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Antioxidant, Antimalarial and Antimicrobial Activities of Tannin-Rich Fractions, Ellagitannins and Phenolic Acids from *Punica granatum* L.

Original Paper

Abstract

The *Punica granatum* L. (pomegranate) by-product POMx was partitioned between water, EtOAc and *n*-BuOH, and the EtOAc and *n*-BuOH extracts were purified by XAD-16 and Sephadex LH-20 column chromatography to afford ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**). Compounds **1 – 4** and the mixture of tannin fractions (XAD-16 eluates) were evaluated for antioxidant, antiplasmodial, and antimicrobial activities in cell-based assays. The mixture of tannins (TPT), XAD-EtOAc, XAD-H_2O, XAD-PJ and XAD-BuOH, exhibited IC_{50} values against reactive oxygen species (ROS) generation at 0.8 – 19 μg/mL. Compounds **1 – 4** showed IC_{50} values of 1.1, 3.2, 2.3 and 1.4 μM, respectively, against ROS generation and no toxicity up to 31.25 μg/mL against HL-60 cells. Gallagic acid (**2**) and punicalagins (**4**) exhibited antiplasmodial activity against *Plasmodium falciparum* D6 and W2 clones with IC_{50} values of 10.9, 10.6, 7.5 and 8.8 μM, respectively. Fractions XAD-EtOAc, XAD-BuOH, XAD-H_2O and XAD-PJ compounds **1 – 4** revealed antimicrobial activity when assayed against *Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Cryptococcus neoformans,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Aspergillus fumigatus* and *Mycobacterium intracellulare*. Compounds **2** and **4** showed activity against *P. aeruginosa, C. neoformans,* and MRSA. This is the first report on the antioxidant, antiplasmodial and antimicrobial activities of POMx isolates, including structure-activity relationships (SAR) of the free radical inhibition activity of compounds **1 – 4**. Our results suggest a beneficial effect from the daily intake of POMx and pomegranate juice (PJ) as dietary supplements to augment the human immune system's antioxidant, antimalarial and antimicrobial capacities.

Key words
Punica granatum · Punicaceae · ellagitannins · phenolic acids · antioxidant · antiplasmodial · antimicrobial activity

Supporting information available online at
http://www.thieme-connect.de/ejournals/toc/plantamedica

Introduction

The pomegranate plant is an erect shrub and its fruit is known to be a rich source of bioactive ellagitannins. It has been used for centuries in ancient cultures for medicinal purposes. The fruit is a globose berry, crowded by persistent calyx lobes, having a leathery pericarp filled with numerous seeds, which are surrounded by a pink to red, transparent, juicy, acidic, pleasantly tasting pulp [1]. Pomegranate's use has been mentioned in the ancient literature, including Ayurvedic texts, Ebers papyrus and Greek, Unani and Egyptian documents. It has been used as a vermifuge, astringent, bacteriocide, refrigerant, stimulant, stomachic, styptic, hair dye, and to alleviate the adverse effects of asthma, bronchitis, cough, cardiac problems, dysentery, diarrhea, dys-

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pepsia, fever, inflammation, bleeding disorders, piles, wounds, ulcers, bruises, sores, mouth lesions, stomatitis, vaginitis, respiratory and urinary tract infections, and as a febrifuge to ameliorate malaria and seasonal fevers [1], [2], [3], [4]. In recent years, the biological activities of pomegranate fruit rind polyphenols have received the increased attention of researchers and industry, as well as consumers. There have been a number of indications that dietary antioxidants offer effective protection from peroxidative damage caused by "reactive oxygen species" (ROS) and other free radicals. The dietary antioxidants are effective inhibitors of oxidative damage and mutagenicity induced during lipid peroxidation [5]. POMx and some of its chemical constituents were evaluated for antioxidant activity [6], atherogenic modifications [7], hepatoprotective [8], antiproliferative, and apoptotic [9] activities. ROS such as hydrogen peroxide (H_2O_2), superoxide anion radical ($O_2^{\cdot -}$), singlet oxygen, hydroxyl radical (·OH), and peroxynitrite ($ONOO^-$) are perceived to indiscriminately attack lipids, proteins, carbohydrates, causing cellular damage by peroxidation of the cell membrane [10], [11]. Several reports suggested that oxidative stress may lead to pathological conditions like ageing, cancer, liver damage, and heart and Alzheimer's disease [12], [13]. The cellular injury caused by excess of free radicals due to oxidative stress has been linked to over 200 clinical disorders [14]. Malaria is responsible for three million casualties annually in more than 100 countries [15], [16].

