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Absorption, Metabolism, and Antioxidant Effects of Pomegranate (*Punica granatum* L.) Polyphenols after Ingestion of a Standardized Extract in Healthy Human Volunteers

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The intake of polyphenols has been demonstrated to have health-promoting and disease-preventive effects. The pomegranate (*Punica granatum* L.), which is rich in several polyphenols, has been used for centuries in ancient cultures for its medicinal purposes. The potential health benefits of pomegranate polyphenols have been demonstrated in numerous in vitro studies and in vivo experiments. This study investigated the absorption and antioxidant effects of a standardized extract from pomegranate in healthy human volunteers after the acute consumption of 800 mg of extract. Results indicate that ellagic acid (EA) from the extract is bioavailable, with an observed C_{max} of 33 ng/mL at t_{max} of 1 h. The plasma metabolites urolithin A, urolithin B, hydroxyl-urolithin A, urolithin A-glucuronide, and dimethyl ellagic acid-glucuronide were identified by HPLC-MS. The antioxidant capacity measured with the oxygen radical absorbance capacity (ORAC) assay was increased with a maximum effect of 32% after 0.5 h, whereas the generation of reactive oxygen species (ROS) was not affected. The inflammation marker interleukin-6 (IL-6) was not significantly affected after 4 h after the consumption of the extract. Overall, this study demonstrated the absorbability of EA from a pomegranate extract high in ellagitannin content and its ex vivo antioxidant effects.

KEYWORDS: Pomegranate extract; absorption; antioxidant; metabolism; punicalagins; ellagitannins; ellagic acid

INTRODUCTION

The intake of polyphenols has been inversely correlated to the incidence of several chronic diseases, such as several types of cancer and cardiovascular disease, which has been demonstrated in several cohort and case-control studies (*1*, *2*). Specifically, the intake of polyphenols has been shown to be associated with an increased antioxidant potential in plasma and vascular protection (*3*, *4*). Several studies have been performed in the assessment of absorption of polyphenols from beverages and dried extracts.

Recent studies with tea polyphenols, administered as either tea extract supplements or as beverage, showed differences in pharmacokinetic parameters and ex vivo biological activities in human volunteers (*5*, *6*). Overall, very little information is available regarding effective amounts of antioxidant dietary supplements (*7*).

The pomegranate (*Punica granatum* L.) tree originated in the Middle East and India and has been used for centuries in ancient cultures for its medicinal purposes. Major areas of pomegranate cultivation are India, Spain, Israel, and the United States, where it has gained recent economic importance due to increased consumption. They are consumed fresh and in processed form as juice, wines, flavors, and extracts. Commercial pomegranate juice (PJ) has one of the highest antioxidant activities compared to other fruit juices, red wine, and green tea (*8*). This can be attributed to its high content of polyphenols and in particular to its ellagitannins, the major ones being punicalagins (*8*, *9*). (Punicalagins are often noted in the singular *punicalagin*; however, it is found naturally as two reversible anomers, α and *β*, hence the plural.) Polyphenols such as condensed tannins, anthocyanins, and minor flavonoids are also present in pomegranates (*10*).

The potent bioactivity of punicalagins and other ellagitannins can be explained by its ability to hydrolyze into ellagic acid (EA) and other smaller polyphenols in vivo and across the mitochondrial membrane in vitro (*11*, *12*) (**Figure 1**). EA concentration above 5% in the juice and extracts of pomegranate and grapes may be explained by hydrolysis of ellagitannins formed during processing and extraction (*13*, *14*). Since detection of EA in human plasma may be dependent on the consumption of larger ellagitannins such as punicalagins (*11*),

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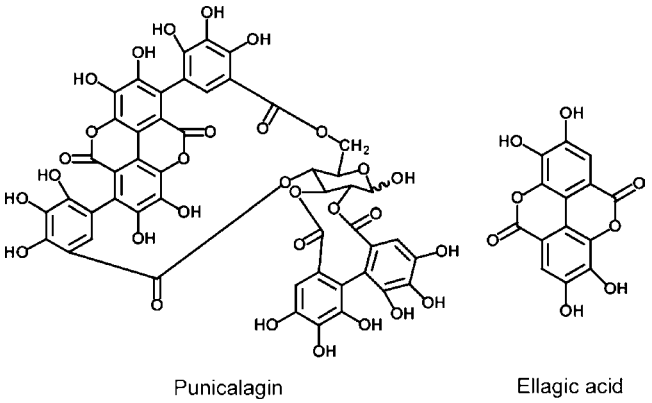

Figure 1. Chemical structure of punicalagin and EA.

quantitative pharmacokinetic studies using pomegranate can utilize punicalagins as the fruit's naturally existing biomarker.

