Antiplasmodial activity of *Punica granatum* L. fruit rind

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A B S T R A C T

Aim of the study: Sun-dried rind of the immature fruit of *Punica granatum* L. (Punicaceae) (*Pg*) is presently used as a herbal formulation (OMARIA) in Orissa, India, for the therapy and prophylaxis of malaria. The aims of this study were (i) to assess in vitro the antiplasmodial activity of the methanolic extract, of a tannin enriched fraction and of compounds/metabolites of the antimalarial plant, (ii) to estimate the curative efficacy of the *Pg* extracts and (iii) to explore the mechanism of action of the antiplasmodial compounds. Urolithins, the ellagitannin metabolites, were also investigated for antiparasitic activity.

Materials and methods: Chloroquine-susceptible (D10) and -resistant (W2) strains of *Pf* were used for in vitro studies and the rodent malaria model *Plasmodium berghei*—BALB/c mice was used for in vivo assessments. Recombinant plasmepsins 2 and 4 were used to investigate the interference of *Pg* compounds with the metabolism of haemoglobin by malaria parasites.

Results: The *Pg* methanolic extract (*Pg*-MeOH) inhibited parasite growth in vitro with an *IC₅₀* of 4.5 and 2.8 µg/ml for D10 and W2 strain, respectively. The activity was found to be associated to the fraction enriched with tannins (*Pg*-FET, *IC₅₀* 2.9 and 1.5 µg/ml) in which punicalagins (29.1%), punicalins, ellagic acid (13.4%) and its glycoside could be identified. Plasmepsin 2 was inhibited by *Pg*-MeOH extract and by *Pg*-FET (*IC₅₀* 7.3 and 3.0 µg/ml), which could partly explain the antiparasitic effect. On the contrary, urolithins were inactive. Both *Pg*-MeOH extract and *Pg*-FET did not show any in vivo efficacy in the murine model.

Conclusions: The in vitro studies support the use of *Pg* as antimalarial remedy. Possible explanations for the negative in vivo results are discussed.

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1. Introduction

Pomegranate (*Punica granatum* L., Punicaceae) is used in the traditional medicine of different Asian cultures for the treatment of a variety of ailments. In Ayurvedic medicine the plant, described under its Sanskrit name “dalima” (fruit), is considered as a “blood purifier” and used to cure parasitic infections, aphthae (mouth ulcers), diarrhoea and ulcers (Jurenka, 2008). The root and stem barks are reported to have astringent and antihelmintic activity (Gracious Ross et al., 2001), and the fruit rind is traditionally used to treat diarrhoea and dysentery (Chopra et al., 1992). The decoction of the rind was found beneficial in fevers and chronic debility due to malaria (http://www.bpi.da.gov.ph/Publications/mp/pdf/g/granada, Reddy et al., 2007).

In the last decade some of the medical properties attributed to the *Punica granatum* (*Pg*) have been investigated by pharmacological in vitro studies. Antimicrobial activity of pomegranate extracts against *Staphylococcus aureus* (Braga et al., 2005), and *Escherichia coli* (Voravuthikunchai and Limsuwan, 2006) has been demonstrated in vitro. Moreover, the fruit rind powder was found to possess immunomodulatory properties (Gracious Ross et al., 2007).

The eastern province of Orissa (India) is an area endemic for *Plasmodium falciparum* (*Pf*) and *Plasmodium vivax* (*Pv*) and malaria constitutes a major health problem for the population, in particular for those living in rural areas. For several years, malaria patients...
referred to the Ayurveda dispensary and received a Pg herbal preparation named OMARIA (Orissa Malaria Research Indigenous Attempt). OMARIA is also distributed as a home based economic remedy for prophylaxis. Clinical application started in 1998 by the Indian Red Cross Society Charitable Ayurveda dispensary (c/o District Magistrate Koraput) on behalf of D. Bhattacharya. Since then, more than 1000 cases have been treated, from which the medical officer has generated 531 dispensary records. Dispensary records indicate that OMARIA can successfully control Pf and Pv infections in all patients including infants and pregnant women. According to the OMARIA promoter no cerebral malaria was recorded in individuals treated with OMARIA and no complaints of side effects were registered from the time when the program was started (Bhattacharya, 2003, 2004). OMARIA is administrated as gelatine capsules (m/s Sunil Health Care Ltd., Jaipur, India), containing each 825–850 mg of sun-dried rind of the immature Pg fruits, and 20% NaCl. The therapeutic dose is 1 capsule every 8h for 3 consecutive days. For prophylaxis, 1 capsule is taken in every day (children receive half the dosages). Records from the Ayurveda health structure indicate a positive impact on the health status of the population under OMARIA coverage: the prophylactic intervention appears not only to reduce malaria episodes but also to reduce the incidence of infectious diseases, such as measles, chicken pox and conjunctivitis (Bhattacharya, 2004).