Despite several previous studies on the antioxidant properties of the mixture of PJ polyphenols, ellagic acid and the punicalagins (*vide supra*), POMx and its constituents have not been evaluated comprehensively for their antioxidant, antiplasmodial and antimicrobial effects. Recent studies have been done on pomegranate fruit ellagitannins and anthocyanins in crude extracts or purified fractions, but not on pure individual compounds. Fractionation and column chromatographic purification of POMx afforded ellagic acid (**1**), gallagic acid (**2**), punicalins (**3,** α- and β-anomers), and punicalagins (**4,** α- and β-anomers) (Fig. **1**) [17], [18], [19].

Herein, we present an overview of the isolation, identification, and assessment of antioxidant activity in a cell-based assay, and antimicrobial and antiplasmodial activities as well as SAR correlations of compounds **1 – 4** for their inhibition of ROS generation. This report represents the first comparative evaluation of antioxidant activity in a cell-based assay, and antimicrobial and antiplasmodial activities of compounds **1 – 4,** the POMx mixture, and the mixture of PJ anthocyanins.

Materials and Methods

General experimental procedures

All chemicals were of analytical grade. NMR spectra were recorded on Bruker 400 and 500 MHz spectrometers, and chemical shifts (δ) were referenced to TMS. Mass spectra (HR-ESI-MS) were run on an Agilent 1100 spectrometer. Millipore water was collected from a Millipore Millipack® Express, 0.22 μm water purifier (Millipore; Billerica, MA, USA). Column chromatography was performed on Amberlite® XAD-16® resin (Supelco; Bellefonte, PA, USA) and Sephadex LH-20 (Sigma-Aldrich; St. Louis, MO, USA). Normal TLC was conducted on precoated silica gel 60 F_{254} plates (Merck; Darmstadt, Germany) with $CHCl_3$:MeOH:-HOAc (7 : 2.5 : 0.5), sprayed with 5 % $FeCl_3$ solution, which gave a characteristic bluish-black coloration for gallo- and ellagitannins. 2D TLC was performed on cellulose plates (Fluka; Buchs, Switzerland), with fluorescent indicator 254 nm, size 20 cm × 20 cm with *sec*-BuOH saturated with H_2O for one direction, and 2 % HOAc in H_2O for the second direction. Roswell Park Memorial Institute-1640 (RPMI-1640) medium, penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA).

Plant material

Pomegranate juice by-product (70 Brix) (POMx) and pomegranate juice (PJ) were provided by POM Wonderful LLC (Los Angeles, CA, USA) in July, 2005.

Extraction and isolation

The commercial POMx (100 mL) was diluted to 500 mL with Millipore purified H_2O and successively partitioned with EtOAc

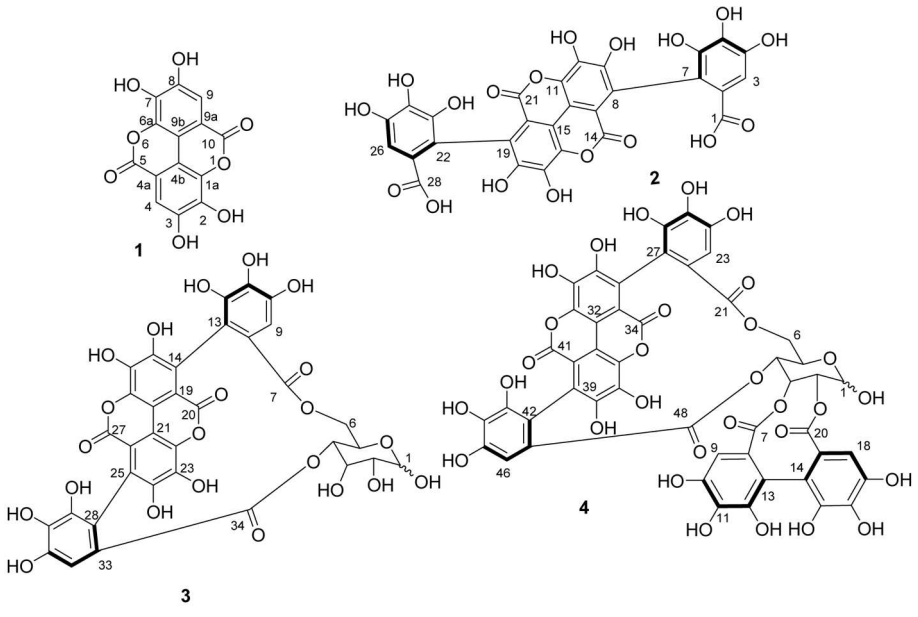

Fig. **1** Structures of ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**).