While several studies investigate the effects of pomegranate extracts in vitro and in animals (*8*, *9*, *15*, *16*), clinical studies on pomegranate, particularly those examining its benefits on cardiovascular health, have utilized commercial PJ (*10*, *17*, *18*). Commercial PJ can be obtained by pressing whole pomegranate fruits and its peels, whereby water-soluble compounds present in the whole fruit, including the major ellagitannins punicalagins, are extracted in significant amounts into PJ. Punicalagin levels are widely variable in PJ and can range from as low as 0.017 to 1.5 g/L of PJ depending on the fruit cultivar as well as processing and storage conditions. Therefore, the regular consumption of PJ may provide significant amounts of this water-soluble hydrolyzable ellagitannin (*8*, *19*). However, there are limitations of using PJ as a means to enhance health for those concerned about sugar and caloric intake. In light of the recent proposed guidelines on beverage consumption, there may be a need to explore alternate ways to consume the beneficial compounds contained in the recommended 5−9 servings of fruits and vegetables per day (*20*, *21*).

Several studies have demonstrated the anti-inflammatory properties and immunomodulatory effects of polyphenolic compounds, where many studies show a decreasing effect of polyphenols on a previously increased biomarker for inflammation, such as IL-1, IL-6, and TNF-alpha (*22*−*24*). Specifically, EA has been shown to inhibit IL-1beta- and TNF-alpha-induced activation of activator protein-1 and mitogen-activated protein kinases in activated pancreatic stellate cells in vitro (*25*). Some studies demonstrate the inductive effect of polyphenols on immune factors including secretion of cytokines IL-2 and IL-4 (*26*, *27*). To determine the effects of pomegranate extract on a particular marker of immune health, this study investigated the influence of the consumption of pomegranate extract on plasma concentrations of interleukin-6.

There have been a few human bioavailability studies using PJ (*11*, *28*, *29*); however, no human intervention studies have been conducted with an oral administration of a pomegranate extract standardized to punicalagins. Due to the health benefits now attributed to pomegranate consumption and the rising popularity of pomegranate botanical supplements, human intervention studies using pomegranate extract supplements are crucial for determining the efficacy of pomegranate extracts in the prevention of chronic diseases and establishing science-based dosing recommendations. The current study is the first to investigate pharmacokinetic parameters, absorption, metabolism, and ex vivo biological activities of a whole-fruit extract of pomegranate standardized to punicalagins in healthy human volunteers.

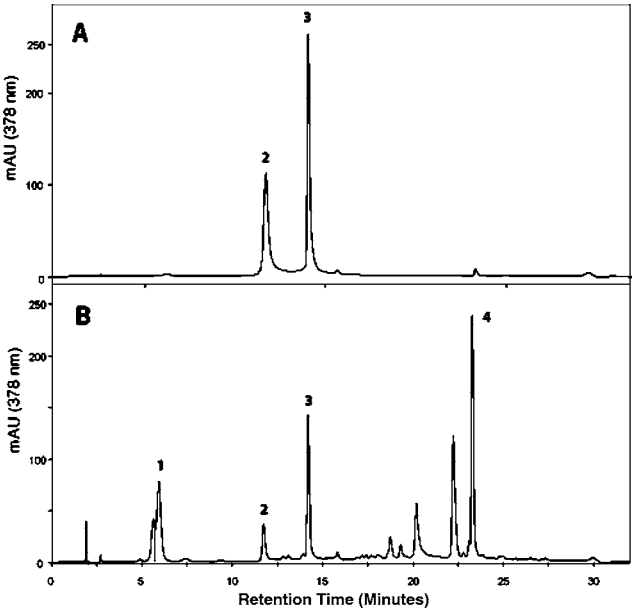

Figure 2. HPLC analysis of punicalagin standard (A) and pomegranate extract standardized to punicalagins (B), containing gallic acid (*1*), punicalagin α (*2*), punicalagin β (*3*), and ellagic acid (*4*).