Thus, the aim of this study was to characterize the antimalarial activity of Pg by (i) determining in vitro the activity of crude extracts, purified fractions and isolated compounds on sexual blood stages of Pf, both chloroquine-susceptible and -resistant strains; (ii) assessing in vivo the activity of extracts on asexual and sexual stages developing in the vertebrate and mosquito host, using the rodent malaria model Plasmodium berghei; (iii) identifying the active compounds and (iv) investigating the inhibitory effect of Pg extracts and isolated compounds on the activity of Pf plasmapesins 2 and 4, two aspartic proteases devoted to haemoglobin degradation. Since ellagitannins were shown to be converted by the human gut microflora into urolithins A, B and urolithin-8-methylether (Cerda et al., 2004, 2005; Seeram et al., 2004, 2006, 2008; Mertens-Jose CA, USA) equipped with an ESI source operating in the negative mode. The column was a Zorbax SB-CN 150 mm × 2.1 mm, 5 μm (Agilent Technologies, Santa Clara, CA, USA), with flow 0.25 ml/min. A gradient elution was performed using 1% formic acid/ammonium formate 5 mM (phase A) and 0.1% ammonia in methanol (phase B). The gradient program was t_min 0–2 min, 99% of A; t_min 20, 1% of A; t_min 25, 15% of A; t_min 26–46, 99% of A. The operating conditions for MS analysis were as follows: spray voltage −4 kV; capillary temperature and voltage were 250 °C and −14 V, respectively; sheath gas and auxiliary gas flow were 60 and 15 arbitrary units, respectively; tube lens offset 150 V. Collision energy was set at 35% of 5 V. Ellagic acid and punicalagins were identified by comparison of the retention times and mass spectra with those of authentic compounds. Punicalins were identified by comparison of the mass spectra with that previously described (Tzulker et al., 2007).

Quantification of EA was performed by monitoring specific ions (m/z 173, 185, 201, 229, 257, 284) derived from the collision of the [M−H]− ion as follows: LC system Surveyor Pump Plus coupled with a linear ion-trap mass spectrometer (LTQ ThermoElectron Co., San Jose CA, USA) equipped with an ESI source operating in the negative mode. The column was a Zorbax SB-CN C18 150 mm × 2.1 mm, 5 μm (Agilent Technologies, Santa Clara, CA, USA), with flow 0.25 ml/min and an isotropic elution with 0.1% ammonia in methanol. The operating conditions for MS analysis were as follows: spray voltage −4 kV; capillary temperature and voltage were 250 °C and −42 V, respectively; sheath gas, auxiliary gas and sweep gas flow were 40, 10 and 1 arbitrary units, respectively; tube lens offset −80 V. Collision energy was set at 30% of 5 V.

Quantification of punicalagins was performed by monitoring specific ions (m/z 301, 601 and 781) derived from the collision of the [M−2H]2− ion as follows: LC system Surveyor Pump Plus coupled with a linear ion-trap mass spectrometer (LTQ ThermoElectron Co., San Jose CA, USA) equipped with an ESI source operating in the negative mode. The column was a Zorbax SB-CN C18 150 mm × 2.1 mm, 5 μm (Agilent Technologies, Santa Clara, CA, USA), with flow 0.25 ml/min and a gradient elution using 1% formic acid/ammonium formate 5 mM (phase A) and 0.1% ammonia in methanol (phase B). The gradient program was t_min 0–2 min, 99% of A; t_min 20, 1% of A; t_min 25, 15% of A; t_min 26–46, 99% of A. The operating conditions for MS analysis were as follows: spray voltage −3.5 kV; capillary temperature and voltage were 300 °C and −9 V, respectively; sheath gas, auxiliary gas and sweep gas flow were 40, 10 and 1 arbitrary units, respectively; tube lens offset −100 V. Collision energy was set at 35% of 5 V.