(3×200 mL) and *n*-BuOH (3×200 mL). The EtOAc and *n*-BuOH fractions were separately concentrated under reduced pressure to yield EtOAc (2.50 g) and *n*-BuOH extracts (9.42 g), respectively. The EtOAc extract (1.2 g) was subjected to column chromatography on Amberlite XAD-16 (500 g, 6×35 cm) and eluted with H_2O (1.5 L) and MeOH (1.5 L). The MeOH fraction upon removal of solvent under vacuum afforded a mixture of tannins fraction, XAD-EtOAc (1.0 g). This was subjected to column chromatography on Sephadex LH-20 with H_2O-MeOH gradient (700 mL) and MeOH (500 mL) as eluents to afford ellagic acid **1** (21 mg).

The *n*-BuOH extract (2.0 g) was subjected to Amberlite XAD-16 column chromatography (500 g, 6×35 cm) and eluted with H_2O (2.0 L) and MeOH (2.0 L), successively. The MeOH fraction on removal of solvent under reduced pressure afforded a tannin fraction (XAD-BuOH) (1.3 g). This was further purified on Sephadex LH-20 CC (6×55 cm), eluted with H_2O:MeOH, 2 : 8 (350 mL), 1 : 9 (500 mL), MeOH (450 mL) and MeOH:Me_2CO, 1 : 1 (600 mL), to give nine fractions. Subfraction 4 was further purified on a small Sephadex LH-20 column with H_2O:MeOH, 1 : 9 (350 mL), and MeOH (400 mL) to yield gallagic acid **2** (18.0 mg). Subfractions 5 and 6 were combined and rechromatographed on Sephadex LH-20 with H_2O-MeOH gradient, 3 : 7 (250 mL), 1 : 9 (500 mL), and MeOH (300 mL) to give punicalins **3** (19 mg).

Subfractions 8 and 9 were combined and further purified on Sephadex LH-20 with MeOH (350 mL), and MeOH:Me_2CO, 1 : 1 (400 mL) as eluent to yield punicalagins **4** (35 mg). The purification process was monitored by 1D and 2D TLC.

The aqueous layers were lyophilized, the residue (1.5 g) was absorbed on XAD-16, and eluted with H_2O (2.0 L) and MeOH (1.5 L), consecutively. The MeOH eluate yielded the XAD-H_2O tannin mixture (0.8 g) upon removal of solvent.

Crude POMx (1.0 g) was chromatographed on Amberlite XAD-16 eluting consecutively with H_2O (1.5 L) and MeOH (1.5 L). The methanol eluate was evaporated to dryness to give the total pomegranate tannins (TPT) (600 mg).

The crude pomegranate juice (PJ) (50 mL) was diluted with H_2O (50 mL) and was chromatographed on Amberlite XAD-16 eluting consecutively with H_2O (2.0 L) and acidified MeOH (pH, 3) (2.0 L), to afford semi-pure anthocyanins (XAD-PJ).

Characterization of isolates

Ellagic acid (**1**) was obtained after lyophilization of appropriate aqueous fractions as a pale yellow powder (21mg). It showed a dark green color with alcoholic ferric chloride, and an $[M - H]^-$ molecular ion at *m/z* = 301.0123 in high resolution electrospray ionization mass spectrometry (HR-ESI-MS), confirming a molecular formula of $C_{14}H_6O_8$. The ^{1}H- and ^{13}C-NMR spectra of **1** were identical with published data [17].

