [ellagitannins (ETs) and gallotannins] and condensed tannins (proanthocyanidins), and anthocyanins and other flavonoids make up a large proportion [2-4]. Suggested mechanisms of anticancer effects of polyphenols include antioxidant, anti-inflammatory, and antiproliferative activities as well as their effects on sub-cellular signaling pathways, induction of cell cycle arrest and apoptosis [5,6].

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and in beverage forms as juice and wines [7]. Commercial pomegranate juice (PJ) shows potent antioxidant and anti-atherosclerotic properties attributed to its high content of polyphenols including ellagic acid (EA) in its free and bound forms [as ETs, and EA-glycosides (EAGs)], gallotannins, and anthocyanins (cyanidin, delphinidin and pelargonidin glycosides) and other flavonoids (quercetin, kaempferol and luteolin glycosides) [7-12]. The most abundant of these polyphenols is punicalagin (Fig. 1), an ET implicated as the bioactive constituent responsible for >50% of the juice's potent

antioxidant activity [7]. Punicalagin is abundant in the fruit husk and during processing is extracted into PJ in significant quantities reaching levels of > 2g/L juice [7,11-13].

We are interested in the potential health benefits of phytochemicals and evaluating the multifactorial effects and chemical synergy of the action of multiple compounds, as found naturally in their unique compositions in foods compared to single purified active compounds [14]. Because pomegranates are widely consumed and implicated with potential human health benefits [8,15], we have investigated the antiproliferative, apoptotic and antioxidant activities (lipid peroxidation inhibitory and Trolox Equivalent Antioxidative Capacity) of its polyphenols. Pomegranate was evaluated in the form of PJ, a popularly consumed beverage, as a standardized total pomegranate tannin (TPT) extract (contains 85% punicalagin anomers, 1.3% EA, ~12% minor ETs and EAGs) [13], and as its reported active ingredients, punicalagin and EA.

EA has previously been shown to exhibit anticarcinogenic properties such as induction of cell cycle arrest and apoptosis, as well as the inhibition of tumor formation and growth in animals [16-18]. Hydrolysable and condensed tannins have also been reported to show in vitro and in vivo anticancer properties [19-20]. However, this is the first report on the evaluation of PJ and TPT and their major purified polyphenols, punicalagin and EA, for antiproliferative activity against this panel of human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, 22Rv1) cancer cell lines. This is also the first report on the inhibition of lipid peroxidation by pomegranate polyphenols using a model of liposome oxidation by fluorescence spectroscopy and on the evaluation of their apoptotic effects against human colon cancer cells.

1 **2. Methods and materials**

2

3 *2.1. General materials*

4 All solvents were HPLC grade and purchased from Fisher Scientific Co. (Tustin,
5 CA). Dimethylsulphoxide (DMSO), dimethyl formamide and ellagic acid (EA) were
6 purchased from Sigma Aldrich Co. (St. Louis, MO). Pomegranate juice (POM®
7 Wonderful LLC, Los Angeles, CA, USA) is commercially available for human
8 consumption and was used in concentrate form (contains 1.74 mg/mL punicalagin and
9 0.14 mg/mL EA; quantification data not shown).

10

11 *2.2. Purification of total pomegranate tannins (TPT) extract and punicalagin*

12

13 Ellagitannins were purified from fruit husk as previously reported and analyzed for
14 purity by high performance liquid chromatography (HPLC) and liquid chromatography
15 electrospray ionization mass spectroscopy (LC-ESI/MS) [13]. TPT contains 85%
16 punicalagin anomers (M-H m/z 1083), 1.3% EA (M-H m/z 301), and ~12% minor ETs
17 and EAGs [13].

18

19 *2.3. Cell culture materials*

20

21 The KB and CAL27 oral cancer, SW480, SW620, HT29 and HCT116 colon cancer
22 and RWPE-1 prostate cancer cell lines were obtained from American Type Culture
23 Collection (ATCC, Rockville, MD). The 22Rv1 prostate cancer cell line was obtained

1 from the laboratory of P. Cohen (Division of Pediatric Endocrinology, UCLA Medical
2 Center, Los Angeles, CA). KB oral cancer cells were grown in Minimum Essential
3 Medium (MEM); CAL27 oral cancer cells were grown in Dulbecco's Minimum Essential
4 Medium (DMEM); SW480 and SW620 colon cancer cells and 22Rv1 prostate cancer
5 cells were grown in RPMI 1640; HT-29 and HCT116 colon cancer cells were grown in
6 McCoy's 5A Medium, Modified. All media contained 10% fetal bovine serum (FBS) in
7 the presence of 100 U/mL penicillin and 0.1 g/L streptomycin. RWPE-1 prostate cells
8 were grown in Defined Keratinocyte Serum Free Medium (DKSFM) containing
9 epidermal growth factor (EGF), insulin and fibroblast growth factor (FGF). Cells were
10 incubated at 37°C with 95% air and 5% $CO_2$. All cells were maintained below passage
11 20 and used in experiments during the linear phase of growth.