2. Materials and methods

2.1. Chemicals

Amberlite XAD16 resin was purchased from Sigma–Aldrich (Milan, Italy) and Kollidon® from BASF Chemical Company (Germany). Ellagic acid (EA) was acquired from Fluka–Sigma–Aldrich (Milan, Italy), urolithin B and urolithin A-8-methylether were synthesized, according to known procedures (Bruggink and McKillop, 1975; Ghosal et al., 1989) by coupling the appropriate bro-mobenzonic acid with resorcinol in the presence of NaOH and CuSO4. Urolithin A was obtained by methylether removal using HBr in acetic acid. Final products gave satisfactory 1H NMR, HRMS and HPLC analysis. All reagents were purchased from Fluka–Sigma–Aldrich (Milan, Italy). Punicalagin was obtained from AvaChem Scientific LLC, San Antonio, TX, USA.

2.2. Plant material and extract fractionation

The immature fruit of Pg was manually plucked from plants growing in tropical forest area of India. A voucher specimen was deposited at the Department of Pharmacological Sciences, University of Milan, Italy. The fruits were cut and the arils discarded. The rind was sun-dried, finely ground, delipidized by petroleum ether (40:60) and extracted twice with methanol (MeOH, 1 g/10 ml). The w/w yield of the MeOH extract was 38% with respect to the starting crude material. For the tannin removal, the extract was treated with Kollidon® 1:25 (w/w).

For the preparation of a fraction enriched in tannins (Pg-FET), 5 g of the Pg-MeOH extract were dissolved in 500 ml of water and chromatographed on Amberlite XAD16 resin as described (Seeram et al., 2005). The w/w yield of Pg-FET was 60% with respect to the Pg-MeOH extract.

2.3. LC–MS–MS analysis of Pg-FET

Qualitative analysis was carried out by LC–MS–MS using a LC system P4000 (Thermo Separation Products) coupled with an LCQ ion-trap mass spectrometer (Thermo Finnigan, USA) equipped with an ESI source operating in the negative mode. The column was a Zorbax SB-CN C18 150 mm × 2.1 mm, 5 μm (Agilent Technologies, Santa Clara, CA, USA), with flow 0.25 ml/min. A gradient elution was performed using 1% formic acid/ammonium formate 5 mM (phase A) and 0.1% ammonia in methanol (phase B). The gradient program was t_min 0–2 min, 99% of A; t_min 20, 1% of A; t_min 25, 15% of A; t_min 26–46, 99% of A. The operating conditions for MS analysis were as follows: spray voltage −4 kV; capillary temperature and voltage were 250 °C and −14 V, respectively; sheath gas and auxiliary gas flow were 60 and 15 arbitrary units, respectively; tube lens offset 150 V. Collision energy was set at 35% of 5 V. Ellagic acid and punicalagins were identified by comparison of the retention times and mass spectra with those of authentic compounds. Punicalins were identified by comparison of the mass spectra with that previously described (Tzulker et al., 2007).

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Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>ng/μl added</th>
<th>ng/μl found</th>
<th>CV %</th>
<th>Error %</th>
<th>R²</th>
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<td></td>
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<tr>
<td>EA</td>
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<td>0.49</td>
<td>7.2</td>
<td>-2.6</td>
<td>0.998</td>
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<td>0.97</td>
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<td>5.0</td>
<td>5.07</td>
<td>0.05</td>
<td>1.4</td>
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<tr>
<td>Punicalagins</td>
<td>5</td>
<td>5.3</td>
<td>6.4</td>
<td>6.0</td>
<td></td>
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<td></td>
<td>25</td>
<td>24.9</td>
<td>3.6</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>EA</td>
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<td>0.47</td>
<td>2.6</td>
<td>-5.2</td>
<td>0.999</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.98</td>
<td>5.4</td>
<td>-2.3</td>
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<tr>
<td></td>
<td>5.0</td>
<td>5.02</td>
<td>1.09</td>
<td>-0.3</td>
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</tr>
<tr>
<td>Punicalagins</td>
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<td>5.2</td>
<td>2.4</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25</td>
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<td>1.9</td>
<td>-2.0</td>
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<tr>
<td></td>
<td>50</td>
<td>50.0</td>
<td>0.12</td>
<td>0.04</td>
<td>1.0</td>
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</table>

Calibration curves were prepared with increasing concentrations of the EA (0–5 ng/μl) and punicalagins (0–25 ng/μl). Linearity of the calibration curves was assessed by R². Intraday and interday precision (CV %) and accuracy (error %), calculated as (obtained value – true value)/true value × 100 of the analytical method were calculated over a series of blank samples spiked with different amount of the authentic compounds estimated on the basis of the calibration curves (Table 1).