Gallagic acid (**2**) crystallized from methanol as dark brown crystals (18 mg). It gave a dark green color with 5% alcoholic ferric chloride solution, and analyzed for $C_{28}H_{14}O_{18}$ which is consistent with an $[M + H]^+$ molecular ion at *m/z* = 639.2425 and an $[M - 2H]^-$ ion at *m/z* = 636.2433, in positive and negative mode HR-ESI-MS, respectively. The ^{1}H-NMR spectra of **2** showed two characteristic singlets at δ = 7.08 and 6.92 for H-3 and H- 26, respectively. The ^{13}C-NMR spectrum corroborated the molecular formula showing resonances for 28 carbon atoms at δ = 167.50 (C-1), 115.01 (C-2), 107.85 (C-3), 144.07 (C-4), 139.08 (C-5), 144.29 (C-6), 113.48 (C-7), 109.08 (C-8), 144.80 (C-9), 142.18 (C-10), 135.94 (C-11), 112.63 (C-12), 124.99 (C-13), 157.91 (C-14), 110.35 (C-15), 135.94 (C-16), 142.90 (C-17), 144.67 (C-18), 109.58 (C-19), 124.99 (C-20), 158.89 (C-21), 114.08 (C-22), 143.90 (C-23), 143.17 (C-24), 143.25 (C-25), 106.80 (C-26), 125.20 (C-27), and 169.12 (C-28). Thus, the structure of compound **2** was confirmed as gallagic acid [18], [19].

Punicalins (**3**) analyzed for an empirical formula $C_{34}H_{22}O_{22}$ (m/z = 782). It showed an $[M + H]^+$ ion at *m/z* = 783.5722 in the positive HR-ESI-MS, and an $[M - H]^-$ ion at *m/z* = 781.0331 in the negative mode spectrum. The ^{1}H- and ^{13}C-NMR spectra of **3** were identical with published data [18], [19].

Punicalagins **4** analyzed for $C_{48}H_{28}O_{30}$ by an $[M + Na]^+$ ion at *m/z* = 1107.0586 and an $[M + K]^+$ ion at *m/z* = 1123.0311 in the positive mode HR-ESI-MS, and the $[M - H]^-$ ion at *m/z* = 1083.0369 in the negative mode HR-ESI-MS. The ^{1}H- and ^{13}C-NMR spectra were in accordance with literature data [18], [19].

Antioxidant assay

Antioxidant activity was determined by the DCFH-DA (2′,7′-dichlorodihydrofluorescein diacetate) method. Myelomonocytic HL-60 cells (1×10^6cells/mL, ATCC; Manassas, VA, USA) were suspended in RPMI-1640 medium with 10% fetal bovine serum, penicillin (50 units/mL) and streptomycin (50 μg/mL). The cell suspension (125 μL) was added to the wells of a 96-well plate. After treatment with different concentrations of the test compounds for 30 min, cells were stimulated with 100 ng/mL phorbol 12-myristate-13-acetate (PMA; Sigma) for 30 min. DCFH-DA (5 μg/mL; Molecular Probes; Eugene, OR, USA) was added and cells were further incubated for 15 min. Vitamin C was used as the positive control. Levels of fluorescent DCF (produced by ROS catalyzed oxidation) were measured on a PolarStar with excitation wavelength at 485 nm and emission at 530 nm.

DCFH-DA is a non-fluorescent probe that diffuses into cells, where cytoplasmic esterases hydrolyse the DCFH-DA to the non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH). The ROS generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells is measured in comparison to PMA-treated vehicle control. The cytotoxicity to HL-60 cells was also determined after incubating the cells (2×10^4 cells/well in 225 μL) with test samples for 48 h by the XTT method [20].

Antimicrobial assay

All organisms were obtained from the American Type Culture Collection (Manassas, VA. USA) and included *C. albicans* ATCC 90028, *C. neoformans* ATCC 90113, *A. fumigatus* ATCC 90906, MRSA ATCC 43300, *E. coli* ATCC 35218, *P. aeruginosa* ATCC 27853, and *M. intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the NCCLS methods [21], [22], [23]. *M. intracellulare* and *A. fumigatus* growth were monitored using Alamar Blue™ (BioSource International; Cama-

rillo, CA, USA) [21], [22], [23]. Samples dissolved in DMSO were serially diluted using 20%/0.9% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula were prepared by diluting saline suspensions of colonies with assay broth to afford recommended colony forming units/mL. Growth (saline only), solvent and blank (media only) controls were included on each test plate. Drug controls [ciprofloxacin (ICN Biomedicals; Aurora, OH, USA) for bacteria and amphotericin B (ICN Biomedicals) for fungi] were included in each assay. All organisms were read at either 630 nm using the EL-340 Biokinetics Reader (Bio-Tek Instruments; Winooski, VT, USA) or 544 ex/590 em, (*M. intracellulare*, *A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies; Offenberg, Germany) prior to and after incubation. The MIC (minimum inhibitory concentration) was defined as the lowest test concentration that allowed no detectable growth.