12

13 *2.4. Cell proliferation assay*

14

15 Proliferation was measured utilizing the CellTiter-Glo® Luminescent Cell
16 Viability Assay (Technical Bulletin # 288, Promega Corp., Madison, WI). When added
17 to cells, the assay reagent produces luminescence in the presence of ATP from viable
18 cells. Cells were plated in 96-well plates at a density of 10,000 cells/well and incubated
19 for 24 hours. Test samples were solubilized in DMSO by sonication, filter sterilized and
20 diluted with media to the desired treatment concentration. Cells were treated with 100
21 μL control media, ascorbic acid (100 μM, used as an antioxidant standard), or test
22 samples and incubated for 48h drug exposure duration. Punicalagin, EA and TPT were
23 tested at 12.5, 25, 50 and 100 μg/mL concentrations. PJ was tested at concentrations

normalized to deliver equivalent amounts of punicalagin (w/w) to evaluate the additive and/or synergistic effects of other pomegranate phytochemicals towards its antiproliferative activity. At the end of 48 h, plates were equilibrated at room temperature for 30 min, 100 μL of the assay reagent was added to each well and cell-lysis was induced on an orbital shaker for 2 min. Plates were incubated at room temperature for 10 min to stabilize the luminescence signal and results were read on an Orion Microplate Luminometer (Bertholds Detection Systems, Pforzheim, Germany). All plates had control wells containing medium without cells to obtain a value for background luminescence. Data are expressed as percentage of untreated cells (i.e. treatment value-blank/vehicle value-blank), mean ± SE for three replications.

*2.5. Assessment of apoptosis*

Apoptosis was assessed utilizing the Cell Death Detection ELISA$^{PLUS}$ Assay (Boehringer Mannheim, Indianapolis, IN). This assay is a photometric enzyme-linked immunoassay that quantitatively measures the internucleosomal degradation of DNA, which occurs during apoptosis. Specifically, the assay detects histone associated mono- and oligonucleosomes, which are indicators of apoptosis. HT-29 and HCT116 cells were plated in 60mm dishes at a density of 100,000 cells/dish and allowed to attach for 24 hours. Cells were treated with vehicle control (100% DMSO; 0.3% final concentration), EA, punicalagin, TPT or PJ (100 μg/mL) for 48 hours. Following treatments, non-adherent cells were collected and pelleted at 200 x g for ten minutes. The supernatant was discarded; the cell pellet was washed with cold CMF-PBS and re-centrifuged.

1 Adherent cells were washed with cold calcium magnesium free- phosphate buffered
2 saline (CMF-PBS, 137 mmol/L sodium chloride, 1.5 mmol/L potassium phosphate, 7.2
3 mmol/L sodium phosphate, 2.7 mmol/L potassium chloride, pH 7.4), trypsinized,
4 collected and combined with non-adherent cells into a total of 1 mL DMEM. Both live
5 and dead cells were then counted via trypan blue exclusion (Pierce, Rockford, IL) and
6 equal number of cells were added to the microtiter plate for all treatment groups and
7 apoptosis assay was performed according to the manufacturer's instructions. Data are
8 expressed as absorbance at 405 nm of each sample over vehicle controls as follows =
9 treatment value-blank/vehicle value-blank.

10

11 *2.6. Inhibition of lipid peroxidation*

12

13 The assay was conducted by analysis of model liposome oxidation using
14 fluorescence spectroscopy as previously reported [21]. Briefly, the lipid, 1-stearoyl-2-
15 linoleoyl-sn-glycero-3-phosphocholine and fluorescent probe, 3-[p-(6-phenyl)-1,3,5-
16 hexatrienyl]-phenylpropionic acid were combined in dimethyl formamide and used to
17 prepare Large Unilamellar Vesicles (LUVs). The final assay volume combined HEPES
18 buffer, test sample or DMSO (control) and a 20 μl aliquot of liposome suspension in a
19 test tube. Peroxidation was initiated by addition of $FeCl_2.4H_2O$ (0.5 mM) for positive
20 controls, [tert-butylhydroquinone (TBHQ), butylated-hydroxyanisole (BHA) and
21 butylated-hydroxytoluene (BHT); all at 10μM] and test samples (all at 10 μg/mL; 20 μl
22 aliquot volume). Each sample was assayed in triplicate. Fluorescence was measured at
23 384 nm and monitored at 0, 1, 3 and every 3 min thereafter up to 21 min using a Turner