MATERIALS AND METHODS

Pomegranate Extract. Standardized pomegranate extract in capsule form was provided by Geni Herbs, (POMELLA, Noblesville, IN). Each capsule contained 400 mg of pomegranate extract. The 800 mg of extract used in this study contained 330.4 mg of the major ellagitannins punicalagins and 21.6 mg of EA as shown in the HPLC-PDA profile (**Figure 2**). The HPLC system included a LC-10ATVP pump, Rheodyne-7725i sampler, and SPD-10AVP detector (all Shimadzu, Japan) with Winchrome software and a Luna C-18 column (4.6 × 150 mm) (Phenomenex, USA). The mobile phase, solvent A (2% glacial acetic acid in water) and solvent B (2% aqueous acetic acid in water), was used in binary linear gradient conditions as follows: 0−5 min, 99% A in B; 5−20 min, 99−40% A in B; 21−30 min, 40−10% A in B with a flow rate of 1.0 mL/ min. The wavelength was monitored at 378 nm for punicalagins and 366 nm for EA.

Study Design. *Washout Period.* Thirteen healthy male and nonpregnant/nonlactating female subjects (BMI = 32.6 ± 0.98, age = 37.6 ± 3.6) were asked to abstain from polyphenol-containing foods, such as tea, wines, berry fruits, etc., for 3 days according to a detailed list they were given, after approval from the University of Florida Institutional Review Board. Moreover, subjects were asked not to consume large amounts of alcohol and antioxidant supplements, not to exercise excessively, and to sleep at least 6−8 h during the night before the study day. The washout period ended with an overnight fast (at least 8 h) before the study day.

Study Day. Eleven subjects completed the study day. Two subjects withdrew for personal reasons. Subjects received two capsules, each containing 400 mg of pomegranate extract with 6 oz of water. Blood was drawn at baseline, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, and 24 h after the consumption. Subjects received 2−3 crackers with 8 oz of water 2−3 h after ingestion, a polyphenol-free low-fat sandwich 4−5 h after ingestion, and a frozen polyphenol-free pasta dinner to take home.

HPLC-MS Analysis of Plasma Samples. EDTA-blood samples were centrifuged at 800*g* and plasma was stored at −80 °C until analysis. Plasma was processed for LC-MS analyses as previously described (*29*). LC-MS conditions were as follows: The HPLC system (Agilent Technologies, Waldbronn, Germany) was equipped with a diode array detector (DAD) and mass detector in series with a binary pump and autosampler and controlled by software (v. A08.03) from Agilent Technologies (Waldbronn, Germany). The HPLC column was a C_{18} LiChroCART column (25 × 0.4 cm, particle size 5 m, Merck) and the solvent system consisted of a gradient system with water (5% formic acid, v/v) (A) and methanol (B) as the mobile phases at a flow

rate of 1 mL/min. The linear gradient started with 1% B, 5 min to 20% B in A at 20 min, 40% B in A at 30 min, and 95% B in A at 35−39 min. The mass detector consisted of an ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS−MS) spectra were measured from *m*/*z* 100 to *m*/ *z* 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. All analyses were performed in triplicate. EA and EA-metabolites were identified by comparison of their respective ions and MS/MS ions as reported (*30*). Briefly, control plasma was spiked with individual solutions and extracted as previously described (*11*). For example, EA standard (25 μg/mL) was solubilized in DMSO and serially diluted to eventually provide 15.625, 7.8125, 3.90625, 1.953125, and 0.97656 ng/mL solutions. Each plasma sample was separately extracted (×3) and each sample was injected in triplicate on the HPLC-MS. Concentrations were then determined from the peak area by using the equation for linear regression obtained from the calibration curve. The lower limit of quantification for EA was 1.6 ng/mL.