Antiplasmodial assay

A suspension of red blood cells infected with *P. falciparum* (D6) or (W2) strains (200 μL, with 2% parasitemia and 2% hematocrit in RPMI-1640 medium supplemented with 10% human serum and 60 μg/mL amikacin; obtained from the Walter Reed Army Institute of Research; Silver Sping, MD, USA) was added to the wells of a 96-well plate containing 10 μL of test samples at various concentrations. The plate was flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2, in a modular incubation chamber (Billups-Rothenberg, 4464 M; Del Mar, CA, USA) and incubated at 37 °C, for 72 h. Plasmodial lactate dehydrogenase (LDH) activity was determined by using Malstat™ reagent (Flow Inc.; Portland, OR, USA) as described earlier [24], [25]. IC_{50} values were computed from the dose-response curves generated by plotting percent growth against test concentrations. DMSO (0.25%), artemisinin, and chloroquine were included in each assay as vehicle and drug controls, respectively.

All the bioassay experiments were conducted in duplicate for each concentration.

Supporting information

The dose effect of TPT on ROS inhibitory activity and the effects of XAD-EtOAc, XAD-BuOH, XAD-H_2O, XAD-PJ, ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**) on ROS inhibitory activity are available as Supporting Information.

Results

TPT, mixture of tannins XAD-EtOAc, XAD-BuOH, XAD-H_2O, phenolic acids (**1**, **2**), ellagitannins (**3**, **4**), as well as XAD-PJ were evaluated for antioxidant, antiplasmodial and antimicrobial activities. As shown in Fig. **1A** (see Supporting Information) and Table **1** TPT exhibited antioxidant activity at an IC_{50} of 11 μg/mL. Among the mixture of tannins, XAD-EtOAc, and XAD-BuOH were much more potent (IC_{50} values 1.5 and 0.8 μg/mL, respectively) than XAD-H_2O (IC_{50} of 19 μg/mL). The mixture of anthocyanins (XAD-PJ) exhibited antioxidant activity at 7.0 μg/mL (Fig. **2B** Supporting Information**,** Table **1**). Among the purified compounds, ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**) showed antioxidant effects and strongly inhibited ROS generation with IC_{50}'s of 1.1, 3.2, 2.3 and 1.4 μM, respectively (Fig. **2B** Supporting Information**,** Table **1**) in comparison to the IC_{50} value of 1. 9 μM for the positive control, vitamin C.

Table **1** Antioxidant activity of TPT, XAD-EtOAc, XAD-BuOH, XAD-H_2O, XAD-PJ, ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**) on ROS scavenging activity

Sample	*Antioxidant Activity [IC_{50} µg/mL]*	*Cytotoxicity (HL-60 Cells) [IC_{50} µg/mL]*
TPT	11.0	NC*
XAD-EtOAc	1.5	NC
XAD-H_2O	19.0	NC
XAD-PJ	7.0	NC
XAD-BuOH	0.8	NC
Ellagic Acid (**1**)	0.33 (1.1 μM)	NC
Gallagic Acid (**2**)	2.1 (3.2μM)	NC
Punicalins (**3**)	1.8 (2.3 μM)	NC
Punicalagins (**4**)	1.6 (1.4 μM)	NC
Vitamin C	0.35 (1.9 μM)	NC
Doxorubicin	NT	0.06

NC = No cytotoxicity up to 31.25 μg/mL
NC* = No cytotoxicity up to 62.5 μg/mL
NT = Not tested

In the antiplasmodial assay TPT, XAD-EtOAc, XAD-BuOH, XAD-H_2O, and XAD-PJ did not inhibit the growth of *P. falciparum*. However, punicalagins (**4**) and gallagic acid (**2**) showed activity with IC_{50} values of 7. 5 and 8.8, and 10.9 and 10.6 μM against *P. falciparum* D6 and W2 strains, respectively (Table **2**).