For intraday, values are the mean of three replications/day. For interday, values are the mean of 3 days replications. Linearity for calibration curves was assessed by R².

2.4. In vitro drug susceptibility assay on Plasmodium falciparum

The CQ-sensitive (D10) and the CQ-resistant (W2) strains of Pf were cultured in vitro as described by Trager and Jensen (Trager and Jensen, 1976). Parasites were maintained in human type A-positive red blood cells at 5% hematocrit in RPMI 1640 (Gibco BRL, NaHCO₃ 24 mM) medium with the addition of 5% albumax, 0.001% hypoxanthine, 20 mM Hepes (Euroclone) and 2 mM glutamine (Euroclone). The cultures were maintained at 37°C in a standard gas mixture consisting of 1% O₂, 5% CO₂, and 94% N₂.

Test compounds were dissolved in either water or DMSO and then diluted with medium to achieve the required concentrations (final DMSO concentration <1%, which is non-toxic to the parasite). Compounds were placed in serial dilutions to 96 well flat-bottom microplates (COSTAR). Asexual parasite stages derived from asynchronous cultures with parasitemia of 1–1.5% were aliquoted into microplates (COSTAR). Asexual parasite stages derived from asynchronous cultures with parasitemia of 1–1.5% were aliquoted into microplates (COSTAR). Asexual parasite stages derived from asynchronous cultures with parasitemia of 1–1.5% were aliquoted into microplates (COSTAR). Asexual parasite stages derived from asynchronous cultures with parasitemia of 1–1.5% were aliquoted into microplates (COSTAR). Asexual parasite stages derived from asynchronous cultures with parasitemia of 1–1.5% were aliquoted into microplates (COSTAR).

Cytotoxicity was evaluated using human dermal fibroblasts (European Collection Cultures, Porton Down, Salisbury, UK). Fibroblasts (6 × 10⁴/well) were grown in 24-well plates with DMEM containing 10% foetal calf serum, 1% penicillin/streptomycin, and 1% l-glutamine as previously described (Verotta et al., 2001). Cells were incubated for 24 h with test compounds and proliferation was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyloxazolium bromide) test (Denizot and Lang, 1986), comparing cell growth in test and control wells.

2.8. In vivo antimalarial efficacy evaluation

The murine malaria model Plasmodium berghei ANKA (CQ-sensitive) /Anopheles stephensi was used for the in vivo assessments. The parasite strain was maintained in BALB/c mice by weekly acyclical passages, transferring blood from parasitemic to healthy mice. Cyclic parasite passages were regularly performed by infecting Anopheles mosquitoes through a blood meal on gametocytic mice followed by a challenge on uninfected mice after 3 weeks, i.e. the completion of the sporogonic cycle.

The Anopheles stephensi colony was kept at 30°C, >95% RH and a photoperiod of 12 h. Groups of 5–6 BALB/c mice weighing between 16 and 20 g were used as experimental hosts. Experimental animal rearing and handling were fully compliant with the Italian and EU regulations and guidelines regarding ethical aspects in animal research.

The curative activity of the Pg-MeOH extract was assessed using the Peters 4-day test (Peters, 1970), modified in accordance with the clinical application scheme of OMARIA (Bhattacharya, 2003, 2004).

Briefly, 18 mice were infected by intraperitoneal (ip) inoculation of 10⁷ parasitized red blood cells, and then randomized into three groups of 6 mice each. Mice were treated orally three times per day with Pg-MeOH extract at 50 mg/kg containing 20% NaCl, in order to mimic the human therapy, or the salt (control), dissolved in distilled water.
water. On day 4 post-infection, parasitemia was assessed by Giemsa stained blood smears.

Pg-FET was assayed for curative activity as described for Pg-MeOH with the difference that treatments were carried out twice a day, and started 48 h before infection. Pg-MeOH was administrated in a vehicle consisting in a 5% Tween 80, 5% ethanol aqueous solution.