Antioxidant Capacity (ORAC Assay). Plasma samples were acidified 3:1 with 0.44 M TFA and centrifuged, and the deproteinated supernatant was diluted with phosphate buffered saline for the ORAC analysis as previously described by Cao et al. and subsequently modified by Ou et al. with fluorescein as fluorescent probe (*31*, *32*). Peroxyl radicals were generated by 2,2′-azobis(2-amidinopropane) dihydrochloride, and fluorescence was monitored at 485 nm excitation and 538 nm emission on a Bio-Tek Synergy KC4 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT).

Generation of Reactive Oxygen Species. To determine the extent of intracellular ROS induced in peripheral blood mononuclear cells, a fluorescence method using dichlorofluorescein-diacetate as probe was adapted from Wang and Joseph (*33*). Cell concentration was adjusted to 1×10^6 cells/mL. Cells were washed twice with PBS and incubated with 10 μmol/L DCFH-DA for 30 min at 37 °C to preload cells with DCFH-DA substrate. After the cells were washed, they were incubated with plasma and with 100 mmol/L hydrogen peroxide. Fluorescence was assessed at 60 min after the incubation on a Bio-Tek Synergy KC4 fluorescence plate reader (Bio-Tek Instruments, Winooski, VT).

Interleukin-6. Plasma concentrations of interleukin-6 were determined with the human IL-6 Ultra Sensitive Kit, Biosource, Camarillo, CA, following the manufacturer's protocol.

Statistical and Pharmacokinetic Analysis. All values are presented as means and where indicated the ratio to the control baseline is given. Statistical analysis was performed with a one-way analysis of variance ANOVA with a Tukey-Cramer comparison of all means as post hoc analysis with the JUMP software (SAS Institute, Cary, NC), which was performed for 0−2 h for the ORAC assay and for 0−8 h for the Il-6 assay. Pharmacokinetic analysis of EA concentrations in plasma was performed using the mean of all subjects by noncompartmental analysis using NCA model 200 of the WINNONLIN 3.1.168 software (Pharsight, Mountain View, CA).

RESULTS AND DISCUSSION

EA and metabolites were found in plasma after the consumption of pomegranate extract, with intersubject variability. The extract potentially induced a significant antioxidant effect in plasma after consumption. Overall, this exploratory study with pomegranate extract demonstrated bioavailability for EA and its derivates after consumption of a pomegranate extract high in ellagitannins, indicating its potential in the promotion of health.

HPLC-MS Analysis of Plasma Samples. Eleven healthy subjects consumed 2 capsules of pomegranate extract (330.4 mg of punicalagin isomers and 21.6 mg of free EA). EA and its in vivo generated metabolites were measured by LC-MS/MS methods. In addition, ex vivo antioxidant activity of plasma (ORAC assays) was determined. Punicalagins were not detected in plasma as previously reported for animals (*29*). However, consistent with previous studies involving consumption of ellagitannins, EA and EA-derived metabolites were detected as follows: 3,8-dihydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one glucuronide (urolithin A, M−H *m*/*z* 389), hydroxy urolithin A (M−H *m*/*z* 243), urolithin A-glucuronide (M−H *m*/*z* 403), 3-hydroxy-6*H*-dibenzo[*b*,*d*]pyran-6-one glucuronide (urolithin B; *m*/*z* 227), and dimethyl ellagic acid-glucuronide (M−H *m*/*z* 505) (*29*, *30*). Urolithins A and B were detected after 8 and 24 h in altogether 3 of the 11 subjects, whereas urolithin A-glucuronide was detected in 6 of the 11 subjects. Hydroxyl-urolithin A was found in 3 subjects at several time points from 2 to 24 h. Also urolithin A-glucuronide was found over a period of 2−24 h in 6 of the subjects. Dimethyl ellagic acid-glucuronide was detected in 2 subjects after 8 and 24 h. Urolithins have been proposed as microbial metabolites that are biosynthesized in the colon and can circulate in plasma up to 24 h after the intake (*29*, *34*). The presence of these metabolites in early plasma samples (<8 h) in our study may be attributed to either the action of colonic microflora on ellagitannins previously consumed by the subjects or may be due to plasma enzymatic action on circulating ellagic acid, although the latter possibility remains to be investigated.