Model 450 Digital Fluorometer (Barnstead Thermolyne, Dubuque, IA). The decrease of relative fluorescence intensity with time indicated the rate of peroxidation according to the following formula: % relative fluorescence = (Fta+Ftb)/(F0a+F0b). Fta and Ftb represent the two measurement of the fluorescence of sample at selected times (0, 3, 6 min...). F0a and F0b represent the two measurements at time 0 of the same sample.

*2.7. Trolox Equivalent Antioxidative Capacity (TEAC)*

The assay was performed as previously reported [22]. Briefly, 2’2’-azinobis(3-thylbenzothiazline-6-sulfonic acid)diammonium salt (ABTS) radical cations were prepared by adding solid manganese dioxide (80 mg) to a 5 mM aqueous stock solution of $ABTS^{\cdot+}$ (20 ml using a 75 mM Na/K buffer of pH 7). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble analog of Vitamin E, was used as an antioxidant standard. A standard calibration curve was constructed for Trolox at 0, 50, 100, 150, 200, 250, 300, 350 μM concentrations. Samples (10 μg/mL concentrations) were mixed with 200 μl of $ABTS^{\cdot+}$ radical cation solution in 96 well plates and absorbance was read (at 750 nm) after 5, 15, 30, 45, 60, 75 and 90 min in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). TEAC values were calculated as reading x volume/1000. Trolox equivalents (in μM) were derived from the standard curve at 5 minutes incubation.

1 *2.8. Statistics*

2

3 Data for the antiproliferative and apoptosis assays were analyzed by either
4 student's t-test, one-way ANOVA followed by Dunnett's Multiple Range test ($\alpha$=0.05)
5 with Graph Pad Prism 3.0 (Graph Pad Software Inc.) as appropriate.

6

7 **3. Results**

8

9 The biological properties associated with pomegranate fruits [7-10] prompted us
10 to evaluate their major phytochemical ingredients as single purified compounds,
11 punicalagin and EA (Fig. 1), and as combinations, TPT and PJ. We have previously
12 reported that TPT contains 85% punicalagin, 1.3% EA-hexoside and minor EAGs and
13 ETs (punicalin and gallagic acid) [13]. The minor pomegranate ETs and EAGs were not
14 quantified in TPT due to the unavailability of commercial standards. The PJ used in our
15 experiments contained 1.74 mg/mL punicalagin and 0.14 mg/mL EA.
16 Test samples were evaluated for antiproliferative activity against human oral (KB,
17 CAL27), colon (SW460; SW620; HT-29; HCT116) and prostate (RWPE-1; 22Rv1)
18 tumor cells. At concentrations normalized to deliver equivalent amounts of the major
19 pomegranate polyphenol, punicalagin (w/w), PJ showed greatest antiproliferative activity
20 against all cell lines by inhibiting proliferation from 30-100% at treatments between 12.5-
21 100 μg/mL (Figs. 2-4). Punicalagin, EA and TPT inhibited cell proliferation in a dose
22 dependent manner in all cell lines tested, but to a lesser degree than PJ. In KB oral
23 cancer cells, EA inhibited proliferation from 45-88%, punicalagin from 0-42% and TPT

from 0-27% (Fig. 2A). In CAL27 oral cancer cells, EA inhibited cell proliferation from 26-69%, punicalagin from 10-96% and TPT from 17-97% (Fig. 2B). SW480 non-metastatic colon cancer cells also showed sensitivity to pomegranate polyphenols with EA inhibiting cell proliferation from 49-76%, punicalagin from 1-65% and TPT from 1-67% (Fig. 3A). In SW620 metastatic colon cancer cells, EA inhibited proliferation from 14-35%, punicalagin from 0-57% and TPT from 0.02-40% (Fig. 3B). Proliferation of HT-29 colon cancer cells was inhibited from 0-21% by EA, from 1-55% by punicalagin and from 2-71% by TPT (Fig. 3C) and in HCT116 colon cancer cells, EA induced inhibition of proliferation from 53-87%, punicalagin from 0-72% and TPT from 13-87% (Fig. 3D). Similarly, in RWPE-1 immortalized prostate epithelial cells, EA inhibited proliferation from 78-92%, punicalagin from 64-94% and TPT from 44-88% (Fig. 4A). In 22Rv1 metastatic prostate cancer cells, EA inhibited proliferation from 43-94%, punicalagin from 68-90% and TPT from 47-89% at treatments between 12.5-100 μg/mL (Fig. 4B).