To assess the transmission blocking activity of Pg-FET two groups of 3 gametocytemic mice each were treated orally with 50 mg/kg of the extract, or with solvent control solution. Two hours after receiving the treatment the mice were narcotized by ip inoculation of 100 μl of a 1:1 mixture xylazine and acepromazine, 13% in PBS, and placed over cages containing ~150 four to five days old Anopheles stephensi females. Mosquitoes were allowed to feed for 1 h, and the following day engorged mosquitoes were transferred into new cages. Ten days after the infective blood meal, 25 mosquitoes per group were dissected and their midguts examined under the light microscope (160×, 400×) to assess prevalence and density of Plasmodium berghei oocysts.

3. Results

3.1. In vitro effect of Pg-MeOH and Pg-FET on Pf growth and plasmepsin activity

The Pg-MeOH extract inhibited the growth of both strains of Pf to a considerable extent. IC50 values of 4.5 ± 1.2 and 2.8 ± 1.0 μg/ml were obtained with the D10 and W2 strain, respectively (mean ± sd, Table 2). When the extract was deprived of tannins, the inhibition was greatly reduced. This result indicated that tannins were likely to be the active principles. Therefore, a fraction enriched in tannins was prepared as described in Section 2 and assayed on both strains. Pg-FET inhibited D10 and W2 growth with IC50 of 2.9 ± 0.6 and 1.5 ± 0.5 μg/ml, respectively (Table 2).

In order to investigate on the mechanism of action, the activity of PLM 2 and PLM 4 were assayed in the presence of the crude Pg-MeOH extract and Pg-FET. A concentration-dependent inhibition of both proteinases was found (Table 2). However, PLM 4 was less affected by the Pg-MeOH extract than PLM 2. Unfortunately, the activity of PLM4 could not be evaluated in the presence of Pg-FET due to spectrophotometric interference at the wavelength used for the assay.

Since Pg-FET represents 60% of the whole crude extract, the recovery of inhibitory activity in Pg-FET vs. Pg-MeOH corresponds to the enrichment in tannins.

To verify whether the observed inhibition of plasmepsins corresponded to the inhibition of haemoglobin digestion by Pg-FET, the cleavage of haemoglobin was investigated on SDS-PAGE in the presence of either PLM 2 or PLM 4 with or without Pg-FET. The results in Fig. 1 show that Pg-FET prevented in a dose-dependent manner the cleavage of haemoglobin by either PLM 2 or PLM 4.

3.2. Analysis of Pg-FET by LC–MS

Fig. 2 reports the TIC chromatographic profile of Pg-FET. On the basis of the mass spectra and the retention times as described in Section 2, the structures attributed to the peaks 1–6 were punicalin isomers (1–2), punicalagin isomers (3–4), and ellagic acid (EA) (6). The attribution of the structure to peak 5 as EA exoside was done on the basis of the ions at m/z 463 [M−H]−, m/z 301, 257, and 229, as described by Mertz et al. (2007). The quantitative analysis revealed that EA and punicalagins represented 13.4% and 29.1% of Pg-FET, respectively.

![Fig. 1](image)

**Fig. 1.** Effect of Pg-FET on haemoglobin degradation assay by PLMs 2 and 4. Haemoglobin was incubated with preactivated PLM 2 or PLM 4 in the presence of the vehicle or of the compounds to be tested. After 30 min, the reaction was stopped by the addition of SDS loading dye. Samples were denatured by boiling for 5 min and loaded onto 18% polyacrylamide gel. Results are expressed as the mean ± standard deviation of four experiments performed in duplicate. Panel A: Pg-FET on PLM 2; panel B: Pg-FET on PLM 4. *p < 0.05; ***p < 0.001.
Fig. 2. TIC chromatographic profile of Pg-FET. Peaks 1–2: punicalin isomers; peaks 3–4: punicalagin isomers; peak 5: ellagic acid exoside; peak 6: ellagic acid.