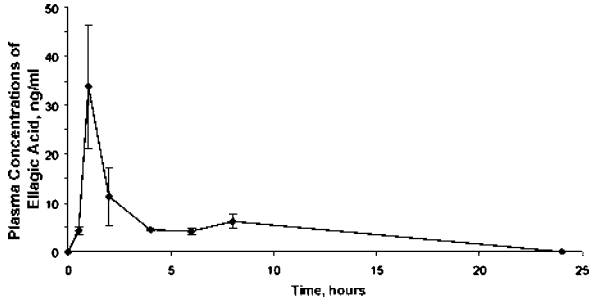

Figure 3. Plasma concentrations of EA after the consumption of 800 mg of pomegranate extract ($n = 11$). Values are means ± SEM.

Pharmacokinetic Analysis. High interindividual variability was observed, as the metabolism of polyphenols is known to vary in human subjects (*34*). For several subjects a complete data set was not available due to concentrations being below the lower limit of quantification of 1.6 ng/mL. For this reason pharmacokinetic parameters were estimated using the mean for all subjects. Three of the subjects had extraordinarily high concentrations of EA in their plasma after 24 h. These values were eliminated, as noncompliance was assumed. The observed mean C_{max} and t_{max} for EA in plasma were determined as 33.8 ± 12.7 ng/mL at 1 h (Figure 3).

In a previous study with PJ, where C_{max} was 31.9 ng/mL after 1 h, the administered juice contained 25 mg of EA and 318 mg of ellagitannins (*11*). The 800 mg of extract used in this study contained 330.4 mg of the major ellagitannins punicalagins and 21.6 mg of EA. The similar content of both ellagitannin-rich treatments is reflected in a comparable C_{max} at 1 h after the consumption. These results would indicate that the bioavailability of EA derivates of ellagitannins from PJ and extracts is comparable at the administered doses.

However, a previous comparison between a beverage and an extract from the beverage (tea and tea extract) demonstrated a difference in absorbance of polyphenolic compounds when comparing naturally dissolved compounds to a dried extract (*5*, *6*). In this study the AUC (area under the curve), MRT (mean residence time), and terminal half-life were estimated as 118.01 ng h/mL, 5.5 h, and 0.942 h, respectively. These data overall compare well to the previously conducted trial; however, no pharmacokinetic parameters were estimated in that previous

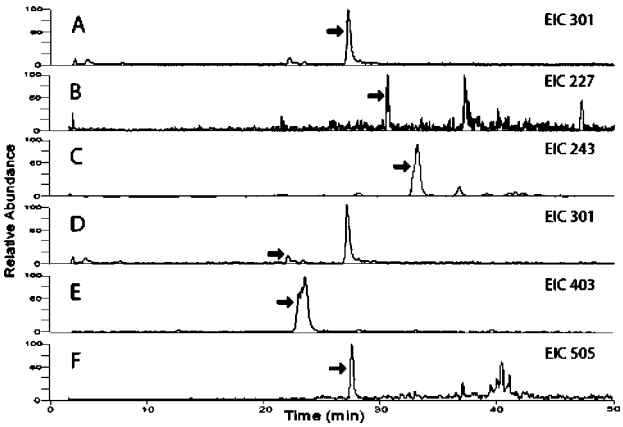

Figure 4. LC-MS extracted ion chromatograms showing the presence of ellagitannin metabolites in human plasma corresponding to previous report (*29*, *30*). Figure (A) ellagic acid (*m*/*z* 301); (B) urolithin A (*m*/*z* 227); (C) hydroxyl-urolithin A (*m*/*z* 243); (D) urolithin B (*m*/*z* 211); (E) urolithin A-glucuronide (*m*/*z* 403); (F) dimethylellagic acid glucuronide (*m*/*z* 505).