The fractions and isolated compounds were tested for activity against a panel of fungi (*C. albicans, C. neoformans* and *A. fumigatus*) and bacteria (*E. coli, P. aeruginosa,* MRSA, and *M. intracellulare*). As shown in Table **3** XAD-EtOAc exhibited IC_{50} values against *E. coli* of 50 μg/mL, *P. aeruginosa* of 45 μg/mL, and MRSA of 50 μg/mL. XAD-H_2O showed 50% growth inhibition against *E. coli* (30 μg/mL), *P. aeruginosa* (30 μg/mL), *C. neoformans* (15 μg/mL), and MRSA (50 μg/mL). XAD-PJ showed IC_{50} values against *E. coli* (25 μg/mL), *P. aeruginosa* (20 μg/mL), *C. neoformans* (25 μg/mL), and MRSA (25 μg/mL) (Table **3**). Compounds **1 – 4** were also tested for their antibacterial and antifungal activity. Compounds **1** and **3** demonstrated no inhibitory activity against all test organisms at the highest test concentration of 20 μg/mL. Compound **2** showed IC_{50} values against *E. coli* (23.5 μM, 15 μg/mL), *P. aeruginosa* (9.3 μM, 6.0 μg/mL), *C. neoformans* (15.6 μM, 10 μg/mL), and MRSA (31.3 μM, 20 μg/mL). Punicalagins (**4**) showed IC_{50} values against *E.coli* of 9.2 μM (10 μg/mL), *P. aeruginosa* of 3.2 μM (3.5 μg/mL), *C. neoformans* of 6.4 μM (7.0 μg/mL) and MRSA of 18.4 μM (20 μg/mL), respectively. Compounds **2** and **4** showed MIC values against *C. neoformans* of 31.3 (20 μg/mL) and 18.4 μM (20 μg/mL), respectively (Table **3**).

Discussion

Previous reports on phenolic acids and ellagitannins showed that they are effective antioxidants [6], [12]. However, the comparative antioxidant activity studies of pure compounds and mix-

Table **2** Antiplasmodial activity of gallagic acid (**2**) and punicalagins (**4**)

Sample	*P. falciparum D6 clone IC_{50} (μM)*	*P. falciparum W2 clone IC_{50} (μM)*	*Cytotoxicity (Vero cells)*
Ellagic acid (**1**)	NA	NA	NC
Gallagic acid (**2**)	10.9	10.6	NC
Punicalins (**3**)	NA	NA	NC
Punicalagins (**4**)	7.5	8.8	NC
Chloroquine	0.05	0.41	NT
Artemisinin	0.03	0.02	NT

NA = Not active at the highest concentration of 11.9 μg/mL.
NT = Not tested.
NC = No cytotoxicity at the highest concentration of 11.9 μg/mL.

tures of pomegranate fruit polyphenols **1 – 4** have not been reported. In addition, SAR correlations of antioxidant activities of compounds **1 – 4** are also lacking. In the present study ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), punicalagins (**4**), XAD-EtOAc, and XAD-BuOH showed significant antioxidant activity (Table **1**). However, TPT, XAD-H_2O and XAD-PJ did not exibit noticeable antioxidant activities (Table **1**). The antioxidant efficacy of pomegranate fruit total tannins and mixture of ellagitannins is explicable in terms of the efficiencies of ellagic acid (**1**) and punicalagins (**4**). XAD-PJ is less active than XAD-EtOAc and XAD-BuOH and compounds **1 – 4**, indicating that the ellagitannins are superior antioxidants compared to anthocyanins.

The strong antioxidant potency of tannins has been explained in terms of the number of phenolic hydroxy groups, and formation of stable reaction products. Polyphenols act as scavengers of ROS, peroxide decomposers, quenchers of singlet oxygen, electron donor, labile hydrogen donor, and inhibitors of lipoxygenase [26]. The ROS scavenging mechanism of the galloyl moiety is shown in Fig. **2**.