Table 3

<table>
<thead>
<tr>
<th>Curative test*</th>
<th>% Parasitemia (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.8 (6.3–11.3)</td>
</tr>
<tr>
<td>Pg-MeOH 150 mg/kg/day</td>
<td>8.7 (7.4–9.9)</td>
</tr>
<tr>
<td>Control</td>
<td>8.6 (2.2–15.0)</td>
</tr>
<tr>
<td>Pg-FET 100 mg/kg/day</td>
<td>9.8 (6.7–12.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Transmission blocking test*</th>
<th>No. of mosquitoes (proportion) with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;50 oocysts &gt;50 oocysts 0 oocysts (neg)</td>
</tr>
<tr>
<td>Control</td>
<td>16 (0.6) 2 (0.1) 7 (0.3)</td>
</tr>
<tr>
<td>Pg-FET 50 mg/kg</td>
<td>11 (0.5) 9 (0.4) 3 (0.1)</td>
</tr>
</tbody>
</table>

* Parasitemia counts on thin blood smears were made on day 4 post-infection.

3.3. Effect of punicalagin and urolithins on Pf growth and plasmepsins

Punicalagins inhibited Pf growth, as well as Pf-PLM 2 and 4. The IC50 for the D10 and W2 strain were similar, amounting: 72 ± 25 and 62 ± 22 μM, respectively. The IC50 for Pf-PLM 2 was observed at 11 ± 0.4 μM; Pf-PLM 4 could not be evaluated in the presence of punicalagin due to an interference at the wavelength used in the spectrophotometric assay. However, the inhibition of PLM 2 and 4 by punicalagin was be confirmed by the prevention of haemoglobin degradation on SDS-PAGE (Fig. 3).

Urolithins A, B and 8Me, the in vivo metabolites of ellagitannins, were inactive on Pf growth (IC50 > 100 μM) and on PLM inhibition (no inhibition at concentrations up to 50 μM).

Among the constituents identified in Pg-FET, the punicalins were not tested since they are not commercially available and EA was already shown to inhibit Pf growth, most likely by blocking the detoxification of haem into the haemozoin (Verotta et al., 2001; Dell’Agli et al., 2003), a process by which Pf gets rid of the excess of haemoglobin-derived haem.

3.4. Cytotoxicity of the compounds used in this study

Pg-MeOH extract, Pg-FET and isolated compounds were not toxic to human dermal fibroblasts at the concentrations used in the described experiments (data not shown).

3.5. In vivo assessments of Pg-MeOH and Pg-FET in the Plasmodium berghei rodent malaria model

Oral treatment of Plasmodium berghei infected mice with Pg-MeOH and Pg-FET did not have any curative effect. The development of parasitemia was similar in treated and control mice reaching on day 4 after infection values of about 10% (Table 3). Also, Pg-FET did not show transmission blocking potential: mosquitoes that fed on Plasmodium berghei gametocytic mice, treated orally with the extract at 50 mg/kg, displayed normal sporogonic development of the parasite, showing numerous oocysts on day 10 after infection (Table 3).

4. Discussion

In this study we have shown that the methanolic extract of Punica granatum (Pg-MeOH) and its fraction enriched in tannins (Pg-FET) possess in vitro antiplasmodial activity. Based on our previous studies (Verotta et al., 2001) and work published by Sturm et al. (2009) and Soh et al. (2009), ellagic acid (EA) is likely to be the constituent responsible for the antiparasitic effect. The IC50 of EA reported in the cited studies was in the nanomolar range.
well, such as the generation of pro-oxidant activity or the inhibition of the haemoglobin metabolism of the parasite. FET inhibits the conversion of haematin into haemoglobin. PAGE suggest that this enzyme is also inhibited. In addition, in vitro experiments with paratubulin and the parasites' targets show that the enzyme is inhibited by FET. All together, the results of the in vitro assays support the use of the immature fruit rind of Punica granatum as herbal antimarial remedy like OMARIA.

Our data are at variance of those published by Reddy et al. (2007), who found no antiplasmodial activity by fractions obtained from the pomegranate juice or the total pomegranate tannins. We believe that this discrepancy may be explained by the different composition/preparation of the extracts used in Reddy’s compared to our studies. It has to be underlined that the same authors did not find any antiplasmodial effect of purified ellagic acid, as well, a finding that is well documented by several studies (Verotta et al., 2001; Banzouzi et al., 2002; Soh et al., 2009; Sturm et al., 2009).