Because of our interest in colon cancer, the apoptotic effect of PJ and its purified polyphenols on the HT-29 and HCT116 colon cancer cell lines were evaluated to ascertain whether the observed reduction in viable cell number was due to the induction of apoptosis (Fig. 5). At doses held equivalent to that found in PJ, punicalagin, EA, and TPT did not exhibit apoptotic activity in HT-29 and HCT116 colon cancer cell lines (data not shown). However, when treated at equivalent doses of 100 μg/mL, PJ, EA, punicalagin and TPT induced apoptosis in HT-29 cells by 2.66, 2.44, 2.65 and 2.59-fold, respectively, over vehicle controls. Similarly, in the HCT116 cells, EA, punicalagin and TPT induced apoptosis by 2.85, 1.52 and 2.87-fold over vehicle controls. Interestingly,

1 although PJ decreased viable cell number at 100 μg/mL, it did not exhibit significant
2 apoptotic activity in this cell line.

3 Punicalagin, EA, TPT and PJ (all at 10 μg/mL concentrations) were also
4 evaluated for the ability to inhibit lipid peroxidation induced by Fe (II) ions in a
5 liposomal model and for Trolox Equivalent Antioxidative Capacity (TEAC). The
6 abilities of the samples to inhibit lipid peroxidation were compared to that of the
7 commercial synthetic antioxidants, TBHQ, BHT and BHA (all at 10 μM concentrations)
8 (Fig. 6A). In the lipid peroxidation assay, the relative decrease in fluorescence showed
9 that PJ was the most active sample among the pomegranate polyphenols tested (Fig. 6B).
10 In the TEAC assay, PJ, TPT, punicalagin and EA had values of 25,591; 100; 90 and 40
11 μM Trolox equivalents, respectively. TEAC is the concentration of Trolox required to
12 give the same antioxidant capacity as 1mM test substance. The total antioxidant activity
13 of PJ was equivalent to that of a solution of 31.8 mM of Trolox calculated experimentally
14 by the TEAC method. The order of antioxidative potency of the pomegranate
15 polyphenols in our assays was PJ>TPT>punicalagin>EA, showing that PJ is a more
16 effective antioxidant than its separated and purified components.
17
18 **4. Discussion**
19
20 Pomegranate fruits are widely consumed in fresh and beverage forms and have
21 been used extensively in ancient cultures for various medicinal properties [23].
22 Pomegranate juice (PJ) and extracts have been shown to have potent in vitro antioxidant
23 [7,24] and in vivo anti-atherosclerotic properties [8,9,15], attributed to its high content of

polyphenols including ETs and EA. Recently, there have also been numerous reports on the in vitro and in vivo anti-cancer properties of pomegranates [10,25-30]. The major pomegranate ET, punicalagin, is reported as the active ingredient responsible for > 50% of the juice's antioxidative potential [7,24] and can reach levels of > 2g/L of juice (7). However the synergistic and/or additive effects of the individual purified polyphenols present in PJ and also in a well standardized extract form are yet to be evaluated for anti-proliferative and apoptotic activities. In addition, although hydrolysable tannins and EA have been reported to have anticancer activities [19,20,31], punicalagin, has never been evaluated for its antiproliferative and apoptotic properties. These in vitro studies are necessary since punicalagin has been shown to release EA in vivo, which is then metabolized to its glucuronides and sulfates in animal and human bioavailability studies [11,12,32,33].

In the present study, punicalagin, EA and TPT decreased viable cell number of human oral (Fig. 2), prostate (Fig. 3) and colon (Fig. 4) tumor cells, however, superior activity was obtained with pure PJ. Similarly, in the apoptosis studies, PJ induced apoptosis in HT-29 cells when concentrations of punicalagin, ET and TPT equalized to amounts found in PJ had no effect. Only when the concentration of these compounds was raised to equivalent amounts (w/w) with PJ were they able to induce apoptosis. PJ was also the most active antioxidant sample that was tested. It is noteworthy that among the polyphenols present in PJ, punicalagin is the most potent antioxidant ingredient contributing largely to the observed antioxidant properties of PJ, as previously reported [7, 24].