Compounds **2 – 4** all possess an ellagic acid moiety, such moiety exhibiting significant antioxidant activity compared to other ellagitannins and vitamin C (Fig. **2S** Supporting Information, Table **1**). The pronounced antioxidant activity of **1** has been explained in terms of its stable digallate unit and phenolic hydroxy groups [27]. The antioxidant ability of *P. granatum* fruit polyphenols ellagic acid (**1**), punicalagins (**4**), punicalins (**3**), and gallagic acid (**2**) is evident from their IC_{50} values of 1.1, 1.4, 2.3 and 3.2 μM, respectively. The observed SAR may be explained in terms of the ellagic acid, gallagyl and hexahydroxydiphenoyl (HHDP) moieties. Ellagic acid (**1**) and punicalagins (**4**) are more potent antioxidants compared to punicalins (**3**) and gallagic acid (**2**), the latter three compounds possessing the gallagyl moiety. Punicalagins (**4**) have an additional HHDP moiety compared to the structures of **2** and **3**. In the inhibition of ROS generation, gallagic acid (**2**) and punicalins (**3**) were weaker compared to punicalagin (**4**). The presence of an HHDP moiety in **4** may explain the superior antioxidant activity of **4**. Compounds **2** and **3** are also significant antioxidants compared to vitamin C. The multiple phenolic hydroxy groups in the HHDP and gallagyl moieties are assumed to increase antioxidative activity by the potential to form *o*- or *p*-quinones hence increasing additional resonance stabilization. Thus, it explains the significant role of the gallagyl and HHDP hydroxy groups in antioxidant activity. Previously, the antioxidant activity of polyphenols has been explained in terms of the number of phenolic hydroxy groups and particularly *o*-dihydroxy groups [28], [29]. The high antioxidant activities of **1 – 4** have been explained in terms of the higher number of phenolic hydroxy groups [30]. Thus, the presence of the ellagic acid moiety, considerable number of phenolic hydroxy groups, hydrophilic nature, and ability to penetrate the cell membrane may explain the antioxidant efficacy of compounds **1 – 4**, and the mixture of tannin fractions of pomegranate. Compounds **1 – 4**, and the mixture of tannins did not exhibit any cytotoxicity up to 31.25 μg/mL. Based on previous reports [12] water soluble antioxidants also showed mutagenic inhibition, hence highlighting the importance of water soluble dietary antioxidants. The mechanisms for inhibition of mutagenicity may be due to inhibition of free radical chain reactions as well as inhibition of the formation of by-products which may cause damage to DNA [12].

Purified ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**) and punicalagins (**4**) were assayed for their antiplasmodial activities against the *P. falciparum* D6 and W2 clones. Compounds **2** and **4** inhibited both *P. falciparum* D6 and W2 strains, with IC_{50} values of 10.9 and 10.6, and 7.5 and 8.8 μM, respectively (Table **2**). This

Table **3** Antimicrobial activity of XAD-EtOAc, XAD-H_2O, XAD-PJ, ellagic acid (**1**), gallagic acid (**2**), punicalins (**3**), and punicalagins (**4**)

	IC_{50} (μg/mL) for fractions and IC_{50}/MIC (μM) for pure compounds						
Sample	*C. albicans*	*E. coli*	*P. aeruginosa*	*C. neoformans*	*MRSA*	*M. intracellulare*	*A. fumigatus*
XAD-EtOAc	NA	50	45	NA	50	NA	NA
XAD-H_2O	NA	30	30	15	50	NA	NA
XAD-PJ	NA	25	20	25	25	NA	NA
Gallagic acid (**2**)	NA	23.5/NA	9.3/NA	15.6/31.3	31.3/NA	NA	NA
Punicalagins (**4**)	NA	9.2/NA	3.2/NA	6.4/18.4	18.4/NA	NA	NA
Amphotericin B	0.11/0.34	NT	NT	0.54/1.35	NT	NT	0.87/1.35
Ciprofloxacin	NT	0.03/0.06	0.21/0.75	NT	0.30/1.51	0.91/2.26	NT

NA = Not active at the highest test concentration of 50 μg/mL (XAD-EtOAc, XAD-H_2O and XAD-PJ).
NA = Not active at the highest test concentration of 31.3 (**2**) and 18.4 (**4**) μM, respectively.
NT = Not tested.