Regarding the mechanism of action and the pharmacological targets of Pg and ellagitannins, our attention was focused on the process of haemoglobin digestion, which includes both the globin cleavage by plasmins (PLMs) and the detoxification of haem into haemoglobin in Plasmodium falciparum (Egan, 2008). The Pg-MeOH extract and Pg-FET both inhibited the activity of PLM 2 at concentrations close to those necessary for parasite killing in vitro. The contribution to the antiparasitic effect of an inhibitory activity directed specifically to PLM 4 could not be estimated due to methodological problems. However, the data obtained from SDS-PAGE suggest that this enzyme is also inhibited. In addition, Pg-FET inhibits the conversion of haematin into β-haematin in vitro (data not shown) as previously shown for EA (Dell’Agi et al., 2003). Therefore, the components of Pg-FET likely interfere with different steps of the haemoglobin metabolism of the parasite.

We cannot exclude, however, that EA exert different functions, as well, such as the generation of pro-oxidant activity or the inhibition of glutathion S-transferase, as recently suggested (Soh et al., 2009; Sturm et al., 2009).

In the present study, punicalagin inhibited PLM 2 in vitro at a concentration six to seven times lower than the IC50 against Pf, implying that the compound might not reach the food vacuole cell compartment. This might be due to the large size of the molecule.

Since the relevance of PLMs as targets for antiplasmodial compounds is presently under debate (Liu et al., 2006), at this stage the pharmacological target of Pg ellagitannins remains an open question.

The biotransformation of punicalagin and punicalin investigated in humans seems to support their conversion to EA, which is in turn converted to urolithins by the intestinal microflora. Urolithins were detected in human plasma and urines after pomegranate consumption (Cerda et al., 2003, 2004, 2005; Seeram et al., 2004, 2008; Mertens-Talcott et al., 2006). Since urolithins were found to be unable to kill the parasite in vitro, it appears that the metabolism of EA may lead to a quick loss of the antiparasitic activity.

Experiments performed to check the curative effect of Pg-MeOH extract and Pg-FET in the rodent malaria model gave conflicting results with the in vitro data. Both extracts were ineffective either as curative or as transmission blocking agents. In our protocols, animals were treated by the oral route, a condition mirroring the human treatment. In a recent report (Soh et al., 2009), very poor activity of EA as prophylactic-curate agent was found when the compound was administered orally to mice infected with Plasmodium vinckei petteri, while it was active by the ip route. The authors ascribed the absence of oral efficacy to a low bioavailability of EA. This observation helps to explain the lack of efficacy of the Pg-MeOH extract and Pg-FET in our animal experiments. Assuming a concentration of 13% EA in Pg-FET, the dose of EA administered to mice was 20 mg/kg/day for the curative assay and 13 mg/kg/day in the transmission blocking assay, a dose that was inactive when used orally in the in the Plasmodium vinckei petteri mouse model (Soh et al., 2009).

The low bioavailability as well as the kinetic of conversion of EA to inactive metabolites urolithins may explain the failure of Pg-MeOH extract and Pg-FET to control parasitemia in mice. Alternatively, it could be argued that Plasmodium berghei unlike Pf and unlike Plasmodium vinckei petteri is not responding to the treatment.

Despite the negative results in the rodent malaria model, records from the Ayurveda dispensary indicate a positive impact on malaria disease and on the general health status of the population receiving OMARIA. Unfortunately, no data are available on the pharmacokinetcis of ellagitannins, in individuals receiving the OMARIA formulation of Punica granatum fruit rind and as Orissa. So far, data on plasma and urine levels of EA and its metabolites in humans derive only from studies with healthy western volunteers after fruit juice consumption or other formulations of pomegranate extracts (Cerda et al., 2003, 2004, 2005; Seeram et al., 2004, 2008; Mertens-Talcott et al., 2006). However, the intestinal absorption may differ between Caucasian and Asian populations or between individuals according to their health status. It has also to be considered that the content of ellagitannins in Punica granatum growing in a tropical region like India may differ in comparison to that of plants from temperate regions. In conclusion, the apparent discrepancy between human and rodent parasites may be ascribed to a variety of factors, still to be investigated.

5. Conclusions

Our in vitro results support for the effectiveness of Punica granatum immature fruit rind for the treatment of malaria and sustain that EA is the main active compound of Pg extracts. EA glycosides and ellagitannins (punicalagins and punicalins) may contribute as pro-drugs to a more efficient delivery of EA in vivo. Urolithins, the metabolic products of EA biotransformation by colon microflora, have no antiplasmodial activity.

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