1 Our finding that PJ is more potent than its separated and individual polyphenols
2 suggests synergistic and/or additive effects from the other phytochemicals present in PJ.
3 This finding is not surprising, as PJ also contains proanthocyanidins, anthocyanins
4 (glycosides of delphinidin, peonidin and cyanidin), and flavonoid glycosides [10-13],
5 phytochemicals that have all been shown to have antioxidant and anti-proliferative
6 activities [14,21]. The limitations in this work are true of most in *vitro* studies. Cell line
7 specific sensitivity and reactions have been shown in many trials and this should be
8 considered when evaluating experimental results. Additionally, in *vitro* trials also do not
9 always reflect the outcome of in *vivo* studies; however, in *vitro* experiments can be a
10 valuable screening tool for future animal and human studies.

11 Cancer cells exist under a state of oxidative stress, as this increases their survival
12 potential by inducing mutations [34], activating redox signaling that may lead to the
13 inactivation of tumor suppressor genes such as p53 [35] and the activation of pro-survival
14 factors such as NFκB and AP-1 [36]. Mild levels of reactive oxygen species (ROS) have
15 been shown to induce proliferation in cancer cells [37,38]. Therefore, foods rich in
16 antioxidant phytochemicals are important for the prevention of diseases related to oxidant
17 stress such as heart disease and cancer. In this study, our focus on cancer cell lines
18 investigated the potential of pomegranate juice and its purified polyphenols as anti-cancer
19 agents by evaluating their effect on oxidation, viable cell number and the sensitivity of
20 colon cancer cells to apoptosis.

21 Other natural antioxidants, such as curcumin, have been shown to stimulate the
22 expression of the tumor suppressor gene p53 [39]. Vitamin E and quercetin also show
23 promise as anticancer agents as they exhibit inhibition of the expression of mutant p53 in

human cancer cell lines [40-42]. In addition, vitamins C and E were shown to decrease nuclear binding and activation of NF*k*B and AP-1 in LNCaP prostate cancer cells. In our studies, we show that PJ and its purified polyphenols are potent antioxidants which may be a mechanism whereby they inhibit cancer cell proliferation and induce cancer cells to undergo apoptosis. Although the purified polyphenols showed significant antiproliferative, apoptotic and antioxidant effects alone, the superior bioactivity of PJ suggest multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients.

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Fig. 1. Structures of punicalagin, the major polyphenol antioxidant ingredient in PJ, and ellagic acid (EA).

Fig. 2. Antiproliferative activities of Punicalagin (—●—), EA (—○—), TPT (—▽—) and PJ (—▼—) against human oral tumor cell lines: 2A = KB; B = CAL27. Cells were exposed to punicalagin, EA or TPT (at 100 –12.5 μg/mL concentrations) and PJ (normalized to punicalagin content) for 48h. Data are expressed as percentage of untreated cells, mean ± SE (n= 3). Asterisk indicates a significant difference compared to untreated controls, $p \leq 0.01$, 2 tailed t-test.

Fig. 3. Antiproliferative activities of Punicalagin (—●—), EA (—○—), TPT (—▽—) and PJ (—▼—) against human colon tumor cell lines: 3A = SW 460; 3B = SW 620; 3C = HT 29; 3D = HCT 116. Cells were exposed to punicalagin, EA or TPT (at 100 –12.5 μg/mL concentrations) and PJ (normalized to punicalagin content) for 48h. Data are expressed as percentage of untreated cells, mean ± SE (n= 3). Asterisk indicates a significant difference compared to untreated controls, $p \leq 0.05$, 2 tailed t-test.

Fig. 4. Antiproliferative activities of Punicalagin (—●—), EA (—○—), TPT (—▽—) and PJ (—▼—) against human prostate tumor cells: Fig. 4A = RWPE-1; Fig 4B = 22 Rv-1. Cells were exposed to punicalagin, EA or TPT (at 100 –12.5 μg/mL concentrations) and PJ (normalized to punicalagin content) for 48h. Data are expressed as percentage of untreated cells, mean ± SE (n= 3). Asterisk indicates a significant difference compared to $p \leq 0.01$, 2 tailed t-test.

1 Fig. 5. Effects of Punicalagin, EA, TPT and PJ on apoptosis in human colon HT29 and
2 HCT 116 cells. Cells were treated with samples at 100 μg/mL for 24 h before they were
3 harvested for analyses using the Cell Death Detection ELISA$^{PLUS}$ Assay. Values are
4 means ± SD, n = 3. Asterisk indicates a significant difference compared to p≤ 0.01, 2
5 tailed t-test.
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7 Fig. 6. Inhibition of lipid peroxidation induced by Fe(II) of A) commercial synthetic
8 antioxidants, BHA, BHT and TBHQ; B) Punicalagin, EA, TPT and PJ. Results are
9 expressed as the mean percent inhibition of triplicate measurements ± SD.
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