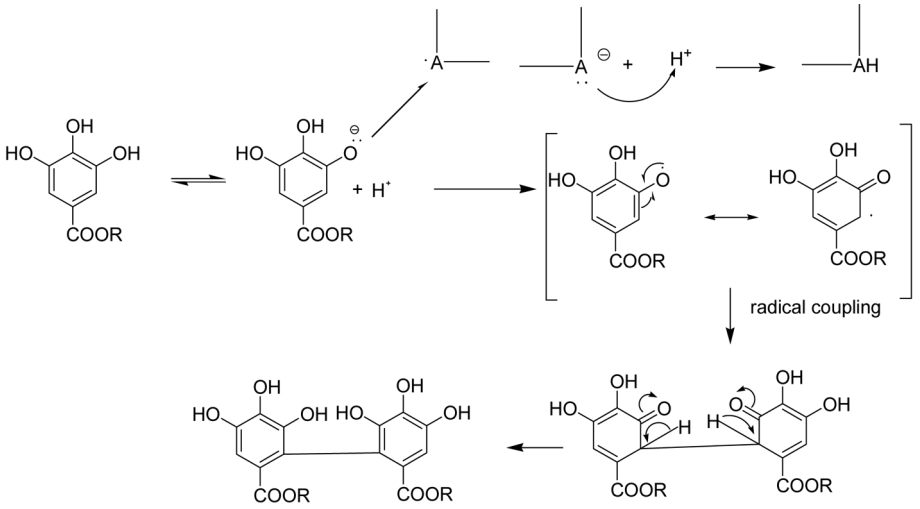

Fig. **2** The reaction of free radicals with alkyl gallate.

study thus rationalizes the traditional use of pomegranate in the treatment of plasmodial fevers [4].

In the antimicrobial assays (Table **3**) XAD-EtOAc showed low activity against *E. coli* (50 μg/mL), *P. aeruginosa* (45 μg/mL) and MRSA (50 μg/mL). XAD-H_2O and XAD-PJ weakly inhibited *E. coli* (30 and 25 μg/mL), *P. aeruginosa* (30 and 20 μg/mL), *C. neoformans* (15 and 25 μg/mL), and MRSA (50 and 25 μg/mL). XAD-H_2O and XAD-PJ exhibited stronger inhibition than XAD-EtOAc against the microbes tested (Table **3**). Compounds **2** and **4** exhibited moderate activity against *E. coli* (23.4 and 9.3 μM), *P. aeruginosa* (9.3 and 3.2 μM), *C. neoformans* (15.6 and 6.4 μM), and MRSA (31.3 and 18.4 μM), respectively, but compounds **1** and **3** were inactive against the microbes assayed at all test concentrations (Table **3**). We cannot explain the inactivity of punicalins (**3**) with its gallagyl structural moiety. Compounds **2** and **4** exhibited MIC (31.2 and 18.4 μM, respectively) against *C. neoformans*. Metabolites **2** and **4** had the strongest effect on the growth of bacteria and fungi. The antimicrobial results indicate that the role of the ellagic acid moiety is not significant compared to the gallagyl and HHDP moieties in the inhibition of microbes. Although the tannin-enriched fractions and the pure compounds **1 – 4** exhibit low to moderate activities against several microbes and fungi, this must be evaluated in terms of the use of pomegranate products as dietary supplements. When used as part of the human diet, these products may feasibly boost the human defence systems against some microbial and fungal infections.

In summary, compounds **1 – 4,** and mixture of tannins showed significant antioxidant activity. In the antiplasmodial studies gallagic acid (**2**) and punicalagins (**4**) showed inhibitory activity. Gallagic acid (**2**) and punicalagins (**4**) were also effective even at lower concentrations in the inhibition of microbes. Even though POMx and its isolates are considered to be dietary supplements, they exhibited noticeable activity all in antioxidant, antimalarial, and antimicrobial assays. Neither POMx nor the mixture of ellagitannins or compounds **1 – 4** showed cytoxicity against mammalian cells. This contrasts with the claims of toxicity of α- and β-punicalagin on liver necrosis of male albino mice [18], [19]. In these reports it was emphasized that hepatotoxicity only occurred at high punicalagin doses. In fact, at low doses, punicalagin is considered an antihepatotoxic compound. Thus, an adequate dosage of POMx polyphenols or PJ as part of a regular diet may be beneficial to human health and quality of life. However, it remains to be established whether the POMx polyphenols or PJ in the right dose provide the expected potency *in vivo* when consumed in combination.

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