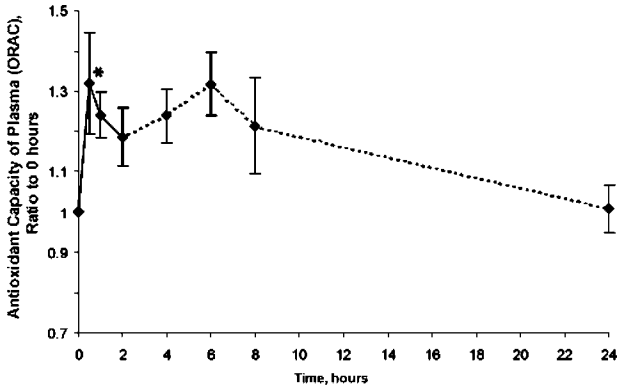

Figure 5. Antioxidant capacity in plasma after the consumption of 800 mg of pomegranate extract ($n = 11$). Values are means ± SEM. Values are deemed significantly different from the baseline (0 h) at $p \leq 0.05$ and are marked with an asterisk. Post hoc analysis was performed for 0–2 h, to exclude the effect of food consumption. The dashed line connects data points excluded from the post hoc analysis.

study (*11*). In another clinical trial with 18 human subjects who consumed 180 mL of PJ containing 318 mg of ellagitannins, pharmacokinetic parameters of EA in plasma were observed as $C_{max} = 18.64$ ng/mL and $T_{max} = 0.98$ h and estimated as AUC = 50.07 ng/h/mL and $t_{1/2} = 0.75$ h (*28*). Pharmacokinetic parameters determined in this study are within a similar range compared to the previous studies performed with PJ.

Antioxidant Capacity (ORAC Assay). After the consumption of the pomegranate extract, the antioxidant capacity of plasma was significantly increased after 0.5 h (31.8%) (Figure 4). The individual increase in antioxidant capacity was up to 2.55-fold after 0.5 h, up to 1.62-fold after 1 h, and up to 1.43-fold after 2 h. A second peak in antioxidant capacity was determined after 6 h (31.7%). Subjects were allowed to consume food after the 4 h blood draw; therefore, this second increase in antioxidant capacity is likely to be influenced by the consumption of food or potentially by more slowly absorbed antioxidant compounds from the pomegranate extract. Since t_{max} of EA was observed after 1 h, the peak effects of antioxidant capacity in plasma may not have been caused by EA but rather by other phenolic compounds, metabolites, or a combination of these with EA. Previous studies demonstrated the correlation of fructose consumption to uric acid concentrations in plasma and uric acid concentration to plasma antioxidant capacity (*35*, *36*). The extract used for this study did not contain any fructose or other sugars, so that an increase in uric acid caused by fructose can be excluded for this study for the initial period of 4 h after the consumption. Several studies demonstrated antioxidant effects in plasma of polyphenol-rich extracts and fruit juices (*37*−*39*). Specifically, in a previous study with 18 human volunteers a maximum EA concentration in plasma of 18.5 ng/mL (compared to a C_{max} of 33.8 ng/mL in this study) has been found; no significant changes in the antioxidant potential of plasma were observed after the consumption of 180 mL of PJ (*28*). Overall, the pomegranate extract administered in this study potentially caused an increase in plasma antioxidant potential, characterized by a high intersubject variability. A more comprehensive study including a placebo group is required to confirm the antioxidant effects of the pomegranate extract.

Generation of Reactive Oxygen Species (DCF-DA Assay). The generation of ROS was determined in PBMC after 1 h of incubation with 100 mM hydrogen peroxide and 10% plasma collected from 0−4 h after the consumption. Previously, polyphenols have been demonstrated to increase as well as decrease the generation of ROS (*40*−*42*). Conversely, in this study no change in the generation of ROS has been observed (data not shown). Potentially, the concentrations of polyphenols in plasma were not high enough to have a significant influence on the generation of ROS.

Interleukin-6. Concentrations of interleukin-6 were determined at 4 h after the consumption of the extract. Overall, plasma concentrations of IL-6 were not significantly increased after 4 h. A follow-up assessment of IL-6 in plasma samples collected after 6 and 8 h indicated a significant increase by 3.36, 6.5, and 6.92-fold, ($p \leq 0.05$), respectively (data not shown). However, since food was consumed after 4 h, this increase cannot be ascribed to the pomegranate extract in this study. Moreover, IL-6 concentrations in plasma are characterized by a circadian rhythm with zenith at 19.00 and 05.00 h (*43*). Previous studies have determined polyphenols to influence immune-relevant biomarkers in a decreasing or increasing manner (*26*, *27*, *44*, *45*). In this study, the baseline concentrations of 0.07 ± 0.02 pg/mL Il-6 can be considered as being within a normal low range for healthy subjects according to their age range (*43*).

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